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Tumor-Associated Macrophages as a Paradigm of Macrophage Plasticity, Diversity, and Polarization

Lessons and Open Questions

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Abstract—Macrophages are present in all body compartments, including cancerous tissues, and their functions are profoundly affected by signals from the microenvironment under homeostatic and pathological conditions. Tumor-associated macrophages are a major cellular component of cancer-related inflammation and have served as a paradigm for the plasticity and functional polarization of mononuclear phagocytes. Tumor-associated macrophages can exert dual influence of cancer depending on the activation state, with classically activated (M1) and alternatively activated (M2) cells generally exerting antitumoral and protumoral functions, respectively. These are extremes in a continuum of polarization states in a universe of diversity. Tumor-associated macrophages affect virtually all aspects of tumor tissues, including stem cells, metabolism, angiogenesis, invasion, and metastasis. Progress has been made in defining signaling molecules, transcription factors, epigenetic changes, and repertoire of microRNAs underlying macrophage polarization. Preclinical and early clinical data suggest that macrophages may serve as tools for the development of innovative diagnostic and therapeutic strategies in cancer and chronic nonresolving inflammatory diseases **(*Arterioscler Thromb Vasc Biol*. 2013;33:1478-1483.)**

Key Words: activation analysis ■ immunity, innate ■ inflammation ■ macrophages ■ polarization microscopy ■ stem cells ■ tumor-associated macrophages

Among cells of hematopoietic origin, cells of the monocyte–macrophage lineage are probably the most plastic ones.[1–6] Mononuclear phagocytes in tissues have long been held to originate from hematopoietic precursors through the intermediate stage of circulating monocytes. However, recent evidence has challenged this view, indicating that tissue macrophages can originate from yolk sac, fetal liver, as well as from bone marrow.[7–9] Under homeostatic physiological conditions, in tissues, macrophages acquire distinct morphological and functional features (eg, alveolar macrophages in lungs, Kupffer cells in liver). In response to tissue damage, microbial invasion, and cytokines produced by innate or adaptive lymphoid cells, macrophages undergo activation in its various versions. Furthermore, the response of mononuclear phagocytes can also undergo long-term reshaping, an adaptive component of innate immunity which has been referred to as memory, training, imprinting, or adaptive.[10–12]

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Macrophages are present in tumor tissues and constitute a major component of the inflammatory microenvironment of cancer.[13–15] Adaptive antitumor immune responses and tumor-derived and stroma-derived cytokines shape the function of tumor-associated macrophages (TAM) during cancer progression, and TAM have served as a paradigm for polarized activation of macrophages.[6,16,17] Here, we will review selected aspects of polarized activation of macrophages and the function of TAM, emphasizing points that may be of general relevance to pathology and outstanding open questions.

Polarized Activation

Classically and alternatively activated macrophages have been referred to as M1 and M2, mirroring T helper (Th) 1 and Th2 T cells characterized by differential production of the activation signals interferon-γ (IFNγ) and interleukin 4 (IL-4). Classically activated (M1) macrophages had long been known to be induced by IFNγ, alone or in concert with microbial stimuli (eg, lipopolysaccharide) or cytokines (eg, tumor necrosis factor α and granulocyte-macrophage colony-stimulating factor). IL-4 and interleukin 13 were subsequently found to induce an alternative (M2) form of macrophage activation.[2] Other cytokines associated with Th2 polarization can activate macrophages in an M2 direction, and in response to various signals macrophages can also acquire M2-like states, which share a variable proportion of the signature features of M2 cells.[6]

Key features of M1-activated cells include an interleukin 12 (IL-12)^{high}/interleukin 23^{high} phenotype, efficient production of effector molecules (eg, reactive nitrogen intermediates) and inflammatory cytokines (interleukin 1β, tumor necrosis factor α, interleukin 6), and a distinct chemokine and chemokine

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receptor profile [16] These cells participate as inducer and effector cells in polarized Th1 responses and mediate resistance against intracellular parasites and tumors. The various versions of M2 and M2-like macrophages usually have an IL-12^{low}/interleukin 23^{low} phenotype, with variable capacity to produce inflammator cytokines depending on the signal used. M2 cells generally express high levels of scavenger, mannose, and galactose-type receptors, and the arginine metabolism is shifted to ornithine and polyamines. M2 cells can contribute to parasite clearance[18] and tissue remodeling[19,20] and have immunoregulatory functions.[17] Finally, it should be emphasized that M1 and M2 fully polarized cells should be viewed as extremes in a spectrum of activation states and that considerable differences exist between mouse and human cells in terms of molecules associated with macrophage polarization.[6,21]

