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Review article

Targeting myeloid cells in the tumor sustaining microenvironment

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ABSTRACT

Myeloid cells are the most abundant cells in the tumor microenvironment (TME). The tumor recruits and modulates endogenous myeloid cells to tumor-associated macrophages (TAM), dendritic cells (DC), myeloid-derived suppressor cells (MDSC) and neutrophils (TAN), to sustain an immunosuppressive environment. Pathologically overexpressed mediators produced by cancer cells like granulocyte-macrophage colony-stimulating- and vascular endothelial growth factor induce myelopoiesis in the bone marrow. Excess of myeloid cells in the blood, periphery and tumor has been associated with tumor burden. In cancer, myeloid cells are kept at an immature state of differentiation to be diverted to an immunosuppressive phenotype. Here, we review human myeloid cells in the TME and the mechanisms for sustaining the hallmarks of cancer. Simultaneously, we provide an introduction into current and novel therapeutic approaches to redirect myeloid cells from a cancer promoting to a rather inflammatory, cancer inhibiting phenotype. In addition, the role of platelets for tumor promotion is discussed.

1. Introduction

Myeloid cells are one of the main cell branches generated in the medulla of the bone marrow during hematopoiesis. Since the discovery of hematopoietic stem cell(s) (HSC) in 1961, the classical model of blood stem cell differentiation has been under continuous revision. HSC are multipotent, have a strong power to self-renew, and are the progenitors of all blood cells. In the classical model, HSC develop into multipotent progenitor cells (MPP), differentiate into the oligopotent common myeloid progenitors (CMP), further into oligopotent precursor cells (MEP, megakaryocyte-erythroid progenitors; GMP, granulocyte-monocyte progenitors) and finally into unipotent myelo-monocytic (monocyte) and granulocytic cells (basophil, eosinophil, neutrophil), mast cells, megakaryocytes and erythrocytes (Fig. 1A) [1,2]. According to the revised model of human blood stem cell differentiation in adults, multipotent HSC, also referred to as continuum of low-primed undifferentiated hematopoietic stem and progenitor cells (CLOUD-HSPC) [3], differentiate continuously into unipotent cells of the myeloid lineage (monocytes, granulocytes (basophil, eosinophil), neutrophils, mast cells), megakaryocytes (platelets) and erythrocytes (Fig. 1B) [3–5]. In the tissue, monocytes can differentiate further into macrophages and dendritic cells (DC). To summarize the new findings, the intermediate states of oligopotent progenitors and the tree like structure of HSC differentiation were superseded by a continuous differentiation, illustrated in a landscape, which was originally developed by Conrad Hal Waddington in 1958 for epigenetic studies [3]. Notta et al. found that megakaryocyte progenitor cells may originate directly from HSC [4].

In this review, we focus on the most prominent myeloid cells in the TME derived from the CMP as defined by the classical definition to discuss the phenotype and underlying mechanisms of important cell types in the tumor microenvironment (TME) (Fig.2). Recent studies showed that also mast cells play an increasing role in some tumors regarding tumor initiation and development [6]. Nevertheless, in several tumor entities they also possess protective capacity. As there are many conflicting results and unanswered questions so far (excellently reviewed in [7]), we do not focus on this type of cells in the present review.

Myeloid cells are key players of the innate immune system. In healthy individuals, their functions include phagocytosis of foreign

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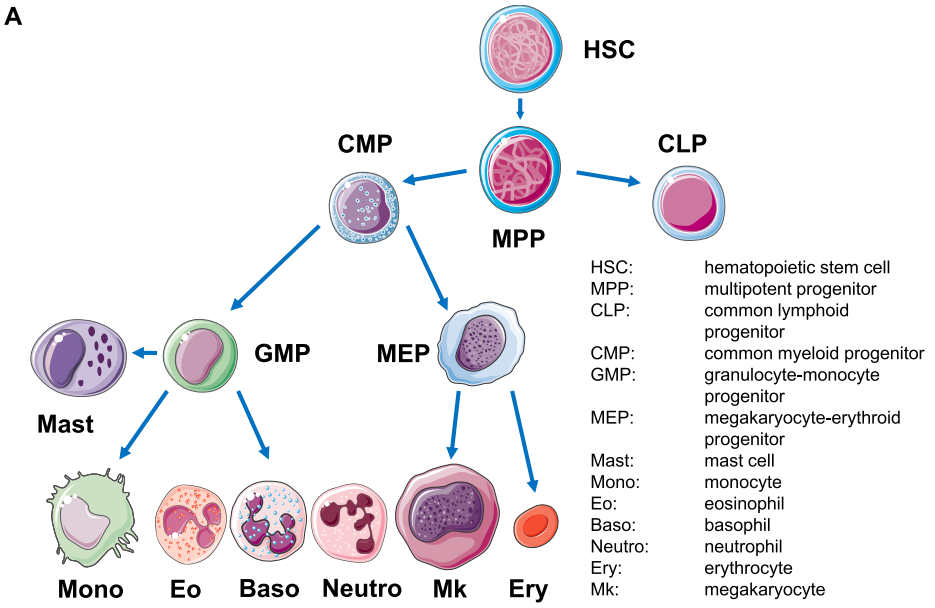

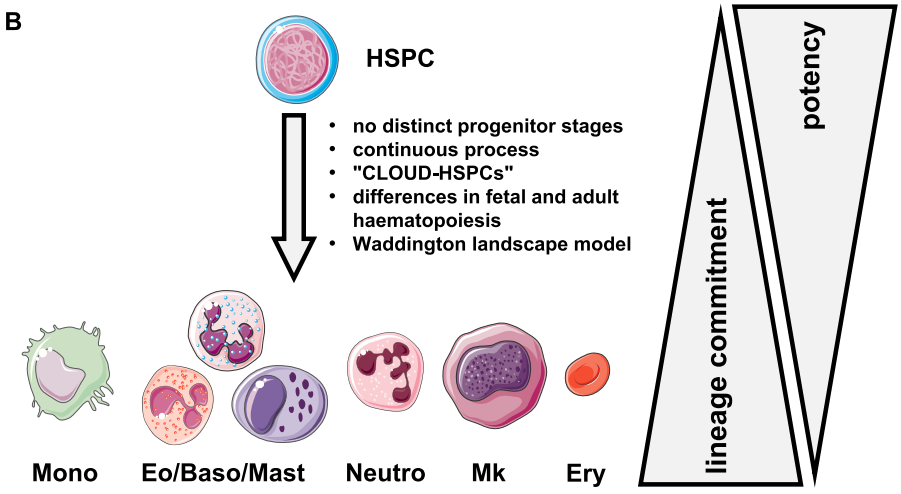

Fig. 1. Hematopoiesis. (A) The "classical" view of hematopoiesis includes oligopotent progenitor cells for the myeloid and lymphoid lineages originating from multipotent hematopoietic stem cells (HSC). (B) Current hypothesis. New findings indicate that bone marrow hematopoiesis is a continuous process in which hematopoietic stem and progenitor cells (HSPC) gradually gain their lineage commitment through complex gene regulatory networks without distinct progenitor states. In early stages, HSPC display a high plasticity, in later stages a switch between lineages is more unlikely. Due to gene expression signatures, monocytes and lymphocytes as well as megakaryocytes and erythrocytes share a close relationship. Derived from the common lymphoid progenitor cell (CLP), lymphocytes mainly develop outside the bone marrow and hence are not depicted. CLOUD-HSPCs, continuum of low-primed undifferentiated hematopoietic stem and progenitor cells.

bacteria (macrophages), processing and presenting antigen (DC), first response to inflammation (neutrophils), and homeostasis and wound healing (platelets). Soluble factors, such as cytokines and chemokines, regulate the migration of myeloid cells from the bone marrow to the blood, and finally into tissues including cancers. These and other short range mediators can affect the total turn-over and functional development of myeloid cells.

The term tumor microenvironment includes the cancer cells, the associated vasculature, recruited immune cells (myeloid cells, lymphoid cells), platelets, (desmoplastic) fibroblasts, and the extracellular matrix (ECM). An illustration of the tumor microenvironment as a whole is included in *Balkwill* et al. [8]. The TME develops simultaneously with the tumor. In the beginning, cancer stem cells utilize inflammatory chemotaxis to gather endogenous hematopoietic (lymphoid, myeloid cells) and mesenchymal cells (fibroblasts/myofibroblasts, mesenchymal stem cells (MSC), pericytes, adipocytes, endothelial cells). Moreover, cells in the tumor are embedded in an ECM [9–11]. The structure of the tumor ECM consists of fibrillar and nonfibrillar collagens, proteoglycans, glycosaminoglycans, and noncollagenous glycoproteins, all which have not only structural functions to maintain a multicellular tissue organization by anchoring cells, but that also provides highly complex cues for their differentiation, migration and metabolism. While a particular role has been assigned to the multiple ECM components of basement membranes, it has become clear that also classical ECM molecules and their degradation products significantly modulate the adjacent stromal and epithelial cells [10–12]. In this setting, the tumor not only gives rise to newly generated cancer cells, but also manipulates attracted cells to remodel the ECM, creating an environment reminiscent of wound healing and chronic inflammation, which is typical for the TME. The particular TME also depends on the stage of malignancy and tissue/organ site of the tumor [13,14].

