

Review Article

Hypoxia: A Key Feature of the Tumor Microenvironment Triggers Several Mechanisms of Evasion from Natural Killer and Cytotoxic T Lymphocytes Surveillance

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Abstract

Since many years, the tumor microenvironment is recognized as an important promoter of cancer initiation and progression. Although tumors primarily consist in cancer cells, various components/factors of the microenvironment and more specifically the immune landscape, dramatically impact cancer progression. Comprehensive analyses conducted on diverse tumors have identified and characterized the most relevant components/factors of the tumor microenvironment that support the malignant behavior of a growing primary tumor. Tumor hypoxia is a common characteristic of the tumor microenvironment that is associated with tumor progression, metastasis, treatment failure and escape from immune surveillance. Although immune cells are usually efficiently recruited into the tumor bed, hypoxic microenvironment was reported to compromise immune cell functions and, in some cases, switch immune cell activity towards a pro-tumorigenic profile. Mechanistic studies have highlighted that hypoxia acts as double-edged sword: it simultaneously impairs the function of immune cells in the tumor microenvironment and activates intrinsic cell resistance mechanisms in tumor cells. The present article aims at reviewing some recent findings on how hypoxia impairs the anti-tumor immune response by focusing on emerging mechanisms by which hypoxia damps the function of immune effectors cells and activates intrinsic immune resistance mechanisms in tumor cells including autophagy and actin cytoskeleton remodeling.

Keywords: Cancer; Hypoxia; Immune response; Tumor microenvironment; Autophagy; Actin cytoskeleton

Abbreviations

AFs: Actin Filaments; APCs: Antigen Presenting Cells; ARP: Actin-Related Protein; ATG: Autophagy-Related Genes; Bcl-2: B Cell Lymphoma 2; CAFs: Cancer-Associated Fibroblasts; Cdc42: Cell Division Cycle 42; CTL: Cytotoxic T Lymphocyte; ECM: Extracellular Matrix; EMT: Epithelial-to-Mesenchymal Transition; FAK: Focal Adhesion Kinase; HIF: Hypoxia-Inducible Factors; HOXA1: Homeobox A1; HRE: Hypoxia-Response Element; IS, Immunological Synapse; LFA: Lymphocyte Function-Associated Antigen; MAPK: Mitogen-Activated Protein Kinases; MHC: Major Histocompatibility Complex; MICA: MHC Class I Polypeptide-Related Sequence A; miR: micro RNA; NK: Natural Killer; NKG2D: Natural Killer Group 2 Member D; PHD2: Prolyl Hydroxylase Domain Protein 2; PTPN1: Protein Tyrosine Phosphatase Non-Receptor Type 1; Rac1: Ras-Related C3 Botulinum Toxin Substrate 1; Rho: Ras Homolog; ROCK1: Rho-Associated Protein Kinase 1; STAT3: Signal Transducer and Activator of Transcription 3; TCR: T Cell Receptor; TNF: Tumor Necrosis Factor; TP5311: Tumor Protein p53-inducible Protein 11; VEGF: Vascular Endothelial Growth Factor; VHL: Von Hippel-Lindau; WAS: Wiskott-Aldrich Syndrome; WASp: WAS Protein; YAP: Yes-associated Protein.

Introduction

Research in tumor immunology has validated the concept of

cancer immune surveillance which predicts that the immune system can recognize precursors of cancer and, in most cases, destroy them or slow their growth before they become clinically apparent [1, 2]. Several types of immune cells are involved in tumor immune surveillance. Briefly, key cells of the adaptive immune system recognizing cancer cells are cytotoxic T lymphocytes (CTL) which are able to identify tumor antigens *via* the T cell receptor (TCR) [3]. Some of these antigens are expressed exclusively by tumors and thus are called tumor-specific antigens [3]. Natural killer (NK) cells of the innate immune system also play an important role in tumor immune surveillance [3] by mechanisms called “missing-self” and “induced-self” recognitions [5]. In addition to CTL and NK cells, macrophages and neutrophil granulocytes are also involved in anti-tumor immunity [6]. Macrophages are antigen presenting cells (APCs) that display tumor antigens and stimulate other immune cells such as CTL, NK cells and other APCs [7]. While the molecular mechanism by which CTL and NK cells recognize their target tumor cells is fundamentally different, both immune cells kill their target following the establishment of immunological synapse (IS) [8]. The formation of IS requires cell polarization and extensive remodeling of the actin cytoskeleton at various stages [9]. It is now well established that CTL and NK cells recognize and kill target cells by two major pathways: either through the release of cytotoxic granules containing perforin and granzymes to the cytosol of target cells [10], or through tumor necrosis factor (TNF) super family-dependent killing [11].

