Trends in Immunology

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Focus: Macrophage states

Review

Tumor macrophage functional heterogeneity can inform the development of novel cancer therapies

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Macrophages represent a key component of the tumor microenvironment (TME) and are largely associated with poor prognosis. Therapeutic targeting of macrophages has historically focused on inhibiting their recruitment or reprogramming their phenotype from a protumor (M2-like) to an antitumor (M1-like) one. Unfortunately, this approach has not provided clinical breakthroughs that have changed practice. Emerging studies utilizing single-cell RNA-sequencing (scRNA-seq) and spatial transcriptomics have improved our understanding of the ontogeny, phenotype, and functional plasticity of macrophages. Overlaying the wealth of current information regarding macrophage molecular subtypes and functions has also identified novel therapeutic vulnerabilities that might drive better control of tumor-associated macrophages (TAMs). Here, we discuss the functional profiling of macrophages and provide an update of novel macrophage-targeted therapies in development.

Highlights

Single cell transcriptomics and spatial profiling have revealed that tumor-associated macrophages comprise multiple subtypes (molecularly), and harbor different functions within the tumor microenvironment. This can depend on their spatial localization, tumor type, and disease stage.

Six major macrophage clusters might exist in tumors including: inflammatory macrophages, angiogenic macrophages, immunoregulatory macrophages, interferon-mediated regulatory macrophages, immunostimulatory macrophages, and a CD169^{+} macrophage cluster whose distinct function remains an active area of investigation.

Putative therapies aiming to specifically target these functional classes of macrophages are actively being tested preclinically and might begin to show clinical impact. The aim is to block the immunosuppressive, inflammatory, or angiogenic activities of these macrophages.

Significance

A deeper understanding of the molecular, spatial, and functional heterogeneity of tumor-associated macrophages, within the local TME has driven the development of a new class of therapies directed against such diverse functional subsets. This is leading to impressive antitumor responses in preclinical models and is now being tested in patients when given as monotherapies or in combination with targeted and T cell-based immunotherapies.

A framework for classifying TAMs

Macrophages are a functionally heterogeneous population of innate immune cells that orchestrate various aspects of mammalian immunity depending on their phenotype. Historically, macrophages were arbitrarily classified as either inflammatory (M1-like) or anti-inflammatory (M2-like) [1], with M2-macrophages being linked to cancer development [2]. Consequently, therapeutic efforts to date have largely focused on reducing [3–5], depleting [6–10], or reprogramming M2-like macrophages [11–17] but have yielded limited success (Table 1).

One of the earliest scRNA-seq studies on the immune compartment of breast cancer (BCa) showed that human TAMs expressed a mix of both canonical M1-like genes (*INOS*, *IL1B*, *MHCII*, and *CD80*) and M2-like genes (*ARG1*, *CD163*, *MRC1*, and *VEGFA*) [18]. This questioned the original polarization theory of macrophages (M1 vs. M2) which had proposed that the two polarization states were situated at opposite ends of the spectrum. These observations have now been confirmed across numerous cancer types including additional BCa studies as well as colorectal cancer (CRC), gastric cancer (GC), and lung and liver cancer samples [19–23]. This has driven the field to closely examine and define the spectrum of TAM phenotypes.

A new framework for categorizing macrophages by including information regarding their function has been suggested [24] based on two comprehensive pan-cancer scRNA-seq analyses of TAMs in human lung adenocarcinoma (LUAD), CRC, melanoma (MEL), hepatic carcinoma, pancreatic adenocarcinoma, ovarian cancer (OVC), nasopharyngeal cancer, BCa, lymphoma, stomach cancer, urethral cancer, renal cancer, thyroid cancer, and head and neck cancer

[19,25]. This framework has now also been endorsed by others [26,27] and can be applied to mouse models and other cancers models such as gliomas [28–30], in which functionally distinct clusters of macrophages can be identified. We expand on this framework by highlighting the effect that spatial localization and tissue specific programming play in determining macrophage functions [29,31–35]. We also discuss biological data (some of which is preliminary and some of which is published), establishing functional differences in macrophage subsets. This holistic appreciation of macrophage classification is already driving a new era of therapeutic targeting with improved success.

Expanding classification of TAMs

Molecular characterization of TAMs

scRNA-seq has identified novel subsets of macrophages in tumors (e.g., in humans and mice) but the classification of these novel subsets relies on defining macrophages by the highest expressing genes within their assigned scRNA-seq cluster. There is some lack of consensus among different studies on naming the highest expressing transcripts in each subtype. For example, an extensive analysis across various human cancer types indicated that half of all cancers displayed distinct *SPP1* (**osteopontin**, see Glossary) expressing clusters of macrophages. However, in the tumors lacking *SPP1*$^{+}$ macrophage clusters, a common angiogenesis signature was shared with *SPP1* macrophages, as identified by the upregulation of different expression markers (*INHBA*, *VCAN*, or *FN1*) [25]. This highlights the dynamic nature of markers within angiogenic macrophage populations, where their archetypal marker (i.e., *SPP1)* might not be present, but for which the general function of the subset is still present. Moreover, categorizing single-cell clusters based solely on highly upregulated gene subsets might inaccurately lead us to perceive a diversity in phenotypes that is not genuinely present. For instance, macrophages expressing the C1 complement component *C1QC* have been reported to constitute a unique macrophage subset in different human tumors [36]; however, in a pan-cancer analysis of macrophages in several human cancers, *C1QC* expression was highly expressed across several unique subsets of macrophages and across various cancers; in addition, it did not represent a unique macrophage subpopulation [19]. Further work is required to determine if there are numerous complement-regulated macrophage subtypes. Therefore, we and others propose a framework of macrophage classification that also includes some of the functions of these macrophage subsets [24].