Progress has been recently made in defining the molecular mechanisms underlying polarized activation of macrophages (summarized in the Figure). A first network tipping the balance in macrophage activation is represented by transcription factors, with signal-transducer and activator of transcription protein (STAT)-1 driving M1 polarization downstream of interferons and toll-like receptor (TLR) signaling and STAT6 directly supporting M2 polarization downstream of IL-4 and interleukin 13.[22] STAT6 also synergizes with a panel of transcription factors involved in M2 polarization, including the nuclear receptors peroxisome proliferator-activated receptor-γ[23] and peroxisome proliferator-activated receptor-δ,[24,25] Kruppel-like factor-4,[26,27] and c-Myc.[28] STAT3 is involved in the expression of genes associated with an M2-like phenotypes, including interleukin 10 and transforming growth factor (TGF) β,[29] and STAT5 promotes M2 polarization downstream of interleukin 3 signaling.[30] Production of several inflammatory mediators downstream of TLR engagement is controlled by the p50/p65 nuclear factor (NF)-κB heterodimer, a key element in M1 polarization,[31] whereas p50 NF-κB homodimers sustain M2 polarization in vitro and are observed in TAM and lipopolysaccharide-tolerant macrophages.[32,33] Finally, different isoforms of hypoxia-inducible factors have been linked to macrophage polarization, with hypoxia-inducible factor-1α driving induction of inducible nitric oxide synthase 2 (M1 polarization) and hypoxia-inducible factor-2α driving induction of arginase 1 (M2 polarization).[34] A complex network of regulatory elements controls the activity of transcription factors, as exemplified by interferon regulatory factor (IRF) and suppressor of cytokine signaling (SOCS) proteins. The emerging M1-associated transcription factor recombination signal binding protein for immunoglobulin kappa J region mediates the ability of Notch to induce a set of genes associated with M1 polarization via IRF8,[35,36] while inducing IL-12 p40 in an IRF-independent manner via direct activation of NF-κB.[37] IRF5 has a positive effect on the induction of IL-12 p40 downstream of interferon signaling, and its activity is counterbalanced by IRF4, which is activated during M2 polarization.[38] The activity of STAT proteins is also directly regulated by SOCS family members, with IL-4 upregulating SOCS1, which, in turn, inhibits the action of STAT1 and IFNγ in concert with TLR agonists upregulating SOCS3, which, in turn, negatively regulates STAT3.[39,40] Finally, Notch also controls M1 polarization in an SOCS3-dependent manner.[41]

Epigenetic mechanisms are also emerging as regulatory elements operating in macrophage polarization.[42–45] Histone modifications affect chromatin states and influence the interaction and functions of selected transcription factors at distinct loci during macrophage polarization. Binding of PU.1 and CCAAT/