Many microenvironmental factors (e.g. hypoxia, composition/organization of the extracellular matrix (ECM), inflammation, immune suppressive tumor-associated cells) contribute to various aspects of cancer progression, including immune evasion of tumor cells [12, 13]. Recently, it has been reported that the immune system also sculpts the immunogenic phenotype of an established tumor to favor the emergence of resistant tumor cell variants [14]. Accumulating experimental and clinical evidence indicates that multiple mechanisms suppressing the anti-tumor immune functions are directly evolved in the tumor microenvironment [15]. Recently, attention has been focused on the mechanisms by which hypoxic stress within the tumor microenvironment alters tumor transcriptional profiles to modulate glycolysis, proliferation, survival and invasion [16].

In this review, we focus on recent progresses in understanding the influence of hypoxic stress on the tumor survival mechanisms and tumor escape from immune surveillance. We discuss how hypoxia impairs tumor cell killing mediated by both innate and adaptive immune cells. We also review how hypoxia confers resistance to immune attack by activating intrinsic resistance mechanisms in tumor cells. Particular attention is given to hypoxia-induced immune escape mechanisms that rely on actin cytoskeleton remodeling in tumor or immune cells. Finally, we briefly address how hypoxia-targeted strategies may have potential clinical application for restoring an efficient anti-tumor immune response.

Hypoxia and Hypoxia-inducible Factors (HIF) Regulation

Hypoxic stress plays a major role in the acquisition of tumor resistance to immune cell-mediated apoptosis and in the impairment of immune cell activity. Tumor cells adapt to hypoxic microenvironment by inducing transcription factors of the hypoxia inducible factor (HIF) family [17]. The HIF family comprises three members, HIF-1, -2 and -3. HIF-1 is a heterodimeric protein composed of a constitutively expressed HIF-1 β subunit and an oxygen-regulated HIF-1 α subunit. In the presence of oxygen, HIF-1 α is hydroxylated on proline residue 402 and/or 564 by prolyl hydroxylase domain protein 2 (PHD2). Hydroxylated HIF-1 α interacts with the Von Hippel-Lindau (VHL) tumor suppressor protein [18] and recruits an E3 ubiquitin-protein ligase to catalyze the polyubiquitination of HIF-1 α and its subsequent targeting to the ubiquitin proteasome system for degradation [19] (Figure 1). Under hypoxic condition, the hydroxylation of HIF-1 α is inhibited leading to HIF-1 α accumulation. Subsequently, HIF-1 α translocates to the nucleus, dimerizes with HIF-1 β subunit, binds to the hypoxia-response elements (HRE) present in the promoter of target genes, and recruits co-activators to activate gene transcription (Figure 1). Similar to HIF-1 α , HIF-2 α is regulated by oxygen-dependent hydroxylation [20]. Although they share structurally similar DNA-binding and dimerization domains, HIF-1 α and -2 α differ in their transactivation domains. Accordingly, HIF-1 α and -2 α share overlapping target genes, and each also regulates a set of specific targets [21]. HIF-3 α lacks the transactivation domain and was proposed to function as an inhibitor of HIF-1 α and HIF-2 α . Interestingly, its expression is transcriptionally regulated by HIF-1[22].