Spatial localization of macrophages is important for defining function

An important consideration when studying macrophages in cancer is the role of sublocalization within the TME, which plays a role in determining the function of macrophages. Chronic inflammatory conditions within the TME are known to generate both intrinsic and extrinsic signals that trigger macrophage activation, and the polarization of these macrophages is greatly shaped by specific signaling events that are unique to their niches [37]. This includes the tumor nest, tumor stroma, hypoxic core, and perivascular (PV) regions (Figure 1, Key figure) [21,29,31,32,38]. Thus, the macrophages can adopt different functional states in response to these niches. For instance, the hypoxic core induces **angiogenic TAMs (Ang-TAMs)** through vascular endothelial growth factor (VEGF) and *ANG2* secretion [39]. In human CRC, spatial analysis indicated that *IL1B* and *VEGF* expression – derived from the hypoxic core of the tumor – can drive maturation of CD14^{+} monocytes to macrophages expressing angiogenic markers such as *MARCO*, *SPP1*, and *TIE2* [22]. Multicolor immunofluorescence of human cervical cancer samples showed that tumor-derived exosomes recruited and increased the chemotaxis of Ang-TAMs into tumors in response to hypoxia [40]. The tumor nest has been preliminarily reported to promote **inflammatory TAMs (Inf-TAMs)** via interleukin (IL)-1β, but requires further validation (in preprint) [31]; of note, two pan-cancer analyses of human tumors identified IL-1β as a key cytokine involved in inducing Inf-TAMs. Cell fate analysis of TAMs indicated that *FCN1*$^{+}$ monocyte-like cells overexpressed *IL1B* and gave rise to TAMs

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expressing Inf-TAM signatures [19,25]. Preliminary data requiring validation (in preprint) of human BCa and CRC using immunohistochemical analysis combined with spatial transcriptomics suggested that Inf-TAMs were concentrated within the tumor nest, although some localized to hypoxic regions [31]. This pattern has also been observed in other types of cancer, such as LUAD, [34] and GC, where the tumor periphery has exhibited a low percentage of Inf-TAMs, while the majority localize to the tumor nest [41]. Peripherally, **immunoregulatory TAMs (Reg-TAMs)** might be sustained by interferon (IFN)-γ (TAM subsets expressing *IRF1* and *ISG15*) or **TREM2 signaling** [19,31]. In human BCa and CRC, preliminary data on spatial transcriptomics requiring further validation (in preprint) suggested that Reg-TAMs localized to the stroma and juxtatumoral region where they might control immune infiltration into the tumor [31]. Reg-TAMs can also be induced by inflammatory factors such as nuclear factor (NF)-κB [42], or STAT family transcription factors [43]. Of note, proximity of TAMs to the tumor influences their function, with those near the tumor nest generally being associated with worse prognosis and tumorigenic roles, while those further away might be linked to a better prognosis [21,29]. scRNA-seq studies have indicated that TAMs in the PV region of human and mouse BCa are generally associated with good prognosis. Also, an *in vitro* T cell assay concurred with functional analyses of these subsets that suggested they might be involved in priming $CD8^+$ T cells [21,29]. This might indicate that a key factor maintaining TAM tumorigenic functions could be the proximity of TAMs to tumor-derived signaling. In line with this, macrophages found close to the tumor nest in human BCa tissues have been associated with a worse prognosis, while those furthest away from the tumor may be found interacting with T cells and associated with better prognosis [44–46]. Direct contact between macrophages and endothelial cells in the breast duct has been documented to induce Wnt-1 upregulation that in turn downregulates E-cadherin junctions, promoting metastasis in the MMTV-HER2-neu BCa mouse model [47]. This was also seen in an earlier study that identified direct contact between **$TIE2^+$ macrophages** and macrophage-derived VEGF-A with endothelial cells. This promoted local loss of vascular junctions, transient vascular permeability, and tumor cell intravasation in the same MMTV-$HER2^+$ BCa mouse model, and has correlated with metastasis in BCa patients [47,48]. This suggests that macrophages located distantly from the tumor might harbor tumoricidal properties that contrast the tumorigenic characteristics of those close to the tumor. This possibility merits further attention.

Macrophages can be divided into distinct functional subtypes

The original functional classification of macrophages proposed functional subsets comprising intermediate monocytes (monocyte populations that represent an intermediate stage before maturation to macrophages), lipid-associated macrophages (LAMs), and tissue-resident macrophages (TRMs) [24]. However, recent evidence suggests that LAMs, TRMs, and intermediate monocytes can influence a variety of protumor or antitumor functions in macrophages but that they may not ultimately correspond to a specific function. For example, a report indicated that BCa consists of *APOE*$^+$ LAMs that are either immunosuppressive (*TREM2*$^+$) or inflammatory (*FOLR2*$^+$), based on scRNA-seq of both human and mouse tissues [21]. Of note, the various protumor or antitumor functions that TRMs and LAMs might play within the TME have been extensively reviewed [49,50]. Such a dichotomy in function must be accounted for when studying macrophage heterogeneity but may not be suitable for a functional classification, but rather, potentially considered as an influence (Figure 1).

Along with others [24], we posit that the human TAMs described in most scRNA-seq experiments might fall into six major classes. These include Inf-TAMs, Ang-TAMs, Reg-TAMs, Inf-Reg TAMs, **immunostimulatory macrophages**, and a cluster of **$CD169^+$ macrophages** (Figure 1). $CD169^+$ (*SIGLEC-1)* macrophages are a functionally plastic subset in lymph nodes and have been found in multiple human and mouse cancer models (human mismatch-repair deficiency

Glossary

Angiogenic tumor-associated macrophages (Ang-TAMs): promote angiogenesis through secretion/expression of factors such as VEGF-A and COX-2.
Chimeric antigen receptor T cell therapy (CAR-T cell therapy): comprising T cells harboring single-chain Fv receptors inserted into their genome; these are directed against cell surface molecules on tumor cells.
$CD169^+$ macrophages: guardian macrophages found in lymph nodes/spleens which bind and phagocytose foreign pathogens and cell debris.
Immune checkpoint blockade (ICB): immunotherapeutic modality using monoclonal antibodies to block immunoregulatory molecules; for example, PD-1, hence directing immune cells against immunosuppressive tumor cells.
Immunoregulatory tumor-associated macrophages (Reg-TAMs)
: macrophages that can regulate immune activation through receptor signaling; for example, by PD-L1 expression.
Immunostimulatory macrophages: macrophages that stimulate immune activation through expression of cytokines such as IFNγ.
Inflammatory tumor-associated macrophages (Inf-TAMs): macrophages that promote an inflammatory microenvironment; for example, by secreting type I IFNs.
NLRP3 inflammasome: complex made of NLRP3, ASC, and caspase-1, which when activated, cleaves pro-IL-1β and pro-IL-18, thus promoting local inflammation through IL-1β signaling.
Osteopontin: secreted protein expressed by macrophage subsets involved in chemotaxis of other immune cells, inhibition of apoptosis, and angiogenesis.
$TIE2^+$ macrophages: proangiogenic; expressing TIE2 which is a receptor tyrosine kinase that promotes proliferation and inhibits apoptosis of angiogenic macrophages.
Trajectory analysis: subtype of scRNA-seq analysis defining the developmental trajectory of cell types from progenitor/stem cells.
TREM2 signaling: TREM (triggered receptor expressed on myeloid cells-2) is used for macrophage recognition of extracellular debris, pathogen-associated molecular pattern molecules,

