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Differential Macrophage Programming in the Tumor Microenvironment

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Abstract

Of the multiple unique stromal cell types common to solid tumors, tumor-associated macrophages (TAMs) have been recognized as significant for fostering tumor progression. The protumor properties of TAMs are derived from their ability to regulate angiogenic programming, provide soluble mediators to malignant cells for proliferation, survival and invasion, and for directly and indirectly suppressing activity of cytotoxic T cells. These varied activities are dependent on the polarization state of TAMs that is regulated in part by local concentrations of cytokines and chemokines, as well as varied interactions of TAMs with normal and degraded components of the extracellular matrix. Targeting molecular pathways regulating TAM polarization holds great promise for anti-cancer therapy.

Macrophages in solid malignancies

Macrophages are important residents of all tissues, where they play critical roles in regulating tissue homeostasis. In this context, tissue-resident macrophages assist with combating infection [1, 2], resolving acute inflammation [3], and in regulating metabolic response to tissue stress [4, 5]. This broad range of functions, and the accompanying plasticity required to permit such adaptive responses, also implicates macrophages in a number of chronic pathological conditions including diabetes and atherosclerosis [5–7]. Solid tumors represent an extreme example of a dysregulated tissue, and multiple characteristics of tumors, including hypoxia [8] and abundant cell death [9], help direct macrophage function towards attempting homeostatic restoration. In the context of a tumor however, this represents a maladaptive response that instead helps drive tumor growth through promotion of angiogenic programs, tissue remodeling, ectopic survival of malignant cells, and development of immunosuppressive microenvironments that blunt cytotoxic T cell activities [10]. More recently, it has been demonstrated that polarization of macrophages towards tumor promoting phenotypes, is not exclusively the result of thwarted tissue homeostasis, but instead a more active process driven by what are likely reciprocal interactions with both malignant and stromal cells in the local microenvironment [10, 11].

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Thus in addition to discussing well accepted functions for tumor-associated macrophages (TAMs; Box 1), this review will also focus on recently recognized molecular and cellular mechanisms underlying TAM polarization within tumor microenvironments, and the therapeutic implications of these findings.

TAM Function

With the exception of non-small cell lung carcinoma [12, 13], patient prognosis in solid tumors is generally described as correlating inversely with TAM density and TAM expression signatures [10, 14]. TAMs have also been related to particular functional roles in human tumors, with an established association between TAM presence and density of tumor vasculature in several carcinomas [15–18] including breast [19–24], as well as increased local invasion and/or metastasis in melanoma [25], breast [26, 27], ovarian [15, 28], colorectal [29, 30], pancreatic neuroendocrine [31] and bladder cancer [32]. Less established in humans is a role for TAMs in local immune suppression, although there have been several reports in ovarian cancer [33–35]. A potential caveat of these studies is that most rely on immunohistochemical detection of CD68 for measurement of TAM density. However, CD68 is not a specific marker of macrophages and is expressed by other stromal populations (Box 1), including those that may have overlapping function with TAMs (Box 2).

Angiogenesis

Experimental studies using murine carcinoma models have clearly demonstrated that TAMs regulate vascular programming of tumors. Important for this activity is TAM production of vascular endothelial growth factor A (VEGFA), because overexpression of VEGFA partially reverses the effects of macrophage-depletion [36], and macrophage-specific loss of VEGFA results in vascular normalization [37]. In some tumor models, production of matrix metalloproteinase (MMP)-9 by TAMs mediates VEGFA bioavailability, thus providing an alternative, but still VEGF-dependent route for promoting angiogenesis [38, 39]. Similarly, the TAM production of placental growth factor (PlGF), a homologue of VEGFA that selectively binds VEGF receptor 1 (VEGFR1), also stimulates angiogenesis in tumors [40] and may in part explain resistance to VEGFA and VEGFR targeted therapies in the clinic [41–43]. Multiple other factors produced by macrophages are also known to regulate vascular programming [44–46], but their roles in tumorigenesis are not firmly established and therefore will not be discussed herein.

