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Review article

Immune cells as tumor drug delivery vehicles

Francis Combes[a,b], Evelyne Meyer[b,c], Niek N. Sanders[a,b,*]

[a] Laboratory of Gene Therapy, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, 9820 Merelbeke, Belgium
[b] Cancer Research Institute Ghent (CRIG), 9000 Ghent, Belgium
[c] Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

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ABSTRACT

This review article describes the use of immune cells as potential candidates to deliver anti-cancer drugs deep within the tumor microenvironment. First, the rationale of using drug carriers to target tumors and potentially decrease drug-related side effects is discussed. We further explain some of the current limitations when using nanoparticles for this purpose. Next, a comprehensive step-by-step description of the migration cascade of immune cells is provided as well as arguments on why immune cells can be used to address some of the limitations associated with nanoparticle-mediated drug delivery. We then describe the benefits and drawbacks of using red blood cells, platelets, granulocytes, monocytes, macrophages, myeloid-derived suppressor cells, T cells and NK cells for tumor-targeted drug delivery. An additional section discusses the versatility of nanoparticles to load anti-cancer drugs into immune cells. Lastly, we propose increasing the circulatory half-life and development of conditional release strategies as the two main future pillars to improve the efficacy of immune cell-mediated drug delivery to tumors.

1. Off-target side effects limit drug efficacy

The current view of cancer is clearly that of a systemic disease [1]. Consequently, the containment of a cancerous mass or a tumor relies on its interaction with non-cancerous cells of the body. Back-and-forth communication between cancer cells and the cells in close proximity establishes a tumor microenvironment (TME) which consists of the extracellular matrix (ECM), fibroblasts, blood vessels and immune cells [2]. Ultimately, the co-evolution and dynamic interaction of these TME components will dictate the clinical progression of cancer.

Due to its heterogeneous nature, treatment of solid tumors requires a multimodal approach where surgery and radiation therapy has to be succeeded by adjuvant chemotherapy [3]. In search for decreasing the sometimes severe side effects due to off-target action on healthy cells, researchers in the 1980's experimented with drug delivery vehicles such as liposomes loaded with doxorubicin [4]. This coincided with the large scale manufacturing of monoclonal antibodies [5] which opened the gates to the identification of many additional therapeutic targets and was the onset of tumor targeted drug delivery [6]. Mapping of the human genome in the 1990s [7] facilitated the in-depth understanding of the genetic basis of cancer and revealed many novel therapeutic targets associated with this disease [8]. The increased knowledge on preventing, diagnosing and treating cancer is reflected in the substantially improved clinical outcome compared to that of half a century ago (WHO-IARC). Nevertheless, there is still a formidable task lying ahead since the majority of cancer-related mortality is currently associated with metastatic disease [9–11], a far more elusive target than the primary tumor.

When administrating drugs *in vivo*, the pharmacokinetics (i.e. the mechanisms governing the absorption, distribution, metabolism and excretion) of these compounds has to be carefully considered [12,13]. Typically, limited bioavailability and activity of therapeutics are a direct result of their inappropriate physicochemical properties and their fast clearance by e.g. enzymatic degradation, glomerular filtration and phagocytosis by cells of e.g. the mononuclear phagocyte system (MPS) [14]. Employing higher dosages, other administration routes or more frequent administrations can increase the drug plasma concentration, but also elicits more side effects. A common practice to treat cancer is administrating the maximum tolerated dosing (MTD) of the chemotherapeutic [15], which is the highest dose that does not cause unacceptable side effects within a specified time period [14,16–18]. However, drug dosages that are below complete efficacy (even at MTD) cause selective pressure on the cancer cells when repeatedly used. By evolutionary principle, sensitive cancer cells are weeded out while resistant cancer cells remain intact, proliferate and compose a new tumor resistant to the used therapeutic [18]. The genetic heterogeneity of

* Corresponding author at: Laboratory of Gene Therapy, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, 9820 Merelbeke, Belgium.
E-mail address: Niek.Sanders@ugent.be (N.N. Sanders).

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cancer cells is even more pronounced when they spread across multiple organs [11]. Employing combination therapies or frequently switching to other drugs helps addressing this problem [19], but oncologists are always limited by the MTD since off-target effects on healthy cells cause side effects and thus diminish the dose at which the therapeutic agent can be used. Moreover, as most anti-cancer drugs are administered systemically, they come into contact with numerous healthy cells.

2. The tumor microenvironment as a more reliable therapeutic target

Compared to the ever-changing cancer cells themselves, more stable tumor properties can be found in the TME of both primary tumors and metastatic niches [20–22]. For example, rapid tumor growth requires an increased influx of nutrients and efflux of waste products. The formation of new blood vessels to cope with this demand is frequently inadequate and hence results in characteristic tumor-associated phenomena such as hypoxia [23], high interstitial fluid tension [24] and low pH [25]. In addition, tumor-associated blood vessels are frequently leaky in nature [26] and exhibit morphological abnormalities such as a lack in pericyte coverage, a serpentine course, irregular branching, arterial-venous shunts and irregular perfusion (Fig. 1) [27,28]. Moreover, the lymphatic drainage of many solid tumors is often heavily impaired [29]. Combined with the production and remodeling of the ECM at the tumor margin [30,31], these anomalies lead to an increased interstitial fluid pressure within the tumor [32,33].

As first described by Matsumura and Maeda [34], the leaky properties of the tumor vasculature can be exploited to more selectively deliver biocompatible particles into solid tumors. This concept became known as the "enhanced permeability and retention (EPR) effect" [26]. To achieve successful EPR-driven drug delivery, the used particles mainly need long circulation times. Though distinct tumor accumulation is already seen as soon as 30 minutes, exploitation of the EPR effect requires drug delivery vehicles to circulate at least several hours in mice. This is obtained when the particles are prevented from clearance by the kidneys or the MPS, do not interact with blood vessels, are not immunogenic and remain intact [26]. The resulting drug retention in the tumor is reported to last several days to weeks [35]. In practice, the used particles should be smaller than 780 nm to pass through the pores of the tumor endothelium but larger than 40 kDa/5.5 nm to avoid their rapid renal clearance [36–38]. A frequently used strategy to prolong nanoparticle circulation time is to provide them with a shell of polyethylene glycol (PEG), a polymer known to reduce opsonisation and premature clearance by the MPS [39]. However, repeated administration of PEGylated agents can elicit an immune response leading to accelerated blood clearance due to formation of anti-PEG antibodies [40].

About three decades ago, the EPR effect sparked the emergence of the nanoparticle research field which was heavily reliant on the "passive targeting" provided by this effect. Since then, many strategies have been devised to provide a complementary "active targeting" component to the used nanoparticles (i.e. adding specific ligands which target overexpressed receptors on cancer cells or in the TME) [36,41]. Despite the initial enthusiasm, this field is currently confronted with skepticism due to failure of providing relevant enhanced tumor accumulation in the clinic [42,43]. Moreover, the added benefit of targeting ligands are minimal compared to the physicochemical properties of nanoparticles [36,44]. A comprehensive literature review corroborated these findings and stated that even in mouse tumors, only 0.6% (median) of the injected nanoparticles reach the tumor via passive targeting [45]. Employing active targeting strategies modestly raised this value to almost 1%, but no evolution in the delivery efficiency has been seen over the span of 10 years [45]. This setback is even more pronounced in humans and can be attributed to differences in tumor manifestation in preclinical animal models compared to tumor growth in humans [45]. The most notable differences relate to the relatively more heterogeneous nature of human tumors compared to experimental rodent tumors and include the smaller tumor-to-body size in humans, the absence of hyperpermeable vasculature, the high interstitial fluid pressure, the presence of avascular regions and the chaotic TME in human tumors [42,46]. These unfortunate developments highlight the limitations of animal models and emphasize the diverse nature of tumor biology. They also generate novel opportunities such as normalization of the tumor vasculature to increase tumor drug delivery [47–50]. In addition, novel studies such as the one by Sindhwani et al. (2020) suggest that almost all injected nanoparticles enter solid tumors via active endothelial transcytosis instead of EPR [51]. Although these mechanisms of nanoparticle transcytosis and their ability to specifically target tumors still need clarification, they bear the potential to elicit a paradigm shift in the nanoparticle research field [52].

Unlike small circulating compounds such as drugs, antibodies and nanoparticles, larger immune cells do not rely on passive diffusion to enter the TME but use their complex cellular machinery to navigate to and through the tumor-associated endothelium to occupy specific regions within the TME [53–57]. Consequently, immune cells potentially provide solutions to the obstacles that emerged in the field of targeted drug delivery. For example, immune cells do not require the presence of endothelial pores to initiate extravasation [58], are able to migrate into areas exerting high interstitial pressure and can even populate avascular regions deep inside solid tumors [59]. Furthermore, since tumor manifestation is often a form of unresolved chronic inflammation, immune cells are continuously recruited to this site [60,61].

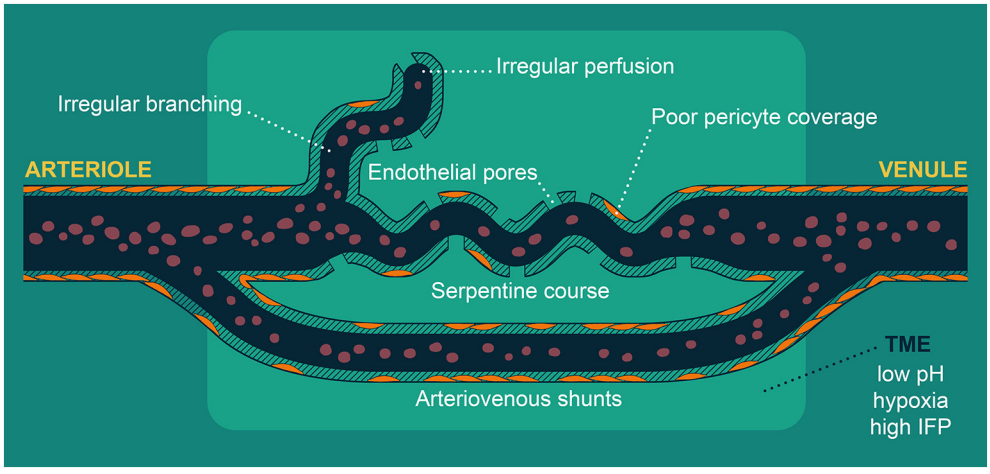

Fig. 1. Tumors display aberrant blood vessels. In normal blood vessels, arteries size down to arterioles and perfuse a capillary bed which then drains into venules of the venous system. A continuous layer of pericytes within the basement membrane encircles these small blood vessels. In contrast, the vasculature of solid tumors demonstrates a severely disarranged capillary bed with typical tortuous (serpentine) course, irregular branching and endothelial pores. Moreover, tumor blood vessels are poorly covered by pericytes and exhibit arteriovenous shunts that bypass certain regions of the capillary bed.

