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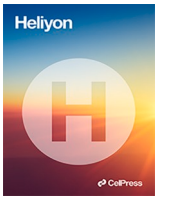

Review article

Macrophage diversity in human cancers: New insight provided by single-cell resolution and spatial context

Militsa Rakina [a,b,1], Irina Larionova [a,b,1,2,*], Julia Kzhyshkowska [a,c,d,**]

[a] Laboratory of Translational Cellular and Molecular Biomedicine, National Research Tomsk State University, Tomsk, 634050, Russia
[b] Laboratory of Molecular Therapy of Cancer, Cancer Research Institute, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, 634009, Russia
[c] Institute of Transfusion Medicine and Immunology, Institute for Innate Immunoscience (MI3), Medical Faculty Mannheim, University of Heidelberg, Mannheim, 68167, Germany
[d] German Red Cross Blood Service Baden-Württemberg – Hessen, Mannheim, 68167, Germany

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ABSTRACT

M1/M2 paradigm of macrophage plasticity has existed for decades. Now it becomes clear that this dichotomy doesn't adequately reflect the diversity of macrophage phenotypes in tumor microenvironment (TME). Tumor-associated macrophages (TAMs) are a major population of innate immune cells in the TME that promotes tumor cell proliferation, angiogenesis and lymphangiogenesis, invasion and metastatic niche formation, as well as response to anti-tumor therapy. However, the fundamental restriction in therapeutic TAM targeting is the limited knowledge about the specific TAM states in distinct human cancer types. Here we summarized the results of the most recent studies that use advanced technologies (e.g. single-cell RNA sequencing and spatial transcriptomics) allowing to decipher novel functional subsets of TAMs in numerous human cancers. The transcriptomic profiles of these TAM subsets and their clinical significance were described. We emphasized the characteristics of specific TAM subpopulations – TREM2+, SPP1+, MARCO+, FOLR2+, SIGLEC1+, APOC1+, C1QC+, and others, which have been most extensively characterized in several cancers, and are associated with cancer prognosis. Spatial transcriptomics technologies defined specific spatial interactions between TAMs and other cell types, especially fibroblasts, in tumors. Spatial transcriptomics methods were also applied to identify markers of immunotherapy response, which are expressed by macrophages or in the macrophage-abundant regions. We highlighted the perspectives for novel techniques that utilize spatial and single cell resolution in investigating new ligand-receptor interactions for effective immunotherapy based on TAM-targeting.

* Corresponding author. Laboratory of translational cellular and molecular biomedicine, National Research Tomsk State University, Tomsk, 634050, Russia.
** Corresponding author. Institute of Transfusion Medicine and Immunology, Institute for Innate Immunoscience (MI3), Medical Faculty Mannheim, University of Heidelberg, Mannheim, 68167, Germany
E-mail addresses: larionovaiv@onco.tnimc.ru (I. Larionova), julia.kzhyshkowska@medma.uni-heidelberg.de (J. Kzhyshkowska).
[1] These authors equally contributed.
[2] Lead contact Irina Larionova larionovaiv@onco.tnimc.ru

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1. Introduction

Macrophage diversity has been studied since late 1960s, and in 2000, Mills and colleagues proposed the M1/M2 macrophage paradigm [1]. According to this concept, macrophages are divided into two groups due to unrivaled plasticity that allows them to modulate their phenotype in response to external activating stimuli. The M1 type corresponds to "classically" activated pro-inflammatory macrophages that are induced by pro-inflammatory signals, including IFN-γ, TNF-α, LPS and Toll-like receptor (TLR) ligands. M1 macrophages have an increased ability to secrete cytokines such as IL-1, TNF, IL-12 and IL-18; phenotypically they express high levels of major histocompatibility complex class II (MHC-II) antigens, CD80/CD86 costimulatory molecules, HLA-DR, and CD197 [2]. The M2 type represents "alternatively" activated anti-inflammatory macrophages that are induced by IL-4, IL-10, IL-13, TGF-β, M-CSF and produce IL-10, TGF-β, VEGF, MMPs, and other anti-inflammatory cytokines. Phenotypically, this population is characterized by the expression of the macrophage mannose receptor CD206, scavenger receptor CD163, CD209, and CCL2 [3–5].

TAMs can stimulate cancer cell proliferation and primary tumor growth, angiogenesis, lymphangiogenesis, cancer cell invasiveness in vessels and metastatic niche formation, as well as tumor response to anti-cancer therapy [6–9]. According to the M1/M2 paradigm tumor-associated macrophages (TAMs) mostly resemble M2-type cells [4,10]. In the early stage of tumor development, local inflammation is sustained in the tumor microenvironment (TME) by secreted pro-inflammatory cytokines and chemokines, which induce TAM polarization to the M1 type. A prolonged M1-like TAM activity can drive tumor growth due to fostering chronic inflammation and promoting genomic instability in malignant cells [11,12]. Once tumor is established, TAMs are "re-educated" to an immunosuppressive anti-inflammatory M2 phenotype supporting tumor growth and facilitating tumor progression by producing tumor growth factors (e.g. EGF, FGF, TGFb, PDGF), pro-angiogenic molecules (e.g. VEGF-A, SPP1, YKL-40, TIE2), immunosuppressive factors (e.g. IL-10, PD-L1), and matrix remodeling factors (e.g. matrix metalloproteases [MMPs], uPA) [11–14].

When studying tumors of various localizations, the main markers of TAMs were identified: CD163, CD204 (MSR1), CD206 (MRC1), MARCO, SIGLEC1 (CD169), stabilin-1 (Stab1), and Tie2 (TEK) [13]. They are M2-type markers and are associated with unfavorable prognosis in cancer patients [13]. According to the existing findings, CD206 and CD163 are the most popular markers of M2-skewed TAMs, and correlate with metastasis and poor disease outcome in many cancer types [13]. CD204-expressing TAMs are involved in tumor progression in gastric, colorectal, breast, lung, ovarian, pancreatic and esophageal cancers [15–22]. MARCO+ TAMs were found to be associated with poor prognosis in lung, hepatocellular, breast and pancreatic cancers [23–26]. High stabilin-1 expression (identified frequently by RS1 antibody) was associated with poor patient survival in pancreatic, gastric and bladder cancers [27–29].

In the last 10–15 years, the technological advances in biomedical field have made possible a more thorough investigation of tumors allowing for the search of new tumor progression mechanisms and novel tumor-associated markers. Single cell RNA sequencing (scRNA-seq) in comparison to bulk sequencing has drastically improved our understanding of cellular functions and heterogeneity through providing a more comprehensive transcriptome information at the single cell resolution [30]. In recent years, most cancer

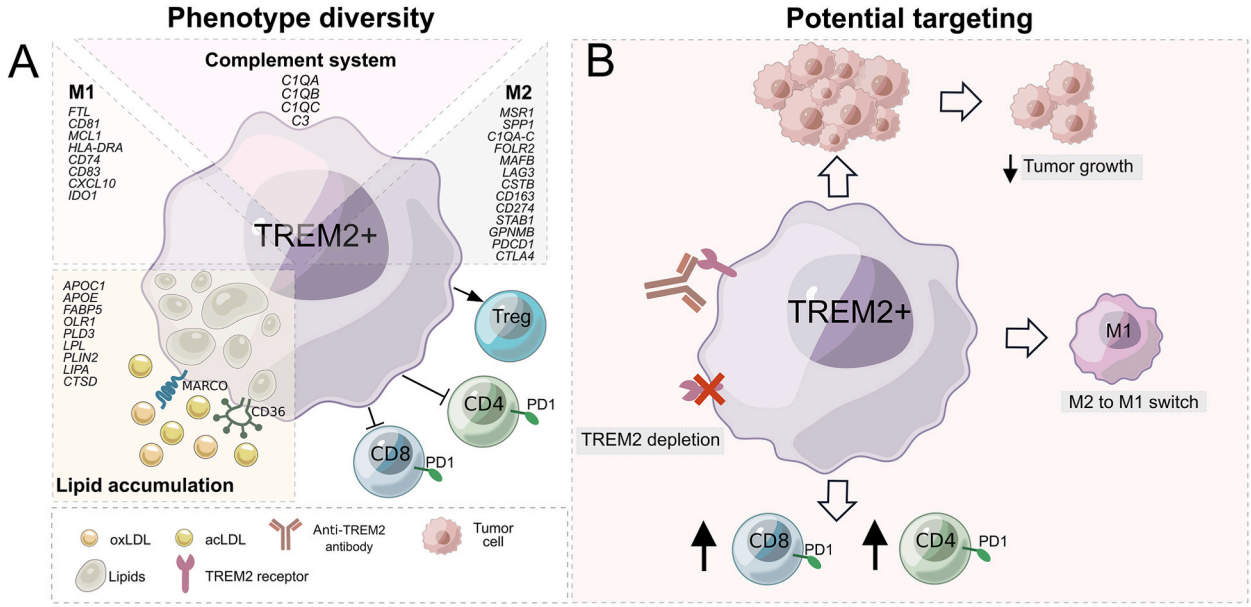

Fig. 1. Phenotypic diversity of TREM2+ macrophage subpopulation in cancer. **A.** The major functional features of TREM2+ TAMs include lipid accumulation via active scavenging of modified lipoproteins (acLDL, oxLDL) by scavenger receptors (e.g. CD36 and MARCO), immunosuppression via suppressing of PD1-expressing $CD4^+$ and $CD8^+$ T cells and Treg activation, as well as the enrichment of genes involved to a great extend in lipid metabolism and complement activation. In many studies, TREM2 is a central marker of lipid-associated macrophages (LAMs). Mixed M1 and M2 genes are integrated in the profile of TREM2+ TAMs. **B.** Targeting/depletion of TREM2 result in inhibition of immunosuppression via activation of PD1-expressing $CD4^+$ and $CD8^+$ T cells, tumor growth suppression and switching M2 phenotype of intratumor macrophages to M1.

Table 1
Key features of TAM subpopulations in human cancers.

