



Beyond the EPR effect: Intravital microscopy analysis of nanoparticle drug delivery to tumors[☆]

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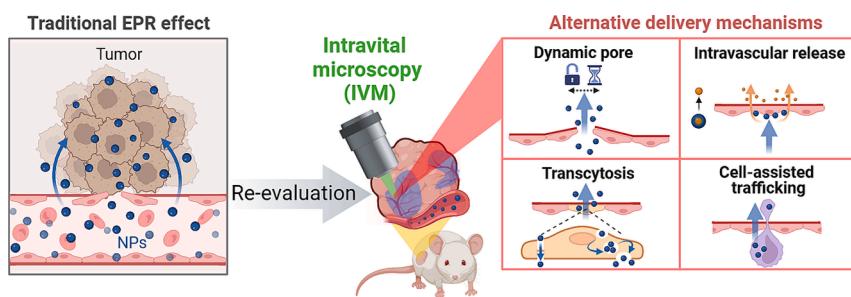
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GRAPHICAL ABSTRACT



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ABSTRACT

Delivery of nanoparticles (NPs) to solid tumors has long relied on enhanced permeability and retention (EPR) effect, involving permeation of NPs through a leaky vasculature with prolonged retention by reduced lymphatic drainage in tumor. Recent research studies and clinical data challenge EPR concept, revealing alternative pathways and approaches of NP delivery. The area was significantly impacted by the implementation of intravital optical microscopy, unraveling delivery mechanisms at cellular level *in vivo*. This review presents analysis of the reasons for EPR heterogeneity in tumors and describes non-EPR based concepts for drug delivery, which can supplement the current paradigm. One of the approaches is targeting tumor endothelium by NPs with subsequent intravascular drug release and gradient-driven drug transport to tumor interstitium. Others exploit various

Abbreviations: CAM, cell adhesion molecule; CD, cluster of differentiation; ECM, extracellular matrix; EPR, enhanced permeability and retention; ESL, E-selectin ligand; EV, extravascular space; FlaRE, flash release in endothelium; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; HSA, human serum albumin; IFP, interstitial fluid pressure; IL, interleukin; IVM, intravital microscopy; MDN, membrane-derived nanovesicle; MMP, matrix metalloproteinase; MPO, myeloperoxidase; MPS, mononuclear phagocyte system; NIR, near-infrared; NP, nanoparticle; N-TEC, nanoparticle transport endothelial cell; PEG, polyethylene glycol; PLGA, poly lactic-co-glycolic acid; PLVAP, plasmalemma vesicle-associated protein; PSMA, prostate-specific membrane antigen; RBC, red blood cell; TAM, tumor-associated macrophage; TEM, tumor endothelial marker; TGF, transforming growth factor; TLR, toll-like receptor; TLs, thermoresponsive liposomes; TME, tumor microenvironment; VE, vascular endothelial; VEGF, vascular endothelial growth factor; VL, vessel lumen; VVOs, vesiculo-vacuolar organelles.

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immune cells for tumor infiltration and breaking endothelial barriers. Finally, we discuss the involvement of active transcytosis through endothelial cells in NP delivery. This review aims to inspire further understanding of the process of NP extravasation in tumors and provide insights for developing next-generation nanomedicines with improved delivery.

1. Introduction

Nanoparticles (NPs) have emerged as a versatile tool for targeted drug delivery and bright imaging of malignant tissues. NPs can serve as carriers for both molecular therapeutics and larger biomolecules, protecting the payload from degradation and increasing its bioavailability [1]. They can be engineered to respond to specific stimuli in the tumor microenvironment, such as acidic pH, hypoxia, or certain enzymes, to perform selective and localized drug release [2]. Decoration of NPs with targeting ligands, recognizing receptors overexpressed on tumor cells, improves precision of therapeutics and minimizes toxicity to healthy tissues [3,4].

The performance of NPs *in vivo* largely depends on their delivery to tumor tissue, which, for several decades, was believed to occur solely through the “enhanced permeability and retention” (EPR) effect. The EPR effect, first observed by Matsumura and Maeda in 1986, refers to the enhanced accumulation of NPs via the abnormal leaky vasculature of tumors and the subsequent retention due to impaired lymphatic drainage [5–7]. NPs with a size from 10 to 500 nm have been shown to traverse the intercellular gaps and accumulate in tumors, imposing constraints on the design of drug carriers [8,9]. Transport via the EPR effect generally belongs to passive delivery routes, meaning it does not require binding to receptors or energy-dependent internalization. In contrast, active delivery routes involve receptors or cells in the process of NP extravasation. Passive transport is an attractive concept for tumor treatment, since it has the potential to be universal for broad classes of nanomedicines, including drug carriers, mRNA vesicles and oncolytic viruses.

However, the clinical translation of EPR-based nanomedicines faced substantial challenges. Despite promising preclinical outcomes, many NPs have failed to demonstrate consistent therapeutic efficacy in human cancers [10]. One of the reasons for this is the significant heterogeneity of the EPR effect, which varies between different types of tumors, between patients, and even within different regions of the same tumor. This variability can be attributed to factors such as the tumor’s vascular density, number and size of intercellular gaps, thrombogenesis, differences in tumor’s interstitial fluid pressure, and functional state of the lymphatic system [11,12]. Such heterogeneity is lower for artificial human xenografts than for patient-derived tumors in murine models [13]. Moreover, the EPR effect is typically more uniform in small, rapidly growing tumors, similar to preclinical models [11]. This led to a re-evaluation of the reliance of drug delivery on the EPR concept, prompting researchers to explore complementary mechanisms for enhanced NP accumulation in tumors, particularly as the understanding of tumor biology and nanoparticle interactions expands [14].

Intravital microscopy (IVM) has emerged as a powerful tool to discover and refine strategies for enhanced NP delivery in tumors. IVM is an optical imaging technique that enables real-time visualization of NP behavior in live animals at the cellular level [15]. Starting from the early years after discovery of the EPR effect, fluorescence microscopy was used to analyze influence of the size and coating of liposomes on the microvascular permeability in tumors [16,17]. Over the past decade, IVM revealed additional processes involved in the delivery of nanomaterials [18]. For instance, polymeric NPs, dendrimers, and liposomes with cationic charge were shown to induce adsorptive-mediated transcytosis in tumor blood vessels. Similarly, IVM revealed a special subtype of tumor endothelial cells, participating in NP transport [19]. These transendothelial mechanisms may provide a more consistent pathway for NP delivery, especially in tumors with limited EPR-based

accumulation [20].

IVM led to the exploration of other passive and active mechanisms of transport by focusing on microvascular dynamics of NPs and their permeation through the tumor vessels. For instance, IVM data demonstrates potential of targeting NPs to the features of tumor vasculature. The reduced blood flow rate in solid tumors enables adhesion and passive accumulation of sub-micrometer drug vesicles [21]. Functionalization of NPs with ligands that bind to receptors overexpressed on tumor endothelial cells (*e.g.*, pro-angiogenic factors or cell adhesion molecules) enables active targeting and retention at the tumor site [22]. For instance, NPs recognizing vascular endothelial growth factor (VEGF) have been shown to remain in C6 glioma for one day [23]. Membrane-derived nanoghosts or membrane-coated NPs can exploit the natural ability of certain cell types to home to the tumor or to the inflamed tumor endothelium [24,25]. Subsequent localized drug release in blood vessels can improve cancer therapy even without NPs permeation [26].

Intravital fluorescence tracking of single cells shows the involvement of cell-mediated active delivery of NPs in overcoming endothelial barriers. Immune cells, including monocytes, neutrophils and lymphocytes naturally home to tumors and can be engineered to carry NPs [27]. Macrophages have shown the ability to transport NPs across the blood-brain barrier, and into challenging tumor regions far from blood vessel wall [28,29]. Circulating leukocytes and marginated neutrophils normally uptake part of intravenously injected nanomaterials and can significantly alter their biodistribution [30,31]. In addition, leukocyte trafficking creates leakiness in basement membrane, enabling NPs to use this route for entrance into the tumor [32]. Red blood cells (RBCs) lack cancer-targeting characteristics but are capable of vascular organ-specific targeting. RBCs have been shown to deliver NPs to the lungs and brain [33] and to perform specific treatment of pulmonary metastases [26]. By leveraging the natural migratory, tumor-homing properties and prolonged circulation of cells, cell-mediated NP delivery bypasses the limitations of EPR-dependent accumulation.

This review provides a comprehensive analysis of the diverse pathways which are utilized by NPs to enter tumors and deliver drugs to cancer cells. We present the EPR effect as a cornerstone principle governing the design of NPs for drug delivery, while also discussing the reasons behind the heterogeneity of this effect in clinical settings. In view of this, we emphasize the role of non-EPR-based transport mechanisms in overcoming heterogeneity of tumor biology. We focus on insights gained from IVM methods for real-time analysis of NP behavior within the tumor microenvironment. Non-EPR-based delivery mechanisms, such as targeting of tumor endothelium, transcytosis, and cell-mediated delivery, offer new perspectives to drive NP design for cancer therapy. These mechanisms are complementary to the EPR concept and all of them may coexist within a single tumor. The review aims to shape the development of next-generation nanomedicines that move beyond the constraints of EPR-dependent uptake, paving the way for personalized, effective, and adaptable delivery strategies for patients with variable tumor profiles.

2. EPR effect in preclinical and clinical studies

2.1. The phenomenon of EPR effect in nanoparticle delivery

Discovery of the EPR effect undoubtedly boosted design of nanomaterials with more than 60 nanomedicines that have been approved for clinical use [34]. The EPR-based accumulation for now remains the fundamental principle underlying passive targeting for imaging and

therapy of solid tumors. The enhanced accumulation of NPs in tumors in comparison to healthy tissues was observed in rodents, rabbits, dogs and other vertebrate animals [16,35,36]. In clinical settings, the EPR effect has been shown in human cancers such as those of the colon, rectum, breast, stomach, and ovaries [37]. However, not all tumors exhibited a strong EPR effect. For example, human pancreatic tumors are known for poor vascularization and a dense microenvironment, which limits tissue permeability [38,39].

EPR mechanism involves the enhanced accumulation of macromolecules through a leaky tumor vasculature and retention via restricted blood and lymphatic elimination pathways (Fig. 1A) [6]. In the pioneering study of Matsumura and Maeda, this was supported by post-mortem optical visualization of delivery of albumin-Evans blue complexes to Sarcoma 180 tumor [5]. Modern IVM techniques allow real-time and high-resolution imaging in living animals, enabling both whole-body cancer screening and microscopic visualization of the tumor vessels (Fig. 1B). For instance, Qi *et al.* utilized short-wave infrared imaging to observe the dynamics of EPR-based delivery of 33-nm fluorescent dots in prostate xenografts, with minimal interference from endogenous fluorescence of tissues [40].

The EPR-based accumulation of NPs is primarily driven by pathological openings between endothelial cells in the tumor vasculature. Immunohistochemistry of blood vessels in MCa-IV murine mammary carcinomas has shown that 14 % of the vessel surface is lined by defective and poorly connected cells. Electron microscopy study of these vessels revealed that endothelium has intercellular openings with a median size of 1.5 μm (ranging from 0.3 to 4.7 μm) and transcellular holes with a median size of 0.6 μm (ranging from 0.2 to 0.9 μm) [41]. A series of studies showed size-dependent accumulation of liposomes in subcutaneous tumor models, where most tumors exhibited a vascular cut-off pore size ranging from 380 nm to 780 nm [9,16,42]. The leaky tumor vasculature facilitates the deposition of drug-loaded nanoparticles, leading to enhanced delivery to tumors compared to normal tissues.

A second key component of the EPR effect is the prolonged retention of NPs within the tumor tissue. This retention has largely been attributed to impaired lymphatic drainage, due to the collapse of lymphatic vessels or the absence of functional lymphatics far from the tumor margin [7,43]. However, lymphatics functionality is usually accessed by monitoring the interstitial drainage of labeled macromolecules [7,44]. A

recent study by Nguyen *et al.* examined the lymphatic drainage of NPs in common murine tumor models (B16-F10, 4 T1, MMTV-PyVT) [45]. All studied tumor lymphatic vessels were not fully collapsed and had a lumen size larger than 286 nm, which is sufficient for NP exit. Optical imaging confirmed that both peritumoral and intratumoral lymphatic vessels participate in NP elimination, while clearance enhances with the decrease of NP size. Large 100-nm particles experienced only minimal clearance, with just 2.1 % elimination of injected dose. This study provides evidence of lymphatic drainage of NPs in tumors, while also supporting the general concept of NP retention, which can be attributed to NP interactions with both cellular and acellular components of tumors [45,46].

