Macrophages and Therapeutic Resistance in Cancer

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http://dx.doi.org/10.1016/j.ccell.2015.02.015

How neoplastic cells respond to therapy is not solely dependent on the complexity of the genomic aberrations they harbor but is also regulated by numerous dynamic properties of the tumor microenvironment. Identifying and targeting critical pathways that improve therapeutic efficacy by bolstering anti-tumor immune responses holds great potential for improving outcomes and impacting long-term patient survival. Macrophages are key regulators of homeostatic tissue and tumor microenvironments. Therefore, therapeutics impacting macrophage presence and/or bioactivity have shown promise in preclinical models and are now being evaluated in the clinic. This review discusses the molecular/cellular pathways identified so far whereby macrophages mediate therapeutic responses.

Macrophages are represented in all tissues by functionally and phenotypically distinct resident populations that are critical for development and homeostasis (Wynn et al., 2013). Under non-pathological conditions, most resident macrophage populations derive from embryonic progenitors and are maintained through local proliferation (Epelman et al., 2014). Exceptions to this include intestinal, dermal, and alveolar macrophages at barrier sites (Bain et al., 2014; McGovern et al., 2014; Perdiguero et al., 2015; Yona et al., 2013) and macrophages in the adult heart that are replaced by circulating bone marrow-derived Ly6C^{+} inflammatory monocytes over a timescale of several weeks (Molawi et al., 2014). Under pathological conditions, there is evidence for both local proliferation and recruitment, with differences observed by tissue location and type of inflammatory insult (Epelman et al., 2014).

Solid tumors appear to be unique. Preclinical studies indicate minimal macrophage proliferation and shorter half-lives compared with resident macrophages in counterpart homeostatic tissues, measurable in days to weeks (Movahedi et al., 2010; Strachan et al., 2013). That said, CD68^{+} cells also positive for proliferating cell nuclear antigen (PCNA) expression have been observed in breast cancers, where they are associated with a poor clinical outcome (Campbell et al., 2011). Whether the macrophage lifespan in this context is reflecting diminished tissue integrity and the extent of damage/inflammation or, instead, represents an adaptive process engaged by tumors to support growth is unclear, but production of the C-C chemokine ligand 2 (CCL2) and/or colony-stimulating factor 1 (CSF-1) are necessary to sustain their numbers (Noy and Pollard, 2014). With the critical role for CCL2 and CSF-1 in recruiting macrophages to neoplastic tissue, there is growing interest in therapeutics targeting these ligands and/or their respective receptors in an effort to ablate the pro-tumorigenic properties of macrophages. This therapeutic approach has led to improved outcomes in a range of pre-clinical models, particularly for agents targeting CSF-1 or the CSF-1 receptor (CSF-1R), the results of which have spurred several clinical trials (Table 1).

As monotherapy, CSF-1R inhibition alone impedes the growth of orthotopically implanted pancreatic ductal adenocarcinoma (PDAC) cell lines (Mitchem et al., 2013), prevents cervical carcinogenesis (Strachan et al., 2013), and induces regression of glioblastoma multiforme (GBM) (Pyonteck et al., 2013). In other tumor models CSF-1R inhibition is without consequence as monotherapy. However, synergism with other modalities, including chemotherapy (DeNardo et al., 2011; Mitchem et al., 2013; Paulus et al., 2006; Ruffell et al., 2014), radiation therapy (Shiao et al., 2015; Xu et al., 2013), angiogenic inhibitors (Priceman et al., 2010), adoptive cell transfer (Mok et al., 2014), and immune checkpoint blockade (Zhu et al., 2014) have been revealed. Together, these findings implicate macrophages in regulating therapeutic responses and indicate that durable responses may be obtained by augmenting standard of care or emerging therapies with "macrophage antagonists." This review focuses on the mechanisms underpinning these observations and concludes with a discussion of targeting approaches that extend beyond inhibiting macrophage recruitment.

Clinical Significance of Macrophages

For many solid tumor types, high densities of cells expressing macrophage-associated markers have generally been found to be associated with a poor clinical outcome (Figure 1; Komohara et al., 2014; Zhang et al., 2012). There are conflicting data for lung, stomach, prostate, and bone, where both positive and negative outcome associations have been reported (Zhang et al., 2012), possibly related to the type/stage of cancer evaluated, (e.g., Ewing sarcoma versus osteosarcoma) (Buddingh et al., 2011; Fujiwara et al., 2011) or to the type of analysis performed (e.g., quantitation of stromal versus intratumoral macrophages). Some discrepancy may also reflect the use of different macrophage markers. CD68, a glycoprotein predominantly resident in intracellular granules, represents a fairly specific marker for murine macrophages and, in combination with F4/80, identifies a majority of tumor-associated macrophages. In humans, however, CD68 expression is widespread and includes granulocytes, dendritic cells, fibroblasts, endothelial cells, and some lymphoid subsets (Gottfried et al., 2008; Hameed et al., 1994; Ruffell et al., 2012b). The use of CD68 for association studies in this context is therefore of variable utility. A clear example of