Subpopulation	Gene signature	Cancer	Correlation with clinical parameters
TREM2+	APOC1(M2) APOC2 (M1) APOE (M2) MSR1 (M2) SPP1 (M2) OLR1 (M1) HK3 (M1)	Colorectal cancer	Correlation of *TREM2* mRNA expression with worse overall patient survival according to TCGA data [37].
	APOC1 (M2) APOC2 (M1) C1QA (M2) C1QB (M2) C1QC (M2) FOLR2 (M2) MARCO (M2) SPP1 (M2) MAFB (M2) CTLA4 (M2) LAG3 (M2) FTL (M1) CD81 (M1) MCL1 (M1)	Non small cellular lung cancer	Association of TREM2+ TAM signature expression with higher tumor stages, advanced tumor growth, tumor grade and lymph node metastasis (according to immunofluorescence staining). Association of TREM2 expression with worse response rate to PD-1-based immune checkpoint (according to IHC staining) [38].
	APOC1 (M2) APOE (M2) C1QB (M2) C1QC (M2) FOLR2 (M2) CD163 (M2) MAFB (M2) CSTB (M2) HLA-DRA (M1) CD74 (M1) CD14 (M1) CD83 (M1)	Renal cancer	Association of three gene signature (*C1Q, TREM2, APOE*) in macrophages with disease recurrence [39].
	APOC1 (M2) MSR1 (M2) C1QB (M2) C1QC (M2) FTL (M1) LILRA2 (M1)	Breast cancer	Correlation of *TREM2* mRNA expression with worse OS and RFS according to TCGA data [37]. In vitro inhibition of $CD4^+$ and $CD8^+$ T cells by TREM2+ macrophages [40].
	APOC2 (M1) C1QA (M2) C1QB (M2) C1QC (M2) FOLR2 (M2) SPP1 (M2) HLA-DRA (M1) CCL5 (M1) HLA-DQB2 (M1)	Basal cell carcinoma	Association of TREM2-high macrophage signature with worse response rate to anti-PD-1 therapy [41].
	APOC1 (M2) CD163 (M2) SPP1 (M2) C1QA (M2) CXCL10 (M1) IDO1 (M1) TNF (M1)	Hepatocellular carcinoma	Correlation of gene signature of TREM2+ TAMs with advanced tumor grade, pathologic stage and T stage as well as shorter OS, DFS, PFS and DSS [42].
	APOC2 (M1) C1QA (M2) C1QB (M2) C1QC (M2) SPP1 (M2) RNASE1 (M1) SEPP1 (M1)	Melanoma	Enrichment of TREM2-high Mφ cluster in non-responders to immune checkpoint therapy [41].
C1QC+	APOC1 (M2) APOE (M2) HLA-A (M1) HLA-DRB1	Non small cellular lung cancer	High mRNA expression of *C1QC* correlate with poor prognosis [43].

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Table 1 (*continued*)

Subpopulation	Gene signature	Cancer	Correlation with clinical parameters
	(M1) RNASE1 (M1) CD40LG (M1) ADORA2A (M2) CTLA4 (M2) IL2 (M2) LAG3 (M2) PDCD1 (M2) TIGIT (M2) CD40 (M1) IDO1 (M1)	Cervical cancer	Association of C1QC^{high} and SPP1^{low} TAM gene signatures with better OS and DSS [44].
	C1QA (M2) C1QB (M2) MAF (M2) TREM2 (M2) MSR1 (M2) HLA-DMB (M1) CTSC (M1) UCP2 (M1)	Colorectal cancer	Correlation of low C1QC+ and high SPP1+ TAM signatures with poor prognosis [45].
MARCO+	*SPP1 (M2)* ALOX5AP (M2) RNASE1 (M1)	Lung adenocarcinoma and lung squamous cell carcinoma	Association of high *MARCO* mRNA expression with a poor prognosis in LUSC [46].
	OLR1 (M1) *SPP1 (M2)*	Prostate cancer	MARCO+ macrophage gene signature significantly correlates with shorter DFS (according to TCGA data) [47].
	HLA class II genes (M1)	Glioblastoma multiforme	Association of *MARCO* mRNA expression with lower rates of OS and DFS (according to TCGA data) [48].
	PD-L1 (M2) *OLR1 (M1)*	Non small cellular lung cancer	Association between *MARCO* gene expression and general immune response pathways with immunosuppressive TAMs, T-cell infiltration and immune checkpoint molecules [24].
SPP1+	MARCO (M2) C1QA (M2) C1QB (M2) C1QC (M2) MSR1 (M2) TREM2 (M2) CD68 (M1) HLA-DRA (M1) HLA-DRB1 (M1) CD74 (M1) CD14 (M1) CD83 (M1) CCl5 (M1)	Breast, ovarian cancer, pancreatic ductal adenocarcinoma, squamous cellular carcinoma and non-small cellular lung cancer	Association of *SPP1* mRNA expression with worse prognosis (according to TCGA) [49].
	APOE (M2) MARCO (M2) C1QA (M2) C1QB (M2) C1QC (M2) MSR1 (M2) TREM2 (M2) OLR1 (M1) CD68 (M1) MRC1 (M1) CD86 (M1)	Colorectal cancer	Association of *SPP1* mRNA expression with worse prognosis and worse NAC response (according to TCGA data) [50].
	IGHG4 (M2) IGKC (M2) IGLC2 (M2) IGHG3 (M2) PLAU (M1) MMP14 (M1) CCL5 (M1)	Lung squamous cell carcinoma	Correlation of SPP1 macrophage gene signature with worse OS (according to TCGA data) [46].
CTSB+	CD40 (M1) CD80 (M1) CD86 (M1) IDO1 (M1) TNFSF4 (M1) CD70 (M1) PDCD1LG2	Colorectal cancer	Along with CD8$^+$ T cells and B cells, CTSB+ macrophages form the memory immune hub involved in anti-tumor response against liver metastasis according to cell-cell interaction analysis of scRNA-seq data [51].

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Table 1 (*continued*)

Subpopulation	Gene signature	Cancer	Correlation with clinical parameters
	(M2)		
	B7–H3 (M2)		
	PD-L1 (M2)		
FOLR2+	SEPP1 (M1)	Breast cancer	Positive correlation of FOLR2+ macrophage density with better patient survival (according to IF analysis) [52].
	CD80 (M1)		
	CD40 (M1)		
	IL6 (M1)		
	SLC40A1 (M2)		
	LYVE1 (M2)		
	CD163 (M2)		
	MRC1 (M2)		
	IL10 (M2)		
	MRC1 (M2)	Lung adenocarcinoma	Association of *FOLR2* mRNA expression in TAMs with the most aggressive tumor type [53].
	C1QA (M2)		
	APOE (M2)		
SIGLEC1+	C1QC (M2)	Breast cancer	mRNA expression of *SIGLEC1* together with *CCL8* is prognostic for poor patient survival [54].
	C1QA (M2)		
	C1QB (M2)		
	APOE (M2)		
	MARCO (M2)		
	MSR1 (M2)		
	CCL18 (M2)		
	SIGLEC10 (M2)		
	CD84 (M1)		
	CCl7 (M1)		
	HLA-DOA (M1)		
	CD40 (M1)		
	IRF8 (M1)		
SIGLEC10+	PD1 (M2)	Hepatocellular carcinoma	Association of intratumoral Siglec-10 protein expression with poor prognosis, in vitro inactivation of $CD8^+$ T cell [55].
	CTLA4 (M2)		
	LAG3 (M2)		
	MRC1 (M2)		
	CD80 (M1)		
	CD86 (M1)		
	HLA-DR (M1)		
	IL-12 (M1)		
	TNF-α (M1)		
GPNMB+	CCL24 (M2)	Glioblastoma multiforme	Association of *GPNMB* mRNA expression with the more aggressive tumor subtype as well as poor prognosis [56].
	SAA3 (M2)		
	ARG1 (M2)		
	MCL1 (M1)		
APOC1+	CCL18 (M2)	Colorectal cancer	Not studied [51].
	IDO1 (M1)		
	APOC1 (M2)	Glioblastoma multiforme	Not studied [57].
	HLA-DRB1 (M1)		
IL-10+	MAF (M2)	Muscle invasive bladder cancer	Association of *IL10* mRNA expression in TAMs with better RFS (according to TCGA) [58].
	MSR1 (M2)		
	TGFB1(M2)		
	TNF (M1)		
	VEGFA (M2)		
	MMP14 (M1)		
	EGF (M2)		
	MRC1 (M1)		
	CCl5 (M1)		
DC-SIGN+	TNFα (M1)	Muscle invasive bladder cancer	According to IF and flow cytometry data, DC-SIGN protein expression is predictive for unfavorable prognosis [59].
	PD-L1 (M2)		
	CCL2 (M2)		
	IL10 (M2)		
	TGFβ (M2)		
	CD206 (M2)		
	IL12 (M1)		
	HLA-DRB5 (M1)		
	CD207 (M1)		

types have been subjected to the analysis via scRNA-seq since the method has become more available. The amount of valuable data that could be obtained after bioinformatic analysis has grown [31].

Despite the achievements of scRNA-seq, the spatial context of analyzed tissue remains missing. Spatial transcriptomics (ST)

combined with scRNA-seq allows for visualization of cell RNA transcripts in the context of tissue architecture without disrupting its structural integrity [32]. Spatial molecular omics mainly define positional relationship and interactions among cells within the tissue and reveal the impact of spatial cell distribution on the pathogenesis of diseases [33].

Recent single cell omics technologies have significantly advanced our understanding of the molecular diversity and functional plasticity of TAMs, and their interaction with other TME components. In this review we focused on the clinical advances made by scRNA-seq and spatial transcriptomics technologies (NanoString DSP and 10x Visium) in the field of immune-oncology. In particular, the subpopulations of TAMs, which can be potential predictors of disease progression and outcome, were described.

2. New specific subpopulations of TAMs in human cancers revealed by scRNA-seq

Intimate interactions of diverse cell populations within tumor form a unique TME that facilitates tumor growth, metastasis and therapy resistance. Non-tumor cells also have distinct transcriptional programs that determine intratumor heterogeneity associated with tumor-supporting or tumor-inhibiting activities. scRNAseq technology has become a valuable tool for dissecting cell transcriptome, that can be helpful for discovering cell types and their functional state that could be essential in determining patients' prognosis [34]. Below we demonstrate how scRNA-seq contributed to the novel insight in the macrophage population content in human cancers by revealing TAM subpopulations with specific gene signatures.

2.1. Lipid-laden macrophages and their marker gene signature

A specific subset of TAMs, defined as lipid-associated macrophages (LAMs), was detected by scRNA-seq analysis in numerous cancers (Fig. 1, Table 1). Several recent experimental studies demonstrated that lipid accumulation results in the formation of foamy lipid-laden macrophages showing immunosuppressive features. Increased lipid intake or lipid-induced signaling pathways facilitate pro-tumor functions of TAMs, thus supporting tumor proliferation and invasiveness [35,36]. Despite the fact that lipid accumulation is an established feature of TAMs in several cancers, the mechanisms of lipid intake and the source of lipids, as well as the specific functional phenotype of these TAMs remain mostly unexplored.

In human breast cancer tissue, LAMs highly express macrophage markers (*SPP1, C1QC*), free fatty acids (FFA) transporters (*FABP3, FABP4* and *FABP5*) and lipid-associated genes (*LPL, LIPA, LGALS3* and *TREM2*) [60]. LAMs were widely dispersed among multiple subtypes of breast cancer, and patients with an increased infiltration of LAMs had poor prognosis [60]. Two unique macrophage populations resembling TREM2-high LAMs were also revealed in breast cancer tissue in another study – LAM1:*FABP5* and LAM2:*APOE* [61]. LAM1 and LAM2 comprised 30–40% of total myeloid cells in tumor tissue and highly expressed *TREM2* and lipid/fatty acid metabolic genes, including *FABP5* and *APOE,* consequently. Analysis of the CITE-seq data demonstrated a broader distribution of PD-L1 and PD-L2 protein expression across the LAM1 and LAM2 clusters. Based on spatial transcriptomic analysis, authors proposed that among lipid-associated macrophages, LAM2 could have more prominent immunoregulatory features. Spots enriched for LAM2 cells and CD4+/CD8+ T cells co-expressed PD-L1/PD-1 (CD274/PDCD1) and PD-L2/PD-1 (PDCD1LG2/PDCD1) [61].

TREM2 was manifested previously as a marker of immunosuppressive macrophages [62–64]. Immunosuppressive phenotype of TREM2+ TAMs revealed by experimental models was confirmed in cancer patients (Fig. 1A). In obesity experimental models, for the first time TREM2 was indicated as a critical factor for LAM cell formation [65]. Below we presented comprehensive characterization of TREM2+ TAMs detected by scRNA-seq in human tumor tissue.

