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Tumor Macrophage Redox and Effector Mechanisms Associated with Hypoxia

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Abstract

Monocytes are recruited from the circulation into solid tumors where they differentiate into macrophages with unique phenotypes. While macrophages utilize oxygen in a broad range of immune effector functions, the generation of reactive oxygen and nitrogen oxide species is less clear in the setting of hypoxia, which can be a prominent feature of solid tumors. The relationships between innate immunity, redox systems and the plasticity of phenotypic changes tumor-associated macrophages undergo in conjunction with tumor hypoxia will be examined.

Keywords

Tumor associated macrophage; Innate; iNOS; Hypoxia; Nitric oxide; Oxygen; NOX-2; antimicrobial peptide; M2

Introduction

A hallmark of most solid tumors is the accumulation of macrophages, which are often the most abundant of infiltrating leukocytes [1-5]. Tumor-associated macrophages (TAM) are bone marrow-derived leukocytes solicited and directed by cancer cells to adopt unique phenotypes that can facilitate tumor growth and survival. While the formation of innate redox effectors is thought to contribute to genetic instability and carcinogenic transformation of pre-neoplasia, less well characterized is the role redox species play in the maintenance and progression of tumors once they are established. Because oxygen is inherently tied to the generation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitrogen oxide species, the participation of redox effectors in cancer is further complicated by the situation of hypoxia that develops regionally in most types of solid tumors. In this review, we will highlight the role oxygen tension can play in the redox biology and heterogeneity of TAM.

Heterogeneity of TAM immuno-phenotypes

Immunologists often use the so-called M1 to M2 classification scheme to phenotypically subdivide macrophages along a spectrum with cells displaying distinct patterns of function in association with lymphocytic T-helper1 versus T-helper2 driven responses. The TAM phenotype has been described as skewed along the M2 axis [6-11]. Prototypic M2-polarizing agents are interleukin (IL)-4, IL-10, IL-13, TGF-β1, steroids and prostaglandin-E2; each agent capable of rendering distinct phenotypic changes. M2-oriented macrophages generally express high levels of galactose, mannose, and scavenger-type A receptors and have IL-12^{low}, IL-10^{high} or IL4/13^{high} cytokine phenotypes. In addition to solid tumors, macrophage

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responses skewed toward M2 are associated with certain parasitic infections, asthma and wound healing.

In contrast to M2, macrophages exposed to microbial products and interferon-γ can be activated toward the M1 phenotype. M1-biased macrophages have prototypic IL-12^{high}, IL-10^{low} or IL4/13^{low} cytokine phenotypes and are enriched in their responses to opsonized ligands and toll-like receptor engagement. M1 macrophage populations are generally recognized for an enhanced capacity to form reactive oxygen and nitrogen oxide species, which they utilize for bactericidal and tumoricidal activities.

A third type of TAM known as myeloid suppressor cells (MSC) can be defined by the Gr-1^{high},CD11b^{high}, F4/80^{low} pattern of cell surface receptors and are represented by a heterogeneous myeloid progenitor population of macrophages, granulocytes and dendritic cells originating in bone marrow [12]. MSC in the peripheral lymphoid organs facilitate tolerance in adaptive immune responses. MSC are often enriched in the lymph nodes and spleen of cancer patients where they are effective suppressors of tumoricidal CD4$^+$ and CD8$^+$ T lymphocytes [12-15]. While the majority of TAM are Gr-1^{low}, F4/80^{high}, MSC can also infiltrate tumors comprising approximately 5% of total cells [15]. One report suggests that MSC in particular may have the capacity to acquire endothelial cell properties at perivascular sites [15]. Hypoxia can promote selective myeloid infiltration to these positions [16]. MSC are emblematic of the problem in distinctly ascribing M1/M2 classifications for TAM as they employ generation of reactive oxygen and nitrogen species, a feature of M1, and foster immunosuppression, a feature of M2.