Recently a novel subset of macrophages expressing Tie2, a receptor for angiopoietins (ANG) was described [47]. Tie2⁺ TAMs tend to be closely associated with tumor vasculature, and have been found crucial for angiogenesis in orthotopic [47] and transgenic tumor models [48]. This activity depends in part on endothelial cell-produced ANG2 and the Tie2 receptor that direct localization of Tie-2-expressing cells along the vasculature [48]. Notably, antagonists that block ANG2 restrict tumor growth in mouse models of de novo mammary carcinogenesis, i.e., MMTV-PyMT mice [49], a property that has thus far not been observed in tumors following blockade of TAM infiltration [50], or by myeloid-specific *Vegfa* ablation [37], thus hinting at a unique mode of action either downstream or parallel to VEGFA bioactivity [48].

Invasion and metastasis

The most comprehensively described mechanism through which TAMs promote solid tumor development is by providing factors that enhance invasion of malignant cells into ectopic tissue. This activity centers around a paracrine interaction loop involving macrophage-expressed epidermal growth factor (EGF) and epithelial cell-expressed colony-stimulating factor (CSF)1, also known as macrophage CSF [51]. Increased expression of CSF1 is a

significant mechanism underlying macrophage recruitment into tissues [50, 52–54] after CSF1 binding to its high affinity receptor (CSF1R) expressed on resident macrophages and some macrophage precursors. This interaction promotes macrophage proliferation, survival, and tissue recruitment during development (e.g., branching morphogenesis in the mammary gland) [55], homeostasis [56], and pathological tissue remodeling processes such as those associated with acute tissue injury [53,57–59] and during solid tumor development [50]. Chemokines such as CCL2 and stromal-derived factor-1 (SDF-1; CXCL12) are also important for TAM recruitment with demonstrated roles in models of glioblastoma, melanoma, cervical and prostate cancer [38, 39, 60–65]. Crucially, in addition to supporting TAM recruitment, activation of intracellular signaling pathways downstream of the CSF1-CSF1R interaction significantly enhance EGF expression, which in turn regulates epithelial cell migration in some tissues [51, 66–68].

Macrophages also regulate composition and structure of extracellular matrix (ECM) through their deposition of ECM components, e.g. various types of collagens, and breakdown of these same components via their release of MMPs, serine proteases and cathepsins [69–71]. Migration on and through ECM is a necessary aspect of cell migration [72], and thus by extension, TAMs are thought to regulate tumor and stromal cell migration/invasion through ECM. This activity has been directly demonstrated *in vitro* [73, 74] and *in vivo* with mice genetically deficient for cathepsin B, cathepsin S and urokinase/plasminogen activator (uPA), all of which derive primarily from TAMs in the tumor microenvironment [75–77]. MMP-dependent cleavage of the ECM [69], collagen deposition [78], and alteration of collagen structure [79] are also potential sources of regulation mediated by TAMs, but formal demonstration of these is lacking.

Immune suppression

Solid tumors are well recognized for repressing the activity of cytotoxic T cells, and are thus characterized grossly as immunosuppressive. While regulatory T (T_{reg}) cells have garnered much attention owing to this capability, recent literature also recognizes macrophages as major determinants of immune suppression in solid tumors. Although the mechanisms underlying these activities are less characterized as compared to myeloid-derived suppressor cells for example [80], TAMs do typically express several genes with immunosuppressive potential [68, 81, 82], and are capable of directly limiting T cell proliferation during classic *in vitro* suppressive assays [8, 34, 50, 83]. TAMs also act through intermediates to regulate immune suppression, as has been reported for recruitment of regulatory T cells by CCL22 [33], and may be important mediators of T cell recruitment based upon the inverse correlation between the presence of $CD68^+$ TAMs and $CD8^+$ T cells in human breast cancer and the enhanced $CD8^+$ T cell infiltration observed during chemotherapy in the MMTV-PyMT model following blockade of the CSF1-CSF1R pathway [50].

