

## Mechanisms of Hypoxia-Mediated Immune Escape in Cancer

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### Abstract

An important aspect of malignant progression is the acquired ability of tumor cells to avoid recognition and destruction by the immune system (immune escape). Clinical cancer progression is also associated with the development of tumor hypoxia, which is mechanistically linked to the acquisition of malignant phenotypes in cancer cells. Despite the well-established role of hypoxia in tumor cell invasion and metastasis, and resistance to therapy, relatively few studies have examined the contribution of hypoxia to cancer immune escape. Accumulating evidence reveals that hypoxia can impair anticancer immunity by altering the function of innate and adaptive immune cells and/or by increasing the intrinsic resistance of tumor cells to the cytolytic activity of immune effectors. Here, we discuss certain aspects of the contribution of hypoxia to tumor immune escape and provide evidence for a novel role of cyclic guanosine monophosphate (cGMP) signaling in the regulation of hypoxia-induced immune escape. Thus, we propose that activation of cGMP signaling in cancer cells may have important immunotherapeutic applications. *Cancer Res*; 74(24); 7185–90. ©2014 AACR.

### Introduction

Hypoxia, a characteristic of many solid cancers, develops from an imbalance between oxygen consumption and oxygen supply. Although hypoxia is an important driver of tumor invasion and metastasis, as well as resistance to therapy (1), there is limited knowledge on the contribution of hypoxia to tumor cell escape from destruction by innate and adaptive immune effector mechanisms.

Immune escape in cancer is a multifaceted process resulting from the suppression of immune effector mechanisms and/or the acquisition of intrinsic tumor cell resistance to the cytotoxic activity of immune effectors. Hypoxia can influence these aspects of immune escape by modifying the intrinsic properties of tumor cells and of the stromal compartment. Here, we review some of the mechanisms by which hypoxia contributes to immune escape in cancer. Furthermore, we propose that activation of cyclic guanosine monophosphate (cGMP) signaling in cancer cells, via administration of low doses of nitric oxide (NO) mimetic drugs, may be a novel therapeutic approach to interfere with hypoxia-induced immune escape.

### Hypoxia-Induced Release of Immunosuppressive Molecules by Tumor Cells

Upon exposure to hypoxia, tumor cells release a variety of immunosuppressive molecules. For example, in the severely

hypoxic tumor microenvironment, dying cells release ATP that is metabolized to adenosine by the ectonucleotidases CD73 and CD39 (2). Soluble adenosine in the extracellular matrix binds specific receptors on T cells to increase their intracellular levels of cAMP, which, in turn, suppresses T-cell functions (3). Tumor-derived cytokines released under hypoxic conditions, such as IL10 and TGF $\beta$ , induce the differentiation of tumor-associated macrophages (TAM) into M2 macrophages with immune-suppressive activities (4). TGF $\beta$  released by tumor cells also inhibits T-cell proliferation and effector function, promotes the generation of regulatory T cells (Treg), and blocks the expression of receptors required for the cytotoxic function of natural killer (NK) cells (5). In addition, TGF $\beta$  negatively regulates the antigen presentation function of dendritic cells (DC), resulting in the inhibition of T-cell function and differentiation (5).

Interestingly, emerging evidence links hypoxia-induced angiogenesis with immune tolerance (6, 7). Hypoxia drives angiogenesis within the tumor microenvironment by inducing the secretion of vascular endothelial growth factor (VEGF) and other proangiogenic molecules by tumor cells. Tumor-derived VEGF suppresses the maturation of DCs and blocks the presentation of tumor-associated antigens to helper T cells, thereby promoting immune escape (6). Moreover, in response to tumor-derived VEGF, DCs increase their expression of the programmed death ligand 1 (PD-L1 or B7-H1), a negative regulator of T-cell function (7). VEGF promotes the accumulation of myeloid-derived suppressor cells (MDSC) in tumor tissues and secondary lymphoid organs (6). MDSCs are potent suppressors of anticancer T-cell responses and also contribute to tumor progression by releasing factors that promote angiogenesis and metastasis (for a review on MDSCs see refer. 8). Consequently, VEGF is a potential target for immune therapy. In support of this, anti-VEGF therapy was shown to be associated with increased numbers of activated DCs and heightened T-cell function in patients with cancer (9). However,

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doi: 10.1158/0008-5472.CAN-14-2598

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targeting VEGF as an immunotherapeutic approach may lead to tumor hypoxia via inhibition of angiogenesis, thereby resulting in the activation of other hypoxia-induced immune escape pathways.