It should be stressed that the M1/M2 axis is a conceptual framework to assist in categorizing macrophage mediation of immune responses. An overwhelming percentage of either M1- or M2-biased TAM within a tumor is usually not evident unless a model system specifically devised to drive either phenotype is used. *In vitro* studies have shown that the sequence in which polarized macrophages are exposed to cytokines can redistribute them into a variety of functional phenotypes [17,18]. These studies suggested that macrophage M1/M2 polarization was not static; rather a pliable spectrum of responsiveness exists that permits macrophage adaptation to multiple microenvironment cues. Distinct differences in the transcriptome of MSC derived from peripheral sites versus TAM isolated from tumor were found following IL-4 stimulation [19]. This finding reinforces the viewpoint that tissue environment clearly influences the functional phenotype of TAM subpopulations. In addition to the cytokine milieu, oxygenation status can also serve a significant determinant cue in directing macrophage phenotypes [20]. This makes evolutionary sense when considering the advantage such flexibility provides in macrophage acute immune responses and subsequent resolution of inflammation where changes in oxygen tension play a prominent role; for instance, our response to both infectious facultative anaerobes and wounds, which occurs on a regular basis relative to cancer. In contrast to acute inflammation, resolution in cancer often is not forthcoming without aggressive therapeutic intervention. The combination of longevity of response, tumor cell signaling and changes associated with oxygen tension divert inflammatory macrophage immunity toward the unique characteristics of TAM.

Solid malignancies on the micro-scale also undergo constant change, with outgrowth of subclonal tumor cell populations, parenchymal remodeling and neovascularization in a recapitulation of morphogenesis. Hypoxia is a salient feature of solid tumors and the levels of tissue oxygenation have a profound influence on the biology of TAM [2,4,5,9-11,19,20-26]. The need for the tumor to maintain adequate nutrient and oxygenation status can drive invasive growth, angiogenesis, metastatic intravasation and development of necrotic pockets. The aggregate effect of the continuous cycle of change on TAM is to become usurped by the tumor into numerous supporting rather than tumoricidal roles. TAM differentiation is determined by

proximal cues from tumor cells, which vary dependent upon their location within the tumor mass. TAM phenotype can be quite heterogeneous and specialized for a particular micro-region of the tumor. Oxygen tension can be a powerful determinant in the paracrine relationships between TAM and tumor cells. Despite the opinion that the M2-type bias of TAM often denudes the involvement of redox effector species, we will review findings that suggest redox regulation for TAM exists at numerous levels in tumor biology and merits a second look.

Oxygen and TAM localization

TAM are thought to extravasate from the blood circulating pool of monocytes [20,28]in response to a variety of chemokine and cytokine substances. TAM accumulation often occurs in margination zones that surround islands of burgeoning tumor cells and in association with the tumor vasculature [1,11,18,19,28-31]. TAM can also be prominent in hypoxic regions proximal to sites of necrosis and poor vascularization. The steps that determine TAM differentiation into distinct local subpopulations within tumors and the degree to which the nature of tumor hypoxia influences TAM position cueing remain key issues to resolve.

The presence of blood vessels does not necessarily ensure that a region of tumor is receiving appropriate perfusion and oxygenation. Likewise, hypoxia is not an absolute condition [28-33]. Tumor blood flow may be transiently compromised in regions where microvessels are immature, occluded or supplying deoxygenated blood [24,68,69]. Over time, TAM subpopulations near vessels may be exposed to sporadic cycles of hypoxia. In contrast to intermittent hypoxia, chronic tumor hypoxia develops in areas where cells are beyond the diffusion distance of oxygen from microvessels; importantly, a gradient for oxygen along the radial distance from the vessel(s) exists. Therefore, TAM in chronically hypoxic regions can experience a range of abnormally low oxygen tensions dependent on their position relative to microvessels.

The dynamic nature of oxygen in tumors can influence TAM trafficking through several redox-related mechanisms. Hypoxic regulation of TAM motility is consistent with macrophage function as an innate responder to areas of necrosis during infection, wound damage and repair. Although amazing improvements have been made in techniques to visualize TAM traffic *in situ* [34], questions on the influence of oxygen tension remain open. Two-photon microscopy studies show that tumor cells and TAM utilize type-1 collagen fibers for movement within tumors [34]. The degree of TAM mobilization into and egress from chronically hypoxic compartments is currently unclear.