The described EPR concept has set restrictions on NP design to maximize tumor accumulation. All the current cancer nanomedicines are designed with a size lower than the endothelial openings cut-off. In addition, NPs should be larger than 6 nm to avoid renal clearance [47] and preferably smaller than 200 nm to reduce uptake by tissue-resident macrophages [48,49]. Consequently, it is generally accepted that NPs within the 10–200 nm range are ideal for passive accumulation in solid tumors [50]. Beyond NP size, surface chemistry has been modified to diminish the uptake by circulating cells, macrophages, and endothelial cells. Surface of NPs is modified with stealth polymers like polyethylene glycol (PEG), shielding interaction with biomolecules, in the vast majority of clinical liposomal and polymeric cancer pharmaceuticals [51]. Recent data, reinforced by IVM observations, suggest multiple alternative mechanisms to the EPR-based permeation of NPs through static pores in endothelium [19,32,52]. If the role of the EPR effect as the primary route of NP delivery to tumor is compromised, restrictions on NP engineering can be reduced.

2.2. Challenges of EPR effect in clinical translation

Over several decades of the pre-clinical development of anti-cancer nanomedicines, numerous NPs demonstrated efficiency in treating solid tumors in animals. Clinical studies showed limited success. For instance, liposomal formulations of doxorubicin significantly reduced cardiotoxic effects for patients compared to free drug administration [53]. However, the clinical assessment of liposomal doxorubicin has shown only modest or no improvement in objective response rates when compared to standard chemotherapy, with no effect on progression-free

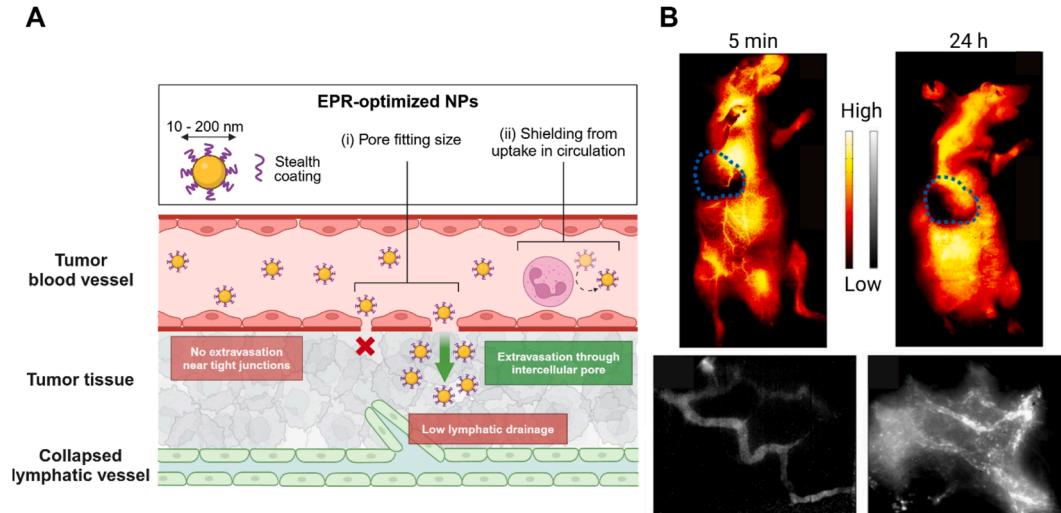


Fig. 1. The enhanced permeability and retention (EPR) effect in nanoparticle delivery. (A) Principle of the EPR effect: circulating NPs passively enter tumors through the pores in abnormal tumor vasculature and retain in tissue due to low lymphatic drainage. The EPR principle restrains NP design. To enhance passive accumulation, the size of NPs should be lower than pore size in endothelium, while surface is shielded from the uptake by any non-target cells. Created with BioRender.com (B) Visualization of the EPR effect in tumor site with the short-wave infrared fluorescence imaging. Whole-body biodistribution (top) and NPs spread near the tumor vessel (bottom) were accessed 5 min and 24 h after intravenous injection of fluorescent NPs. Adapted with permission from ref. [40].

survival [54]. Other NP-based drug delivery systems have encountered clinical setbacks. BIND-014 docetaxel-loaded polymeric NPs targeting prostate-specific membrane antigen (PSMA) in metastatic prostate cancer failed to demonstrate significant therapy improvement over standard docetaxel therapy in a phase II clinical trial [55]. While NPs in general offer favorable blood circulation, their therapeutic effects in humans have often been underwhelming, highlighting the challenges in translating preclinical successes to clinics.

These challenges can be largely attributed to the differences in structure of murine tumor models and clinical samples (Fig. 2). Human tumors are typically larger and exhibit more heterogeneous and diverse genetic patterns compared to animal models [56]. This heterogeneity, in particular, is reflected in the non-uniform vascularization and vessel fenestration, and variations in the parenchymal microenvironment [57,58]. Furthermore, blood flow in human tumors is frequently suppressed and vessels are blocked due to the formation of thrombi and occlusions, which can hinder sufficient NP accumulation in the peritumoral vasculature [11]. Tumor size is another aspect influencing the EPR status. The analysis of clinical trials by Natfji *et al.* concluded that large tumors ($140 \text{ cm}^2 > \text{area} > 25 \text{ cm}^2$) generally exhibit greater nanomedicine accumulation compared to medium-sized ($\text{area} \leq 25 \text{ cm}^2$) or very large tumors ($\text{area} \geq 140 \text{ cm}^2$). However, the enhanced permeation was observed in only about half of the patients with large tumors, whereas for medium-sized tumors it occurred in most cases [37]. This contradicts murine models, where the tumor concentration of NPs tends to decrease with the increase of tumor size [59,60].

Active proliferation of cancer cells and deposition of extracellular matrix (ECM) create solid stresses, which compress blood and lymphatic vessels and induce hypoxia [43,61]. This compression, along with high vascular permeability and reduced lymphatic drainage, results in elevated IFP, creating a diffusion barrier that impedes the penetration of macromolecules and NPs into deep regions of the tumor [62]. Consequently, even when NPs successfully extravasate into the extravascular space, they may not be able to distribute evenly through the tumor volume, which is important for achieving therapeutic efficacy [63].

Moreover, tumor cells tend to populate more densely the space near the blood vessels [64]. These dense perivascular layers may impede the permeation of NPs, which is similar to the effect of limited diffusion of molecular drugs through multicellular layers [65].

Other components of tumor microenvironment (TME) play a critical role in EPR-based NP delivery. The TME includes a network of stromal cells, including fibroblasts, pericytes, smooth muscle cells, and various immune cells, embedded within a rigid ECM primarily composed of collagen [66]. This high-density matrix acts as a significant physical barrier, impeding the diffusion of NPs and macromolecules within the tumor [67]. Moreover, stromal cells contribute to the solid stress and compress blood vessels [61]. TME generally features a highly hypoxic and acidic extracellular environment due to irregular blood flow and oxygen deprivation. However, the levels of acidity and oxygenation are not uniform within the tissue [68], which results in different NP behavior in interstitial regions [69,70]. These variability factors severely compromise the uniform distribution of nanomedicines within solid tumors, particularly in areas distant from blood vessels [71,72].

IVM can be an informative tool to report physical parameters of the TME, which is important for any drug delivery concept. Intraoperative IVM of patient tumor vessels in clinics has shown that they are disorganized, tortuous, and half of them do not support blood flow [73]. Wang *et al.* presented a novel hypoxia probe and intravitaly examined the activity and extravasation of hypoxic and normoxic cell phenotypes near the blood vessels. Zhang *et al.* recently established the method to evaluate the progression of intratumoral solid stress over time at single-cell resolution, achieved by probing the deformation of polyacrylamide particles with intravital fluorescence microscopy [74]. Cell migration, pH, enzyme activity and other functional and anatomical features of tumor can be accessed using fluorescent probes to provide valuable insights about TME in clinical samples [75].

Challenges in the translation of EPR-based nanomedicines can be partially overcome by implementation of personalized medicine concept. The TME characteristics and EPR-based accumulation should be analyzed before therapy with nanomedicines. IVM with the ability to

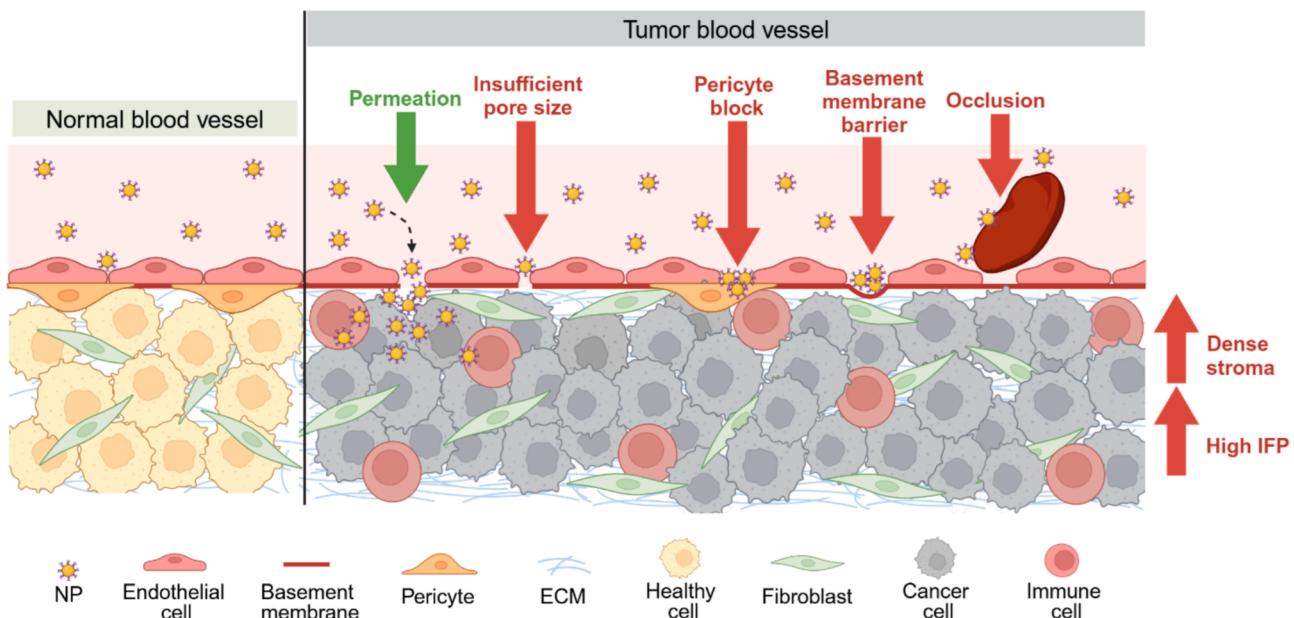


Fig. 2. Barriers preventing the efficient extravasation of NPs via the EPR effect. In normal blood vessels, cells are connected by tight junctions and lack intercellular pores, which limits the permeation of NPs larger than 5 nm. In tumor blood vessels, NPs extravasate from the lumen through the pathological interendothelial pores via convection. Pericyte coverage and basement membrane impede NP leakage into the tumor interstitium. Tumors build a dense microenvironment consisting of rapidly proliferating cancer cells, infiltrating immune cells, and fibroblasts. Combined with a dense extracellular matrix (ECM), the solid stress created by disorganized and compact cell arrangement establishes a diffusion barrier for NPs. Permeation and diffusion are further hindered by elevated interstitial fluid pressure (IFP) due to the imbalance between liquid influx and lymphatic outflow. Finally, the flow of NPs from tumor vessels can be blocked by vascular occlusions, such as thrombi. Created with BioRender.com.

monitor NP behavior in real-time remains attractive for investigations of the correlation between vascularization properties and particle delivery [76]. Miller *et al.* demonstrated that some magnetic and fluorescent NPs can be used as companion imaging agents during nanomedicine trials to predict drug accumulation and treatment response in solid tumors [77]. Alternatively, animal avatars with patient-derived tumors can be used for clinical analysis of EPR status of tissue and its heterogeneity [78]. Jeon *et al.* applied near-infrared fluorescent imaging to analyze tumor-targeting efficiency of NPs in cellular pancreatic tumor model, xenografts from patient-derived cells and patient-derived tissues [13]. Targeting of malignant tissue was closely related to the characteristics of TME, including blood vessel density and ECM content. The model from patient-derived tissues better displays TME of clinical tumors and shows faithful heterogeneity of NP accumulation in tissue.

3. Non-EPR based mechanisms of nanoparticle delivery

The enhanced accumulation of nanoparticles, observed in certain tumor types or specific tumor regions, drives investigation into the reasons behind this phenomenon. The passive accumulation of NPs through the tumor's leaky vasculature is likely not the sole mechanism of NP delivery. IVM reveals that immune and endothelial cells actively participate in the delivery and retention of NPs. Multiple mechanisms are involved in NP transport and the dominant pathways in specific tumor types or microenvironments have yet to be determined.