In multiple cohort study, TREM2+ macrophages were found in several human cancers, including skin, liver, lung, breast, bladder, colon, stomach, pancreas, and kidney cancers, as well as in nodal lymphoma, cutaneous melanoma, and brain glioma [37]. In tumor tissue, morphology of TREM2+ tumor macrophages varied from small monocytoid to large foamy or multinucleated giant cells. TREM2+ tumor macrophages co-expressed CD68, CD163, CSF1R, and nuclear MAFB; foamy TREM2+ macrophages also co-expressed MITF [37]. Data from TCGA cohorts showed correlation of *TREM2* expression with worse survival in colorectal (CRC) and triple negative breast (TNBC) cancers. In CRC tissue, *TREM2* correlated with scavenger receptor *MSR1*, the Fc receptor *FCGR1A*, the TREM2 adaptor protein *TYROBP*, the exhaustion marker *HAVCR2*, and several genes associated with lipid metabolism (*APOC1, APOE*, and *OLR1*). In TNBC patients, *TREM2* significantly correlated with *MSR1, FCGR1C, TYROBP*, monocyte-macrophage receptors (*SIGLEC8* and *LILRA2*), genes associated with macrophage activation and phagocytosis (*LY86, LY96,* and *RNASE6*), macrophage metabolism (*APOC1, ADORA3, TBXAS1*, and *FTL*), and complement activation (*C1QB* and *C1QC*) [37].

Single-cell transcriptomic analysis performed in human hepatocellular carcinoma (HCC) tissue detected the TREM2+ macrophage subpopulation that was characterized by high expression of the marker genes *TREM2*, *FOLR2*, and *CD163,* and their transcriptome profile resembled LAMs [42]. Dual-stain immunofluorescence analysis confirmed that TREM2+CD163+ macrophages were highly enriched in HCC tissue compared to adjacent liver tissue [42]. *SPP1* and *CCL2* were both significantly upregulated genes in TREM2+ LAM-like cells in tumor. HCC patients with advanced tumor grade, pathologic stage and T stage had significantly increased accumulation of TREM2+ LAM-like cells. The amount of TREM2+ LAM-like cells correlated with unfavorable overall survival (OS), progression-free survival (PFS), disease-free survival (DFS), and disease-specific survival (DSS) in patients from TCGA-LIHC cohort. Pseudotime analysis showed that TREM2+ LAM-like cells exhibit preferential expression of genes involved in angiogenesis and higher M2 polarization scores [42]. Both subpopulations of C1Q+ and TREM2+ TAMs were also discovered in tumor tissue of renal cancer [39]. The accumulation of *C1Q+/TREM2+/APOE+* macrophages were detected more closely to tumor cells. Moreover, the expression of *TREM2* and *C1Q* were significantly enriched in the tumor stroma of patients with disease recurrence compared to patients without recurrence, and C1Q+ macrophages were significantly associated with disease recurrence [39]. In another cohort of HCC, TAM cell cluster in tumor tissues mainly expressed *C1QC, CD163*, and *APOC1*. *APOC1* was highly enriched in TAMs in the overall cell cluster

analysis, partially expressed in mast cells, and less expressed in other cell clusters such as $CD8^+$ T cell, $CD4^+$ T cell, and NK cells [66].

The presence of TREM2+ TAMs in non-small cell lung cancer (NSCLC) tissues was also detected [38]. The infiltration of TREM2+ TAMs increased with higher tumor stages and was associated with advanced tumor growth, tumor grade and lymph node metastasis. Single-cell sequencing revealed abundant expression of *TREM2* across TAM clusters, but not in monocytes or lung-associated macrophages. TREM2+ TAM population significantly expressed high levels of apolipoprotein-comprising genes (*APOC1, APOC2* and *APOE*) and higher levels of immunomodulatory proteins and chemokines, including *MARCO* and *FTL*. TREM2+ TAMs highly expressed both *SPP1* and *C1QC* genes, and M2 macrophage markers, including *CD206*, *ARG-1*, and *IL-10*. Enhanced expression of *MAF/MAFB* in TREM2+ TAMs was also detected. In cohorts of patients with NSCLC, TREM2+ TAMs, rather than total TAMs, predicted both shortened OS and relapse free survivals (RFS). High proportion of immune checkpoint molecules, including *PDCD1*, *CD274, CTLA4, LAG3, HAVCR2*, *TIGIT*, and *ENTPD1*, as well as immunomodulatory cytokines *IL10* and *TGFB1,* were positively correlated with TREM2+ TAMs. Accordingly, the objective response rate to PD-1-based immune checkpoint blockage (ICB) was 31.58% in TREM2+ TAM-low patients compared to 14.29% in TREM2+ TAM-high patients that can indicate the immunosuppressive state of TREM2+ TAMs [38]. One more scRNAseq analysis of advanced-stage NSCLC samples taken from patients before and after initiating systemic targeted therapy revealed a TREM2+ TAMs subpopulation that was enriched for lipid metabolism genes (*APOE*, *APOC1*, *CTSD*, *PLD3*), complement components (*C1QA, B, C*), as well as *SPP1* and *FOLR2* genes [67]. This population slightly decreased upon treatment, but no difference was detected in progressive disease versus residual/stable disease [67].

Results of single-cell analysis of treatment-naive patients with NSCLC also revealed TAM subset with core genes including *TREM2, GPNMB, SPP1, CTSB, RNASE1,* and *GPR183* [68]. In contrast to alveolar macrophages, TAMs expressed higher levels of *PD-L1*, lipid metabolism genes (*APOE, LIPA, PLIN2, LPL* and *HILPDA*), genes involved in antigen uptake and trafficking (*LRP12, RAB7, VAT1,* and *MFGE8*) and genes encoding machinery proteins for degrading phagocytosed cargo (*CTSB, CTSD* and *CTSS*). In lung cancer animal model, TREM2 expression was induced by efferocytosis of cell debris. TREM2+ macrophages reduced NK cell activity by modulating interleukin (IL)-18/IL-18BP decoy interactions and IL-15 production [68] (Fig. 1A).

In triple-negative breast cancer, among HLA-DR+CD11c+ cell fraction, the subset of LAMs, sharing the expression of *APOE, TREM2, GPNMB* and *APOC1* genes, was sub-divided on TREM2-STAB1 (additionally expressing *MRC1, MAFB, CD163, LYVE-1, STAB1, FOLR2, CD209* as well as *CD276* and *PDCD1* immune checkpoints) and TREM2-APOC1 (*SPP1, C1QA, C1QB, C1QC, LAG3, PDCD1LG2,* and *CD200*) macrophages [40]. APOE was expressed in all macrophage clusters. In vitro TREM2-STAB1 macrophages differentiated from monocytes by co-culture with FAP+CD29+ primary fibroblasts demonstrated immunosuppressive phenotype inhibiting CD4+ and CD8+ T cells expressing PD1 and CD25 [42].

Cluster of TREM2-high Mφ was enriched in patients with melanoma who didn't respond to immune checkpoint therapy (ICT) versus responders [41]. TREM2-high Mφ displayed a unique signature with overexpression of *SPP1*, *RNASE1*, *MT1G*, *SEPP1*, *FOLR2*, *NUPR1*, *KLHDC8B*, *CCL18*, *MMP12*, and *APOC2* along with key complement system genes (*C3*, *C1QA*, *C1QB*, and *C1QC*). A single-cell RNA-seq dataset of basal cell carcinoma (BCC) patients confirmed decreased expression of the TREM2-high macrophage signature in the responsive BCC tumors after anti-PD-1 therapy compared to the pretreatment BCC samples [41].

Immunosuppressive phenotype of TREM2+ TAMs was also demonstrated in ovarian cancer [63]. In human ovarian tumor samples, scRNAseq detected that within CD45+ cells, TAMs highly expressed *TREM2*, and *TREM2* expression correlated to T cell exhausted state. In mouse models of breast and colon cancers, combination of anti-TREM2 and anti-PD-1 mAb treatment resulted in sustainable reduction of M2-like TAMs and an increase in neutrophils and activated CD8+ T cells, as well as phenotypic alterations in the residual TAMs toward a pro-inflammatory phenotype [63]. A depletion of TREM2+ macrophage was achieved using an effector-enhanced anti-TREM2 antibody (eFc mAb) [69]. In the ID8 ovarian cancer and the EMT6 breast cancer models, anti-TREM2 treatment hindered tumor growth and reduced the number of TREM2+ macrophages [69] (Fig. 1B). In the anti-PD-1 resistant colon carcinoma model CT26, the simultaneous anti-TREM2 and anti-PD-1 therapies led to tumor rejection in 20–60% of treated mice, along with increased infiltration of activated CD8+ T cells [69].

In presented above results, *APOE* and *APOC1* were indicative for TREM2+ LAM subset in renal cancer, TNBC, CRC, HCC and NSCLC [33–35,60,38] (Table 1). *APOC1* and *APOE* form *TOMM40/APOE/APOC1* gene cluster on chromosome 19q13 [70]. In malignant glioblastoma (GBM), *APOC1,* which was found in other cancers among core genes of TREM2 cluster, was expressed in microglia/macrophage and TAMs. Single-cell sequencing also confirmed that *APOC1* was principally expressed in the macrophage cluster [57]. In a co-culture with renal cell carcinoma cells the expression of *APOC1* was elevated in the M2 TAMs and *APOC1* promoted M2 macrophage polarization via interaction with *CD163* and *CD206* [57]. Several studies revealed that *APOC1* acts as an oncogene in the progression of breast, pancreatic, colorectal, and lung cancer [71–75]. *APOC1* regulate monocyte-macrophage differentiation and can be implicated in the formation of foam cells [70,76].

Similar to *APOC1, APOE* which was found in LAMs, also belongs to apolipoproteins and involved in cholesterol metabolism [77]. *APOE* overexpression was associated with tumor progression, poor survival and deeper tumor invasion in CRC, NSCLC and gastric cancer [78–80]. In macrophages, *APOE* promotes cholesterol efflux and reduces pro-inflammatory activity [77,81,82]. APOE was a specific marker that distinguished macrophages from CD14+ monocytes and CD1c+ dendritic cells among CD11c+HLA-DR+ cells in primary luminal BC tumors and metastatic lymph nodes [52]. Discriminant analysis revealed that APOE+ TAMs comprise two distinct populations: the TREM2/CADM1+ (along with *SPP1* and *ISG15*) macrophages and the FOLR2+ (along with *SEPP1, SLC40A1, MRC1*, and *LYVE1*) macrophages. Morphological analysis showed that FOLR2+CADM1− macrophages resembled the typical macrophage shape and are filled with vacuoles. In contrast, FOLR2lowCADM1+ macrophages were closer to monocytes [52].