Emphasis to date has been on TAM recruitment into tumors. *In vitro* studies have shown that hypoxia can cause TAM migratory arrest. Hypoxic conditions (1% O_2) decreased murine macrophage mRNA expression and protein secretion of monocyte chemoattractant protein-1 (CCL-2) and CCR5, the receptor for macrophage inflammatory protein-1α and monocyte chemoattractant protein-2 [35,36]. Studies with human peripheral blood-derived macrophages and THP-1 cells suggested that TAM chemoreceptor signaling (e.g., CCR2) through p42/p44 mitogen activated protein kinases (MAPK) can be rapidly attenuated by hypoxia-induced activation of MAPK phosphatase-1 [37]. These experiments suggest that hypoxia diminishes facets of paracrine signaling amongst TAM and between TAM and tumor cells resulting in weakened chemokine responsiveness.

Two-way trafficking, however, implies a need for both stop and start signals under tumor hypoxia. Matrilysin matrix metalloproteinase (MMP-7) is illustrative of how differential redox regulation may govern aspects of itinerant TAM that may enable transit both into and out of hypoxic regions. Hypoxia strongly induces MMP-7 expression in TAM [21], however, the presence of oxygen is necessary for pro-enzyme processing and activity via cysteinyl oxygenation in the enzyme pro-domain [38]. In this manner, TAM movement from hypoxic

regions via MMP-7 may be forwardly directed along gradients of increasing oxygen concentration. Interestingly, the basement membrane degrading activity of mature MMP-7 becomes inhibited through cross-linking of a tryptophanyl residue in markedly oxidative environments [39]. Hypoxic induction, oxygen dependent processing and oxidative stress inhibition for MMP-7 taken together show a specialized adaptation to changing redox microenvironments. Selective impairment of chemoattractant production by hypoxic conditions [35-37] may create a gradient well for these agents that strengthens chemotaxis-driven emigration of cancer cells and allied TAM chaperones adapted to move out of chronically hypoxic regions. Initiation of metastatic disease therefore may be inherently tied to oxygen tension and redox regulation of cell motility.

Evolution, oxygen and innate immunity

It is somehow fitting that the first description of macrophages and the concept of innate immunity was born from experimental observations by Metchnikoff of a relatively simple invertebrate, a starfish larva, which he had irritated with a rose thorn [40,41]. The origins of macrophages in irradicating pathogenic invaders are indeed primordial, stretching backwards to our earliest metazoan ancestors. The usage of oxygen as a substrate for specialized immune functions through NADPH oxidase (NOX-2) and nitric oxide synthase (NOS-2 or *i* nducible NOS) occurred relatively late in evolution. The pattern of molecular and phylogenetic changes in both enzymes suggests that they were the products of gene duplication, perhaps developing in chordates with the separation of mammals and fishes [42-46].

Features of TAM biology under low oxygen tensions may be rooted in our innate immune responses to facultative anaerobes. With the emergence of NOX-2 and iNOS enzymes, a new dimension was added to the more ancient antimicrobial peptide defense network. Secreted chiefly by phagocytes and epithelial cells, defensins and cathelicidins are small peptides typically organized into highly cationic and hydrophobic domains that directly promote membrane lysis when in contact with bacteria [47-49]. Members of this diverse family participate in macrophage chemoattraction [50] and promote angiogenesis [51,52]. Antimicrobial peptides can act as opsonizing agents to enhance superoxide formation by NOX-2 [53]. Pro-α-defensin is a substrate for MMP-7 [54], which is activated by low concentrations of myeloperoxidase-derived HOCl [38]. The proline-arginine-rich antimicrobial PR-39 was found to directly inhibit NOX-2 through direct binding of $p47^{phox}$ subunit [55], while serving as an inducer of iNOS expression and activity [56].