Table 1. Macrophage Therapeutic Targeting

Pathway	Target[a]	Efficacy in Murine Models	Clinical Compounds	Clinical Trials in Solid Tumors[b]
Recruitment	CD11b	radiation, chemotherapy	rovelizumab	
	CSF-1R	single agent (GBM, PDAC), chemotherapy, radiation, angiogenesis inhibitors	PLX3397, AMG820 IMC-CS4/LY3022855, RG7155/RO5509554	NCT01596751 (O); NCT01444404 (C); NCT01349036 (O); NCT01004861 (O); NCT01346358 (O); NCT02265536 (O); NCT01494688 (O); NCT02323191 (O)
	CCL2	single agent (metastasis, PDAC)	carlumab	NCT00992186 (C); NCT01204996 (C)
	Neuropilin-1	angiogenesis inhibitors	MNRP1685A	NCT00747734 (C); NCT00954642 (C)
	ANG2	single agent (mammary), chemotherapy, angiogenesis inhibitors	nesvacumab	NCT01271972 (O); NCT01688960 (O)
Polarization	IL-4	single agent (metastasis), chemotherapy, radiation	pascolizumab	
	IL4Rα		dupilumab	
	IL-13	chemotherapy	lebrikizumab, tralokinumab, GSK679586,	
	FcγR	chemotherapy	rituximab (CD20), ibrutinib (BTK), R788 (Syk)	
Function	IL-6		clazakizumab, olokizumab, siltuximab, sirukumab	NCT00433446 (C); NCT00385827 (C) NCT00841191 (C)
	IL-6R		tocilizumab, sarilumab	
	TNF-α	mitogen-activated protein kinase inhibitors	adalimumab, certolizumab, etanercept, golimumab, infliximab	
Activation	CD40	single agent (PDAC), chemotherapy	CP-870,893	NCT00711191 (C); NCT01456585 (C) NCT02157831 (C); NCT01008527 (O) NCT02225002 (C); NCT00607048 (C) NCT01103635 (O)

O, ongoing; C, completed.
[a]Only targets with clinical compounds are listed.
[b]Data obtained from https://clinicaltrials.gov.

this is non-small-cell lung cancer, where detection of the macrophage scavenger receptors CD163 and CD204, but not CD68, yielded correlations with negative outcome (Chung et al., 2012; Hirayama et al., 2012; Ohri et al., 2011; Quatromoni and Eruslanov, 2012).

In addition to potentially representing more selective macrophage biomarkers, both CD163 and CD204 are associated with activation of macrophages toward an alternative or tumor-promoting and immunosuppressive phenotype, and, accordingly, significant correlations between CD163/CD204 and negative outcomes have been reported across multiple tumor types (Komohara et al., 2014). These correlations may indicate that macrophage polarization can direct clinical outcome, as also supported by the positive association between the presence of CD68$^+$ cells and survival in colorectal adenocarcinoma (Roxburgh and McMillan, 2012). Unlike most populations of tumor-associated macrophages that possess pro-tumor and immunosuppressive properties (Biswas and Mantovani, 2010), macrophages in human colorectal cancer have been found to be functionally (and phenotypically) anti-tumor (Edin et al., 2012; Ong et al., 2012; Zhang et al., 2014). Together, these data collectively support the tenet that repolarizing macrophages toward an anti-tumor phenotypic state, either by impeding activities/signals that drive pro-tumor polarization or by delivering exogenous signals that enhance anti-tumor polarization, could act as an alternative and perhaps more efficacious approach to blocking macrophage recruitment, even though these activities and responses are all dynamically regulated in vivo. Indeed, an agonist monoclonal antibody against CD40, a co-stimulatory protein found on professional antigen-presenting cells, has demonstrated efficacy in mouse models of PDAC (Beatty et al., 2011) and patients with PDAC (Beatty et al., 2013) when delivered in combination with the chemotherapeutic agent gemcitabine, ostensibly via the anti-tumor activities of macrophages (Vonderheide et al., 2013). In addition to the use of an anti-CSF-1R antibody in diffuse-type giant cell tumors (Ries et al., 2014), these are the first clinical studies to demonstrate the potential efficacy of macrophage-targeted agents.

