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AUTHOR'S VIEW

"Suffocating" tumors by blocking adaptation to hypoxia: a new headway in melanoma immunotherapy

Bassam Janji[a] and Salem Chouaib[b,c]

[a]Tumor Immunotherapy and Microenvironment (TIME) Group, Department of Oncology, Luxembourg Institute of Health (LIH), Luxembourg City, Luxembourg; [b]Inserm Umr 1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, Villejuif, France; [c]Thumbay Research Institute of Precision Medicine, Gulf Medical University, Ajman, UAE

ABSTRACT

We recently reported that inhibiting Hypoxia-inducible Factor-1α (*Hif1a*) transcriptional activity improves melanoma immunotherapy by driving immune cells into the tumor microenvironment (TME). This Author's View provides additional perspectives on how hypoxia inhibitors combined with immunotherapy can be used as innovative approaches to improve the therapeutic benefit of melanoma patients.

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Given its role in tumor resistance to therapies, hypoxia is an important target in cancer treatment. However, deploying hypoxia inhibitors in the clinic requires a full understanding of hypoxia signaling. After 30 years of research on the mechanisms by which cells adapt to hypoxia, William G. Kaelin Jr., Sir Peter J. Ratcliffe, and Gregg L. Semenza have been awarded the Nobel Prize in Medicine 2019. Their seminal discoveries have contributed to the development of strategies to target hypoxia in tumors using hypoxia-activated prodrugs or blocking the transcription activity of hypoxia-inducible factors (*HIFs*).

The wealth of clinical and preclinical data accumulated over a decade underlined the central role of hypoxia in impairing the efficacy of cancer immunotherapy.[1] Hypoxia modulates the immune landscape of tumors and harms the tumor-killing functions of immune cells.[2] Depending on the hypoxia grade, and regardless of their presence in the tumor microenvironment (TME), immune cells are unable to fulfill their cytotoxic function and may be corrupted to support tumor growth. Therefore, developing hypoxia inhibitors has inspired significant interest in combination with immunotherapy. The ultimate aim of such a combination is to extend the therapeutic benefit of cancer immunotherapy approaches to a large number of cancer patients. Indeed, clinical data showed that the remarkable benefit of immune checkpoint blockade (ICB)-based immunotherapy was only observed in a low proportion (30%) of melanoma patients, while the majority of patients have a short-term benefit or no benefit at all. Although the low response rate to ICB is not clearly attributed to hypoxia, it should be highlighted that melanoma contains large regions of hypoxia and anoxia (estimated to be up to 50–60%).[3] Hypoxia contributes to many aspects of melanoma development and progression by inducing melanocyte transformation[4] and increasing the heterogeneity of melanoma cell populations in a *HIF1A*-dependent manner.[5] Moreover, hypoxia resulted in the activation of signaling pathways involved in cancer cell invasiveness and epithelial-to-mesenchymal transition (EMT).[6, 7] Furthermore, hypoxia directly or by inducing metabolic shifts affects the function and the trafficking of immune cells to the TME. Therefore, there is a pressing need to better understand how hypoxia affects antitumor immunity and how it can be manipulated to improve the treatment outcome of immunotherapy.

We investigated the impact of targeting the transcriptional activity of *Hif1a* on improving the immunotherapy benefit in a melanoma mouse model. By CRISPR/Cas9, we deleted in *Hif1a* the domain responsible for its interaction with Hypoxia-inducible Factor-1β (*Arnt*) and generated B16-F10 melanoma cells expressing truncated *Hif1a* (termed *Del-Hif1a*) unable to form a heterodimer with *Arnt*. We showed that *Del-Hif1a* is transcriptionally inactive under hypoxia in B16-F10 cells and corresponding tumors.