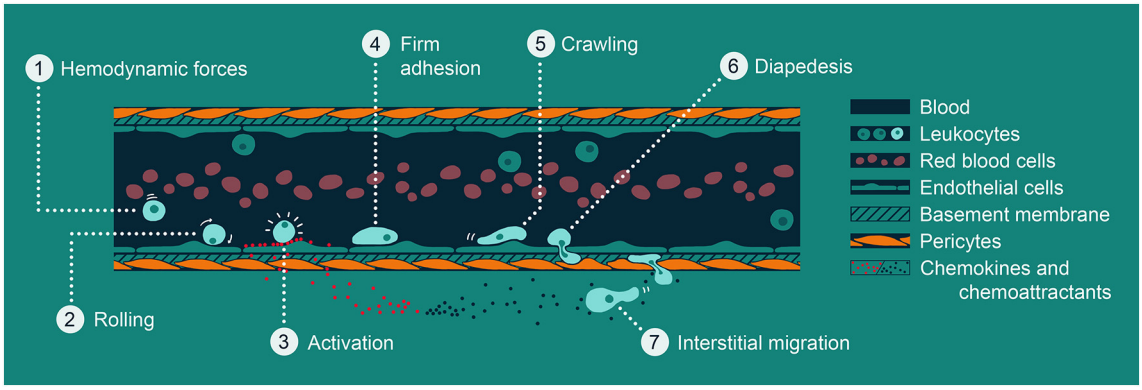

Fig. 2. The uniformly used migration cascade in normal postcapillary venules. (1) RBC concentration at the center of the venule and slower blood flow due to an increased vessel diameter drive leukocytes to the vessel wall. Due to the laminar flow of blood, leukocytes slow down further when they come in close proximity with the endothelium. (2) Leukocytes expressing L-selectin reversibly bind sugar moieties containing the SLE^x motif on endothelial cells. Conversely, P- and E-selectin expression on endothelial cells bind sugar moieties on leukocytes. In addition, the rolling of leukocytes is further mediated by low-affinity integrins. (3) Inflammation-associated chemokines are presented on the luminal surface of activated endothelial cells. Rolling leukocytes that bind these chemokines on cognate receptors become activated and express integrins in a high-affinity conformation. (4) High-affinity integrins on the leukocyte cell surface bind ligands on the endothelial cells (e.g. Cellular Adhesion Molecules). This induces firm adhesions which keep the leukocyte stationary. (5) Firmly attached activated leukocytes will spread out and start Mac-1-mediated crawling along the vessel wall to find a suitable extravasation site. Crawling occurs with or against the blood flow. (6) Leukocytes squeeze their way in between (or through) endothelial cells via PECAM-PECAM interactions. Next, the leukocytes slowly navigate their way through the basement membrane until they find a gap in between pericytes. (7) Traversing the ECM requires coordinated integrin expression and apical proteolysis. Alternatively, the leukocyte can crawl through ECM pores in an amoeboid way. The direction of migration follows a gradient of chemoattractant molecules in the ECM. RBC: red blood cells, SLE^x: Sialylated Lewis X, Mac-1: Macrophage-1 receptor, PECAM: platelet endothelial cell adhesion molecule, ECM: extracellular matrix.

3. How immune cells reach their target

Circulating immune cells respond to inflammation-associated cues in a relatively uniform manner. However, some important distinctions exist between different immune cell populations that have an effect on their response time and trafficking patterns. For example, in contrast to granulocytes, naive lymphocytes are poorly responsive to inflammatory signals. They migrate efficiently to secondary lymphoid tissue where they first need to be activated before they acquire the capacity to be recruited to inflammation sites [62]. Immune cell trafficking occurs in sequential steps where each step is conditional for the next and thus, generates high targeting specificity (Fig. 2) [63]. These steps often take place in postcapillary venules, small veins with a diameter of about 50 μm which are located directly after the sites of increased vascular permeability during inflammation. The leakage of plasma and the wider lumen increase hemoconcentration, slowing the blood flow and allowing more interactions between the endothelium and leukocytes [64]. In addition, hemodynamic forces confine red blood cells (RBCs) to the center of the blood vessel, thereby "marginating" other large particles such as immune cells to the vessel walls [65].

Passing immune cells that come into contact with the endothelial layer loosely bind sugar moieties, thereby inducing a reversible leukocyte rolling along the endothelial surface lasting several seconds to minutes [66,67]. This first step in the migration cascade is mediated by selectins (CD62), cell surface molecules that bind distinct terminal carbohydrate residues (a tetrasaccharide motif known as "sialylated Lewis X") of glycoproteins or glycolipids in a calcium-dependent fashion [62,66]. Selectins are composed of three families named after the cell type on which they were originally identified: E-selectin on activated endothelium, P-selectin on activated platelets, but also on activated endothelium and L-selectin constitutively on lymphocytes, but also expressed on hematopoietic stem/progenitor cells [62,66]. The carbohydrate selectin ligands are present on endothelial cells and other leukocytes, thus facilitating leukocyte-endothelium or leukocyte-leukocyte interactions [68]. Of all the available ligands, P-selectin glycoprotein ligand 1 (PSGL-1) appears to be the main ligand for all these selectins [69]. The non-covalent bonds forming between the rolling leukocyte and the vessel wall must counteract the shear stress, the horizontal force and torque exerted by the blood flow [70]. Interestingly, this involves selectin-associated "catch bonds" which require a certain amount of shear stress for leukocytes to attach to the endothelium and may prevent inappropriate leukocyte aggregation or extravasation during vascular stasis [68]. After reaching a maximal shear stress value, selectin-ligand interactions change from "catch bonds" to regular "slip bonds" [70]. This delicate balance makes the cell pivot over the remaining binding sites and hence roll to a position further downstream at about 10 μm/s. In addition to mediating reversible cell-cell adhesion, selectin binding also activates intracellular signaling pathways leading to e.g. β2-integrin activation associated with intermediate affinity [71,72]. Consequently, selectin-mediated rolling further reduces rolling velocities to about 5 μm/s by facilitating conformational changes in integrins and thus providing more opportunities for rolling cells to encounter endothelium-bound chemokines [72,73]. In turn, the encountered inflammation-associated chemokines which are displayed on the endothelium surface will further stimulate integrin activation that mediates leukocyte arrest and subsequent transendothelial migration [64].

Integrins are transmembrane cytoskeleton-linked heterodimers consisting of paired α and β subunits. These subunits assemble into many different receptors with different binding properties and tissue distribution [74]. Essentially all nucleated cells express integrins [73], but the β2- or CD18-integrins are only expressed on leukocytes [73,75]. For example, lymphocyte function-associated antigen 1 (LFA-1, αLβ2 or CD11a/CD18) is expressed on all leukocytes and is the predominant integrin on lymphocytes while macrophage-1 (Mac-1, CR3, αMβ2 or CD11b/CD18) dominates on myeloid leukocytes [75]. Integrin activation induces rapid (i.e. within 1 s) conformational changes from a completely bent form with low ligand affinity to an intermediate or fully extended form with very high ligand affinity [74,76,77]. Most integrins typically bind a wide variety of ligands, making these receptors essential in many cell-cell and cell-matrix interactions [74]. Though integrins can be involved in the rolling of leukocytes [78], they are mostly important for inducing firm adhesions. Conformational change of integrins is initiated by other cell surface receptors (e.g. selectins, chemokine receptors, Toll-like receptors or the T cell receptor) and occurs through cytoplasmic activation of the integrin β subunits [75,79]. Once activated, the integrins can bind their endothelial ligands such as members of the immunoglobulin superfamily (e.g. ICAM-1,

VCAM-1 and MAdCAM-1) [75]. At inflammatory sites, this is followed by cell spreading and Mac-1-mediated crawling along the vessel wall to find suitable extravasation sites [80,81].

Most of the immune cells that pass through the vasculature of the inflammation site do not roll, most of those that roll do not adhere and most of the adherent immune cells do not extravasate [82]. However, the commitment of immune cells to initiate extravasation marks a certain “point of no return” [82]. Transendothelial migration or diapedesis is initiated by clustering of leukocyte integrins with their endothelial ligands and occurs in between (para-) or to a lesser extent through (trans-) endothelial cells [83,84]. The resulting actin-rich endothelial docking structures then form a tight ring around the transmigrating leukocyte [64]. VE-cadherin (CD144) normally connects endothelial cells at adherens junctions, but is cleared from the site of transmigration to form a gap. In addition, a dynamic vesicle complex known as the lateral border recycling compartment at the intercellular junctions between endothelial cells has to assemble around the extravasating leukocyte and continuously recycle to facilitate transendothelial migration [64]. This requires PECAM-PECAM or CD31 interactions between the leukocytes and the endothelium as well as an essential increase of intracellular calcium in the endothelial cells [64]. Once the immune cell has successfully crossed the endothelial layer within less than 5 minutes, it must also find a way through the basal lamina. In general, this can take up to 15 minutes as the leukocyte has to navigate its way to areas with less collagen IV and laminin 511 deposition [85–88]. These areas frequently occur at gaps in pericyte coverage and squeezing through these gaps involves many of the molecules seen for transendothelial migration [64,89]. In addition, leukocytes require deformable nuclei to traverse these narrow pores and can rely on protease-mediated remodeling of the basement membrane to clear the path [89].

After immune cells have penetrated the dense sheet-like basement membrane, migration through the rest of the interstitial mesh-like ECM starts. The ECM is a highly complex organ-specific network of fibrillary and non-fibrillary collagens, glycoproteins and proteoglycans assembled in supramolecular structures which provide structural support, but also bind and regulate the distribution of soluble factors such as growth factors and chemokines [87,90]. Immune cell migration through the ECM is achieved by coordinated expression of certain collagen-binding and –sensing integrins [87]. In addition, secreted and membrane-bound proteases e.g. matrix metalloproteinases can help in clearing the path [91]. In turn, this localized proteolysis of the extracellular matrix exposes more integrin ligands, cytokines and chemokines which elicit a combined directional response in the migrating leukocyte [92,93]. Active breakdown of the ECM and integrin-mediated attachment is not always a requirement as leukocytes can also navigate through pre-existing matrix gaps in an amoeboid way independent of matrix-degrading enzymes or even integrins [94]. Nonetheless, cell type-specific limitations in nuclear stiffness impose protease activity as ECM pores are typically smaller than 1 μm [87,95].