Influence of Hypoxia on Cancer Cell Sensitivity to CTL and NK-mediated Lysis

As previously mentioned, hypoxia modulates both the activity of immune effectors and the response of tumor cells to these effectors. It has been reported that hypoxia decreases the sensitivity of tumor cells to CTL-mediated killing by several mechanisms involving HIF-1. For example, Noman *et al.* showed that HIF-1 α induces the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in tumor cells by a mechanism involving at least in part, vascular endothelial growth factor (VEGF) secretion. Following its translocation to the nucleus, HIF-1 α cooperates with pSTAT3 to alter the susceptibility of non-small cell lung carcinoma to CTL-mediated

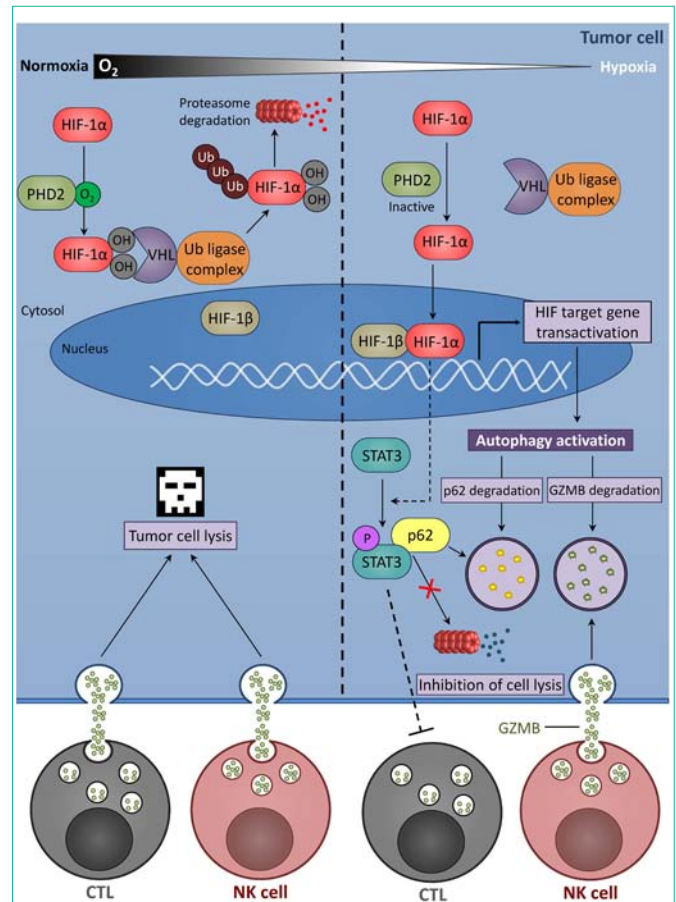


Figure 1: Hypoxia induces tumor cell resistance mechanisms leading to escape from CTL- or NK-mediated lysis. Under normoxic condition, the oxygen-sensitive prolyl hydroxylase domain protein 2 (PHD2) hydroxylates HIF-1 α subunit which is bound to Von Hippel-Lindau protein (VHL), ubiquitinated and subsequently degraded by the ubiquitin-proteasome system. Under hypoxia, PHD2 protein is inactivated, and HIF-1 α is therefore stabilized. Stabilized HIF-1 α translocates to the nucleus and forms with HIF-1 β , a heterodimeric complex responsible for transcriptional activation of HIF-target genes including those responsible for the activation of autophagy. In addition, HIF induced the phosphorylation of the signal transducer and activator of transcription (STAT) 3 by a mechanism which is not fully described. The activation of autophagy leads to the degradation of the cargo protein p62, responsible for the elimination of phospho-STAT3 by the ubiquitin proteasome system. The degradation of phospho-STAT3 inhibits CTL-mediated lysis of tumor cells. Furthermore, excessive autophagy in hypoxic target cells leads to the selective degradation of granzyme B (GZMB), a serine protease contained into the cytotoxic granules released by natural killer (NK) cells, thereby inhibiting NK-mediated lysis.

killing [23] (**Figure 1**). A follow-up study has reported that the level of pSTAT3 is tightly controlled by the activation of autophagy in hypoxic cells. Indeed, the accumulation of pSTAT3 was no longer observed when the autophagic flux was inhibited in hypoxic tumor cells [24]. Briefly, autophagy is a catabolic process where a cell self-digests its own components. It can be activated in response to multiple stresses including hypoxia, nutrient starvation, growth factor withdrawal and endoplasmic reticulum stress. Autophagy primarily serves as an adaptive metabolic response providing nutrients, preventing accumulation of altered cell components [25], shaping the anti-tumor immune response [26] and the acquisition of resistance to TNF α [27]. Studies aimed at investigating the mechanisms by which autophagy controls pSTAT3 revealed that targeting autophagy decreases the level of pSTAT3, and that this decrease was mediated by the ubiquitin proteasome system [24, 28].

