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1 **Hypoxia as a driver of resistance to immunotherapy**

2

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24

25 **Abstract**

26 Hypoxia, a hallmark of solid tumors, determines the selection of invasive and aggressive  
27 malignant clones displaying resistance to radiotherapy, conventional chemotherapy or

28 targeted therapy. The recent introduction of immunotherapy, based on immune checkpoint  
29 inhibitors (ICPIs) and chimeric antigen receptor (CAR) T-cells, has markedly transformed the  
30 prognosis in some tumors but also revealed the existence of intrinsic or acquired drug  
31 resistance. In the current review we highlight hypoxia as a culprit of immunotherapy failure.  
32 Indeed, multiple metabolic cross talks between tumor and stromal cells determine the  
33 prevalence of immunosuppressive populations within the hypoxic tumor microenvironment  
34 and confer upon tumor cells resistance to ICPIs and CAR T-cells. Notably, hypoxia-triggered  
35 angiogenesis causes immunosuppression, adding another piece to the puzzle of hypoxia-  
36 induced immunoresistance. If these factors concurrently contribute to the resistance to  
37 immunotherapy, they also unveil an unexpected Achille's heel of hypoxic tumors, providing  
38 the basis for innovative combination therapies that may rescue the efficacy of ICPIs and CAR  
39 T-cells. Although these treatments reveal both a bright side and a dark side in terms of  
40 efficacy and safety in clinical trials, they represent the future solution to enhance the efficacy  
41 of immunotherapy against hypoxic and therapy-resistant solid tumors.

42

43 **Keywords:** drug resistance; immune checkpoint inhibitors; CAR T-cells; tumor hypoxia

44

## 45 **1. Introduction: the impact of hypoxia on tumors and response to therapy**

46 Notwithstanding the compensatory neo-angiogenesis, hypoxic areas are a hallmark of rapidly  
47 growing tumors, because of the chaotic architecture of the neo-vessels, and the tendency to  
48 undergo vascular collapse under the pressure of growing tumor and stroma (Gacche &  
49 Assaraf, 2018; Huijbers et al., 2016; Kleibeuker et al., 2012; Nussenbaum & Herman, 2010).  
50 Hypoxic areas are heterogeneously distributed within the tumor bulk, because the continuous  
51 alternation between vessel formation and collapse determines conditions of cycling hypoxia

52 and re-oxygenation (Vaupel et al., 2004). While the physiological pressure of O<sub>2</sub> (pO<sub>2</sub>) in  
53 normal tissues is between 1 and 11%, the mean tumor pO<sub>2</sub> is below 2% (Li Petri et al., 2020;  
54 Mckeown, 2014; Muz & Azab, 2015; Raz et al., 2014). Depending on pO<sub>2</sub>, hypoxic  
55 oscillations, concomitant shortage of other nutrients such as glucose and amino acids, cancer  
56 cells growing in hypoxic areas can either slow their proliferation rate, hence undergoing  
57 necro-apoptosis, or adapt to the hypoxic conditions. This adaptation selects certain  
58 phenotypic features – increased cell cycling, migration, stemness, epithelial mesenchimal  
59 transition (EMT), resistance to stress – that confer a selective advantage over the less  
60 adaptable clones (Erin et al, 2020; Santoro et al, 2017). This natural selection renders the  
61 tumor more aggressive and difficult to be eradicated by radiotherapy and chemotherapy  
62 (Gacche & Assaraf, 2018; Huijbers et al., 2016; Kleibeuker et al., 2012; Suh et al., 2014).

63 The adaptation to hypoxia is coordinated by the up-regulation of the hypoxia-inducible factor  
64 (HIF) proteins, a family of transcription factors sensing intra-tissue pO<sub>2</sub> and controlling more  
65 than 200 genes (Gacche & Assaraf, 2018; Godet et al., 2019; Raz et al., 2014; Semenza,  
66 2013b). HIF proteins are heterodimers, composed of the O<sub>2</sub>-sensitive  $\alpha$  subunits (namely  
67 HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ ), which are degraded under normoxia conditions, and the stable,  
68 O<sub>2</sub>-insensitive  $\beta$  subunit (Kaelin & Ratcliffe, 2008). Most of the transcriptional programs  
69 driven by hypoxia in tumors are controlled by HIF-1 $\alpha$  and HIF-2 $\alpha$ , while the role of HIF-3 $\alpha$   
70 is still poorly known (Duan, 2020). Under normoxic conditions,  $\alpha$  subunits are hydroxylated  
71 on proline 402 and 564 by the O<sub>2</sub>-depending prolyl hydroxylase dioxygenases (PHDs)  
72 (Semenza, 2001). This process creates a binding site for the von Hippel Lindau tumor  
73 suppressor protein (pVHL), which promotes the ubiquitination and proteasome degradation of  
74  $\alpha$  subunits (Kaelin, 2008; Shen and Kaelin, 2013). Conversely, under hypoxia conditions, the  
75 activity of PHDs is low and  $\alpha$  subunits are stabilized up to one hour: they hence  
76 heterodimerize with  $\beta$  subunits and translocate as active transcription factors to the nucleus.

77 Also miRNAs (Pugh & Ratcliffe, 2017), oncogenic pathways active in tumors - as the  
78 Ras/phosphatidylinositol 3'-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)  
79 pathway (Semenza, 2013) -, inactivating mutations in the oncosuppressor TP53 (Sethi, 2019),  
80 BRCA1 DNA repair associated (BRCA1) or tensin homolog deleted on chromosome 10  
81 (PTEN) (Li et al., 2018) proteins, intratumor reactive oxygen species (ROS), which inactivate  
82 PHDs (Kaelin & Ratcliffe, 2008), metabolites produced by the cancer associated fibroblasts  
83 (CAFs) (Assaraf et al., 2019; Raz et al., 2014) and diffused paracrinely within the tumor  
84 microenvironment (TME) such as glutamate (Briggs et al., 2016), stabilize HIF $\alpha$  in an O<sub>2</sub>-  
85 independent manner. Hence, multiple cross-talks can finely tune – either enforcing or  
86 attenuating HIF-driven programs – the response of cancer cells to hypoxia.