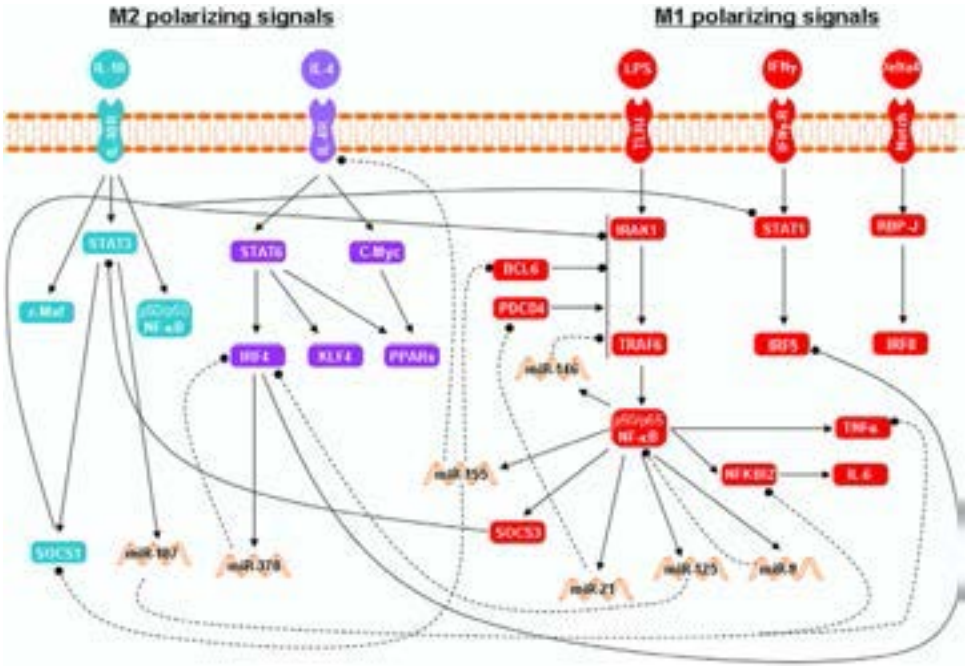

Figure. Transcription factors and microRNA (miR) networks in macrophage polarization. Macrophage polarized activity results from the balance activation of signal-transducer and activator of transcription protein (STAT) proteins, finely regulated by suppressor of cytokine signaling (SOCS) and interferon regulatory factor (IRF) proteins. M1 macrophage polarization, characterized by cytotoxic and inflammatory functions, is supported by predominance of nuclear factor (NF)-κB and STAT1 activation downstream of toll-like receptor (TLR)/interferon (IFN) receptors and by the activation of recombination signal binding protein for immunoglobulin kappa J region subsequent to Notch activation. In contrast, M2 macrophage polarization, associated with immune suppression and tumor progression, results from interleukin 4 (IL-4)–dependent STAT6 activation, with the cooperation of Kruppel-like factor (KLF)-4, c-Myc, and peroxisome proliferator-activated receptor transcription factors for distinct aspects of M2 macrophage activation. Interleukin 10 (IL-10) promotes M2 polarization through the induction of p50 NF-κB homodimer, c-Maf, and STAT3 activities. A complex network of miR induced by different polarizing stimuli overlays and cross-talk with the transcription factor network. Arrows indicate positive regulatory effects (induction/activation), bullet-end lines indicate negative regulatory effects (inhibition/inactivation), and dotted lines indicate direct targets of indicated miRs. For details refer to text.

enhancer binding protein α to regulatory regions controls the transcription of several genes by TLR agonists, which also removes negative histone marks by promoting dissociation of the B-cell CLL/lymphoma 6 repressor and supporting demethylase activity.[46–48] M1 polarization downstream of IFNγ signaling is also supported by STAT1-dependent events, leading to chromatin remodeling,[49] whereas IRF4 downstream of IL-4 induces upregulation of jumonji D activity, thus promoting chromatin modifications driving M2 polarization and inhibition of M1 genes.[6,50] Finally, distinct microRNA (miR) profile are specifically enriched in macrophages exposed to polarizing agents and regulate their polarization and their gene expression profile [51] In particular, miR-155, which is induced by TLR activation, targets the interleukin 13 receptor α chain,[52] the TGFβ signaling component SMAD family member 2,[53] and the interleukin 3 receptor α,[54] thus interfering with different forms of M2 polarization.[55] Similarly, miR-125 and miR-29b interfere with M2 activation by targeting IRF4 and sustain M1 activation by unleashing NF-κB signaling as a consequence of A20 downregulation,[51,56] and miR-378 operates a negative feedback loop on M2 polarization by targeting the IL-4 signal transducer AKT1.[57] Conversely, miR-511 is highly expressed in M2 and TAM and controls several associated genes by direct targeting of rho-associated, coiled-coil containing protein kinase 2.[58]

As polarized phenotypes are reversible, both in vitro and in vivo,[32] reeducating TAM is considered a promising strategy to interfere with tumor promotion. Although infusion of in vitro activated autologous macrophages in early clinical trials in the 1990s only showed limited success,[59] intraperitoneal injection of IFNγ in patients with ovarian cancer showed evidence of clinical responses and TAM phenotype switch.[60] Evidence also supports the notion that anticancer agents may function, at least in part, by acting on TAM. The antitumor effect of agonist anti-CD40 antibodies in a model of pancreatic ductal adenocarcinoma was accompanied by the induction of M1 markers in macrophages[61]; TAM depletion has been recently shown to represent a major determinant of the clinically approved anticancer agent trabectedin[62]; zolendronic acid, an agent used for preventing recurrence of breast cancer bone metastasis, has been shown to reverse the M2 polarity of TAM to M1.[63,64] The increasing comprehension of molecular mechanisms underlying macrophage polarized activation is expected to improve our ability to design innovative therapeutic strategies aimed at modulating macrophage functions.