and microsatellite instability-high CRC, stage 2 primary BCa, and orthotopic mouse models with GL261 mouse glioma cell lines) [51–53]. The latter have been associated with both good and poor prognosis breast and bladder cancers, depending on their localization (tumor vs. lymph node). These monocyte derived macrophages have been reported to aid the recruitment of $CD8^+$ T cells and prevent metastasis when found in tertiary lymphoid structures. However, when these localized to the tumor, they adopted an immunosuppressive role by recruiting regulatory T cells (Tregs) into the tumor [52,54], thus making a clear determination of their function difficult (at least for this subtype) (Figure 1).

and damage-associated molecular pattern molecules; this ultimately results in proliferation and phagocytosis via PI3K/Akt activation.

Triple-negative breast cancer: histological type of breast cancer lacking the expression of HER2, estrogen receptor, and progesterone receptor; typically the most aggressive form of breast cancer.

Factors influencing the functional subtypes of macrophages

Functions associated with different macrophage subtypes can be influenced by their LAM, TRM, or monocyte-derived transcriptional programming, as well as by their spatial localization within the TME (Figure 1). For instance, LAMs have been described extensively in BCa [20], and multiple scRNA-seq studies in human and mouse BCa indicate that monocyte-derived LAMs are found specifically within the tumor nest, expressing a tumorigenic LAM signature [21,29,55]. These studies show that monocyte-derived LAMs exhibit an increase in LAM genes such as *APOE*, *CD9*, and *APOC1*, while expressing immunosuppressive *TREM2*, and inflammatory *GPNMB* expression associated with tumorigenesis [21,29,55]. In contrast, tissue-resident LAMs found in the stroma and tumor nest seemingly have not been affected by tumor-specific signaling, demonstrating tumoricidal immunostimulatory signatures regardless of location. Considering the apparent influence of ontogeny (developmental origin) and spatial localization on determining function, these parameters must be accounted for when discussing specific macrophage subsets.

As different TAM subtypes can adapt a spectrum of functional states, it is hard to clearly distinguish them using transcriptional signatures alone, especially because they can exhibit overlapping gene signatures and/or gradients of gene expression [19,25,56]. Therefore, the development of functional assays to assess macrophage functions should work alongside molecular profiling to allow a better characterization of macrophage subtypes. An example of this type of characterization is the expression of *FOLR2*. scRNA-seq studies conducted in mouse and human BCa tissues identified a subset of macrophages marked by *Folr2/FOLR2* expression (initially categorized as T cell-priming subsets) [21]. However, recent spatial analysis of human BCa and CRC tissues has uncovered a second subset of *FOLR2*$^+$ macrophages exhibiting tumor-promoting properties based on immunofluorescence (CODEX imaging) and on scRNA-seq datasets [31]. Although requiring validation (preprints), preliminary data on the analysis of scRNA-seq datasets from seven different human cancers suggested that *TREM2*, commonly used to define immunosuppressive TAMs [57], did not solely predict prognosis, given that different macrophage subsets can express this marker in a context-dependent manner [58]. Thus, within each functional group, predominant gene signatures and pathways involved in tumorigenic functions might be identified to better pinpoint potential targets to inhibit these subtypes, but this should be interpreted with caution, and many of these analyses remain to be verified via other means [24].

Of note, the genetic signatures of macrophage subsets overlap significantly, leading to a spectrum of diverse functions. TAM functional states may also transition into one another at various times and locations within the TME. This phenomenon has been observed via **trajectory analysis** of **triple-negative breast cancer** tissue. Specifically, monocytes recruited into the tumor and which highly expressed *NLRP3* and *IL1B* at first (key Inf-TAM signatures), developed into IFN-stimulated monocytes that matured into *TREM2*$^+$ Reg-TAMs [55]. Potentially, external influences such as IFN expression within the TME may have redirected inflammatory monocytes towards a trajectory

Table 1. Summary of selected clinical trials targeting TAMs in solid tumors[a]