In murine tumor models, suppression of $CD8^+$ T cell proliferation by TAMs is at least partly dependent on metabolism of L-arginine via Arginase-1 or inducible nitric oxide synthase (iNOS) [8, 83], and resulting production of oxygen radicals or nitrogen species [84, 85]. In contrast, suppression of $CD8^+$ T cells by human TAMs can occur independent of L-arginine metabolism [34], and instead may rely on the B7 family of molecules as has been described for B7-H1 (PD-L1) in hepatocellular carcinoma [86], and B7-H4 in ovarian cancer [34]. This distinction may be a result of differences between human and murine macrophages, as expression of *arginase-1* by the former does not correlate with macrophage polarization. Alternatively, the tissue specific microenvironment could dictate the mechanism of TAM suppression. As an example of this, it was described that ovarian TAMs are exposed to minimal levels of IL-4, which in other circumstances may down-regulate B7-H4 expression [34]. TAMs from murine mammary and human breast carcinomas on the other hand are

exposed to significantly higher amounts of IL-4/13 [68, 87], and thus one would anticipate minimal expression of B7-H4 in TAMs from these tissues.

Mechanisms of TAM Polarization

The description of macrophage activation as either classical (M1; IFN γ /LPS-dependent) or alternative (M2; IL-4/IL-13/IL-10/Fc γ R-dependent) has provided a necessary framework for the understanding of TAM polarization [88]. However, even though the M1/M2 designations represent extreme ends of a scale, the concept is an oversimplification of the diversity of TAM phenotypes evident simply based on their localization within tumors (Figure 1). TAMs do not become polarized by virtue of their location per se, but instead receive signals from the particular microenvironment they reside (Figure 2). These heterotypic signals may overlap, for example, IL-4 promotes both *Egf* and *Arginase-1* expression by TAMs [68], whereas other signals may be unique to particular microenvironments, such as hypoxic zones within solid tumors that also induce *Arginase-1* expression [8], but which are not found along vasculature where invasive macrophages are localized. From these two examples, all murine TAMs would be anticipated to express higher levels of arginase-1 than normal tissue macrophages, but the suppressive capabilities of intratumoral TAMs in hypoxic regions would be even further enhanced. Thus, in addition to differentiation of TAMs, from either Tie2⁺ [89] or other monocyte precursors [83] that may preferentially home to specific localizations, one can envision that integration of these distinct signals result in production of an array of TAM populations/phenotypes with unique tumor-regulating properties [10]. It is important to note that the composition of the immune microenvironment [68, 90, 91] and the overall polarization state of TAMs becomes more favorable towards growth during tumor progression [92]. The functional role of macrophages during tumor initiation [93, 94] therefore differs from that during tumor progression.

Immune Microenvironment

Consistent with the original description of alternative activation, the type 2 cytokine IL-4 [68, 77], and immunoglobulin (Ig) signaling through activating-types of Fc γ receptors [91], exert significant regulation on TAMs in their ability to direct TAM pro-tumor phenotype. IL-13 derived from malignant epithelial cells or TH2-polarized CD4⁺ T cells [87], or CD1d-restricted natural killer T (NKT) cells [95], may have similar effects on TAM polarization due to overlapping IL-13 and IL-4 signaling cascades that lead to STAT6 activation, but this has yet to be proven *in vivo*. Likewise, activation of STAT3 by IL-10 suppresses IL-12 [96] and tumor necrosis factor- α (TNF- α) expression [97] by TAMs, but the source of IL-10 and the relevance of this pathway to tumor development is unclear. Intriguingly, while IL-4 from TH2-polarized CD4⁺ T cells is necessary for TAM programming in the MMTV-PyMT mammary carcinoma model for EGF expression and promotion of metastasis, the absence of Igs in B cell-deficient mice does not affect tumorigenesis [68]. Conversely, autoantibodies in the K14-HPV16 mouse model of squamous carcinogenesis drive TAM-dependent angiogenesis by FcR γ -dependent mechanisms [91], whereas CD4⁺ T cell-deficient animals have only mildly reduced tumor incidence [98]. These paradoxical findings likely indicate tissue-specific dependencies in each respective tumor model, and argue for detailed analysis of counterpart human tissue as a precursor to clinical translation because human tumors could vary considerably in leukocyte composition.