Another study showed that the transcription factor NANOG, which is involved in stem cell self-renewal, is a new mediator of hypoxia-induced resistance to CTL-mediated lysis [29, 30]. NANOG is induced at both transcriptional and protein levels under hypoxic stress and its targeting leads to significant attenuation of hypoxia-induced tumor resistance to CTL-dependent killing. NANOG depletion results in inhibition of STAT3 phosphorylation and nuclear translocation. Furthermore, it has been shown that HIF-1 induces hypoxia-inducible micro RNA (miR)-210. Overexpression of miR-210 in tumor cells targets protein tyrosine phosphatase non-receptor type 1 (PTPN1), homeobox A1 (HOXA1), and tumor protein p53-inducible protein 11 (TP53I11), and thereby decreases tumor cell susceptibility to CTL [31]. Hypoxia has also been reported to increase the shedding of the major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA), a ligand for the activating receptor natural killer group 2 member D (NKG2D), at the surface of prostate cancer cells. This shedding is related to the impairment of NO signaling [32] and leads to tumor escape from NK and CTL cells. Furthermore, HIF-1 is able to downregulate MICA expression in osteosarcoma cells resulting in tumor resistance to NK-mediated lysis [33]. Recently, we showed that targeting autophagy under hypoxia restores NK-mediated lysis in breast cancer cells. We provided mechanistic evidence that activation of autophagy under hypoxia leads to the degradation of NK-derived granzyme B making hypoxic tumor cells less sensitive to NK-mediated killing [34, 35] (**Figure 1**). Taken together, it is now well documented that hypoxic microenvironment is an important determinant involved in the control of anti-tumor immune response. Indeed, its immune suppressive effect contributes not only in damping the immune cell function but also in conferring resistance mechanisms to tumor cells to resist immune cells attack [36].

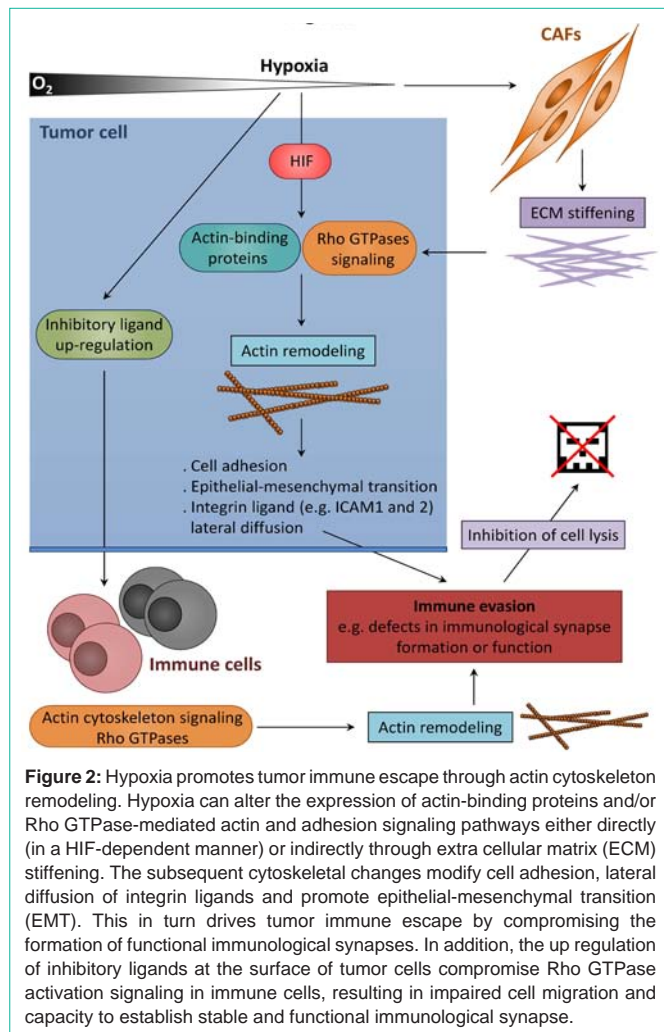
The Actin Cytoskeleton: A Point of Convergence for Various Processes Involved in Tumor Immune Evasion

