The role of hypoxia in shaping the recruitment of proangiogenic and immunosuppressive cells in the tumor microenvironment

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Hypoxia characterizes growing tumors and contributes significantly to their aggressiveness. Hypoxia-inducible factors (HIFs 1 and 2) are stabilized and act differentially as transcription factors on tumor growth and are responsible for important cancer hallmarks such as pathologic angiogenesis, cellular proliferation, apoptosis, differentiation and genetic instability as well as affecting tumor metabolism, tumor immune responses, invasion and metastasis. Taking into account the tumor tissue as a whole and considering the interplay of the various partners which react with hypoxia in the tumor site lead to reconsideration of the treatment strategies. Key limitations of treatment success result from the adaptation to the hypoxic milieu sustained by tumor anarchic angiogenesis. This raises immune tolerance by influencing the recruitment of immunosuppressive cells as bone marrow derived suppressor cells (MDSC) or by impairing the infiltration and killing of tumor cells by cytotoxic cells at the level of the endothelial cell wall of the hypoxic tumor vessels, as summarized in the schematic abstract.

Key words: angiogenesis, hypoxia, immunosuppression, microenvironment.

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Microenvironmental hypoxia in shaping the angiogenic reaction controls tumor immune resistance

During tumor growth, endothelial cells first react to hypoxia. The early signals that reach the endothelial cells are produced as a response of the cancer cells to the hypoxic stress to which they are submitted while they develop. The hypoxia-mediated response of tumor cells to such lack of oxygen resulted in the stabilization of the transcription factor HIF-1 and the transcription cascade that starts upon binding of the heterodimer HIF-1$\alpha\beta$ to the hypoxia response element (HRE) [1]. The synthesis of the main factors which turn on angiogenesis is induced to fight the hypoxic conditions inside the tumor site. Indeed, angiogenesis is the only mechanism by which the blood can be introduced into the tumor site to bring nutriments and oxygen to the tumor cells and allow tumor progression [2]. It is, by now, clear that tumor angiogenesis is pathologic and cannot establish efficient blood flow inside the tumor [3]. This, consequently, maintains chronic hypoxia and a permanent proangiogenic state characteristic of the tumor microenvironment (TME). According to the antiangiogenesis-based approaches long used for tumor treatment, angiogenesis should be totally destroyed to starve and exhaust the tumor [4].

This strategy could not be applied as the Warburg effect brings the means for the tumor cells to overtake the lack of nutriment to produce energy by the process of anaerobic glycolysis [5]. The tumor cells set their rescue mechanism through the induction of glucose receptors which start the glycolysis cascade [6]. This energetic rescue metabolism uses the glucose-containing substrates in the tumor milieu and glucosamine/N-acetyl glucosamine pathway leading to the production of lactate and the reduction of pH of the tumor microenvironment [7].

The second reason why such an antiangiogenic strategy could not be applied results from the similarities in endpoints of both the pathologic angiogenesis and the antiangiogenic treatments, the second being the limit of the first. The antiangiogenic treatment effect pushes forward the rescue mechanisms that the cells have to raise in order to survive.

In such conditions, the antiangiogenic treatments have been shown to favor the selection of resistant tumor cells [5]. They are dedifferentiated and, in a quiescent state, remain able to survive in very hypoxic and acidic conditions [8]. We have directly demonstrated that not only are these cells

selected and their phenotype maintained by hypoxia, but they are also, in the tumor, responsible for the selective recruitment of endothelial progenitor cells as opposed to mature endothelial cells [9]. Such stem-like cells are prone to produce the whole tumor repertoire as soon as the microenvironment conditions allow it. They are turned on for differentiation and growth by cooperation with given stromal cells such as endothelial progenitor cells (EPCs) and carcinoma-associated fibroblasts (CAFS) [9, 10].