Such human/mouse differences have been described in breast/mammary cancer. Notably, leukocyte composition in murine mammary carcinomas are dominated by TAMs, while lymphocytic infiltrate, predominately CD4⁺ T cells, comprise the majority of immune cells in human breast carcinomas [68, 99]. CD4⁺ T cells from human breast cancers express high levels of interferon (IFN) γ resulting in protein concentrations over 10-fold higher than IL-4

or IL-13 in human tumors [87]. This suggests divergent cytokine milieu between human and mouse tissue, as tumor CD4⁺ T cells from MMTV-PyMT-derived mammary tumors exhibit high expression of IL-4, IL-13 and IL-10, but produce minimal IFN γ [68]. Given the strong polarizing effects of these cytokines, and the potential for synergistic effects on macrophage phenotype [100], transcriptional profiling of purified human TAMs should prove informative in relating functional data in mouse models to correlative studies in patients.

Tumor derived

In addition to its potential production by multiple leukocyte subsets, IL-10 is secreted *in vitro* by many human carcinoma cell lines [101], which in some instances actually reflects its origin *in vivo* [102]. Well established as a broad immunosuppressive molecule, *in vitro* administration of IL-10 to macrophages inhibits production of proinflammatory cytokines and chemokines [103], and reduces surface expression of major histocompatibility complex (MHC)II, and the costimulatory molecules CD80 and CD86 [104]. IL-10 also synergizes with IL-4 to induce *Arginase-1* expression in macrophages, possibly through induction of IL-4R α [103]. As mentioned however, the source of IL-10 in tumor microenvironments is unclear, and may even derive from TAMs themselves [96, 97, 105]. Interestingly, the ability of TAMs to produce IL-10 has been associated with another molecule produced by tumor cells, prostaglandin E2 (PGE2), suggesting that this may also regulate TAM polarization [97, 106] through EP2 and EP4 receptors [107]. The importance of PGE2 in cancer is inferred from the preventative effects of cyclooxygenase-2 (COX-2) inhibitors including aspirin in colon [108, 109] and respective transgenic mouse models [108], as well as a less prominent effect in multiple other cancers [110]. In the APC^{Min/+} mouse model of intestinal tumorigenesis, COX-2 inhibition reduces expression of *Arginase-1* and increases that of *CXCL1* [111], both changes that are also associated with repolarization of TAMs. Whether this repolarization is important for therapeutic efficacy of COX-2 inhibitors is unknown however, as PGE2 has pleiotropic effects on multiple aspects of tumor development [108].

Homeostatic imbalance

Hypoxic conditions exist within solid tumors in areas distal from functional vasculature (Figure 1). Although nutrient deprivation is the goal of anti-angiogenic therapy, the resulting hypoxia actually seems to promote malignant conversion and metastasis [112]. This response to oxygen availability is mediated primarily through hypoxia-inducible factor (HIF)-1 α and HIF-2 α , both of which also regulate macrophage function [113]. Using LysM-cre mice to induce myeloid-specific loss of either HIF-1 α or HIF-2 α , two recent reports have established that the pro-tumor functions of TAMs are likewise dependent on HIFs [8, 114]. Loss of HIF-1 α limited *arginase-1* expression and the suppressive capabilities of TAMs in the MMTV-PyMT model [8], while loss of HIF-2 α reduced TAM recruitment through lower chemokine receptor expression in models of inflammatory hepatocellular and colon carcinoma [114]. Despite the divergent mechanisms implicated in each study, both observed reduced tumor volume and progression, suggesting either tissue specific roles for TAMs and/or involvement of overlapping pathways regulated by HIF-1 α or HIF-2 α . This includes the possible induction of an angiogenic response that was not thoroughly evaluated in either study, but which has been associated with TAMs localized to hypoxic regions of tumors [83, 115]

