

# Chapter 6

## Macrophages and Tumor Development

Suzanne Ostrand-Rosenberg

**Abstract** Macrophages are malleable cells that can adapt varying phenotypes and functions. They are critical components of the innate immune system that have the potential to eliminate intracellular pathogens, to facilitate wound healing, and to activate T cells and natural killer (NK) cells. Their specific phenotype and functions are regulated by the cytokines and chemokines in their microenvironment. Although macrophages have the potential to be tumoricidal, the inflammatory milieu of the tumor microenvironment co-opts macrophages and renders them tumor promoting. Tumor-associated macrophages (TAMs) are present in most solid tumors where they facilitate tumor progression by enhancing angiogenesis, promoting tumor cell invasion and metastasis, protecting tumor cells from chemotherapy, and inhibiting antitumor immunity. Macrophage infiltrates in solid tumors have long been recognized as indicators of poor prognosis. However, current studies are exploring novel strategies for reprogramming TAMs and converting them into cells that facilitate tumor rejection.

**Keywords** M1 · M2 · Tumor-associated macrophages · Immune suppression · Angiogenesis · Tumor invasion and metastasis · Polarization · Inflammation

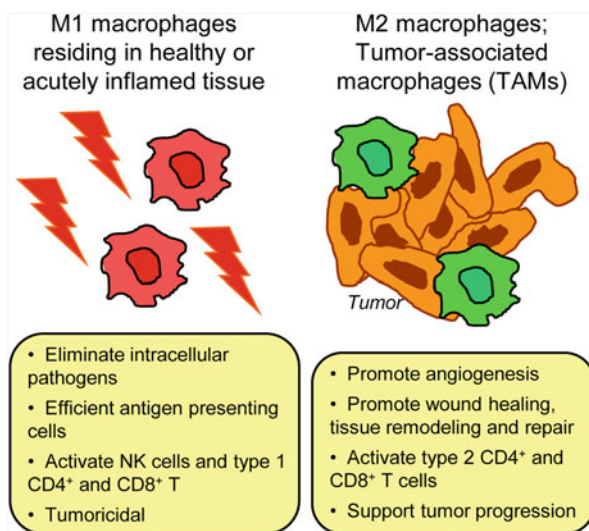
### 1 Introduction

Macrophages are an exceptionally diverse population of cells. They are critical players in both innate and adaptive immunity and their functions impact responses to pathogens, to self-antigens (autoimmunity), and to cancer cells. Macrophages were first identified in the late 1800s by Elie Metchnikoff, a Ukrainian pathologist. He observed that if starfish were injected with dye particles, cells within the starfish would engulf the particles. He called these cells “phagocytes” based on the Greek words

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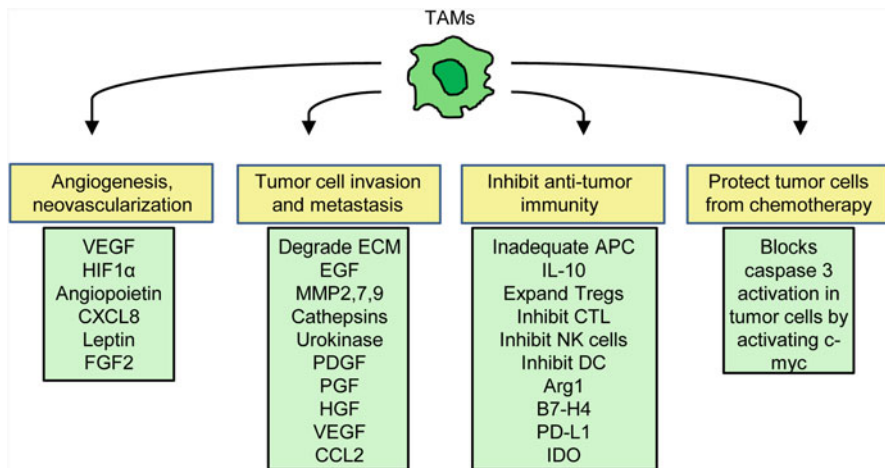
S. O.-Rosenberg (✉)  
Department of Biological Sciences, University of Maryland Baltimore County,  
Baltimore, Maryland, USA  
e-mail: srosenbe@umbc.edu

**Fig. 6.1** Macrophages residing in healthy or pathogen-infected tissues (M1 macrophages) and tumor-associated macrophages (TAMs, M2 macrophages) have distinct functions



*macros* (large) and *phagein* (to eat). He subsequently observed similar cells in human blood, and in 1892 he proposed his “cellular (phagocytic) theory of immunity,” stating that white blood cells are the key elements of the immune system that protect against pathogens [1], [2]. At that time, this theory was both groundbreaking and controversial because it contradicted the prevailing concept that humoral immunity, mediated by soluble proteins (antitoxins) and serum, was responsible for immune protection. It was not until the 1940s that immunologists appreciated the dual role of humoral and cellular immunity, despite the recognition of both cellular and humoral immunity by the awarding of a shared Nobel Prize to Metchnikoff and Paul Ehrlich, the discoverer of antitoxins (antibodies), in 1908.

The phagocytic cells seen by Metchnikoff were actually a mixture of multiple cell types, including what we now know as dendritic cells (DC), neutrophils, and macrophages. In addition to phagocytosis, macrophages also mediate their effects through their production of soluble factors including cytokines and chemokines, and by direct cell-to-cell contact with their cellular targets. Macrophages are now recognized as central players in the immune system and as having extensive plasticity. Their role and function depend largely on their anatomical location and microenvironment since their plasticity is largely driven by factors produced by surrounding cells. In healthy individuals, macrophages play an important role in facilitating wound healing, in regulating adaptive immunity, in eliminating infectious agents, and in regulating metabolism (reviewed in [3]–[6]). Macrophages have been categorized as “M1-like” and “M2-like” to reflect this apparent dichotomy of function (Fig. 6.1). M1-like macrophages typically reside in healthy tissue or at sites of acute inflammation. They eliminate intracellular pathogens, activate type 1 cluster of differentiation 4 (CD4<sup>+</sup>) and CD8<sup>+</sup> T cells by functioning as antigen-presenting cells (APC), and may be cytotoxic for tumor cells. In contrast, M2-like macrophages (also referred to as “alternatively activated macrophages”) mediate wound healing,



**Fig. 6.2** Tumor-associated macrophages (*TAMs*) facilitate tumor progression through diverse mechanisms. Tumor-promoting mechanisms are listed in *yellow boxes*; factors mediating the mechanisms are listed in *green boxes*

promote angiogenesis, and drive type 2 immunity. The conditions and factors that drive the development of M1 and M2 macrophages are described in Sect. 5. In individuals with cancer, macrophages are usually co-opted by tumor-secreted factors and facilitate the development and progression of tumors. These tumor-associated macrophages (*TAMs*) are immune suppressive and share many characteristics with M2-like macrophages.