87 Among the main genes up-regulated by HIFs, there are the pro-angiogenic vascular  
88 endothelial growth factor (VEGF), the pro-invasive metalloproteinase 9 (MMP9) and  
89 urokinase-type plasminogen activator (uPA) factors (Schito & Semenza, 2016), several  
90 glycolytic enzymes – such as glucose transporters 1 and 3 (GLUT1 and GLUT3), hexokinase  
91 (HK), phosphofructokinase-1 (PFK1), aldolase, triose-phosphate isomerase (TPI),  
92 glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase, lactate dehydrogenase A  
93 (LDHA), pyruvate kinase M2 (PKM2) –, pyruvate dehydrogenase kinase 1 (PDK1) (Sethi et  
94 al., 2019), the amino acid transporters xCT (SLC7A11) and L-type amino acid transporter 1  
95 (LAT1/SLC7A5) (Elorza et al., 2012; Lu et al., 2015), as well as the multidrug resistance 1  
96 (mdr1) gene (Comerford et al., 2002; Li et al., 2016).

97 The coordinated up-regulation of these genes in tumor cells, CAFs, endothelial cells and  
98 immune cells as tumor-associated macrophages (TAMs), favours tumor growth, invasion and  
99 resistance to therapy. For instance, the uptake of glucose and amino acids is strongly  
100 promoted by HIF, granting excellent energy sources and building blocks for rapidly dividing  
101 cells. Hypoxia and acidosis within the TME, caused by the increased extrusion of lactic acid

102 and H<sup>+</sup> as end-product of glycolysis (Kung-Chun Chiu et al., 2019), favor the maintenance of  
103 cancer stem cells (CSCs) (Ayano Kondo et al., 2017; Corbet et al., 2014; Likus et al., 2016;  
104 Koren & Fuchs, 2016; Sharifzad et al., 2019; Taylor et al., 2015) that contribute to the self-  
105 renewal and expansion of tumor mass. CSCs growing in hypoxic conditions have an EMT  
106 phenotype (Joseph et al., 2015; Yang et al., 2016; Liu et al., 2020) and result more invasive.  
107 Also, by synergizing with the hepatocyte growth factor (HGF)/Met receptor (Rankin et al.,  
108 2014) and the VEGF/VEGF receptor (VEGFR) (Wang et al., 2020) axes, HIF-1 $\alpha$  and HIF-2 $\alpha$   
109 further enhance the invasive nature of hypoxic cells.

110 Hypoxia creates the proper conditions for a dominant resistance to multiple systemic  
111 anticancer treatments. Hypoxic tumors often display multidrug-resistance (MDR), resulting  
112 simultaneously resistant to *Vinca* alkaloids, anthracyclines, cisplatin, etoposide, actinomycin-  
113 D, 5-fluorouracil, gemcitabine and antifolates like methotrexate and pemetrexed (Doktorova  
114 et al., 2015; Li Petri et al., 2020; Raz et al., 2014). One reason explaining MDR (Kopecka et  
115 al., 2020) is the transcriptional up-regulation of genes encoding for drug efflux transporters,  
116 such as *mdr1*/ATP binding cassette (ABC) transporter B1/P-glycoprotein (ABCB1/Pgp)  
117 (Comerford et al., 2002; Dong et al., 2020; Kathawala et al., 2015; Li et al., 2016; Stark &  
118 Assaraf, 2017), MDR related protein 1/ABC transporter C1 (MRP 1/ABCC1) (Su et al, 2021;  
119 Wang et al., 2021; Zhu H et al., 2005) and breast cancer resistance protein/ABC transporter  
120 G2 (BCRP/ABCG2) (Bram et al., 2006; Bram et al., 2007; Bram et al., 2009; Ifergan et al.,  
121 2005; Shafran et al., 2005; Xiaodan He et al., 2016). Interestingly, the up-regulation of Pgp  
122 has been reported also in normoxic cells with acquired or constitutive MDR, characterized  
123 by a constitutively active HIF-1 $\alpha$ , which is stabilized by the Ras/extracellular signal regulated  
124 kinase 1/2 (ERK1/2) and RhoA/RhoA kinase axes (Kopecka et al., 2015; Kopecka et al.,  
125 2016; Rigoni et al., 2015; Salaroglio et al., 2015). The activity of pathways favoring stemness  
126 (e.g. Wnt- and Notch-dependent pathways) or cell survival – such as Ras/mitogen activated

127 kinase (MAPK)-, PI3K-, Akt/mTOR-, nuclear factor-kB (NF-kB)-dependent pathways – in  
128 hypoxia also confer chemoresistance, by preventing the apoptotic effects of chemotherapeutic  
129 agents (Doktorova et al., 2015). Indeed, the hypoxic environment selects highly resilient  
130 tumor clones, rich in anti-apoptotic proteins, such as inhibitor of apoptosis protein 3 (IAP3)  
131 and B-cell lymphoma 2 (Bcl-2) (Coffey et al., 2005; Shahar and Larisch, 2020) that are more  
132 resistant to chemotherapy. Hypoxic tumors also have a strong destabilization of TP53, caused  
133 by the down-regulation of TP53 exerted by HIF-1 $\alpha$  and HIF-2 $\alpha$ . The destabilization of TP53,  
134 coupled with the HIF-1 $\alpha$ -induced up-regulation of topoisomerase 2A (Sullivan & Graham,  
135 2009) and DNA repair machinery, such as DNA-PKs, Ku80 and Ku70 (Wirthner et al.,  
136 2008), protect cancer cells from chemotherapeutic drugs which damage DNA, such as  
137 cisplatin, anthracyclines and etoposide. The low levels of mitochondrial ROS, consequent to  
138 the reduced oxidative phosphorylation (OXPHOS) in hypoxic cells (Rohwer et al., 2010),  
139 determines lower TP53-mediated apoptosis in response to cisplatin (Cao et al., 2020; Hao et  
140 al., 2008; Stiewe & Haran, 2018).