Polarization and Macrophage Function

Macrophages produce an array of cytokines, chemokines, polypeptide growth factors, hormones, matrix-remodeling proteases, and metabolites, many of which possess tumor-promoting activities (De Palma and Lewis, 2013; Noy and Pollard, 2014; Ruffell et al., 2012a). A caveat to some of these reported
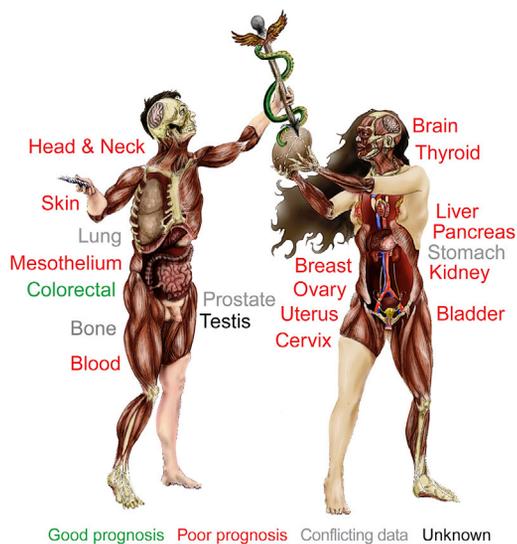
Figure 1. Clinical Implications of Macrophage Density
Organs where tumor progression and/or clinical outcome are negatively associated with increased macrophage density are shown in red. Green indicates a positive association. Organs where the implications of macrophages density are unclear or unknown are shown in gray and black, respectively. Image created by Tarot Walker.

Figure 2. Macrophage Polarization as a Dynamic System
The integration of multiple signals emanating from the tumor microenvironment (outer circle) dictates the functional role of macrophages (inner circle). Integrins and toll-like receptors (TLRs) will be engaged by multiple ligands.

activities is that many findings originate from cell culture studies utilizing neoplastic myeloid cell lines or bone marrow-derived macrophages and, therefore, cannot account for the complex milieu of polarization signals to which macrophages would be exposed in vivo (Figure 2). This includes the aforementioned CSF-1 and CCL2, prostaglandin E$_2$ (PGE$_2$), and damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), extracellular ATP, and degraded extracellular matrix components (Ruffell et al., 2012a; Zelenay and Reis e Sousa, 2013).

Stabilization of hypoxia-inducible factor (HIF) 1α and 2α is also important in mediating the pro-tumor properties of macrophages, as evidenced by the use of LysM-cre mice to induce myeloid-specific loss of either factor (Doedens et al., 2010; Imtiyaz et al., 2010). As might be expected, hypoxic conditions drive an angiogenic phenotype in macrophages, and, in vivo, this occurs specifically in a subpopulation of macrophages found within hypoxic regions of tumors that express low levels of major histocompatibility complex (MHC) II (Laoui et al., 2014; Movahedi et al., 2010). The recruitment of macrophages (presumably MHCII^{LO}) into hypoxic regions through Neuropilin-1 also supports an immunosuppressive phenotype (Casazza et al., 2013) that is likely dependent upon HIF-1α (Doedens et al., 2010). Surprisingly, however, although hypoxia can induce HIF-1α-dependent expression of arginase-1 in macrophages (Doedens et al., 2010), neither improving tumor oxygenation nor preventing macrophage recruitment into hypoxic areas alters arginase-1 expression (Casazza et al., 2013; Laoui et al., 2014). This discrepancy might be explained by the recent finding that lactic acid promotes arginase-1 expression by macrophages in an HIF-1α-dependent manner (Colegio et al., 2014).

Finally, the use of immune-competent murine models has firmly established that macrophage polarization and function within tumors are strongly influenced by lymphocytes through the production of multiple factors, including interleukin (IL)-4, IL-10, IL-13, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and immunoglobulins (Andreu et al., 2010; DeNardo et al., 2009; Gocheva et al., 2010; Guiducci et al., 2005; Kang et al., 2011). These activities include mediating responses to therapy because B cells coated in anti-CD20 antibody can suppress their phagocytic removal from circulation by Kupffer cells through the secretion of IL-10 (Horikawa et al., 2011), whereas B cell production of immunoglobulins (Affara et al., 2014) and CD4$^+$ T cell expression of IL-4 (Shiao et al., 2015) suppress responses to cytotoxic therapy by altering macrophage polarization. Even the efficacy of CSF-1R inhibition depends upon altered macrophage polarization, rather than depletion, in certain models (Pyonteck et al., 2013).
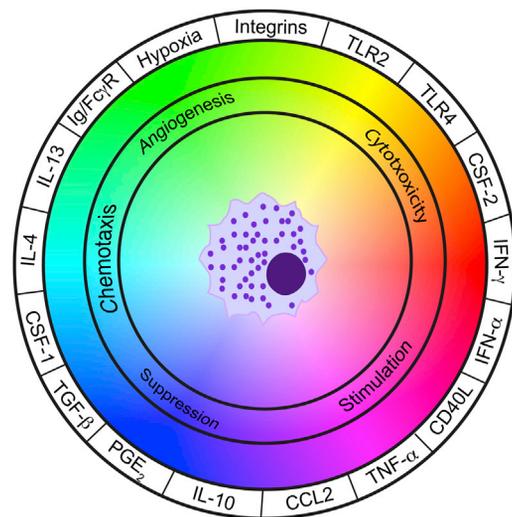
Macrophage Function and Therapeutic Resistance
Regulation of Tumor Cell Survival Pathways by Macrophages
The general concept that neoplastic cell-extrinsic factors mediate resistance to cytotoxic therapy is due to 3D cell culture models evaluating microenvironmentally derived factors (Correia and Bissell, 2012), but in vivo studies have revealed that macrophages also mediate chemotherapy resistance by providing survival factors and/or activating anti-apoptotic programs in malignant cells. Although macrophage-secreted soluble factors have usually been implicated, it is also possible that extracellular matrix deposition and/or remodeling or direct cell-cell interactions are involved (Castells et al., 2012; Correia and Bissell, 2012; Meads et al., 2009).