The mislocalization of cellular and extracellular components is another prominent feature of tumors due to cell death and dysregulated tissue architecture, respectively. The presence of extracellular ATP, high-mobility group box 1 protein (HMGB1), and other normally intracellular molecules is detected by a class of receptors on the surface of macrophages called Toll-like receptors (TLR) [6]. Although TLR signaling in dendritic cells is important

for generating an adaptive immune response following cytotoxic therapies through TLR4 recognition of HMGB1 [116], both TLR4 and TLR2 signaling promote growth of cell lines in the lung through induced TNF α production by macrophages [117, 118]. For TLR2, this can be mediated by tumor-derived versican [118], but other ECM components including biglycan and hyaluronan also induce proinflammatory cytokine expression by macrophages via TLR2 and TLR4 [119], and are potentially important in dictating TAM polarization. Crucially, these ECM components do not bind TLRs in their native form in non-inflamed tissue, but become TLR ligands following degradation by protease cleavage or interaction with reactive oxygen or nitrogen species, thereby forming putative sensory pathways for detection of inflammation and tissue disruption.

Conclusion: Targets for therapy

The pathways that engage and mediate the maladaptive response of TAMs present attractive therapeutic targets, several of which have already shown promise in the preclinical arena, and to improve therapeutic responses to chemotherapy [37, 50, 120]. Therapeutic strategies directed at TAMs can be grouped crudely into four prospective themes: blocking effector function, limiting recruitment, reprogramming, or preventing pro-tumor polarization. Monoclonal antibodies and small molecular inhibitors targeting the VEGF and EGF pathways are already approved for treatment of various carcinomas alone or in combination, although none were designed specifically to target TAM function and their clinical efficacy has been mixed [121, 122]. It has recently been established that blocking TAM recruitment and/or survival in solids tumors (in murine models) improves efficacy of cytotoxic therapies [50, 123], in a manner dependent upon CD8⁺ T cells [50]. Though monoclonal antibodies against CD11b have been unsuccessful as single agents for the treatment of inflammatory disorders, antagonists targeting the CSF1-CSF1R pathway in breast cancer and the CCL2-CCR2 axis in prostate cancer are now in early phase clinical trials.

We anticipate emerging therapeutics to focus on repolarization as a method to invoke the anti-tumor potential of TAMs, as has been reported in pancreatic ductal adenocarcinoma for agonist antibodies against the costimulatory molecule CD40 [120] and the use of Tie2⁺ monocytes to delivery IFN α to tumors [124]. Additional approaches include synthetic TLR ligands such as CpG [125] or imiquimod [126], although reports that TLR signaling and NF- κ B activation in TAMs promotes tumor growth [117, 118, 127] suggest that TLR ligands must be used in a multi-targeted approach [125]. Based upon our findings in mouse models of mammary and squamous carcinogenesis, we are currently evaluating whether blocking pro-tumor polarization of TAMs, as opposed to direct repolarization, is similarly efficacious in pre-clinical models in combination with chemotherapy. Although this may seem like a distinction without a difference, we hypothesize that this approach may be less prone to refractory responses and adverse autoimmune side effects.