Taken together, these observations highlight a complex level of cross regulation between different members of our innate effector systems that are predisposed to act under varied oxygen tensions during infection. Knockout studies showed that expression of antimicrobial peptides in macrophages was coordinated by the action of hypoxia-inducible factor-1 (HIF-1) [57]. Therefore, HIF-1 represents an important link between our inherent innate response strategy to infectious agents and a key transcription factor involved in both TAM and tumor cell biology [58]. The contribution of antimicrobial peptides in tumor biology and TAM phenotypes may be most relevant in tumors of epithelial origin where production of these agents by malignant cells and TAM could be activated by dysregulation of mucosal immunity processes, necrosis and hypoxia.

Hypoxic iNOS and NOX-2

The functional affects of TAM catalyzing the generation of reactive species (e.g., O_2^-, H_2O_2, NO) derived from oxygen will be strongly influenced by local tumor oxygen gradients. The macrophage iNOS Michaelis-Menten constant (K_m) for oxygen has been studied in detail [59,60]. Kinetic models and experimental data show that the K_m for oxygen shifts from 2.5 μM in the absence of arginine to 7.5 μM (or 0.85%) in the presence of arginine due to NO

intrinsically (near-geminate NO) binding within the iNOS heme pocket as the enzyme is in the ferric state. Subsequent to heme reduction to the ferrous form, the iNOS apparent K_m for oxygen increases (25 to 130 μM) as a function of NO concentration in the surrounding milieu, which can enter the enzyme to form ferrousnitrosyl complexes that rapidly consume oxygen in a futile cycle to produce alternate products (e.g., nitrate, NO_3^-) in lieu of NO. From a kinetic viewpoint, NO formation from iNOS at relatively low oxygen tensions would be compromised by scant oxygen availability for arginine (and *N*-hydroxyarginine) oxidation. On the other hand, decreased NO output would render iNOS less prone to NO feedback inhibition lowering the apparent K_m for oxygen. The net effect for hypoxic TAM would be an iNOS equilibrium optimized for a minimal oxygen K_m and maximal productive NO biosynthesis. Further complicating this scheme is the possibility for the formation of either O_2^- or H_2O_2 from iNOS via uncoupled electron flow [61]; however, this alternate enzymology may be related primarily to the neuronal and endothelial NOS isoforms [62,63].

In addition to genes involved in glycolysis and angiogenesis [58], HIF has an association with iNOS in TAM. Murine B16 melanoma tumors engineered to constitutively express GM-CSF resulted a large TAM infiltrate that showed high immunoreactivity for both HIF-1 and iNOS [64]. However, clinical studies suggest that TAM expressing iNOS and/or HIF in human tumors represent more select subpopulations of TAM that vary widely depending on the tumor type [e.g., 65-68,]. Interestingly, human tumors with a relatively lower numbers of TAM were observed to have a higher proportion of TAM with HIF-1 immunoreactivity [68].

These studies suggest that the relationship between HIF-1 and iNOS may serve a variety of specialized functions in TAM depending on their tissue location and nadir of hypoxia. HIF-1 can drive the expression of iNOS [69,70] and NO product can differentially regulate HIF protein stability dependent upon oxygen tension, redox tone and the absolute level of NO exposure [71-74]. This delicate system of feedback regulation operates at several levels. Moderate steady state levels of NO under aerobic conditions were shown to stabilize HIF protein [71,74]. In contrast, HIF degradation is prompted by NO exposure in cells under hypoxia [72,73]. One hypothesis proposes that by virtue of NO inhibition of mitochondrial respiration, micro gradients of increased oxygen availability are created, which in turn is permissive for prolyl hydroxylase mediated destabilization of HIF [72]. In the context of TAM, glucose metabolism under hypoxia is shifted from oxidative phosphorylation to glycolysis [20]. Reactive oxygen species produced during intermittent hypoxia and/or within the penumbra of chronically hypoxic regions of tumor could also shift the balance of nitrosylative chemistry in such cells toward an more oxidative character that results in HIF destabilization [73,74]. The aggregate effect of this system is one of feedforward where either NO or other agents promote HIF stabilization, which mediates transactivation of iNOS gene expression and NO generation to strengthen HIF stabilization. Negative feedback regulation under hypoxia can occur from by NO chemistry scaled to the concentration of oxygen and presence of reactive oxygen species within the immediate microenvironment resulting in HIF destabilization.