Tumor cells can secrete proteins such as CC-chemokine ligand 22 (CCL22) and various chemokines that inhibit effector T-cell responses and promote the generation and recruitment of immunosuppressive Tregs (10). In an ovarian cancer model, hypoxia was shown to promote the recruitment of Tregs via increased tumor cell expression of CCL28 (11). Tregs in turn can also secrete VEGF, thereby contributing to the VEGF pool in the tumor microenvironment that contributes to immune tolerance (11).

Tumor cells can also produce galectin-1 and galectin-3 to induce apoptosis of activated lymphocytes (12, 13). In patients with melanoma, there was a strong correlation between expression of galectin-2 and apoptosis of tumor-infiltrating lymphocytes (TIL; ref. 12). In Wilms tumors and Schwannomas, galectin-3 was shown to colocalize with the transcription factor hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ; ref. 14). In addition, it was reported that galectin-1 expression is transcriptionally regulated by HIF1 in colorectal cancers (15) and head and neck squamous cell carcinomas (16).

Hypoxia was also shown to induce immunosuppression by upregulating COX-2 expression in tumor cells; and HIF1-mediated upregulation of COX-2 increased colorectal tumor cell survival and VEGF production (17). COX-2 is a proinflammatory enzyme that converts arachidonic acid into prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). The latter causes immunosuppression by increasing adenosine/cAMP signaling in effector T cells (18). PGE<sub>2</sub> secreted by tumor cells can also inhibit antitumor immunity by inhibiting the maturation of DCs (19). Also, PGE<sub>2</sub> enhances the suppressive activity of Tregs and supports the differentiation of Tregs (20). Finally, PGE<sub>2</sub> can stimulate the immunosuppressive functions of MDSC by binding to EP-4 receptors on these cells (21). Hypoxia-induced immune suppression via COX-2 can explain why chronic administration of indomethacin, a COX-2 inhibitor, in the drinking water of mice led to significant reduction in the growth rate and metastasis of mammary tumors as well as restoration of splenic NK cell activity (22). A recent study revealed that use of NSAIDs reduced recurrence of breast cancer in overweight and obese women (23).

The above studies indicate that the secretion of immunosuppressive molecules by tumor cells under conditions of hypoxia provides a survival advantage, and therefore support the concept that hypoxia represents a selection pressure driving immune escape.

### Direct Effects of Hypoxia on Immune Effectors

Hypoxia can also directly impair antitumor immune responses. For example, hypoxia in the tumor microenvironment can induce the release of VEGF by M2 macrophages (24). Furthermore, TAMs suppress T-cell function in a manner dependent on HIF1 (25), and TAMs in hypoxic regions of tumors exhibit increased expression of M2-promoting molecules, such as TGF $\beta$  (26). Hypoxia inhibits the *in vitro* cytolytic activity of other immune effectors such as the NK cell-mediated

ated killing of hepatocellular carcinoma cells and multiple myeloma cells (27, 28).

Hypoxia was shown to decrease T-cell survival (29), and incubation of naïve T cells under hypoxia decreases their secretion of the trophic cytokine IL2 in a HIF1-dependent manner (30). CD4<sup>+</sup> and CD8<sup>+</sup> T cells derived from HIF1 $\alpha$ -deficient mice exhibit increased proliferation, produce higher levels of interferon- $\gamma$ , and display increased antitumor responses (31). HIF1 was also shown to mediate Treg differentiation via increased expression of FoxP3 (32). Increased numbers of Tregs in the tumor stroma have been associated with poor survival of patients with various cancers (33, 34).