Box 1: Identity crisis

As their name implies, tumor-associated macrophages (TAMs) are found within or proximal to primary tumors, and represent a mature population of terminally differentiated myeloid-lineage cells [55]. This location distinguishes them from metastasis-associated macrophages [128], and they are phenotypically distinct from the heterogeneous population of immature myeloid cells that predominantly accumulate in the periphery of tumor-bearing individuals, and are associated with immune suppression [80]. Identifying TAMs can be difficult however, as there are no lineage-defining markers for macrophages [128], and marker expression can vary by activation status and tissue localization [129]. In general, both human and mouse TAMs can be identified via flow cytometry through high surface expression of CD11b, CD14, and MHCII/HLA-DR,

in addition to the common leukocyte antigen CD45. High expression of MHCII differentiates TAMs from immature myeloid cells, as does low expression of Ly6C in mice and CD34 in humans [128]. Murine TAMs are also commonly identified by expression of F4/80, an EGF-transmembrane 7 family molecule of unknown function. However, not all macrophage populations express F4/80, and it has been observed on Langerhans cells in the skin and on eosinophils in adipose tissue. Dendritic cells also express MHCII, and subsets express CD11b and CD14, while the most commonly used marker for dendritic cells, CD11c, is expressed constitutively by certain tissue macrophages and induced by inflammatory conditions such as those found in the tumor microenvironment. The problem of accurately identifying TAMs is more acute in humans as studies rely almost exclusively on single marker detection of CD68 via immunohistochemistry. In addition to other leukocyte populations, CD68 is expressed by fibroblasts, and at least for breast cancer is not a specific marker for TAMs [99]. Thus while human studies will be referenced here, the functions ascribed to TAMs based on correlations between TAM density and clinical parameters require validation in some tissues.

Box 2: Tumor commune

Although TAMs may constitute the majority of immune cells in some tumors, additional myeloid and mesenchymal-derived cells pervade tumors and are known to functionally overlap with TAMs. This includes granulocytic mast cells, neutrophils and immature myeloid cells that can be angiogenic, immune suppressive, and promote metastasis through pathways akin to those employed by TAMs [128]. Cancer-associated fibroblasts have also been shown to promote vascularization through SDF-1 [130] and IL-1 β [131], while mesenchymal stem cells promote metastasis through CCL5 [132] and are potentially immunosuppressive [133]. Monocyte-derived cells with a fibroblast morphology, or fibrocytes, are increased during chronic inflammation, and based upon their dual macrophage/fibroblast phenotype [70] are likely an emerging cell population involved in tumorigenesis.