Chemotaxis is the movement of an immune cell in response to chemical substances present in tissues (chemoattractants). Secreted cytokines that elicit such chemotaxis are termed chemokines and typically bind cellular G protein-coupled receptors [96]. They are subdivided into four classes based on the spacing between the first 2 cysteine (C) residues at the amino terminus. These 2 cysteines are separated by either zero, one or three amino acids in respectively C, CXC and CX_3C chemokines. C chemokines have only one cysteine at the N-terminus [97]. Chemokines are constitutively expressed under homeostatic conditions to maintain immune surveillance, but activated cells can also produce inflammatory chemokines to recruit additional immune cells to inflamed tissues [96].

Leukocytes first encounter chemokines, produced by interstitial or vascular cells, while rolling on the endothelial surface at sites of inflammation [98]. This interaction activates integrins, thereby initiating transendothelial migration. Upon arriving in the ECM, immune cells sense gradients of chemotactic cues in a cell type-specific manner and migrate towards the region of highest concentration [96,98]. As many chemoattractants influence the chemotaxis simultaneously, migrating immune cells have the capacity to hierarchically prioritize certain chemokine gradients [96]. Interestingly, migrating immune cells may also release chemokines themselves, allowing other immune cells to follow their trail [99]. To establish a gradient, soluble chemokines need to diffuse away from their source. To prevent chemokines from washing away, they are immobilized in the ECM and on the luminal surface of endothelial cells via glucosaminoclycans [96,100]. Nevertheless, dislodged chemokines are also detected in the blood during inflammation. These blood-borne chemokines are suspected of activating immune cells off-site and by doing so, causing immune cell accumulation in organs such as the lungs and the liver [96].

4. Hijacking cells for drug delivery purposes

Immune cells are continuously recruited to establish a TME that can be either anti- or pro-tumorigenic. Tumors can be pathologically categorized according to the relative presence of these different recruited immune cell populations and the character of their inflammatory response [101–103]. For example, tumors that are heavily populated by T lymphocytes are considered immunologically “hot” whereas “cold” tumors are characterized by an absence of these cells. In between these 2 extremes, we find “infiltrated-excluded” TMEs which are heavily populated by lymphocytes at their periphery but lack cytotoxic lymphocytes (CTLs) in the tumor core [104]. In addition, “infiltrated-immunosuppressed” TMEs contain a low number of lymphocytes with suppressed effector function in the tumor core [101]. Tumor eradication requires combined innate and adaptive immune responses after successful recognition of the malignant cells. Likewise, tumor growth also relies on hematological cells to establish an immune-suppressive TME. Therefore, both scenarios require immune cells to be recruited [101]. This premise forms the main argument for using cells as drug delivery vehicles. In addition, due to the ubiquitous nature of this approach, it has the potential to result in targeting strategies that are widely applicable in cancer patients.

Like other drug carriers, cells can be used to protect their therapeutic cargo from immune recognition and degradation, thereby prolonging its circulation time [105]. In addition, the inherent migration capacity of immune cells can be used to specifically target inflammatory regions and the TME (Fig. 3). Even metastases can potentially be targeted using this approach, since they are also associated with increased immune cell infiltration [106]. Unlike the diffusion-driven delivery used by other carriers (e.g. liposomes, synthetic polymers and protein-DNA complexes), cells can truly actively penetrate solid tumor tissues, even when they are characterized by high interstitial pressure. Moreover, when DNA or RNA are used as launching platforms for therapeutic proteins, such cellular carriers can effectively be used as local production factories [105]. The possibilities of using bioengineering tools to develop and iteratively improve molecular sensors and switches further accentuates the advantages and future potential of intelligent cell delivery vehicles [107–110].

5. Red blood cells

In theory, red blood cells (RBCs or erythrocytes) are ideal drug carriers with the drugs either residing inside the RBCs or adsorbed to their surface. These simple cells are readily available in vast quantities, are very biocompatible even in allogeneic settings and are easily stored [111]. RBCs have virtually unrestricted access to vascularized tissues because of their relatively small size (7 μm diameter and 2 μm thick) and capacity to deform (Table 1). In humans, they travel through the circulatory system every 20 seconds and exhibit a lifespan of about 3–4 months in circulation while mouse RBCs have a lifespan of only 1 month [105,112–114]. Due to the lack of a nucleus, RBCs can be

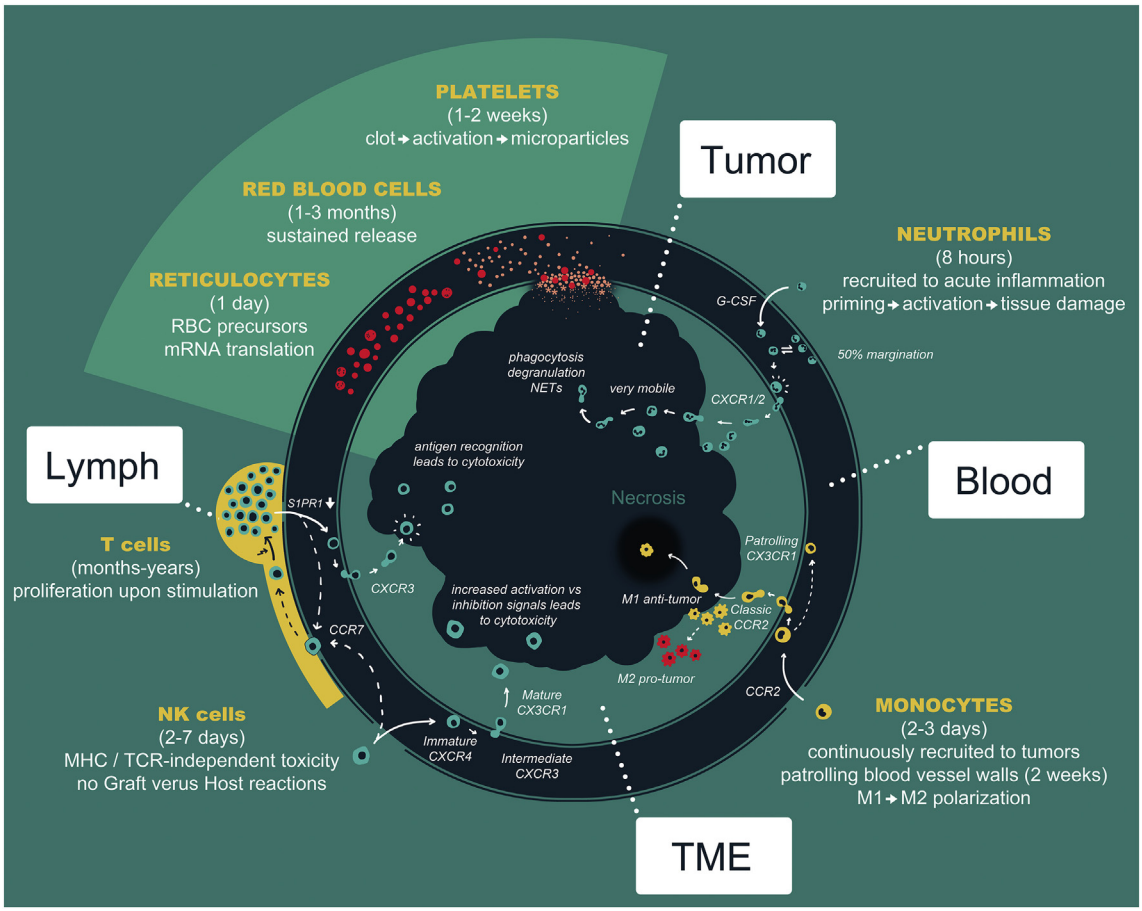

Fig. 3. Blood circulating cells as drug delivery vehicles. Non-immune cells such as reticulocytes, red blood cells and platelets can be used for intravascular release of drugs but do not actively migrate to the interstitial space (green sector). Immune cells such as neutrophils, monocytes, NK cells and T cells traverse the blood (and lymphatic) vessels before initiating transmigration into the TME and tumor tissue. The circulation time, the most distinctive properties and main chemoreceptors for each cell type is depicted. RBC: red blood cells, MHC: major histocompatibility complex, TCR: T cell receptor, S1PR1: Sphingosine 1-phosphate receptor 1.

viewed as biological microparticles that can be used to compartmentalize drugs, e.g. to avoid renal clearance [14]. However, their size and lack of migration machinery confines the use of RBCs to intravascular applications such as circulating bioreactors for enzymes, as reservoirs for slow drug release, as carriers for imaging agents and as "super-carriers" for nanoparticles [14,113]. Alternatively, RBC precursors (reticulocytes) contain translation machinery and can therefore be exploited as production factories for mRNA-based therapeutics [115]. Some degree of targeting of the MPS is described e.g. via cell surface modifications which mimic damaged or senescent RBCs [116,117]. Since a large portion of the MPS resides in the spleen and the liver, the MPS-mediated clearance of RBC drug carriers is sometimes seen as active targeting of these organs [111]. Nevertheless, since virtually all circulating particles, debris and compromised cells are eventually cleared by cells of the MPS, the added benefit of using RBCs for this goal over liposomes can be questioned [118].

Most drug delivery strategies try to minimize RBC elimination [113]. Apart from avoiding membrane alterations due to the drug loading protocols, CD47 as a macrophage "do not eat me" signal or complement inhibitory molecules can be introduced in the RBC

Table 1
Main properties of blood cells that have been considered as drug delivery vehicles.