Using a CSF-1-neutralizing antibody in combination with chemotherapy, Paulus et al. (2006) reported increased chemosensitivity of subcutaneous MCF-7 breast cancer xenografts. Co-culture studies utilizing mammary carcinoma cell lines and bone marrow-derived macrophages revealed

macrophage-mediated resistance to paclitaxel, doxorubicin, and etoposide (Shree et al., 2011) and to gemcitabine in murine PDAC cells (Mitchem et al., 2013). At least with PDAC cells, this resistance is dependent on the activation of signal transducer and activator of transcription 3 (STAT3), implicating macrophage IL-6 or other macrophage-derived factors such as milk fat globule-epidermal growth factor VIII, found to promote resistance to carboplatin in vivo and to synergize with IL-6 to enhance tumor cell growth (Jinushi et al., 2011). STAT3 activation promotes neoplastic cell proliferation and survival, and multiple tumor cell lines exhibit IL-6- or STAT3-dependent chemoresistance in vitro (Taniguchi and Karin, 2014; Yu et al., 2014). Although autocrine production of IL-6 is common, tumor-associated macrophages produce IL-6 in vivo (DeNardo et al., 2009; Movahedi et al., 2010; Song et al., 2009), with expression induced in bone marrow-derived macrophages by co-culture with neoplastic cells (Mitchem et al., 2013). However, because the only in vivo evidence of IL-6 being chemoprotective derives from a murine lymphoma model where IL-6 is expressed by thymic endothelial cells (Gilbert and Hemann, 2010), the source and relevance of IL-6 during chemotherapy for solid tumors remains incompletely described.

Surprisingly, macrophage production of soluble chemoprotective factors is in part dependent on cathepsin protease activity, specifically cathepsin B and S, where inhibition of cathepsin activity in vivo enhances the response of mammary carcinomas to paclitaxel (Shree et al., 2011). A possible underlying mechanism may derive from the cathepsin B-dependent activation of inflammasomes in myeloid cells following treatment with gemcitabine or fluorouracil, leading to IL-1β release and enhancement of a T$_H$17 immune response (Bruchard et al., 2013). Tumor-associated macrophages isolated from ovarian cancer patients also direct IL-17 production in memory T cells through IL-1β and IL-23 production (Kryczek et al., 2009). Because IL-17 induces IL-6 expression in multiple cell types, including melanoma, mesenchymal, endothelial, and immune cells (Wang et al., 2009), cathepsin B could, therefore, be linked indirectly to STAT3 activation. However, IL-17 has also been found to direct anti-tumor responses to subcutaneously implanted tumor cell lines following treatment with anthracycline chemotherapy (Ma et al., 2011), and a role for IL-17 fails to explain macrophage chemoprotection in the absence of T cells. A more direct pathway may be through IL-1β-induced IL-6 expression, which occurs in multiple cell types, including monocytes and osteoblasts (Mori et al., 2011; Tosato and Jones, 1990).

Alternatively, because cathepsin B activity is important in the trafficking of TNF-α-containing vesicles to the surface of macrophages (Ha et al., 2008), TNF-α may be one of the critical factors mediating chemoprotection, either directly through NF-$\kappa\beta$ activation (Li and Sethi, 2010), or indirectly through induced IL-6 expression and subsequent STAT3 activation (Mori et al., 2011). Macrophages can be a critical source of TNF-α in vivo, as it has recently been demonstrated that macrophage-derived TNF-α imparts resistance to MAPK inhibitors in melanoma through NF-$\kappa\beta$-dependent expression of microphthalmia transcription factor (Smith et al., 2014). Further research is warranted to determine whether macrophages indeed mediate resistance to cytotoxic therapies via these pathways and to extend these finding to other targeted therapeutics because with the complexities of TNF-α and NF-$\kappa\beta$ in cancer development/growth, the efficacy of targeting these pathways may be context-specific (Balkwill, 2009).