Similarly to tumor cells, endothelial cells use the glycolytic mechanism for growth even in normal, non-hypoxic, conditions [11]. In the tumor but also normoxic (physiological value of oxygen in tissues) [12] conditions the endothelial cells use the glucose and glucosamine degradation pathways to perform angiogenesis in response to stimulatory mechanisms [13]. The paracrine effect of proangiogenic molecules produced by the tumor cells such as VEGF-A, -B, -C, IL-8, PDGF, and angiopoietins 1 and 2, in response to hypoxia join, during endothelial cell angiogenic process, the hypoxic response and the glycolytic degradation process [14].

Endothelial cells coordinate the proper expansion of the vessels through the NOTCH/DLL4 mechanism of stalk/tip signaling [15]. Notch is controls activation of the tumor suppressor PTEN in the endothelial cells [16]. In the absence of PTEN phosphatase activity, angiogenic growth is constantly stimulated because of the constant PI3K/AKT/mTOR active pathway which results in anarchic tumor angiogenesis [17].

Consequently, the initial angiogenic switch coming from the hypoxic tumor cells is addressed to the non-hypoxic endothelial cells and recruits them to form vessels inside the tumor by the chemotactic effect of hypoxia response molecules such as VEGF-A and IL-8. Endothelial cells are thus recruited into the tumor by a non-hypoxia-dependent mechanism from the preexisting vessels close to the tumor and the bone marrow from where the endothelial progenitors are mobilized [15, 18].

The endothelial cells' response to the tumor proangiogenic switch is first indirectly related to hypoxia. In a second step, the process of tumor vessel formation occurs and is maintained by the hypoxic microenvironment [19]. In these conditions the endothelial reaction to produce the tumor induced angiogenesis is totally regulated and dependent on the constancy of the hypoxia parameter. We have shown that PTEN activation in endothelial cells is directly related to hypoxia reoxygenation and the maintenance of PTEN activation is a key condition which allows vessel changes from pathologic to stably normalized [19]. The importance of angiogenesis normalization was later confirmed, inhibiting the glycolytic activity of the endothelial metabolism, leading to the same anti-metastatic effects [20].

At the endothelial cell level normalization translates into a series of rearrangements and differential expression of antigens as well as secreted molecules playing a strong role in the immunomodulation. Under hypoxic conditions the molecules expressed on the endothelial cell surface participate in the intercellular recognition with cancer cells as well as with immune cells. As such the endothelial barrier not only helps the intravasation and further dispersion of metastatic cells but also modulates the adhesion properties and extravasation of immunocompetent cells and/or immunosuppressive cells. The resulting cellular and molecular composition of the tumor microenvironment allows a relative classification of cancers for further personalized immunotherapeutic protocols [21].

The endothelial reaction in hypoxia contributes to selection, differently than in normoxia, of the immune cells' entry inside the tumor. We could show that although the direct interaction of NK cells with endothelial cells [22] is increased upon NK cell activation by IL-2 [23] as well as IL-15 and IL-27 [24], hypoxia modulates the recognitions in an opposite manner by reducing NK cell adhesion to the tumor endothelium in the mammary carcinoma [10], similarly to the case of multiple myeloma-associated endothelial cells (Fig. 1) [24].

Contributing to the hypoxia-dependent immunosuppressive effect of tumor angiogenesis, the expression of soluble molecules such as VEGFs exerts a strong chemoattraction towards efficient suppressive cells such as MDSCs, as described in detail below. Immune cells can express VEGF receptors as $CD4^+$ forkhead box protein P3 $(FOXP3)^+$ regulatory T cells which are chemoattracted by VEGF inside the tumors, where they suppress the anti-tumor immune response by CTLs and NKs [25]. Vessels normalization reduces it [18]. Linked to this, endothelial cells in the tumor are ruled for their immunosuppressive effect to the reduction of PTEN activation. The tumor suppressor effect indeed controls PI3K activation, which activates the mammalian target of rapamycin (mTOR), which is stimulated in a hypoxic and nutrient-poor environment. PTEN antagonizes the PI3 kinase pathway mainly, but also has phosphatase-independent functions. In cancer, the PTEN status determines the response to chemotherapy through the normalization effect of vessels on endothelial cells, highlighting the advantage of monitoring PTEN expression and activity and developing PTEN-targeted therapies. PTEN inactivation by hypoxia has a direct immunosuppressive effect by the expression of immune checkpoint molecules as PD-L1 and PD-L2 on the endothelial cell surface [26–29]. Activity of immune checkpoints' PD-L1 expression is directly involved in the reduced activity of NK cells which as T cells are PD1+ [30] and PD-L1 expression and the immunosuppressive effect is under the control of PTEN [31].