Cell type	Size (μm)	Lifespan	Main chemokine/receptor	Remarks
RBC	6–7	3–4 months (Mouse: 1 month)	-	only for intravascular use, no nucleus, no activation
Platelet	1–3	9 days	-	Interesting for injuries involving hemostasis, no nucleus
Neutrophil	7–11	6–8 hours ($t_{\frac{1}{2}}$)	CXCL8/CXCR1–2	Widely applicable, rapid recruitment, collateral damage
Eosinophil	12–17	8–12 hours	CCL11/CCR3[a]	Collateral damage, allergens
Basophil	5–7	1–2 days	CCL11/CCR3[a]	Collateral damage, allergens
Monocytes	10–18	$Ly6C^{high}$: 17 hours ($t_{\frac{1}{2}}$) $Ly6C^{low}$: 2–14 days ($t_{\frac{1}{2}}$)	$Ly6C^{high}$: CCL2/CCR2 $Ly6C^{low}$: CX3CL1/CX3CR1	Widely applicable, versatile functions, penetration into necrotic tumor core React to ex vivo manipulation
T cell	8–10	2 weeks after activation	PSGL-1/CXCR3	Local proliferation and memory cells, cytotoxic upon antigen recognition
NK cell	10	1 week	CXCL8/CXCR1[b]	MHC-independent cytotoxicity

[a] Eosinophils and basophils use very similar chemokine receptors but both cell types rely on specific combinations of receptors to achieve successful recruitment into tissues and their main receptor CCR3 binds a large array of ligands [151].
[b] CXCR1 is an example of a chemokine receptor that can only be found on the cytotoxic NK cell subset.

membrane. Moreover, RBC "immune-camouflaging" via PEGylation offers some interesting prospects [113,119]. Accumulation of RBCs in specific organs can be achieved by incorporating magnetic materials into drug-loaded RBCs and subsequently applying an external magnetic field on the organ of interest [120]. Similar strategies include the induction of microvessel rupture through targeting microbubbles via ultrasound [121] or coating of the RBC surface with targeting moieties such as antibodies [122]. However, since hydrodynamic forces drive RBCs to the center of the vascular lumen and RBC-endothelial interactions are minimized by the 0.5 μm thick endothelial glycocalyx [123], the use of targeting moieties may not be efficient. It is currently unclear how these approaches can be applied to situations where the pathological region is unknown such as in metastatic disease.

Within the context of cancer therapy, RBC drug carriers can provide the same benefits as liposomal drug formulations such as Doxil®, a commercial PEGylated liposomal formulation of doxorubicin, but with a decreased risk of mounting immune responses [124,125]. Interestingly, although doxorubicin-loaded RBCs demonstrated virtually equal tumor targeting compared to free doxorubicin, other differences in biodistribution were noticed. Indeed, RBC-doxorubicin demonstrated increased accumulation in the liver, the spleen and the lungs while free doxorubicin resulted in a higher accumulation in the heart and skin of tumor-bearing mice. Despite the prolonged release of doxorubicin from the circulating RBCs that resulted in a significantly decreased tumor volume, less cardiotoxicity and myelosuppression was seen [124]. Therefore, the effects of RBC-mediated drug delivery seem to be twofold: sustained release which maintains drug concentrations within the therapeutic window and skewing biodistribution towards organs of the MPS. However, a main limitation is that RBCs cannot be used to actively deliver drugs to extravascular targets.

6. Platelets

Platelets or thrombocytes are smaller (1–3 μm) than RBCs and only circulate for about 9 days followed by their clearance via the MPS [126]. They have a key role in blood clotting and secrete bioactive molecules such as growth factors, chemokines, cytokines and matrix metalloproteinases upon their activation. Moreover, they are also involved in inflammation, wound healing and tumor progression [126–128]. Activation takes place when circulating platelets encounter damaged endothelial walls, where they change shape, shed off platelet microparticles and aggregate to form clots through the interaction with fibrin [129]. Pathological release of excessive platelet activators can cause platelets aggregation within intact blood vessels or thrombosis [126]. Like RBCs, platelets do not possess a nucleus and cannot actively penetrate tissues. Nevertheless, their advantage as drug delivery vehicles is that activation allows them to acquire more selectivity compared to RBCs. As such, they can be used to selectively deliver therapeutic cargo into platelet-activating tumors [130,131]. For example, platelets have been loaded with doxorubicin to treat lymphoma-bearing mice [132]. Similar to RBCs, this targeting strategy maintained the tumor inhibiting effect of doxorubicin while avoiding its cardiotoxicity [132,133]. Perhaps the most efficient strategy to utilize platelets for drug delivery is post-surgical treatment because this medical intervention is always associated with bleeding and hence, hemostasis. In this context, a recent study demonstrated substantially increased anti-PDL-1 efficacy through platelet-mediated drug delivery against residual melanoma cells after surgical removal of the primary tumor [134,135]. Anti-PDL-1 antibodies were conjugated to the cell surface allowing them to be dispersed on platelet-derived microparticles after activation of the carrier platelets. This approach was also successfully demonstrated after thermal ablation of the primary tumor [136].

7. Myeloid cells

Immune cells of the myeloid lineage mostly comprise cells involved in the innate immune system (e.g. granulocytes, monocytes, macrophages, dendritic cells), but provide an essential bridge to the adaptive immune system as well. They are implicated in all inflammation processes and form important members of the TME. The modern view on cell function incorporates a spectrum of functions ranging from immune stimulatory to -suppressive, depending on the input the cell receives [137]. In this regard, solid tumors frequently produce soluble factors which favor the production and recruitment of (immature) myeloid cells to the TME [138]. Paradoxally, these innate immune cells are submerged in an array of tumor-derived factors, instructing them to adopt phenotypes that are favorable for tumor survival, progression and eventually metastasis [138]. Therefore, myeloid cells are essential components in establishing an immunosuppressive TME reminiscent of a state of chronic inflammation [139,140]. Due to the plastic nature of myeloid cells in such an environment i.e. the ability to switch phenotypes, much debate arises concerning their classification [141,142]. Most of these cells derive from circulating immune cells which were recruited via the classic migration cascade. Of note, myeloid cells are also suspected of shaping the "pre-metastatic niche", indicating that their recruitment can even precede the arrival of metastatic tumor cells [143,144]. Besides the large amount of chemotaxins that recruit multiple immune cell populations, the occurrence of post-translationally modified (e.g. glycosylation) chemokines in the TME was reported [145,146]. These altered chemokines can demonstrate a change in receptor specificity and affinity, thereby selectively recruiting certain leukocyte populations while dismissing others [147]. It is therefore possible that additional selectivity can be obtained by focusing on those populations that specifically react to these tumor-associated altered chemokines. Given the high number of chemotaxins and corresponding receptors involved in leukocyte recruitment, this will most likely require elaborate integrated analysis to predict their combined effect. Still, myeloid cells have the inherent capacity of trafficking to solid tumors. Those myeloid cell populations that have been reported as drug delivery vehicles will be discussed below.

7.1. Granulocytes

Bone marrow-derived granulocytes are a group of innate immune cells characterized by an irregular nucleus and the presence of granules in their cytoplasm. They are typically further subdivided into 3 families: neutrophils, eosinophils and basophils. Neutrophils have historically been extensively studied due to their relative abundance i.e. 40–75% of the total leukocyte count in human blood and their central role as "first responders" in most inflammatory situations [148]. The vast amount of available data on their biodistribution and migration properties provides us a template to which other immune cell populations can be compared.

7.2. Neutrophils

In the bone marrow, granulocyte colony stimulating factor (G-CSF) is the main stimulator of neutrophil differentiation, proliferation and release [149]. Neutrophils (7–11 μm) are kept in the bone marrow through the interaction with chemokine SDF-1, which is constitutively expressed by bone marrow stromal cells. Binding of SDF-1 on neutrophil receptor CXCR4 then modulates integrin-ligand binding VLA-4/VCAM-1 [150]. G-CSF inhibits this interaction by down-regulating the expression of both SDF-1 and CXCR4, resulting in the release of neutrophils [149]. After leaving the bone marrow, neutrophils circulate in the blood with a short half-life of only 6–8 hours. In the blood they either slowly transit through intravascular "margination pools" such as the liver, the spleen, the bone marrow and the lungs, get cleared from the circulation or enter inflamed tissues [149,151]. About 50% of the intravascular neutrophils will reversibly bind/roll along the endothelium of postcapillary blood vessels [152–155]. This marginated pool is in dynamic equilibrium with the circulating pool, a balance that

can be manipulated to mobilize neutrophils back into circulation. Of note, organ sequestration of cells also includes irreversibly bound cells, trapped cells (e.g. by the MPS) and cells that migrated in the interstitium [155]. Therefore, calculating the size of the marginated pool in an organ is challenging, but most reports indicate that besides the liver, especially the spleen and the bone marrow contain many of these marginated neutrophils [155,156]. Moreover, a large portion of the marginated neutrophils reside in the lungs, most likely due to the high number of capillaries which all cells ejected from the heart have to squeeze through [155,157–159]. Hence, increasing cardiac output via e.g. adrenaline forces neutrophils into circulation [157,158].

Sequestration in a particular organ also depends strongly on the maturation and activation status of the neutrophils [160]. The majority of immature mouse neutrophils isolated from the bone marrow home back to the bone marrow after infusion, but can later be redistributed to sites of inflammation, while mature neutrophils localized equally between the liver and the bone marrow after injection, with no observed remobilization to an inflammation site [160]. In fact, bone marrow can act as a clearance organ for senescent neutrophils where they are phagocytized by macrophages, which in turn produce G-CSF to stimulate the production and release of other neutrophils [156]. In contrast, apoptotic neutrophils are mainly cleared by the liver Kupffer cells after exposing phosphatidylserine on their cell surface [161]. Noteworthy, inflammation-induced remobilization also causes neutrophils to accumulate in the lungs, which sometimes leads to acute lung injury during inflammatory states [162,163].

Activation of neutrophils occurs in 2 stages. Upon encountering certain inflammation-associated factors (e.g. cytokines, chemokines, bacterial and viral products), neutrophils are first 'primed' after which they can demonstrate an enhanced activation by interaction with e.g. pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) [151,164]. Priming induces cytoskeleton changes, making the neutrophils more rigid and consequently more prone to accumulating in capillary beds [159,165,166]. It also induces many other phenotypic changes, including adhesion to endothelium through increased expression and activation of β2 integrins, extravasation, increased responsiveness to chemokines (CXCL8 (IL-8) in particular) [167] and resistance to apoptosis [164,168,169]. Thus, neutrophils that underwent transendothelial migration are by definition primed [170]. Interestingly, circulating neutrophils can also be de-primed in the pulmonary blood vessels by currently unknown mechanisms, and this reversible activation state influences the kinetics and biodistribution of neutrophils [166,169]. Thus, relatively nonresponsive neutrophils become primed after encountering a first stimulus after which they become fully activated upon encountering a second stimulus. This state is characterized by phagocytic activity, degranulation and production of neutrophil extracellular traps (NETs) [171].