3.1. Approaches for intravascular targeting and drug release

3.1.1. Drug release in the tumor vasculature

The high heterogeneity and instability of NP delivery to tumors raise the question: "Is the NP extravasation across tumor vasculature even necessary for cancer treatment?" In certain cases, the answer might be "No." Delivering NPs "as close as possible" to the tumor can still be effective for cancer diagnostics and therapy. IVM reveals that dense

collagen matrix in tumors hinders the penetration of most nanomedicines deep into malignant tissue [46,79]. As a result, in most cases, tumor imaging with NPs represents only visualization of the tumor margin. On the other hand, Miller *et al.* reported a multispectral IVM analysis of biodistribution of NPs and their therapeutic cargo within tissue. Although the NPs did not cross the blood vessel wall, their released payload permeated tumor tissue within 30 min after particle injection (Fig. 3A) [80].

In consistency with this fact, a novel drug delivery concept was recently proposed that does not require extravasation of NPs through the endothelium. This approach, termed Flash Release in Endothelium (FlaRE)-based drug delivery, involves the rapid delivery and enhanced accumulation of drug-loaded NPs in the tumor vasculature, followed by their fast degradation within the vessels. The flash release of the cargo in the tumor vasculature creates high concentration of the drug in blood vessel and drives its diffusion along the gradient from the endothelium into the interstitium. Thus, the released payload can reach distant cancer cells, with ensuing therapeutic effects (Fig. 3B) [26].

According to the EPR-based drug delivery paradigm, slow and gradual release is expected to induce better treatment outcomes, since NPs require time to reach the interstitium. Under this rationale, most drug delivery systems developed over the past decades have been designed for controlled and prolonged drug release over hours or days [82]. Novel NPs were engineered to release payload under external stimuli specific for TME, such as acidic pH, increased oxygenation or high redox potential [82]. The therapeutic efficiency of anti-cancer drugs can be predicted by the peak drug concentration at the affected site [83]. A theoretical comparison of drug delivery using NPs with slow (3 h) and rapid (3 min) drug release, has shown the advantages of NPs with rapid drug release mode. After fast intravascular drug release, the highest peak concentration of drug was achieved in the distant malignant cells (Fig. 3C), which further allowed to perform treatment of pulmonary metastases in mice [26].

The feasibility of the FlaRE drug delivery principle existed

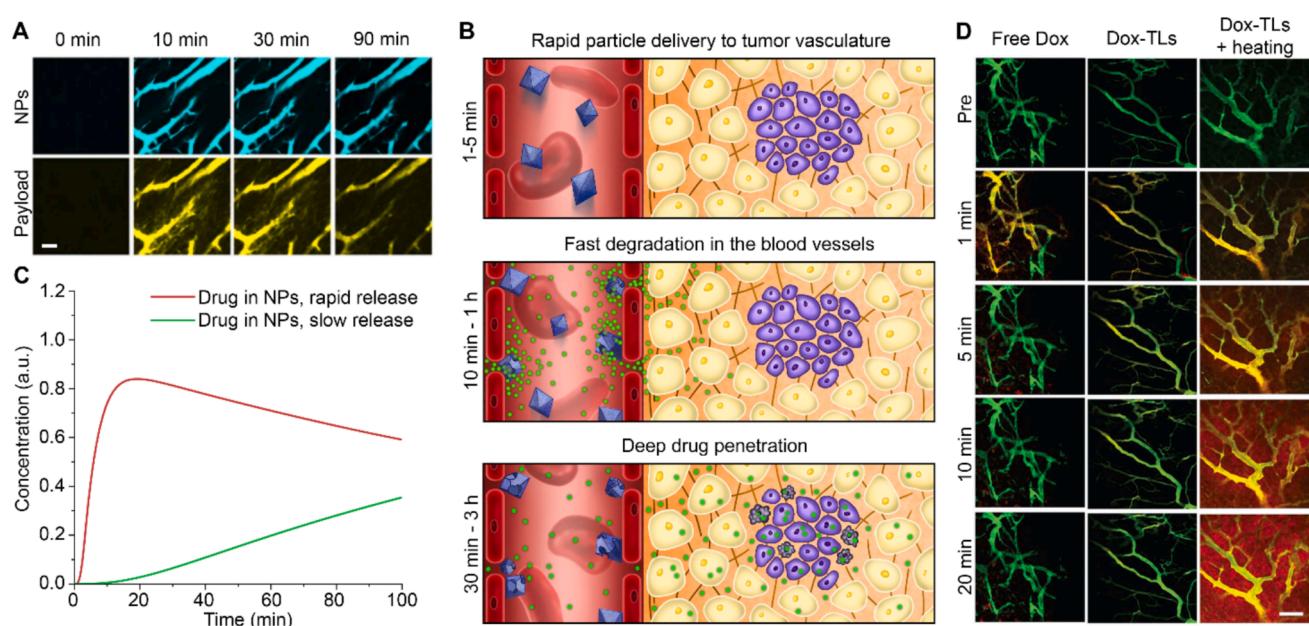


Fig. 3. Drug release in tumor vasculature as an alternative to EPR-based extravasation. (A) Time-lapse intravital images of NPs (blue) and their payload (yellow) accumulation in a xenograft tumor model. Scale bar is 50 μ m. Adapted with permission from ref. [80]. (B) Schematic diagram of FlaRE drug delivery concept: NPs bind to tumor vasculature and rapidly release encapsulated drug (green). The high intravascular concentration of drug drives diffusion to the distant tumor interstitium. Adapted with permission from ref. [26]. (C) Theoretical modeling of drug concentration at the 50- μ m distance from the endothelial wall for different drug delivery modes: delivery with NPs degrading within 3 min (rapid release) and 3 h (slow release). Adapted with permission from ref. [26]. (D) Doxorubicin delivery via triggering of intravascular release from thermoresponsive liposomes (TLs). Blood vessels are stained with fluorescein-labeled dextran (green) or fluorescein-labeled liposomes (green) in corresponding columns, while doxorubicin permeation is marked with red. Scale bar is 100 μ m. Adapted with permission from ref. [81].

throughout the literature for nanomaterials activated by external stimuli. Manzoor *et al.* reported triggering of rapid intravascular drug release from thermally activated liposomes. Real-time confocal imaging of doxorubicin illustrates that intravascular drug release increases both the drug permeation distance and the duration of cancer cell exposure (Fig. 3D) [81]. Li *et al.* used thermosensitive liposome, which released 95 % of doxorubicin upon mild heating from 37 °C to 42 °C to treat human melanoma xenografts and significantly improved the survival of mice [84]. Modification of clinically used liposomes with temperature-sensitive polymers can transform NPs from slow to rapid drug release systems [85].

Other external stimuli such as light, X-ray, and ultrasound, can be used to trigger drug release in blood vessels. Luo *et al.* developed liposomes activated under near-infrared light by incorporating porphyrin-phospholipids in the NP structure. Light irradiation induced singlet oxygen-mediated oxidization of unsaturated lipids, leading to a drug release [86]. The FlaRE strategy can be further enhanced by triggering drug release with ultrasound. Ho and Yeh demonstrated that acoustic vaporization of nanodroplets induces intravascular release of doxorubicin, but also disrupts tumor vessels smaller than 30 μm [87]. Acoustic cluster therapy utilized sound-activated large microbubbles to exert intense mechanical effects on endothelial cells, further improving drug delivery [88]. Real-time multiphoton IVM has shown the potential of ultrasound to increase extravasation of large macromolecules, such as dextran, at distances of up to 46 μm from the vessel. This covers most tumor regions, since the intercapillary distances in cancer tissues are lower than 100–150 μm [89]. Ultrasound has also shown potential as cancer monotherapy. Ultrasound irradiation at specific wavelengths can damage rapidly dividing cancer cells during mitosis, thereby slowing down tumor growth [90,91]. Combining ultrasound-mediated nanoparticle delivery with passive ultrasound-induced damage to cancer cells could result in synergistic therapeutic effects.

Liposomes with intravascular drug release are currently undergoing clinical studies. A phase I trial of ThermoDox demonstrated the safety of using focused ultrasound to trigger drug release from thermosensitive liposomes. Drug transport in clinical solid tumors mirrored results from murine studies, showing significant permeation of malignant tissue with doxorubicin after liposome activation. The non-invasive thermal drug release was precise and safe for patients [92,93].

IVM is one of the few methods that may be used for real-time experimental validation of different intravascular drug delivery approaches. For instance, ten Hagen *et al.* developed a theoretical model for drug transport after different drug release modes and validated it through the IVM data in mice [94]. This study underscores the importance of identifying materials that exhibit first-pass accumulation in tumor vessels and rapid drug release within minutes.

3.1.2. Active targeting to receptors of tumor vasculature

Drug delivery concepts with flash intravascular release of payload may benefit from rapid binding of drug-loaded NPs to tumor endothelial cells. Active targeting receptors overexpressed on the surface of these cells is a straightforward method to enhance specific NP accumulation near the malignant tissue. To ensure specific delivery, molecular targets should be limited to those found only on pathological endothelial cells rather than normal ones [95]. Tumor endothelial cells express various tumor endothelial markers (TEM), some of which encode membrane proteins available for targeting [96]. Molecular targets commonly used for vascular targeting are listed in Table 1. However, not all such proteins are endothelial-specific and may be present on other cell types. Non-specific targeting of tumor endothelial cells induces high accumulation of drug-loaded NPs in inflamed tissues, such as joints, which is not beneficial for treatment of elderly patients [97]. In addition, expression of receptor can be upregulated on endothelium during several cardiovascular diseases, such as atherosclerosis, coronary artery disease, myocardial infarction, and ischemic stroke. Certain receptors can be also overexpressed in different tumor cells (Table 1). However, in the context

Table 1
Endothelial markers for tumor vasculature targeting.

Target	Presence on vasculature	Presence on tumor cells	Reference
B7-H3, CD276	Tumor endothelial cells	Expressed, not specific	[98,99]
4-1BB, CD137	Tumor endothelial cells	Expressed, not specific	[100]
TEM1, CD248	Fibroblasts and a subset of pericytes associated with tumor blood vessels	No	[101,102]
TEM5, Gpr124	Tumor endothelial cells	No	[96,103]
TEM7	Tumor endothelial cells	No	[96,103]
TEM8	Tumor endothelial cells	No	[96,103]
MMP-2	Tumor endothelial cells	Expressed, not specific	[104,105]
MMP-9	Tumor endothelial cells	Expressed, not specific	[105]
MMP-11	Tumor endothelial cells	Expressed, not specific	[106]
PV1, PLVAP	Tumor endothelial cells and endothelial cells during cardiovascular diseases	No	[107,108]
VCAM-1, CD106	Ubiquitous. Upregulated in inflamed endothelium, tumor endothelial cells and endothelial cells during cardiovascular diseases	No	[109]
ICAM-1, CD54	Ubiquitous. Upregulated in inflamed endothelium, tumor endothelial cells and endothelial cells during cardiovascular diseases	No	[109]
E-selectin, CD62E	Inflamed endothelium, tumor endothelial cells and endothelial cells during cardiovascular diseases	Expressed, not specific	[110]
P-selectin, CD62P	Inflamed endothelium, tumor endothelial cells and endothelial cells during cardiovascular diseases	Expressed, not specific	[110,111]
APN, CD13	Tumor endothelial cells and angiogenic vasculature	Expressed, not specific	[112]
Integrin αvβ3	Tumor endothelial cells and angiogenic vasculature	Expressed, not specific	[113,114]
Integrin αvβ5	Tumor endothelial cells and angiogenic vasculature	Expressed, not specific	[113]
Integrin α5β1	Tumor endothelial cells and angiogenic vasculature	Expressed, not specific	[115,116]

of low efficiency of EPR-based permeation of nanomedicines, other cells except endothelial are unlikely to be accessible for targeting with FlaRE delivery concept.

B7-H3, also known as CD276, is a transmembrane protein from the B7 family of immune checkpoint proteins that regulates the adaptive immune response [117]. CD276 was found to be overexpressed on endothelial cells during pathological, but not physiological, angiogenesis. For example, expression level of CD276 was upregulated in the vasculature of clear cell renal cell carcinoma in 95 % of patient samples [118] and in adrenocortical carcinoma in 91 % of samples [119]. This receptor is also overexpressed in a wide range of tumor types, including cancers of bladder, breast, colorectal, ovarian, prostate, and melanoma, among others [98,99]. A meta-analysis of RNA sequencing data and clinical data shows that CD276 is overexpressed in most malignant tissues, and high receptor expression is correlated with low survival probability [98]. CD276 can be a target for NPs and drug conjugates. CD276-positive endothelial cells promoted tumor delivery of gold NPs in mature vessels with proper pericyte coverage [120]. This study contradicts the EPR concept and indicates that CD276 tumor endothelial cells can participate not only in binding targeted NPs in vasculature, but in their further transport toward tumor cells. Seaman *et al.* applied anti-CD276 drug conjugates for the treatment of several models of breast, lung and colon cancer xenografts [99]. They demonstrate that CD276 is expressed on both newly formed and established vasculature, co-opted by the tumor during growth. The treatment shows complete eradication of lesions for lung and colon carcinomas and potent anti-tumor and

anti-metastatic activity for other cancer models [99]. Another study demonstrated precise accumulation of anti-CD-276 targeted gold nanocages in small lung carcinoma and destruction of B7-H3-positive tumor cells and tumor-associated vasculature through synergistic chemo- and photothermal therapy [121].