Therefore, endothelial cells in the hypoxic tumor site establish a physical barrier that prevents homing of effective tumor-rejecting cells such as NK cells and CTLs, and suppresses effector lymphocytes through the recruitment and activation of immunosuppressive cells such as myeloid-derived suppressor cells, tolerogenic monocytes and T regulatory cells. The anti-tumor strategies which aim at endothelial PTEN as a molecular target may open new adjuvant approaches for immunotherapy.

Role of microenvironmental hypoxia in shaping tumor MDSC recruitment and immunosuppression

It has been well documented in the recent decade that various tumors were able to develop immune es-

cape mechanisms dealing with structural and functional changes both in tumor and stroma cells, leading finally to the inability of even activated effector immune cells to reject the tumor [32]. These mechanisms were reported to include i) alterations of MHC class I expression and components of the antigen processing machinery [33, 34], ii) intensive, long-term secretion of inflammatory and immunosuppressive factors such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, interleukin (IL)-1β, IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), reactive oxygen and nitrogen species or prostaglandins [35, 36], and iii) hypoxic conditions inducing the expression of transcription factor hypoxia inducible factor (HIF)-1α [37]. In this regard, tumor cells were found to express HIF-1α released chemokines such as CCL2, CCL3, C-X-C chemokine receptor (CXCR)-4, CCL5, CCL7, CCL8, CCL9, and CXCL12, as well as growth factors such as CSF-1, macrophage stimulating protein (MSP), GM-CSF, semaphorin 3A and TGF-β, that will help attract monocytes from the circulation [37, 38].

It has been demonstrated in numerous reports that myeloid-derived suppressor cells (MDSC) could play a critical role in the development of the immunosuppressive tumor microenvironment [35, 39–42]. This extremely heterogeneous population of immature myeloid cells representing precursors of granulocytes, macrophages, and DCs has recently attracted much attention as one of the key cells promoting tumor progression and creating the immunosuppressive tumor microenvironment. Importantly, MDSC acquire strong immunosuppressive functions that allow them to efficiently inhibit T-cell mediated anti-tumor reactivity by various mechanisms [35, 40, 41, 43]. In mice, MDSCs express Gr1 and CD11b surface molecules and consist of two major subsets: polymorphonuclear $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ (PMN-MDSCs) and monocytic $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ (M-MDSCs) [40, 44, 45]. In humans, MDSCs are characterized as $Lin^{-}HLA\text{-}DR^{-/lo}CD33^{+}$ or $Lin^{-}HLA\text{-}DR^{-/lo}CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ for PMN-MDSCs and $CD14^{+}HLA\text{-}DR^{neg/lo}$ or $Lin^{-}HLA\text{-}DR^{neg/lo}CD11b^{+}CD14^{+}CD15^{-}$ for M-MDSCs [45–47]. MDSCs have been shown to derive from bone marrow hematopoietic precursors due to the alteration of myelopoiesis by chronic inflammatory mediators such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), VEGF, IL-6, IL-1β as well as chemokines CCL2, CCL3, CCL4, CCL5 etc. [35, 36, 40, 42, 48, 49]. These factors are known to be produced both by tumor and stroma cells, and the signaling involves mainly the signal transducer and activator of transcription 3 (STAT3), preventing MDSC differentiation and promoting their proliferation [50, 51]. However, this is not sufficient for the induction of MDSC immunosuppressive activity that is provided by pro-inflammatory molecules such as interferon (IFN)-γ, IL-1β, IL-4, IL-13, TNF-α, toll-like receptor (TLR) ligands, and prostaglandin (PGE) E2 and is mediated by STAT1, STAT6 and nuclear factor (NF)-κB transcription factors as well as by cyclooxygenase (COX)-2 upregulation [35, 36, 43, 50, 52].