## 2 Macrophages Promote Tumor Progression Through Multiple Mechanisms

It is well established that macrophages localize to tumor sites and many studies demonstrate a strong correlation between quantity of macrophages and poor prognosis [7], [8]. *TAMs* are recruited to solid tumors because of the hypoxic and inflammatory tumor microenvironment. These conditions retain macrophages within tumors while additional factors modulate macrophage phenotype and enable them to promote tumor progression [9], [10]. *TAMs* facilitate tumor progression through diverse mechanisms. Figure 6.2 summarizes these mechanisms which are described in the following sections.

### 2.1 *TAMs Drive Angiogenesis and Neovascularization*

As solid tumors enlarge, they become too large to obtain their nutrients and oxygen through simple diffusion and must induce neovascularization and establish a vascular network. This process, known as the “angiogenic switch,” as well as continued

neovascularization of growing tumors, is mediated by macrophages through their production of vascular endothelial growth factor A (VEGF-A) and other pro-angiogenesis factors [11]–[14]. Macrophages expressing Tie2, the receptor for angiopoietin-2 (ANG2), are particularly associated with neovascularization [12]. Induction of VEGF in macrophages is partially regulated by hypoxia [15], [16]. Poorly vascularized regions of solid tumors become hypoxic and chemoattract macrophages [17]. The hypoxic environment turns on the transcription factors, hypoxia-inducible factors (HIF) 1 $\alpha$  and 2 $\alpha$ , which in turn upregulate VEGF synthesis. VEGF then chemoattracts more macrophages [18], [19]. Macrophages also produce matrix metalloproteinases (MMP) and other proteases that degrade the extracellular matrix (ECM) causing the release of additional pro-angiogenic factors [20].

Inflammation through the action of the pro-inflammatory cytokine interleukin-1 beta (IL-1 $\beta$ ) also induces VEGF synthesis by stabilizing HIF1 $\alpha$ . This upregulation occurs in both normoxic [21] and hypoxic [22] conditions and involves nuclear factor kappa B (NF- $\kappa$ B) upregulation of cyclooxygenase-2 [21]. Other factors also contribute to macrophage-induced angiogenesis. Urokinase-type plasminogen activator and its receptor, molecules that increase vascularization, are upregulated on TAMs [23]. Macrophage-induced angiogenesis has also been attributed to fibroblast growth factor-2, angiopoietin, chemokine (C-X-C motif) ligand 1 (CXCL8 or IL-8), and leptin [24], as well as more than 30 other genes that are upregulated by hypoxia [25].

## ***2.2 TAMs Promote Tumor Cell Invasion and Metastasis***

Tumor cell invasion and metastasis are complex processes that require tumor cells to leave their primary site, invade their surrounding normal tissue and ECM, intravasate into and traffic via either the circulatory or lymphatic system, extravasate at a distant location, and acquire nutrients and oxygen at their final destination. Macrophages facilitate several of these steps. Early experiments using knockout mice deficient for colony-stimulating factor 1 (CSF-1), a factor required for macrophage differentiation, demonstrated that macrophages in the local microenvironment are essential for tumor cell invasion and metastasis [26]. Elegant multiphoton microscopy studies demonstrated that TAMs facilitate tumor cell intravasation into surrounding normal tissue and blood vessels [27]. In vivo studies demonstrated that resistance to established murine mammary carcinoma metastatic disease requires macrophage depletion or polarization of macrophages towards a tumor-rejecting M1-like phenotype [28], [29]. Several factors produced by macrophages contribute to these processes. In addition to CSF-1 serving as a differentiation factor, it also stimulates macrophage production of epidermal growth factor (EGF) which is a chemoattractant for tumor cells and promotes tumor cell invasiveness [30]. The release of macrophage inhibitory factor (MIF) also promotes tumor cell trafficking [31]. TAMs also synthesize and secrete multiple MMP (MMP-9, -2, and -7) [32], [33], cathepsins [34], [35], urokinase [36], and other proteases [37] which degrade the ECM and basement membrane, thereby allowing tumor cells to migrate to distant sites. Macrophages

also produce platelet-derived growth factor, placental growth factor (PIGF), and hepatocyte growth factor which directly support tumor cell proliferation [25]. Recent studies in multiple mouse tumor systems and in immune-deficient mice with human tumors definitively identified CD11b<sup>+</sup> F4/80<sup>+</sup> macrophages as essential for promoting tumor cell extravasation and for “conditioning” a metastatic niche prior to arrival of migrating tumor cells [38]. Subsequent studies revealed that the progenitors of these macrophages are Gr1<sup>+</sup> inflammatory monocytes that express C–C chemokine receptor type 2 (CCR2) and are chemoattracted by tumor and stromal cells producing chemokine (C–C motif) ligand 2 (CCL2), and that extravasation involves monocyte and macrophage production of VEGF. These studies demonstrate the link between inflammation and cancer in the metastatic process and provide a mechanistic explanation for the poor prognosis of patients with high levels of CCL2 and tumor-infiltrating macrophages [39].

### 2.3 TAMs Inhibit Antitumor Immunity

TAMs inhibit both innate and adaptive antitumor immunity and are themselves impacted by the immune system of tumor-bearing individuals. Unlike M1-like macrophages which have high levels of major histocompatibility complex (MHC) class II molecules and are effective APC, TAMs are poor APC because they have reduced levels of MHC II molecules as well as low levels of the costimulatory molecules CD80 and CD86. Hypoxia contributes to these decreased levels [40], [41], as does interaction with myeloid-derived suppressor cells (MDSC) [42].

TAMs are characterized by their high production of IL-10 and their minimal production of IL-12. IL-10 polarizes immunity towards a type 2 response whereas IL-12 polarizes towards a type 1 response. Type 1 responses favor tumor rejection while type 2 responses promote tumor progression. IL-10 production drives CD4<sup>+</sup> Th2 cells at the expense of CD4<sup>+</sup> Th1. Optimal activation of potentially tumoricidal CD8<sup>+</sup> T cells requires help from CD4<sup>+</sup> Th1 cells, so the absence of Th1 cells usually results in sub-optimally activated cytotoxic CD8<sup>+</sup> T cells. IL-10 also inhibits CD8<sup>+</sup> T-cell function by inducing the expansion of natural T regulatory cells (Tregs) and the development of induced Tregs. The Tregs, in turn, directly inhibit CD8<sup>+</sup> cytotoxic activity [43]. Since IL-12 contributes to the activation and efficacy of both natural killer (NK) cells [44] and DC [45], TAMs also impact antitumor immunity by diminishing NK-mediated cytotoxicity and antigen presentation by DC.

TAMs directly impact T-cell activation and proliferation through several mechanisms. They produce high levels of arginase 1, an enzyme that degrades arginine. Arginine degradation not only deprives T cells of L-arginine needed for protein synthesis but also results in the generation of toxic catabolic products including oxygen and nitrogen radicals [46], [47].