To fulfill their biological functions, immune cells require exceptional capabilities to proliferate, move through the entire body, infiltrate tissues, establish cell-cell contacts and exchange various types of compounds/signals with both other immune cells and their targets. The related cellular processes, including cell division, cell migration and invasion, cell shape plasticity, intracellular transport,

endocytosis and exocytosis, membrane trafficking and phagocytosis, largely rely on dynamic re-arrangements of the actin cytoskeleton. Accordingly, dysregulation of the actin cytoskeleton organization or dynamics can compromise immune cell function and lead to severe diseases, including cancer [37]. A typical illustration is provided by the Wiskott-Aldrich syndrome (WAS) which is caused by alterations of WAS protein (WASp) activity. One major function of WASp is to stimulate actin polymerization through the actin-related proteins (ARP) 2/3 nucleation complex in response to signals from the cell surface [38, 39]. Although some signaling activities of WASp are independent of actin remodeling, most immune cell defects induced by the loss of WASp are at least to some extent, related to defective actin responses, including cell migration, activation, IS formation, and phagocytosis [40-43].

One important aspect of the anti-tumor immune response is the establishment of the IS between cytotoxic immune cells, such as CTL and NK cells, and their targets. Although the signaling pathways activating CTL and NK cell-mediated tumor cell death differ, the establishment of a functional IS and the directed secretion of cytolytic granules toward the tumor cell are relatively well conserved processes that require extensive cytoskeletal remodeling. Following immune cell activation, actin polymerization is activated [44, 45] and granules polarize toward the cell-cell contact region in a microtubule network and microtubule motor activity-dependent manner [46-48]. A critical feature of this process is the translocation of the microtubule-organizing center (upon which granules converge) from the rear of the immune cell to the leading edge where the immunological synapse is established. The presence of pre-docked granules at the cell cortex of CTL and NK cells, that can be exocytosed before centrosome polarization has been reported [49-51]. Both pharmacological and genetic studies indicate that immune cell activation, IS formation, and the steps following granule polarization largely rely on actin cytoskeleton remodeling [46, 48, 52-54]. A substantial remodeling event occurring simultaneously with centrosome translocation is the accumulation of actin toward the edge of the cell-cell contact region in the so-called distal supra molecular activation cluster where integrin proteins concentrate to mediate cell adhesion [55-57]. The central region of mature synapses was initially proposed to function as an actin-free clearance through which granules are passively released [47, 56]. Such a model was however challenged by studies supporting that granules associate with actin in a myosin IIA dependent manner prior to their delivery to the precise de-granulation sites [58, 59]. Recently, high-resolution microscopy has provided compelling evidence that an actin meshwork is present at the center of the IS from NK cells and that it functions as a facilitator for granule secretion rather than as a barrier [46, 60-62].

In contrast to the well-characterized configurations and roles of actin filaments (AFs) in NK and CTL cells during the cytolytic process, there is a relative gap of knowledge about AF organization and dynamics in target tumor cells. However growing evidence indicate that AF reorganization in tumor cells can promote resistance to anti-tumor immunity (**Figure 2**). Thus, knock-down of two actin-regulatory proteins, namely the actin-severing factor scinderin [63, 64] and the signaling molecule ephrin-A1 [65] that are overexpressed in resistant lung tumor cells, was sufficient to restore normal cell morphology and to increase cell sensitivity to CTL-mediated cell



death [66]. A follow-up study established that the focal adhesion kinase (FAK) signaling pathway, which controls actin organization and modulates cell adhesion and proliferation was defective in tumor resistant cells [67]. Remarkably, the suppression of FAK in tumor cell was sufficient to promote their resistance to CTL by blocking IS formation. Furthermore, increased AF rearrangement and adhesion following pharmacological activation of Ras homolog (Rho) GTPases has been reported to induce tumor cell susceptibility to CTL mediated killing. These data further support the role of actin remodeling as a switch in the control of tumor cell susceptibility to cytotoxic immune cells. In addition, depolymerization of AFs in NK-sensitive target cells abrogates the conjugate formation mediated by lymphocyte associated antigen (LFA)-1 and subsequently affects granule polarization [68]. Experimental data point to a mechanism where actin cytoskeleton-dependent tethering of LFA-1 ligand such as intercellular adhesion molecules (ICAM)-1 and -2, is required for proper integrin signaling in NK cells [68]. Thus, the modulation of the lateral diffusion of integrin ligand by actin remodeling at the tumor cell cortex represents a potential facet of the immune evasion.