Despite their abundance (50–70% of white blood cells in humans, 10–30% in mice), rapid recruitment and capacity to fight cancer cells [172,173], neutrophils are rarely used as cell delivery vehicles because of their reactive nature and short lifespan. Moreover, neutrophils are notoriously known to cause substantial collateral damage to healthy tissues after degranulation [174]. Nevertheless, to combat antibiotic resistance, Wendel et al. (2015) devised an interesting strategy involving the encapsulation of chlorhexidine by dead bacteria used as stable liposome-like drug carriers [175]. These bacteria were loaded into neutrophils, infused in mice with liver abscesses and their subsequent infiltration into the liver significantly reduced the bacterial burden. Unfortunately, this study did not report biodistribution data. Moreover, the absence of a chlorhexidine only-control prevents interpretation of the added effect of using neutrophils as delivery vehicles. Such a control was probably omitted from the study due to the toxic properties of this topical antiseptic agent.

Overall, the short lifespan of neutrophils might enable to use these innate immune cells as delivery vehicles in acute therapeutic settings, provided that their off-target effects are genuinely restrained. In addition, the concept of loading therapeutic agents (e.g. via nanoparticles) in immune cells within a patient is definitely worth pursuing as it eliminates the need for collecting, isolating, loading and reinfusing immune cells [176]. In this regard, Chu et al. (2017) demonstrated that intravenously injected anti-CD11b-coated nanoparticles are taken up by neutrophils which then accumulate in tumor tissue after induction of acute inflammation [177]. In contrast, only low levels of accumulation was seen upon injection of PEGylated nanoparticles or when no acute inflammation was induced [177]. Similarly, neutrophils were used to deliver paclitaxel-loaded liposomes to surgically resected gliomas [178].

7.3. Eosinophils and basophils

After release from the bone marrow by IL-5, eosinophils (12–17 μm) circulate only 8–12 hours in blood, where they comprise 1–3% of the total leukocyte count and rely heavily upon CCR3 for their recruitment into tissues(Table 1) [179,180]. Eosinophils have many homeostatic functions, but can also be recruited in response to inflammation where they are best known for their IL-5-mediated interactions with Th2 lymphocytes during parasitic infections and in response to allergens [179]. Inflammation-induced degranulation of these cells is typically associated with cell toxicities and disruption/remodeling of the ECM [179]. They are also implicated in establishing the TME of solid tumors, demonstrating their potential use as drug delivery vehicles [181]. Eosinophil recruitment to tumors occurs in all stages of tumor growth and independently from Th2 lymphocytes. Their accumulation is mainly restricted to the fibrous tumor capsule and to areas of necrosis [182]. The capacity of adoptively transferred bone marrow-derived eosinophils to be efficiently recruited to inflammatory lungs upon allergen-induction hints at a potential therapeutic use in this specific organ [183]. However, it has been demonstrated that this type of inflammation is not restricted to eosinophils, but also recruits neutrophils and lymphocytes [184].

Basophils (5–7 μm) represent the lowest fraction of leukocytes in the blood (< 1%). They have an estimated lifespan of 1–2 days and are mostly implicated in inflammation reactions associated with allergies and parasites (Table 1) [180,185,186]. However, this stereotypical view is currently discarded in favor of a complex immunomodulatory and tissue remodeling function that is strongly dependent on its environment [185,187]. Their capacity to produce IL-4 and subsequently elicit Th2 cell responses after adoptive transfer suggests therapeutic potential of these cells to modulate immune responses [188]. Like eosinophils, recruitment of basophils into tissues uses multiple chemokine receptors and largely relies on the same chemokine receptors [189].

Eosinophils and basophils have not been explored as drug delivery vehicles most likely due to their low presence in the blood, and hence the problems associated with obtaining enough cells. Moreover, these cells can potentially cause extensive tissue damage [182,186]. Nevertheless, since eosinophils and basophils are specialized cells implicated in specific disease situations, they might have therapeutic potential in future delivery strategies. Alternatively, parts of their migration machinery such as selected chemokine receptors could also be incorporated in other cells to acquire their specific tissue tropism.

7.4. Monocytes and macrophages

A population of self-maintaining tissue-resident macrophages and classical dendritic cells (DCs) are established before birth. In addition, bone marrow-derived cells of the monocytic lineage are continuously produced to replenish and assist these cells in maintaining tissue integrity after birth [190,191]. The macrophage-DC precursor (MDP) or monoblast, a common founder cell residing in the bone marrow, differentiates into dedicated DC precursors and "common monocyte progenitors" [192,193]. Monocyte development mostly relies on the interaction of M-CSF or CSF1 with its receptor M-CSFR or CD115 and

monocytes subsequently require CCR2 to exit the bone marrow [194,195]. The postnatal bone marrow constitutes the main production site of monocytes, but relocation of hematopoietic stem cells and progenitor cells to the spleen can form supplementary sites of extramedullary hematopoiesis [196].

Monocytes represent about 10% of the circulating leukocytes in human and 4% in mice. They comprise a highly dynamic cell population that is vital in ensuring rapid injury detection, inflammation and resolution. Like neutrophils, a substantial number of intravascular monocytes can be found in marginal pools within organs such as the lungs and the spleen [197,198]. Again, this marginated monocyte pool is in dynamic equilibrium with circulating monocytes and can be mobilized on demand [197,199]. Multiple monocyte populations are found within the blood vessels. In mice, these populations can be discriminated via their level of Ly6C expression, a cell surface marker variably expressed by many immune cell populations except B cells [200]. The Ly6C^{high} (Gr-1^{+}) monocytes also highly express the chemokine receptor CCR2 and are the first subset of monocytes to be released from the bone marrow [200]. Conversely, Ly6C^{low} monocytes typically express the chemokine receptor CX3CR1 [201]. Cell surface phenotyping consequently reveals two distinct mouse monocyte populations: “immature”, “classical” or “inflammatory” Ly6C^{high}/CX3CR1^{mid}/CCR2^{+}/CD62L^{+}/CD43^{low} monocytes, and “mature”, “patrolling” or “resident” Ly6C^{low}(Gr-1^{-})/CX3CR1^{high}/CCR2^{-}/CD62L^{-}/CD43^{high} monocytes (Table 2) [194]. The Ly6C^{high} are termed “classical” because they typically enter inflamed tissues to further differentiate into peripheral mononuclear phagocytes i.e. macrophages or monocyte-derived DCs [194]. Of note, the murine Ly6C^{high} monocyte subset is considered to be the precursor of smaller Ly6C^{low} monocytes through an intermediary Ly6C^{int} population [193,202]. Ly6C^{high} monocytes can even home back to the bone marrow before maturing into Ly6C^{low} monocytes [203]. The latter can be seen as terminally differentiated blood-resident macrophages that survey endothelial integrity, hence they are termed “patrolling” monocytes [190,194,202]. While the circulatory half-life of Ly6C^{high} monocytes is estimated at about 17 hours (Table 1), intravascular Ly6C^{low} monocytes exhibit far longer half-lives ranging from 2 days up to 2 weeks [190].

Like their mobilization from the bone marrow, recruitment of Ly6C^{high} monocytes to sites of inflammation (including in the central nervous system) is highly dependent upon the CCL2-CCR2 axis [204]. There, these inflammation-induced monocytes transiently assist the self-maintaining pool of tissue macrophages [194]. Dependent on the context of the microenvironment, monocyte-derived macrophages subsequently polarize to a spectrum of cells between 2 opposing phenotypes: the classically-activated or pro-inflammatory “M1” macrophages versus the alternatively-activated or anti-inflammatory, tissue repairing, “M2” macrophages [205]. It is currently unclear whether some of these monocyte-derived macrophages can further establish in the tissues through self-maintenance [194]. Nevertheless, it has been demonstrated that tissue macrophages can proliferate at low levels during steady-state and that their proliferative capacity is enhanced through environmental cues such as IL-4 and M-CSF [206–208]. This has also been observed for monocyte-derived macrophages in the gut and in atherosclerotic plaques [209,210].

As mentioned earlier, to successfully migrate from the bone marrow into inflamed tissues, Ly6C^{high} monocytes rely on L-selectin-mediated rolling and CCR2-mediated activation and chemotaxis based on CCL2 and CCL7 gradients [193,211–213]. In addition to these well-known migration receptors, monocyte recruitment further involves many other integrins and chemokine receptors such as CCR1 and CCR5, a comprehensive list is provided in the excellent review by Shi and Pamer (2011) [214]. In contrast to these “inflammatory” Ly6C^{high} monocytes, Ly6C^{low} monocytes do not express L-selectin nor CCR2 but primarily rely on CX3CR1 and β2-integrins such as LFA-1 which engage with endothelial ICAMs [193,213]. Instead of rapidly rolling, steady state Ly6C^{low} monocytes continuously “crawl” over relatively long distances on the luminal side of mouse blood vessels i.e. at about 12 μm/min, independent of blood flow direction [193]. Interestingly, the ideal location of Ly6C^{low} monocytes makes them swiftly (< 1 hour) extravasate into tissues upon induction of inflammatory stimuli, a response found to precede that of neutrophils [193].

Intravenous injection of mouse macrophages results in a typical distribution pattern [215]. This pattern is characterized by rapid disappearance from the circulating pool with an initial arrest in the lungs, followed by redistribution to mainly the spleen and the liver [215,216]. When a distant site of inflammation is present e.g. in the footpad, specific accumulation of a small portion of the injected macrophages is observed for at least 1 week [216]. Bromodeoxyuridine labeling demonstrated that Ly6C^{high} monocytes take 2–3 days to fully accumulate in tumor tissue after arriving in the circulation [217].

Due to their well-known ability to kill tumor cells, their antigen-presenting properties and their accumulation in necrotic/hypoxic regions of solid tumors, classically activated (by IFN-γ and/or LPS) macrophages were explored as cancer treatment already in the 1990s [110,218,219]. Proper macrophage culture upscaling techniques were even developed to facilitate their clinical use in humans [218]. Indeed, the natural tendency of macrophages to be attracted to hypoxic regions in tumors presented an opportunity to target these hard-to-access areas frequently associated with resistance to chemo- and radiotherapy [220,221]. Moreover, injected macrophages had previously been shown to enter small lung metastases of B16 melanoma [222]. More recently, intraperitoneal injected macrophages were found to traffic into an experimentally-induced inflamed pancreas, indicating their potential use in a broad range of inflammation-associated pathologies [223]. The latter experiment also indirectly demonstrated that organs in the abdominal cavity are likely to be targeted with much higher efficiency after intraperitoneal injection compared to systemic injection [223]. However, since similar inflammation site-targeting efficiencies can be achieved with macrophage cell lines such as the mouse RAW264.7 as opposed to primary macrophages, a rather non-selective accumulation is to be suspected [224].