Macrophages and Tumor Angiogenesis
Macrophages are well described regulators of tumor angiogenesis, with supporting evidence derived from both clinical and experimental studies (Murdoch et al., 2008; Ruffell et al., 2012a) in which much of their capability is associated with vascular endothelial growth factor (VEGF) signaling. This includes macrophage production of VEGF-A (Lin et al., 2007; Stockmann et al., 2008), production of VEGF homologs such as placental growth factor (Fischer et al., 2007; Rolny et al., 2011), enhancement of VEGF-A bioavailability through matrix metalloproteinase (MMP)-9 activity (Bergers et al., 2000; Du et al., 2008; Giraudo et al., 2004; Nakasone et al., 2012), and induction of VEGF-A production by endothelial cells via WNT7B expression (Yeo et al., 2014). VEGF-A drives the formation of abnormal vasculature in tumors, consisting of excessive branching, dead-end vessels, and vessel leakiness, that, together, impact tumor hemodynamics and drug delivery (Heldin et al., 2004; Trédan et al., 2007). VEGF antagonists induce vascular normalization (Greenberg et al., 2008; Jain, 2005), and several studies have reported increased uptake of chemotherapeutics associated with this process, likely because of reduced vessel leakiness and interstitial fluid pressure (Chauhan et al., 2012; Tong et al., 2004; Turley et al., 2012). Although macrophages are not necessarily a dominant source of VEGF-A in all tumor tissues, specific deletion of VEGF-A in macrophages via lysozyme M promoter-driven Cre recombinase revealed their role in driving abnormal vascular phenotypes in tumors (Stockmann et al., 2008). Importantly, similar to the use of VEGF antagonists, tumors in these mice were more sensitive to chemotherapy, although, unexpectedly, they also grew at a faster rate because of improved tissue perfusion and reduced hypoxia in the absence of therapeutic intervention (Stockmann et al., 2008).

Although CSF-1 neutralization enhances the response to chemotherapy in mammary carcinomas (DeNardo et al., 2011), this is not due to the increased delivery of chemotherapeutic agents, at least for small molecules such as paclitaxel and doxorubicin (Ruffell et al., 2014). Why then does macrophage depletion not phenocopy specific VEGF-A inhibition? One possible explanation is that blockade of the CSF-1/CSF-1R pathway only partially depletes macrophages in tumors, with macrophages surrounding the remaining vasculature (DeNardo et al., 2011; Pyonteck et al., 2012; Ruffell et al., 2014). This residual subset has not been analyzed in detail, but at least a portion of the remaining cells are composed of Tie2^{+} macrophages (Mitchem et al., 2013) associated with vascular programming that are important mediators of tumor angiogenesis (De Palma et al., 2005; Mazzieri et al., 2011). Although CSF-1 neutralization could functionally impair the angiogenic potential of Tie2^{+} monocytes (Forget et al., 2014), neutralizing angiopoietin-2, the ligand for Tie2, inhibits the growth of mammary carcinomas (Mazzieri et al., 2011), a phenotype not observed following therapeutic inhibition of CSF-1 or CSF-1R, and exhibits efficacy in xenograft models when used in combination with chemotherapy or VEGF antagonists (Brown et al., 2010). Interfering with Tie2^{+} macrophage recruitment via CXCR4 blockade also enhances the effects of the vasculature-disrupting agent CA-4-P (Welford

et al., 2011), and macrophage depletion further suppresses tumor growth in the context of VEGF/VEGFR inhibition (Priceman et al., 2010; Zeisberger et al., 2006). Although Tie2 is also expressed by endothelial cells and pericytes (De Palma et al., 2005), and, therefore, the results with angiopoietin-2 neutralization cannot be entirely ascribed to the role of Tie2 expression by macrophages in regulating vascular architecture (Mazzieri et al., 2011), it would be interesting to evaluate combination angiopoietin-2 and CSF-1R-blockade for synergistic efficacy.

Macrophages as Mediators of Immune Suppression

In murine tumor models, macrophages contain immunosuppressive transcriptional profiles (Biswas et al., 2006; Ojalvo et al., 2009) and, accordingly, can directly suppress CD8+ T cell proliferation in vitro (DeNardo et al., 2011; Doedens et al., 2010; Movahedi et al., 2010; Ruffell et al., 2014). Based on macrophage expression of CD163, CD204, and CD206 in human tumors, it is presumed that macrophages will exhibit similar profiles, although this has yet to be evaluated in cells isolated directly from tumors. That said, CD14+ myeloid cells from hepatocellular and ovarian carcinomas suppress autologous T cell proliferation and IFN-γ expression in vitro and nullify anti-tumor T cell activity during in vivo adoptive transfer experiments (Kryczek et al., 2006; Kuang et al., 2009).