Studies published in recent years demonstrated that the generation, enrichment and activation of MDSCs could be mediated not only by chronic exposure to inflammatory factors but also by hypoxia typical for the tumor microenvironment [53, 54]. It was found that HIF-1α induced in the tumor microenvironment under hypoxia conditions could strongly up-regulate expression of inducible nitric oxide (NO) synthase and arginase (ARG)-1 [55, 56]. NO has been found to induce T cell apoptosis as well as the nitration of chemokines and T cell receptors (TCR) that block T cell migration and cytotoxic effects against tumor cells; finally, these molecules could inhibit production of cytokines (such as IL-2), which are crucial for T cell anti-tumor functions [35, 41, 43, 57–59]. ARG-1 activation results in the deprivation of arginine, which is important for multiple T cell functions [35, 39, 60]. In particular, the lack of arginine induces the down-regulation of TCRζ-chain expression, blocking the transmission of activation signals from the cell membrane [42, 43, 48]. Such hypoxia-related upregulation of NO production and ASRG-1 expression was reported to be mediated by the binding of HIF-1α. Such hypoxia-related upregulation of NO production and ASRG-1 expression was reported to be mediat [56]. Interestingly, miR-210 overexpression was sufficient to enhance MDSC-mediated T-cell suppression under normoxic conditions, while targeting hypoxia-induced miR-210 was sufficient to decrease MDSC function against T cells [56]. In addition to the ability to suppress T cell functions, hypoxia promotes differentiation of MDSC to immunosuppressive tumor-associated macrophages, further supporting the immunosuppressive network in the tumor microenvironment [55].

Recently, it has been demonstrated that HIF-1α (but not HIF-2α) could also strongly upregulate expression of the negative immune checkpoint molecule programmed death ligand (PD-L)-1 (also known as B7-H1 or CD274 on MDSC from tumor bearing mice [61]. The interaction between PD-L1 and its receptor PD-1 originally discovered as a protein involved in programmed cell death [62] and expressed on activated T cells [63] has been reported to downregulate the anti-tumor reactivity of T cells infiltrating tumor lesions [64, 65]. Importantly, PD-L1 expression was significantly increased not only on MDSC [63] but also on macrophages, dendritic cells, and tumor cells via direct binding of HIF-1α to a hypoxia-response element in the PD-L1 proximal promoter [61].

Hypoxic tumor cells were demonstrated to secrete various soluble factors that could condition pre-metastatic niches by recruiting $CD11b^{+}/Ly6C^{med}/Ly6G^{+}$ polymorphonuclear MDSC that inhibited NK cell functions [66]. A more hypoxic tumor microenvironment induced by tumor pericyte abnormalities leading to defective tumor vasculature has been found to increase the recruitment of MDSC to the tumor site [67]. Another mechanism of MDSC recruitment to the tumor microenvironment stimulated by HIF-1α and HIF-2α includes activation of chemokine (C-C motif) ligand (CCL) 26 production in cancer cells interacting with its receptor CXCR1 on MDSC [68]. Knockdown of CCL26 in cancer cells, and inhibition of its production by the HIF inhibitor or blockade of CXCR1 with respective neutralizing antibodies, could substantially suppress MDSC recruitment angiogenesis, and tumor growth in MDSC [68].