4-1BB or CD137 belongs to the tumor necrosis factor receptor family. It is a membrane surface protein expressed on activated T-cells, B-cells, natural killers, and other immune cells. The use of CD137 agonists in cancer treatment promotes tumor shrinkage by enhancing the cytotoxicity of T-cells [122]. CD137 is expressed on tumor endothelial cells, does not present on normal endothelium, and is minimally expressed on tumor cells or in inflamed tissues, as observed in human tissue samples. Positive staining to CD137 was observed in the vessels of 32 % of malignant tumor tissue samples [100]. Analysis of human melanoma brain metastases showed the expression of CD137 on both cancer and endothelial cells [123]. Expression of CD137 at the protein level can be increased under hypoxic conditions [124]. The presence of CD137 on pathological endothelium is not specific to a particular tumor type and can be found in colon and breast tumors, both in transplanted and spontaneous models [124]. Anti-CD137 nanomedicines were not yet used for chemotherapy but promoted immunotherapy through receptor activation and increasing trafficking of T-cells to the tumor site [124]. Liposomes decorated with anti-CD137 and interleukin-2 have shown higher NP accumulation in tumor and anti-cancer activity without systematic toxic effects [125]. However, CD137 is also found in inflamed atherosclerotic lesions, suggesting that future therapies targeting CD137 should consider the potential for mistargeting tumors and side effects on the cardiovascular system [126]. Utilizing NPs decorated with CD137 agonists may not only enhance NP delivery to tumor blood vessels but also improve the cytotoxic effects of chemotherapy by stimulating immune cells.

Serial analysis of gene expression in normal and tumor endothelial cells identified several genes that are upregulated in tumor vasculature. Some of these TEMs encode cell surface proteins available for NP targeting, such as TEM1, TEM5, TEM7 and TEM8 [96]. Recent studies indicate that TEM1 (CD248) is expressed on the surface of fibroblasts and a subset of pericytes associated with tumor blood vessels, but not on the tumor endothelial cells [102]. TEM5, or the endothelial G-protein-coupled receptor (Gpr124), plays a role in embryonic development of brain blood vessels and function of blood-brain barrier [127]. Although TEMs are general markers of angiogenesis, they are not specific to tumors and can be found during embryonic development. However, in adults, cell surface TEMs are largely restricted to tumor endothelial cells and are absent in the blood vessels of healthy tissues [103]. Therefore, TEM5, TEM7 and TEM8 are potential targets for selective delivery to tumor vasculature. To the best of our knowledge, anti-TEMs NPs are not yet have been studied for targeted delivery of drugs to the tumor vessels and cancer treatment.

Matrix metalloproteinases (MMPs) are a family of both soluble and membrane-anchored enzymes, whose primary function is the cleavage of the extracellular matrix. MMPs are associated with angiogenesis, both normal and pathological, as well as with the metastatic processes [128]. MMP-2, MMP-9, and MMP-11 are overexpressed on the surface of tumor vasculature and play a crucial role in tumor angiogenesis [96,104,106]. Modification of the NP surface with MMP-sensitive polymers or encapsulating drugs within MMP-cleavable polymers enhances drug delivery to tumors through the activity of enzymes. These MMP-sensitive NP delivery systems improve the treatment of both primary tumors and metastases [129,130]. High efforts have been made to create anti-MMP-2 antibodies [131,132], which could be used to modify the NP surface for active targeting of MMP-2-positive endothelial cells.

Plasmalemma vesicle-associated protein (PLVAP) is specifically expressed on the cell surface of endothelium and participates in cardiac development in embryos. PLVAP is not found in the heart of adult mice. However, PLVAP is upregulated in abnormal endothelial cells in cancer, heart injuries, traumatic spinal cord injury and several other diseases

[107,108]. Anti-PLVAP treatment of hepatocellular carcinoma administrated through tumor-feeding artery resulted in thrombosis, blockade of tumor vasculature, reduced blood flow and tumor necrosis [133]. Conjugation of anti-PLVAP antibodies to NP surface may provide sufficient carrier accumulation in tumor vessels.

Other receptors are less specific to the tumor neo-vasculature and are also present on endothelial cells in inflamed tissues. However, several studies demonstrated the potential of using these molecular targets to improve cancer treatment with controlled and manageable side effects [134–136]. Cell adhesion molecules (CAMs) are a family of cell-cell recognition proteins found on the membranes of endothelial and immune cells [137]. Expression of several CAMs is upregulated in tumor vessels making them viable molecular targets: vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), selectins, and integrins [134,136,138]. For instance, liposomes functionalized with anti-VCAM-1 antibodies showed preferential accumulation and retention in tumor vessels, while non-modified liposomes were able to diffuse into the tumor interstitium [136]. P-selectin is an intracellular CAM that can become extracellular after activation of endothelial cells. Shamay *et al.* reported the induction of P-selectin expression in tumor vessels after X-ray radiation, followed by localized chemotherapy with fucoidan-based NPs [139].

Numerous nanomedicine cancer treatments were demonstrated with targeting by RGD (Arg-Gly-Asp) peptides, a minimal motif binding integrins [135]. RGD-decorated NPs accumulate highly in tumors, for example, in breast cancer [140] and glioma [141] models. Thermosensitive liposomes modified with the RGD motif showed high accumulation in tumor vasculature in melanoma, enhancing drug penetration into the tumor interstitium after triggered intravascular release [142]. Moreover, integrin activation with RGD motif can promote endocytosis and transport of nanoparticles. Modification of drug-loaded dendrimers with RGD-based peptides resulted in a 3-fold improvement of nanocarrier permeation in a spheroid cancer model. After systemic administration, the modified dendrimers demonstrated increased tumor vascular permeability of doxorubicin, with significant prolongation of mice survival [143].

3.1.3. Design of nanoparticles for passive targeting of tumor vasculature

Along with active targeting receptors of endothelial tumor cells, NPs can be designed to have passive accumulation at the vasculature without subsequent extravasation into malignant tissue. Tumor blood vessels have elevated viscous and geometrical resistance, which reduces blood flow compared to normal tissue [144,145]. Therefore, NPs of submicron size are delivered and retained in tumor endothelium due to enhanced shear stress. This is beneficial for the concept of drug delivery via intravascular flash drug release [21,146]. Other characteristics of NPs and blood vessels can influence NP retention in vasculature and can be used for passive tumor targeting [147].

To adhere to endothelial cells, NPs should marginate from the blood flow towards the vessel wall. Numerous experimental and theoretical studies have shown that the shape and size of NPs significantly influence their margination behavior [148]. Once injected, NPs collide with each other and with cells present in the blood, primarily erythrocytes. RBCs tend to move toward the center of blood flow, influencing the distribution of NPs within the bloodstream. Vahidkhah and Bagchi theoretically analyzed the shape-dependent behavior of submicron-sized particles (spherical, cylindrical, and discoidal) in the presence of RBCs. Discoidal particles align with the shear flow plane, leading to more frequent collisions and greater lateral drift than microspheres. Cylindrical particles align with the vorticity axis and have fewer collisions and reduced lateral drift. Once marginated, cylindrical particles are more likely to contact the wall due to their alignment and large angular fluctuations. Simulations indicate that disk-like particles have the highest probability of firm adhesion to the vessel wall, followed by elongated particles and microspheres [149].

Dynamic analysis with IVM highlights the margination-adhesion

behavior exhibited by individual particles with different shapes within the tumor vasculature [146]. Among spherical, hemispherical, and discoidal particles, the latter demonstrated a higher tendency to adhere to the blood vessel wall and exhibited longer circulation times [150]. Another study optically visualized the movement of spherical NPs with sizes from 10 to 1000 nm *in vivo*. Particles smaller than 100 nm moved uniformly with RBCs and showed limited near-wall accumulation, whereas larger particles tended to accumulate preferentially along vessel walls, with approximately 70 % of 1000-nm particles found near the endothelium [151]. Further *in vivo* tracking of discoidal and cylindrical silicon NPs using IVM revealed that discoidal particles preferentially accumulated within the tumor vasculature. Discoidal particles with 1000×400 nm size showed the highest tumor-to-liver accumulation ratio among the studied particles [146]. Thus, discoidal particles of submicron size are preferred for enhanced margination and adherence to the tumor vasculature.

Mechanical properties of NPs can significantly influence the margination and adherence to endothelium [152]. From theoretical calculations, stiff particles, i.e. those featuring higher Young's elastic moduli, have a higher probability to marginate towards the blood vessel wall than soft analogs [153]. Nevertheless, adhesion of microparticles can greatly depend on the wall shear rate in blood vessels. Fish *et al.* investigated adhesion of ICAM-1-targeted soft and stiff microgels on human endothelial monolayers under different blood flow conditions. Soft microgels demonstrated better adhesion at low wall shear rate (200 s^{-1}), while stiff microgels better bound to the endothelium at high shear rate (1000 s^{-1}) [154]. This finding led to the development of a multistep delivery system, where deformable micro-sized particles are used to deliver smaller NPs to the tumor vasculature. This system enhanced the delivery of 50-nm polystyrene NPs 30-fold in comparison to the freely injected NPs [154]. For active methods of NPs targeting, soft NPs tend to demonstrate longer interaction with endothelial cells via enhanced avidity of particles after flow-stress deformation [155].

In addition to longer retention in tumor vessels, soft nanoparticles have lower interaction with immune cells and macrophages. As a consequence, soft nanomaterials usually have longer blood circulation time than hard counterparts [156,157]. Key *et al.* compared the biodistribution of 1000×400 nm discoidal polymeric NPs with Young's modulus of 1.3 and 15 kPa [158]. Stiffer NPs showed higher deposition in Kupffer cells and along vascular walls of liver. Soft counterparts were distributed with 6-fold higher area density in vasculature of B16-F1 melanoma model [158]. Similar results were obtained by comparing biodistribution of polymeric nanogels, where liver uptake increased with the increase of Young's modulus of NPs from 80 kPa to 420 kPa [157]. Uptake by tumor cells is also lower for soft NPs. Guo *et al.* analyzed uptake of NPs with moduli varied from 45 kPa to 19 MPa and found up to a five-fold *in vitro* cellular uptake and 2.6-fold higher *in vivo* tumor uptake for soft NPs [159]. However, small soft nanoparticles were shown to permeate tumor due to lower interaction with extracellular stroma and deformability [160,161].

One of the most effective ways to target a specific site is to mimic natural solutions already existing in the body. Exosomes or extracellular vesicles are enclosed compartments with sizes up to 200 nm, produced by various cell types and used for cell-cell communication through the transport of biological molecules [162]. While exosomes can naturally recognize specific cell types, their isolation in high quantity is complicated. Thus, artificially engineered membrane-derived nanovesicles (MDNs), or cell nanoghosts, were proposed for tumor homing. These MDNs combine targeting moiety from the surface molecules of the parent cell, incorporation of cytotoxic component for cancer treatment, and reproducibility of nanovesicles production [163]. As MDNs preserve the biological function of the cell membrane, their biodistribution and blood circulation depend on the parent cell type [24]. A comparison of biodistributions of neutrophil- and erythrocyte-derived nanovesicles by means of IVM revealed that only neutrophil-derived MDNs exhibited specific accumulation in inflamed endothelium, whereas RBC-derived

did not [164]. Several other cell types can target human tumors. For instance, MDNs derived from mesenchymal stem cells demonstrated specific targeting of cancer tissue with no accumulation in human primary benign cells, owing to the natural ability of parental cells for chemotaxis and surface interactions with susceptible tumors [165]. Nanoghosts derived from cancer cell membranes have been shown to self-recognize homotypic tumors, which can be utilized for drug delivery [166].

Alongside their application as drug carriers, MDNs can be used for camouflaging NPs of different nature, including polymeric and inorganic materials [167]. Platelet membrane-coated nanogels have shown a higher accumulation at tumor site compared to non-modified NPs, with a 4-fold reduced tumor volume 16 days after the treatment. Additionally, platelet-coated NPs significantly reduced the number of metastatic nodules in lungs, while bare NPs did not show any anti-metastatic activity [168]. Cancer cell-membrane coated PLGA NPs recognized homologous tumors *in vivo* [169]. This recognition is specific to homologous pair of membrane-camouflaged NPs and cancer cells type. For instance, coating magnetic iron oxide NPs with cancer cell membranes, provided 3-fold increase in doxorubicin tumor-specific delivery by homotypic NPs, compared to heterotypic ones [170].