Interestingly, a recent study of human colorectal liver metastasis (CRLM) samples identified two major TAM types by morphology – large TAMs (L-TAMs) and small TAMs (S-TAMs) [83]. Transcriptomic analysis revealed foam-like phenotype of L-TAMs that is characterized by the activation of cholesterol metabolism, including the up-regulation of the LXR gene (NR1H3) and LXR-downstream

genes involved in cholesterol transport (ABCA1, ABCG1, and CETP), extracellular lipid acceptors (APOE, APOA1, and APOA2), and lipid metabolism-associated genes (CD5L and FASN), as well as down-regulation of genes regulating lipid uptake and biosynthesis (LDLR, CYP27A1, and HMGCR). L-TAMs also presented up-regulation of phagocytic and scavenger receptors, including MERTK, MSR1, MRC2, and members of the complement family (C1QA, C1QB, and C1QC). Single-cell analysis confirmed the diversity of TAMs associated with «S» and «L » morphology: S-TAM signature included S100A8, S100A9, S100A12, FCN1, VCAN, THBS1 and SERPINB2 genes, while L-TAM signature CD5L, SLC40A1, C1QA, C1QB, MARCO, CETP, APOE, and VCAM1 genes. Notably, L-TAMs were more frequently found in patients with worse prognosis compared to patients with favorable outcome, suggesting that TAM morphology is a critical feature of distinct clinical outcomes in CRLM patients [83].

Thus, single-cell analysis allowed for identifying lipid-loaded TAMs gene profile that we demonstrated above. In almost all investigated cancers, the most commonly described genes in LAM cluster include *TREM2, SPP1, APOE, APOC1, MARCO, FOLR2, MSR1,*

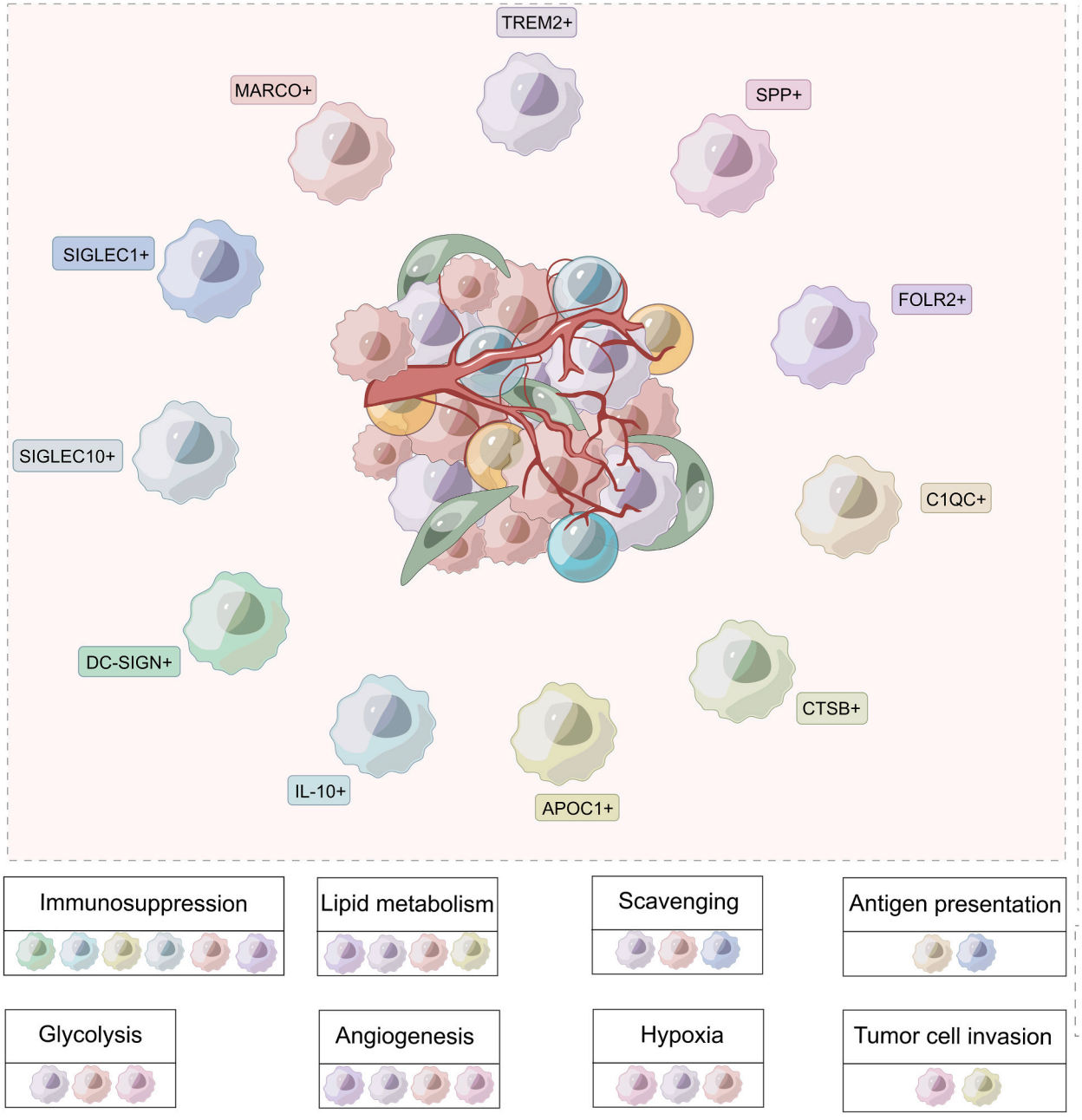

Fig. 2. Major TAM subpopulations and their functional states dissected by single-cell analysis. ScRNAseq revealed the diversity of TAM phenotypes characterized by the involvement in different processes: immunosuppression, angiogenesis, hypoxia, glycolysis, tumor cell invasion, scavenging and antigen presentation.

MAFB, CD163, and genes of complement system (*C1QA, C1QB*, and *C1QC*) (*Table 1*). For example, scRNAseq of nasopharyngeal carcinoma (NPC) patients' samples confirmed that macrophage cluster expresses almost all of these genes (*APOE, APOC1, C1QA, C1QB, C1QC, SPP1*, *CD163, MAFB,* and *TREM2*) among the 47-gene signature [84].

The subset of LAMs expresses key genes involved in M2 activation state, immunosuppression, lipid metabolism and formation of foam cells, mediating tumor progression (Fig. 2). Gene signature of LAMs can be a valuable criterion for cancer prognosis and prediction of the efficacy of anti-cancer therapy. Foamy lipid-laden macrophages were described before most extensively in atherosclerosis and tuberculosis, although they were also found in the following cancer types – NSCLC, ovarian, esophageal, CRC, prostate and gastric cancers [35,47,85–89]. One of the first studies showing abundant accumulation of foam-like TAMs in tumor tissue was the study of renal cancer [90]. Lipid accumulation that exceeds the homeostatic capacity of macrophages triggers lipid droplet formation, which results in the foamy appearance of these macrophages [85]. This process polarizes TAMs to a pro-tumor or an immunosuppressive phenotype [91]. The mechanisms of lipid intake remain barely explored, and the most known source of lipids is low-density lipoproteins (LDLs) [92]. Previously, lipid-loaded TAMs were found to express high levels of scavenger receptors CD36 and MARCO that are implicated in lipid scavenging and consequently augmented FAO and OXPHOS, which are both M2 metabolic features [92,93]. However, there is no clear understanding whether LAMs revealed by scRNA-seq and foam macrophages detected by histological analysis are the same cells. Only a few studies have validated the morphology of LAMs in tumor tissue. However, the specific

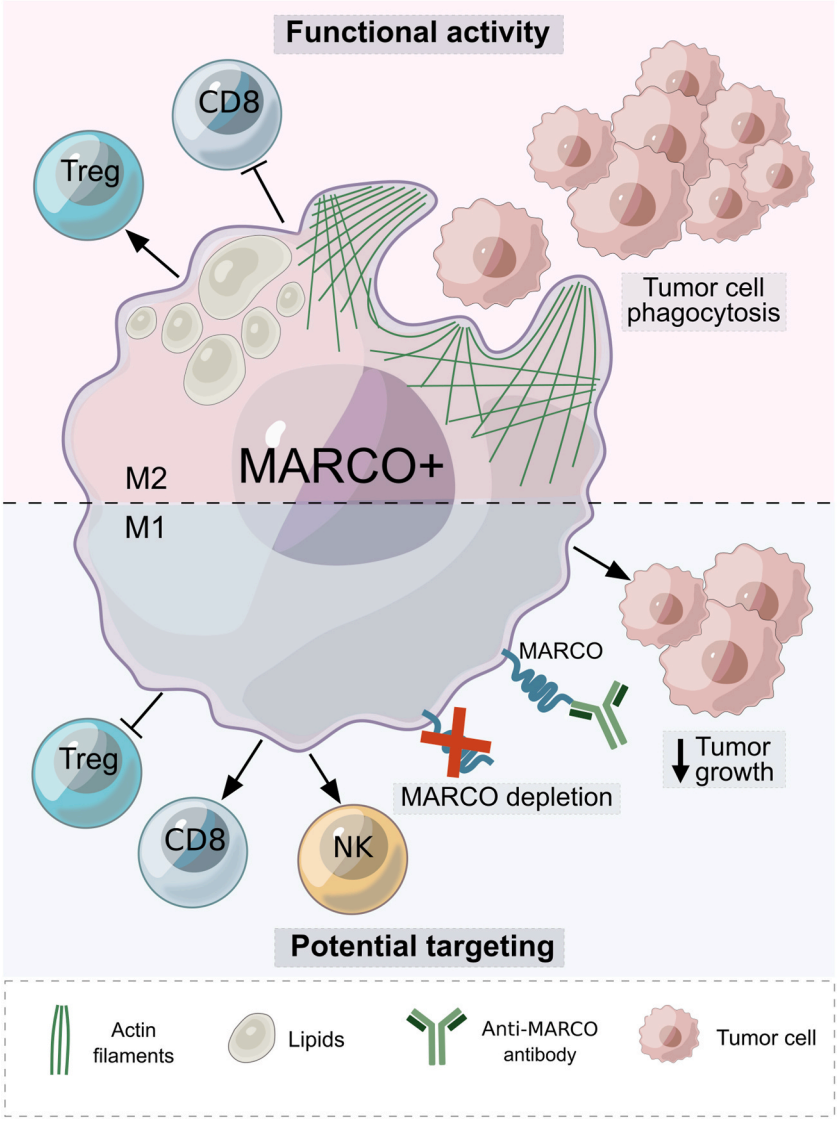

Fig. 3. Functional characteristics of MARCO+ macrophages in cancer. Schematically, MARCO defines immunosuppressive subset of TAMs that inhibits cytotoxic CD8+ T cells and activates Tregs. MARCO+ TAMs are involved in macrophage-mediated phagocytosis to clear tumor cells via cytoskeletal rearrangement. Potential targeting of MARCO was developed and may result in inhibition of tumor growth and reverse the immunosuppressive microenvironment to the cytotoxic one by activating NK cells and CD8+ T cells.

mechanisms of the connection between LAM lipid accumulation and tumor progression remain unclear, which calls for further analysis using functional models.

The next step of LAMs investigation should be their phenotypic characterization in tumors by protein analysis as well as the spatial distribution of these types of TAMs in different tumor compartments, in particular in close proximity to blood vessels, inside the tumor nest or in the stroma. The spatial data have to be complemented by the information about the cell-cell interactions that can be derived from extended bioinformatic analysis of spatial transcriptomic data as well.