141 The metabolic rewiring induced by HIF-1 $\alpha$  also plays an active role in resistance to  
142 chemotherapy. The acidosis characterizing hypoxic tumors (Taylor et al., 2015) neutralizes  
143 the efficacy of weak bases such as anthracyclines and many other chemotherapeutics that  
144 are protonated and entrapped within lysosomes (Assaraf et al., 2019; Guo et al., 2016;  
145 Hussein et al., 2021; Stark et al., 2020; Zhitomirsky & Assaraf, 2015; Zhitomirsky & Assaraf,  
146 2016; Zhitomirsky & Assaraf, 2017; Zhitomirsky et al., 2018). The high ratio between  
147 anaerobic glycolysis/OXPHOS-based metabolism (Kung-Chun Chiu et al., 2019) prevents  
148 the anti-cancer effects of drugs – such as 5-fluorouracil, cisplatin (Rohwer et al., 2010),  
149 doxorubicin, etoposide (Sinha, 2020), gemcitabine (Wang et al., 2019) – that exert part of  
150 their cytotoxic effects by generating mitochondrial ROS (Mai et al., 2019). The high levels of  
151 mitophagy induced by HIF-1 $\alpha$  correlate with resistance to 5-fluorouracil (Liu et al., 2009),

152 gemcitabine (Wang et al., 2019) and cisplatin (Mai et al., 2019) because mitophagy is an  
153 effective mechanism to recover ATP, building blocks and oxide-reductive cofactors, three  
154 elements that are vital for cell proliferation and resilience to exogenous stresses. Overall,  
155 hypoxia triggers several and concurrent molecular circuitries that make tumors more  
156 aggressive and resistant to chemotherapy (Figure 1).

157 The introduction of immunotherapy in the oncological treatments has improved the prognosis  
158 of patients in specific tumors, such as melanoma, non-small cell lung cancer (NSCLC) and  
159 haematological disorders, but the presence of patients unresponsive to immunotherapy has  
160 been documented as well (Dal Bo et al., 2020; Diesendruck and Benhar, 2017; Hays &  
161 Bonavida, 2019; Kon & Benhar, 2019; Leonetti et al., 2019; Pérez-ruiz et al., 2020). How the  
162 hypoxic TME impacts on the efficacy of immunotherapy, and how resistance to  
163 immunotherapy is related to hypoxia, are hot topics in the preclinical and clinical oncological  
164 research. In this review, we critically discuss the evidence suggesting a diminished efficacy  
165 of immune checkpoint inhibitors (ICPIs) and chimeric antigen receptor (CAR) T-cells in  
166 hypoxic tumors, dissecting the molecular circuitries linking hypoxia and poor efficacy of  
167 immunotherapy. We also analyze the clinical impact of this resistance and suggest possible  
168 strategies to target hypoxic and refractory tumors as novel immune-sensitizing approaches.

169

## 170 **2. The imprinting of hypoxia on tumor microenvironment reduces the efficacy of** 171 **immune checkpoint inhibitors**

172 Hypoxia may impair the efficacy of immunotherapy by acting at multiple levels. A hypoxic  
173 environment decreases the ratio between anti-tumor immune cells and immunotolerant or  
174 immunosuppressive cells. Furthermore, hypoxia directly increases the expression and activity  
175 of ICPs and ICP ligands (ICPLs) on immune-cells and tumor cells. The concurrent presence



176 of immunosuppressive cells, anergic effector cells and immunoevasive cancer cells  
177 unequivocally reduces the efficacy of ICPIs.

## 178 **2a. Hypoxia induces an immunosuppressive environment**

179 A hypoxic and acidic TME facilitates immunosuppression, by reducing the expansion of anti-  
180 tumor cells as CD8<sup>+</sup> T-lymphocytes, natural killer (NK) cells and M1-polarized TAM (de la  
181 Cruz-López et al., 2019), and/or favoring the expansion of tumor-tolerant populations, as M2-  
182 polarized TAMs, myeloid-derived suppressor cells (MDSC) and T-regulatory (Treg) cells  
183 (McDonald et al., 2016) (Figure 2).

184 Hypoxia induces apoptosis of CD8<sup>+</sup> T-lymphocytes and reduces their recruitment within the  
185 tumor bulk (Mpekris et al., 2020). Firstly, the abnormal blood vessels characteristic of  
186 hypoxic regions may reduce the recruitment of circulating T-lymphocytes. Second, the  
187 stroma of hypoxic tumors is particularly rich in collagen and is stiffer than in normoxic areas  
188 (Kuczek et al., 2018; Xu et al., 2019). Together, these physical barriers reduce the  
189 extravasation and infiltration of CD8<sup>+</sup> T-lymphocytes. Moreover, hypoxia decreases  
190 cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-2 (IL-2) (Wang et al., 2021) that  
191 support the expansion and activation of effector cells. As a result, T-lymphocytes display  
192 reduced proliferation and secretion of cytolytic factors, resulting in an attenuated anti-tumor  
193 response (Rangel Rivera et al., 2021). HIF-1 $\alpha$  also regulates the degradation of forkhead box  
194 P3 (FoxP3), a transcription factor that physiologically converts effector T-cells into Treg  
195 cells instead of Th-helper 17 (TH17) cells, reducing the anti-cancer activity of tumor  
196 infiltrating lymphocytes (TILs) (Dang et al., 2011).