Hypoxia has also been reported to induce in the tumor microenvironment production of adenosine, which is known to inhibit T cell anti-tumor functions via interaction with A2 adenosine receptors on the surface of effector T cells [69–72]. Adenosine accumulation outside the cell has been reported to be induced by increased expression of ectonucleotidases CD73 and CD39 [73, 74]. Although the activation of these enzymes was attributed initially to tumor cells and regulatory T cells [71, 75, 76], tumor infiltrating MDSC have been recently demonstrated to upregulate the expression of CD73, leading to elevated adenosine production and thereby to immunosuppression [72]. Moreover, it has been found that extracellular adenosine induced MDSC expansion and stimulation through the receptor A2B [77]. High CD73 levels were detected on the surface of PMN-MDSC, and the substrate of CD73, adenosine monophosphate, could elevate immunosuppressive activity of MDSC *in vitro* [72, 78]. Importantly, the observed upregulation of CD73 on tumor-infiltrating MDSC could be induced in a HIF-1α-dependent manner similar to such upregulation reported for regulatory T cells [79].

Taken together, hypoxic conditions typical for the tumor microenvironment could not only induce the trafficking of MDSC into the tumor site but also stimulate their capacities to inhibit anti-tumor reactivity of T and NK cells by various mechanisms.

Role of microenvironmental hypoxia in shaping immune reaction and tumor resistance to killer cells

Hypoxia – a key determinant of microenvironment hostility

The crosstalk between stromal cells and malignant cells within the tumor microenvironment crucially determines the fate of tumor progression, its hostility and heterogeneity. It is well established that the tumor microenvironment (TME) supports tumor growth and limits the effectiveness of solid tumor immunotherapies by promoting neoplastic transformation and cell plasticity and by inducing tumor cell resistance to host immunity. Accumulating data suggest that hypoxic stress in the TME promotes several tumor escape mechanisms, including immune suppression and the emergence of tumor-resistant variants. Highly aggressive and rapidly growing tumors are exposed to hypoxia or even anoxia that occurs as a consequence of inadequate and/or irregular blood supply. It is widely reported that gradients of hypoxia occur in most solid tumors and cells found in hypoxic regions are associated with the most aggressive and therapy-resistant fractions of the tumor. Under hypoxia or pseudohypoxia, cells activate a number of adaptive responses coordinated by various cellular pathways. Despite the ubiquity and importance of hypoxia responses, little is known about the impact of hypoxic stress on the anti-tumor cytotoxic immune response.

Hypoxia induces resistance to cell-mediated cytotoxicity

Although the advent of new immunotherapeutic approaches has improved the survival of many patients with advanced malignancies, the high prevalence of non-responders also provides a strong reminder that we possess only a partial understanding of the events underlying the immune resistance of tumors. The ultimate goal of most cancer immunotherapy strategies is to induce a strong cytotoxic T lymphocyte (CTL) response. The prevailing view is that the generation of a sufficiently high frequency of CTL response will result in tumor regression. However, it has become increasingly apparent in both preclinical models and patient trials that tumors can efficiently evade or inactivate even substantial immune responses by denying T cells access to the tumor, by establishing a metabolically hostile microenvironment, and through selection of immune-resistant tumor cell variants. In this regard, we showed that hypoxia induced tumor resistance to CTL-induced killing [80]. We next provided evidence indicating that hypoxia-induced autophagy impairs CTL-mediated tumor cell lysis by regulating phospho-STAT3 in target cells [81]. Additionally, boosting the CTL response, using a TRP-2-peptide vaccination strategy, and targeting autophagy in hypoxic tumors improves the efficacy of this cancer vaccine and promotes tumor regression *in vivo* [81]. More recently, we have reported that attenuation of miR-210 in hypoxic