2.2. *MARCO-positive macrophages*

MARCO defines pro-tumor, immunosuppressive subset of TAMs and is implicated in tumor progression [23,24,26,94,95] (Fig. 3, Table 1). Experimental models demonstrated that blocking of MARCO induced repolarization of TAMs into pro-inflammatory phenotype, activated anti-tumoral capacity of NK cells and T cells, and inhibited Treg activities, as well as decreased tumor growth in murine models, indicating that MARCO can be a target for immunotherapy [26,94–96]. MARCO is involved in

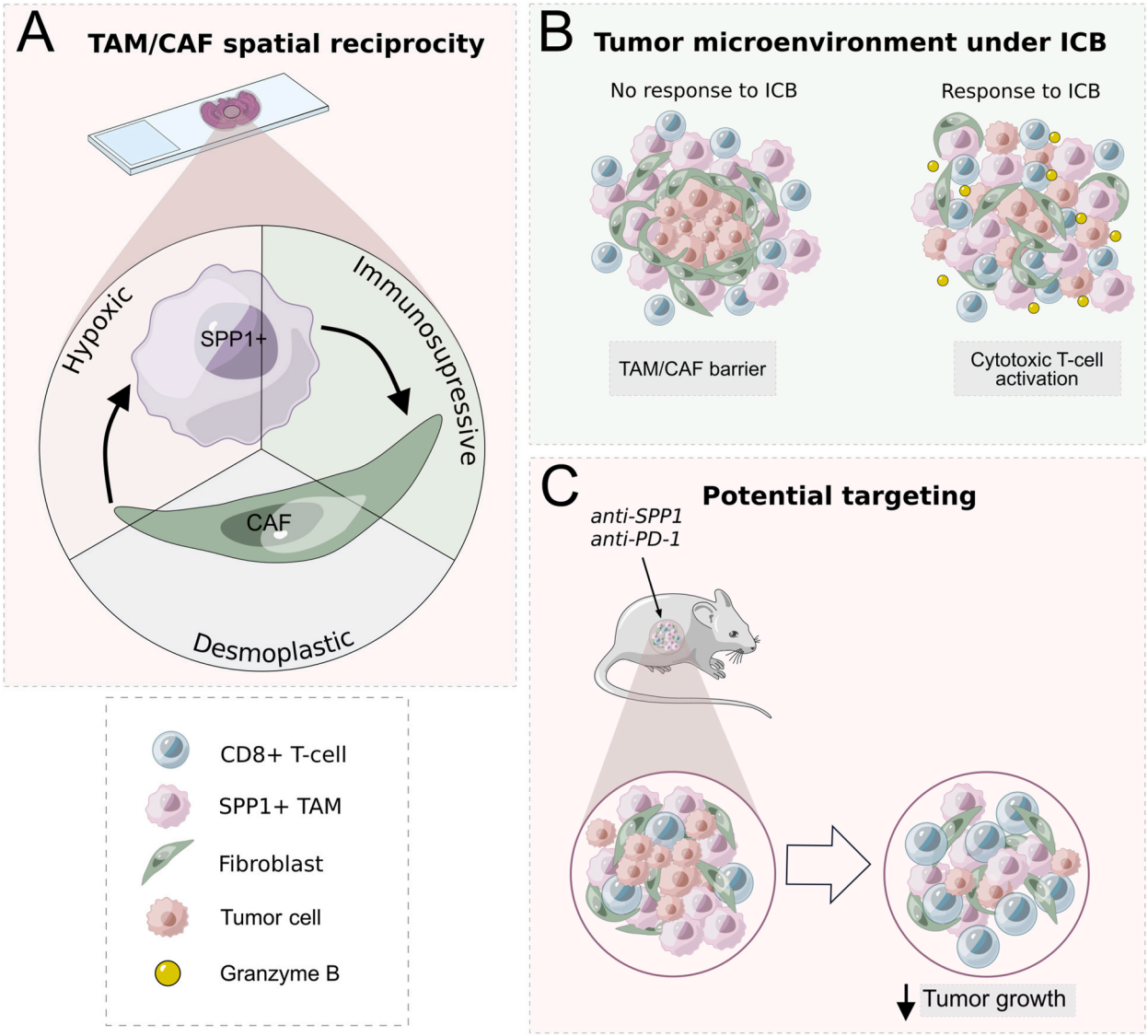

Fig. 4. The role of SPP1+ in tumor microenvironment. **A.** For SPP1+ TAMs, their intercellular interaction with CAFs were revealed by spatial transcriptomics. The intercommunication of SPP1+ TAMs and CAFs leads to the formation of immunosuppressive, desmoplastic and hypoxic stroma. **B.** In patients who did not respond to immune checkpoint blockade (ICB), tumors formed a histological barrier built by SPP1+ macrophages/CAFs that prevented the interaction of cytotoxic immune cells with tumor cells. In contrast, immune cells interacted easily with tumor cells as well as with SPP1+ macrophages/CAFs in the TME of responders. **C.** SPP1 blockade results in inhibition of tumor growth together with decreased amount of immunosuppressive SPP1+TAMs and activating cytotoxic T cells.

macrophage-mediated phagocytosis responsible for tumor cells clearance by regulating cytoskeletal rearrangement in macrophages and binding to β5 integrin on tumor cells via PI3K-Rac pathway [97] (Fig. 3).

In the study of prostate cancer, single cell analysis revealed MARCO_Mac cluster that was specifically enriched in tumor versus normal tissue [47]. Interesting that gene signature of *MARCO*+ TAMs was aligned with lipid-loaded cells since it was related to lipid metabolism and lipid intake, including LXR/RXR activation, FXR/LXR activation, and atherosclerosis signaling. This data overlap with the transcriptomic profile of LAM expressing *MARCO* that was described above in NSCLC and CRC [38,83]. Immunofluorescence staining of prostate cancer (PCa) tissue demonstrated MARCO co-expression in CD68+ TAMs, and BODIPY staining of the lipid content of cells correlated with MARCO expression, confirming a link between MARCO and lipid accumulation in TAMs in PCa [47].

In NSCLC patients, a distinct subpopulation of TAMs expressing MARCO aggregated in close proximity to tumor cell nests was found [24]. Multiplex immunofluorescence staining confirmed the co-expression of CD68, CD163 and MARCO. Co-staining of PD-L1, MARCO and CD68 revealed MARCO-positive macrophages in direct contact with PD-L1-positive tumor cells. On the transcriptomic level, *MARCO* gene expression positively correlated to immunosuppressive TAMs, T-cell infiltration and immune checkpoint molecules [24]. MARCO+ macrophages were also found in another patient cohort of lung adenocarcinoma (LUAD). ALOX5AP and MARCO were highly expressed in immune cells in tumor tissue of LUAD [46]. The subpopulation of MARCO+ macrophages was exclusively found in IDH1-wild-type (Isocitrate dehydrogenase 1 (NADP+), soluble) GBM, rather than IDH1-mutated or lower-grade counterparts [48]. The transcriptome of MARCO+ macrophages was enriched with epithelial-mesenchymal transition, angiogenesis, glycolysis, and hypoxia (Fig. 2). The expression of pro-inflammatory molecules, including HLA class II genes (*HLA-DRB1, DRA, DPA1*, and *DPB1*) and *CD74* (MHC class II invariant chain) was highly downregulated in MARCO+ macrophages. Patients with high *MARCO* expression had lower OS and DFS rates compared to MARCO-low patients [48].

2.3. SPP1-positive subsets of TAMs

Two distinct TAM subpopulations exerted immunosuppressive phenotype were identified in NSCLC patients: CCL18+ macrophages and SPP1+ macrophages [43]. Although both of TAM subtypes had the anti-inflammatory signature, their transcriptome characteristics were actually different. Specifically, CCL18+ macrophages express factors responsible for the inhibiting the production of inflammatory factors and manifest high levels of fatty acid oxidative phosphorylation metabolism. Conversely, glycolysis is the main metabolic pathway for SPP1+ macrophages contributing to tumor metastasis by promoting angiogenesis and matrix remodeling [43]. Accumulating data demonstrate that SPP1 can be expressed by TAMs, and its expression strongly correlates with macrophage infiltration in human cancers [98–100].

Subtype of TAMs expressing *SPP1* was universal for breast cancer, colorectal cancer, ovarian cancer, lung cancer, pancreatic ductal adenocarcinoma, and squamous cell carcinoma [49]. According to single-cell data, *SPP1* expression was higher in hypoxia-high macrophages, and, vice versa, the hypoxia score was higher in such SPP1+ TAMs (Fig. 4A). The expression of *SPP1* was also higher in hypoxia-high tumor samples than that in low ones. A positive correlation was found between SPP1+ TAM signature, *SPP1* expression with glycolysis, and EMT program in multiple cancer types (Fig. 2). Clinical data collected from the TCGA data base, demonstrated that patients with a higher level of *SPP1* gene expression as well as a higher proportion of SPP1+ TAMs showed worse prognosis in six cancer types [49]. In lung cancer, several macrophage clusters were identified: *SPP1*-Mφ cluster with elevated expression of immunoglobulin-related genes IGHG4, IGKC, IGLC2, IGHG3, and PLAU; *FCN1*-Mφ cluster highly expressed S100A8, ANXA1, VCAN, EMP1 and AREG; *FABP4*-Mφ enriched by genes associated with fatty acids and obesity, including FABP4, CES1, HPGD and IGFBP2; and *SELENOP*-Mφ, highly expressed SELENOP, FOLR2, IL32, CD3D and LTC4S contributed to the local antioxidant capabilities and lymphocyte-related function [46]. Genes of FABP4-Mφ and FCN1-Mφ were enriched with phagocytosis, while the SPP1-Mφ cluster exhibited angiogenesis. Survival analysis of each macrophage subcluster-specific markers showed that increased *AREG, IGHA1, MMP14, PLAU,* S100A8 and decreased *HPGD* were inversely correlated with the OS in lung cancer patients. Among these genes, *MMP14, PLAU,* and *IGHA1* were specifically upregulated in the SPP1-Mφ, suggesting the role of this cluster in the lung tumorigenesis [46].

C1QC+ TAMs and SPP1+ TAMs gene signatures were proposed to be used for cancer patient prognosis, rather than M1 and M2 gene signatures, at least in colon cancer and advanced basal cell carcinoma [44] (Table 1). In cervical cancer, patients with C1QC-high and SPP1-low TAMs gene signatures have the best OS and DSS, highest proportion (71.79%) of locally advanced cervical cancer and lowest immune cell infiltration, whereas patients with C1QC-low and SPP1-high TAMs gene signatures have the worst OS and DSS, higher expression of most of the immune checkpoint molecules (ICMs) [44]. Based on the transcriptomic data in colon cancer tissue, these hallmark subsets of TAMs express distinct gene profiles [45]. C1QC+ TAMs gene signature included the following genes: *C1QA, C1QB, ITM2B, C1QC, HLA-DMB, MS4A6A, CTSC, TBXAS1, TMEM176B, SYNGR2, ARHGDIB, TMEM176A, UCP2, CAPZB, MAF, TREM2*, and *MSR1*, whereas SPP1+ TAMs gene signature includes the following genes: *SPP1, PCSK5, SLC11A1, VCAN, SLC25A37, FLNA, UPP1, BCL6, AQP9, TIMP1, VEGFA, ADM, MARCO, FN1*, and *IL1RN*. Functionally, an enrichment of tumor angiogenesis, ECM receptor interaction, and tumor vasculature pathways was found in SPP1+ TAMs, while the complement activation, antigen processing and presentation pathways were significantly increased in C1QC+ TAMs [45]. The analysis of scRNA-seq data sets demonstrated the distribution of the cluster of SPP1+ and C1Q+ macrophages across 13 types of cancer [101].