197 Part of the hypoxic effect is mediated by the metabolic reprogramming characterized by  
198 increased intratumor acidosis, production of kynurenine and adenosine (Pietrobon &  
199 Marincola, 2021). The acidification produced by surface carbonic anhydrase (CA) IX and  
200 XII, Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> antiporter or Na<sup>+</sup>/H<sup>+</sup> exchanger, under the transcriptional control of HIF-1 $\alpha$

201 (Boedtkjer, 2019; Brand et al., 2016; Cardone et al., 2019; Sedlakova et al., 2014), reduces  
202 the survival and the cytolytic activity of CD8<sup>+</sup> T-lymphocytes and NK cells (Brand et al.,  
203 2016). Moreover, at low pH, the nuclear factor of activated T-cells (NFAT), which promotes  
204 T-cell differentiation and activation, is blunted (Brand et al., 2016). Lactate, produced either  
205 by tumor cells or immune-infiltrating cells, also impairs the maturation of dendritic cells  
206 (DCs) (Sangsuwan et al., 2020) that support CD8<sup>+</sup> T-lymphocytes expansion. Sometimes,  
207 vicious regulatory loops occur; for instance, M1-polarized TAMs and DCs (Kopecka et al.,  
208 2020) are high producers of lactate in hypoxic tumor areas. Contrarily to the expectations,  
209 blocking the lactate exporter monocarboxylate transporter 4 (MCT4) in these cells increases  
210 the M2/M1 ratio and reduces the ability of DCs to recruit anti-tumor cytotoxic CD8<sup>+</sup> T-  
211 lymphocytes (Sangsuwan et al., 2020). Therefore, potential antitumor strategies relieving the  
212 hypoxia-associated acidosis may act as a double edge sword, paradoxically favouring intra-  
213 tumor immunosuppression. Moreover, anti-tumor CD8<sup>+</sup> T-lymphocytes are strongly  
214 glycolytic in hypoxic tumors and export lactate through MCT1 (Cretenet et al., 2016).  
215 However, the high production and efflux of lactate by tumor cells leads to the accumulation  
216 of this metabolite within the hypoxic TME: this unfavourable gradient slows down the efflux  
217 of lactate from CD8<sup>+</sup>T -lymphocytes, causing an intracellular acidosis that reduces cytolytic  
218 activity and secretion of anti-tumor cytokines (Fischer et al., 2016).

219 In hypoxic TME, glucose supply from blood is low and there is a strong competition for  
220 glucose and glutamine between tumor cells and lymphocytes. HIF-1 $\alpha$  increases the  
221 expression GLUTs as well as glutaminase 1, which catabolizes glutamine into glutamate, in  
222 tumor cells (Belisario et al., 2020), depriving rapidly proliferating T-lymphocytes of the key  
223 metabolites necessary to fuel their activity (Wood et al., 2007; Xiang et al., 2019). Notably,  
224 tumor-associated programmed-death-1 ligand (PD-L1), which is the main ligand of the ICP  
225 programmed death-1 (PD-1), increases the glycolysis in cancer cells by recruiting its

226 downstream effectors Akt/mTOR. Anti-PD-L1 antibodies reduce the glycolytic rate of cancer  
227 cells, sparing glucose for CD8<sup>+</sup> T-lymphocytes. In this way, ICPIs achieve two goals: they  
228 reduce the competition for glucose between tumor cells and T-lymphocytes, and relieve the  
229 functional energy of lymphocytes induced by the interaction between the PD-1 and PD-L1  
230 (Chang et al., 2015). By contrast, PD-1 present on T-lymphocytes forces them to use fatty  
231 acid  $\beta$ -oxidation (FAO) as main fuel pathway alternative to glycolysis, as demonstrated by  
232 the increase in the lipolytic enzyme adipose triglycerides lipase (ATGL) and of the FAO-  
233 limiting enzyme carnitine palmitoyl transferase 1A (CPT1A) in PD-1-expressing  
234 lymphocytes (Patsoukis et al., 2015). This metabolic rewiring that makes T-lymphocytes less  
235 tumoricidal, is reversed by anti-PD-1 antibodies, which turn off FAO and increase glycolytic  
236 rate (DePeaux & Delgoffe, 2021), restoring a metabolic phenotype more convenient for  
237 activated and proliferating T-cells.

238 Another competition between tumor cells and T-lymphocytes occurring in hypoxia is for  
239 tryptophan, an essential amino acid that supports T-cell proliferation (Liu et al., 2019). HIF-  
240  $1\alpha$  up-regulates the indoleamine 2,3 dioxygenase (IDO) enzyme in tumor cells and CAFs.  
241 IDO catabolizes tryptophan, leading to the depletion of this amino acid and to the production  
242 of kynurenine, which suppresses T-cell activity (Liu et al., 2019).

243 Adenosine is another immunosuppressive metabolite mainly produced by CD39 and CD73,  
244 two ecto-nucleotidases abundantly expressed on CAFs (Giatromanolaki et al., 2020). Not  
245 only CD39 and CD73 (Eltzschig et al., 2009; Petruk et al., 2021), but also adenosine receptor  
246 A2 on T-lymphocytes (Leone et al., 2018), are up-regulated in hypoxia. Adenosine impairs  
247 the activity of NK cells (Sitkovsky et al., 2014; Wang et al., 2021), induces apoptosis of T-  
248 cells and increases the expression of PD-1, cytotoxic T-lymphocyte associated protein 4  
249 (CTLA-4) and lymphocytic activating-3 (LAG-3) ICPs (Leone et al., 2018), reducing the  
250 anti-tumor potential of CD8<sup>+</sup> T-lymphocytes. Moreover, HIF- $1\alpha$  and HIF- $2\alpha$ , or the

251 knockdown of pVHL in T-lymphocytes, directly up-regulate ICPs, such as PD-1, CTLA-4  
252 and LAG-3 (Chen et al., 2015; Cubillos-Zapata et al., 2017; Doedens et al., 2013; Koh et al.,  
253 2016). At the same time, HIF-1 $\alpha$  up-regulates PD-L1 on stromal cells (Cubillos-Zapata et al.,  
254 2017; Koh et al., 2016), enforcing the immunosuppression induced by the PD-1/PD-L1 axis.