In the context of gene therapy, macrophages can be transduced with viral vectors coding for prodrug-activating enzymes. Additional layers of selectivity can be obtained by incorporating hypoxia response elements (HRE), driven by transcription factors such as hypoxia-inducible factors [225,226]. The *in vivo* validation of obtaining increased selectivity by combining HRE with therapeutic genes stalled about 20 years ago, but is still worth exploring since the clinical efficacy of systemic macrophage-based cell therapies turned out to be limited, despite being well-tolerated [218,227]. The potential of multi-layered selectivity using tumor-homing macrophages as vehicles has been described for a hypoxia-regulated oncolytic adenovirus, whose proliferation is additionally restricted to prostate tumor cells by prostate-specific promoter elements [228]. When the criteria are met, local amplification of the viral agents can then compensate for the low number of transduced cells that were able to reach the target site [229]. Additional therapeutic efficiency can be obtained when cell-delivered oncolytic virus therapy is initiated directly after tumor radiation therapy

Table 2
Commonly used cell surface markers of mouse and human blood cells.

Cell type	Mouse	Human
RBC	CD235a, TER-119	CD235a
Platelet	CD41, CD62P[a]	CD42b, CD62P[a]
Neutrophil	CD11b, Ly6G	CD15, CD16, CD66b, MPO
Eosinophil	CD11b, Siglec-F	CD11b, Siglec-8
Basophil	CD200R3	CD123
Monocytes	CD11b, CD115, Ly6C, CCR2, CD62L	CD14, CD16, CD64
T cell	CD3, CD25[a], CD69[a]	CD3, CD25[a], CD69[a]
NK cell	CD3(-), CD11b, CD27	CD3(-), CD56, CD16

[a] These markers are only present after cell activation.

[230,231]. Unfortunately, no data on the biodistribution of these vectors in organs other than the prostate tumor were included [228]. Of note, systemically administered oncolytic viruses are impeded by the same barriers as nanoparticles, e.g. interaction with blood cells, compromised access to non-vascularized regions within the tumor, dense collagen deposition and elevated interstitial pressure [232].

Summarized, much is known about *ex vivo* culture of primary monocytes. Employing this versatile cell type as drug delivery vehicles is tempting because they are involved in many pathologies and are well-tolerated after administration. Furthermore, the ability of monocytes to traffic deep into badly perfused tumors makes them appealing in these settings as well. However, monocytes are hardwired to react heavily to foreign substances and can therefore be difficult to manipulate for therapeutic purposes.

7.5. Myeloid-derived suppressor cells

Tumor-associated soluble factors induce and release immature immune cells of myeloid lineage from the bone marrow. These cells cause immunosuppression in the TME and are therefore named myeloid-derived suppressor cells (MDSCs) [233,234]. They constitute a heterogeneous population that can be broadly subdivided into polymorphonuclear (PMN-MDSC) and monocytic (M-MDSC) subsets. However, solely based on immunophenotypic markers, MDSCs are difficult to distinguish from their more mature counterparts and therefore require functional evaluation of their immature and immunosuppressive nature [233,235,236]. Interpretation of the already vague distinction between MDSC and mature myeloid cells is further complicated by non-standardized methods of MDSC isolation and storage [237,238]. Furthermore, MDSCs can differentiate into mature myeloid cells and vice versa within the TME [239]. Nevertheless, atypical myeloid subsets with distinct properties such as decreased cellular density have been clearly identified in cancer patients [240].

In tumor-bearing mice, a highly increased number of mostly PMN-MDSCs are mobilized from the bone marrow and accumulate in organs such as the lungs, the liver and the spleen [241]. The latter represents an important organ in myeloid cell dissemination and even forms a major site of extramedullary hematopoiesis in some cancer models. Splenectomy delays tumor growth by reducing aberrant myeloid cell responses in the blood and tumor tissues [242,243]. Adoptively transferred MDSCs traversed the lung and liver capillary networks more rapidly in tumor-bearing mice than in healthy control mice and subsequently massively accumulated in the spleen [241]. In addition, tumor-bearing mice supported MDSC survival, decreased their susceptibility to apoptosis and stimulated their proliferation in the spleen [241]. Hypothetically, tumor-associated factors inhibit the expression of apoptotic markers on adoptively transferred MDSCs and consequently prevent their sequestration in organs associated with cell clearance.

The increased production, survival and mobilization of MDSCs results in more circulating and recruited cells into the tumor via a multitude of chemokines, cytokines and mediators of inflammation [244]. Interestingly, increased blood PMN-MDSCs do not always correlate with increased numbers in the TME due to impaired migration functions resulting from e.g. downregulation of chemokine receptors CXCR1 and CXCR2 [240]. Conversely, the highly CXCR2-dependent recruitment of PMN-MDSCs into the TME can be blocked either by monoclonal antibodies [245] or by small molecule inhibitors [246]. Utilizing such inhibitors leads to improved therapeutic outcomes when combined with anti-PD-1 treatment, anti-CTLA-4 treatment or adoptive transfer of T cells [246,247].

Bone marrow-derived MDSCs can be generated *in vitro* via GM-CSF and IL-6 supplementation to standard culture medium [248]. This method was used to investigate the biodistribution of adoptively transferred PMN-MDSCs in an orthotopic PyMT-MMTV mammary tumor mouse model [249]. Intravenously injected DiD-labeled PMN-MDSCs mostly accumulated in the liver, the spleen and the lungs but fluorescence was also observed at the primary tumor, both at 48 hours and at 7 days after injection [249]. Moreover, injected MDSCs also co-localized with tumor metastases in the adrenal gland but off-target splenic accumulation was seen as well [249]. However, histological verification should be performed to evaluate whether this acquired fluorescence originated from intact labeled MDSCs [249,250].

The tumor homing tendency of mouse Ly6C$^+$ MDSCs has been exploited to more efficiently deliver oncolytic viruses into the TME [251]. However, this study used an intrahepatic tumor model in which the obtained “tumor-specific” MDSCs are likely confounded with MDSCs residing in the liver. Another study reported delivery of attenuated *Listeria monocytogenes* bacteria engineered to express tumor antigens via MDSCs into mouse primary tumors and metastases [252]. In addition to their ability to elicit antigenic responses, these bacteria infect and kill tumor cells as well [253]. Despite infecting both MDSC populations, bacterial proliferation and subsequent delivery of *L. monocytogenes* occurred mostly via M-MDSCs and resulted in equally efficient delivery at the primary tumor and tissue metastases of both young and old mice. In contrast, a much lower bacterial load was demonstrated in the spleen [252] although assessing the contribution of the MDSC as vehicles is difficult since much of the selectivity might be related to the bacteria alone.

In conclusion, it remains unclear whether MDSCs demonstrate higher tumor recruitment potential compared to mature myeloid cells. Further studies are needed to answer this question and to unravel how the migration of MDSCs differs from other immune cell populations.

8. T cells

The trafficking properties of T cells are currently intensely investigated due to their potent capacity to eradicate tumor cells, certainly since the availability of clinically applicable methods to genetically engineer CTLs that are able to kill cells expressing an antigen of choice [254,255]. Some of the specific properties of lymphocytes in relation to myeloid cell trafficking will be summarized (Table 1), for more detailed information readers are kindly referred to the excellent up-to-date reviews on this subject [62,256–259].

Cells of the adaptive immune system show some remarkable differences with innate immune cells. T cells develop in the thymus instead of the bone marrow, enter the circulation in a “naive” state and require multistep activation to initiate their effector functions. Moreover, they have the capacity to proliferate upon activation thus amplifying their immunological contribution and to produce long-lasting memory cells. Importantly, this property is exploited to acquire sufficient cells for T cell-based therapies [260]. In further contrast to myeloid cells, naive T cells mainly traffic through lymphoid tissues via L-selectin and CCR7-mediated interactions with corresponding ligands i.e. PNAd and CCL19/21, respectively, on specialized high endothelial venules in lymph nodes [261]. Once activated in these lymphoid tissues, T lymphocytes downregulate their surface expression of CCR7, egress the lymph node via their sphingosine 1 phosphate receptor 1 and upregulate other adhesion receptors to acquire the capacity to migrate into non-lymphoid tissues [56,62]. Interestingly, these newly expressed adhesion receptors partly differ depending on the tissue location of the priming lymph node and antigen-presenting dendritic cells, thereby bestowing a certain vascular “ZIP code” on the activated T lymphocyte. This combination of acquired adhesion receptors/ligands corresponds to adhesion ligands/receptors in the tissue where the priming occurred [62,256]. Consequently, eliciting adaptive immunity through vaccination preferentially occurs in the targeted tissue. Some homing receptors/ligands such as CXCR3 and PSGL-1 are upregulated independent of the activation site and inflammation utilizes many of the homing receptors/ligands normally restricted to specific tissues in steady state conditions [256]. This inflammation-induced upregulation of chemokines and vascular receptors is often actively inhibited (e.g. by

VEGF) in solid tumors, but can be reinitiated by experimentally introducing stimulators of inflammation such as Toll-like receptor (TLR)-agonists [256,262,263].

Aberrant tumor blood vessel morphology also contributes to defective T cell homing. In this regard, low-dose anti-angiogenic therapy might be more beneficial in treating solid tumors than high-dose therapies as the former allows normalization of the tumor vasculature which allow more efficient recruitment, adhesion and extravasation of T cells [48]. In addition to normalizing the tumor vasculature, tumor-matching chemokine receptors such as CXCR2 [264], CX3CR1 [265], CCR4 [266] and CCR2 can all be genetically introduced into T cells [257,267,268]. Unfortunately, clinical studies with therapeutic T cells have indicated that addition of these chemokine receptors only modestly improved the infiltration in humans [257]. Yet, an interesting link between myeloid cells and lymphocyte recruitment was recently demonstrated: infiltration of immunosuppressive PMN-MDSCs in mouse tumors was substantially reduced upon administration of a small molecule inhibitor of both CXCR1 and CXCR2 [246]. In turn, this greatly enhanced T cell accumulation in these tumors, indicating that myeloid cells residing in the TME contribute to constraining lymphocyte recruitment.