The effect of the TME on myeloid cells is best reflected in their state of differentiation, migration, and activation. Myeloid cells like monocytes patrol the blood and mature into macrophages and DC in the tissue, where they can be activated by bacteria and viruses to prominently phagocytose bacteria (macrophages) or present (foreign) antigens (DC) to T cells. Under normal conditions, myeloid cell activation is transitory, and myeloid cells reacting to the presence of bacteria and viruses are activated as long as the inflammatory state is maintained by a strong stimulus like pathogen-associated molecular patterns (PAMPs).

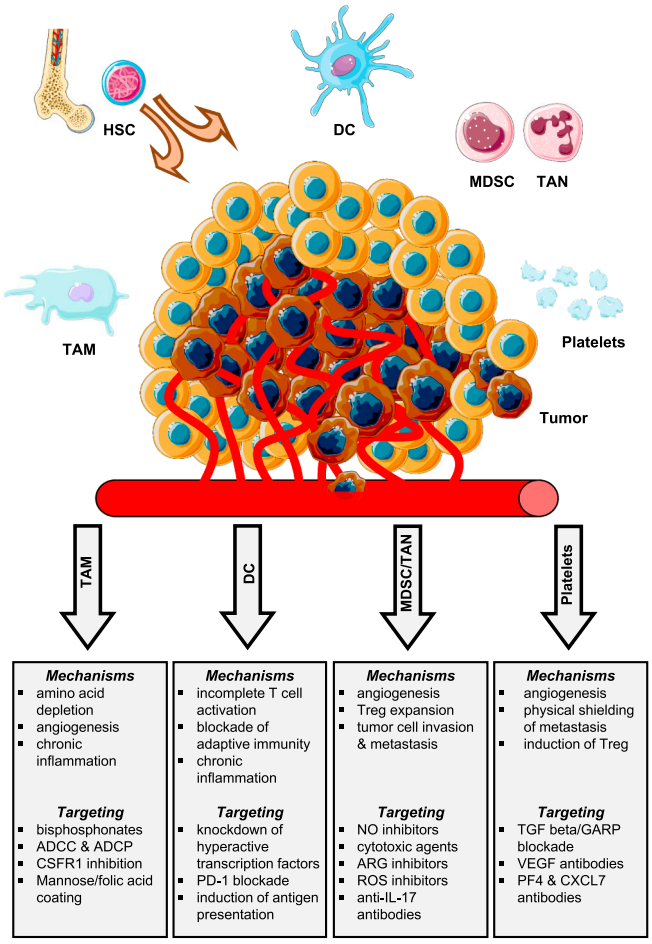

Fig. 2. Myeloid cells in the tumor microenvironment (TME). The upper part shows the most prominent CMP-derived cells in the TME consisting of tumor-associated macrophages (TAM), tolerogenic dendritic cells (DC), myeloid-derived suppressor cells (MDSC) and tumor-associated neutrophils (TAN) and platelets. Platelets can recruit other immune cells and surround tumor cells if they enter the bloodstream and thereby support metastasis. The lower part depicts immune suppressive mechanisms used by these cells and strategies to target them. CMP, common myeloid progenitor cell; HSC, hematopoietic stem cell; PD-1, programmed death receptor-1; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ROS, reactive oxygen species; NO, nitric oxide; ARG, arginase; TGF, tumor growth factor; GARP, glycoprotein A repetitions predominant.

As a result, the activated cells unleash pro-inflammatory cytokines to recruit other immune cells and function, with strong phagocytotic activity and high reactive oxygen species (ROS) production to destroy and process the engulfed material. Overall, the process is directed, effective, and rapidly occurring and most importantly transient. In contrast, in the presence of chronic inflammation like in the tumor, myeloid cells are continuously activated by a weak stimulus which leads to low phagocytic activity, a consistently strong respiratory burst, and the release of anti-inflammatory cytokines. Therefore, the pathologic activation presents as a continuous and rather undirected activation. Due to the immunosuppressive capacity of these cells, it has been argued that this form of activation is meant to provide protection against chronic inflammation in “wounds that do not heal” [15]. Overall, in the TME most myeloid cells display an immature phenotype. Pathologically enhanced turnover of myeloid cells and constant migration of new immature myeloid cells to the tumor leads to a measurable increase of tumor-infiltrating myeloid cells [16].

Thereby, the tumor exploits pathologically modulated myeloid cells to sustain constituent inflammatory mechanisms supporting the hallmarks of cancer [17,18]: tumor proliferation, invasion and metastasis, immune escape, prevention of cell death, induction of neoangiogenesis, and the reorganization of cellular metabolism. Genetic or epigenetic traits such as promotion of inflammation and autophagy, and destabilization/mutation of DNA are serving as tumor promoters [19,20].

In our review, we highlight the role and relevance of myeloid cells in the TME as promoters of tumor progression and as potential targets for novel therapeutic approaches.

2. Tumor-infiltrating myeloid cells and platelets

2.1. Macrophages

Macrophages are known as large phagocytotic cells which aid in innate immune defense by engulfing and degrading pathogens, like bacteria or microbes, which are recognized by pathogen-recognition-receptors (PRRs). In recent years, the historic view of the mononuclear-phagocyte-system [21] has been overthrown by the discovery that tissue macrophages originate from embryonic precursors, and maintain their numbers without replenishment by monocytes in the steady state. The monocytes in the blood only replenish macrophage or DC numbers in certain situations like inflammation or cancer [22]. Similarly, like the closely related DC, they bridge innate and adaptive immunity, as macrophages are professional antigen-presenting cells (APC) which obtain the help of antigen specific T cells in fighting pathogens. However, this function is just one of many. Their distribution in the (human) body in almost all tissue types, even in the brain, results in many different names for one cell type and stresses their importance. Macrophages are responsible for tissue homeostasis by removing the remains of dead or old cells, like the erythrocyte turnover in the spleen. They are also important for the initiation and resolution of inflammation. Tissue regeneration and angiogenesis is guided by macrophages, too. These numerous processes controlled by macrophages illustrate the plasticity of this cell type. It is understandable, that pathogen killing cells and wound healing cells differ in their signature of expressed genes and secreted cytokines. These two different states represent the extremes of macrophage polarization and have been described as M1 (proinflammatory, classical) and M2 (wound healing, alternative) macrophages.

Markers commonly used to identify the most polar phenotypes of the macrophage spectrum are a combination of CD68, CD64, CD40, CD80 and CD86 for M1 macrophages and CD68, CD163 and CD206 for M2 macrophages. Even the pan-macrophage marker CD68, which is widely used in immunohistochemistry (IHC) is under debate and non-exclusively expressed on macrophages [23,24]. Markers like CD11c, CD11b, HLA-DR, CD14 and interleukin-4 receptor alpha (IL-4R alpha) are found on multiple cell types [25]. The concurrent marker expression of both polarization states albeit to a different extend complicates this situation, especially in staining techniques which are not capable of dissecting differing levels of development.

A different and probably better approach is the identification of certain macrophage phenotypes by function. Inflammatory M1 macrophages secrete a different cytokine signature than their M2/TAM counterpart. M1 cytokines include tumor necrosis factor alpha (TNF alpha), IL-12, IL-1, interferon gamma (IFN gamma), IL-6, IL-23 and chemokine (C-X-C motif) ligand 10 (CXCL10). M2 factors include tumor growth factor beta (TGF beta), IL-10, interleukin-1 receptor antagonist (IL-1RA), chemokine (C−C motif) ligand 22 (CCL22), CXCL12, vascular endothelial growth factor A (VEGFA) and matrix metalloproteinase-9 (MMP-9). M2 macrophages also favor immune suppression through arginase mediated L-arginine and indoleamine 2,3-dioxygenase (IDO) mediated tryptophan metabolism. The former suppresses T cell responses by L-arginine depletion whereas the latter acts by depleting extracellular tryptophan. In contrast, M1 macrophages use L-arginine to generate ROS via inducible nitric oxide synthases (iNOS) thereby fighting pathogens.

In vitro M1 macrophages are generated by the addition of lipopolysaccharide (LPS), IFN gamma, or growth factors like granulocyte-

macrophage colony-stimulating factor (GM-CSF) or a combination of these factors. Through signaling via toll-like receptor 4 (TLR4) and interferon gamma receptor (IFNGR) and the subsequent action of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and phosphorylated signal transducer and activator of transcription 1 (pSTAT1), M1 genes are activated. Macrophages are shifted into the M2 direction *in vitro* if they encounter IL-4, IL-13 and/or IL-10.

These cytokines induce the phosphorylation of STAT3 (IL-10) or STAT6 (IL-4/IL-10). IL-10 is able to block TLR4 induced signals by forming a p50/p50 homodimer NF-κB which is a repressor and competes with the p50/p65 activator [26]. Macrophage polarization and nomenclature has already been reviewed in detail [27–29].

2.1.1. Macrophages and cancer

Knowing these versatile properties of macrophages, their importance in the tumor setting is evident. Tumor-associated macrophages are a major part of the immune cell infiltrate of tumors. They support chronic inflammation, which is carcinogenic in itself [30,31], suppress anti-cancer immune responses by T cells through cell-contact or by secreted factors, support tumor growth by neoangiogenesis, and form niches to enable metastasis.

The significance of macrophage infiltration of tumors is demonstrated by its evaluation as prognostic marker. Many studies showed that the existence of TAM in different tumor types results in poor prognosis [32,33]. To foreclose a major obstacle in the analysis of myeloid tumor infiltrating cells, it should be mentioned that some groups also detect opposing results [34,35]. These different findings might originate from the general obstacle to identify certain closely related immune cell types and to assess a specific phenotype or polarization by analyzing surface markers only respectively [34,35].