255 Apart from the effects on T-lymphocytes, HIF-1 $\alpha$  also impairs the efficiency of NK cells, by  
256 preventing the increase of the major receptors activated in NK cells, as NKp46, NKp30,  
257 NKp44, and NKG2D (Balsamo et al., 2013). On the other hand, hypoxic tumors are enriched  
258 in immunosuppressive populations, because cancer cells with high levels of HIF-1 $\alpha$  highly  
259 secrete chemokines, as C-C motif chemokine ligand 5 (CCL5), CCL28 and C-X-C motif  
260 chemokine ligand 12/stromal cell-derived factor (CXCL12/SDF-1) that recruit Treg cells  
261 (Pietrobon & Marincola, 2021) and MDSCs (Du et al., 2008; Lin et al., 2012). CCL28 is one  
262 of the main recruiter of Treg cells in hypoxic ovarian and liver cancers (Vignali et al., 2008):  
263 the progressive enrichment with Treg cells, which in turn secretes immunosuppressive  
264 cytokines as transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-10, inhibits CD8<sup>+</sup>T-lymphocyte  
265 cytotoxic activity, and promotes the expansion of anergic clones T-cells, rich of CTLA-4 and  
266 LAG-3 (Vignali et al., 2008). Also the TGF- $\beta$  produced by tumor cells has a role in attracting  
267 Treg cells and reducing M1 TAMs in hypoxia: in melanoma, this mechanism has been  
268 attributed to the increased signalling downstream Nanog that enhances the paracrine  
269 production of TGF- $\beta$  (Pietrobon & Marincola, 2021; Wang et al., 2021).

270 MDSCs are other group of immunosuppressive cells abundant in the hypoxic TME which  
271 reduce CD8<sup>+</sup> T-lymphocyte activation by releasing the inhibitory cytokines IL-10 and IL-6.  
272 In the same time, HIF-1 $\alpha$  increases PD-L1 and PD-L2 on MDSCs (Noman et al., 2014),  
273 making these cells a sort of immunosuppressive hub.

274 Both HIF-1 $\alpha$  and HIF-2 $\alpha$  favour macrophage infiltration (Imtiyaz et al., 2010): the main  
275 mechanism seems to be due to the up-regulation of the HIF target gene PDK1, a moonlight

276 enzyme that controls the anaerobic glycolysis/OXPHOS metabolic flux and stimulates the  
277 migratory capacity of macrophages (Semba et al., 2016). Among TAMs, M2-polarized  
278 macrophages predominate in hypoxic tumors, because HIF-1 $\alpha$  (Raggi et al., 2017) and lactate  
279 (Mu et al., 2018) activate a transcriptional program favouring the polarization of M1 to M2.  
280 By producing platelet-derived growth factor (PDGF), VEGF and TGF- $\beta$ , M2 TAMs promote  
281 tumor progression, neoangiogenesis and immunosuppression (Lewis et al., 2016). Moreover,  
282 the hypoxia-induced production of CCL20 stimulates macrophages to secrete kynurenine,  
283 thus impairing CD8<sup>+</sup> T-lymphocyte activation (Lequeux et al., 2019). Moreover, the  
284 phagocytic capacity of macrophages is impaired under hypoxia, in consequence to the up-  
285 regulation of the “do not eat me” molecule CD47 on tumor cells, elicited by HIF-1 $\alpha$   
286 (Veillette & Chen, 2018; H. Zhang et al., 2015).

287 Overall, these experimental evidence are indicative of the strongly immunosuppressive  
288 environment characteristic of hypoxic tumors. Under these conditions, the activity of  
289 cytotoxic T-cells, including CAR T-cells, is markedly diminished. Such T-cell anergy is the  
290 premise for the low efficacy of ICPIs. The metabolic cross-talks between tumor and TME-  
291 associated cells, as well as the competition for essential energy sources and building blocks,  
292 also reduce the anti-tumor potential of T-lymphocytes, further decreasing the ability of ICPIs  
293 to prevent T-lymphocytes' exhaustion.

## 294 **2b. Hypoxia renders cancer cell more immunoresistant**

295 Beside decreasing the ratio between effector and immunotolerant cells, hypoxia directly  
296 modulates expression and activity of ICPs and their ligands, exploiting pleiotropic circuitries  
297 in tumor and immune cells (Figure 3).

298 Specific pathways activated by hypoxia are also pathways that control the expression of  
299 ICPLs or act downstream ICPLs in tumor cells. For instance, the *PD-L1* promoter has a  
300 hypoxia response element (HRE) and is a direct target of HIF-1 $\alpha$ , as proved by the down-

301 regulation of PD-L1 in oral squamous cell carcinoma (OSCC) and adenocarcinoma cells  
302 treated with HIF-1 $\alpha$  inhibitors (Chen et al., 2015; Koh et al., 2016; Noman et al., 2014). In  
303 addition, the activation of NF- $\kappa$ B (Antonangeli et al., 2020), elicited by inflammatory  
304 cytokines as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or IFN- $\gamma$  (Asaka Kondo et al., 2010), or the  
305 inactivation of PTEN (Kohnoh et al., 2016), two conditions often associated with a  
306 constitutively activated HIF-1 $\alpha$  (Semenza, 2013b), up-regulate PD-L1. In parallel, the  
307 inactivation of PTEN triggers the EMT program, making tumor cells more invasive, more  
308 resistant to chemotherapy and less susceptible to T-lymphocyte killing (Kohnoh et al., 2016).  
309 Curiously, different reports have shown that PD-L1 is up-regulated during EMT and that PD-  
310 L1 signaling maintains EMT. These observations suggest that the EMT program and PD-L1  
311 are reciprocally regulated, and contribute concurrently to tumor resistance (Chen et al., 2015;  
312 Jiang & Zhan, 2020; Song et al., 2013). As proof of concept, the downregulation of PD-L1  
313 increases the sensitivity to cisplatin (Li et al., 2012), although it has not been investigated if  
314 the mechanisms depend on the reduced amount of HIF-1 $\alpha$  and/or reduced EMT program.