Chimeric antigen receptors (CARs) were originally engineered to confer selective T cell-mediated killing of target cells bearing the antigen of choice independent of MHC. However, the same principle can be used to elicit expression of a certain gene of choice after recognition of the antigen. In essence, this makes CAR T cells drug delivery vehicles for therapeutic genes such as cytokines and they are therefore termed "T cells redirected for universal cytokine killing" (TRUCKs) [269]. For example, TRUCKs can be used to locally express IL-12 after recognition of its cognate antigen [270]. Indeed, this is a very elegant system because IL-12 is a cytokine that elicits potent Th1 cellular immune responses but cannot be administered systemically due to unacceptable toxicity [271]. Its expression can be linked to either CAR or TCR activation through incorporation of a nuclear-factor of activated T cells (NFAT) minimal promoter [269]. Due to the proliferative property of T cells, TRUCKs which constitutively express IL-12 can also be constructed [272]. Systemic injection of a small number of such IL-12 TRUCKs will not elicit toxic effects [273]. However, after encountering the targeted cells, T cells massively proliferate and exponentially increase local IL-12 concentrations until therapeutic effects are achieved [272]. Nevertheless, induced expression of IL-12 is considered more safe than constitutively expressed IL-12 [269]. Cytotoxic T cells can also be used to deliver drugs adsorbed to their surface. For example, Jones et al. (2016) conjugated drug-loaded lipid nanoparticles to the surface of T cells [274]. Upon recognition of their cognate antigen, the T cells secrete perforins which not only lyse the target cells but also release the drug from the adsorbed lipid nanoparticles. The drawback of CAR or TCR-mediated release is that it requires prior knowledge on the targeted antigen. Choosing the wrong antigen can result in severe "on-target off-tumor" side effects. Moreover, targeting a single antigen is often insufficient in constantly evolving heterogeneous pathologies such as cancer [56,256]. Alternatively, more stable TME-associated antigens such as VEGFR-2 can be targeted as well [275].

The development of multiple generations of CAR T cells have provided extensive insight into the possibilities of genetic engineering. In fact, CARs have also been inserted in other cell types [276]. Facilitated by gene-editing technology, researchers are exploring genetic knock-out of MHC-associated genes to prevent host immune recognition of adoptively transferred cells [277,278]. A complementary strategy is to remove native T cell receptors from CAR T cells to prevent graft versus host reactions. Consequently, allogeneic cells can be used to create "off the shelf" cell delivery vehicles.

9. Natural killer cells

Treating tumors with bioengineered CAR T cells requires sufficient functional T cells to reach the tumor. This poses a problem in treating immunosuppressive tumors or "cold tumors", in which T cells cannot permeate [279]. Natural killer (NK) cells are currently explored to overcome some of the limitations associated with T cells [280]. TCR/CD3-negative NK cells are lymphocytes which possess essential functions for the innate immune system. This makes NK cells interesting for cancer immunotherapy [281]. Their cytotoxic effector mechanisms include perforin/granzyme-containing granules, antibody-dependent cell-mediated cytotoxicity (via FcγRIII or CD16) and cell death through death-receptor and interferon-γ mediated pathways [282]. Through both activating and inhibitory receptors, they can recognize target cells based on reduced expression of inhibitory ligands and/or increased expression of activating ligands and thus exert broad and rapid cytotoxicity against e.g. virus-infected cells or tumor cells [283]. For example, loss of MHC class I molecules (inhibitory signal) and/or increased expression of stress ligands such as MICA, MICB and ULBPs (activating signal) makes cells susceptible to NK cell-mediated lysis [283].

Fueled by the success of CAR T cells, NK cells are now also genetically modified with CARs [284]. In essence, this makes CAR-NK cells function as adaptive immune cells via their tumor antigen-recognizing CAR and as innate immune cells through their native receptors. This combination makes tumor escape through downregulation of CAR targets less likely. Moreover, no cytokine release syndrome is expected since NK cells do not proliferate upon stimulation [285]. Importantly, because NK cell-mediated cytotoxicity is MHC-independent, they lack the potential to cause graft-versus-host disease, which allows NK cells to be used as off-the-shelf allogeneic therapeutics [283].

Whereas all lymphoid and non-lymphoid tissues contain NK cells, most human NK cells in the lymph nodes are of the immature $CD56^{bright}/CD16^{low}/perforin^{-}$ subset and about 90% of the NK cells found in the blood and the spleen are of the more mature cytolytic $CD56^{dim}/CD16^{+}/perforin^{+}$ subset(Table 2) [282,286]. The non-cytotoxic $CD56^{bright}$ and cytotoxic $CD56^{dim}$ NK cell subsets are also characterized by a different set of chemokine receptors [287,288]. In mice, the functional classification occurs through CD27/CD11b characterization. Immature mouse NK cells are double negative CD27-/CD11b-. Next, they mature into CD27+/CD11b- to CD27+/CD11b+ and finally CD27-/CD11b+ NK cell subsets [289].

In vitro cultivation of primary NK cells is technically challenging. Current strategies therefore rely on infusing irradiated NK cells from the human NK-92 cell line which retain their cytotoxicity but exhibit suppressed proliferation to prevent their permanent engraftment [283]. As a result, CAR-NK-92 cells remain in circulation for about 48 hours [290] and are no longer detectable within 7 days of adoptive infusion (Table 1) [291]. Alternatively, human embryonic stem cells [292] or CAR-NK cells derived from induced pluripotent stem cells demonstrate equal anti-tumor capacities as CAR T cells, but with a better safety profile [293,294]. Of note, activating the NK cells prior to adoptive transfer significantly improves their tumor trafficking in mice 1 hour after administration but also causes them to accumulate in off-target organs such as the lungs, the liver and the spleen [282]. Such activated NK cells also demonstrate accumulation at pulmonary metastases. This interesting property of NK cells was used to deliver membrane-linked doxorubicin to B16 melanoma metastases in the lung thereby severely concentrating doxorubicin at these sites [295].

10. Employing nanoparticles to load tumor-targeting immune cells

The focus of cell-based drug delivery is gradually merging with the field of nanoparticles [296]. Careful nanoparticle design considerably improves drug pharmacokinetics, but still lacks efficient targeting capacity. However, when combined with the inherent properties of immune cells to target inflamed regions, a marked increase in delivery

efficiency has been reported [148]. For example, in a recent study Ly6C^{high} monocytes loaded with paclitaxel-containing pH-sensitive micelles demonstrated increased paclitaxel accumulation in the primary tumor and pulmonary metastases when compared to micelles directly injected in the blood [297]. Moreover, about one decade ago macrophages loaded with nanoparticles containing an antiviral drug severely prolonged drug release and brain deposit in an HIV-1 encephalitis mouse model [298]. However, *ex vivo* culture of monocytes and loading them with nanoparticles or reporter genes can negatively impact the delivery efficiency of these cells [299].

Another example is to coat nanoparticles with anti-CD11b to target circulating myeloid cells. After inducing an acute inflammation reaction in the TME by photosensitization therapy, neutrophils that have taken up these anti-CD11b nanoparticles are recruited into the TME [300]. Moreover, since neutrophil infiltration has been observed after radiation therapy it can complement this frequently used cancer treatment modality [301]. Although this strategy resulted in higher delivery of nanoparticles in the TME, radiation therapy still requires knowledge about the exact position of the TME and hence, is not practical for targeting metastases of unknown locations. Nevertheless, systemic immunotherapy using antibodies directed against melanoma antigens is also associated with neutrophil influx and could therefore benefit from this approach as well [302]. Still, two additional problems arise when employing immune cells to deliver therapeutic nanoparticles on site: [1] drug leakage from the nanoparticle into the immune cell can compromise its function and viability, [2] immune cell recruitment inevitably results in excessive drug deposition into sequestration organs such as the lungs, the liver and the spleen [303].

For example, in a recent study Ly6C^{high} monocytes loaded with paclitaxel-containing pH-sensitive micelles demonstrated increased paclitaxel accumulation in the primary tumor and pulmonary metastases when compared to micelles directly injected in the blood [297]. Moreover, about one decade ago macrophages loaded with nanoparticles containing an antiviral drug severely prolonged drug release and brain deposit in an HIV-1 encephalitis mouse model [298]. However, *ex vivo* culture of monocytes and loading them with nanoparticles or reporter genes can negatively impact the delivery efficiency of these cells [299].

Multiple strategies have been described to load cells with synthetic or biological therapeutic agents. For example, enzymes can be encapsulated into red blood cells via hypotonic dialysis and isotonic resealing [304] or can be attached to cell membranes via integration of biotin-phospholipid integration followed by binding of avidin-conjugated agents [305]. Alternatively, cell-penetrating peptides are used to facilitate intracellular delivery of hydrophilic agents or macromolecular substances [306]. Of particular interest is the use of nanoparticle drug carriers which can be coated with cell membranes [307,308] or attached/loaded into the cellular vehicles. Membrane coating transfers beneficial properties of the source cells (e.g. targeting ligands/receptors and “don't-eat-me” signals) to the used nanoparticles. This strategy is covered in the review by Zhang et al. (2019) [309]. Attaching nanoparticles to endogenous circulating cells is known as “cellular hitchhiking” [310] and can be achieved by non-covalent adsorption via hydrophobic, electrostatic, Van der Waals or hydrogen bonding [310,311]. In addition, drug carriers can be attached to cells via receptor-ligand interactions [312] or they can even be covalently attached to functional thiol [313] or amine [314] residues on the cell surface [315]. Internalization of adherent nanoparticles through endocytosis, pinocytosis or phagocytosis is considered a limiting factor but can also be used to load therapeutic cargo in phagocytes [315,316]. However, the latter approach requires proper protection against intracellular degradation mechanisms.

11. Challenges and future perspectives

11.1. The lungs, the liver and the spleen

To scout the entire organism for possible target sites such as cancer metastases, systemic administration is mandatory, but also obligates the injected cells to pass through certain checkpoints. Typically, the lungs are the first checkpoint encountered after intravenous injection [303]. Priming or activation of immune cells leads to cytoskeleton rearrangements which makes them more rigid and exhibit a higher tendency to get stuck into capillary beds such as in the lungs [159,165,166,317]. Likewise, a clear size-dependent pulmonary accumulation of intravenously injected nanoparticles has been described in rats [318,319]. Simultaneous treatment with vasodilation agents such as sodium nitroprusside helps cells to pass through the lungs more rapidly, further indicating that physical obstruction is at least partly responsible for the pulmonary retention of injected immune cells [320,321]. After their release from the lungs, systemically injected cells are distributed among all other organs.