In mouse models, T cell suppression by immature myeloid cells is typically linked to nutrient depletion via the metabolism of L-arginine or production of free radicals (Gabrilovich and Nagaraj, 2009). However, although hypoxia promotes macrophage-suppressive activity via expression of arginase-1 (Doedens et al., 2010) and thyioglycollate-induced peritoneal macrophages suppress T cell proliferation through L-arginine depletion (Rodriguez et al., 2003), inhibition of arginase activity does not blunt in vitro suppressive functions of tumor macrophages (Movahedi et al., 2010). This seems to be the case even for MHCII^LO macrophages associated with hypoxic areas of tumors. To date, only inhibition of inducible nitric oxide synthase (NOS) has been reported to reduce suppression by tumor macrophages isolated from subcutaneously implanted lung carcinomas (Movahedi et al., 2010). Whether macrophages directly suppress T cell activity in vivo remains compelling, albeit speculative, but one role may simply be to overwhelm T cells with non-productive interactions (Broz et al., 2014).

In humans, there is no evidence for a role of nutrient depletion in mediating immune suppression by macrophages because macrophages conditioned by ovarian carcinoma ascites suppress T cell proliferation independent of arginase and NOS activity (Kryczek et al., 2006). Instead, macrophages directly suppress T cell responses through programmed death ligand 1 (PD-L1) in hepatocellular carcinoma (Kuang et al., 2009) and B7-H4 in ovarian carcinoma (Kryczek et al., 2006). This is perhaps fortuitous because immune checkpoint blockade is therapeutically more attractive (Pardoll, 2012), with monoclonal antibodies against PD-1, PD-L1, and PD-L2 all in clinical trials. Notably, the response rates in the PD-1/PD-L1 trials relate, at least partially, to PD-L1 expression in tumor stroma (Herbst et al., 2014; Tumeh et al., 2014), consistent with a role for macrophages and/or other stromal cells in blocking anti-tumor T cell responses. Could macrophage targeting enhance checkpoint blockade therapy? At least one study to date has reported this using an orthotopic implant model of PDAC, with CSF-1R in-

hibition providing additive efficacy to either PD-1 or cytotoxic T lymphocyte-associated protein 4 (CTLA-4) blockade in combination with gemcitabine (Zhu et al., 2014). Importantly, CSF-1R inhibition also enhanced the response to combined PD-1/CTLA-4 blockade in the absence of chemotherapy (Zhu et al., 2014). Therefore, it would not be surprising for CSF-1R antagonists to be combined with checkpoint blockade antibodies in future clinical trials (e.g., clinical trial NCT02323191).

Rather than directly suppressing anti-tumor T cell responses, macrophages may also regulate the immune microenvironment so that T cell responses are controlled indirectly through an intermediate cell type, as first suggested in human ovarian carcinoma with regulatory T (T_Reg) cell recruitment via CCL22 (Curiel et al., 2004). In vitro, T_Reg cells induce IL-6 and IL-10 expression by macrophages, leading to autocrine upregulation of B7-H4 and a suppressive phenotype (Kryczek et al., 2006, 2007). Macrophages are also a key source of IL-10 in murine mammary carcinomas, but, in this system, macrophages do not express detectable levels of B7-H4 (B.R. and L.M.C., unpublished data), and IL-10 was not a significant mediator of macrophage polarization or suppressive function (Ruffell et al., 2014). Instead, in mammary carcinomas exposed to paclitaxel, macrophage IL-10 suppresses the capacity of dendritic cells to express IL-12, thereby blocking productive cytotoxic CD8+ T cell responses (Ruffell et al., 2014). An increased understanding of interactions between macrophages and other immune cells in tumor microenvironments and deconstruction of the molecular pathways underlying these interactions will undoubtedly provide additional therapeutic targets to fine-tune an immune response during therapy.