To assess the outcome of anti-cancer treatments, it is not sufficient to look only at numbers or the sole existence of macrophages at the site of tumor. Putting treatments in relation to immune cell populations in the TME is a necessity. Studies estimating TAM as a prognostic marker have had variable results [36,37]. Heterogeneous responses of patients to treatments arise from different reactions of TAM due to their variable numbers, phenotypes, and drug responsiveness.

Chemotherapies, radiotherapies, or a combination of both are influenced by TAMs and vice versa. The nature of this influence can be cancer promotion or healing. TAM can enhance the effect of chemotherapeutic agents like doxorubicin [38]. Additionally, chemotherapeutics can have a direct effect on macrophages e.g. trabectedin, which depletes TAM via caspase-8 activation augmenting the direct therapeutic outcome on tumor cells [39]. On the other hand, TAM can reduce the efficiency of chemo- or radiotherapy by boosting tissue repair and guarding cancer stem cells, which are responsible for relapse after treatment [40].

Since macrophages are responsible for orchestrating neoangiogenesis by secreting vascular endothelial growth factor (VEGF) [41], they are capable of limiting antiangiogenic therapies aimed at VEGF signaling.

The context of TAM and immune-checkpoint inhibitors is discussed in Section 2.1.3.4.

2.1.2. Role of macrophages in the TME

2.1.2.1. Tumor associated macrophages. Macrophages are essential for carcinogenesis before even supporting tumor growth and metastasis, because they are key mediators of tissue inflammation during infection by bacteria or viruses, autoimmunity, or because of different irritants like tobacco smoke, alcohol abuse, or UV irradiation. If this inflammation persists after the resolution of the causative agent, then it enters the state of chronic inflammation, which has been shown to be a key promotor of tumor development. The chronically inflamed tissue reveals a high selective pressure and acts as a perfect niche for somatic mutations causing cells to transform into malignant tumor cells by promoting survival and proliferation, e.g. via STAT3 and NF-κB [42,43]. This leads to enhanced DNA damage, reduced DNA repair, suppressed anti-cancer immune responses, augmented invasive growth and metastasis by inducing epithelial-mesenchymal plasticity (EMT). Cancer cells support this on-going chronic inflammation via secretion of several pro-inflammatory chemokines and cytokines [31].

Since tissue-resident macrophages have a broad distribution in the body, they are involved from the beginning of carcinogenesis to the end of tumor metastasis. Their numbers may be enhanced during the course of the disease by invading monocytes and monocytic MDSC (M-MDSC) which evolve into mature TAM [44]. This equates to a hijacking of tissue repair and growth supporting phenotype of macrophages by tumors, which simulate the need for growth support, resulting in angiogenesis, modification of the ECM, adaption to hypoxic conditions, avoidance of immune responses in the tissue, and the favoring of stem cell like properties.

2.1.2.2. TIE-2 expressing monocytes. As described above, macrophages can promote neoangiogenesis. A new subclass of monocytes emerged in the last decade. These monocytes express the tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE-2) receptor, which can bind the growth factors Angiopoietin 1–4 and the monocyte surface markers CD14, CD16 and HLA-DR. They exist in the peripheral blood of cancer patients and in healthy people. These cells migrate to the tumor by chemotaxis via angiopoietin 2 (ANG-2) and act as immunosuppressants by secretion of IL-10 and VEGF. Depending on the tumor type, they may constitute the majority of myeloid cells in the tumor [45]. TIE-2 expressing monocytes could be detected in the invasive tumor edge of glioblastoma xenografts in mice after anti-VEGF therapy and in human surgical specimens [46]. Since our knowledge of this particular cell type is very limited, their role in the TME is largely unknown. Research needs to be done to distinguish between the capabilities of conventional macrophages and TIE-2 monocytes/macrophages.

2.1.3. Strategies to exploit TAMs in anti-cancer therapy

2.1.3.1. ADCC & ADCP. Antibody-dependent cell-mediated cytotoxicity (ADCC), a mechanism best known in natural killer (NK) cell effector function, can also be used by macrophages to lyse target cells after Fcγ receptor engagement. Binding of the antibody coupled to its target results in the activation of the Fc receptor, FcγRIIIa (CD16a), which leads to the killing of the tumor cell. The killing mechanism seems to depend on the attacking cell type [47]. NK cells rather kill extracellularly by releasing toxic proteins like perforin and proteases into the immunological synapse (ADCC), whereas macrophages primarily engulf and phagocytose target cells via antibody-dependent cellular phagocytosis (ADCP). It should be noted, that neutrophils and eosinophils are also capable of ADCC. The outcome of antibody-mediated anti-tumor therapies like cetuximab (alpha epidermal growth factor receptor-antibody (alpha EGFR-Ab)) and trastuzumab (alpha human epidermal growth factor receptor 2-antibody (αHER2-Ab)) is partly mediated by these mechanisms. The expression of CD47 on almost every cell acts as a “do not eat me” signal by binding to signal-regulatory protein alpha (SIRP alpha) on phagocytotic or cytotoxic cells. Since CD47 is overexpressed on many tumor cells, its blockade is necessary for efficient anti-tumor therapies. Overexpression of CD47 might be a point of attack using bispecific antibodies against CD47 and a further tumor antigen [48]. This matter has been reviewed extensively elsewhere [49–51].

2.1.3.2. CSF1R inhibition. The macrophage colony-stimulating factor (M-CSF, CSF1) drives the differentiation of hematopoietic stem cells into monocytes/macrophages. Furthermore, it is responsible for macrophage proliferation, survival, and polarization. Its receptor CSF1R (CD115) is a tyrosine kinase receptor closely related to the platelet-derived growth factor receptor (PDGFR), and the fibroblast

growth factor receptor (FGFR) which signals through several pathways like PI3K/AKT (phosphatidylinositol-3-kinase/protein kinase B) and RAS-ERK (rat sarcoma/extracellular signal-regulated kinase). Blocking of this axis is possible by antibodies like the humanized mAb emactuzumab or a variety of CSF1R kinase inhibitors, like BLZ945 and PLX3397, which are currently employed in clinical trials (PLX3397: NCT02584647, NCT02071940, NCT02975700, NCT02452424 and NCT02371369; BLZ945: NCT02829723). BLZ945 has been shown to repolarize macrophages and block tumor progression in a glioblastoma multiforme (GBM) mouse model and human GBM xenografts [52]. Further findings indicate significant benefits of BLZ945 treatment in murine models of cervical and mammary carcinomas [53]. It reduces TAM numbers and enhances $CD8^+$ T cell infiltration. In regard to the GBM model, it should be mentioned, that the same group discovered an acquired resistance to BLZ945 in more than 50% of the animals due to IL-4 mediated upregulation of insulin-like growth factor 1 (IGF1) secretion which drives tumor growth via the PI3K-AKT pathway [54]. Therefore, treatment with IGF1R inhibitors improved the survival.

An analogous situation occurs with the treatment of melanoma with $BRAF^{V600E}$ inhibitors, like Vemurafenib where promising results are spoilt by acquired resistance and recurrence [55]. Thus, current treatments consisting of a combination of inhibitors is a standard approach which might be combined with immune checkpoint inhibitors in the future [56]. These results highlight that blocking only one redundant pathway will most probably lead to resistance. PLX3397 shows promising results in mesothelioma [57] and hepatocellular carcinoma [58] and $BRAF^{V600E}$ melanoma mouse models, but failed to show effects against recurrent glioblastoma in a phase II study [59]. Emactuzumab is used in a phase 1 clinical trial, but patients with high levels of IL-4 may not benefit from this treatment [60,61].

2.1.3.3. Recruitment. To avoid accumulation of TAM, it is a reasonable strategy to block their reinforcement through bone marrow derived monocytes. Obviously, this strategy will not target TAM which are already present at the tumor, similar to blocking the CSF-1/CSFR1 axis.

The chemokine CCL2 acts as chemo attractant for monocytes expressing its receptor C−C chemokine receptor type 2 (CCR2). CCL2 is produced by cancer cells [62], tumor associated neutrophils, [63] and macrophages in an autocrine fashion [64]. Hypoxic conditions favor CCL2 secretion via hypoxia-inducible factor 1 (HIF-1) signaling [65]. High levels of CCL2 in tumor patients lead to poor prognosis in different tumor types [66–68]. CCR2 inhibition in combination with FOLFIRINOX as treatment of pancreatic cancer has been used in a phase Ib clinical trial where it leads to stable disease or partial response in almost every patient who received the combination therapy [69]. The number of TAM was reduced compared to the FOLFIRINOX group.

Alternative recruitment of monocytes can be achieved via the CCL5-CCR5-axis. Knockdown of the autocrine CCL5 secretion by MDSC and TAM resulted in a decrease of MDSC and TAM numbers and an immune response [70]. CCL5 may be regulated via raf kinase inhibitor protein (RKIP) [70].

2.1.3.4. Immunosuppression. Inhibition of immune checkpoint molecules cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 receptor (PD-1), which are expressed on T cells, aim to decrease the immune responses (see Section 3.3).