TAMs also suppress T-cell activation through their expression of immune suppressive co-inhibitory molecules. B7-H4 is a member of the B7 family of genes and is induced in macrophages by IL-6 and IL-10. A subset of TAMs from ovarian

cancer patients express B7-H4. When B7-H4 interacts with its receptor on T cells, T cells fail to progress through the cell cycle and therefore neither proliferate nor produce the cytokines essential for tumor cell killing [48]. Some TAMs also express the co-inhibitory molecule programmed cell death ligand-1 (PD-L1, also called B7-H1). PD-L1 causes T-cell apoptosis when it binds to its receptor, PD-1, which is expressed on activated T cells. IL-10 and tumor necrosis factor alpha (TNF $\alpha$ ) in the tumor microenvironment of hepatocellular carcinoma patients induce expression of cell surface PD-L1 in TAMs, and these macrophages suppress antitumor immunity [49].

The enzyme indoleamine 2,3-dioxygenase (IDO) is an immune suppressive molecule produced by macrophages and other cells of myeloid origin. IDO degrades tryptophan and therefore depletes the microenvironment of tryptophan making surrounding cells unable to progress through the cell cycle [137]. Tumor cells themselves secrete IDO which to some extent reduces tumor growth. However, DC and macrophages also produce IDO, and this IDO suppresses T-cell activation and outweighs the direct effects of IDO on tumor cells. As a result, lymphocytes in the lymph nodes draining solid tumors are tolerized or nonresponsive to tumor antigens, thereby eliminating adaptive antitumor immunity [138], [139]. Recent studies in mouse models have established that the immune suppressive effects of IDO significantly contribute to the progression of both primary and metastatic lung and mammary tumors [50].

## ***2.4 TAMs Protect Tumor Cells from Chemotherapy***

Macrophages also promote tumor growth by interfering with chemotherapy [51]. In patients with multiple myeloma, large quantities of macrophages are present in the bone marrow. Chemotherapy of multiple myeloma with dexamethasone and melphalan induces apoptosis of tumor cells by cleaving and activating caspase 3. Cell-to-cell contact between macrophages and myeloma cells prevents caspase 3 activation and therefore protects tumor cells from apoptosis [52]. Interactions between myeloma cells and macrophages depend on binding of myeloma cell-expressed P-selectin glycoprotein-1 and ICAM-1/CD18 to their respective partners on macrophages (selectins and CD11b, respectively). Binding of these ligand–receptor pairs activates the c-myc pathway and kinases Src and Erk1/2 in myeloma cells, rendering them resistant to caspase activation [53].

## **3 Origin and Identification of Macrophages**

Macrophages are a type of white blood cell (leukocyte) that reside in both lymphoid and non-lymphoid tissues. In the mouse, macrophages are identified by their expression of the cell surface markers CD11b (a subunit of Mac-1) and F4/80, a G protein-coupled receptor. CD11b is also expressed by macrophages in humans,

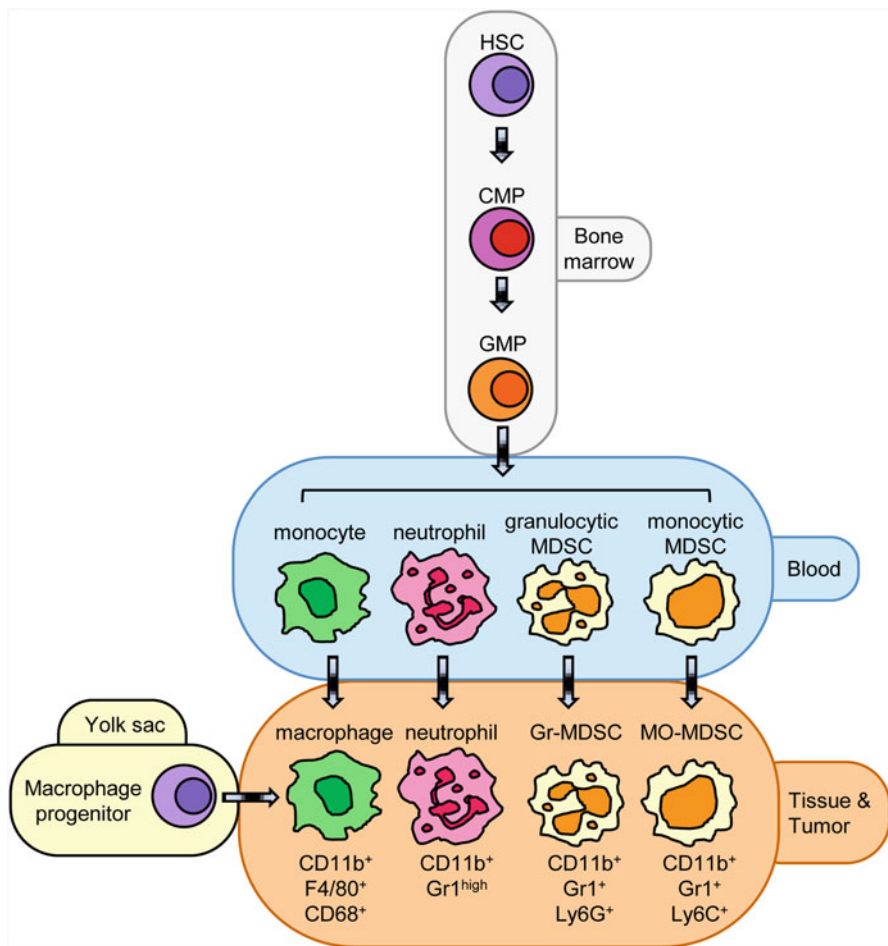
along with the cell surface protein CD68. Mouse and human macrophages are also characterized by their expression of other markers which are acquired in response to their microenvironment (see the following sections on macrophage polarization and inflammation).

Macrophages may have specialized names depending on their location. For example, macrophages within the central nervous system are called microglia while those in the lung are termed alveolar macrophages. Most macrophages are derived from pluripotent hematopoietic stem cells (HSC) that originate in the bone marrow (Fig. 6.3). HSC give rise to a common myeloid progenitor (CMP) that in turn gives rise to a granulocyte/macrophage progenitor (GMP) which serves as the progenitor for multiple myeloid cells including monocytes that circulate in the blood, bone marrow, and spleen [54]. Circulating monocytes become macrophages when they migrate into and become resident in tissues. Recent studies have demonstrated that some tissue-resident macrophages are present and proliferate in the absence of HSC and have identified a second lineage of macrophages that arises from the yolk sac in 9.5–10.5-day-old mouse embryos. The transcription factor Myb was found to be essential for the development of classical hematopoietic-derived macrophages, but was not necessary for the development of microglia or macrophages in the liver [55]. Although studies have not specifically been conducted on the origin of tumor-infiltrating macrophages, it is likely that these cells are of hematopoietic origin since they are derived from circulating monocytes.

#### **4 Inflammation Drives Malignant Transformation and Tumor Progression and Recruits TAMs**

Inflammation is now appreciated as one of the hallmarks of cancer [56]–[58]. Inflammation in the tumor microenvironment facilitates the growth of established tumors, and approximately one quarter of cancers are thought to be the result of chronic inflammatory conditions [59]–[64]. The tumor microenvironment is a highly complex milieu of pro-inflammatory cytokines, chemokines, and bioactive lipids that are produced by tumor cells themselves, as well as by infiltrating host cells, including macrophages [65]. Pro-inflammatory cytokines such as IL-6 and TNF $\alpha$  produced by many tumor cells are key drivers of inflammation in the tumor microenvironment [9], [63], [66]–[68]. In addition to their inherent inflammatory properties, TNF $\alpha$  and IL-6 stimulate production of pro-inflammatory chemokines such as CCL2, CXCL1, CXCL8, and CXCL12, as well as MMP [65]. These cytokines and chemokines establish a setting that not only maintains inflammation but also recruits macrophages, other immune cells, and stromal cells that produce inflammatory mediators. The result is a network of autocrine factors that sustain an inflammatory tumor microenvironment.