In the immunocompetent, but not in immunocompromised, there was a significant reduction of tumor growth and weight as well as improvement of survival in mice bearing Del *Hif1a* tumors compared to those bearing full-length (Fl) *Hif1a* tumors. These data highlight that blocking the transcription activity of *Hif1a* impacts tumor development by involving the host immune system. Profiling the immune landscape and the chemokine network of tumors revealed a significant increase in the infiltration

CONTACT Bassam Janji bassam.janji@lih.lu Tumor Immunotherapy and Microenvironment (Time) Group, Department of Oncology, Luxembourg Institute of Health, 84, Val Fleuri, L-1526, Luxembourg City, Luxembourg

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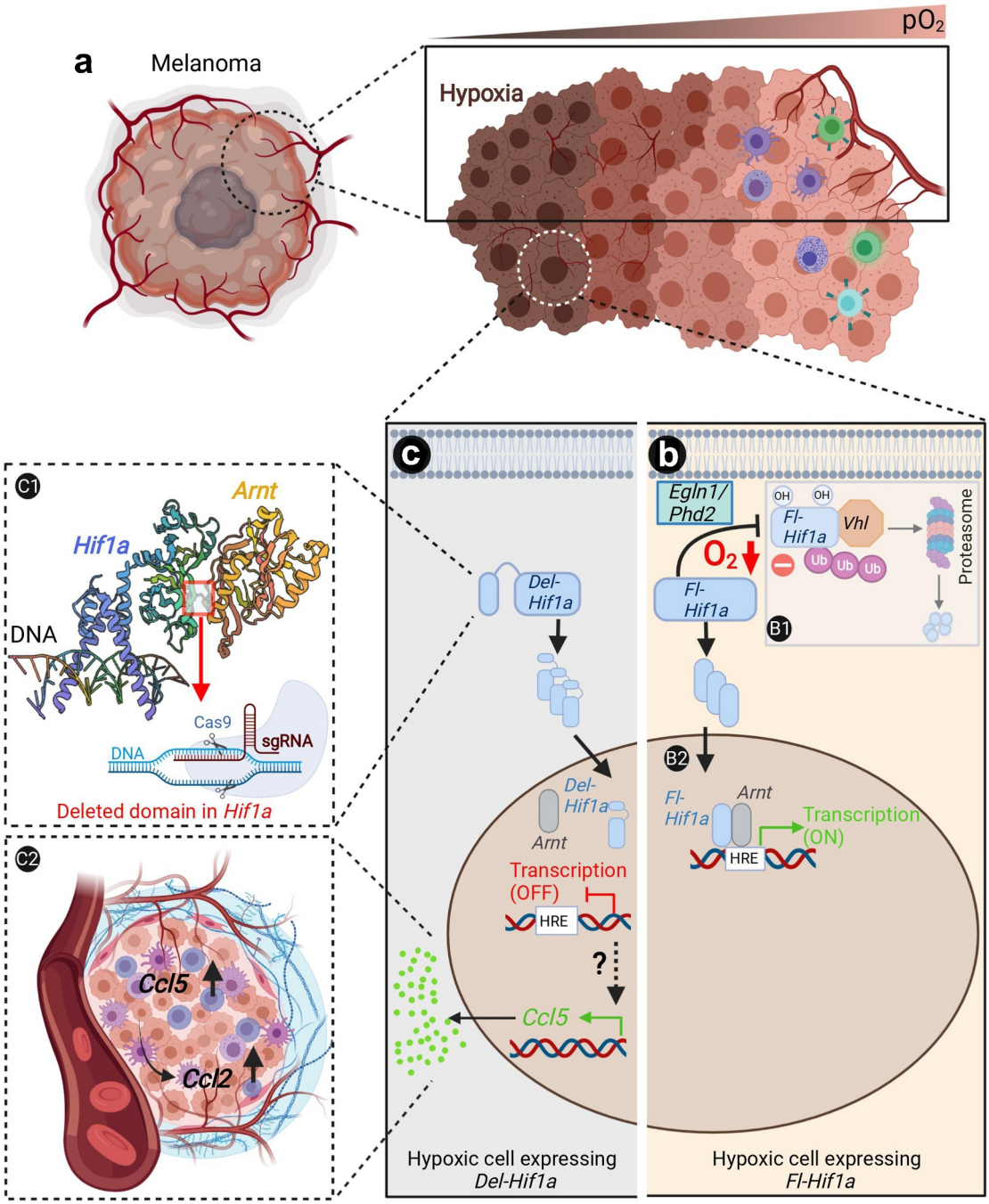