Metabolism and Macrophage Polarization

Intrinsic metabolic features are instrumental in fulfilling the different effector function of macrophages, and these cells play a role in the orchestration of various metabolic pathways.[65] Arginine metabolism is probably the first and best known example of dichotomic metabolic pathways in M1 and M2 macrophages, as in M1 its catabolism by the IFNγ-inducible isoform of nitric oxide synthase generates the prominent bactericidal mediator nitric oxide, whereas in M2 the upregulation of arginase 1 shifts its metabolism to urea, which is required for collagen synthesis, and ornithine, which is then converted by a second M2-related enzyme, ornithine decarboxylase, in polyamines that sustain cell proliferation.[66] The TEK tyrosine kinase-expressing TAM subset has been reported to express high levels of arginase 1, whose selective depletion in this TAM subset was associated with angiogenesis inhibition and reduced tumor growth.[67,68] More recent evidence indicates that the receptor tyrosine kinase Ron is responsible for arginase 1 expression in TEK tyrosine kinase-expressing TAM because its ablation decreased arginase 1 expression and reduced syngeneic tumor growth, thus indicating that targeting arginase 1 activity or mechanisms responsible for its expression in TAM could have important therapeutic implications.[69]

Still on the amino acid metabolism, it is of note that the metabolism of tryptophan via indoleamine 2,3-dioxygenase is a major immunosuppressive mechanism used by myeloid-derived suppressor cells. Polarized macrophages also profoundly differ in energy supply. In M1, glucose metabolism is mainly oriented to the anaerobic glycolic pathway as an effect of the switch from the liver type to the ubiquitous type and more active 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, whereas M2 macrophages mainly use the oxidative glucose metabolism.[70,71] To provide the substrate for energy supply via fatty acid oxidation, M2 macrophages improve their fatty acid uptake.[71] However, it is of note that fatty acids also have a direct effect in macrophage polarization because the fatty acid sensors peroxisome proliferator-activated receptor-γ and peroxisome proliferator-activated receptor-δ are also involved in the regulation of expression of alternative activation genes, including pattern-recognition receptors.[24] Finally, polarized macrophages handle iron in different ways as a consequence of a coordinated differential expression of genes that control iron metabolism.[72] M1 macrophages operate a bacteriostatic iron withholding activity sustained by the upregulation of genes implicated in iron uptake and storage. Conversely, M2 macrophages are equipped with a set of molecules, allowing them to actively capture heme iron by means of various heme receptors (the hemoglobin receptor CD163, the hemopexin receptor CD91, the heme carrier protein 1) and catabolize it via the heme oxigenase 1 enzyme, which generates the immunoregulatory molecules CO and biliverdin/bilirubin[73] and free iron, which is then exported, thanks to the M2-restricted expression of ferroportin. Iron trafficking in M2 macrophages not only allows removal of the toxic heme molecule from the environment consistent with the scavenger function of M2 macrophages but also provides a significant contribution to their anti-inflammatory and tissue repair–prone characteristics. Thus, divergent expression of different classes of molecules involved in iron management significantly impacts both intramacrophage iron availability and the macrophage interplay with parenchymal cells. Decreased intracellular iron availability in M2 macrophages has an anti-inflammatory outcome by negative regulation of NF-κB activity and translation of tumor necrosis factor α and interleukin 6.[74] Conversely, the increased availability of iron in the extracellular milieu, supported by M2 macrophages, influences the growth rate of adjacent parenchymal cells and fibroblasts and contributes to collagen biosynthesis during the repair phase.[75] Finally, M2-like TAM could support tumor growth through increased iron bioavailability.

The Yin Yang of TAM

Several growth factors and chemokines have been associated with TAM differentiation and chemotaxis. In particular, both macrophage colony-stimulating factor, the main regulator of the macrophage lineage, and CC chemokine ligand (CCL)-2, a

potent monocyte chemoattractant, have been found to be overexpressed in various tumor types, and in several cases their expression levels have been shown to be directly correlated with increased TAM content and poor prognosis.[76] CCL2 is also involved in preferential recruitment in metastatic sites of Gr1-positive inflammatory monocytes and metastasis-associated macrophages, which promote seeding and growth of tumor cells. Recruitment and subsequent interaction with metastasizing tumor cells are dependent on the production by both the tumor and the stroma of CCL2, and inhibition of CCL2–CC chemokine receptor 2 signaling blocks the recruitment of inflammatory monocytes, inhibits metastasis, and prolongs survival, indicating its potential relevance as therapeutic target.[77]