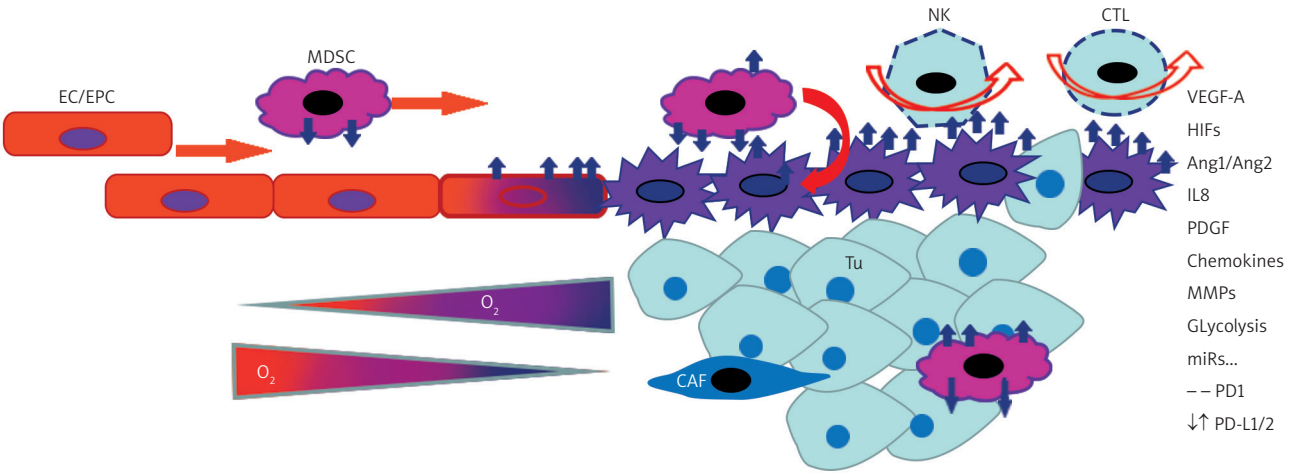

Fig. 1. Hypoxia vs normoxia in the tumor microenvironment balancing the immunosuppressive cells and cytokine production

cells can significantly restore susceptibility to autologous CTL-mediated lysis, independent of tumor cell recognition and CTL reactivity [82]. A comprehensive approach using transcriptome analysis, Argonaute protein immunoprecipitation and a luciferase reporter assay revealed that the genes *PTPN1*, *HOXA1* and *TP53I11* were miR-210 target genes regulated in hypoxic cells. Further analysis showed that silencing of *PTPN1*, *HOXA1* and *TP53I11* dramatically decreased tumor cell susceptibility to CTL-mediated lysis [82]. We have also demonstrated that hypoxic tumor cells can escape NK-mediated immune surveillance by activating autophagy under hypoxia [83, 84]. Janji *et al.* [83, 84] have shown that Granzyme B is selectively degraded upon activation of autophagy in hypoxic cells, thereby inhibiting NK-mediated target cell apoptosis [84]. More recently, the role of autophagy in regulating NK-mediated immune responses was investigated in a clear cell renal cell carcinoma cell model harboring mutation in the *VHL* gene and known to be resistant to NK-mediated killing [85].

Conclusions

The novel immunotherapeutic approaches have provided durable remission in a significant number of cancer patients with cancers previously considered rapidly lethal. Nonetheless, the high degree of non-responders, and in some cases the emergence of resistance in patients who do initially respond, represents a significant challenge in the field of cancer biology and immunotherapy.

Besides the several mechanisms known to alter the immune responses to tumors, the presence of intratumoral hypoxia is significantly associated with increased risk of treatment failure, invasion, metastasis, and patient mortality. This is, in part, due to inappropriate local immune reaction and resistance of hypoxic tumor cells to cytotoxic treatments including immune cell-mediated cytotoxicity.

Since intratumoral hypoxia has long been considered as a driving force of tumor progression and to play a key role in remodeling the tumor stroma and favoring the emergence of tolerance, immune suppression and tumor resistance, efforts to incorporate components of the hypoxic microenvironment are necessary to guide the successful design of future cancer immunotherapeutic approaches. Targeting the hypoxic tumor microenvironment to awaken or reawaken immune cells, or to redirect it from a pro-tumor to an anti-tumor state, is at present a pertinent strategy.

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