The transcription factor NF- $\kappa$ B is the major intracellular regulator of the inflammatory program and is activated in macrophages and in tumor cells through a Toll-like receptor-myeloid differentiation primary response gene (88) (TLR-MyD88)



**Fig. 6.3** Macrophages differentiate from hematopoietic stem cells in bone marrow or from yolk sac. Hematopoietic stem cells (*HSC*) in the bone marrow give rise to a common myeloid progenitor (*CMP*) which gives rise to a granulocyte/macrophage progenitor (*GMP*). The *GMP* differentiates into monocytes, neutrophils, and monocytic and granulocytic myeloid-derived suppressor cells (*MDSC*) which circulate in the blood. When monocytes move into tissue or tumor, they become macrophages. Tissue macrophages also differentiate from a progenitor cell that arises in the yolk sac during embryogenesis. Cell surface markers for neutrophils, granulocytic MDSC (*Gr-MDSC*), and monocytic MDSC (*MO-MDSC*) are for murine cells

pathway. Ligands include bacterial products, cellular debris, IL-1 $\beta$ , and TNF $\alpha$  [56]. In tumor cells, it induces a more aggressive phenotype and regulates the production of chemokines and cytokines that chemoattract macrophages that in turn promote tumor growth [69]. The role of NF- $\kappa$ B in macrophages is less well defined. In agreement with its pro-inflammatory role in malignant cells, inhibition of NF- $\kappa$ B in macrophages of mice that spontaneously develop tumors results in delayed tumor



onset [70], as well as in mice with chronic inflammation that progresses to malignancy [62], [64], [71]. However, in late stage tumors, TAMs have defective NF- $\kappa$ B and restoring NF- $\kappa$ B activity in these TAMs delays tumor growth [72]. Therefore, whether activated NF- $\kappa$ B is pro-tumor or antitumor may depend on the stage of disease and the type of cancer.

## **5 Macrophage Polarization and Phenotype: M1-like Macrophages have Antitumor Activity While M2-like Macrophages Facilitate Tumor Progression**

Depending on environmental cues, macrophages can either facilitate the destruction of tumor cells or promote the progression of tumor growth. This dichotomy of function is the result of the extreme plasticity of macrophages and their phenotypic adaptation to their environment. Similar to the Th1 vs. Th2 paradigm for CD4<sup>+</sup> T lymphocytes, Mills proposed that macrophages also polarize into two categories based on their phenotype and function [73]. This nomenclature was further developed by Mantovani and colleagues and has now become the accepted jargon to describe macrophages [3], [74]–[76]. M1-like macrophages (also known as “classically activated” macrophages) are activated by bacterial products such as lipopolysaccharides (LPS) in combination with interferon gamma (IFN $\gamma$ ). M1 polarization has also been attributed to TNF $\alpha$ . These cells produce high levels of IL-12 and low levels of IL-10 and are able to lyse tumor cells. They also synthesize and contain inducible nitric oxide synthase (iNOS or NOS2) and typically express high levels of cell surface CD86 and MHC class II molecules. In contrast, M2-like macrophages are activated by IL-4, IL-13, IL-10, and glucocorticoid hormones. They produce low levels of IL-12, high levels of IL-10, and support tumor growth. They also synthesize and contain high levels of arginase 1 and express low levels of MHC class II and high levels of the cell surface molecules FIZZ1 and Ym1. Their high level of expression of IL-4R $\alpha$  facilitates their polarization towards an M2 phenotype by the binding of IL-4 and IL-13 [6].

M1 macrophages eliminate intracellular pathogens and mediate tumor cell destruction by their production of a variety of reactive intermediate species that result from the production of nitric oxide (NO) and its reaction with molecular oxygen [77]. M1 macrophages also contribute to tumor destruction through their skewing of immunity towards a type 1 response that activates tumor-reactive T cells. Their production of the chemokines Mig (CXCL9) and IP-10 (CXCL10) chemoattract Th1 cells [65], [78]. Type 1 immunity is further facilitated by their production of pro-inflammatory cytokines including IL-1 $\beta$ , IL-6, IL-23, and TNF $\alpha$  combined with their ability to function as APC to activate Th1 and Tc1 CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively [79].

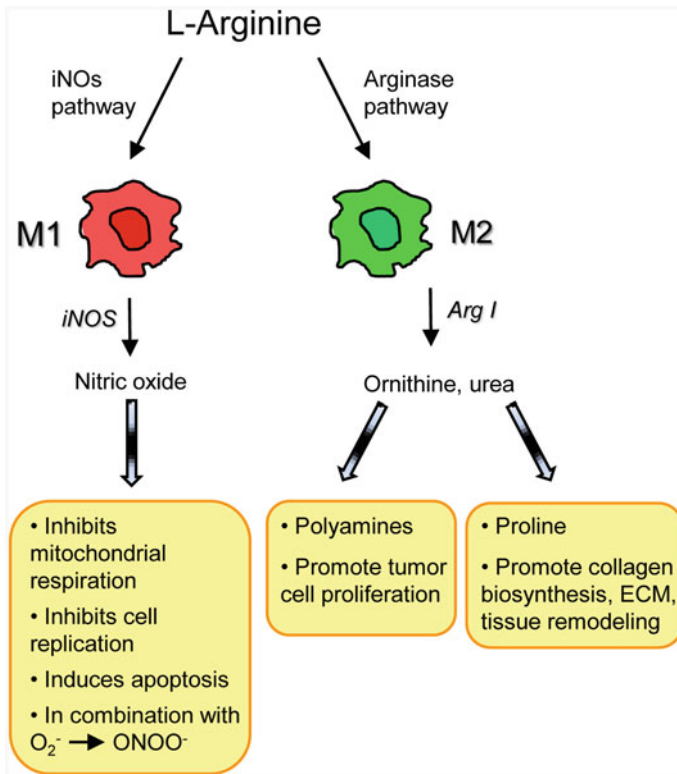
In contrast, M2 macrophages are predominately anti-inflammatory, although they secrete some pro-inflammatory molecules. They promote type 2 immunity that facilitates the elimination of parasites, promotes wound healing, and drives tumor

progression. TAMs are polarized towards an M2 phenotype. The production of CCL22 by M2 macrophages chemoattracts Tregs which inhibit T-cell activation [80]. M2 macrophages also secrete prostaglandin E2 and transforming growth factor beta (TGF $\beta$ ), both of which are immunosuppressive [81], and they express PD-L1 which binds to its receptor PD-1 on activated T cells and causes T-cell apoptosis [49]. Their production of IL-10 and their lack of IL-1 $\beta$  production due to their high expression of IL-4 receptor antagonist and decoy type II receptor promote their anti-inflammatory effects [82]. M2 macrophages also stimulate angiogenesis which facilitates tumor growth [74] and they produce high levels of arginase 1 (Arg1) which prevents T-cell proliferation by depriving T cells of arginine [83], [84].