Interestingly, *C1QC* and *SPP1* were both the hallmark genes in TREM2+ LAMs, described in HCC, NSCLC, BCC and melanoma [35,60,61]. Moreover, the correlation of *C1QC* and *SPP1* expression with poor prognosis is found for glioma, melanoma, breast, colon, ovarian, lung and colorectal cancers [60,102–107]. It means that all together they can form a gene signature associated with the parameters of tumor progression and can be potential targets for immunotherapy.

ScRNA-seq of 20 matched primary CRC and CRLM tissue samples revealed two major immunosuppressive macrophage subtypes

specifically presented in liver metastasis compared to primary foci, among which – SPP1+ TAMs and MRC1+CCL18+ TAMs [50]. These two subsets are enriched in treatment-naïve liver metastasis compared to primary tumors. In a TCGA cohort, high scoring of MRC1+CCL18+ macrophages and SPP1+ macrophages in primary tumors both predicted a significantly worse prognosis. Liver metastasis–enriched MRC1+CCL18+ macrophages highly expressed key molecules of M2 macrophage polarization, *APOE* and *MARCO,* whereas in primary tumors MRC1+CCL18+ TAMs showed higher expression of inflammatory cytokines such as *TNF, IL1B, CCL3* and *CCL4*. The same observation was made for SPP1+ TAMs. Interestingly, in CRLM samples of patients with partial response to neoadjuvant chemotherapy (NAC) the amount of SPP1+ and MRC1+ CCL18+ macrophages decreased while the number of cytotoxic T cells such as GZMK+ CD8 T cells and XCL1+ CD8 T cells was upregulated, compared to non-responsive samples (progressive disease/stable disease). This results indicate that NAC reprogrammed intratumor immune balance and activated systemic antitumor immune responses and support the potential use of NAC in resectable CRLM [50].

2.4. Other key subpopulations of TAMs

Other prominent subsets of TAMs were revealed by scRNA-seq analysis in cancer patients (Fig. 2, Table 1). Single cell analysis of liver metastasis of CRC allowed to distinguish 6 clusters of $CD68^+$ macrophages: cluster 2 (*C1QA* + Mφ); cluster 4 (*CTSB* + Mφ); cluster 5 defined metabolic activated macrophage (MAMφ); cluster 6 (*FOLR2*+ Mφ); cluster 7 (*APOC1*+ ResMφ); cluster 8 defined proliferating macrophages (ProlMφ) [51]. In liver metastasis tissue, authors found memory CD8+ T cells, B cells, and CTSB+ macrophages forming the memory immune hub which was important for the anti-tumor response against liver metastasis. *CD40,* CD80*,* CD86*, IDO1, TNFSF4, PDCD1LG2,* CD70*,* CD276, and *CD274* positively correlated to CTSB+ macrophages indicating that CTSB+ macrophages may play crucial role in suppressing immune memory response of the liver metastasis microenvironment [51].

FOLR2+ macrophages were distinguished from the APOE+ macrophages in breast cancer [52]. The gene signature of FOLR2+ macrophages consisted of top genes *SEPP1*, *SLC40A1*, and *LYVE1* as well as M1 genes (*CD80, CD40*, and *IL6*) and M2 genes (*CD163, MRC1*, and *IL10*). Tumor-associated FOLR2+ macrophages in BC were not found to be an immunosuppressive population, but a potent antigen presenting cell population displaying the functional ability to trigger CD8+ T cell activation. Live confocal analysis of fresh tumor tissue demonstrated that tumor-infiltrating CD8+ T cells actively interact with FOLR2+ macrophages. Moreover, FOLR2+ macrophages located near CD31+ vessels along with CD8+ T cell aggregates. In patients with BC, FOLR2 gene signature was strongly associated with better survival [52].

In contrast, TAM-FOLR2 can form the immunosuppressive microenvironment in invasive lung adenocarcinoma by triggering the maturation of CD4+NR4A3+ cells into intratumoral Tregs [53]. To demonstrate this, bioinformatical analysis based on mapping the interactions between chemokine ligands and receptors was applied. The limitations of this study may be the lack of protein validation on a large patient cohort (only 5 patients in tumor vs non-tumor tissue) as well as the lack of functional modelling to confirm the TAM-T-cell interactions [53]. In another study, the same authors also indicated *FOLR2* as a marker gene of TIM4+ TAMs in different cancers [108]. Gene signature of TIM4+ TAMs localized in tertiary lymphoid structures positively correlates with a better prognosis [108].

In breast cancer tissue, SIGLEC1 encoding CD169 was one of the top upregulated genes in breast cancer TAMs compared with resident macrophages [54] (Fig. 2, Table 1). *SIGLEC1* correlated with expression of the pan-macrophage marker CD163. In the METABRIC cohort, univariate analysis showed that SIGLEC1 high expression was significantly associated with shorter DSS. Flow cytometric analysis determined that SIGLEC1 was expressed on TAMs, but not on other immune cells or CD45–non-immune cells, indicating its specificity to macrophages/TAMs [54]. Single cell RNA sequencing of another breast cancer cohort also demonstrated Mac:*SIGLEC1* macrophage cluster, resembling the M2-like phenotype [61]. Intratumoral subset of Siglec-10-high TAMs exhibit mixed M1/M2 phenotype and exert immunosuppressive function associated with the pro-tumor immune contexture in patients with HCC [55]. Tumors with high Siglec-10-high CD68+ cell infiltration exhibited higher levels of CD4+FoxP3+ regulatory T cells compared to tumors with low Siglec-10-high CD68+ TAMs. HCC specimens with high Siglec-10-high TAM infiltration exhibited lower proportions of granzyme B+ (GZMB+), IFN-γ+, IL-2+ and perforin-1+ (PRF-1+) $CD8^+$ CTLs, but higher proportions of CTLA-4+, LAG-3+, PD-1+, TIGIT+ and TIM-3+ CD8 CTLs compared to samples with low Siglec-10-high TAM infiltration [55].

Using TCGA data sets of scRNA-seq, a population of macrophages expressing inflammatory markers *GPNMB, CCL24, SAA3, ARG1* in glioblastoma multiforme (GBM) was revealed [56] (Fig. 2, Table 1). *GPNMB*+ macrophages were highly presented in a more aggressive mesenchymal (MES) subtype of GBM. High expression of *GPNMB* was predictive for poor prognosis in both low- and high-grade gliomas [56]. Single-cell analysis of CD163+CD66b– cells isolated from colorectal liver metastasis (CLM) tissues allowed for uncovering two major macrophage clusters [109]. One cluster consisted of more mature macrophages with low expression of monocytic markers *ITGAM* and *SERPINB2,* and high expression of *CD68*, *MSR1*, and *HLA* genes. Among other genes uniquely expressed by TAMs were *GPNMB, TREM2, LGALS3*, *SPP1* and *FABP4.* Another cluster was represented by monocytic macrophages specifically expressing *SERPINB2* and genes related to leukocyte recruitment and cytokine production (*CXCL8*, *CXCL3,* S100A8, S100A9, and *S100A12*). High infiltration of SERPINB2+ cells detected by multiplex fluorescent immunohistochemistry was associated with longer DFS, whereas both shorter DFS and OS correlated with a high density of GPNMB+ TAMs [109].

In patients with muscle-invasive bladder cancer (MIBC), specific population of *DC-SIGN*+ TAMs, but not total TAMs, predicted unfavorable prognosis [110] (Fig. 2, Table 1). The amount of *DC-SIGN*+ TAMs correlated with enhanced tumor stages in MIBC. Flow cytometry and transcriptomic analysis demonstrated that *DC-SIGN*+ TAMs exhibit an M2-like immunosuppressive phenotype highly expressing M2 macrophage marker *MRC1* as well as expressing low levels of pro-inflammatory cytokines (*IL12* and *TNFα*) and high levels of anti-inflammatory cytokines (*CCL22, IL10, TGFβ*, and *CD274*) [110].

A subpopulation of IL10+ TAMs was found in patients with muscle-invasive bladder cancer (Fig. 2, Table 1) [58]. Notably, M1

macrophage markers (including *CCR7, CCL5* and *TNF*) were significantly downregulated, while M2 macrophage markers (including *CD163, MRC1, MSR1*, *VTCN1, ARG1, TGFB1, MMP14* and *EGF*) were upregulated in IL10+ TAMs. The administration of adjuvant chemotherapy increased RFS rate in patients with high IL10+ TAMs infiltration in TCGA cohort and experimental cohort, but patients with low IL10+ TAMs infiltration had inferior RFS after receiving ACT in TCGA cohort [58].

Other interesting recent findings show new subsets of TAMs expressing markers uncommon for these cells. Thus, previously unknown TUBB3+ TAM subset showed a neuron-like transcriptome signature in lung cancer that was similar to the TUBB3+ neuronal cells associated with human neural diseases, but unlike the one revealed in TAMs by scRNA-seq [111]. TUBB3+ CD68+ TAM subset strongly expressed neuronal genes including *BMP7, SHANK, CHL1*, and *PAX6* in human non–small cell lung cancer (NSCLC) biopsy samples. The abundance of the TUBB3+ TAMs was significantly associated with poor survival of older NSCLC patients (age >60). Authors used trajectory analyses to show that TAMs can directly transit into neuron-like cells via a previously unknown phenomenon "macrophage to neuron-like cell transition" (MNT). This study discovered new potential direct mechanism of TAMs for promoting nociception in cancer, which can kickstart the development of new approaches for cancer pain management [111]. The same authors also detected the phenomenon of macrophage–myofibroblast transition (MMT cells) where TAMs can further differentiate into protumoral cancer-associated fibroblasts (CAFs) in NSCLC tumor tissue [112]. The transcriptional profiles of MMT cells were more homologous to αSMA+CD68− CAFs than to αSMA−CD68+ TAMs. In vivo the protumoral activity of the MMT-derived CAFs was also