315 PI3K/mTOR is another point of intersection between HIF-1 $\alpha$  and PD-L1: indeed, PI3K  
316 increases the transcription of HIF-1 $\alpha$  gene, either in a mTOR-dependent or independent way  
317 (Pietrobon & Marincola, 2021). On the other hand, PD-L1 activates mTOR, promoting cell  
318 survival and cell cycle progression (Clark et al., 2016), and fueling a feed forward circuit  
319 increasing HIF-1 $\alpha$  levels. Consistently, the reduction of PI3K, Akt or mTOR results in  
320 decreased PD-L1 amount in NSCLC, glioma, prostate and breast cancer (Crane et al., 2009;  
321 Lastwika et al., 2016; Parsa et al., 2007), as well as in aggressive melanomas, resistant to v-  
322 raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors (Jiang et al., 2013).  
323 PI3K/Akt/mTOR-dependent pathways increase PD-L1 at transcriptional or post-  
324 transcriptional level. For instance, while in OSCC the PI3K/Akt/mTOR/HIF-1 $\alpha$  axis up-  
325 regulates PD-L1 transcription (Chen et al., 2015; Koh et al., 2016; Noman et al., 2014), in

326 colon cancer cells PI3K/Akt pathway increases PD-L1 protein without changing the mRNA  
327 levels (Chen et al., 2016). In clear cell renal cell carcinoma (ccRCC) the up-regulation of PD-  
328 L1 is specifically due to the biallelic inactivation of pVHL, a genetic alteration typical of this  
329 tumor that allows the HIF-2 $\alpha$ -mediate transcription of PD-L1 transcription (Lequeux et al.,  
330 2019). The simultaneous presence of other factors typical of hypoxic tumors, such as PTEN  
331 loss or STAT1/STAT3 activity that also increase PD-L1 amount (Wu et al., 2019), makes the  
332 molecular mechanisms linking PD-L1 and HIF-1 $\alpha$  expression highly variable and tumor-  
333 dependent. Overall, the evidence collected clearly indicate the presence of multiple cross-  
334 talks between PD-L1- and HIF-1 $\alpha$ -dependent pathways, that contribute to tumor invasion,  
335 resistance to chemotherapy and low efficacy of ICPIs.

336 Hypoxia influences ICP conformation and the consequent binding of ICPIs also by inducing  
337 post-translational modifications or altering the lipid environment where ICPs are embedded.  
338 The ICPs CTLA-4 and PD-1, and their ligand PD-L1, are all glycosylated proteins.  
339 Glycosylation regulates ICPs stability in the plasma membrane, the trafficking and the  
340 expression of PD-1 (He & Xu, 2020). Hypoxia impairs protein glycosylation (Greville et al.,  
341 2020), potentially altering the 3-D structure of ICPs and the binding of ICPIs. Hypoxia also  
342 increases protein palmitoylation that stabilizes PD-L1 in the plasma membrane and reduces  
343 its trafficking toward the endo-lysosomal compartment (Sikarwar et al., 2014; Wang et al.,  
344 2020; Yang et al., 2019). The presence of PD-L1 on the cell surface promotes breast cancer  
345 growth (Yang et al., 2019), likely favoring the immunoevasion of tumor cells.

346 Two other pathways regulate the distribution of PD-L1 between plasma membrane and  
347 endosomal compartment. First, CKLF-like MARVEL trans-membrane domain-containing  
348 protein 6 (CMTM6) protects PD-L1 from lysosomal degradation as the deletion of CMTM6  
349 decreased the levels of PD-L1 on the cell surface without affecting PD-L1 mRNA.  
350 Consistently, CMTM6-deficient tumor cells are more susceptible to killing by antigen-

351 specific cytotoxic T-lymphocytes (Burr et al., 2017; Mezzadra et al., 2017), which are  
352 relieved by an ICP-dependent energy. Second, the ADP ribosylation factor 6 (ARF6) and its  
353 GTPase activating protein ArfGAP with an SH3 domain, ankyrin repeat and PH domain 1  
354 (AMAP1) prevent the intracellular recycling and the consequent lysosomal degradation of  
355 PD-L1 (Tsutaho et al., 2020). While CMTM6 levels do not vary in hypoxia, ARF6 is  
356 increased in hypoxic areas (Abdul-Salam et al., 2019; Marquer et al., 2016), where it  
357 maintains high PD-L1 on cell surface (Tsutaho et al., 2020) and makes the tumors more  
358 resistant to ICPIs.