Since all ligands for CTLA-4 and PD-1 are also expressed by macrophages and can be upregulated in the TME, the fate of immune checkpoint therapies is also influenced by them. Programmed cell death 1 receptor ligand (PD-L1) is found to be expressed by certain tumors. Opsonization of activated T cells, Tregs, and melanoma cells which do express CTLA-4 by Ipilimumab results in ADCC exerted by monocytes/macrophages and NK cells [71,72]. PD-1 blockade is dependent on intratumoral expression levels of PD-L1 [73,74] and the amount of PD-1 positive T cells along with a quick rise in $CD8^+$ T cells and $CD68^+$ macrophages shortly after start of the therapy [75]. Checkpoint inhibitors are prone to resistance, just like other inhibitors (of CSF1R or $BRAF^{V600E}$). This problem has been reviewed recently [76]. One possible mechanism of resistance may be the removal of anti-PD-1 antibody bound to T cells by monocytes or macrophages, as discovered *in vivo* in mice and *in vitro* using human cells [77].

2.1.3.5. Bisphosphonates. The drug class of bisphosphonates (or diphosphonates) shares a common motif of two phosphonate groups ($–PO(OH)_2$) bound to one central carbon atom. The simple bisphosphonates of the first generation (e.g. clodronate) contain no nitrogen and are converted to non-hydrolysable analogs of adenosine triphosphate (ATP). This disruption of energy metabolism results in apoptosis. The second class of bisphosphonates (e.g. zoledronate) contains nitrogen and blocks the enzyme farnesyl diphosphate synthase (FPPS) which leads to reduced levels of farnesyl and geranylgeranyl diphosphate resulting in interference with lipid modification and anchoring of proteins to the cell membranes. Originally, these compounds have been used to target osteoclasts, the macrophages of the bones, to achieve less bone absorption and therefore reduced loss of bone mass in pathologic situations like osteoporosis, Paget's disease, and bone metastasis. These drugs have a high affinity towards bone tissue, hence osteoclasts are targeted. The use and effect of bisphosphonates on cancer not only towards osteoclasts was discovered later and has been nicely reviewed [78,79].

To achieve higher doses in other tissues than bone, bisphosphonates have been encapsulated in nanoparticles, liposomes, or red blood cells [80]. However, unfortunately uptake of the free substance is insufficient because it is negatively charged. Ablation of macrophages in mice by clodronate liposomes is a common technique [81]. Depletion of TAM by clodronate-containing liposomes induces growth of human cutaneous T cell lymphoma xenografts [82] and of B16/F10 melanoma in mice [83]. Zoledronic acid impairs TAM recruitment via monocyte chemoattractant protein 1 (MCP-1) in human breast cancer xenografts, breast cancer cell invasion, skews macrophages towards the M1 phenotype [84], and renders human M1 and M2 macrophages susceptible towards γδ T cell mediated cytotoxicity [85]. Although even in nano-based delivery methods zoledronic acid is also taken up by tumor cells [86] and the delivery agent itself can have adverse effects in mice [87].

2.1.4. How to target TAM?

2.1.4.1. Passive targeting. Since macrophages are highly phagocytotic cells, they uptake particles ranging from micro- to nano-size. For a long time, the consensus was that this attribute combined with the Enhanced Permeability and Retention (EPR) effect would be sufficient to achieve high local doses of carriers in the tumor and subsequently high local doses at the tumor or in surrounding macrophages. However, the EPR effect has no validity for all tumor types and species. It is especially less prominent in humans compared to rodents [88].

Moreover, liver and spleen resident macrophages (Kupffer cells) act as a filter-system for the bloodstream [89,90], especially because the complement system opsonizes nanoparticles [91]. Taken together, therapeutic nanoparticles need active targeting and stealth properties to evade premature clearance [92].

Another form of passive targeting is the use of drugs which act only or predominantly on macrophages because of exclusive signaling pathways or receptors, (see Sections 2.1.2 and 2.1.3.3).

2.1.4.2. Active targeting. To circumvent high unspecific uptake and high doses of possible toxic cargo in non-target organs or tissues, active drug targeting to macrophages or other immune cells is inevitable for a high local drug concentration. Classical targeting involves antibodies or antibody fragments. High availability favors their use, but due to their size antibodies alter the nanoparticle properties significantly and it requires specific markers which can be targeted. As already mentioned, cell-type specific markers are rare in context of immune cells. If the antibody still possesses an Fc-part, it can

result in undesired immune activation or uptake via Fc-receptors.

Surface modifications with low molecular weight ligands have benefits over antibodies. Since mannose is one of the natural ligands for the CD206 receptor, it can be used as targeting vector. Unfortunately, monomeric mannose has only low affinity towards CD206 and the receptor is not exclusive for TAM, but binding to CD206 leads to receptor-mediated endocytosis [93]. The folate receptor beta (FR beta) has been shown to be overexpressed in TAM [94,95] and hence folic acid, analogs or antibodies against FR beta have been used as modification for nanocarriers, but mostly to target tumor cells that have also a high folate receptor expression.

Nanoparticle development and modifications regarding targeting and stealth effect is an active field of research [96–100].

2.2. Dendritic cells

Dendritic cells are the most potent APC. They are often referred to as professional APC since their main function is the presentation of antigens to T and B cells. Besides, DC also provide costimulatory signals and cytokines that are crucial for an efficient antigen-specific T cell activation and differentiation [101,102]. They also interact with other immune cells like B cells, NK cells, MDSC, neutrophils and platelets and therefore shape immune responses [103,104]. During T cell activation, DC present captured antigens to T cells major histocompatibility complex (MHC) dependently. Upon DC contact, naïve $CD4^+$ and $CD8^+$ T cells get activated in an antigen-specific manner and differentiate into corresponding subsets. $CD4^+$ T cells can differentiate into T helper cell 1 (Th1), Th2, Th9, Th17 and Treg, while $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTL). Thus the T cell response is greatly dependent on the activating DC subpopulation [105].

2.2.1. Plasticity of the DC subsets

In steady state conditions, DC reside in peripheral tissues in an immature state and constantly take up antigens. Immature DC exhibit a high expression of antigen uptake receptors but only slightly express costimulatory molecules, and reveal a limited production of cytokines leading to a low T cell stimulatory capacity [101,102,106]. Immature DC induce tolerance by deletion of T cells and the induction of T cell anergy [107]. They are also able to induce, expand, and activate regulatory T cells (Treg) [108–110]. DC can be activated by stimulation of their pattern recognition receptor (PRRs) e.g. TLR, membrane C-type lectins, or cytoplasmatic nucleotide-binding oligomerization domain (NOD)-like receptors by conserved pathogen associated molecular patterns (PAMPs), as for example bacterial LPS that activates TLR4. Upon recognition of pathogens, like viruses or bacteria, and tissue damage, DC become activated and undergo a maturation step. The maturation of DC is characterized by decreased potency to capture antigens, increased expression of MHC-class-II and costimulatory molecules, plus the vigorous expression of numerous inflammatory cytokines (e.g. TNF alpha, IL-1 beta, IL-6 and IL-12). Additionally, they show an expression of lymph-node homing receptors like CCR7, which allows the active migration into secondary lymphatic organs [101,106]. Mature DC can induce strong T cell responses and are therefore critical for tumor rejection.

2.2.2. Heterogeneity of DC

There are different subtypes of DC that can be distinguished by their function, localization, and phenotype. The identification of DC subsets is difficult due to various reasons. Firstly, DC subsets are quite heterogeneous, and secondly, differing marker sets have been used in different studies to identify the DC subsets. Beside the challenging phenotype characterization, the DC population can be differentiated into three major groups: conventional DC (cDC), myeloid DC (mDC), and the plasmacytoid DC (pDC). In humans, two subsets of the cDC can be distinguished. One subset is characterized by the expression of CD11c, CD11b, CD1c (blood dendritic cell antigen 1, BDCA1) and CD172a. The other can be identified through their expression of CD11c, CD141 (BDCA3), CD370 (Clec9a/DNGR1) and CXCR1. Human $CD141^+$ DC are highly potent in capturing antigens. These antigens are processed and presented on MHC-class-I molecules. This is called cross-presentation and [111,112] enables the DC subpopulations to induce a strong cytotoxic $CD8^+$ T cell-mediated immune response, which e.g. enhances the expansion of tumor antigen specific CTL. $CD141^+$ cDC in tumors are known to correlate with a better clinical outcome [113].

In contrast, pDC are multifunctional BM-derived DC, specialized in type I interferon secretion [114]. pDC represent only a small fraction of all DCs. They show the morphology of plasma cells in humans and express CD4, HLA-DR, CD123, BDCA2, TLR 7 and 9, but not CD11c. After stimulation of TLR 7/9, the pDC start to produce IL-12, IL-6, TNF alpha, and secrete high amounts of type I IFN, besides other inflammatory cytokines [115]. pDC also function as APC, but they are not as effective as cDC. They are capable of inducing inflammation or tolerance depending on their surrounding cytokine milieu. However, their role in the TME remains elusive. There is evidence that pDC can induce an immune response towards cancer throughout by production of IFN alpha [116]. In one study, pDC loaded with tumor antigens were able to enhance the $CD4^+$ and $CD8^+$ mediated T cell responses in melanoma patients [117].