In contrast to iNOS, NOX-2 in TAM biology and hypoxia is relatively unexplored. Studies on NOX-2 and hypoxia have focused on this isozyme in the context of neutrophils and innate host defense against infectious agents. The NOX-2 Michaelis-Menten constant (K_m) value for oxygen in neutrophils was estimated to be approximately 5 to 10 μM, with an affinity increase of 2 to 3-fold following activation [75,76]. Therefore, formation of reactive oxygen species via NOX-2 in activated TAM would be plausible even at very low oxygen tensions; however, net output would still be low dependent on the absolute availability of oxygen. This raises the issue that reactive oxygen species generated at both high concentrations and sufficient duration are generally required to elicit cytotoxic effects, while low levels may contribute pro-growth signals to tumor cells. In this manner, hypoxia could convert TAM reactive oxygen-based cytolytic mechanisms into one that fosters tumor cell survival. Always inherently tied into this

dynamic is the relative concentration of NO, which serves to modulate the balance in O_2^- and H_2O_2 actions toward either cytotoxic or pro-growth [71,73,74,77].

Interestingly, MSC isolated from tumor bearing mice were found to have a tonic elevation in H_2O_2>production relative to those obtained from naïve and immunized mice that was insensitive to superoxide dismutase treatment [78]. In addition to peripheral lymphoid tissues, the potential generation of H_2O_2>by MSC within tumors may suppress cytotoxic effector function of tumor infiltrating lymphocytes. TAM extrinsic sources of reactive oxygen species derived from tumor and endothelial cells certainly contribute to the overall redox microenvironment. Indeed, other NOX isoforms can be overexpressed in many types of cancer cells [79]. While formation of reactive oxygen species plays a major role in cytotoxicity associated with immunosurveillance and carcinogenesis, generation of these agents in established tumors is weighted toward immunosuppression and growth progression affects.

Significant formation of reactive oxygen species in tumors would be most involved in zones of intermittent hypoxia and reperfusion. The anionic character of O_2^- limits it diffusion through membranes; therefore, H_2O_2 may be considered as the traveler among reactive oxygen species thereby affecting areas more distal to its point of formation through peroxidase-driven chemistry. In the presence of peroxidases or metal catalysts, H_2O_2 in combination with NO autoxidation product nitrite (NO_2^-) generates another relatively motile species NO_2 [80], which is the chief agent responsible for formation of nitrated moieties such as 3-nitrotyrosyl residues on proteins [81]. An exciting area of investigation is the reductive conversion of nitrite (NO_2^-) to NO under conditions of tumor hypoxia, which would relieve the need for O_2 consumption to activate soluble guanylyl cyclase (unpublished observations).

Wound repair, hypoxic tumors and arginine metabolism

In step with the view that aspects of tumors are similar to wounds that do not heal [82], insight from the relationship between oxygen and macrophage arginine metabolism during wound repair can be gained [83]. Rat macrophages derived from hypoxic wounds displayed marked shift in arginine catalysis from iNOS to that mediated via the polyamine enzyme arginase-1 at oxygen tensions of less than 5% (45 μM) [84]. Upon reoxygenation of rat wound derived macrophages, the absolute level of arginine catabolism was augmented due to increased expression of both arginase-1 and iNOS. At the substrate level, arginase-1 may loose out in competition with iNOS due to a relatively higher K_m for arginine and the potent competitive inhibition of arginase-1 by *N*-hydroxyarginine produced during iNOS catalysis [84,85]. Likewise, arginase activity is independent of oxygen tension.

These data suggest that hypoxia can elicit induction of both arms of the arginine axis (oxidation and hydrolysis) in macrophages with catabolic fate toward either NO or ornithine biosynthesis dictated by oxygen tension. In the rat wound model, temporal segregation of arginine metabolism was observed consistent with an inflammation-wound repair sequence [83]. Both HIF-1 induction and iNOS activity were evident primarily in the initial 48-72 hours of wound formation, which preceded development of wound hypoxia and arginase activity occurring only after 4 to 5 days of injury in this model [86].