To increase NP accumulation in tumor vasculature their surface can be modified with neutrophil or leukocyte membranes [25,171]. Neutrophil membrane-coated polymeric PEG-PLGA NPs have shown enhanced internalization by inflamed endothelial cells *in vitro* and improved retention in both orthotopic and ectopic pancreatic tumor models. Site-specific release of celastrol from NPs significantly inhibited primary tumor growth, prolonged mice survival and minimized liver metastases [172]. In another study, decoration of discoidal silicon NPs with leukocyte membrane increased NP attachment to tumor endothelium. Modified NPs mimicked leukocytes and had longer blood circulation time and higher accumulation in inflamed tumor endothelium, as confirmed by IVM [173,174]. Incorporation of leukocyte membrane proteins into liposomes increased accumulation of NPs in breast and melanoma tumors [175,176]. Quantification from IVM images showed a 4.5-fold increase in liposomes concentration in vessel lumens and a 14-fold increased presence in vessel walls after modification. This delivery can be reduced or prevented by blocking the surface of liposomes with antibodies against LFA-1 and CD45 proteins [175]. Further progress in membrane-modified NPs could come from using hybrid cell membranes [25]. For instance, camouflaging NPs with mixed membranes from RBCs and immune cells can improve blood circulation by inhibiting phagocytosis via CD47-signal regulatory protein alpha (SIRP α) pathway, while maintaining endothelial targeting. However, the targeting of inflamed tissues can result in off-target accumulation in conditions such as atherosclerosis, stenosed vessels, and other vascular diseases [177,178]. Therefore, a thorough evaluation of the risks and benefits is essential before clinical implementation of vascular-targeted nanomedicines.

3.2. Cell-mediated nanoparticle delivery

In the EPR paradigm, many stealth NPs were designed to avoid non-target interactions during circulation. Nevertheless, various types of blood-circulating cells can enhance NP delivery to tumors [32,179]. Neutrophils, lymphocytes, and monocytes naturally migrate to inflamed tissues through chemotaxis, while infiltrating macrophages form up to half of the tumor mass at advanced stages of cancer [180]. NPs can utilize the natural routes of these cells to cross endothelial barriers, either by anchoring to their surface or via internal uptake. In addition, immune cell permeation through vascular barriers can promote delivery of non-bonded NPs by inducing micoleakages.

Other cell-targeting strategies focus on delivering NPs to affected organs. T-cells can transport drug-loaded NPs to lymphoid organs, which are difficult to access from circulation [181]. RBCs can enhance NP delivery to organs with high number of small capillaries, such as lungs and brain [33]. These approaches can increase the specificity of

metastasis treatment in selected organs. The cell-mediated “hitchhiking” of NPs can be achieved through *ex vivo* loading but can also occur through *in situ* interactions within the bloodstream, influencing delivery of non-stealth nanomaterials (Fig. 4A) [30,182]. The approaches promoting assembly of NPs with cells are summarized in Fig. 4B, C.

IVM has become a valuable tool to study interactions of NPs with circulating blood cells and their subsequent transport [32,179,183]. With IVM, the insights on the uptake of NPs by phagocytes in circulation and at the endothelial interface were provided, in particular for neutrophils [32] and monocytes [183]. IVM proved feasibility for tracking the dynamics of the NP transport bound to neutrophils via diapedesis. This mechanism has emerged as critical for permeation of a wide variety of nanomaterials into tumor [184,185]. Methods of intravital fluorescence imaging are evolving to address the migration of phagocytes in challenging environments, such as respiratory system, where motion artifacts can hinder clear imaging [186]. As a result, IVM currently emerges as a main method unlocking the potential of cell-mediated NP delivery for improved cancer therapies.

3.2.1. Delivery with neutrophils

Neutrophils are the major population of circulating leukocytes (50–70 %) and are the first responders to inflammation or injury. About 10^{11} neutrophils are produced in bone marrow daily and then cleared with a short lifespan of 7–20 h primarily by the bone marrow and liver [188]. In certain tumor types, neutrophils are a major infiltrating immune cell population, playing a key role in acute inflammation. Their recruitment is driven by the expression of CXC chemokines (e.g., CXCL5, CXCL2, CXCL6), which mediate neutrophil migration through a process known as the extravasation cascade (Fig. 4E) [189]. This begins when circulating neutrophils are captured by endothelial E- and P-selectins, which interact with glycoprotein ligands on the neutrophil surface (e.g., P-selectin glycoprotein ligand-1, CD44, E-selectin ligand-1). Neutrophils then roll along the endothelial wall until activated β 2 integrins on their surface form stable bonds with ICAM-1 glycoproteins on the endothelium, leading to deceleration and strong adhesion. Once adhered, neutrophils extravasate into the interstitial space either by rearranging their filamentous actin to form protrusions or by crossing the endothelium via diapedesis. These protrusions respond to chemotactic gradients, allowing neutrophils to navigate through the endothelium, pericytes, and basement membrane to reach the interstitium [190].

Building on their role in inflammation and tumor infiltration, neutrophils demonstrated the ability to enhance NP delivery. They can induce leakages in the endothelial wall during transvascular migration, facilitating the exit of NPs from circulation. This mechanism has been shown across different human and murine tumor models, with treatment efficiency positively correlated to neutrophil quantity in the peritumoral vasculature [32]. Migrating neutrophils are also capable of breaching the tumor vascular basement membrane surrounding the endothelium, enabling deeper NP permeation into tumors [184].

Neutrophils can sequester any systemically administered nanomaterials, as was shown with 500-nm polystyrene particles, polylactic-co-glycolic acid (PLGA) NPs of 100 and 260 nm sizes, 150-nm PEGylated liposomes, and 35-nm magnetic NPs [185,191–193]. This association alters the pharmacokinetic properties of both neutrophils and NPs and works as a double-edged sword. First, it reduces the adhesion of neutrophils to the endothelium and reroutes part of NPs to the liver [191,194]. This feature was applied to mitigate fast infiltration of neutrophils and therefore prevent acute inflammation in tissues [194]. On the other hand, neutrophils and other marginating leukocytes can act as reservoirs transporting NPs from vasculature to tumor interstitium. The example of NP uptake and extravasation to tumor *in vivo* is shown in Fig. 4F. Targeting activated neutrophils with anti-CD11b antibodies 35-fold increased tumor delivery of gold nanorods compared to PEGylated nanorods [195]. Targeting neutrophils can promote their activation, where the antitumor N1 phenotype is advantageous for cancer treatment, as it enhances the release of primary granules with

myeloperoxidase (MPO). MPO release remained unchanged when neutrophils were loaded with anti-CD11b-functionalized 240-nm spheric polymeric NPs [196], but increased significantly when larger disk-shaped microparticles were attached (6 μ m diameter, 625 nm thickness). The mechanism of activation is mediated by providing cells with surface for adhesion and for fragment antigen-binding region (Fab) mediated cross-linking of neutrophil's receptors.

Attachment of NPs onto neutrophils *ex vivo* remains an attractive idea but presents challenges due to their short lifespan, potential changes of their adhesion and activation, and adverse effects associated with their transfusion. Large-scale neutrophil extraction can lead to neutropenia syndrome in donor, characterized by a cytokine storm and increased susceptibility to lung infections, while transfusion may cause hypoxia [197]. Nevertheless, cancer treatment with chemotherapeutic NPs (silica [198] and liposomes [199]) loaded into neutrophils was shown to be significantly improved compared to standard neutrophil infusion or NP administration in mice. In these studies, neutrophils released NPs within the tumor interstitium. This process was concomitant with the release of neutrophil extracellular traps, an anti-microbial extracellular fiber composed primarily of DNA and globular proteins.

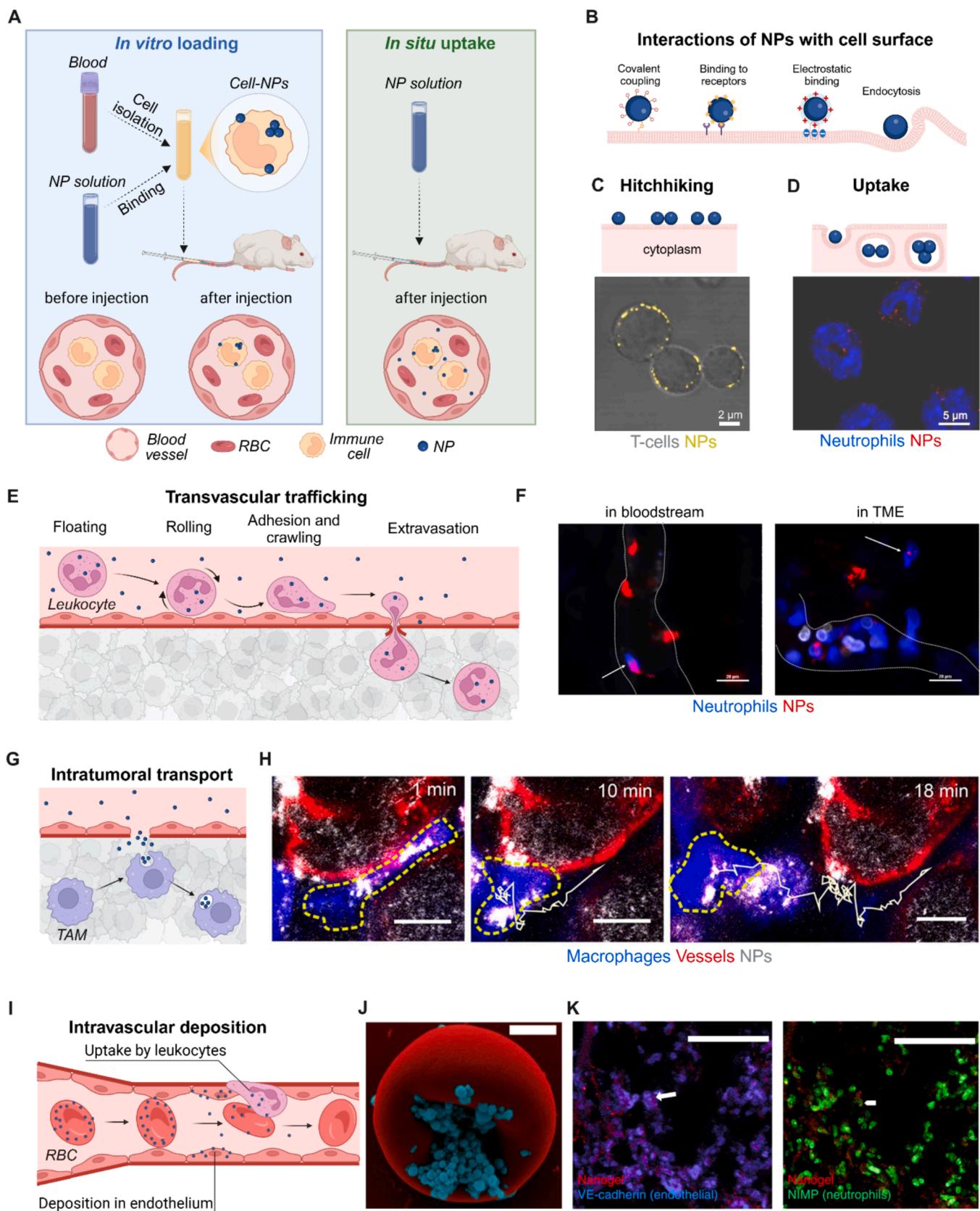
3.2.2. Delivery with monocytes and macrophages

Monocytes circulating in the blood are key players of the innate immune system, differentiating into macrophages and dendritic cells. Their transport presents both challenges and opportunities for NP-based drug delivery. Monocyte-derived macrophages rapidly sequester NPs from the bloodstream into tissues of the mononuclear phagocyte system (MPS). To address this, strategies such as stealth coating of NPs or MPS blockade have been developed to improve NP delivery in tumors [49,200]. Nevertheless, monocytes can also be recruited to sites of inflammation in tumor, where they differentiate into TAMs. This opens a pathway for NPs to reach tumor tissues by “hitchhiking” on these cells [201].

Deprez *et al.* reported that myeloid-cell-mediated transport accounts for 50–60 % of the delivery of lipid nanocarriers to inflamed tissue. Importantly, PEGylation of liposomes decreased the uptake by MPS cells, but the longer blood circulation time favored the uptake by myeloid cells [193]. Another study by Garanina *et al.* showed that liposomal and polymeric NPs were found in 80 % of circulating monocytes, highlighting the importance of these cells in interaction with NPs. Huang *et al.* advanced monocyte targeting via the design of a series of monocyte-binding aptamers anchored at lipid-based nanocarriers. The aptamer functionalization significantly improved NP delivery in an orthotopic pancreatic cancer model, characterized by high monocyte infiltration [202]. Kuang *et al.* developed MMP-2 peptide-liposomes loaded with doxorubicin and modified with lipoteichoic acid, a molecule recognized by CD14 and toll-like receptor 2 (TLR2) on monocytes. These NPs successfully reached glioblastomas after systemic administration by hitchhiking on monocytes [203].