2.2.3. DC in the tumor microenvironment

There is evidence that the presentation of tumor antigens by cDC happens not only in the lymphatics, but also in the TME. Within the TME, DC are exposed to numerous tumor antigens. The classical cancer treatment approaches like radiation and chemotherapy lead to massive cancer cell death resulting in massive release of tumor-associated antigens (TAAs) [118]. These antigens are taken up by DC and can be presented to T cells, resulting in an antigen-specific anti-tumor T cell response. Thus, it becomes apparent that cDC in the TME are able to influence the anti-tumor T cell response highlighting their important role in the tumor microenvironment. Mature DC can infiltrate the tumor and are able to increase the effective anti-tumor immune response, by recruiting tumor-specific immune cells. However, tumor cells have acquired strategies to suppress DC function and/or recruit tolerogenic DC (DCtol), like TAM and MDSC. Tumors can evade the antitumor specific response (tumor immune escape) through mutations in the TAAs by downregulation of the expression of MHC I molecules, and prevention of antigen processing in DC [119]. For example, the lipid accumulation of DC in the TME is one of the main mechanisms that contributes to altered DC function [120]. The accumulation of lipids in DC is due to stress in the ER. This leads to an activation of the endoplasmic reticulum (ER) stress response factor spliced X-box-binding protein 1 (sXBP). The activation of this factor leads to the abnormal lipid accumulation through the induction of a triglyceride biosynthesis [121,122].The accumulation of lipids leads to an impaired antigen cross presentation, inducing an inadequate $CD8^+$ T cell activation [120,123–126]. In addition, tumors can surround themselves with a tolerogenic environment, promoting the attraction and induction of tolerogenic immune cells like Treg, DCtol, M2 macrophages leading to a defective mDC function. Tumors also actively express factors that are known to inhibit DC maturation like IL-10, TGF beta, VEGF, IL6 and chemokines like CCL2, CXCL1, and CXCL5. Together these mechanisms result in an ineffective antigen presentation by DC and lead to an induction of tolerance by T cell death and/or generation of Treg [108,109]. Additionally, the induced expression of the inhibitory surface molecule CTLA-4, which is upregulated after T cell stimulation, competes with activating CD28 molecules for binding to DC CD80/CD86 resulting in an ineffective T cell activation. Tumors can induce an immunosuppressive phenotype in tumor-infiltrating DC (TIDC). This is e.g. correlated with an overexpression of the transcription factor (TF) forkhead box O3 (FOXO3) in TIDC. FOXO3 can induce the expression of IDO, arginase, and TGF beta. It also suppresses the expression of costimulatory molecules [127,128]. A lot of tumors express enhanced levels

of phosphorylated STAT3 which can also be induced by TIDC. On TIDC, STAT3 induces downregulation of MHC and costimulatory molecule expression, which ultimately inhibits tumor cell apoptosis. Moreover, STAT3 induces an expression of immunosuppressive cytokines and downregulates the expression of inflammatory cytokines, inhibiting Th1 response [129].

It has been shown that the interaction of DC with CTLA-4 on Tregs or exhausted T cells leads to an expression of IDO, resulting in arrest of T cell proliferation, apoptosis and Treg induction [130,131]. Expression of PD-L1 by tumors is known to contribute to the abnormal DC function in the TME and tumor draining lymph nodes (dLN). Blockade of PD-L1 and PD-1 partially restores the T cell-stimulatory capacity and cytokine production of TIDC [132]. Furthermore, tumors can even inhibit DC generation. Instead of monocytic differentiation into DC, monocytes may differentiate into TAM and M-MDSC [133–135]. DC maturation can also be reduced by tumor derived exosomes (TEX). Some studies have shown that TEX are able to induce MDSC instead of DC in $CD14^+$ peripheral monocytes and induce low HLA-DR expression [136]. Additionally, that TEX can induce downregulation of TLR4, and reduce cytokine expression like TNF alpha and IL-12 via miRNA-203 induction [137]. As a result, TIDC are often associated with good and poor prognosis in several cancer types [138]: breast, colorectal, melanoma, lung, renal, head and neck, bladder and gastric and ovarian cancers [133]. Thus, their use for prognosis depends on the composition of the different TIDC subsets, not on their absolute numbers in the tumor.

2.2.4. Recent strategies to address DC dysfunction in the TME

Through their important role in the immune maintenance, TIDC are an ideal target for novel cancer therapies.

One recent strategy to address DC in the TME is the use of nanocarriers. Nanocarriers allow cell-type specific targeting of different suppressive TIDC subsets and hence restoration of their stimulatory capacity by loading with TAAs, TAA encoding RNA, small interfering RNA (siRNA), or small molecules that induce a tumor antigen specific T cell response, or blockade of immunosuppressive pathways. Through cell specific targeting, side effects and degradation of the cargo is greatly reduced. Additionally, the simultaneous co-delivery of encapsulated antigen plus an adjuvant to the same DC is secured, preventing tolerance induction which may occur by antigen delivery alone.

A similar approach is the so-called exosome based therapy that could be used to modulate immunosuppressive mechanisms in the TME. Exosomes can be loaded with specific tumor antigens, siRNAs, or used as a drug delivery system [139]. Currently, not all the necessary components for an artificial exosome synthesis are known. DC-derived exosomes express costimulatory molecules, MHC-class-I and MHC-class-II, on their surface. These exosomes can induce T cell proliferation and activate NK cells. Furthermore, they can induce DC maturation and trigger tumor-antigen specific responses [140,141]. In 2005, two phase one clinical trials based on DC-derived exosomes have been conducted. Notably, there was only a small effect observed, due to a slight NK cell activation [142,143].

2.3. MDSC

The TME diverts migrated endogenous myeloid cells towards an immature non-specific immunosuppressive phenotype, which has been termed myeloid-derived suppressor cells (MDSC) [144]. MDSC are intensely studied cancer promoters [145]. Reminiscent of lymphoid regulatory T cells, MDSC show an immunosuppressive capacity and can inhibit innate and adaptive immune responses. The tumor exploits this characteristic to its advantage by activating immunosuppressive MDSC thereby promoting tumor development, progression, and angiogenesis [146].

2.3.1. Definition and origin of human MDSC

2.3.1.1. Definition. MDSC are defined as a heterogeneous population of immature myeloid or progenitor cells harboring an immunosuppressive capacity. MDSC are subdivided into monocytic (M-MDSC), polymorphonuclear (PMN-MDSC) and early MDSC (e-MDSC). Phenotype, morphology, and biochemical characteristics of each subpopulation are currently under discussion. For reasons of simplicity, we focus on human MDSC, as murine MDSC display a largely different set of phenotypic markers. M-MDSC are phenotypically and morphologically comparable to monocytes; PMN-MDSC are comparable to neutrophils displaying less granules and a modified buoyancy. e-MDSC are comprised of various immature progenitors. Due to overlapping marker expression and cell plasticity, flow cytometric analysis led to a variety of cells classified as MDSC making it difficult to compare the different *in vitro* and *in vivo* studies [145]. Hence, the rationale to distinguish MDSC from other cell populations and the standardization of MDSC gating strategies in fluorescent associated cell sorting (FACS) has been a prominent issue of many publications [147,148]. Interlaboratory comparison of MDSC frequencies also stresses the importance of a standardized gating strategy [147]. In 2016, *Bronte* et al. published a review recommending a minimal phenotype needed to identify the above mentioned MDSC subpopulations: $CD14^-CD11b^+CD15^+$ (or $CD66b^+$) for PMN-MDSC, $CD11b^+CD14^+HLA\text{-}DR^{low/-}CD15^-$ for M-MDSC and Lin^- (CD3, CD14, CD15, CD19, CD56) /$HLA\text{-}DR^-$/$CD33^+$ for e-MDSC [148]. In contrast to neutrophils, PMN-MDSC display less CD62L and CD16, while the expression of CD11b and CD66b is increased.

Functionally, PMN-MDSC exhibit decreased arginase 1 (ARG1) and peroxynitrite levels. However, despite a lot of research there is yet no clear, distinct marker for the unambiguous characterization of MDSC. The authors emphasize the importance of additional functional data such as comparison to healthy donor frequencies and evidence of suppressive capacity when analyzing MDSC in the context of disease. Furthermore, the given minimal marker combinations do not allow for differentiation between PMN-MDSC/neutrophils and M-MDSC/monocytes. Bearing these limitations in mind, density gradient centrifugation can separate phases into (i) low-density PMN-MDSC and activated neutrophils, and (ii) high-density phase neutrophils (see Section 2.4). Notably, many pathologically upregulated proteins, such as transcription factors and cytokines, introduced in the next section are used as functional markers to identify MDSC and may also contribute to MDSC-mediated immunosuppressive capacity.

2.3.1.2. Origin. In the TME, MDSC originate from immature myeloid progenitor cells undergoing aberrant differentiation. MDSC generation occurs primarily in patients suffering from cancer, autoimmune diseases, and chronic inflammation. For MDSC generation in cancer, tumor-associated and cancer cells release cytokines and soluble factors like IL-3, VEGF, GM-CSF, granulocyte colony-stimulating factor (G-CSF), M-CSF, and stem cell factor (SCF), to stimulate further migration of myeloid cells to the tumor, inducing an immature differentiation state and emergency myelopoiesis in the bone marrow, which occurs during bacterial infections to satisfy the high demand of myeloid cells. MDSC activation is promoted by the pro-inflammatory cytokines IL-1 beta and IL-6, and the Ca^{2+}-binding proteins S100A8/9, released by tumor cells, as well as the cytokines IL-4, IL-10, IL-13 and IFN gamma, released as a result of T cell activation [149]. In cancer patients, high frequencies of MDSC are generated, migrate to lymphoid tissues and accumulate in the tumor. In contrast, nearly no MDSC are found in healthy individuals [148]. There is evidence for functional differences depending on their localization. PMN-MDSC are primarily located in peripheral tissues and mediate suppressive capacity by enforcement of antigen-specific T cell tolerance. In comparison M-MDSC have the upper hand at the tumor site and sustain the tumor development by strong suppressive function and further differentiation into tumor associated macrophages (TAM). New findings suggest that micro RNA miR-142-3p plays a role in MDSC differentiation or function reminiscent of macrophages. Decrease of miR-142-3p enhances macrophage maturation by IL-6/CCAAT/enhancer-binding protein

beta (C/EBP beta) signaling increasing their immunosuppressive capacity [150].