Strategy	Drug/dose	Phase/ no. of patients evaluated	Tumor type	Combination partners	Outcome	Trial ID (clinicaltrials.gov)
TAM depletion	AMG 820 (anti-CSF1R ab) 1100/1400 mg q3w	1B (*n* =15), 2 (*n* =101)	Advanced solid tumors	Pembrolizumab (anti-PD-1 antibody) 200 mg q3w	Completed. Phase 1B (0% CR, 27% SD, 53% PD), Phase 2 (0% CR, 3% PR, 35% SD, 40% PD)	NCT02713529 [10]
	Emactuzumab (anti-CSF1R ab) 500–1000 mg q3w	1 (*n* =37)	Advanced solid tumors	Selicrelumab (CD40 agonist antibody) 2–16 mg q3w	Completed. 0% CR, 40.5% SD	NCT02760797 [9]
	Emactuzumab 100–3000 mg q2w	1 (*n* =54)	Advanced solid tumors	Paclitaxel (chemotherapy) 80 mg/m^2 weekly	Completed. 0% CR, 7% PR, 43% SD	NCT01494688 [8]
	PLX3397/pexidartinib (CSF1R inhibitor) 400–2000 mg daily	1B (*n* =54)	Advanced solid tumors	Paclitaxel 80 mg/m^2 weekly	Completed. 3% CR, 13% PR, 34% SD, 45% PD	NCT01525602 [7]
	ARRY382/ PF-07265804 (CSF1R inhibitor) 200–400 mg oral daily	1B (*n* =19), 2 (*n* =57)	Advanced solid tumors	Pembrolizumab 2 mg/kg every 2 weeks	Terminated due to insufficient clinical activity. Phase 1B (0% CR, 10.5% PR, 26.3% SD, 47.4% PD); Phase 2 (0% CR, 3.7% PR, 29.8% SD, 35% PD)	NCT02880371 [6]
Inhibiting TAM recruitment	PF-04136309 (CCR2 inhibitor) 125 mg twice daily	1B (*n* =21)	Pancreatic cancer	Nab-paclitaxel 125 mg/m^2 Gemcitabine 1000 mg/m^2 weekly on a 3-week cycle	Completed. 0% CR, 23.8% PR, 14.2% SD	NCT02732938 [5]
	Carlumab (CCL2 inhibitor) 15 mg/kg intravenous infusion q3w	1B (*n* =48)	Advanced solid tumors	Either docetaxel (chemotherapy) 75 mg/m^2, gemcitabine (chemotherapy) 1000 mg/m^2, or paclitaxel + carboplatin (platinum chemotherapy) 175 mg/m^2 q3w	Completed. 0% CR, 2% PR, 38% SD	NCT01204996 [4]
	BL-8040 (CXCR4 antagonist) 1.25 mg/kg daily for 5 days followed by same dose tiw	2A (*n* =37)	Pancreatic cancer	Pembrolizumab 200 mg once q3w	Completed. 0% CR, 3.4% PR, 31% SD	NCT02826486 [3]
	Maraviroc (CCR5 inhibitor) 300 mg twice daily	1 (*n* =20)	Colorectal cancer	Pembrolizumab 200 mg q3w	Completed. 0% CR, 5% PR, 5% SD.	NCT03274804 [107]
TAM reprogramming	IPI-549 (selective PI3K-γ inhibitor) 20–40 mg daily	1 (*n* =31)	Advanced solid cancers	Nivolumab (anti-PD-1 antibody) 240 mg q2w	Active. 7% PR at first assessment	NCT02637531 [17]
	Imiquimod (topical TR7 agonist) 5 days/week for 8 weeks	2 (*n* =10)	Breast cancer	N/A	Completed. 0% CR, 20% PR, 50% SD, 20% PD	NCT00899574 [16]
	Motolimod/VTX-2337 (TLR8 small molecule agonist) (3 mg/m^2) weekly (six cycles)	II (*n* =195)	Squamous cell of head and neck	Cisplatin (100 mg/m^2) or carboplatin + 5-FU (1000 mg/m^2/d) + cetuximab (400 mg/m^2 followed by 250 mg/m^2 daily)	Completed. No improvement in overall survival.	NCT01836029 [15]
	Sotigalimab/APX005M (CD40 agonist antibody) 0.1 or 0.3 mg/kg 2 days post chemo	1/2 (*n* =24)	Pancreatic cancer	Gemcitabine (1000 mg/m^2) and nab-paclitaxel (125 mg/m^2) weekly for 3 weeks followed by 1 week off ± nivolumab 240 mg q2w	Completed. 0% CR, 58% PR, 29% SD, 4% PD	NCT03214250 [14]

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Table 1. (continued)

Strategy	Drug/dose	Phase/ no. of patients evaluated	Tumor type	Combination partners	Outcome	Trial ID (clinicaltrials.gov)
	CP-870,893 (CD40 agonist antibody) 0.1 or 0.2 mg/kg 2 days after first chemotherapy dose of cycle	1 (*n* =21)	Pancreatic cancer	Gemcitabine 1000 mg/m^2 weekly for 3 weeks followed by 1 week off	Completed. 0% CR, 19% PR, 50% SD	NCT00711191 [108]
	Ibrutinib (BTK inhibitor) 560 mg daily	3 (*n* =424)	Pancreatic cancer	Gemcitabine (1000 mg/m^2) and nab-paclitaxel (125 mg/m^2) weekly for 3 weeks followed by 1 week off	Completed. 0% CR, 29% PR	NCT02436668 [13]
	Galunisertib (TGFβ receptor inhibitor) 50 mg daily or 50, 80, 150 mg twice daily	1B (*n* =32)	Pancreatic cancer	Durvalumab (anti-PD-L1 antibody) 1500 mg q4w	Completed; 0% CR, 3% PR, 21% SD, 47% PD	NCT02734160 [12]
	Cediranib (VEGFR inhibitor) 30 mg daily	2	Advanced solid cancers	Olaparib (PARP inhibitor) 200 mg twice daily	Active, not recruiting. Results pending	NCT02498613
	Zoledronic acid (bisphosphanate) 4 mg every 6 months	3 (*n* =1803)	Breast cancer	Goserelin (LH blocker) 3.6 mg q4w plus tamoxifen (ER antagonist) daily or anastrozole (aromatase inhibitor) 1 mg/day	Completed. Addition of zoledronic acid increased disease-free survival by 3.2% and reduced the risk of disease progression by 36%	NCT00295646 [11]

[a]Abbreviations: CR, complete response; ER, estrogen receptor; LH, luteinizing hormone; PD, progressive disease; PR, partial response; q2w, every 2 weeks; q3w, every 3 weeks; q4w, every 4 weeks; SD, stable disease; tiw, three times a week.

culminating in the development of Reg-TAMs based on transcriptional changes in response to IFNs. This might suggest that the trajectory from which Inf-TAMs develop is shared with Reg-TAMs. Presumably, the switch between an Inf-Mac and a Reg-TAM might be as simple as the expression of IFNs within the TME. Understanding the relationship between different functional subsets and the role that localized niches in the TME play in macrophage maturation might also help us to develop targeted candidate therapies to deplete terminally differentiated subsets or reprogram those that can switch to other subsets. Of note, Ang-TAMs can also arise from Inf-TAMS; the former have shown increased expression of genes encoding inflammatory cytokines such as IL-6, IL-1β, and tumor necrosis factor (TNF)α in a mouse xenograft model of cervical cancer [40]. This, and the presence of inflammatory regulator *FOSL2* in Ang-TAMs in the hypoxic core of several mouse and human scRNA-seq studies, suggests that they might harbor inflammatory functions controlled by hypoxic conditions (Figure 1) [59]. In BCa, CRC, LUAD, and GC, the tumor core and hypoxic region are exclusively enriched by macrophages with inflammatory signatures overlapping angiogenic ones [31,34,41]. This suggests that Ang-TAMs might arise from Inf-TAMs in hypoxic conditions and under tumor-induced VEGF-A, perhaps because Inf-TAMs in the tumor nest are recruited into the hypoxic core by chemokines such as VEGF-A. Understanding such functional switches is relevant because targeting Inf-TAMs might result in depletion of Ang-TAMs. Targeting Inf-TAMs might also result in resistance by Inf-TAMs, generating transcriptionally different Ang-TAMs. Given that Inf-TAMs have been implicated as the only subset playing a possible role in cancer initiation, expressing inflammatory cytokines such as IL-1β in mouse models of LUAD (EGFR$^+$ mouse models exposed to particulate matter) and BCa (MMTV-Her2-neu transgenic model) [47,60,61], perhaps Inf-TAMs can give rise to other subsets upon tumor progression. Therefore, these findings highlight the concept of complex programming in TAMs, a process which must be validated when analyzing scRNA-seq data.