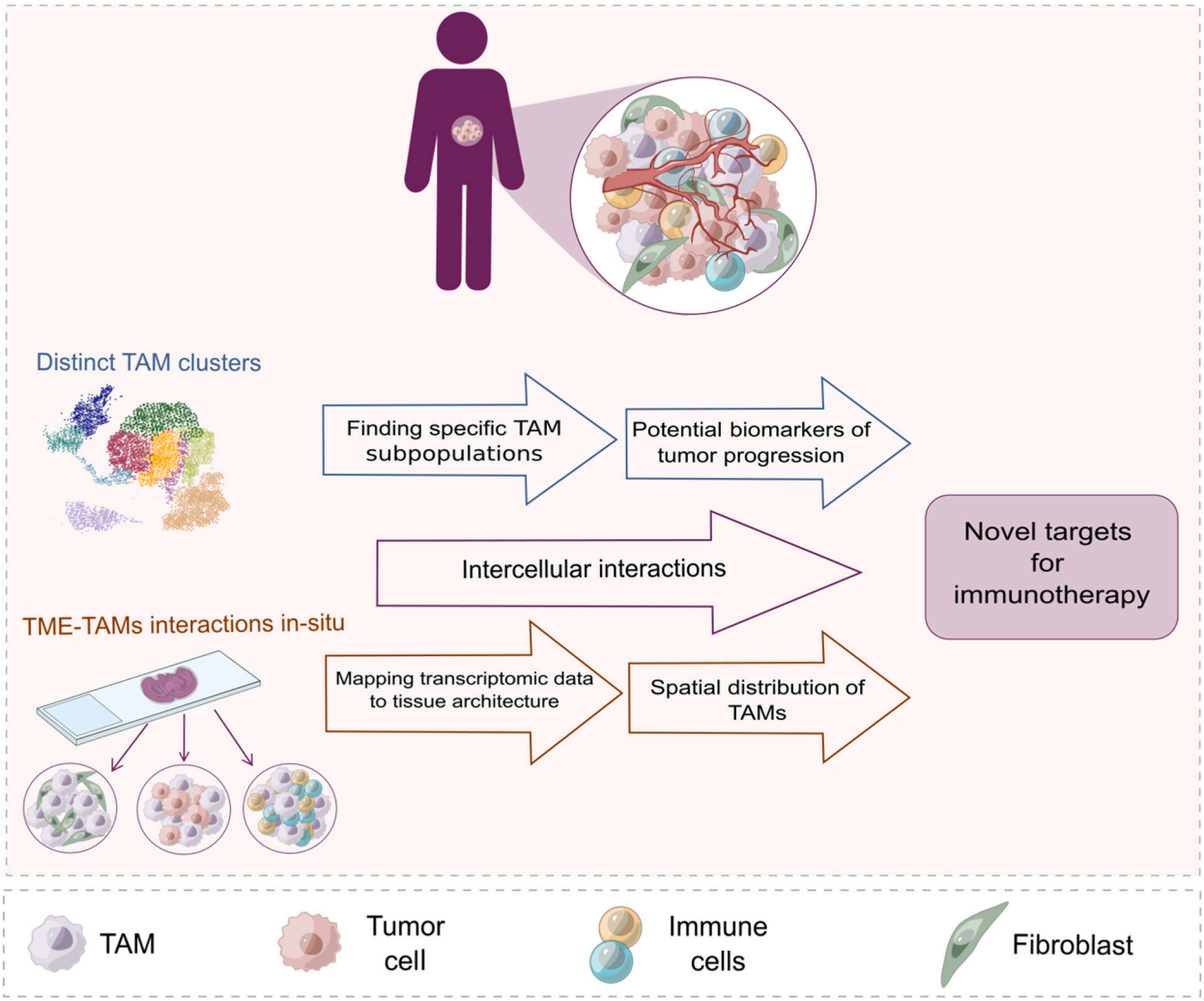

Fig. 5. Single-cell resolution and spatial context providing novel targets for immunotherapy. (Upper line) Single-cell RNAseq dissects cellular function and heterogeneity through providing a more comprehensive transcriptome information at the single cell resolution. ScRNAseq allowed to reveal specific TAM subpopulations which can be potential biomarkers for tumor progression. (Lower line) Spatial transcriptomics (ST) define positional relationship and interactions among cells of the tumor microenvironment within the tissue section. ST techniques (e.g. Nanostring GeoMx-DSP and 10x Genomics Visium) allow mapping transcriptome of TAMs to tissue architecture and detecting spatial distribution of TAMs in relation to other cells (tumor cells, immune cells, fibroblasts, endothelial cells). ScRNAseq and ST data can help finding effective targets for immunotherapy based on TAMs.

demonstrated [112]. This phenomenon was similarly described in renal fibrosis. CD68+αSMA+ cells acquired M2 phenotype and accumulated in progressive fibrotic lesions, but were largely absent in acute inflammatory or sclerotic lesions [113]. In both human chronic kidney disease and heart failure, a population of SPP1+ profibrotic macrophages marked by expression of *Spp1, Fn1*, and *Arg1* aggravated fibrosis. Mechanistically, platelet-derived chemokine CXCL4 drove SPP1+ macrophage activation and macrophage-fibroblast crosstalk [114].

3. Macrophage spatial distribution

Macrophage diversity and functional activity inside the tumor strongly depend on both their localization in particular intratumor compartments and their close interactions with other components of the TME [13]. For example, subpopulations of TAMs at perivascular or hypoxic areas display proangiogenic and immunosuppressive properties, TAMs populating cancer cell nests may support tumor growth, while macrophages in the invasive front may exhibit tumoricidal activity [11,108,115].

Spatial characterization of both transcriptome and protein expression of TME components with retaining tissue architecture would help illustrate the intricate crosstalk between tumor cells and immune components that defines malignant progression [116]. Recently developed spatial transcriptomics technologies include Nanostring GeoMx digital spatial profiling (GeoMx DSP) and 10x Genomics visium [30]. Since these technologies are still at their developing stages and are quite expensive, they have not yet become widely used. Most of the studies are focused on tissue mapping and assigning cell types to certain clusters rather than using it for searching potential prognostic markers (Fig. 5). However, attempts to find markers of immunotherapy response were made in several studies [117,118,119].

GeoMx DSP platform is able to perform whole-transcriptome analysis (WTA) and detect protein expression within a region of interest (ROI) containing hundreds of cells from a single tissue section on a slide [30]. Pretreatment samples from 56 patients with NSCLC treated with ICB were analyzed using the NanoString GeoMx DSP technology [117]. CD66b expression in the $CD45^{+}CD68^{+}$ immune stroma compartment predicted significantly shorter OS. Quantitative immunofluorescent analysis demonstrated that CD66b is associated with ICB therapy resistance [117]. Spatial transcriptomic profiles of 16 NSCLC tumors revealed the upregulation of pro-inflammatory *CCL5* and *CD27* and immune suppressive *ITGAM* genes in tumors with high CD163+ cell infiltration [118]. Furthermore, in high macrophage-enriched tumors, the upregulation of genes associated with IFN-γ signaling pathway and M1 phenotype was associated with better response to immunotherapy. In tumors of responders high expression of *CSF1R* correlated with an increased durable clinical benefit rate, PFS and OS after ICB treatment [118]. In 60 FFPE from immunotherapy-treated patients with melanoma, 44 immune markers were measured in three different compartments: macrophages ($CD68^{+}$), leukocytes ($CD45^{+}$) and melanocytes (S100+ and HMB45+) [119]. DSP analysis showed that high CD8 counts in the CD68 compartment were found to be associated with prolonged OS and PFS as well as complete response to immunotherapy. Notably, PD-L1 expression in macrophages was associated with prolonged OS and PFS. PD-L1 expression in macrophages but not in tumor cells predicted immunotherapy response [119]. Using the Nanostring GeoMX platform, a high-plex profiling of intraepithelial tumor and adjacent stroma of TNBC was performed to characterize and compare the immune protein milieu of PD-L1-positive and PD-L1-negative tumor immune microenvironment (TIME) [120]. PD-L1+ stromal and intraepithelial TIMEs were highly enriched in immune proteins IDO-1, HLA-DR, CD40, and CD163 compared with PD-L1– TIMEs [120].

In order to characterize primary uveal melanoma (pUM) phenotypically and functionally, a high-resolution single-cell analysis of five *BAP1*– pUM samples was performed [121]. Previously *BAP1* loss in pUM was found to be associated with an activation of immunosuppressive microenvironment. Among infiltrating CD45+ cells, a predominant cluster of macrophages had mixed phenotype of M2-like CD68+CD163+CD74+ macrophages and M1-like $CD68^{+}CD163{-}CD74{-}CD11c{+}CD11b{+}$ macrophages. To discover the protein expression profile of the macrophage-abundant ROIs, a DSP assay of two metastatic cases of UM was performed which revealed high expression of CD163, HLA-DR, and CD11c [121]. In addition, high expression of B7–H3, a checkpoint regulator of lymphocyte functions [122], was observed in macrophage-abundant ROIs together with IDO-1, which is known to induce resistance to anti-PD1 and anti-CTLA4 immunotherapies [121].

A spatial analysis using quantitative multiplex immunofluorescence (qmIF) was performed on melanoma samples to understand interactions of tumor cells and macrophages more closely in recurrent and non-recurrent samples [123]. HLA-DR+, but not HLA-DR– macrophages clustered close to tumor cells in the non-recurrent compared to recurrent samples. Multiplex staining revealed that phenotype of HLA-DR– macrophages refers to CD163+CSF1R+ and CD163+CD33+ cells. GeoMX DSP analysis demonstrated that patients with higher stromal density of HLA-DR−macrophages had lower CD45+ cells infiltration. PD-L1 and PD1 levels per CD45+ ROIs were significantly higher in patients with higher HLA-DR− macrophage density [123]. These data suggests an unfavorable tumor immune microenvironment in melanoma cases associated with macrophage HLA-DR– phenotype [123].

Analysis of primary lung cancer and brain metastasis tissues using Nanostring GeoMx-DSP revealed that brain TME undergoes extensive remodeling to create an immunosuppressive and fibrogenic niche in order to sustain progression [124]. In ROIs with abundant fibrosis, the expression of M1 markers (*CXCL10, TNF, IL10, CD80, IL1B,* and *IL6*) significantly decreased, while M2 markers (*CCL18, CD163, HLA-DRA, MRC1,* and *TGFB1*) were upregulated. The expression of *TLR6, TLR2*, and *CSF1* genes associated with inflammatory immune response increased in the brain metastasis TME. In high fibrosis samples, high expression of phagocytosis or antigen presentation markers (*CD68, ITGB2*, and *AIF1*) together with significant reduction in the expression of effector T hallmark genes (*GZMB, GZMA, IFNG* and *IL2)* was observed [124].

One more spatial transcriptomics platform is 10x Genomics Visium that combines close to single-cell resolution (1–10 cells per spot) within tissue architecture [125]. Using scRNA-seq, subpopulations of FAP+ fibroblasts and SPP1+ macrophages correlated with shortest PFS were found in patients with CRC [100]. In order to determine spatial context of FAP+ fibroblasts and SPP1+

macrophages, 10x Genomics Visium technology was applied. Most of SPP1+ macrophages were co-localized with FAP+ fibroblasts at the same spatial spots on tissue (Fig. 4B). CRC tumors with high expression of FAP in fibroblasts and SPP1 in macrophages displayed the lowest lymphocyte infiltration that can explain shorter survival rates after anti-PD-L1 immunotherapy in patients with high expression of both FAP and SPP1 [100]. It was similar to the results obtained by single-cell analysis of tumor tissue in another CRC cohort. There, authors also demonstrated that SPP1+ TAMs showed interactions with cancer-associated fibroblasts (CAFs) and myofibroblasts [45].

Another study identified the spatial structure of the tumor immune barrier (TIB), which is formed by the interaction of SPP1+ macrophages and CAFs in the HCC microenvironment and contributes to decreased immunotherapy efficacy by limiting immune cell infiltration into malignant regions [126]. The SPP1+ macrophages/CAFs was a histological barrier that prevented the interaction of cytotoxic immune cells with tumor cells in tumors of ICB non-responders, while in tumors of responders, immune cells interacted easily with tumor cells as well as with SPP1+ macrophages/CAFs. In tumors from responders without the TIB structure immune cell infiltration was more abundant than in ICB non-responders. In vivo, blocking SPP1 or macrophage-specific deletion of SPP1 in mice lead to the destruction of the TIB structure and sensitization of HCC cells to immunotherapy [126] (Fig. 4B and C).