Tumor cells do not only remodel their own actin cytoskeleton to escape from the immune system but are also capable of promoting cytoskeletal modifications in immune cells to impair their activity (Figure 2). Gene-expression profiling study revealed that genes

involved in actin cytoskeleton formation and organization in peripheral blood CTL are considerably dysregulated in chronic lymphocytic leukemia patients [69]. Thus, leukemia cells were able to induce similar cytoskeletal defects in CTL from healthy allogeneic donors. Interestingly, the underlying process required cell-cell contact and was independent on cytokine release. Follow-up studies established that malignant cells up-regulate inhibitory ligands such as the B7 super family-related ligands CD200, CD274 and CD276, and TNF-receptor super family member CD270, to compromise the activation of key regulators of T cell synapse actin dynamics, including the Rho GTPases RhoA, Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle protein 42 (Cdc42) and eventually compromise the establishment of a functional IS [70, 71]. Remarkably, similar inhibitory ligand signaling axes were found in a range of other hematologic and solid carcinoma cells, suggesting that tumor cell-induced T cell actin synapse dysfunction is a common immunosuppressive strategy. Recently, elegant work by Ramsay *et al* demonstrated that leukemia cells from patients impair T cell LFA-1-mediated migration by altering Rho GTPase activation signaling. Importantly, the immune modulating drug lenalidomide was shown to restore T cell Rho GTPase activity and LFA-1 activation, laying the ground for immunotherapy approaches promoting T cell motility in cancer patients [72].

Tumor Microenvironment-induced Cancer Progression and Immune Evasion Involve Actin Cytoskeleton Remodeling

It has been reported that the physical properties of tumor stroma considerably differ from those of corresponding healthy tissues. Hypoxic stress within the tumor microenvironment induces an increase in ECM stiffness [73] and this increase is largely associated with the ability of hypoxia to induce the expression of ECM genes in cancer-associated fibroblasts, such as those involved in collagen fiber deposition and organization [74-76]. The perception of matrix stiffening by actin cytoskeleton-linked integrins activates signaling pathways that promote Rho GTPase-dependent cytoskeletal tension, focal adhesion assembly and cell migration [73, 77]. Interestingly, a recent study showed that an increase in matrix stiffness and actin cytoskeleton contractibility activates the transcription factor Yes-associated protein (YAP) in cancer-associated fibroblasts and that this activation is required for these fibroblasts to further increase matrix stiffening, cell invasion and angiogenesis [78]. Thus, by inducing a stiff microenvironment, hypoxia promotes actin remodeling in both tumor and cancer-associated fibroblasts (CAFs) leading to increased fibrillar collagen deposition and promoting tumorigenic functions of CAFs (Figure 2).

Hypoxic stress has been described to modify the expression and/or activity of Rho GTPases and/or of their downstream targets in both healthy and cancer cells. Recently, HIF-1 and -2 were shown to directly activate transcription of RhoA and Rho-associated protein kinase 1 (ROCK1) genes, and subsequently promote actin cytoskeleton changes that underlie the invasive tumor cell phenotype [79]. Such coordination between HIF and RhoA-ROCK1 activation also induced FAK activation and focal adhesion formation, indicating that FAK is a major effector of RhoA/ROCK1 in hypoxic breast cancer cells. Rac1 and Cdc42, two other major Rho GTPases