Activation of MDSC is induced by chronic inflammation characterized by continuous stimulation with moderate levels of anti-inflammatory cytokines, ROS, nitric oxide (NO), and low phagocytic activity. Compared to the quick immune response of myeloid cells to bacteria and viruses, activation of immature myeloid cells like MDSC in the tumor microenvironment is a long-lasting process comparable to chronic inflammation.

2.3.2. Functions and mechanisms of MDSC contributing to cancer progression

MDSC promote tumor cell survival, angiogenesis, invasion of tumor cells, and initiation of metastasis formation. MDSC initiate immunosuppressive mechanisms supporting the hallmarks of cancer in the TME.

2.3.2.1. Genes and molecules. Several genes and molecules contribute to the immunosuppressive capacity displayed by MDSC. The enzyme IDO1 is a recommended functional MDSC marker, which blocks T cell activation by degradation of the essential amino acid tryptophan (see Section 3.2) [151] and increasing regulatory T cell (Treg) frequencies by activation of natural Treg and induction of novel Treg [152]. Gene expression of arginase 1 (*arg1*) and nitric oxide synthase 2 (*nos2*) competes for L-arginine as central substrate for either decreasing or increasing the production of NO, respectively. While NO production can increase (tumor cell) cytotoxicity, arginase activity impairs T cell responses by mere depletion of this essential amino acid (see Section 3.1) [153]. Due to high frequencies of NADPH oxidase (Nox2), a membrane-bound enzyme, MDSC show strong production of reactive oxygen and nitrogen species (ROS, RNS), which consist of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (PNT). ROS and RNS partake in the upregulation of tumor-specific T cell tolerance, the impairment of T cell trafficking to the tumor site, thus leading to the escape of tumor cells from cytotoxic T cells [154]. In addition, MDSC play a negative regulatory role in T cell proliferation by expressing the inhibitory molecule PD-L1 (see Section 3.3). By expression of prostaglandin E2 (PGE2) MDSC take a negative effect on the IL-2 and IFN gamma production of T cells and hence impair T cell-mediated immune responses. Moreover, MDSC support tumor angiogenesis by producing VEGF. In mice, a proteomic-based analysis of membrane bound proteins on murine MDSC in tumor bearing mice comparing strong and low inflammatory conditions found statistically significant differences in levels of the tumor biomarkers S100A8/9 [155].

2.3.2.2. Transcription factors and ER stress response markers. Like tumor cells, MDSC show an upregulation and pathological activation of different janus-activated kinase/signal transducer and activator of transcription (JAK/STAT)-mediated pathways, particularly high frequencies of phosphorylated STAT-5 and STAT-3. Hence, MDSC upregulate target genes affecting cell proliferation, inflammation, apoptosis, and genes leading to differentiation [156].

Hypoxia-induced ER stress in the TME upregulates spliced X-box binding protein 1 (sXBP) via the ER stress sensor inositol-requiring enzyme 1 (IRE1) pathway and the C/EBP homologous protein via the protein kinase RNA (PKR)-like ER kinase (PERK)-pathway in MDSC [157]. Thereby, MDSC undergo caspase 8-dependent death receptor 5 (DR5)-mediated apoptosis initiated by cancer cells. Accordingly, MDSC in the spleen, lung, and cancerous tissue of non-small cell lung cancer (NSCLC) patients have lower survival rates than monocytes and polymorphonuclear neutrophils [157]. In addition, one of the main regulators of MDSC differentiation in the tumor microenvironment is HIF-1 alpha, which induces polarization of MDSC towards TAM [44]. Therefore, high MDSC frequencies in tumor tissue are due to constant migration of MDSC from the bone-marrow resulting in a high MDSC turnover.

Retinoic-acid-related orphan receptor 1 (ROR gamma/RORC1) was found in human PMN-MDSC. RORC1 plays a key role as positive transcriptional regulator of granulopoiesis by driving c/EBP beta, a transcription factor important for myeloid cell differentiation. Common myeloid progenitor (CMP) transcription factors like RORC1 and interferon regulatory factor 8 (IRF8) are central initiators of myelopoiesis and differentiation to monocyte precursor cells (MPC), and hence to monocytes and macrophages. In addition, RORC1 deficiency in mice leads to an increase in PMN-MDSC apoptosis, less though in M-MDSC, which could partly be reversed by addition of G-CSF/GM-CSF [158]. As a result, low frequencies of transcription factors RORC1, c/EBP beta, and IRF8 sustain an immature myeloid phenotype necessary for tumor progression and metastasis in the tumor microenvironment.

So far, the contribution of the retinoblastoma gene (Rb) in MDSC has been mainly investigated in mice. Here its suppression led to cells with a PMN-MDSC phenotype but with reduced suppressive capacity. Expression of Rb1 is more prominent in murine M-MDSC than PMN-MDSC. Rb1 downregulation is associated with M-MDSC differentiation into PMN-MDSC in mice. Accordingly, knock-down of Rb1 in murine splenic MDSC is associated with tumor progression [159].

2.3.2.3. Cytokines, chemokines and receptors. MDSC release the anti-inflammatory cytokines TGF beta 1 and IL-10 leading to inhibition of tumor specific $CD8^+$ T cells. In addition, IL-10 promotes the polarization of inflammatory M1 macrophages to tolerogenic M2 macrophages, also known as tumor-associated macrophages (TAM). CD124 (IL-4R alpha), a common regulator of TGF beta 1 production in TAM and MDSC, supports the suppressive function of MDSC via the IL4R alpha-STAT6 pathway. Co-activation of STAT1 and especially the above mentioned STAT3 lead to ROS production and the upregulation of tumor growth and metastasis promoting MMPs. *Roth* et al. demonstrated that IL4R alpha blockade on MDSC in mice results in MDSC apoptosis [160].

2.3.3. Therapeutic methods to target MDSC

Recent trends using targeted anti-cancer therapies have not yet efficiently and specifically addressed MDSC. Despite its proven benefits, systemic anti-cancer therapy comes with side effects and often low efficacy. Even though the active agent is target-specific the molecule of interest often lacks cell-type specificity. The “magic bullet” concept introduced by Paul Ehrlich frequently fails when applied to molecular targets, which are expressed on host as well as on cancer cells.

The network of interactions between the different myeloid cell populations suggests that therapy is only highly effective when targeting multiple cancer-promoting mechanisms at once. Depending on function and marker expression as well as localization, different cells may be affected by the treatment.

Targeted therapies of MDSC have been the subject of several reviews [161–163]. To give an overview, therapeutics were assigned to groups depending on the pursued strategy. According to *Wesolowski* et al., starting points of treatment are prevention of MDSC development, promotion of maturation of immature MDSC and the deactivation of MDSC [163].

2.3.3.1. MDSC development. To impair differentiation of myeloid cells into MDSC, peptidomimetics, small molecule inhibitors, platinum agents, and derivatives of curcumin have been used as JAK2/STAT3 inhibitors. After treatment with curcumin derivative Cucurbitacin B (CuB), a decrease of $CD33^+HLA\text{-}DR^-$ myeloid cells in peripheral blood of lung cancer patients was observed [164]. The VEGF and multi-kinase inhibitor Sunitinib addresses, among others, the proto-oncogene receptor tyrosine kinase (c-kit) and the VEGF receptor (VEGFR), which both regulate MDSC migration and function [165]. Cyclooxygenase (COX)-2 inhibition (Celecoxib) reduces the CXCR4/CXCL12 and CXCR1-CXCR2/CXCL8-mediated MDSC trafficking to the

tumor [166]. Bisphosphonates, like zoledronic acid, downregulate proangiogenic matrix metalloproteinases such as MMP-9, which yield c-kit release [167]. MMP-9 remodels the tumor stroma and releases proangiogenic molecules like VEGF and fibroblast growth factor (FGF)-2 from the ECM [168].

2.3.3.2. MDSC maturation. Vitamins, including vitamin A metabolite all-trans retinoic acid (ATRA), and vitamin D3 have been investigated for their role in MDSC maturation. Being an agonist of retinoid-activated transcriptional factors (RAR alpha, RAR beta), vitamin A regulates target genes of myeloid cell differentiation. In metastatic renal cell carcinoma, patients treated with ATRA experienced enhanced expression of the maturation marker human leukocyte antigen – antigen D related (HLA-DR) on MDSC [169]. Similarly, vitamin D treatment resulted in an upregulation of myeloid cell HLA-DR. After treatment, frequencies of IL-12 and IFN gamma in myeloid cells were upregulated. Likewise, treatment with TLR9 agonist, unmethylated deoxycytosine-deoxyguanine dinucleotide (CpG) oligodeoxynucleotides (ODN), coupled to STAT3 siRNA provided TLR9 specific binding to myeloid cells and silencing of STAT3. Downregulation of STAT3 led to decreased immunosuppressive capacity of MDSC on $CD8^+$ T cells [163,170]. TLR9 agonists are argued to induce release of IL-12 in MDSC, DC, B and natural killer (NK) cells creating an immunogenic environment [171].