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Key figure

A spatial map of human macrophages and their interactions within the tumor microenvironment

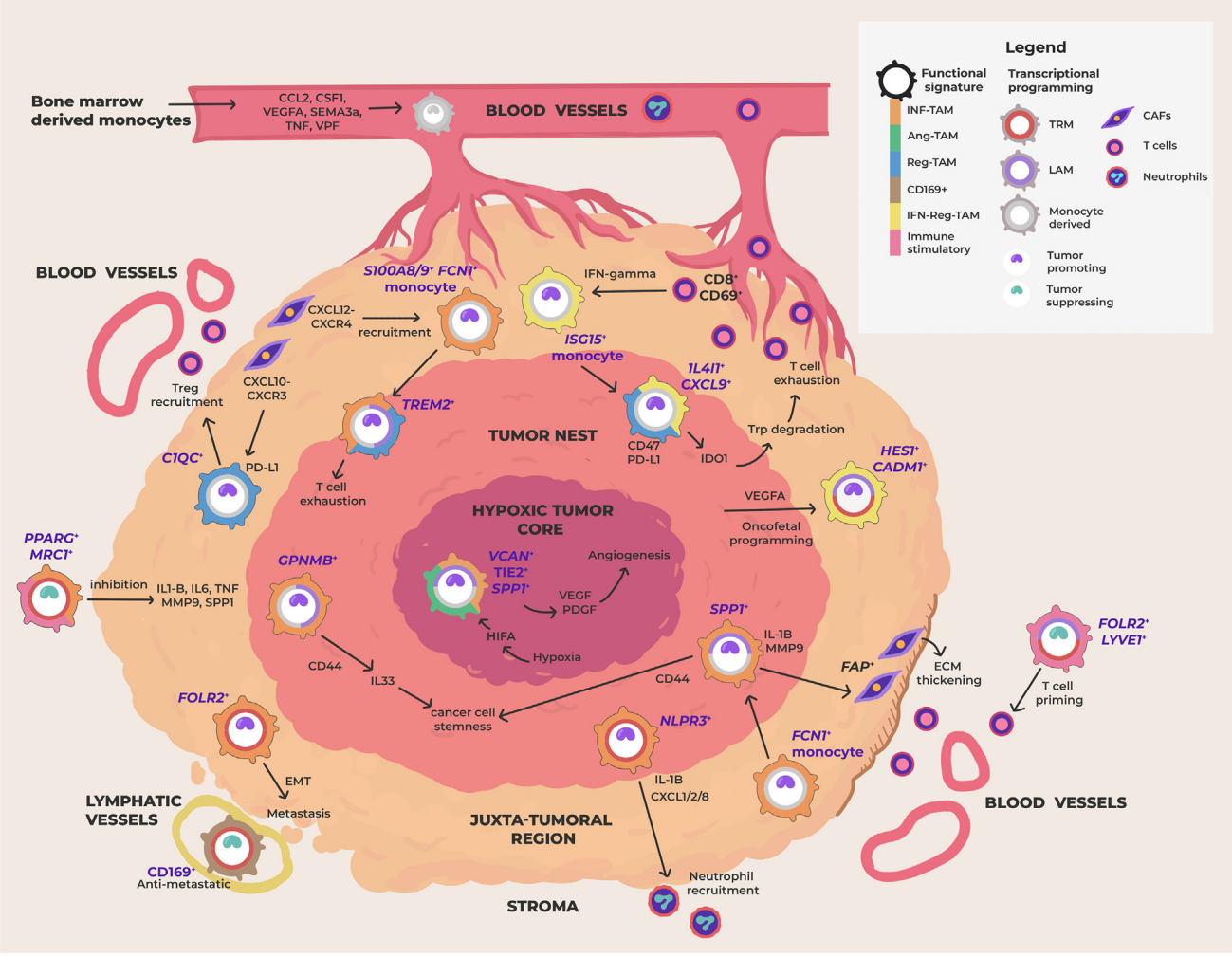

Figure 1. Circulating monocytes are recruited into the tumor by factors such as chemokine CC ligand 2 (CCL-2), colony-stimulating factor 1 (CSF-1), vascular endothelial growth factor A (VEFGA), semaphorin 3A (Sema3A), tumor necrosis factor (TNF), and vascular permeability factor (VPF), and mature into intermediate monocytes and macrophages [103]. Different macrophage functional subsets may include inflammatory tumor-associated macrophages (Inf-TAMs), angiogenic TAMs (Ang-TAMs), immunoregulatory TAMs (Reg-TAMs), interferon-mediated regulatory TAMs (IFN-Reg-TAMs), immunostimulatory macrophages, and CD169$^+$ macrophages (which act to prevent tumor metastasis if present in the lymph node) [51,54]. Ang-TAMs within the hypoxic core are glycolytic and can promote angiogenesis and tumor vasculature [25,31]. In the tumor nest, monocyte-derived Reg-TAMs expressing *C1QC* and *TREM2* interact with T cells, inducing T cell dysfunction (exhaustion) and Treg infiltration [19]. *C1QC*$^+$ TAMs are recruited by cancer-associated fibroblasts (CAFs) via the chemokine CXC ligand (CXCL)10–chemokine CXC receptor (CXCR)3 axis [36]. Monocytes recruited via the CXCL12–CXCR4 axis mature to *Trem2*-expressing Reg-TAMs that in turn can suppress T cells [55]. Monocytes primed by IFNγ signaling from activated CD8$^+$ T cells develop into *ISG15*$^+$ early macrophages that mature into IFN-Reg-TAMs [31]. *NLRP3*$^+$ Inf-TAMs activate inflammasomes via the CXCL1/2/8 axis to recruit neutrophils that promote metastasis [31]. *GPNMB*$^+$ and *SPP1*$^+$ Inf-TAMs interact with tumor-secreted CD44 to induce IL-33 that can promote tumor proliferation [104,105]. Folate receptor 2 (FOLR2)-expressing Inf-TAMs in the tumor nest promote the epithelial-to-mesenchymal transition in metastasis, and dissemination of tumor cells [47]. Moreover, *SPP1*$^+$ Inf-TAMs can also interact with FAP$^+$ fibroblasts to increase fibrosis via MMP-9 [34] and secrete IL-1β maintaining chronic inflammation within the tumor. Tissue-resident tumor-suppressing macrophages reside in the perivascular (PV) regions of the stroma, mostly consisting of FOLR2$^+$, CD206$^+$, LYVE$^+$, or PPARG$^+$ TRMs that can prime T cells to enter the tumor tissue via blood capillaries [21,31]. Alternatively, in the tumor nest, oncofetal programming from the tumor induce HES1$^+$ TRMs to perform Reg-TAM functions [106].