The metabolism of the amino acid arginine by M1 vs. M2 macrophages is a major distinguishing characteristic of the two macrophage phenotypes. M1 macrophages predominantly metabolize arginine via the NO pathway, while M2 macrophages use the arginase pathway [85]. iNOS or NOS2 is upregulated in M1 macrophages by IFN $\gamma$  and TNF $\alpha$ . The NO that is produced is cytotoxic and destroys target cells by inhibiting cell proliferation [86], blocking mitochondrial respiration [87], and inducing apoptosis [88], [89]. NO also reacts with superoxide (O $_2^-$ ) to produce peroxynitrite (ONOO $^-$ ) [88], [90]. In contrast, Arg1 is upregulated in M2 macrophages by IL-4, IL-13, TGF $\beta$ , and IL-10 [91], [92]. Arg1 converts arginine to ornithine which is subsequently converted to polyamines. Ornithine is a precursor for proline, a major constituent of collagen which is necessary for tumor infrastructure. Polyamines promote tumor cell proliferation because they are required for deoxyribonucleic acid (DNA) replication [93]. Figure 6.4 schematically illustrates the two pathways. MDSC metabolize arginase through the same two pathways with monocytic MDSC (MO-MDSC) using the iNOS pathway while granulocytic MDSC use the Arg1 pathway [47]. Interestingly, MDSC production of iNOS does not result in tumoricidal activity as it does for M1 macrophages.

The preceding description of macrophage polarization applies to murine macrophages. Human macrophages are more enigmatic in terms of polarization and distinct classes of macrophages have not been conclusively identified. Whether this is due to a lack of adequate markers or a different program of differentiation is unclear at present.

Macrophages are characterized by their expression of the specific markers discussed above. However, some of these markers are also expressed by other cell types and not all macrophages express all of these markers. For example, murine macrophages are often characterized by their expression of CD11b; however, CD11b is also expressed by other myeloid cells. Likewise, MHC II serves as a marker for macrophages; however, DC also express MHC II. Similarly, F4/80 is expressed by macrophages as well as by eosinophils and Langerhans cells. Expression of functional molecules such as iNOS and Arg1 is also not restricted to macrophages in that subpopulations of MDSC also express these molecules. There are many fewer cell surface markers for human macrophages. CD68 expression is typically used, although it is also expressed by some fibroblasts. Therefore, macrophage identification by marker expression is not always unambiguous and straightforward, although a combination of cell surface markers and functional markers can typically give a reliable identification.



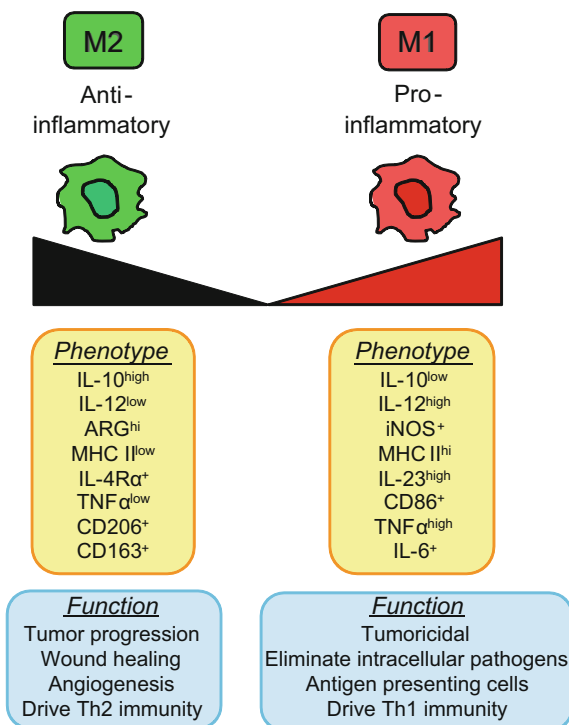
**Fig. 6.4** M1 and M2 macrophages metabolize arginine through different pathways. M1 macrophages metabolize arginine through the *iNOS* pathway which converts arginine to nitric oxide. M2 macrophages metabolize arginine through the arginase pathway and produce polyamines and proline

Figure 6.5 summarizes the phenotypes and functions of M1- and M2-polarized macrophages.

## 6 Macrophages are a Heterogeneous Mixture of Myeloid Cells

The M1–M2 paradigm is a convenient nomenclature for categorizing macrophages. However, most macrophages do not conveniently fit into one or the other of these categories, and the M1 and M2 states represent the extremes of a continuum of macrophage phenotypes that are governed by the local environment. Since tumors include a diversity of microenvironments, it is not unusual that different regions of solid tumors will have macrophages with differing and intermediate phenotypes. Since the microenvironments within solid tumors are generated by factors secreted by tumor cells, and different types of cancers produce different factors, macrophage populations between tumors can vary significantly. Likewise, as solid tumors evolve

**Fig. 6.5** Macrophages display a continuum of phenotypes and functions, with *M1*-like and *M2*-like cells representing the extremes. *M2*-like and *M1*-like macrophages have distinct phenotypes (shown in yellow boxes) and functions (shown in blue boxes). *M2*-like macrophages are anti-inflammatory and facilitate tumor progression, while *M1*-like macrophages are pro-inflammatory and facilitate tumor rejection



in individuals through the process of immunoediting and selection, tumor-secreted factors are likely to change such that macrophage populations will also evolve with tumor progression.

Macrophages also differ significantly within a single tumor. Movahedi and colleagues have identified seven distinct subsets of macrophages in mammary carcinoma and lung adenocarcinoma [94]. These subsets are distinguished by their level of expression of MHC II, the monocyte marker Ly6C, the homing receptor L-selectin (CD62L), and the chemokine receptors CX<sub>3</sub>CR1 and CCR2. The different subsets had varying half-lives as well as different differentiation kinetics. Monocytes with a Ly6C<sup>hi</sup> phenotype were observed to be precursors of all TAMs. Gene expression profiles have been performed on macrophages from multiple different types of tumors [95]–[97]. These studies revealed that overall TAMs are predominantly of an M2 phenotype. Additional profiling studies on macrophage subsets from three different mouse tumors revealed that macrophages with an MHC II<sup>low</sup> phenotype were the dominant TAM population and that these cells express high levels of M2 genes including *Arg1*, *IL4Rα*, and *Il10*. MHC II<sup>low</sup> TAMs were also present, but at a lower level and expressed high levels of M1 genes including *Cox2* and *IL-1β*. RNA levels roughly correlated with protein levels for most genes; however, *NOS2* mRNA was highest in MHC II<sup>low</sup> macrophages, while NOS2 protein was highest in MHC II<sup>high</sup> macrophages [94]. These studies also noted that MHC II<sup>low</sup> macrophages were most