Macrophages and Metastasis

From local invasion, intravasation into vessels, and extravasation at peripheral sites, macrophages (or their monocyte precursors) have been implicated as regulators of all stages of the metastatic process, often through positive feedback pathways involving CCL2 and/or CSF-1 (Joyce and Pollard, 2009). Studies with human tissues have also demonstrated a relationship between epithelial-mesenchymal transition and macrophage expression of CCL18, for which there is no murine homolog (Meng et al., 2015; Su et al., 2014). Preclinical mouse models of tumor development (mammary, pancreas, glioblastoma, etc.) in which macrophages have either been depleted (albeit not completely) or reprogrammed exhibit a diminished metastatic burden in end-stage mice (DeNardo et al., 2009, 2011; Gocheva et al., 2010; Lin et al., 2001; Qian et al., 2009; Rolny et al., 2011; Shree et al., 2011; Welm et al., 2007; Zabuawala et al., 2010). Mechanistically, however, direct evidence for a pro-metastatic role is largely derived from directed migration of neoplastic cells in response to molecules secreted by macrophages (DeNardo et al., 2009; Mizutani et al., 2009; Qian et al., 2009). The combined impact of these studies has been interpreted to indicate that therapies targeting macrophage presence and/or polarization would ameliorate metastasis in late-stage cancer patients. The fact that malignant cells likely already reside in secondary metastatic niches long before the clinical presentation of malignant primary disease (Valastyan and Weinberg, 2011) mandates that this therapeutic approach be evaluated carefully. A recent evaluation of preclinical mammary carcinoma metastasis models with CCL2-neutralizing antibodies revealed
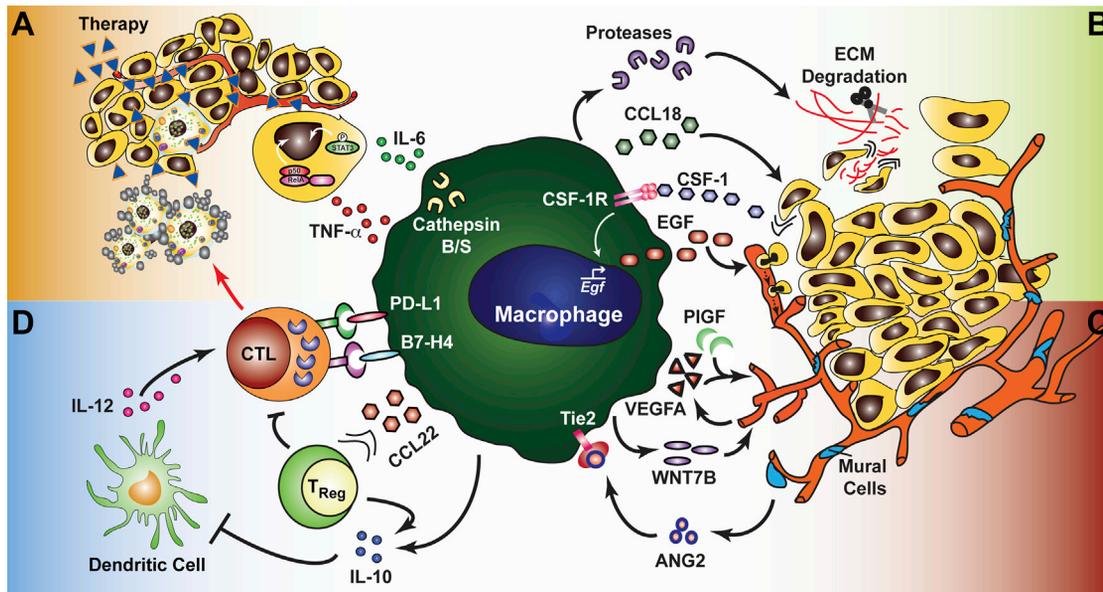
Figure 3. Macrophage Function in the Tumor Microenvironment
(A) Macrophage expression of IL-6 and TNF-α promotes survival signaling in neoplastic cells and resistance to chemotherapy and targeted agents. The expression of survival factors is dependent upon the protease activity of cathepsin B and/or S.
(B) Neoplastic cell invasion of ectopic tissue can be promoted through the directed release of cytokines/chemokines such as epidermal growth factor (EGF) and CCL18 or through protease-dependent extracellular matrix (ECM) remodeling that may directly affect neoplastic migration or increase chemoattractant bioavailability. EGF expression is driven by signaling through the CSF-1R via neoplastic cell production of CSF-1 as well as T cell-derived IL-4 (not shown).
(C) Macrophages directly promote angiogenesis via production of VEGFA and other angiogenic factors and can enhance VEGFA expression by endothelial cells through WNT7B. A subset of macrophages expressing the Tie2 receptor is recruited to the vasculature by mural cell/pericyte expression of ANG2 and is important in regulating vascular structure.
(D) Direct suppression of a cytotoxic T cell (CTL) response can occur via expression of B7 family ligands (PD-L1, B7-H4). Indirect suppression may occur through release of IL-10 or recruitment of IL-10-expressing T_{Regs} via CCL22, whereby IL-10 suppresses the capacity of dendritic cells to produce IL-12 and promote a T_H1/CTL anti-tumor immune response.

an enhanced metastatic burden. Experimental neutralization of CCL2, although limiting early metastatic processes, promoted metastasis following the cessation of therapy by enhancing the recruitment of monocytes to micrometastatic lesions (Bonapace et al., 2014). Therapies targeting the stromal compartment of primary tumors may also prove ineffective at treating metastasis if the pathways regulating the targeted process differ between the tissue of origin and the metastatic site. As a possible example of this, IL-34 mediates the development of Langerhans cells and microglia through CSF-1R (Wang et al., 2012), whereas CSF-1R signaling is mediated in most tissues by CSF-1 (Pollard, 2009). The development of macrophage-directed therapeutics aiming to minimize or eradicate metastasis will, therefore, first require the identification of pathways that drive neoplastic cell survival, proliferation, angiogenesis, and immune suppression in ectopic sites. Some progress has been made in the lung, where both VEGF-A and angiopoietin-2 appear to be important for angiogenesis in metastatic tumors derived from mammary carcinomas (Bonapace et al., 2014; Mazzieri et al., 2011). However, as mentioned, this may not be directly linked to macrophage function. Interestingly, CSF-1R inhibition reverses the effects observed following cessation of CCL2 neutralization (Bonapace et al., 2014) even though CSF-1R inhibition does not alter the number of macrophages in lungs (Strachan et al., 2013). This could hint at a possible role for CSF-1R signaling in mediating macrophage polarization in metastatic lungs, similar to the observations in glioblastoma multiforme (Pyonteck et al., 2013).