Indeed, the presumed functional classification of macrophages that primarily relies on data obtained from scRNA-seq emphasizes the importance of experimentally validating these roles. Thus, detailed studies in preclinical models accurately recapitulating observations from human tumors are required. In addition, this must be complemented with molecular analyses in human tumors that align with functional biological assays such as T cell assays (immunosuppression) [62–64], metabolic assays [65], tube forming assays (angiogenesis) [66,67], tumor proliferation [68], or wound-healing assays (inflammatory functions) [69].

Novel candidate therapeutic approaches targeting macrophages

New insights into macrophage biology are enabling the development of novel putative therapies to inhibit TAM-mediated immunosuppression, angiogenesis, or inflammation. This might be achieved by targeting receptors, transcription factors, or secreted proteins involved in metabolic reprogramming or downstream/upstream signaling pathways, as summarized in the following section.

Targeting TAM-mediated immunosuppression

One of the key functions performed by TAMs is the suppression of the immune system to promote tumor immune evasion. With such functional ability, TAMs can be categorized as Reg-TAMs. Inhibition of Reg-TAMs and their functions can allow the adaptive immune system to infiltrate and attack tumors, as in the case of *Trem2*$^+$ Reg-TAM inhibition with Trem2 small molecule inhibitors, or inhibition of immunosuppressive enzymes such as indoleamine-2,3-dioxygenase (IDO)1 from Reg-TAMs (Figure 1) [70]. In some cases, this has yielded strong tumoricidal effects preclinically (Table 2) that are now being tested in the clinic (Table 3); for example, targeting the IDO1 enzyme in recurrent solid tumors with navoximod (GDC-0919, NLG-919) in a Phase 1 trial showed promise in stabilizing disease (NCT02048709)[i] [71]. Given the immunosuppressive nature of Reg-TAMs, a major area of interest includes using targeted inhibitors of Reg-TAMs in combination with **immune checkpoint blockade (ICB)** in several solid tumors [72,73]. Indeed, treatment with an IDO1 enzyme inhibitor (epacadostat) in combination with ICB in advanced solid tumors was more effective than monotherapy showing an objective response rate of 55% in patients compared with controls (NCT02178722)[ii] [74].

scRNA-seq has identified *Trem2* as an essential gene in Reg-TAMs, contributing to immunosuppression in aggressive orthotopic ovarian and triple negative transgenic BCa mouse models [55,57]. Indeed, targeting *Trem2*$^+$ macrophages with specific inhibitors such as PY314 has shown promise in enhancing the effectiveness of ICB in OVC preclinical models by reducing tumor growth and increasing T cell antitumor activity [57], leading to ongoing clinical trials (NCT04691375[iii], NCT04682431[iv]). The inflammatory COX-2/PGE2 pathway has been implicated in promoting tumor immune evasion by recruiting and activating Reg-TAMs, as seen in mouse models of CRC adenomas; COX-2–derived PGE2 increased the recruitment of TAMs into the tumor as well as PD-1 expression on CD8$^+$ T cells [75]. Overexpression of COX-2 led to TAM recruitment and increased gastric hyperplastic growth in a COX-2/mPGES-1 transgenic GC mouse model [76]. The COX-2/PGE_2 pathway in Reg-TAMs has been targeted to alleviate immunosuppression, with COX-2 inhibitors such as celecoxib and aspirin showing promising results in clinical trials for CRC (Table 3) [77,78] (NCT03026140[v]; NCT03926338[vi]). Another approach has been to target the immunosuppressive enzyme, Arginase (ARG)1, from the arginine catabolism pathway that is upregulated in Reg-TAMs. ARG1 removes arginine from the TME, a key amino acid required for the activation and proliferation of CD8$^+$ T cells, resulting in T cell suppression [79]. A first in class inhibitor of ARG1 has been tested in a Phase 1 trial that demonstrated on-target inhibition of ARG, increased arginine availability, and a good safety profile with only moderate adverse events reported in patients with solid tumors (NCT02903914)[vii]

(Table 3) [79]. Additionally, approaches targeting the folate receptor (FR)β in Reg-TAMs in the ID8 mouse OVC model demonstrated the potential for restricting the tumor [80] via recruitment of proinflammatory monocytes and cytotoxic $CD8^+$ T cells [48].

Targeting TAM-mediated angiogenesis

The prevalence of Ang-TAMs in the hypoxic core of most solid tumors and their ability to stimulate blood vessel formation render these cells an ideal candidate therapeutic target. Therapeutic targeting of Ang-TAMs has so far focused on their recruitment (Tables 2 and 3). Historically, targeting angiogenesis in cancer has been challenging and efforts to normalize tumor vasculature by targeting VEGF-A have resulted in a hypoxic environment that promotes the recruitment of Ang-TAMs via angiopoietin (ANG)2, acting as a facilitator of angiogenesis and leading to therapeutic resistance [81]. Therefore, an approach to improve the therapeutic outcome of VEGF inhibitors has been to combine it with an ANG2 inhibitor. Promising results have been observed in two orthotopic mouse models of glioblastoma (GI261 and U87) with the use of a bispecific ANG2–VEGF antibody, which improved survival over each therapy alone, delaying GI261 growth, increasing U87 necrosis, and reducing tumor burden. The dual therapies also enhanced morphological normalization of vessels. [50,51]. These preclinical findings suggest that further exploration of bispecific ANG2–VEGF antibodies rather than VEGF antibodies alone as putative treatment strategies to improve resistance to anti-VEGF antibody treatments in cancer is warranted [82,83].