controlling actin dynamics [80] and cancer progression [81] are up-regulated and activated by hypoxia in different tumor cells [82, 83]. In this context, it is worth noting that an immune functional screen identified Cdc42 as a key mediator of tumor resistance to CTL-mediated killing [84]. Remarkably, stable expression of constitutively active Cdc42 protected colorectal tumor cells against cytotoxicity and tumor suppression by alloreactive CTL *in vitro* and CTL and NK cells *in vivo*. However, the role of actin remodeling in the underlying mechanism remains uncertain. Instead, Cdc42 has been shown to promote immune resistance, at least in part, by mitogen-activated protein kinases (MAPK)-dependent inhibition of apoptosis and stabilization of the B cell lymphoma 2 (Bcl-2) antiapoptotic factor. In addition to modulating actin regulatory signaling pathways, hypoxia also drives changes in the expression level of specific actin-binding proteins [85]. However, how such changes influence the immune tumor response remains to be assessed.

As previously reported, hypoxia-induced autophagy plays important roles in tumor immune escape [86]. To our best knowledge, the role of the actin cytoskeleton in hypoxia-induced autophagy has not been extensively investigated in the specific context of tumor immune escape. Nevertheless, emerging evidence indicate that epithelial-to-mesenchymal transition (EMT), a biological process associated with dramatic morphologic changes and actin cytoskeleton remodeling, alters the susceptibility of tumor cells to CTL-mediated immune surveillance by a mechanism involving autophagy [87, 88]. There is also clear evidence that Rho family-dependent actin remodeling is required for proper autophagy induced by other environmental stresses, such as starvation [89]. Finally, several autophagy-related genes (ATG) proteins were reported to physically and/or functionally interact with actin cytoskeleton components, such as the actin motor myosin II [90] and the FA protein paxillin [91].

Concluding Remarks

The ability of cancer cells to evade immune surveillance and resist immunotherapy raises fundamental questions about how tumor cells survive in the presence of a competent immune system. To address this issue, studies have primarily focused on the mechanisms by which tumor cells avoid recognition by the immune system without considering the impact of the tumor microenvironment. Thus, despite intense investigation, the relatively modest gains provided by immunotherapy can be in part attributed to the activation of mechanisms suppressing the anti-tumor immunity. It is now clearly established that the majority of these mechanisms are likely evolved in the local tumor microenvironment. In line with this, it may be more accurate to consider cancer, which was initially thought to be a disease of cells, then of genes, and then of genomes, as a disease of the microenvironment. While remarkable and fairly rapid progresses have been made over the past two decades regarding the role of the microenvironment in cancer biology and treatment, our understanding of its actual contribution in tumor resistance to immune cell attack is still fragmented.

Emerging data indicate that hypoxia in the tumor microenvironment plays key role in mediating tolerance to immune cell attack. Therefore, an understanding of the tumor microenvironment, and in particular of hypoxia-induced tumor resistance, may allow better understanding of tumor adaptation and evolution, and ultimately lead to improve the efficacy of potential

therapeutics. Thus, it stands to reason that targeting hypoxia could have a substantial effect in evolving the properties of tumor microenvironment from immunosuppressive to immune permissive. In keeping with this, an extensive number of HIF inhibitory molecules are currently under development and being tested or in use in clinical settings. Such inhibitors comprise HIF- α antisense, dominant negative HIF- α and viral vectors as well as small molecules that inhibit HIF at multiple sites. Briefly, these inhibitors have been shown to block tumor xenograft growth and inhibit HIF activity through a wide variety of molecular mechanisms, including decreased HIF-1 α mRNA levels, decreased HIF-1 α protein synthesis, increased HIF-1 α degradation, decreased HIF subunit hetero-dimerization, decreased HIF binding to DNA, and decreased HIF transcriptional activity. Notably, HIF-1 α inhibitors would be expected to affect some parameters of the antitumor immunity such as CTL activity *in vivo* and *in vitro* (*i.e.*, cytokine production, proliferation, and activation). Indeed, several recent studies highlight that hypoxia is capable of mediating tumor cell resistance to CTL and NK cells [24, 31, 36]. In addition, it should be investigated whether HIF inhibitors could affect several other important mediators of immune tolerance such as myeloid-derived suppressor cells (MDSC) and T regulatory cells (*i.e.*, recruitment, function and differentiation). Overall, hypoxia inhibition in hypoxic tumors can be used as a cutting-edge approach to improve cancer immunotherapy and to formulate more effective cancer vaccine-based therapy.

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