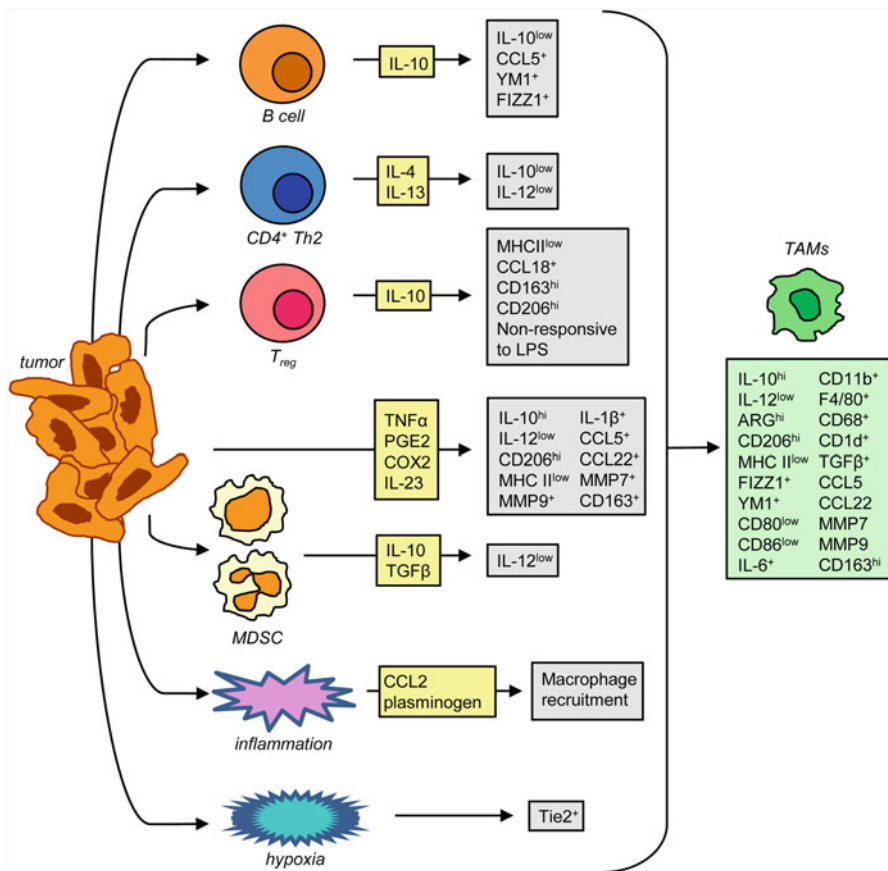
frequently present in hypoxic regions of the tumor, while MHC II<sup>high</sup> cells localized to normoxic areas. Hypoxia has also been shown to drive expression of the angiopoietin receptor Tie2 on a subset of macrophages (TEMs) that are pro-angiogenic [98]. Another subset of TAMs identified by their motility, invasiveness, and wound-healing properties was characterized by their high content of molecules associated with the Wnt signaling pathway [99].

## 7 The Tumor Microenvironment Regulates Macrophage Polarization

In addition to TAMs and tumor cells, the tumor microenvironment includes a complex milieu of host cells such as CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, Tregs, B lymphocytes, DC, mast cells, MDSC, NK cells, natural killer T (NKT) cells, neutrophils, and cancer-associated fibroblasts (CAFs). Many of these host cells are induced by tumor-secreted products to secrete factors that drive the polarization of macrophages towards an M2 phenotype. For example, both solid and ascites human ovarian cancer cells produce soluble mediators that upregulate macrophage production of IL-10 and CSF-1. These cells also produce many chemokines characteristic of the M2 phenotype. TNF $\alpha$ , a cytokine produced in abundance by ovarian cancer cells, upregulates hemoglobin scavenger receptor A (CD163), a marker characteristic of TAMs [100].

CD4<sup>+</sup> T cells also impact macrophage polarization. In vitro coculture studies demonstrated that CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs divert macrophages towards an M2 phenotype by increasing macrophage expression of CD163 and CCL18, and by increasing macrophage phagocytic activity through their production of IL-10. IL-4, IL-13, and IL-10 produced by Tregs downregulate macrophage production of pro-inflammatory mediators produced in response to LPS. Tregs also reduced macrophage expression of MHC class II molecules [101]. Studies in transgenic mice that spontaneously develop mammary carcinoma (*PyMT* mice) confirmed an in vivo role for CD4<sup>+</sup> T cells in polarizing macrophages and further demonstrated that the altered macrophages promote metastatic disease by activating EGF receptor signaling in the mammary epithelial cells. These latter effects were due to CD4<sup>+</sup> T effector cells and not Tregs, indicating that CD4<sup>+</sup> T cells alter macrophage phenotype through diverse mechanisms [102].

Macrophages are also impacted by B lymphocytes and by CAFs. Coculture and in vivo experiments using B1 lymphocytes (B220<sup>low</sup>IgM<sup>high</sup>CD11b<sup>+</sup>) increased macrophage production of IL-10 and decreased macrophage production of M1-type molecules including TNF $\alpha$ , IL-1 $\beta$ , and CCL3. M2 markers FIZZ1 and Ym1 were also increased [103]. In a chemically induced two-stage skin carcinogenesis system, large quantities of fibroblasts accumulate at the site of carcinogen application. The fibroblasts produce high levels of monocyte chemotactic protein 1 (MCP-1) which chemoattracts macrophages to the carcinogenic locale and promotes papilloma progression [104].



**Fig. 6.6** Tumors produce multiple factors that condition their environment and surrounding cells and mold macrophage phenotype. Tumor-produced factors polarize immunity towards a type 2 response and induce *B cells*, *CD4<sup>+</sup> Th2* cells, T regulatory cells (*Tregs*), and myeloid-derived suppressor cells (MDSC) to produce cytokines and chemokines that polarize macrophages towards an M2 tumor-promoting phenotype. Tumor-secreted factors also induce an inflammatory and hypoxic environment that favors the development of TAMs. Cytokines and inflammatory mediators (in yellow boxes) are produced by tumor cells, the indicated *B cells*, *CD4<sup>+</sup> Th2* cells, *Tregs*, *MDSC*, inflammation, and/or hypoxia and induce specific markers and molecules in TAMs (in gray boxes). TAMs may express all or a subset of the markers/molecules shown in the green box

Crosstalk with MDSC also drives macrophage phenotype. In the presence of MDSC, macrophage production of IL-12 and IL-6 and expression of MHC II are reduced. Macrophages, in turn, increase MDSC production of IL-10 via an IL-6-dependent mechanism [42], [105]. This crosstalk is regulated by signaling through the TLR4 pathway in MDSC, involves upregulation of CD14 on MDSC, and is exacerbated by inflammation [106].

Figure 6.6 summarizes how tumor-secreted factors and host cells impacted by tumor-secreted factors drive macrophages towards a tumor-promoting phenotype.