Once in the tumor mass, TAM can express protumoral and antitumoral functions depending on their activation states. There is evidence for a role of innate responses in antitumor resistance early in carcinogenesis[78] or after TAM reeducation in established tumors.[61,79] In established progressing mouse and human tumors, TAM usually express an M2-like phenotype,[16] geared toward promoting tumor growth directly and via angiogenesis, tissue remodeling, and suppression of adaptive immunity. These functions of TAM recapitulate in many respects their functions in tissue repair. Tumor-infiltrating T cells (Th2 cells producing IL-4), immune complexes or cancer cells, or stromal element–derived cytokines (including macrophage colony-stimulating factor, interleukin 10, and TGFβ)[80] can orient TAM in an M2-like state.[61] TAM in murine tumors consist of cells with substantial differences.[81] For instance, in a transplanted mammary carcinoma, TAM were reported to have an M1-like and M2-like phenotype in normoxic and hypoxic areas, respectively.[82] Evidence for intratumor TAM diversity was also obtained in human colon cancer.[83] An additional level of diversity is dictated at the level of the different organs involved in carcinogenesis.[84,85]

Virtually all aspects of tumor growth and progression can be regulated by TAM.[13] By direct interaction with tumor cells, they can influence senescence and stimulate cancer cell growth[86] invasion and metastasis.[87] TAM also engage in a complex cross-talk with stroma cells, thus promoting angiogenesis and lymphangiogenesis.[87,88] In particular, TAM recruitment has been shown to be sustained by CCL2 derived from tumor-associated fibroblasts, which are then stimulated by TAM-derived signals (TGFβ in particular) to remodel extracellular matrix and promote tumor cell growth and dissemination.[89]

TAM usually express low levels of the major histocompatibility complex class II and little tumoricidal activity; finall , they have the potential to suppress antitumoral adaptive immunity. After exposure to IL-4, interleukin 10, TGFβ, and tumor cell supernatants, selectively the fibronectin isoform migration-stimulating factor, a potent mitogen for monocytes, is induced. Its role in ontogeny and immunopathology remains to be defined [90]

TAM and Stem Cells

Evidence suggests that cells of the monocyte macrophage lineage interact with cells with progenitor or bona fide stem cell properties and that this interplay is important in tissue remodeling and cancer.[18,91] In particular, mesenchymal stem (or stromal) cellsengage in a bidirectional interaction with mononuclear phagocytes. M2-like macrophages and their mediators promoted the proliferation of human mesenchymal stem cells.[72] Conversely, mesenchymal stem cells orchestrate the function of macrophages. Mesenchymal stem cells have been reported to induce an interleukin 10^{high} $IL\text{-}12^{low}$ alternative (M2) activation phenotype in macrophages.[92] The interaction of macrophages with stem/progenitor cells is relevant to the tumor microenvironment.

Indeed, TAM interact with cancer stem cells (CSC) or cancer-initiating cells, in particular in mammary carcinoma and gliomas.[93–96] TAM were observed to stimulate CSC tumorigenicity and drug resistance by releasing milk fat globul-E8.[97] Milk fat globul-E8 affected CSC via the Notch and Stat3 pathway. In glioma, CSC shape the function of macrophages/microglial cells that are recruited via chemokines and acquire immunosuppressive activity.[94] However, TAM and resident microglial cells have been reported to favor tumor cell invasion.[95] Thus, as for normal stem/progenitor cells, a ping-pong interaction is likely to take place between CSC and macrophages.

Conclusions

Plasticity is a major characteristic of cells and of the monocyte macrophage lineage and their activation states. Their phenotype and function are shaped by individual tissue microenvironments. Tissue-specific cues, microbial signals, and cytokines dictate differentiation and activation of macrophages. TAM has served as a paradigm for the plasticity of mononuclear phagocytes. Although progress has been made, key questions remained unanswered, including mechanisms sustaining macrophage levels in human tumors, diversity within and among tumors, and diagnostic and therapeutic significance. The same or similar questions are relevant to chronic nonresolving inflammatory conditions

Dissection of the diversity of TAM and macrophages in inflamed tissues will be an important design for antimacrophage or macrophage activating strategies to specific human diseases. The recent demonstration that targeting TAM is a key component of the antitumor activity of an approved anticancer drug against sarcoma and ovarian carcinoma[62] provides general encouragement for the development of macrophage-centered diagnostic and therapeutic strategies.

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