2.3.3.3. MDSC deactivation. Suppressive activity of MDSC is sustained by ARG1 and NOS2/iNOS expression. Though the mechanism is not clear yet, it is argued that phosphodiesterase-5 (PDE-5) inhibitors like sildenafil and tadalafil decrease ARG1 and NOS2. Mechanisms such as (i) inhibition of the STAT6/IL-4R alpha pathway, (ii) disruption of NOS2 mRNA expression, and (iii) decrease of calcium-dependent protein kinase C signal transduction have been suggested [172]. Another approach to reduce NO is nitro-aspirin (NO-Aspirin). NO-Aspirin decreases ROS production and indirectly impairs NOS2 [173]. L-NAME (N(G)-Nitro-L-Arginine Methyl Ester) decreases NOS transcription and thereby NO production in addition to inhibition of arginase. *In vitro*, *N*-hydroxy-L-Arginine (NOHA), a physiologic inhibitor of ARG1, impeded MDSC induced Treg expansion [174]. There has been good evidence that COX-2 inhibitors like Celecoxib may decrease MDSC function by ARG1 downregulation and decrease MDSC frequencies [175]. Synthetic triterpenoids like bardoxolone methyl (CDDO-Me) are used to decrease ROS via upregulation of antioxidant NADPH. CDDO-Me also impedes the STAT3 pathway and thereby MDSC expansion [176]. Anti-glycan antibodies are argued to suspend the receptor for advanced glycation end products (RAGE)-S100A8/9 loop, which is involved in MDSC function and recruitment [177,178]. Other therapeutic agents, intended to modify MDSCs include colony-stimulating factor 1 receptor (CSF-1R) inhibitors (MDSC recruitment and migration), histamine inhibitors (MDSC/ TAM generation), and anti-IL-17 antibodies (MDSC recruitment) [163]. In 2011, Yang et al. found that histidine decarboxylase (HDC), an enzyme to endogenously produce histamine and highly expressed by immature myeloid cells, influences the differentiation of myeloid cells positively. In cancer, there is evidence that the HDC promotor is hypermethylated impeding myeloid cell differentiation [179]. Interestingly, cimetidine, a histamine type-2 receptor antagonist, facilitates MDSC turnover by upregulation of death receptor Fas/FasL expression on MDSC surface and induces MDSC apoptosis caspase-dependently, irreversibly by histamine [180].

2.3.3.4. MDSC depletion. MDSC are sensitive towards cytotoxic agents like gemcitabine, cisplatin, paclitaxel and 5-fluorouracil. Chemotherapeutic agents act by hindering DNA replication, in the case of paclitaxel by affecting microtubule polarization during cell division, eventually leading to cell death [181]. Heat shock protein 90 (HSP90) inhibitor, 17-DMAG (17-Dimethylaminoethylamino-17-demethoxygeldanamycin), renders tumor cells visible to specific $CD8^+$ T cells. Thereby 17-DMAG has been found to positively influence the tumor microenvironment for T cell invasion and reduced MDSC frequencies in a sarcoma murine model [182]. In mice, depletion of MDSC could be achieved by administration of IL-13-PE (IL-13 linked to pseudomonas endotoxin) plus an IL-13R alpha 2 DNA vaccine [183].

2.4. Neutrophils

Neutrophils are the mature subtype of granulocytes and account for 50–60% of the white blood cells in the blood stream. In the bone marrow, myeloblasts differentiate into granulocytes, also referred to as polymorphonuclear cells (PMN), and monocytes. PMN also include basophil and eosinophil granulocytes. Histologically, neutrophils differ from monocytes by their characteristic 2–5 lobular nuclei and a granula-rich cytoplasm. Further classification distinguishes between anti-tumor N1 and pro-tumor N2 neutrophils [184].

2.4.1. Function

In the TME, neutrophils exhibit ambiguous functions (reviewed in [15,185–187]). They support tumor angiogenesis, invasion and progression. Nevertheless, there is evidence that they can also interfere with cancer development. In response to an inflammatory environment, neutrophils engage three major mechanisms to fight pathogens or bacterial infections: phagocytosis, degranulation, and the setting of neutrophil extracellular traps (NET) [188]. Frequently, phagocytic neutrophils are targeted by macrophages. During degranulation, granules release proteins (lysozyme, NADPH oxidase) to invoke destruction of target cells. Nevertheless, granules releasing neutrophil elastase (NE), neutrophil collagenase (MMP-8), and gelatinase B (MMP-9) promote ECM degradation and remodeling, and thus tumor progression. Built from heterochromatin (DNA) complexed with peptides like serine proteases and numerous defense factors like antibacterial LL-37 (Cathelicidin), NETs function as pathogen catchers [189]. Given their capacity to accumulate foreign cells and pathogens, NETs can also collect platelets and hence promote thrombosis [190]. In response to chemotaxis, neutrophils have been reported to produce several tumor-promoting factors. For instance, hepatocyte growth factor (HGF) increases cancer cell migration and therefore progression. Oncostatin M induces the proangiogenic factor VEGF leading to tumor vascularization. In addition, PGE2 favors tumor proliferation, and IL-8 induced ARG1 production sustains the immunosuppressive environment.

Anti-tumor N1 neutrophils target tumor cells and support T cell immune response (reviewed in [191]). There is evidence that N1 act like APC and attract cytotoxic T cells as well as $CD4^+$ T cells using CXCL9/CXCL10 [192]. Due to their high motility neutrophils are the first immune cells entering the site of infection or inflammation. In addition, neutrophils are slightly smaller in size than monocytes rendering them more mobile. Furthermore, they migrate more quickly from the vasculature to the tissue or tumor and, most interestingly, may leave the site of inflammation depending on chemotaxis (IL-8, CXCL-6, IFN gamma, C3a, C5a, *N*-formylmethionyl-leucyl-phenylalanine (fMLP), Leukotriene B4, H_2O_2, lectins, proteins) and cell-to-cell and cell-ECM interactions, including macrophages [193]. Therefore, it is argued that increasing neutrophil reverse migration may be a mechanism worth exploiting for anti-tumor therapy.

2.4.2. Neutrophil targeted therapy

A high neutrophil-to-lymphocyte ratio (NLR) is associated with tumor progression and is used as a prognostic as well as diagnostic marker in several cancer types. Targeting neutrophil recruitment and subsequent induction of angiogenesis, CXCL-8/IL-8, anti-CXCL1/2 (IL-8 receptor) antibody and small molecules, have been used to decrease neutrophil migration to the tumor [194]. Neutrophils can release large quantities of bioactive MMP-9 from its complex formed with tissue inhibitor of metalloproteinases (TIMP)-1, which acts as an inhibitor of

MMP-9 [195]. Hence, neutrophil pro-angiogenic and ECM remodeling MMP-9 is more likely to be activated successfully, releasing also VEGF and FGF-2 from the ECM. Therefore, neutrophils play an important role in tumor neovascularization and ECM remodeling. New approaches explore the reverse migration of neutrophils out of the TME and the targeting of neutrophils using tumor specific nanoparticles [196]. G-CSF therapy depletes neutrophils from the TME as it mobilizes PMN precursors from the bone marrow, which induce an environment of acute, reversible inflammation rather than chronic sustained inflammation. Thus, due to their role in infection and ambiguous function in cancer, appropriate targeting of neutrophils could also lead to a decrease of anti-tumor immune responses [197].

2.5. Platelets

Platelets are primarily known for their indispensable function in hemostasis. Nevertheless, recent work suggests that besides playing an important part in thrombosis, they also have important influence on innate and adaptive immunity, being related to both protective and inflammatory effects. Through their expression and secretion of different immune mediators, they play a key role in inflammation but are also related in cancer and metastasis. In the tumor and the surrounding tissues, platelets are integral for the recruitment of immune cells and therefore contribute to tumor progression. Since the 1970's, it is known that a high platelet count (thrombocytosis) is associated with decreased survival rate and correlates with a poor prognosis in cancer patients [198,199]. Platelets possess different kinds of granules, like for example the most abundant alpha granules. Alpha granules store a variety of different proteins and factors that are required for platelet function and hemostasis. For example, they stock von Willebrand factor (VWF), fibronectin, thrombospondin, factor V, factor viii and glycoprotein IIb/IIIa (GPIIb/IIIa). Additionally, they store factors known for immune modulation like platelet-selectin (P-selectin), platelet factor 4 (PF4)/CXCL4, beta-thromboglobulin (CXCL7), CCL3, CXCL5, CXCL1, CXCL12, platelet-derived growth factor (PDGF), TGF beta and VEGF. P-selectin is expressed on activated platelets and upon contact with its ligand platelet-selectin glycoprotein ligand-1 (PSGL-1), which is known to be expressed on neutrophils and monocytes and can activate those cells [200–202]. Chemokines like PF4 and CXCL7 are released upon platelet activation and enable platelets to recruit endothelial progenitor cells and neutrophils. Furthermore, PF4 and CXCL7 activate and suppress the apoptosis of neutrophils [203,204]. Furthermore, CCL3 and CCL5 are able to recruit monocytes. Additionally, CXCL1 seems to be critical for the recruitment and the positioning of macrophages in the tumor [205–207]. PDGF, TGF beta, epidermal growth factor (EGF), and VEGF, released from alpha granules, have not only immunoregulatory properties but also aid in metastasis through the establishment of adhesive sides for the tumor. For instance, 80% of the VEGF that is found in human blood is released by platelets upon activation [198]. Tumor growth is dependent on angiogenesis, and platelet derived VEGF is one of the major angiogenesis inducers in cancer [208,209]. Platelets are also able to scavenge VEGF from the TME. This is of relevance, because VEGF in cancer is only durable for around 30 min. Thus, scavenging and re-release of VEGF induce a prolonged angiogenesis [210]. Both, platelet-derived TGF beta and the direct tumor cell contact with platelets induce the TGF beta/Smad (Mothers against decapentaplegic homolog 4) and NF-kB pathway, which induces a change of cancer cells into an invasive mesenchymal phenotype with enhanced metastasis [211].