## 8 Multiple Regulatory Elements Control Macrophage Polarization

Macrophage polarization has been attributed to multiple genes and regulatory elements. Deletion of the suppressor of cytokine signaling 3 (*SOCS3*) gene in myeloid cells generated M2-like macrophages, while deletion of the *SOCS2* gene yielded phenotypically M1 macrophages. Targeted deletion of *SOCS* in macrophages also altered macrophage function. *SOCS3*-deficient macrophages were more potent recruiters of Tregs, while *SOCS2*-deficient macrophages were not. Polarization towards an M2 phenotype yielded increased activation via signal transducer and activator of transcription 6 (STAT6), while polarization towards an M1 phenotype increased activation through STAT1 [107]. These changes in signal transduction preference correlate with the known role of STAT1 in transmitting signals from IFN $\gamma$ , a known inducer of M1 phenotype cells, and STAT6 in transmitting signals from IL-4 and IL-13, known inducers of M2 macrophages.

MicroRNAs (miRNA) have also been shown to regulate macrophage polarization. The mannose receptor (CD206), encoded by the *MRC1* gene, is a marker of TAMs and facilitates macrophage phagocytosis of microbial and host glycoproteins [108]. The *MRC1* gene encodes miR-511-3p. This miRNA is constitutively expressed at high levels in TAMs; however, increasing expression inhibits tumor growth and reduces the pro-tumor phenotype of TAMs. Therefore, miR-511-3p levels appear to be important for maintaining TAM phenotype, but overexpression skews macrophages away from an M2 state [109].

Tumor antagonizing/malignancy suppressor genes (TAG/MSG) are a family of genes that suppress tumorigenicity in vivo but have no apparent in vitro effects. *RNASET2*, an extracellular RNase, is a member of this family. Ovarian cancer cells expressing wild type or catalytically inactive *RNASET2* grow much more slowly than cells containing mutated *RNASET2*. Reduced tumor progression is due to the infiltration of iNOS<sup>+</sup> M1 macrophages since supernatants of cancer cells containing wild-type *RNASET2* polarized macrophages towards an M1 phenotype [110]. Thus, *RNASET2* is another gene that regulates macrophage polarization and its ability to polarize is independent of its catalytic RNase activity.

Histone-rich glycoprotein (HRG) is an anti-angiogenic and immunomodulatory factor present in serum and produced by platelets that regulates blood vessel formation. The tumor microenvironment typically contains less HRG than tumor-free corresponding normal tissue. Overexpression of HRG in several mouse tumor cell lines prevented the development of disorganized blood vessel formation associated with wild-type tumor, and delayed tumor progression. HRG overexpression reduced the number of M2-like TAMs within tumors by half and increased the number of M1-like cells. These were direct effects of HRG on macrophages since similar skewing was observed when macrophages were cultured in the presence of HRG. HRG mediates these effects by downregulating PIGF, a known driver of angiogenesis and homolog of VEGF-A. Depletion of TAMs from HRG overexpressing tumors restored blood vessel abnormalities and increased tumor growth, indicating that HRG regulates tumor progression via macrophage polarization [111].

Macrophage polarization is also regulated epigenetically by chromosome remodeling. Proteins containing a Jumonji-C (JmjC) domain, including Jmjd3, are histone demethylases. Many genes that are activated by LPS are targets for Jmjd3. TLR stimulation activates Jmjd3 in macrophages via an NF- $\kappa$ B-dependent mechanism that controls the expression of the *Bmp2* and *Hox* genes. In a parasite model, macrophages attain an M1 phenotype in the absence of Jmjd3 activation, whereas Jmjd3 activation is essential for the generation of M2 macrophages [112], [113]. Jmjd3 is also activated in macrophages by IL-4 through a STAT6-dependent mechanism. Activated STAT6 increases transcription of Jmjd3 which subsequently demethylates M2 marker genes, polarizing macrophages towards an M2 phenotype [114].

## 9 Macrophages as Prognostic Indicators of Tumor Progression

Macrophage infiltrates in solid tumors have long been recognized as indicators of poor prognosis. A recent clinical study in Hodgkin's Lymphoma patients cemented this correlation. Gene profiling and immunohistochemistry revealed that high levels of CD68<sup>+</sup> macrophages predicted poor outcome after primary and secondary therapy. In contrast, low levels of tumor-associated CD68<sup>+</sup> macrophages were associated with a subgroup of patients that had 100 % long-term disease-free survival [115]. In contrast to most cancers, levels of peritumoral CD68<sup>+</sup> macrophages correlate with a good prognosis in colorectal cancer patients [116], [117]. This apparent inconsistency is because most macrophages in colorectal cancer are M1-like macrophages. As for other cancers, high levels of M2-like macrophages correlate with poor prognosis [118]. Expression of CD40 by HLA-DR<sup>+</sup> CD80<sup>+</sup> CD86<sup>+</sup> M1-like macrophages has also been identified as an indicator of favorable prognosis in colorectal cancer patients [119]. Experimental studies in mice indicate that activation of macrophages by antibodies to CD40 in combination with IL-2 therapy induces macrophage production of NO and reduces metastatic disease [120]. High levels of peritumoral macrophages also correlate with good prognosis for patients with high-grade osteosarcoma. These tumors contain both M1-like and M2-like macrophages; however, there was no correlation between macrophage phenotype and patient outcome [121].

These studies suggest that although most tumors induce tumor-promoting M2-like macrophages, some tumors induce M1-like macrophages which may contribute to tumor regression. A better understanding of the conditions that drive M1 vs. M2 polarization is essential to eliminate pro-tumor M2-like macrophages and induce antitumor M1-like macrophages.

## 10 TAMs Can be Reprogrammed Toward an M1-like Phenotype

Given the potent antitumor activity of M1 macrophages, considerable attention has been devoted to strategies to repolarize TAMs. Several studies have identified IL-12 as a key molecule for converting M2 macrophages to an M1 phenotype. Treatment



with IL-12 decreases TAM expression of the tumor-promoting factors IL-10, MCP-1, and TGF- $\beta$  and induces expression of TNF $\alpha$ , IL-15, and IL-18, factors that facilitate tumor rejection [122]. TAMs are also repolarized by IL-12 through treatment with a chimeric antigen receptor (CAR) that redirects cytotoxic T lymphocytes (CTL) to release IL-12 at the tumor site [123]. Delivery of IL-12 by engineered tumor-specific CD8<sup>+</sup> T cells reprograms much of the myelomonocytic compartment of tumor stroma by converting immune suppressive macrophages, DC and MDSC into cells that sustain T-cell activation and promote debulking of large tumors [124]. This latter effect involves IFN $\gamma$  and is consistent with previous findings that IFN $\gamma$  repolarizes TAMs from an immune suppressive M2 phenotype to an antitumor M1 phenotype [125].