359 Of note, ARF6 controls the retrograde trafficking of cholesterol: high levels of this protein  
360 alter the fluidity of membrane microdomains where PD-L1 is embedded (Abdul-Salam et al.,  
361 2019; Marquer et al., 2016). Membrane fluidity, which is dependent on lipid composition, is  
362 an important factor controlling the conformations of integral membrane proteins including  
363 ICPIs. Indirect evidence suggests that changes in membrane fluidity alter the ICPI/ICPL  
364 interactions. Indeed, liposomes rich in phosphatidylcholine reversed choline phosphate,  
365 which increases membrane rigidity, to which anti-PD-L1 antibodies were attached, enhanced  
366 the interaction between anti-PD-L1 and PD-L1 antibodies in melanoma cells (Li et al., 2021),  
367 resulting in immune-sensitizing effects. Hypoxia reduces cholesterol and glycosphingolipids  
368 content in lipid rafts (Király et al., 2013), and this event may impair the binding of ICPIs. A  
369 high cholesterol content, however, does not always produce positive outcome in terms of  
370 treatment efficacy. Indeed, a high plasma membrane cholesterol content is associated with  
371 chemotherapy resistance (Alves et al., 2016; Kim et al., 2018). Furthermore, chemoresistant  
372 cells, characterized by a higher *de novo* cholesterol biosynthesis (Gelsomino et al., 2013),  
373 efflux isoprenoids and cholesterol derivatives within TME and negatively modulate the  
374 activation of the immune-infiltrating cells (Kopecka et al., 2020). Changing lipid  
375 composition, in particular cholesterol levels, or membrane fluidity, produce sometimes



376 opposite effects in terms of sensitivity to ICPIs, to chemotherapy or to the host immune  
377 system. This variegated scenario raises some doubts about the use of agents targeting  
378 cholesterol biosynthesis likes statins or aminobisphosphonates, or membrane fluidity inducers  
379 as polyunsaturated fatty acids as new immune-sensitizer agents. Indeed, if it is true that they  
380 enhance the direct killing effect of chemotherapy and the chemotherapy-elicited  
381 immunogenic cell death (Gelsomino et al., 2013; Kopecka et al., 2016), they may potentially  
382 reduce the efficacy of the immunotherapy based on ICPIs.

383 The big limitation of most studies concerning post-translational modifications, trafficking and  
384 protein-lipid interaction is that they are mainly focused on PD-L1, because the PD-1/PD-L1  
385 axis is currently the most attractive therapeutic target. However, it should be noted that all the  
386 known ICPIs and ICPLs present on tumor cells - CTLA-4, LAG-3, T-cells immunoglobulin  
387 and mucin domain-containing protein 3 (TIM-3), Herpesvirus entry mediator (HVEM),  
388 galectin-9 (GAL-9), T-cells immunoreceptor with Ig and ITIM domains (TIGIT) - are  
389 glycosylated integral membrane proteins, subjected to periodic recycling. Therefore, the same  
390 changes induced by hypoxia on PD-L1 can have an impact on the structure, expression, and  
391 interaction with the respective targets of the other ICPIs. This field is completely open and  
392 may lead to the identification of potentially druggable circuitries that reduce the levels of the  
393 ICPIs/ICPLs, and/or restore the efficacy of ICPIs.

### 394 **2c. Hypoxia limits the efficacy of immune checkpoint inhibitors**

395 Since HIF-1 $\alpha$  up-regulates PD-L1 on tumor and stromal cells, PD-1, CTLA-4 LAG-3 on  
396 immune cells (Chen et al., 2015; Cubillos-Zapata et al., 2017; Doedens et al., 2013; Koh et  
397 al., 2016; Noman et al., 2014), it is not surprising that it attenuates the efficacy of ICPIs.  
398 ICPIs are more active in well-oxygenated areas than in hypoxic areas. For instance, in murine  
399 melanoma models the efficacy of anti PD-1 treatment, in terms of increasing activity of  
400 cytotoxic TILs and tumor regression, is greater at higher pO<sub>2</sub> (Scharping et al., 2017). Similar

401 results were obtained in murine glioma models where the increase in HIF-1 $\alpha$  was associated  
402 with the lower activity of an anti-PD-L1 antibody: both the increase in PD-L1 levels on  
403 glioma cells and the anergy of CD8<sup>+</sup> T-lymphocytes due to the hypoxic environment, may  
404 explain this phenotype (Ding et al., 2021).

405 The findings obtained in animal models are corroborated by few clinical studies. A  
406 retrospective study in squamous cell carcinoma of the head and neck (HNSCC) patients  
407 treated with anti PD-1 ICPIs as second-line treatment after chemotherapy showed that the  
408 less hypoxic and acidic tumors, measured as tumors with lower expression of CAIX, had a  
409 better response to the ICPI in terms of overall survival (OS). In this model, the acidic TME  
410 typical of hypoxic areas seems the only factor predicting a dismal response to ICPIs, because  
411 no correlations were found between ICPI efficacy, intratumor pO<sub>2</sub>, PD-L1 levels, amount of  
412 infiltrating CD8<sup>+</sup> T-lymphocytes or Treg cells (Zandberg et al., 2020). Conversely, another  
413 work on HNSCC demonstrated that higher intratumor pO<sub>2</sub> was directly correlated with the  
414 amount and activity of infiltrating CD8<sup>+</sup>T-lymphocytes and with a better response to anti PD-  
415 1 treatments, evaluated as progression-free survival (PFS) and OS (Zandberg et al., 2021).  
416 HIF-1 $\alpha$  is not the only factor reducing the ICPIs efficacy. In hepatocellular (HCC) patients,  
417 both HIF-1 $\alpha$  and CXCL12 levels were associated with tumor areas characterized by high PD-  
418 L1 expression. Since HIF-1 $\alpha$ , CXCL12 and PD-L1 levels all correlated with a worse  
419 prognosis, this study provides a rational basis to adopt a triple combination therapy based on  
420 sorafenib, ICPIs and anti C-X-C motif chemokine receptor 4 (CXCR4)/CXCL12 agents  
421 against resistant HCCs (Semaan et al., 2017).

422 Overall, these preclinical and clinical studies clearly indicate that tumor hypoxia is an  
423 obstacle to ICPI-based immunotherapy, but targeting HIF-1 $\alpha$  or specific chemokines/growth  
424 factors produced by the hypoxic TME, could be an effective approach to enhance the efficacy  
425 of ICPIs.