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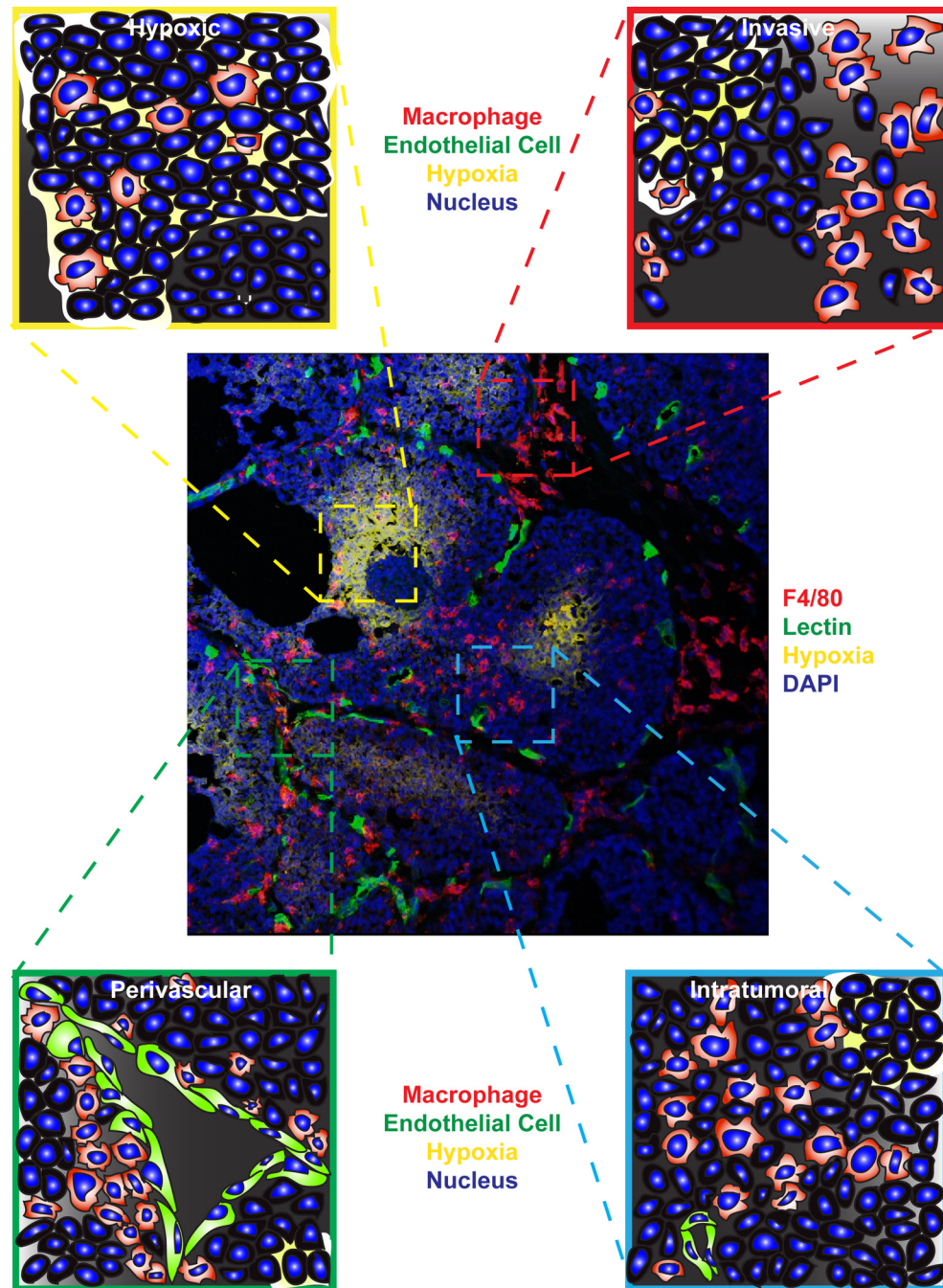


Figure 1.

TAM localization within unique tumor microenvironments. Centrally displayed is an immunofluorescent confocal micrograph of F4/80⁺ macrophages (red) within a late-stage tumor from the MMTV-PyMT mouse model of mammary carcinogenesis. Also shown are areas of hypoxia detected with pimonidazole (yellow), functional vasculature as revealed by perfusion with tomato lectin (green) and DAPI nuclear stain (blue). Clockwise from top left insets display enlarged graphical representations of TAMs within a hypoxic region, at a locally invasive edge, in a normoxic area within the tumor, and associated with the vasculature.