Other conditions also reeducate TAMs. Administration of IL-2 in combination with antibodies to CD40 converts TAMs to an M1 phenotype of high NO production [120]. In the inflammatory tumor microenvironment, macrophages acquire an M2 phenotype by signaling through the IL-1R and activating NF- $\kappa$ B. Inhibition of NF- $\kappa$ B signaling “reeducates” TAMs to an MHC II<sup>high</sup>, IL-12<sup>high</sup>, ARG<sup>low</sup> phenotype. Inhibition of NF- $\kappa$ B also renders them tumoricidal and generates antitumor activity through the recruitment of IL-12-dependent NK cells [126]. Whether inhibition of NF- $\kappa$ B will be generally applicable, however, is unclear because microarray studies demonstrated that TAMs induced by some tumors are constitutively defective in NF- $\kappa$ B signaling [95]. The Notch pathway has also been invoked in macrophage polarization. In mouse tumor models, TAMs have lower levels of Notch pathway activation compared to M1 macrophages. Macrophages with defective Notch signaling or TAMs treated with Notch signaling inhibitors acquire an M2 phenotype [127].

Additional cytokines and growth factors have also been identified as potential reagents for reprogramming TAMs. Restoration of host-produced histidine-rich protein (HRP) converts TAMs to M1 macrophages by downregulating PIGF [111]. TAMs, as well as DC, are repolarized by treatment with anti-IL-10R antibodies plus the TLR9 ligand, CpG [128]. In another study, IL-4 and IL-10 were identified as critical factors for driving macrophage polarization towards an M2 phenotype, and inhibition of the IL-4R $\alpha$  in mice with VEGF-induced skin carcinogenesis prevented M2 polarization of macrophages [129], [130].

Macrophage polarization is also regulated by the transcription factor STAT6 since IL-4 and IL-13 drive M2 polarization by binding to the IL-4R $\alpha$  and signaling through the JAK2/STAT6 pathway. As a result, macrophages in STAT6-deficient mice default develop into M1 cells and these cells contribute to the rejection of established metastatic mammary carcinoma [28]. The nonclassical MHC class I CD1d gene also regulates macrophage polarization. CD1<sup>-/-</sup> mice are IL-13-deficient because they lack NKT cells which produce IL-13. The absence of IL-13 causes macrophages to default to an M1 phenotype. These mice also reject established metastatic mammary carcinoma [29] and are resistant to recurring fibrosarcomas [131].

These reprogramming studies have been done in mouse tumor models and have resulted in innate antitumor immunity and delayed tumor progression and/or reduction of metastasis. Given the heterogeneity of TAMs, and the lack of well-defined markers for M1 and M2 phenotypes in human cancer, it remains to be demonstrated whether the findings in mice will be applicable to human systems. Table 6.1 summarizes

**Table 6.1** Strategies for reprogramming TAMs

Effect on TAMs	Effect on tumor	Strategy	Tumor	Reference
Decrease TAM production of IL-10, MCP-1, TGF $\beta$ ; increase TNF $\alpha$ , IL-15, IL-18; increase NK and CTL		IL-12 in microspheres	Murine 3LL lung	[122]
Increase quantity of tumor-infiltrating macrophages that expressed CD80 and CD86 and produced TNF $\alpha$ (M1 phenotype)	Reduce primary tumor growth	CAR-redirected CTL releases IL-12 at tumor site	Murine MC38 colon carcinoma	[123]
Reprogram TAMs, DC to type 1		IL-12-engineered CTL	Murine B16 melanoma	[124]
Convert macrophages to M1 phenotype	Reduce primary tumor growth	IL-2 plus mAbs to CD40	Murine Renca renal cell carcinoma under kidney capsule	[134]
Convert macrophages to M1 phenotype that secrete high levels of NO; downregulated MMP2, MIMP9	Reduce metastasis	IL-2 plus mAbs to CD40	Murine Renca renal cell carcinoma i.v. and intrasplenic	[120]
Convert macrophages to MHC II <sup>high</sup> IL-12 <sup>high</sup> ARG <sup>low</sup> ; improve APC function; recruit NK cells	Reduce primary tumor growth	Inhibit NF- $\kappa$ B by targeting I $\kappa$ B kinase	Murine ID8 ovarian carcinoma	[126]
Convert macrophages to M1 by upregulating CXCL9, IFN $\beta$ , IL-12; downregulating CCL17, IL-10, MRC1	Reduce primary and metastatic tumor growth	Restore histidine-rich protein which downregulates PGF	Murine T241 fibrosarcoma, Panc02 pancreatic tumor, 4T1 mammary carcinoma; demonstrated HRP decreased in multiple human tumors	[111]
Convert macrophages (and DC) to type 1 that secrete IL-12, TNF $\alpha$	Reject primary tumor and metastasis	Adenovirus delivery of CCL16 plus CpG plus Ab to IL-10R	Murine TSA and 4T1 mammary carcinomas, MCA38 colon carcinoma	[128]
Convert macrophages to M1 that secrete IL-12	Reduce primary tumor growth	Activate notch pathway	Murine B16 melanoma, 3LL lung carcinoma	[127]
Convert macrophages to M1 phenotype	Reduce primary tumor growth	Inhibit NF- $\kappa$ B by overexpressing p50 NF- $\kappa$ B inhibitory homodimer	Murine MN/MCA1 fibrosarcoma, B16 melanoma	[72]

Table 6.1 (continued)

Effect on TAMs	Effect on tumor	Strategy	Tumor	Reference
Convert macrophages to tumoricidal M1 phenotype by inducing NO secretion, reducing ARG secretion and are tumoricidal	Reject metastatic disease; confer survival	Delete STAT6 gene	Murine 4T1 mammary carcinoma	[28]
Convert macrophages to tumoricidal M1 phenotype by inducing NO secretion, reducing ARG secretion and are tumoricidal	Reject metastatic disease; confer survival	Delete CD1 or IL-4R $\alpha$ gene which eliminates IL-13 or IL-13 responsiveness, respectively	Murine 4T1 mammary carcinoma	[29]
Drive macrophages to an ARG <sup>low</sup> , NO <sup>high</sup> M1 phenotype		Increase SHIP expression		[135]
Convert TAMs from ovarian ascites to IL-10 <sup>low</sup> IL-12 <sup>high</sup> CD86 <sup>+</sup> M1 phenotype		IFN $\gamma$	Human ovarian cancer	[125]
Convert TAMs to IL-10 <sup>low</sup> VEGF <sup>low</sup> NF- $\kappa$ B-activated M1 phenotype	Reduce primary tumor growth; extend survival time	Zoledronic acid	Murine TUBO mammary carcinoma	[136]

the therapeutic approaches that have been developed to reprogram M2 to M1 macrophages. Several recent reviews include additional information [9], [79], [132], [133].

## 11 Conclusions

Macrophages can either enhance tumor progression or facilitate tumor rejection. Their ultimate phenotype and function are determined by their tissue microenvironment. Virtually all solid tumors chemoattract monocytes and polarize them to M2-like tumor-promoting macrophages. These TAMs are a major contributor to disease progression through their direct promotion of tumor cell growth, and their indirect effects of suppressing antitumor immunity, promoting angiogenesis, and facilitating tumor cell invasion and metastasis. Strategies for eliminating TAMs are being actively pursued in animal models. However, given the extreme plasticity of macrophages and the tumoricidal properties of M1-like macrophages, therapies that repolarize TAMs towards a tumor-rejecting M1 phenotype may be more beneficial.

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