Monocyte differentiation into TAMs presents another target for drug delivery. Miller *et al.* conducted high-resolution microscopic imaging in tumor-bearing mice with dorsal window chamber to show that TAMs can act as long-term drug reservoirs in tumors [80]. They separately monitored NPs, carrying platinum payload, and DNA damage at the single-cell level and observed TAMs sequestration of the Pt followed by its release into neighboring tumor cells. TAMs depletion significantly reduced both NP accumulation within tumor and their effectiveness [80]. The *in vivo* microscopic visualization of TAMs in this study was achieved by using Cx3cr1^{GFP/+} reporter mice, where expression of GFP-labeled fractalkine receptor (CX3CR1) indicated the population of the host leukocytes on site [204]. Since TAMs could not be identified from other CX3CR1-positive cells, these conclusions were made by synergizing the IVM data with immunofluorescence histology and flow cytometry for macrophage-specific markers.

IVM also demonstrated that TAMs can actively transport NPs from stromal cells into the tumor interstitium (Fig. 4G, H) [29]. Lin *et al.*



(caption on next page)

Fig. 4. Blood cells and immune cells transport nanoparticles. (A) NPs can be attached to specific isolated cells *in vitro* or can interact with them during circulation in blood. (B) The main types of interactions of NPs with the cell membrane. (C,D) NPs can be anchored to the surface of blood cells and hitchhike on them (C) or can be transported inside cells after the uptake (D). Bottom: optical imaging of both transport modes on immune blood cells, adapted with permission from ref. [187] and ref. [185]. (E) Mechanism of extravascular transport of captured NPs by circulating leukocytes. (F) Interaction of 100 nm PLGA NPs (red) with neutrophils (Ly6G, blue) inside the blood vessel (dotted line) and transport of NPs to the tumor microenvironment (TME). Scale bars are 20 μm . Adapted with permission from ref. [185]. (G) Tumor-associated macrophages (TAMs) capture NPs in tumor stroma and redistribute them in microenvironment. (H) IVM images showing the uptake and migration of NPs (grey) captured by macrophages (blue) from the blood vessel (red) into the tumor over time. Scale bars are 10 μm . Adapted with permission from ref. [29]. (I) NPs, hitchhiked on RBCs, can detach at the endothelial wall of capillaries due to shear stress and be endocytosed by endothelial cells. Also, NPs can be transferred from RBC to marginated leukocytes. (J) Electron microscopy image of nanogel adsorption on RBCs. Scale bar is 1 μm . (K) Micrographs of lungs after treatment with nanogel-loaded RBCs. Nanogels (red) are absorbed by pulmonary capillary endothelial cells (VE-cadherin, blue) and resident leukocytes (NIMP, green). Scale bars are 100 μm . Images from (J) and (K) are adapted with permission from ref. [33]. Graphic schemes are created with BioRender.com.

observed that passive diffusion of NPs is limited due to interaction with extracellular matrix and stromal cells. Nevertheless, NPs can be engulfed by TAMs and redistributed at 2–5 farther distances away from tumor vessels, compared to passive diffusion [29]. TAMs-associated transport was size-dependent and enhanced with an increase of NP size from 15 to 100 nm. It is likely that tumor-associated monocytes demonstrate similar mechanisms of intratumoral transport during the infiltration process, as they have higher mobility than differentiated macrophages [205].

Monocyte- and macrophage-mediated NP delivery is a promising approach for boosting efficiency of targeted cancer nanotherapy. However, these strategies depend on tumor's ability to recruit myeloid cells and the viability of transport cells. After uptake of NPs by macrophages, they are processed into endosomes and subsequently into lysosomes for degradation, where the payload can be released and may damage host cells, limiting their movement. Therefore, these transport methods are more appropriate for delivering pro-drugs or NPs activatable by external stimuli.

3.2.3. Delivery with lymphocytes

The high heterogeneity of the EPR effect makes the treatment of highly disseminated diseases and tumors with poor angiogenesis challenging. One such tumor type is lymphoma, which spreads to numerous tissues through the lymphatic system. Lymphocytes demonstrate a physically optimized amoeboid migration that enables flexible transport, and a homing process regulated by adhesion molecules and chemokines [206]. Therefore, healthy lymphocytes, such as T-cells, can serve as vehicles for NPs targeting lymphoma tumors, mimicking the behavior of cancer cells. Huang *et al.* expanded autologous polyclonal T-cells *ex vivo* under conditions that promoted their homing to lymphoid organs and attached NPs to their surface. This resulted in a 100-fold increase in the concentration of NPs in lymph nodes compared to standard intravenous administration [181]. The anti-lymphoma drug SN-38, loaded into these NPs, accumulated in the lymph nodes 90-fold more efficiently than the free drug, even when the latter was administered at a 10-fold higher dose. This significant increase in drug transport was also observed in other lymphoid organs, such as spleen and bone marrow, providing better targeting of disseminated lymphoma sites [181].

T-cells have shown promise in delivering nanotherapeutics to a variety of other tumor types. Lipid NPs loaded with an interleukin-15 super-agonist complex (IL-15Sa) and interleukin-21 (IL-21) were conjugated to CD8 + T-cells, improving drug delivery to melanoma in mice [187]. This strategy resulted not only in the transport of NPs to tumors but also in a 10-fold increase in T-cell expansion and enhanced tumor eradication triggered by the release of bioactive cytokines. Notably, the attachment of NPs to the cells did not affect their proliferation or other key cellular functions, preserving therapeutic potential of the T-cells [187]. Tang *et al.* advanced this strategy by attaching nanogels to T-cells through the CD45 receptor [207]. These nanogels selectively released their drug cargo in response to T-cell receptor (TCR) activation, which occurs when the T-cells recognize antigens in the TME. This method allowed for at least 8-fold higher cytokine doses to be delivered in tumors without toxicity. IL-15Sa drug release from nanogels induced 16-fold greater expansion of T-cells compared to systemic administration

of free cytokine, and improved therapy of murine melanoma allografts and human glioblastoma xenografts. Considering advances of chimeric antigen receptor T-cell (CAR-T) therapy in humans [208], NP transport with T-cells presents an innovative approach capable of synergizing different modes for cancer treatment.

3.2.4. Delivery by red blood cells

RBCs are flexible vehicles with a long circulation time (~120 days in humans), which have long been used for improving pharmacokinetics of drugs, biomolecules and NPs [209]. Unlike immune cells, RBCs do not typically extravasate through endothelial walls or adhere to them. However, the high deformability of RBCs enables them to pass through capillaries smaller than their own diameter (~ 6–7 μm). This gives an opportunity to transport NPs to endothelial walls in certain organs by detachment of particles under shear stress during erythrocyte movement (Fig. 4I) [210].

The feasibility of erythrocytes as shuttles to the endothelium was first demonstrated by groups of Mitragotri and Muzykantov, where intravenous infusion of RBCs with non-covalently anchored polystyrene NPs led to more than a 7-fold increase in NP delivery to lungs [211]. A 40-fold enhancement in lung delivery was further observed for liposomes after optimization of the loading protocols on RBCs [33]. Notably, Brenner *et al.* revealed that adsorption of nanogels onto RBCs facilitates the delivery of these particles to both endothelial cells and lymphocytes in inflamed lungs (Fig. 4J, K) [33]. The enhanced accumulation of NPs in lungs is also physiologically favored by large cardiac output, extensive endothelial surface area (25 % – 30 % of the total endothelial surface in the body), and a slow blood flow relative to systemic circulation [210].

This delivery concept is promising for treating pulmonary metastases, where cancer cells arrest and disseminate near the endothelium. Triggering rapid payload release from NPs can induce diffusion-driven drug permeation through the endothelium. Two different groups demonstrated treatment or prevention of multiple melanoma metastases in lungs via RBC-hitchhiking [212,213]. Erythrocyte-mediated delivery of 100-nm chitosan-coated particles loaded with doxorubicin increased lung accumulation of NPs up to 41 % of the injected dose. Doxorubicin release induces a 3-fold decrease in the total number of pulmonary metastatic nodes and a significant decrease in the average size of the metastases [212]. Similarly, doxorubicin-loaded polymeric NPs assembled on the surface of RBCs demonstrated prolonged blood circulation time and 10-fold higher drug accumulation in the lungs compared to injection of non-bound nanoparticles. RBC-mediated delivery substantially inhibited tumor growth and improved survival outcomes in both early- and late-stage melanoma models of pulmonary metastases [213].

Erythrocytes can interact with certain NPs inside the blood vessel and transport them to non-lung tumor types. Zhao *et al.* designed a library of zwitterionic liposomes containing tertiary amine oxides. These liposomes showed reversible binding to circulating RBCs through interactions with hydrophilic heads of membrane phospholipids and detachment near the tumor site [214]. Two types of these carriers demonstrated the infiltration of tumor via transcytosis through rapid micropinocytosis and endoplasmic reticulum/Golgi-involved exocytosis of NPs. The delivery of anticancer drugs in zwitterionic micelles

improves treatment of both human orthotopic HepG2 xenografts and patient-derived tumors in mice [215]. This strategy illustrates how RBC hitchhiking can be adapted to target a broader range of cancers beyond pulmonary metastases.

3.3. Transendothelial permeation of nanoparticles

3.3.1. Vascular eruptions

Disorganization of peritumoral vessels with the formation of static gaps between endothelial cells with incomplete pericyte coverage was considered the primary route for NP transport in the classical EPR paradigm. One of the key oversights in this early understanding of the EPR mechanism was the failure to account for its dynamic nature. This aspect was highlighted in 2016 when Matsumoto *et al.* demonstrated that, alongside static pores, some endothelial openings exhibit a stochastic and transient behavior (Fig. 5A) [52]. These time-limited dynamic pores can randomly open across tumor vessels, allowing NPs to erupt deeply into the tumor interstitium, driven by pressure gradient. The escape of NPs through these pores creates a plume-shaped front in the perivascular space, which then spreads over the interstitial space as the pore closes. Using IVM, the progression of these eruptions was visualized, showing bright single events in sub-micrometer endothelial openings that later diffused across areas larger than $1000 \mu\text{m}^2$ (Fig. 5B) [216]. These vascular eruptions were observed in mice bearing pancreatic human BxPC3 adenocarcinoma xenografts and 4 T1 mammary carcinoma allografts [217–219].

The effect of dynamic pores on NP extravasation becomes more pronounced with the increase of particle size. Fluorescent NPs of 70-nm diameter generated larger and longer-lived eruption plumes compared to 30-nm NPs [52]. Through a combination of mathematical modeling and *in vivo* imaging of breast cancer tumors, it was concluded that these dynamic pores can reach diameters of at least 625 nm, allowing the effective passage of sub-300 nm particles and even platelets [218]. Dynamic pores coexist with static pores in the tumor-surrounding vasculature, but vascular eruptions were identified as the primary mechanism responsible for the extravasation of clinically approved liposomal nanomedicine Doxil (85 nm) [52]. This highlights the need to rethink and refine NP design strategies that have traditionally been based on the static EPR model.

Studies on murine breast cancer models have confirmed that dynamic pores occur more frequently in vessels located within or adjacent to tumor tissues compared to distant healthy regions. Moreover, formation of dynamic pores can be stimulated by damaging endothelium (Fig. 5C). Naumenko *et al.* described two patterns of NP extravasation through the dynamic pores [32], classified by the degree of NP leakage (Fig. 5D–F). Microleakage, or local deposition of NPs in perivascular space, was found in both cancer and healthy tissues. On the other hand, macroleakages were observed in malignant tissues, characterized by deep penetration of NPs in tissue through the basement membrane. The distribution of leakages was heterogeneous between different tumor types. Few eruptions were observed for human prostate cancer 22Rv1, while an increase of macroleakages was found for orthotopic breast 4 T1