Figure 1. Targeting the transcription activity of *Hif1a* recruits immune cells to the melanoma tumor microenvironment. **a:** Melanoma tumors are characterized by the presence of poorly oxygenated areas [Oxygen pressure (pO_2) less than 8 mmHg]. These hypoxic areas result from an imbalance between low O_2 supply due to an abnormal vascularization and high O_2 consumption by tumor cells. Hypoxic areas are poorly infiltrated by cytotoxic immune cells. **b:** Under a low pO_2 or hypoxic microenvironment, the enzymatic activity of prolyl hydroxylase domain-2 (*Egln1/Phd2*) protein is inhibited. Such inhibition leads to blocking the prolyl hydroxylation (OH) of *Hif1a*, inhibition of both *Hif1a*-dependent ubiquitination (Ub) and Von Hippel-Lindau (*Vhl*) interaction, and subsequent proteasomal degradation of *Hif1a* **(B1)**. Consequently, *Hif1a* accumulates in the cytoplasm, translocates to the nucleus, and forms a heterodimer with hypoxia-inducible Factor-1β (*Arnt*). The *Hif1a*/*Arnt* complex binds to the hypoxia-responsive element (HRE) and activates transcription of several downstream target genes **(B2)**. **c:** Inhibiting the transcription activity of *Hif1a* can be achieved by deleting the domain responsible for the formation of a heterodimer with *Arnt* using CRISPR/Cas9 gene-editing technology **(C1)**. In hypoxic cells expressing deleted *Del-Hif1a*, the formation of heterodimer *Hif1a*/*Arnt* is prevented, and the transcription activity of *Hif1a* is blocked. In these cells, the expression of the pro-inflammatory (C-C motif) ligand 5 chemokine (*Ccl5*) is activated by a mechanism that is not fully understood. The release of *Ccl5* by tumor cells expressing *Del-Hif1a* increases the recruitment of cytotoxic immune cells in the microenvironment, which subsequently release of (C-C motif) ligand 2 chemokine (*Ccl2*) to support the establishment of inflammatory signature in melanoma **(C2)**.

of CD45+, Natural killer (NK), CD4+, and CD8+ cells into *Del-Hif1a* tumors compared to *Fl-Hif1a* tumors. Such infiltration was associated with an increased release of pro-inflammatory (C-C motif) ligand 5 and 2 chemokines (*Ccl5* and *Ccl2*) in the TME of *Del-Hif1a* tumors (Figure 1).

Although much remains to be learned about the mechanism(s) responsible for the infiltration of immune cells into *Hif1a*-defective tumors, we believe that analyzing the quality and integrity of the blood microvascular network could provide more insight into how immune cells have infiltrated the TME. Nevertheless, our data revealed that inhibiting the transcriptional activity of *Hif1a* would enhance the therapeutic benefit of immunotherapy in melanoma. To provide the experimental evidence of this concept, we used acriflavine, reported as a drug suppressing the transcriptional activity of *Hif1a* by preventing *Hif1a*/*Arnt* dimerization, hence mimicking our B16-F10 melanoma cell model. While monotherapy based on anti-PD-1 or TRP-2 vaccination has no or moderate impact on tumor growth, combining acriflavine with both anti-PD-1 and TRP-2 vaccination suppressed B16-F10 tumor growth, presumably by releasing *Ccl5* and *Ccl2* involved in driving cytotoxic immune cells to the TME. The translational aspect of our study is underscored by data generated from melanoma patients described in the TCGA database. Compared to patients having high hypoxia scores, those exhibiting low hypoxia scores express a high level of *CCL5*, which correlates with increased expression of NK, C3, CD4, and CD8 cell markers and improved survival.[8]

The expression of *Hif1a* is closely correlated with glycolytic products (pyruvate, lactate).[9] Under hypoxia, inhibiting the *Mif* (Macrophage Migratory inhibition Factor)/*Cd74* axis that reduces lactate production in *Ctla4* resistant melanoma cells significantly reduces the expression of *Hif1a*.[10] Therefore, it would be interesting to evaluate the expression of glycolytic products in the microenvironment of *Del-Hif1a* tumors and assess the signaling pathway of *Mif*/*Cd74*. In this context, we believe that inhibitors of the *Mif*/*Cd74* pathway can be used as hypoxia modulators to improve the benefit or restore the sensitivity to *Ctla4*-based cancer immunotherapy in melanoma.

Overall, our results highlight the critical role of hypoxia in the establishment of immune-suppressive TME. We believe the hypoxic status of tumors should be considered in immunotherapy protocols in the clinic. This study will contribute to designing an innovative combination with hypoxia inhibitors that might create tremendous enthusiasm in melanoma therapy.

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ORCID

Bassam Janji http://orcid.org/0000-0002-9763-0943

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