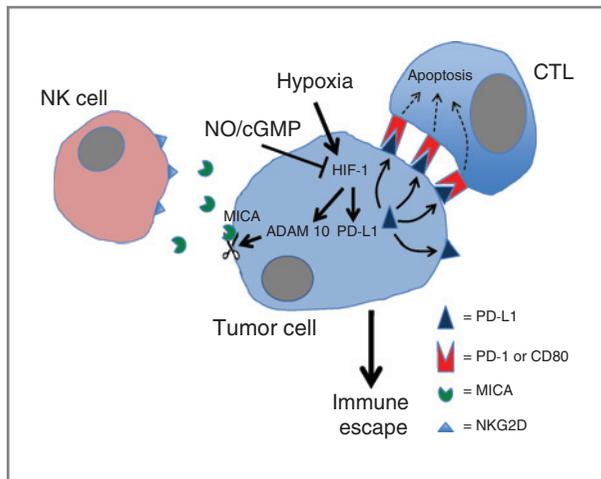
Another mechanism of tumor cell immune escape involves binding of the cytotoxic T lymphocyte antigen-4 (CTLA-4; an immune checkpoint regulator) to its natural receptors, CD80 (B7.1) and CD86 (B7.2). Interestingly, hypoxia was shown to increase the expression of CD86 by bone marrow-derived mouse DCs in a HIF1-independent manner (35). Studies revealed that CTLA-4 blockade attenuates the growth of several mouse tumors (36), reduces tumor-infiltrating Tregs, and promotes effector T-cell function in humans (37).

It is important to note that not all of the reported effects of hypoxia on T cells are detrimental to their function. Hypoxia was reported to upregulate CD137, a member of the TNF receptor family that is known for its costimulatory activity on T cells (38). Expression of CD137 on activated mouse T cells was shown to be stimulated by hypoxia (39), and tumors from HIF1 $\alpha$ -deficient mice exhibited undetectable numbers of CD137<sup>+</sup> TILs (39). In tumor growth assays, hypoxia induced the activation of T cells via the upregulation of surface CD137 in a HIF1-dependent manner, which, in turn, resulted in improved immune response and slower tumor growth (39).

### Hypoxia Induces Immune Tolerance via Regulation of Tumor Cell-Associated Immune Checkpoint Molecules

In addition to decreasing the cytolytic potential of immune effectors, hypoxia increases the intrinsic resistance of tumor cells to immune-mediated killing. One strategy that tumor cells use to avoid immune detection and destruction is to alter their expression of cell-surface immune checkpoint regulators. For example, tumor cells may shed stress-induced MHC class I chain-related proteins A and B (MICA/B) from their surface to avoid interaction with NKG2D receptors on NK cells,  $\gamma\delta$  T cells, and CD8<sup>+</sup>  $\alpha\beta$  T cells (40), thereby escaping cytotoxicity (41). We have shown that exposure of tumor cells to hypoxia leads to the shedding of surface MICA, which, in turn, results in increased resistance to lysis by innate immune effectors (42). We also showed that the hypoxia-induced release of MICA and resistance of tumor cells to lysis required HIF1-mediated expression of the metalloproteinase ADAM 10 in the tumor cells (Fig. 1; ref. 43).

As discussed earlier, there is evidence that tumor cells can suppress cytotoxic T lymphocyte (CTL) function through the interaction of inhibitory costimulatory molecules with their ligands. Certain members of the B7 family of costimulatory molecules expressed on the surface of tumor cells provide



**Figure 1.** Proposed mechanisms of hypoxia-induced tumor cell escape from innate and adaptive immunity. Hypoxia increases the accumulation of HIF1 $\alpha$  in tumor cells, which, in turn, leads to higher levels of ADAM 10 and PD-L1 on the surface of tumor cells. ADAM 10 cleaves MICA from the cell surface to limit binding to NKG2D receptors on NK cells, leading to escape from innate immunity, whereas interaction of PD-L1 with PD-1 or CD80 on activated CTLs causes apoptosis in the CTLs and escape from adaptive immunity. NO/cGMP signaling is proposed to block the effect of hypoxia on ADAM 10 and PD-L1 upregulation by inhibiting HIF1 $\alpha$  accumulation.

signals that suppress CTL responses. For example, binding of PD-L1 with PD-1 or with CD80 (B7.1) on activated CTLs leads to suppression of immune responses via mechanisms that include induction of apoptosis and anergy (nonresponsiveness to antigen) in the T cells (44). Recent clinical studies revealed that therapy with blocking anti-PD-1 antibody (nivolumab) produced objective responses in patients with non-small-cell lung cancer, melanoma, or renal-cell cancer (45). Also, reinduction therapy with anti-PD-1 antibody for late tumor recurrence showed durable remissions in patients with colorectal cancer, renal cell cancer, and melanoma (46). In another study, concurrent therapy with anti-CTLA-4 antibody (ipilimumab) and nivolumab resulted in tumor regression in a substantial proportion of patients with unresectable, stage III or IV melanoma (47). We recently provided evidence that, when exposed to hypoxia, human and mouse cancer cells increased their expression of PD-L1 and acquired resistance to CTL-mediated lysis in a manner dependent on HIF1 $\alpha$  (Fig. 1; ref. 48). Furthermore, the hypoxia-induced expression of PD-L1 in tumor cells led to increased apoptosis of cocultured CTLs as well as Jurkat T cells (48).