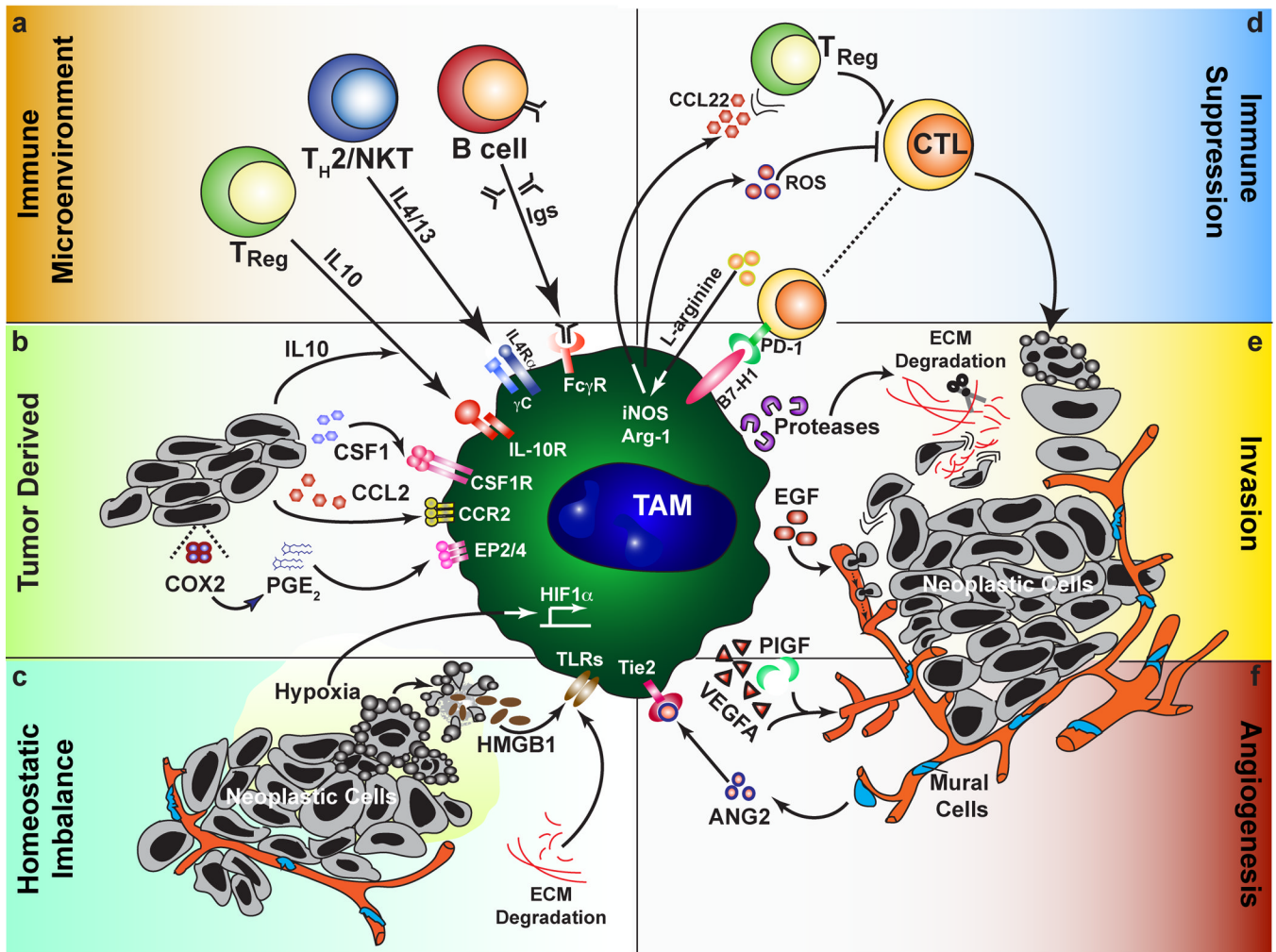


Figure 2.

TAMs as central regulators of the tumor microenvironment. Factors that promote the polarization of TAMs towards a pro-tumor phenotype (a–c) can be subdivided into those derived from the immune system, actively produced by tumor cells, or resulting from tissue stress. (a) From leukocytes, this includes cytokines and other soluble factors such as immune complexes. (b) Neoplastic cells can produce chemokines that recruit macrophages, including CSF1 and CCL2 depending on the tissue involved, as well as directly producing immunosuppressive molecules such as IL-10 and PGE₂. (c) Signs of dysregulated tissues include leaky vasculature, hypoxia, ECM remodeling and cell death. These signals all direct the pro-tumor functions of TAMs (d–f) including immune suppression, tumor cell dissemination, and promoting angiogenesis. (d) Immune suppression can occur through soluble or cell surface mediators, and may be indirect such as through the recruitment of regulatory T cells. (e) Neoplastic cell invasion of ectopic tissue can be promoted through directed release of cytokines such as EGF, or through protease-dependent ECM remodeling that may directly affect neoplastic migration or increase chemoattractant bioavailability. (f) In addition to the interplay of TAMs with endothelial cells through production of VEGFA and other angiogenic factors, subsets of TAMs expressing the Tie2 receptor interact with mural cells/pericytes to regulate vascular structure.