Spatial transcriptomics analysis using 10x Genomics Visium was applied to study PD-L1-positive and PD-L1-negative tumors from patients with TNBC [127]. The profile of PD-L1-negative tumors that had higher infiltration of M0 macrophages was characterized by the expression of *CD163*, *HLA-A*, and *STAB1*. In contrast, PD-L1-positive tumors had an elevated level of M2 macrophages, and the following marker genes: *IL1R2*, *CHI3L2*, and *CHI3L1* [127].

Tissue analysis of pancreatic ductal adenocarcinoma consisting of scRNA-seq and spatial transcriptomics revealed two macrophage subpopulations – inflammatory M1 macrophage state (marker gene *IL1B)* and M2 macrophages (*CD163* and *MS4A4A)* [128]]. M2-like macrophages were most enriched in the ducts, while the M1 macrophages were more enriched in the stroma and cancer regions, pointing to an inflammatory environment in these regions [128].

10x Genomics Visium technology was applied to two CLM samples to assess spatial distribution of monocytic macrophages and GPNMB+ TAMs subsets discovered during scRNA-seq analysis [109]. Spatial transcriptomic analysis revealed a preferential localization of GPNMB+ TAMs in stromal regions of the invasive margin and of the tumor center, as well as in the adjacent liver surrounding bile ducts, whereas the location of monocytic macrophages was most prominent in proximity to blood vessels, possibly suggesting recent recruitment to the tissue [109].

Thus, technologies of spatial transcriptomics were applied for the identification of markers of immunotherapy response, which are expressed by macrophages or in macrophage-abundant regions. This method allowed determining specific spatial interactions between TAMs and cancer-associated fibroblasts in CRC patients.

4. Concluding remarks

For decades now the M1/M2 paradigm has been considered a staple in TAM classification, but application of state-of-the-art methods provide encouraging evidence for reconsidering this idea of the macrophage dichotomy. Recently accumulated data represent new clinically significant subsets of TAMs that have the features of both M1 and M2 macrophages (Table 1). ScRNA analysis allowed to reveal crucial immunosuppressive phenotype of TAMs, which is associated with lipid metabolism – LAMs. In the last 5 years, numerous studies have concentrated on the role of this subpopulation in tumor progression. Multiple recent studies demonstrate the accumulation of LAM subpopulation in tumor tissue. Primary experimental findings indicate that this subset of macrophages correlates with poor prognosis and poor response to immune checkpoint therapy (Table 1), providing the possibility of using LAMs as prognostic marker and prompting their intensive investigations. TREM2 is determined as the major LAM marker. However, even though single cell analysis revealed the presence of LAMs in tumors, their cell morphology has not been adequately investigated in tissue. The specific mechanisms of the relation between LAM lipid accumulation and tumor progression remain unclear, which requires further analysis using functional models.

Other major subtypes of TAMs were also found in different cancers: SPP1+ TAMs with angiogenic and hypoxic features in breast cancer, colorectal cancer, ovarian cancer, lung cancer, pancreatic ductal adenocarcinoma, and squamous cell carcinoma [48,49], MARCO+ TAMs with immunosuppressive phenotype accompanied by activated glycolysis, angiogenesis and lipid metabolism, found in lung cancer, prostate cancer and glioblastoma [24,46,48]; immunosuppressive SIGLEC10+ TAMs in hepatocellular carcinoma and DC-SIGN + TAMs in muscle-invasive bladder cancer (MIBC) [54,59], and others (Fig. 2, Table 1). Validations using independent patient cohorts reveled strong correlations of specific TAM subsets with patient outcome in several cancer types. These observations were made by IHC and mRNA analysis for TREM2+, C1QC+, MARCO+, FOLR2+, Siglec1+, GPNMB+ and DC-SIGN+ TAMs (Table 1). Other studies have limitations due to authors performed validation using TCGA datasets, where mRNA expression are not cell-specific (whole tumor tissue is analyzed) and patient cohorts are very diverse. It is worth noting that these and other scRNAseq-identified subpopulations are not unique and appear in multiple cancer types. Most likely, it will not be possible to isolate a cancer type-specific subpopulation, but will be possible to pinpoint the functional subset that contributes the most to tumor progression in particular cancer as well as has clinical significance specifically for that cancer. Most investigated TAM-targeting that included blocking the recruitment of macrophages into the tumor, depletion or reprogramming macrophages from M2 to M1 have not reached clinical success [129]. This is why broader understanding of the functional diversity of macrophage subpopulations will help find ways to improve therapeutic control of TAMs.

At the same time, revealed TAM subsets express overlapping markers. For example, TREM2+ TAMs express *APOC1, APOE, MSR1, C1QC, SPP1, MAFB, CD163* etc.; SPP1+ TAMs express *MARCO, C1QC, APOC1, APOE, TREM2, MSR1* etc.; MARCO+ TAMs express *SPP1* among other genes; C1QC+ TAMs express *APOC1, APOE, TREM2, MSR1* etc. (Table 1). It means that more thorough investigation of

these particular TAMs is needed to find their more specific phenotypic features and clarify their clinical significance in cancer patients' prognosis. Expression analysis of these subsets couldn't explain the M1/M2 dichotomy. This confirms that TAMs carry a mixed phenotype, but the prevalence of M1 or M2 may vary in different TAM subsets. A greater accumulation of macrophages with pro- or anti-tumor activity, together with certain cellular TME composition determines the outcome of the disease and the effectiveness of anti-tumor therapy. Single cell analysis is clearly an important tool for deciphering cell subpopulations that are significant for tumor progression. However, we among other researchers [129] raise the question of the lack of a unified criterion for characterizing new macrophage subsets. Given the rapidly accumulating data, the diversity of bioinformatics approaches, and the heterogeneity of clinical material on which the scRNA-seq data are based, identification of a common "denominator" (precise gene signature) to identify a specific subpopulation is required. Whether this TAM subset will be the same for all types of cancer is another question that remains to be resolved. This is the only way to obtain a high-quality effective target for TAM-based immunotherapy.

Another aspect of TAM heterogeneity that requires attention is their localization in tumor compartments. It is assumed that TAMs can acquire different functional phenotypes depending on their location in hypoxic or perivascular regions, inside the tertiary lymphoid structures, in the tumor nest or in the invasive front [11,13,108,115]. TAM molecular interactions with other components of the TME are not fully investigated. Considering only certain marker expression as indication of the target subset may not always be an effective approach for targeted therapy. This is where ST methods together with single cell analysis will help identify specific cell-cell interactions formed by target TAM subpopulation and how these interactions may change the TME. These findings will allow us to determine the group of patients for whom TAM-based therapy will be the most effective.

Following from the above, characterization of the active state of TAMs, together with their location in certain regions of the tumor, will help deepen the understanding of reasons for prevalence of one or another macrophage subpopulation in a particular tumor microenvironment (hypoxic, angiogenic, immunosuppressive, etc.), and how it can be used in a personalized TAM-targeting. Moreover, advanced technologies can help to identify TAM-derived biomarkers for patient stratification and monitoring patients' prognosis and the efficacy of anti-cancer therapy.

5. Future perspectives

Today immunotherapy is aimed to reactivate the suppressed immune components via checkpoints – cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and PD-1 ligand 1 (PD-L1) [130]. Almost ten years after the introduction of the therapeutic options based on ICIs, it became clear that there are limitations in the effectiveness of this class of drugs [131]. These obstacles can be explained by several facts: objective responses are only observed in PD-L1-expressing tumors; ICI therapy can be effective in poorly immunogenic tumor subtype; it's effect is also diminished in patients with scarce gut microbiome who took antibiotics; the response to ICI depends on the of individual immune competency and diversity; chemotherapy/radiotherapy administered before ICI affect the competency of the immune system [132–134].

The following types of macrophage-based molecular interactions were proposed recently for immunotherapeutic targeting: CD47 receptor (on tumor cell)/Signal-regulatory protein alpha [SIRPα] (on macrophage) [135], leukocyte immunoglobulin-like receptor B [LILRB] (on macrophages)/β2-microglobulin (β2M) as a part of major histocompatibility complex I [MHCI] (on tumor cell) [136], CD24 receptor (on tumor cells)/sialic acid-binding immunoglobulin-like lectin-10 [Siglec-10] (on macrophages) [137]. "Don't eat me" signals CD47, β2M, and CD24 expressed by tumor cells mediate their escape from surveillance and inhibit clearance by macrophage [138,139]. Blockade of these ligand/receptor interactions in animal cancer models demonstrated promising results, however, no clinical trials with therapeutic targeting have been finalized as of yet. Data obtained by ST technologies can provide new targets for immunotherapeutic interventions that can be reached by finding new ligand-receptor interactions in whole tumor or specific cell-abundant regions (Fig. 5). Recent data obtained by ST demonstrate functional reciprocity between SPP1+ TAMs and CAFs in tumors resulting in the formation of immunosuppressive microenvironment, indicating potential targeting of this interaction (Fig. 4). Targeting of immunosuppressive activity of TREM2+ and MARCO+ macrophages is currently under extensive investigation, and the first in vivo studies demonstrated promising results that are associated with the inhibition of immunosuppression and further decreased tumor growth [26,63,94–96] (Figs. 1 and 3). Depletion of CD169+ macrophages in murine TNBC models resulted in reduced tumor growth and decreased lung metastasis due to a significant expansion of CD8+ T cells and the accumulation of these cells within the tumors [140].

Other promising TAM-based therapies include gene therapy with chimeric antigen receptor (CAR) or non-viral vectors [141,142]. CAR allows gene trafficking to the tumor and phagocytosis of tumor cells [139,143,144]. CAR-M exerted prominent phagocytic and tumor-killing capacity against tumor cell in vitro. CAR-M and CAR-T demonstrated synergistic cytotoxicity by the induction of macrophage M1 polarization via the inflammatory factors secreted by CAR-T [144]. CAR-M demonstrated their efficacy in tumor xenograft mouse models by releasing proinflammatory cytokines and activating innate immune cells that decreased tumor growth and prolonged overall survival [141]. Phase I clinical trial with CAR macrophages (Clinicaltrials.gov identifier number: NCT04660929) are being performed [141]. This trial uses CAR macrophages engineered with chimeric adenoviral vector Ad5f35 and carrying scFv targeting HER2.

A virus-free strategy of TAM-targeted gene therapy was recently developed to target Mincle, a pattern recognition receptor on macrophages responsible for cancer progression [145]. ShRNA sequence specific for Mincle was developed by combining RNA interference with an ultrasound-microbubble-mediated gene transfer system (USMB). USMB-shMincle effectively silenced the expression of Mincle, inhibited the protumoral phenotypes of TAMs as well as the progression [145].

In conclusion, we are at the peak of unprecedented opportunities to comprehensively explore the tumor microenvironment and clarify all the functional and molecular features of each of its components. In this new era, this means that single cell analysis together

with ST will help us understand TAM diversity, investigate novel ligand-receptor interactions, and potential role of macrophages in shaping a particular type of tumor microenvironment. This will be most useful for the development of effective TAM-based targeting in the foci with particular cell-cell interactions in the TME (Fig. 5).

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Data availability statement

The data will be made available on request to the corresponding author.

CRediT authorship contribution statement

Militsa Rakina: Data curation, Writing – original draft. **Irina Larionova:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Julia Kzhyshkowska:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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