Apart from the recruitment of other cell subsets upon activation, platelets also cause direct effects on cancer cells for example by physical protection of tumor cells in the bloodstream during metastasis is suggested. When tumor cells become surrounded by activated platelets through crosslinking by adhesion molecules (e.g. integrin GPIIb/IIIa) and soluble fibrinogen, they can evade immune cells as well as TNF alpha mediated cytotoxicity by NK cells [212,213]. Platelets also protect the tumor cells from the high physical forces in the bloodstream [214,211]. At some point, there is an adhesion at the vascular endothelium, resulting in platelet activation and the release of platelet factors like PDGF, EGF, IGF1, TGF beta and VEGFA from the platelet alpha granules resulting in cell proliferation and angiogenesis. Besides, platelet derived factors themselves can induce a suppressive phenotype in e.g. T cells. Recently *Rachidi* et al., were able to show that the deletion of platelet expressed GARP (glycoprotein A repetitions predominant), a docking receptor on platelets for TGF beta, blunted the TGF beta activity at the tumor site and improved the $CD4^+$ and $CD8^+$ T cell immune response against the tumor [215]. In addition, after interaction with tumor cells, TGF beta downregulates the expression of the activating immunoreceptor natural killer group 2, member D (NKG2D) on NK cells [216]. Recently we could show that GARP itself plays a role in the TME. Membrane bound GARP, expressed on activated Treg and melanoma cells is shed from the surface and is, in its soluble form (sGARP), able to induce peripheral Treg and M2 macrophages. sGARP was also able to inhibit the proliferation and effector function of cytotoxic T cells. So, GARP itself contributes to the TME [217]. However, the exact function of platelets in the microenvironment and their use as target for anti-tumor therapy still remains elusive and needs to be addressed more specifically. Whereas there are some experimental models indicating that inhibitors of platelet-driven effects reduce metastasis, clinical studies show that anticoagulation may promote tumor progression. Taken together, there is an unmet need to investigate the remaining and sometimes contradictory questions considering anti-platelet approaches as anti-cancer treatment in more detail.

3. Common immune suppressive mechanisms mediated by myeloid cells

To assess the mechanisms involved in myeloid cell regulation, it is important to have a closer look at markers giving insight into the activation status, origin of cell, and its function.

3.1. Arginase

In its physiological role, the enzyme arginase catalyzes the reaction of arginine and water to ornithine and urea, thereby neutralizing toxic ammonia which originates from amino acid breakdown [218]. This reaction is the final step in the urea cycle. The human genome encodes two isoforms ARG1 and ARG2. ARG1 is predominantly responsible for the urea cycle, which occurs in the cytoplasm of liver cells, and its role is better understood than that of ARG2, a mitochondrial enzyme.

The enzymatic function of arginase depletes arginine levels and competes with nitric oxide synthases for this shared substrate. High activity of arginase leads to arginine deprivation which hampers T cell responses and oxidative anti-tumor responses of macrophages via nitric oxide synthesized by the inducible nitric oxide synthase (iNOS) [219,220]. Murine macrophages express high levels of ARG1 in response to IL-4, IL-10, and TGF beta [221]. In humans, ARG1 has been detected in M-MDSC of cancer patients [222]. However, in cases where tumor cells, like hepatocellular carcinoma cells, rely on exogenous arginine, arginine depletion via pegylated recombinant human arginase 1 or arginine deiminase is beneficial for patients [223,224].

3.2. IDO1, IDO2 and TDO

A further amino acid that is relevant in regulating immune responses is tryptophan. Its oxidative degradation via the kynurenine pathway is achieved by the enzymes indoleamine 2,3-dioxygenase 1 (IDO1), tryptophan 2,3-dioxygenase (TDO), and IDO2, although the role of the latter is not fully elucidated. These enzymes can be expressed by tumor cells of different origin [225–227] and tumor-infiltrating immune cells like DC and macrophages in humans [228,229]. The tryptophan depletion and the tryptophan metabolites result in impaired

T cell function and induction of apoptosis in T cells and NK cells. Further information can be found in the following publications [219,221,230,231].

3.3. Immune checkpoint inhibitors

On T cells, checkpoint inhibitory molecules like CTLA-4 and PD-1 function as regulators of immune cell proliferation and activation. In healthy individuals, activation of T cells gives rise to a positive feedback loop of checkpoint inhibitor surface expression on T cells to protect the host from overshooting immune responses. In healthy individuals, binding of CTLA-4 and PD-1 to their respective ligands on APC, CD80/CD86 and PD-L1/PD-L2, leads to downregulation of T cell receptor signaling and, hence, proliferation [232]. For PD-1 and CTLA-4, a pathway via PIK3-AKT-mTOR (mechanistic target of rapamycin) is suggested, which decreases cell glycolysis [233–235]. Tumor cells express PD-L1, which binds to PD-1, leading to a reduction of T cell receptor (TCR) signaling and T cell proliferation [236]. Immunotherapy using monoclonal antibodies like anti-CTLA-4 (Ipilimumab), anti-PD-L1 (Atezolizumab) and anti-PD-1 (Nivolumab, Pembrolizumab) aims to level this endogenous precaution by blocking inhibitory receptors on T cells leading to a boost of immune cell proliferation, cytokine production, and activation. Up to 63.8% of patients with untreated advanced melanoma receiving Ipilimumab and Nivolumab and up to 49.5% receiving Ipilimumab alone achieve therapy success and even long-term survival for 2 and 4 years respectively, exhibiting much higher response rates than chemotherapy at these stages [237–240]. Albeit the induction of severe adverse effects due to an unleashed immune system is a downside, immunotherapy with (combinations of) checkpoint inhibitors is a state of the art treatment for stage IV melanoma and various other cancers [241].

3.4. TGF beta & IL-10

TGF beta is a soluble factor expressed by tumors and other cells in the tumor microenvironment [242,243] and is connected with induction of tolerance. The contact of DC with TGF beta is connected with an inhibition of antigen processing, migration, and priming of tumor specific T cell activation in the lymph nodes [244]. TGF beta has also effects on $CD4^+$ and $CD8^+$ T cell function by inhibiting proper tumor specific immune response and inducing Treg [215].

Like TGF beta, IL-10, derived from the tumor, is able to inhibit the maturation of antigen presenting cells and reduces the expression of costimulatory molecules, MHC I and II plus the secretion of inflammatory cytokines [245]. IL-10 also inhibits migration of DC towards secondary lymphoid tissue and is able to induce a tolerogenic phenotype in DC [246]. Beside its effect on DC, IL-10 also leads to an polarization of macrophages into a tolerogenic M2 phenotype [247].

3.5. Prostaglandin PGE2

PGE2 is a prostaglandin produced during inflammation and at high frequencies by cancer cells [248]. In a healthy host, PGE2 often induces a negative feedback-loop to regulate immune responses [249]. Tumor cells exploit this mechanism. Tumor induced immunosuppressive monocytes and macrophages release PGE2 at high levels whereas neutrophils and MDSC show moderate levels. In cancer, high PGE2 levels sustain the chronic inflammatory processes in the tumor microenvironment. This is due to a cyclic adenosine monophosphate (cAMP)-upregulation and the impedement of IL-2 and IFN gamma production by T cells. In addition, the release of IL-1 beta and TNF alpha production of macrophages is inhibited.

4. Conclusion

Although a lot of data dealing with myeloid cells in the tumor environment derive from murine tumor models, their validity for the human system is limited. Therefore, this review focused mainly on human studies, especially when discussing immune cell plasticity.

There are two major obstacles in the analysis of myeloid cells in human tumors and the tumor microenvironment in general:

1. There is a lack of a consensus on unambiguous MDSC marker combinations. Currently, a marker panel consists of several expressed proteins that can be assessed by multicolor flow cytometry, immunofluorescence staining of frozen tissue samples, and novel multicolor immunohistochemistry staining techniques [111]. By isolation of myeloid cells from biopsies, those technologies will facilitate direct analysis of the TME *in vivo*. However, cell function, e.g. suppression of T cell responses, represents the best method to characterize myeloid cell types, but unfortunately this requires a sufficient number of living cells, which is often not possible, and the procedure to isolate homogeneous cellular populations likely generates artefacts. Laser capture technologies and single cell transcriptomics may help to better define these cellular subsets in the future. Defining and using those methods is indispensable to analyze and identify myeloid cells in the tumor microenvironment as otherwise conflicting, non-reproducible data may be generated.
2. Targeting immunotherapies only to one immune cell subset or to a single functional protein may be insufficient, as seen with CSF1R inhibitors. Here, combination therapies, e.g. checkpoint inhibitor therapy using both anti-CTLA-4 and anti-PD-1 have proven more effective than single therapies.

Conflicts of interest

There are no conflicts of interest.

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