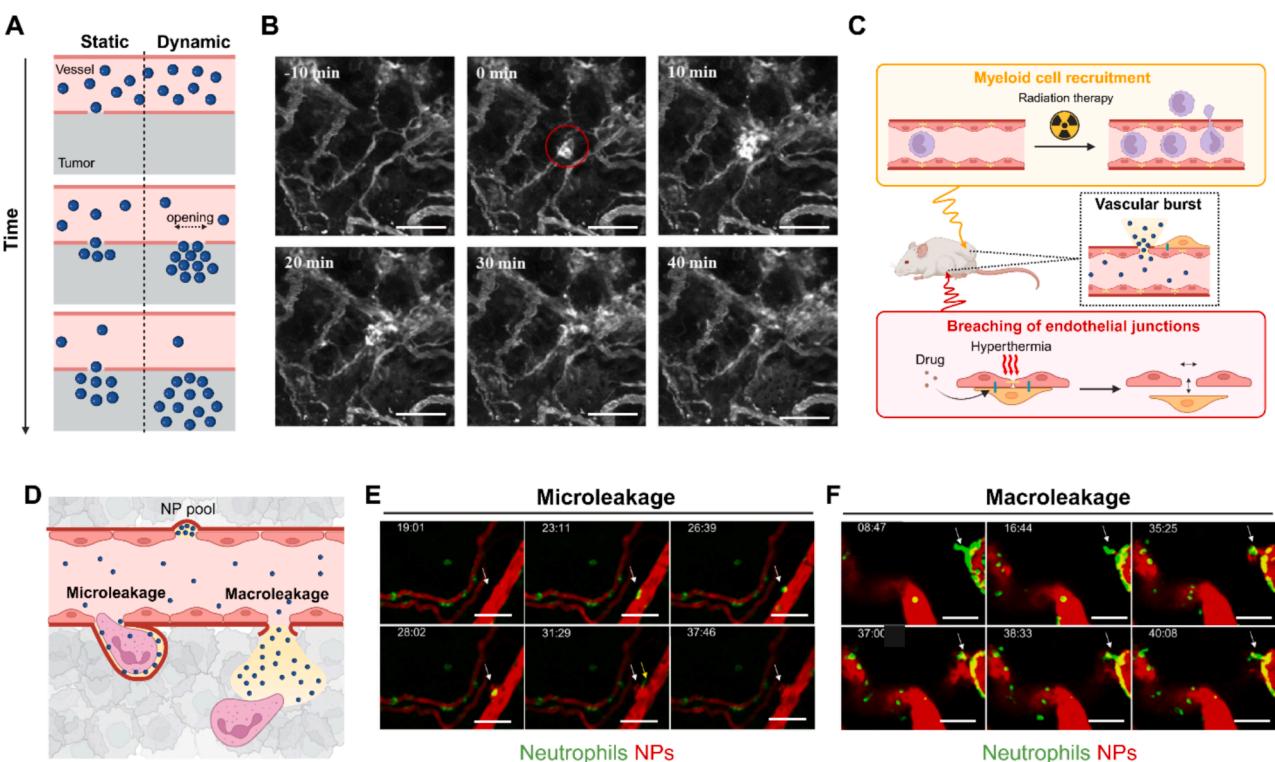


Fig. 5. Dynamic vascular openings participate in NP transport. (A) Illustration of the NP extravasation through static and dynamic pores to the tumor interstitium. Static pores are constantly open for NP transport. Dynamic pores are short-living wide openings in the endothelium, allowing the extravasation of large quantities of nanoparticles. (B) Time-lapse images of vascular burst (red circle) generated by 70-nm polymeric micelles (grey) in BxPC3 human xenografts. Scale bars are $100 \mu\text{m}$. Adapted with permission from ref. [216]. (C) Approaches for stimulation of vascular bursts. Local radiation therapy promotes the recruitment of myeloid cells, such as monocytes and neutrophils, to the tumor site. The extravascular migration of cells is associated with NP eruptions. Endothelial cell-cell junctions can be breached by local hyperthermia, while the detachment of pericytes can be achieved by inhibiting growth factors. (D) Illustration of micro- and macro-leakages of NPs promoted by the extravasation of immune cells. Agglomerates of NPs are distributed along the vessel wall in intraendothelial pores and cannot diffuse deeply into tumor due to basement membrane barrier. In micoleakage, phagocytes cross NP pools but remain in the perivascular zone. Macroleakages occur when extravasating leukocytes break the basement membrane, promoting NP release into the interstitium. (E,F) The time-lapse images of phagocyte-mediated leakages of liposomes (red) in mouse tumor after neutrophil extravasation (Ly6G, green). Scale bars are $50 \mu\text{m}$. Arrow points to migrating neutrophils. Adapted with permission from ref. [32]. Partially created with BioRender.com.

cancer, compared to ectopic model. Moreover, even different blood vessels within one tumor demonstrated heterogeneous extravasation of NPs.

Several studies closely linked dynamic pore formation with the damage in the basement membrane and the recruitment of immune cells to the tumor site. The infiltration of neutrophils and monocytes into the tumor has been shown to drive NP leakage, as these cells induce temporary disruptions in the vasculature [32,217]. Miller *et al.* demonstrated that low-dose radiation therapy, known to promote macrophage recruitment, can trigger vascular bursts and enhance tumor uptake of NPs ranging from 5 to 110 nm [219]. Moreover, the frequency and the area of transient openings can be further increased by inhibiting TGF β , which disrupts intercellular junctions between endothelial cells and pericytes, facilitating nanoparticle extravasation [216]. Wang *et al.* demonstrated that NP eruptions could result from the release of accumulated pools in the space between endothelial cells and the basement membrane. By inducing local hyperthermia in the tumor, they disrupted vascular endothelial (VE)-cadherin junctions, leading to accumulation of NPs at the basement membrane. The following eruptions were associated with neutrophil migration through the endothelial wall [184]. Nevertheless, some reports did not identify a clear correlation between immune cell presence in vasculature and NP eruption. For instance, Hoechst staining of neutrophils in BxPC3 pancreatic cancer model has revealed that NP eruptions can occur without cells nearby [52].

3.3.2. Transcytosis through tumor blood vessels

Transcytosis, an active and dynamic cellular process, enables the transport of NPs across the endothelium. This mechanism involves the internalization of NPs at the apical side, followed by their transport across the cell and their eventual release into the tumor interstitium via exocytosis or diffusion [220]. Unlike passive delivery mechanisms like

the EPR effect, transcytosis leverages cellular machinery, allowing NPs to navigate through various endothelial features, such as intracellular vesicles, vesicular-vacuolar organelles and thin fenestrae (Fig. 6A) [20]. Sindhwan *et al.* quantified the density of intracellular and intercellular gaps, and fenestrae across blood vessel perimeters in U87-MG glioblastoma xenografts. They found that gaps were relatively rare events, with only 1.1 gaps detected per millimeter of vessel length, and that only 27 % of them were classical intercellular channels typically associated with the EPR effect. Vacuoles and fenestrae were observed at densities of 111 and 24 per millimeter, respectively. The frequency of different endothelial features was similar in mouse models and in clinical samples of human cancer (Fig. 6B), while tumor vessels of cancer patients express proteins associated with transcytosis [19]. This study highlights the significant role of transcytosis in NP transport.

IVM allows the capture of the dynamic aspect of NP extravasation by analyzing NP signal colocalization with endothelial cells. Bright fluorescent spots from gold NPs were observed by microscopy in vicinity of endothelial cells, described as different stages of transcytosis [19]. A similar IVM pattern was observed for tumor permeation of albumin-coated NPs [221] and single-walled carbon nanotubes [222]. Transcytosis can account for a substantial portion of NP transport. Sindhwan *et al.* developed “Zombie” animal model obtained through whole-body paraformaldehyde fixation of blood vessels. The accumulation of NPs in this model can be only due to passive pathways, such as gaps, and accounts for only 3–25 % of NP accumulation, dependent on NP size. However, rational criticism exists regarding potential overestimation of transcytosis contribution in “Zombie” model due to unexamined impacts on pore structure by fixation methods [223]. Nevertheless, 3D microscopy demonstrated that gold NPs remained localized in the fixated tumor vessels, while clear active extravasation of NPs was observed in naïve mice.

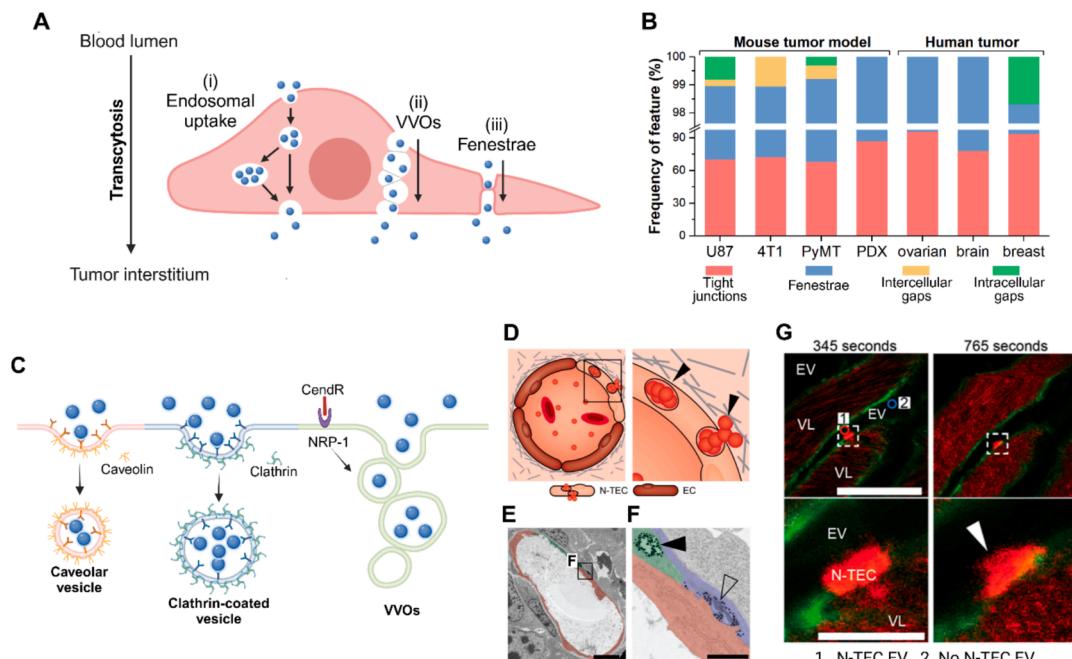


Fig. 6. Transcytosis through the endothelium in NP delivery. (A) Main pathways for transcellular transport of NPs through tumor endothelial cells: (i) recycling of captured NPs from endosomes to the extracellular environment, (ii) migration within vesiculo-vacuolar organelles (VVOs), and (iii) permeation through fenestrae. (B) Relative fraction of permeation-determining features of tumor endothelium (tight junctions, fenestrae, intercellular and intracellular gaps) in murine tumor models and clinical tumor samples. Data reexamined from ref. [224]. (C) Main vesicular uptake pathways mediating transcytosis of NPs. Formation of vesicles is triggered by specific receptors on the cell surface. (D-F) Special nanoparticle transport endothelial cells (N-TECs) responsible for NP transport: scheme of NP delivery (D) and corresponding transmission electron micrographs of a tumor blood vessel (E,F). N-TECs are marked in green, endothelial cells in red, basement membrane in blue. A solid black arrow indicates NP clusters in vacuoles of N-TECs, an open arrow shows NPs in the extravascular space at basolateral side. Scale bars are 5 μ m (E) and 1 μ m (F). (G) IVM imaging of gold NP transport through N-TECs. Tumor blood vessels are pseudocolored in green, NPs in red. VL – vessel lumen. EV – extravascular space. The NP-associated plum moves toward the EV space with time. Scale bars are 50 μ m (top) and 10 μ m (bottom). Images in (D-G) adapted with permission from ref. [224]. Partially created with BioRender.com.

Several mechanisms can induce transcytosis of NPs (Fig. 6C). Caveolae-mediated uptake is considered one of the primary transcytosis initiators for macromolecules. This pathway is particularly active for NPs presenting albumin on their surface, as albumin binds with caveolar glycoprotein receptors (gp60 and gp90) on endothelial cells, facilitating their transport toward the basolateral side [225–227]. Nevertheless, caveolar-mediated endocytosis is also reported to take place for polystyrene and metal NPs [227,228], as well as for PEGylated liposomes [229]. Despite the estimated upper physical diameter of caveolae vesicles is 80 nm, NPs even larger than 200 nm have been reported to follow caveolae-dependent uptake [230]. Clathrin-mediated endocytosis has been identified responsible for the uptake of certain NP types, especially particles functionalized with ligands for transferrin or leptin receptors [231–233]. Liposomes functionalized with antibody against transferrin receptor allow transport of NPs across the blood–brain barrier [231]. The vesicles formed in clathrin-mediated uptake are typically 80–100 nm in diameter, but there is a variability in their size since polystyrene particles larger 500 nm have been reported to internalize through a clathrin-dependent mechanism [234]. Other receptor-dependent and independent endocytosis mechanisms can also play a role in vascular transcytosis. PEGylated liposomes with engineered transcytosis-targeted peptide underwent transfer through monolayers of brain-derived endothelial cells via lipid raft-mediated endocytosis [235]. Endocytosis modulated by ICAM-1 was reported in vascular endothelial cells for targeted NPs [236]. Lin *et al.* reported that NPs can interact with increased concentrations of platelet factor 4 in injured blood vessels to induce uptake into endothelial cells [237]. Further studies required to link transcytosis in tumor endothelial cells with certain uptake mechanisms.

Transendothelial transport of NPs *in vivo* predominantly occurs within a specialized subpopulation of endothelial cells in tumor vessels, known as NP transport endothelial cells (N-TECs) (Fig. 6D) [224]. These N-TECs present only 21 % of tumor endothelial cells located on a small number of vessels. They exhibit elevated levels of caveolin-1 and clathrin-related gene expression, which are associated with the enhanced accumulation of nanoparticles. Their involvement in NP transcytosis was confirmed both by transmission electron microscopy (Fig. 6E, F) and IVM (Fig. 6G).