Notable differences between wound macrophages and TAM with respect to arginine metabolism exist. The prototypic M1 interferon-γ activated transcription factor STAT-1 was found to be crucial for expression of both iNOS and arginase-1 genes in freshly isolated MSC from murine tumor, but not spleen of afflicted mice [87]. This finding again highlights that the M1/M2 scheme cannot be applied broadly to selective TAM subpopulations [19, 87]. This and other studies amply demonstrate that arginine catabolism and phenotypic behavior of TAM can be varied and change dependent on their tissue of origin (e.g., blood, spleen, tumor) and length of time cultured *ex vivo*; debate continues on interspecies differences in macrophage

arginine catabolism [83-92] showing the circumstance of TAM arginine catabolism in human tumors and its relationship to hypoxia to be an important subject of investigation.

Summary

Hypoxia, intermittent hypoxia and associated reperfusion oxidative stress differentially influences TAM behavior. It is currently not known whether TAM diversity is generated as a result of distinct defined lineages, tumor-directed prompting or weighted by physical factors such as oxygen tension. *In vitro* studies show that the sequence in which macrophages are exposed to cytokines can sway them to different functional phenotypes, suggesting that the M1/M2 polarization is not static, rather it is pliable dependent on multiple microenvironment cues [17,18]. The context of oxygen tension within the tumor cytokine/chemokine milieu is an important consideration for studies ascribing mechanism to TAM phenotypic responses.

Innate immunity between mammalian species can be similar, yet distinct in specific ways. The repertoire of antimicrobial peptides in rats and mice is quite different from that in humans [47-49]. A comparison of pulmonary granulomas induced by infection with *M. tuberculosis* in mice and humans found select differences in the spatial organization of macrophages to other leukocytes as well as marked differences in relative oxygen tension [93]. Interspecies comparisons of TAM and their adaptations to hypoxia involving redox mechanisms should be evaluated with caution.

The redox behavior of TAM is a key area of study from the perspective of understanding how to improve treatment strategies and alleviate suffering of patients with solid tumors. The predilection for tumor recruitment and differentiation of peripheral blood monocytes to hypoxic TAM opens the door for Trojan horse tactics that employs manipulation of TAM gene expression and metabolism to enhance tumoricidal effects of current treatment modalities [4, 21,22,68]. Itinerant TAM can improve disbursement of therapeutic cargo in areas that are inaccessible to delivery of chemotherapeutics through the vasculature or reticent to radiation treatment due to hypoxia. Future developments in the use of cancer patient autologous monocytes for TAM-based therapy will require an increased appreciation for redox regulation of their trafficking and effector functions.

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Biography

Brief Biography

Dr. Espey is a native of Clinton, Iowa and received his B.S. in Medical Technology at the University of Iowa in 1987. He is Board certified in Clinical Pathology, Histocompatibility and Immunogenetics. After working in immunology, organ transplant and infectious disease laboratories at Georgetown Hospital and NIH Clinical Center, he obtained a Ph.D. with distinction for his thesis dissertation on the biochemistry and immunology of tryptophan from Georgetown University in 1995. Dr. Espey's postdoctoral research on retrovirus-induced neurodegeneration was in the NIDDK at NIH. He is currently a Staff Scientist in the David Wink group at NCI, NIH where his research focus is on the detection and imaging of redox metabolism as it relates to cancer biology. A goal is to understand the biochemistry of reactive nitrogen and oxygen species and their relationship to signals that govern tumor growth and response to interventions such as irradiation.

Abbreviations

HIF
hypoxia-inducible transcription factor

iNOS
inducible NOS nitric oxide synthase

IL
interleukin

GM-CSF
granulocyte macrophage colony stimulating factor

MMP
matrix metalloproteinase

MAPK
mitogen activated protein kinase

NOX-2
NADPH oxidase

TAM
tumor-associated macrophages

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