The two main sites of subsequent sequestration are the liver and the spleen [303,322–324]. It is difficult to assess what exactly happens to the immune cells in these two organs and whether the observed retention in these secondary “checkpoints” is reversible. One possibility is that the immune cells accumulate for mere physical reasons (e.g. large vascular volume of these organs or small capillary diameters) [325]. Given that spleen contraction is susceptible to α-adrenoceptor agonists, treatment with adrenaline or epinephrine can possibly elicit leukocyte mobilization from this reservoir [326]. Although spleen contraction substantially increases blood hematocrit in horses and dogs, it appears to be far less influential in humans [327]. Nonetheless, catecholamine-mediated demargination combined with expulsion of splenic leukocytes likely contributes to increased circulation of adoptively transferred immune cells [157,158,326]. Moreover, immune cell mobilization can readily be enhanced through exercise, which increases immune cell infiltration in tumors and subsequent therapeutic effect [257]. Removing the spleen before injection of immune cells resulted in increased liver accumulation of these cells, but their short-term trafficking to tumors remained unaffected [328]. This key observation could indicate that the luminal surface of the tumor vasculature is saturated with endogenous immune cells, hindering injected cells to find an available attachment site [329]. Consequently, intratumoral cell accumulation is a gradual process that requires multiple days or even weeks to complete.

Another possibility is that injected immune cells are actively arrested by other cells. The removal of dead or compromised cells from circulation (efferocytosis) by cells of the MPS is a major function of both the spleen and the liver [330]. Any deviation of the cell surface caused by experimental manipulations such as isolation, *ex vivo* culture, transfection and labeling likely activates markers of efferocytosis. This is exemplified by the rapid disappearance of optimally stored red blood cells (RBCs) after transfusion [331]. In addition, other “eat me” signals such as phosphatidylserine externalization, changes in either charge or glycosylation patterns, ICAM-1 alterations and exposure of calreticulin on the cell surface all mark a cell for phagocytosis by the MPS [332]. Interestingly, masking phosphatidylserine exposure via annexin V prevented subsequent phagocytosis and could therefore be explored as a way to decrease the arrest of adoptively transferred immune cells in organs participating in efferocytosis [333]. Our group has recently demonstrated that depleting the MPS via pretreatment with clodronate liposomes drastically increased both the circulatory half-life and tissue accumulation efficiency of infused bone marrow-derived monocytes [329]. In addition, no change in monocyte distribution pattern was observed after depletion of endogenous neutrophils [329]. Alternative strategies could consist of depleting cells that express apoptotic markers before their infusion, or to incorporate cell surface “don't eat me” signals such as CD47 into the cellular vehicles [334]. In addition, adding

anti-apoptotic agents to the culture medium can perhaps diminish the fraction of injected cells that express apoptotic markers [335]. Compared to RBCs, decorating the cell surface with polyethylene glycol (PEGylation) is likely less efficacious on immune cells as these modifications have been shown to interfere with surface molecules. Hence, PEGylation will likely inhibit the migration cascade of immune cells [113].

11.2. Prolonging the circulatory half-life of immune cells as the first pillar

Despite the preference for recruiting certain immune cell types to the TME in the long-term, the short-term accumulation of injected immune cells is mainly of non-specific nature [303,328]. Retention of injected immune cells therefore likely depends primarily on physical properties such as tumor perfusion, total tumor blood volume and cell flow rate. This underscores the importance of optimizing essential physical properties such as the circulatory half-life rather than identifying optimal injection sites [336]. Nevertheless, the rapid recruitment of endogenous neutrophils in acute inflammatory contexts suggests that some level of short-term trafficking of specific immune cell subsets is possible but we should bear in mind the high number of circulating neutrophils in steady-state conditions [337] and the induced reactive neutrophilia in inflammatory conditions which further helps circulating neutrophils to accumulate at the pathological site by ensuring their constant passage [338].

Considering these general remarks, prolonging the circulatory half-life of cell vehicles should be designated as the first pillar in this research field and preventing the immediate recognition of altered cell surfaces should be prioritized in future research endeavors (Fig. 4). Even when injected cells are not immediately sequestered in organs such as lungs, spleen and liver, only a small portion of them will actually travel through the blood vessels of their targets sites. Moreover, interaction with the endothelium through rolling, adhesion and transmigration is only reserved for a minority of passing cells and thus, the majority of the cellular vehicles will return to the heart and the lungs to restart the cycle. Therefore, like passively flowing particles, these injected cells also largely depend on their circulatory half-life to increase their chances of successfully engaging the adhesion cascade at their target sites.

Based on the demargination and consequential leukocytosis mediated by glucocorticosteroid administration, it would perhaps be interesting to further investigate whether glucocorticosteroids can also increase the circulatory half-life of adoptively transferred immune cells [339–342]. Moreover, this class of anti-inflammatory and strongly immunomodulating drugs also prevents apoptosis of neutrophils and can potentially inhibit delayed adaptive immune responses against therapeutic transgenes [343]. Despite the existence of a vascular ZIP-code system for primed effector T cells, specific tissue migration is likely hampered by the shared expression of adhesion molecules in different organs [62]. Therefore, instead of trying to acquire increased specificity via addition of adhesion receptors in cellular vehicles, it would perhaps be more efficient to mask the non-specific receptors/ligands already present on these cells. This would then substantially decrease interaction with adhesion receptors in non-targeted organs and possibly increase circulation half-life and target site accumulation of the injected cells.

One of the many exciting evolutions of the past decade is the merging of the nanoparticle delivery field with research on immune cell trafficking [148,300,302,344,345]. The main strategy is to bestow immune cell-targeting properties to nanoparticles containing a therapeutic agent. After systemic injection, circulating immune cells then adsorb or engulf the nanoparticles, effectively guiding them into diseased tissues such as solid tumors. Given that leakage from the nanoparticles can be constrained, “piggybacking” endogenous immune cells can potentially bypass the clearance problems associated with *ex vivo* manipulations.

11.3. Conditional release as the second pillar

Even if we assume that the hurdles of efferocytosis and circulatory half-life could be adequately addressed, unwanted off-target accumulation by physical means such as differences in total vascular volume between organs, will always hamper drug delivery efficiency. By taking advantage of the many possibilities that cells offer for genetically engineering multilayered selectivity, investing substantial research efforts into developing tools for conditional release should form the second pillar of obtaining true targeted delivery (Fig. 4). Currently pursued

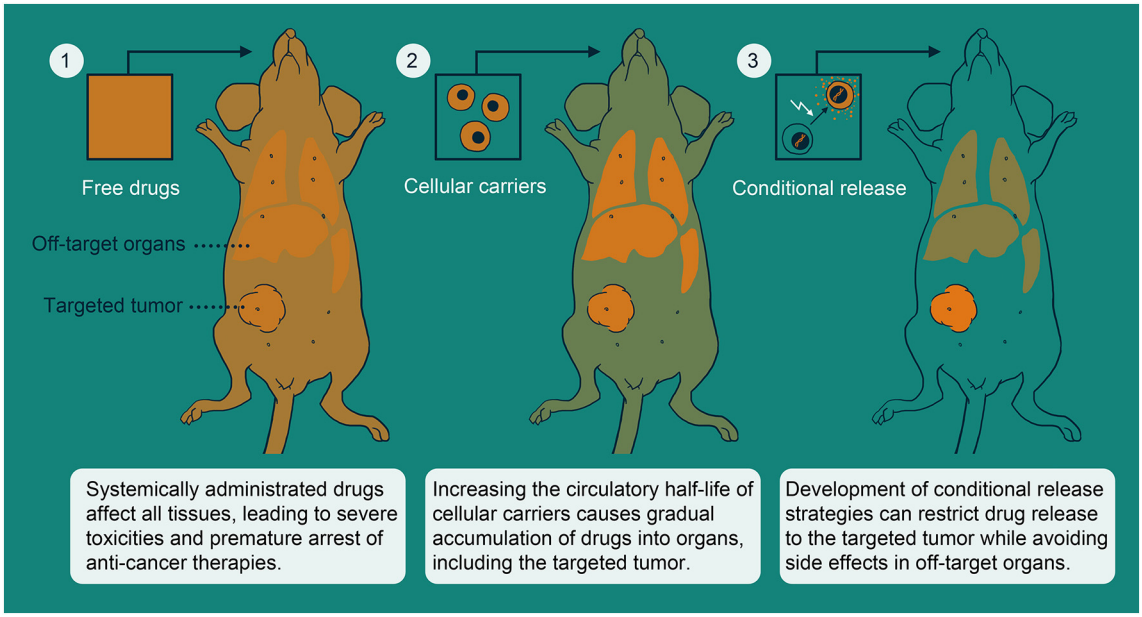

Fig. 4. Future perspectives on using immune cells as targeted drug delivery vehicles. As a first pillar for future research endeavors, we propose increasing the circulatory half-life of cellular carriers to improve non-specific tumor accumulation. As a second pillar, conditional release mechanisms should be developed which release the drug from its carrier based on specific cues in the tumor.

avenues include local amplification of adoptively transferred T cells after recognition of their cognate antigen [346], local amplification of oncolytic viruses [347] and development of genetic switches [348–350] that control transgene expression based on environmental cues. In our opinion, the development of multilayered genetic switches has the most potential in terms of flexibility (i.e. back-and-forth optimization and adaptation). These switches do not necessarily require prior knowledge about a specific antigen, but can link cell phenotypic changes based on environmental cues in the TME with the conditional expression of a therapeutic transgene. For example, combining a miRNA-driven approach [351] with other selective expression techniques such as polarization-dependent promoters [352] or enhancers [353] can undoubtedly ensure specific transgene expression in targeted tissues. In this regard, integrating hypoxia responsive elements (HREs) in the genetic constructs can confine transgene expression to the badly perfused tumor regions [225,226]. Nevertheless, these HREs could also impose too much restrictions (e.g. early metastatic sites do not necessarily contain hypoxic regions) and therefore limit the therapeutic relevance of this construct.

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