In addition, hypoxia may induce immune escape in cancer cells via epigenetic mechanisms. For example, tumor cells can upregulate miR210 in lung cancer and melanoma (49). In turn, miR210 was shown to block the susceptibility of tumor cells to lysis by antigen-specific CTLs. This effect was mediated via increased expression of protein tyrosine phosphatase, nonreceptor type I (PTPN1), homeobox A1 (HOXA1), and tumor protein p53-inducible protein 11 (TP53I11; ref. 49). Further studies are required to elucidate the mechanisms used by these molecules to suppress CTL activity.

Exposure of tumor cells to hypoxia also resulted in resistance to autologous CTL-mediated lysis in a manner dependent on the signal transducer and activator of transcription (STAT) 3 (50). STAT3 modulates the cross-talk between tumor and immune cells (51). A small-molecule inhibitor of STAT3, WP1066, was reported to reverse immune tolerance in patients with malignant glioma (52). Another STAT3 inhibitor, sunitinib, reduced the immunosuppressive phenotype of renal cell carcinomas (53) and reversed MDSC-mediated immune suppression via increased recruitment of CD4<sup>+</sup>CD8<sup>+</sup> cytotoxic T cells (54).

### Regulation of Immune Tolerance via Hypoxia-Induced Autophagy

Cancer cells often rely on autophagy as a mechanism of survival under conditions of stress including hypoxia, nutrient starvation, growth factor withdrawal, and chemotherapy (55, 56). However, the mechanisms by which autophagy enables survival of normal or malignant cells are not well known.

Hypoxia-induced autophagy is partly dependent on the HIF1/BNIP3–BNIP3L–Beclin1 axis (57), and partly on HIF1/platelet-derived growth factor receptor signaling (58). Through the activating transcription factor 4 and C/EBP homologous protein (CHOP), hypoxia increases the expression of microtubule-associated protein 1 light chain 3 (LC3) and autophagy protein 5 (ATG5) involved in formation and maturation of autophagosomes (59).

Hypoxia-induced autophagy is known to promote tumor cell survival via several mechanisms, including the removal of damaged mitochondria that produce cytotoxic reactive oxygen species (57) and the degradation of harmful protein aggregates (59). Activation of autophagy in cancer cells during hypoxia or exposure to other microenvironmental stressors may also lead to inhibition of death signals such as those triggered by CTLs (60). Furthermore, stress-induced release of the molecular pattern molecule HMGB1 induces cytoprotective autophagy and leads to recruitment of Tregs (60).

Autophagy can also promote activation of anticancer immunity. For example, autophagy has been shown to be crucial for proliferation of immune cells as well as for their effector functions such as antigen presentation and T cell-mediated tumor cell cytotoxicity (61). In T cells, autophagy is activated upon TCR engagement in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subtypes (62). The knockdown of the essential autophagy-related genes, ATG5 or ATG7, during TCR stimulation leads to a significant decrease in cellular proliferation demonstrating the importance of autophagy during T-cell activation (62, 63). Furthermore, culture of DCs under low-oxygen results in the stabilization of HIF1 $\alpha$ , which initiates BNIP3 expression and promotes survival of mature DCs, possibly due to induction of autophagy (64). Hypoxia-induced autophagy in antigen-presenting cells infiltrating a tumor can occur via Toll-like receptor (TLR) signaling (65).

Together, the above findings indicate a dual role for autophagy in cancer immune escape. Therefore, immunotherapeutic strategies designed to target autophagy will need to consider its impact on the immune system.