Nevertheless, it remains uncertain whether macrophages are important in mediating therapeutic resistance at metastatic sites or even the degree to which they are involved in mediating metastatic outgrowth. Because the majority of patients succumb to metastatic disease, this is an urgent area of research that has been largely unexplored, in part because of experimental obstacles such maintaining mice with spontaneously metastasizing tumors where primary tumor burden limits the duration of study.

Macrophages as Therapeutic Targets
Based on compelling preclinical data from numerous laboratories indicating that macrophage presence and/or activity are malleable in vivo (Figure 3), clinical studies are now ongoing in several solid tumor types where macrophages are being targeted via CSF-1R inhibitors or by blocking monoclonal antibodies (Table 1). Although a goal of these clinical studies is to reduce the presence of tumor-associated macrophages, based on preclinical and clinical studies, we anticipate that not all macrophages will be eradicated. The hope is, however, that those remaining will be reprogrammed toward an anti-tumor phenotypic state where they would support T cell responses and, together with cytotoxic therapy, limit ongoing tumor growth. CSF-1R antagonists appear well tolerated as single agents in both pre-clinical and clinical studies (Radi et al., 2011; Ries et al., 2014; Ruffell et al., 2014), but because significant macrophage depletion in the colon and liver is observed in nonhuman primates, toxicity

is a significant concern for combinatorial studies moving forward. It should also be noted that although increased CSF-1 serum concentrations resulting from the use of CSF-1R antagonists (Ries et al., 2014) are an excellent biomarker to evaluate on-target efficacy, recent findings with CCL2 inhibition (Bonapace et al., 2014) indicate that recurrence or exacerbation of disease is a possibility after therapy cessation. These potential issues will need to be incorporated into the design of clinical studies for appropriate drug combinations and patient monitoring.

Blocking macrophage recruitment into tumors (DeNardo et al., 2011; Shiao et al., 2015), pro-tumor polarization (Affara et al., 2014; Pyonteck et al., 2013; Shiao et al., 2015), effector function (Ruffell et al., 2014; Shree et al., 2011), or directly promoting macrophage activation (Beatty et al., 2011) have all been used successfully in preclinical models to enhance the response to cytotoxic therapy. The question remains which of these approaches (Table 1) will be most efficacious when combined with cytotoxic, targeted, or immune checkpoint blockade therapy. At least in a murine model of squamous cell carcinogenesis, repolarizing macrophages was more effective than blocking recruitment, and, in fact, repolarized macrophages were necessary for recruitment of CD8$^+$ T cells via CCR5 during paclitaxel chemotherapy (Affara et al., 2014). For this reason, going forward, it will be critical to understand whether depletion, or instead repolarization, is the best therapeutic approach to accompany combination therapy, for which tumor types, and at which stage of tumor progression (primary or metastatic disease). Multiple agents targeting T$_H$2 cytokines and their receptors have gone beyond phase II clinical trials, with demonstrated efficacy in autoimmune disorders and acceptable safety profiles (Beck et al., 2014; Corren et al., 2011; Danese et al., 2015). Although none of these compounds have been re-directed toward therapy in solid tumors, we have found recently that targeting this pathway (IL-4, IL-13, IL-4Rα) improves the response to cytotoxic therapy (Shiao et al., 2015).

One lesson learned from results comparing efficacy of immunotherapy in mice bearing orthotopic versus subcutaneously implanted tumors is that the latter exhibit enhanced sensitivity (Devaud et al., 2014), indicating that context matters. In light of these findings and given the dearth of late-stage metastatic studies, it will be important to be mindful of the fact that a one size fits all approach, although attractive, may not be realistic as we strive to translate preclinical findings to the clinic. A major question to address regarding these preclinical and clinical studies with either macrophage depletion or reprogramming approaches will be to evaluate the durability of resultant anti-tumor immune responses formed against the tumor. Along these lines, evaluating patients longitudinally for indicators of T cell function or T cell receptor diversity could reveal an important diagnostic opportunity to indicate when the addition of combination therapy, e.g., immune checkpoint or anti-tumor vaccine, might be beneficial to the patient.

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