Targeting TAM-mediated inflammation

Inf-TAMs actively induce inflammatory pathways, which in turn sustain the immunosuppressive and proliferative hallmarks of cancer. Consequently, the proteins responsible for initiating these tumorigenic processes have been considered promising therapeutic targets. Macrophage-

Table 2. Selected preclinical tests performed targeting functional endotypes of macrophages

Strategy	Potential inhibitors	Combination partners	Biological effects	Tumor models
Targeting Inf-TAMs	NHWD-870 (BET inhibitor) [91]	Sunitinib	Suppressed melanoma cell proliferation, migration, and invasion	Melanoma
	Wortmannin (PI3K inhibitor) and SP600125 (JNK inhibitor) [92]	None	Reduced tumor cell proliferation	Prostate
	Dapansutrile (NLRP3 inhibitor) (OLT1177) [90]	Anti-PD-1 antibody	Reduced T cell suppression and tumor progression	Melanoma
	Anti-IL-1β antibody [86]	Anti-PD-1 antibody or cabozantinib (RTK inhibitor)	Reduced neutrophils and enhanced antitumor activity of TAMs independent of T cell activity	Renal carcinoma
	I-aptamer (anti C5a) [93]	Anti-PD-1 antibody (RMP1-14)	Increased frequency of $CD8^+$ T cells and reduced frequency of myeloid-derived suppressor cells	Lung cancer
Targeting Reg-TAMs	Anti-TREM2 monoclonal antibody [57]	Anti-PD-1 antibody	Depleted TAMs and enhanced antitumor immunity	Ovarian cancer
	Phenelzine (small molecule monoamine oxidase A inhibitor) [94]	Anti-PD-1 antibody	Reprogrammed TAMs and suppressed tumor growth	Colon cancer Melanoma
	CAR-T cell therapy targeting FOLR2 [80]	None	Reprogrammed TME, promoted endogenous T cell-mediated immunity and reduced tumor growth	Ovarian cancer
	BGB-5777 (IDO1 inhibitor) [70]	Anti-PD-1 antibody and radiotherapy	Reduced tryptophan catabolism by Reg-TAMs resulting in enhanced T cell infiltration and effector function of T cells	Glioblastoma
Targeting Ang-TAMs	Cediranib (VEGFR inhibitor) [82]	MEDI3617 (Ang-2 inhibitor)	Reduced tumor burden, improved vascular normalization, and altered TAMs towards Inf-TAMs	Glioblastoma
	Ang-2/VEGF bispecific antibody [83]	None	Delayed tumor growth, increased numbers of antitumor macrophages, and pruning of immature tumor blood vessels	Glioblastoma

Table 3. Selected clinical trials targeting functional endotypes of macrophages[a]

Strategy	Drug	Phase	Tumor type	Combination partners	Outcome	Trial ID
Targeting Inf-TAMs	Canakinumab (anti-IL-1β antibody)	3 (*n* = 10066)	Lung cancer	None	>60% reduction of incidence and mortality from lung cancer	NCT01327846 [87]
	Anakinra (IL-1Ra antagonist recombinant protein)	1 (*n* = 16)	Pancreatic cancer	Gemcitabine (chemotherapy), nab-paclitaxel (chemotherapy), cisplatin (platinum chemotherapy)	Completed, improved survival, median PFS of 13 months, no recurrence of disease	NCT02550327 [88]
		2 (single-arm) (*n* = 32)	Metastatic CRC	Fluorouracil (chemotherapy) and bevacizumab (anti VEGF-A antibody)	Completed, median PFS of 5.4 months and overall survival of 8.7 months	NCT02090101 [89]
Targeting Ang-macrophages	Cediranib (VEGFR inhibitor)	1 (*n* = 126)	Advanced solid cancers	Olaparib (PARP inhibitor)	Active, not recruiting	NCT02498613
Targeting Reg-TAMs	PY314 (anti-TREM2 antibody)	1 (*n* = 288)	Solid tumors	Pembrolizumab (anti-PD-1 antibody)	Active, not recruiting	NCT04691375
	PY159 (anti-TREM1 antibody)	1 (*n* = 343)	Solid tumors	Pembrolizumab	Active, not recruiting	NCT04682431
	Celecoxib (NSAID)	2 (*n* = 268)	CRC	Ipilimumab (anti-CTLA4 antibody) + nivolumab (anti-PD-1 antibody)	Completed, 100% pathological CR	NCT03026140 [77]
	Celecoxib (NSAID)	1 (*n* = 34)	Microsatellite instability-high, locally advanced, colorectal cancer	Toripalimab (anti-PD-1 antibody)	Completed, 88% pathological CR. Acceptable safety profile	NCT03926338 [78]
	Aspirin (NSAID)	Proof of concept window trial	Triple-negative breast cancer	Avelumab (anti-PD-L1 antibody)	Active, not yet recruiting	NCT04188119
	Navoximod (small molecule IDO1 inhibitor)	1 (*n* = 22)	Recurrent advanced solid tumors	None	Completed, 36% had SD and 46% had PD	NCT02048709 [71]
	BMS-986205 (small molecule IDO1 inhibitor)	1/2 (*n* = 12)	Advanced malignant solid tumors	Nivolumab	Completed, data not shared yet	NCT03792750
	Epacadostat (small molecule IDO1 inhibitor)	1/2 (n = 62)	Advanced solid tumors	Pembrolizumab	Completed, 55% objective response rate, 13% CR, 27% PR, well-tolerated safety profile	NCT02178722 [74]
	CB-1158 (small molecule arginase inhibitor)	1 (*n* = 260)	Solid tumors	Pembrolizumab	Completed, 6% objective response rate, 37% SD.	NCT02903914 [79]

[a]Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; PFS, progression-free survival.

secreted IL-1β is a significant target due to its role in maintaining protumor inflammatory functions in TAMs. Blocking IL-1β in mouse models of prostate, breast [84], and renal carcinoma [85] has led to reduced tumor growth. In a mouse model of renal cancer, inhibiting the IL-1β receptor axis decreased protumorigenic TAMs and tumor growth, independently of T cell antitumor immunity [86]. A large Phase 3 trial of canakinumab (IL-1β-blocking antibody) in patients with cardiovascular conditions showed a >60% reduction in LUAD incidence and mortality compared to patients in the placebo group, highlighting its potential as a possible preventive cancer therapeutic (NCT01327846)[viii] [87]. As a result, clinical trials for pancreatic cancer [88] and CRC [89] targeting IL-1β have shown impressive results (Table 3).