An alternative transcytosis pathway involves trafficking NPs through the network of vesiculo-vacuolar organelles (VVOs) of tumor endothelial cells [20]. VVOs appear as cytoplasmic grape-like clusters, which can form a connection between luminal and abluminal cell sides. VVOs have been observed at least in the ovarian (MOT) and mammary (TA3/St) murine cancer models [238], as well as in patient-derived pancreatic cancer xenografts [239]. Dvorak and colleagues accounted the VVOs as the primary promoters of hyperpermeability of tumor vessels, although their findings of VVO presence in endothelium were reported only in limited solid and ascites tumor models in mice and guinea pigs [240]. Liu *et al.* demonstrated that stimulating tumor endothelial cells with the iRGD (CRGD[K/R]GP[D/E]C) peptide led to a significant increase in VVO formation within 1 day in human pancreatic cancer models in mice [239]. This peptide indirectly targeted the NRP-1 receptor via C-terminal CendR motif, resulting from iRGD cleavage upon binding to the integrins on the tumor capillary endothelial surface, and eventually triggered transcytosis through VVOs [241]. Moreover, co-administration of iRGD with 130-nm lipid-coated silica NPs resulted in a 4-fold increase in NP uptake in tumors [239]. This highlights the critical role that VVO-mediated transcytosis may play in enhancing NP delivery.

Modification of surface properties of NPs can improve their transcytosis. Zhou *et al.* demonstrated that presentation of cationic moieties on zwitterionic micelles enhanced caveolae-mediated endocytosis by promoting interactions with endothelial cells [215,242]. Poly(2-(N-oxide-N,N-diethylamino)ethyl methacrylate) (OPDEA) micelles, conjugated to the anticancer drug SN38, showed tumor accumulation driven by transcytosis. IVM revealed that micellar OPDEA-SN38 formulation

exhibited faster systemic clearance than PEGylated micelles, but rapidly extravasated in the vascular endothelium and accumulated 2-fold greater in tumor tissue [215]. Similarly, Wang *et al.* demonstrated that cationic lipid NPs exhibited the greatest ability to traverse matrix gels, as observed in endothelial (HUVEC) and breast cancer (MDA-MB-231) cell models, compared to neutral and anionic analogs. This enhanced translocation of NPs correlated with their better therapeutic outcomes in models of hyperpermeable, poorly permeable, and metastatic tumors [243]. Another strategy to improve delivery by transcytosis is to provide albumin moieties on the surface of NPs. Wei *et al.* observed that micelles coated with human serum albumin (HSA) achieved 7.2-fold higher intratumoral delivery compared to uncoated micelles [221]. IVM demonstrated that HSA-mediated transport was accompanied by presence of multiple focal spots in the tumor interstitium, suggesting that NPs are delivered by transcytosis route.

Another strategy to improve transcytosis is through the regulation of angiogenesis. Yamamoto *et al.* provided unexpected evidence that silencing the vascular endothelial cell growth factor receptor 2 (VEGFR2) in hypervasculature tumors significantly enhanced the uptake of 100-nm liposomes [244–246]. These findings were counter-intuitive because VEGF is a key protein in cancer angiogenesis, and its knock-down is accompanied with the maturation of the tumor vasculature, including increased pericyte coverage and increased endothelial cell adhesion [246]. Nevertheless, such treatment increased the expression of caveolin-1 protein in tumor endothelial cells, while knocking down the caveolin-1 alongside with VEGFR2 abolished the enhanced accumulation of liposomes [245]. This supports the dominant role of caveolae-mediated transcytosis in NP transport through mature vasculature. However, the approach of VEGFR2 silencing for transcytosis improvement might be effective only in specific types of “vessel-rich” cancers, while tumors with a stromal vessel type, such as colorectal and breast cancer, may not respond to this therapy [247].

4. IVM development for nanoparticle transport analysis

A broad toolkit of optical microscopy techniques is available to analyze the TME, visualize NP transport, and study their interactions with various cells in the living body. A detailed discussion of these techniques is beyond the scope of this review, as they have been extensively reviewed elsewhere [15,248]. However, in this section, we will survey the main achievements in the development of IVM, which boosted progress in the field of NP delivery, and highlight further steps required for the development of this area.

In the pioneering paper describing the EPR effect, one of the proofs was optical analysis of accumulation of Evans blue-albumin complex in intradermal tumor compared to skin in mice [5]. Post-mortem analysis provides limited information about processes involved in NP delivery. In contrast, IVM methods enable the capture dynamics of the accumulation and assess its heterogeneity by analyzing different tumor regions. In 1993, IVM was used to observe higher extravascular accumulation of 80-nm PEGylated liposomes compared to conventional liposomes in rat mammary adenocarcinoma [17]. Yuan *et al.* established a 400-nm cut-off size for effective extravasation of liposomes in LS174T human xenografts [9]. Over the past decade, IVM has demonstrated its feasibility for multiplex monitoring of the transport of NPs and their molecular payloads in relation to various cell populations present in the TME [15].

Despite this potential, high optical scattering and absorption of biological tissues limit the IVM technique to visualizing only the surface tumor vasculature. Multiphoton microscopy provides greater penetration depth by relying on non-linear optical effects and pulsed near-infrared (NIR) excitation (700–1300 nm). NIR light has reduced scattering and absorption in biological tissues compared to light with shorter wavelengths, while non-linear absorption enables narrow localization of emission at the focal plane [249]. A multiphoton microscope excites fluorophores only within the focus volume, reducing photodamage and allowing for the acquisition of optical slices without the use of confocal

pinholes. The combination of multiphoton microscopy and NPs that absorb and emit light in the short-wave infrared region can further increase imaging depth to almost 1 mm [40,250,251]. Such nanomaterials in most cases cannot be used as drug carriers by themselves but can serve as companions to access tumor permeability.

IVM analysis of deep-seated tumors benefits from introduction of imaging windows. The dorsal window chamber technique involves surgically implanting a titanium frame to support a transparent window for microscopic probing of subcutaneous tumors and internal organs [252]. This technology enables high-resolution analysis of NP transport from the same vessels over extended periods but can be impractical for accommodating growing tumors and may obstruct perfusion of the tissues. Moreover, the mechanotransductive effects of the window interface can influence tumorigenesis in these models [253]. The substitution of the glass interface with flexible silicon membranes has recently been proposed to perform microscopy of rapidly growing soft tissues [254]. Perfusion window chambers incorporate microchannels for drug injection and enable IVM studies of drug transport after local administration in tumor surroundings [255]. Alternatively, optical tissue clearing techniques offer an attractive pathway for microscopic imaging without mechanical perturbation. Recently, it was demonstrated that the injection of tartrazine dye can significantly reduce light scattering of lipids, allowing the observation of abdominal organs without the need for imaging chambers [256]. This and other tissue clearing techniques [257] hold promise to improve analysis of NP transport in a minimally disturbed microenvironment.

The challenge posed to IVM as a pre-clinical cancer imaging modality is the extension of its applicability to probing orthotopic tumors. IVM-aided cancer research is predominantly focused on subcutaneous tumors, as their superficial localization facilitates clear imaging with conventional single-photon microscopy systems and imaging chambers. Orthotopic models provide a more reliable landscape for pre-clinical NP delivery evaluation, as their microenvironment is native in vascularity, cellular network and extracellular matrix structure. Some orthotopic tumors can be accessed by developing organ-specific imaging windows [258] and improving the signal-to-noise ratio of microscopy [259]. For example, intravital two-photon microscopy with a transparent cranial window has been used to analyze NP delivery in brain gliomas after TME remodeling [260]. To name a few, pancreatic, liver, and breast tumors can be accessed orthotopically using IVM and mechanisms of NP delivery can differ significantly from those observed in ectopic tumors.

Complex dynamics of NP interaction with cells *in vivo* can be elucidated by advanced fluorescence imaging tools. Standard fluorescence imaging can be enhanced by fluorescence lifetime imaging microscopy (FLIM). FLIM provides information on the interaction of fluorophores with surrounding environment based on the changes in their fluorescence lifetime [261]. A fluorophore's lifetime can be significantly affected by Förster resonance energy transfer (FRET), where the lifetime of an energy donor is shortened in proximity to an acceptor. For example, intravital FRET-assisted FLIM allowed longitudinal imaging of doxorubicin intercalation into GFP-tagged chromatin of cancer cells in tumor nodules in mouse peritoneum [262]. Similarly, FLIM can indicate localization of NPs and their payloads at subcellular level. Furthermore, FLIM can distinguish between released and encapsulated payloads in tumors. For instance, the fluorescence lifetime of free porphyrazine molecules is approximately four times shorter than that of NP-loaded ones, reflecting differences in intramolecular rotation and local viscosity [21].

However, detailed analysis of mechanisms of NP delivery requires the integration of additional imaging techniques to overcome limitations of IVM. While IVM allows for high-magnification observations at the cellular level, it is less suited for capturing accumulation at the organ level. The same fluorescent NPs used for IVM can often be adapted for whole-body fluorescence imaging, establishing a full landscape of NP delivery in animal models [40]. Another challenge with IVM is quantification of NPs via fluorescence, as the signal can be significantly

influenced by the microenvironment. Reliable biodistribution measurement can be achieved through nuclear imaging techniques, particularly single-photon emission computed tomography (SPECT) and positron emission tomography (PET). These methods need the incorporation of suitable tracer probes into NPs for accurate assessment of their delivery in tissues.

Looking ahead, a more precise analysis of NP behavior may be facilitated by the application of artificial intelligence tools. Deep learning-based image restoration can play an important role in improving the signal-to-noise ratio in deep tissue layers [259]. Furthermore, machine learning algorithms can process various types of medical data to perfume large-scale quantification of NP transport mechanisms, critical step in addressing heterogeneity of nanomedicine accumulation in clinical samples [263].

The final challenge in the convergence of IVM with nanomedicine will be validating different NP transport mechanisms in clinical settings. The rapid dynamics and complexity of the processes require the use of advanced microscopy techniques. In the current state, intraoperative IVM technology enables visualization of tumor vasculature and measurement of blood flow [73,264]. To minimize invasiveness of observations, confocal laser endomicroscopy can be employed during laparoscopic procedures [265]. Enhancing the spatial resolution of these techniques in clinical trials could potentially guide clinical decisions regarding tumor stratification and treatment options.

5. Conclusion

Exploiting IVM techniques evolved the understanding of NP transport from belief in EPR-based accumulation through static pores to involvement of multiple diverse pathways for tumor entry. Among the most significant discoveries are the dynamic nature of the EPR effect, the involvement of immune blood cells in NP transport, and the recognition of active transcytosis of NPs via N-TECs. However, these mechanisms have only been observed in animal xenograft models, which poorly reflect the architecture of human tumors. Monocellular xenografts exhibit low heterogeneity and limited genetic diversity. Employing patient-derived xenografts or tumors grown from patient-derived cells could improve the relevance of preclinical studies [13]. Development of multicellular heterogeneous tumors could benefit from the application of IVM to assess the accuracy of these models. The use of advanced fluorescent cellular sensors for measuring acidity (pH), oxygenation (pO_2), solid stress and other parameters can precisely assess tissue heterogeneity [74,266].

Second, the accumulation of EPR-designed nanomaterials in clinic was highly heterogeneous both in different regions of tumor and between patients. The newly discovered routes of active NP transport are likely subject to the same heterogeneity, which needs to be measured. For now, immune cell infiltration levels vary significantly among patients with the same tumor type [267,268]. Endothelial cells capable for transcytosis of NPs are also distributed unevenly along the tumor vasculature [224]. Therefore, it is crucial to evaluate the contributions of different mechanisms to the overall transport of NPs and to develop the capability to predict tumor response to various treatment options. Companion diagnostic nanoagents have been proposed to assess the passive accumulation of therapeutic nanocarriers in tumors via the EPR effect [77,269]. Similar selective tracers should be developed to target active NP transport mechanisms.

Finally, if the EPR effect is not the sole mechanism, the constraints it imposes on nanomedicine design need to be reassessed. For example, stealth coatings of NPs have been developed to prolong circulation time and reduce non-specific interactions with other cells, primarily of the MPS [270]. However, these coatings can significantly affect interactions with endothelial cells, neutrophils, and blood monocytes, potentially limiting active transport of NPs to tumors. Recent studies have shown that prolonged circulation time can be achieved even without stealth coatings by injecting high doses of NPs [271] or by pre-blocking the MPS

[200,272]. In addition, a small NP size below 100 nm is beneficial for both the EPR concept and for delivery via clathrin- or caveolin-mediated transcytosis, due to the size limit of the endocytic vesicles. Nanoparticles with larger sizes may exhibit better margination near tumor vasculature and greater interaction with blood immune cells, facilitating chemotaxis-mediated delivery. Therefore, the optimal size of NPs should depend on the relative contributions of different pathways involved in NP transport. It is essential to investigate the influence of other physicochemical properties of NPs, such as shape, rigidity, and functional modifications, on the efficiency of different delivery mechanisms in tumors.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.addr.2025.115550>.

Data availability

No data was used for the research described in the article.

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