## Nitric Oxide/cGMP-Mediated Inhibition of Hypoxia-Induced Immune Escape

Our research over the last 15 years has revealed that classical NO signaling involving cGMP production functions as an O<sub>2</sub>-sensing mechanism playing a key role in tumor cell adaptations to hypoxia (42, 43, 48, 66–69). On the basis of our findings, we postulated that an important aspect of the mechanism by which cancer cells adapt to hypoxia involves inhibition of endogenous NO/cGMP signaling. Our research demonstrates that low concentrations of NO mimetics [e.g., glyceryl trinitrate (GTN), DETA/NO], known to selectively activate soluble guanylyl cyclase (sGC), inhibit malignant adaptations to hypoxia such as increased invasiveness, metastatic ability, and drug resistance (66–69). Moreover, because NO production is dependent on O<sub>2</sub> availability, endogenous NO generation is severely limited in cells exposed to hypoxia (70, 71). This is despite the fact that hypoxia was shown to increase the expression of inducible NO synthase (iNOS) in the same cells (RAW 264.7 macrophages; ref. 71). We previously reported that cGMP levels are decreased in MDA-MB-231 breast tumor cells incubated for 6 hours in 0.5% O<sub>2</sub> (68). This observation is consistent with the more recent findings of Hickok and colleagues (71), who reported decreased sGC activation in a murine macrophage line incubated under 5% O<sub>2</sub>. It is likely that our observed effects of NO/cGMP signaling on hypoxia-induced malignant phenotypes are at least partly mediated via inhibition of HIF1 transcriptional activity. This conclusion is based on evidence that NO mimetics, including the cGMP analogue 8-bromo-cGMP, inhibit the accumulation of HIF1 $\alpha$  in cells exposed to hypoxia (43, 72). Our research has also revealed that NO mimetics interfere with the HIF1-mediated upregulation of ADAM10 expression involved in the shedding of MICA from the tumor cell surface and resistance to immune-mediated lysis (Fig. 1; ref. 43). In that same study, treatment of mice with GTN attenuated the growth of transplanted prostate tumors via a mechanism dependent on innate immune effectors. More recently, we demonstrated that low concentrations of GTN interfere with hypoxia-induced escape from T cell-mediated immunity in tumor cells by preventing the HIF1-dependent expression of PD-L1 (Fig. 1; ref. 48). Together, these studies indicate that activation of NO/cGMP signaling may have important applications in the prevention and/or treatment of cancer.

## Conclusions

Although there is evidence that hypoxia can activate certain components of pathways involved in antitumor immunity,

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most studies indicate that hypoxia is a major contributor to cancer immune escape. Hypoxia-induced tumor cell escape from innate and adaptive immunity is likely a consequence of multiple mechanisms operating in a complementary, and sometimes redundant, manner. Thus, targeting individual mechanisms of hypoxia-induced immune escape will likely prove to be ineffective as a therapeutic strategy. However, it is clear that several mechanisms of such immune escape rely on the transcriptional activity of HIF1. This raises the possibility that interference with hypoxia response pathways involving HIF1 activity may be a fruitful immunotherapeutic approach. Interference with such pathways could be achieved through the use of molecules that directly inhibit HIF1 activity or block HIF1 $\alpha$  accumulation in hypoxia. Our studies on the inhibitory effect of NO/cGMP signaling on HIF1 $\alpha$  accumulation and malignant adaptations to hypoxia, including tumor cell escape from innate and adaptive immunity, support the therapeutic potential of NO mimetic agents. In this review, we highlighted some key mechanisms of hypoxia-mediated immune escape. However, because tumor cell avoidance of immune destruction is multifaceted, it is likely that hypoxia influences escape mechanisms not described herein. The role of the hypoxic tumor microenvironment on other key aspects of cancer immune surveillance, such as antigen presentation, additional immune checkpoints and effector mechanisms of tumor cell destruction, warrants investigation.

## Disclosure of Potential Conflicts of Interest

C.H. Graham has ownership interest (including patents) in and is a consultant/advisory board member for Nometics Inc. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** I.B. Barsoum, M. Koti, D.R. Siemens, C.H. Graham  
**Development of methodology:** I.B. Barsoum, D.R. Siemens, C.H. Graham  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** I.B. Barsoum  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** D.R. Siemens, C.H. Graham  
**Writing, review, and/or revision of the manuscript:** I.B. Barsoum, M. Koti, D.R. Siemens, C.H. Graham  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** C.H. Graham  
**Study supervision:** D.R. Siemens, C.H. Graham

## Grant Support

Our studies on the role of hypoxia in cancer immune escape are funded by a grant from the Canadian Institutes of Health Research awarded to CHG and DRS (grant number MOP 79267).

Received September 2, 2014; revised October 14, 2014; accepted October 14, 2014; published OnlineFirst October 24, 2014.

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