Other targets for Inf-TAMs include the **NLRP3 inflammasome** and TAM-derived osteopontin (SPP1) – both crucial for Inf-TAM functions. Specifically, inhibiting NLRP3 inflammasome with dapansutrile reduced TAM expansion and improved antitumor immunity in an orthotopic B16F10 melanoma mouse model [90]. BET inhibitors targeting SPP1-mediated pathways reduced tumor burden in an A375 orthotopic melanoma mouse model [91] and specific inhibitors blocking Akt and JNK activation downstream of the CD44-SPP1 pathway eliminated macrophage-induced cell proliferation during tumor initiation (prostate neoplasia) in mouse models of prostate [57,92]. These findings suggest that counteracting Inf-TAM-mediated tumor promotion might be a useful prophylactic approach in addition to its therapeutic potential, warranting further investigation. The examples discussed in this section focus on the shift from previously less responsive treatments (Table 1) to more tailored targeting with higher potential (Tables 2 and 3). Of note, several novel approaches targeting TAMs are still in clinical trials or have only shown efficacy preclinically [80,86,92–94].

Association of functional macrophage subsets with cancer prognosis

To provide further support for the importance of different functional macrophage subsets in cancer progression, it will be important to determine if there are specific subsets that are associated with poor response/resistance to chemotherapy and immunotherapy. Perhaps we can then target them to improve clinical outcomes. While the biological functions or the gain or loss of certain single cell macrophage clusters has yet to be aligned with prognosis, some of the markers we associate with macrophage subsets have been reported. For instance, early recurrence-free survival of pancreatic cancer patients has worse prognosis in tumors harboring $TIE2^{+}$ macrophages [95]. Moreover, high TIE2 expression in monocytes within GC tumor tissue has been associated with poor survival [96]. Also, *TREM2* gene expression has been negatively associated with prognosis in most human cancers [97]. Of note, IL-4- induced 1 (IL4I1) expression (a feature of IFN-primed regulatory TAMs) has been associated with bad prognosis in OVC patients [98]. A last example listed here is the increased expression of SPP1 in tumors, which has been associated with poor prognosis in CRC and LUAD patients [34,99,100].

Concluding remarks

Recent advancements such as scRNA-seq have unveiled a wealth of new information regarding the origin, location, stage, and function of TAMs, along with their unique expression markers and metabolic profiles. However, the fundamental rules that determine the diversity of TAMs in cancer, as well as the roles that ontogeny and tumor programming plays in modulating the functional diversity of TAMs, remain unclear. We also do not fully understand how the functional subsets change as the tumor evolves and spreads to distant organs. We need to understand how the TME in primary and metastatic lesions impacts macrophage functional polarizations. As studies using new tools are now taking an in-depth look at TAMs, and we expect that many of these questions might be answered in coming years, ideally yielding promising targets for the development of candidate therapies.

We are likely to enter a new era of macrophage-targeted therapies as these new technologies evolve to leverage the ubiquitous roles that macrophages play. Also, advancements in macrophage-targeted therapies are on the horizon as medical nanotechnology is harnessed for drug delivery, showing promise in putative anticancer treatments [101]. Macrophage-based cell therapies, such as chimeric antigen receptor (CAR)-macrophages, might offer certain advantages over **CAR-T cell therapy**, perhaps in solid tumor treatments, although this awaits robust investigation. Monocyte and macrophage precursors continually infiltrate tumors, thus enabling possible macrophage-based strategies to deliver cytokines or nanoparticles to the TME, or express engineered receptors [102]. Understanding macrophage biology is therefore necessary for developing such putative

Outstanding questions

Some of the molecular markers identifying particular macrophage clusters are associated with poor prognosis; however, how do changes in macrophage function and spatial distribution impact prognosis? Is this association distinct for different tumor types?

Can we show in preclinical studies that novel candidate therapeutics targeting macrophage function can drive biological inhibition of tumor angiogenesis, ECM deposition, and improve the ability of other innate and adaptive immune cells to acquire antitumor functions?

Are there specific macrophage subsets that are associated with poor response/resistance to chemotherapy and immunotherapy, and can these be targeted to improve clinical outcomes?

CAR-T cell therapy in solid tumors remains challenging due to immunosuppressive tumor microenvironments. Can we identify and target the macrophage subsets present within different solid tumors to improve the efficacy of CAR-T cell therapies?

Are the same functional macrophage clusters present earlier in tumor progression? If so, can we extend macrophage targeting therapies to *in situ* disease? If they are different, what does this tell us about the role of macrophages in early cancer development and how can we bolster their antitumor activity, and/or limit protumorigenic effects?

Can we develop preclinical models that recapitulate the full spectrum of macrophage subsets within the TME, while aiming to match the tissue composition for individual patients?

What are the molecular drivers that switch macrophages from one functional state to another, or drive the changes in function based on localization?

improved therapeutics, whether it be utilizing possible CAR-macrophages or targeting macrophages directly.

It is an exciting time for macrophage tumor immunologists. Key questions to address (see Outstanding questions) include determining whether novel function-specific directed therapies can drive functional changes in the TME. Do certain functional macrophage subsets correlate with responsiveness to targeted therapy, chemotherapy, or immunotherapy, and can macrophage therapies be introduced in combination with these treatments? If we want to extend macrophage therapies at an earlier stage in tumor development, it will be important to determine if the same functional macrophage clusters are present at earlier timepoints in tumor progression.

Molecular profiling together with recent data on spatial localization and functional macrophage subsets are providing a platform for the development of new and more effective antitumor macrophage targeting therapies. It is exciting that certain preclinical results have been impressive and may start to have clinical impact.

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Declaration of interests

No interests are declared.

Resources

[i]https://clinicaltrials.gov/study/NCT02048709

[ii]https://clinicaltrials.gov/study/NCT02178722

[iii]https://clinicaltrials.gov/study/NCT04691375

[iv]https://clinicaltrials.gov/study/NCT04682431

[v]https://clinicaltrials.gov/study/NCT03026140

[vi]https://clinicaltrials.gov/study/NCT03926338

[vii]https://clinicaltrials.gov/study/NCT02903914

[viii]https://clinicaltrials.gov/study/NCT01327846

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