426

427 **3. Mitigating intratumor hypoxia to overcome resistance to immune checkpoint**

428 **inhibitors: a versatile and open therapeutic field**

429 The pharmacological strategies reducing the deleterious effects of hypoxia worked well in  
430 preclinical models to improve the efficacy of chemotherapy, radiotherapy and targeted  
431 therapies (Graham & Unger, 2018). Starting from these premises, inhibitors of HIF-1 $\alpha$ ,  
432 agents mitigating the effects of hypoxia, reoxygenation methods may work as immune-  
433 sensitizer agents as well. Different strategies have been tested.

434 Although pharmacological inhibitors of HIF are apparently the easiest category of drugs to be  
435 tested, they did not reach the expected therapeutic success in clinical trials  
436 (<https://clinicaltrials.gov/>), because of the lack of tumor specificity and the inhibition of  
437 physiological processes controlled by HIF. As a result, most inhibitors have produced  
438 predicted toxicities and only a few of them are now under clinical evaluation to improve  
439 ICPIs efficacy. Belzutifan (PT2977, MK-6482) is one of the latest, potent and selective  
440 second-generation HIF-2 $\alpha$  inhibitor that allosterically disrupts the heterodimerization of HIF-  
441 2 $\alpha$  and HIF- $\beta$  subunits, blocking the transcription of HIF2 $\alpha$ -responsive genes (Choueiri &  
442 Kaelin, 2020; Xu et al., 2019). This small molecule is currently under investigation in 10  
443 trials (<https://clinicaltrials.gov/>) and on March 16, 2021 it received a Priority Review from  
444 the FDA for VHL disease-associated ccRCC not requiring immediate surgery. The review  
445 was based on the objective response rate (ORR) obtained in the open label phase 2,  
446 NCT03401788 trial (Iliopoulos et al., 2021; Srinivasan et al., 2021). After the evaluation of  
447 pharmacodynamics, pharmacokinetics, anti-tumor activity and safety in the first-in-human  
448 phase 1 NCT02974738 study (Choueiri et al., 2021c) (Choueiri et al, 2021a), belzutifan was  
449 evaluated as single agent (NCT02974738) or in combination with the tyrosine kinase receptor  
450 inhibitor cabozantinib (NCT03634540) for metastatic ccRCC previously treated with PD-

451 1/L1 and/or VEGF inhibitors (Bauer et al., 2021; Choueiri et al., 2021b). The most common  
452 adverse events due to HIF-2 $\alpha$  inhibition during belzutifan treatment were hypoxia, related to  
453 an increased pulmonary arterial vasoconstrictive response, and anemia, caused by the reduced  
454 transcription of erythropoietin (Choueiri et al., 2021a). After these studies, belzutifan was  
455 evaluated in combination with the VEGF-TKI lenvatinib or with different ICPIs - the anti-  
456 CTLA-4 quavonlimab, the anti-LAG-3 favezelimab, the anti-PD-1 pembrolizumab, the anti-  
457 immunoglobulin-like transcript 4 (ILT4) (MK-4830), as first line (1L) (MK-3475-03A,  
458 NCT04626479) or second line plus (2L+) (MK-3475-03B, NCT04626518) treatment for  
459 patients with advanced ccRCC as part of the phase 1b/2 umbrella platform study U03. As  
460 presented during 2021 ASCO Annual Meeting, the sub-study 03A (NCT04626479) is  
461 recruiting advanced ccRCC patients, without prior systemic therapy, that will be randomly  
462 assigned 2:1 to one of the experimental arms [I (coformulation of quavonlimab +  
463 pembrolizumab and lenvatinib), II (coformulation of favezelimab + pembrolizumab and  
464 lenvatinib), III (pembrolizumab, lenvatinib and belzutifan)] or to the reference arm. Instead,  
465 the sub-study 03B (NCT04626518) will evaluate patients whose disease progressed after a  
466 previous treatment with PD-1/PD-L1 inhibitors or VEGF-TKIs: patients will be allocated 1:1  
467 to an experimental arm [I (pembrolizumab and belzutifan), II (lenvatinib and belzutifan), III  
468 (coformulation of quavonlimab and pembrolizumab), IV (coformulation of favezelimab +  
469 pembrolizumab), V (pembrolizumab and MK-4830)] or to the reference arm (Plimack et al.,  
470 2021). The primary end points will be safety and ORR, the secondary end points will be  
471 duration of response, PFS, clinical benefit rate and OS. Although the results are not available  
472 yet, belzutifan raised great hope to be a safe and effective antitumor agent, and was further  
473 investigated in combination treatments. Another phase III open label trial (NCT04736706),  
474 which started in April 2021, is testing the combination of belzutifan with an ICPI  
475 (pembrolizumab or quavonlimab), alone or in combination with the VEGF inhibitor

476 lenvatinib as first-line treatment in ccRCC (<https://clinicaltrials.gov/>). The results of all these  
477 ongoing trials are of paramount importance to establish the role of belzutifan either as a  
478 single agent or in combination with ICPIs or TKIs for patients with advanced ccRCC. It is  
479 possible that studies will be extended to other refractory tumor types.

480 Among the FDA-approved HIF inhibitors under evaluation for the possible combination with  
481 ICPIs is vorinostat (suberoylanilide hydroxamic acid, SAHA), a well-known histone  
482 deacetylase (HDAC) inhibitor used for the treatment of cutaneous T-cell lymphoma, capable  
483 of decreasing both HIF-1 $\alpha$  expression (Hutt et al., 2014) and nuclear translocation (Zhang et  
484 al., 2017). Therefore, it represents a multi-target drug endowed with an additional antitumor  
485 mechanism of action beyond its epigenetic effect. Recently, in a randomized phase II study  
486 (NCT02395627), 34 estrogen receptor (ER)-positive breast cancer women who have  
487 progressed on a median of five prior therapeutic regimens, received vorinostat, the anti-ER  
488 tamoxifen and pembrolizumab. Although the study was terminated because of the low  
489 efficacy in the whole population enrolled, among the 27 evaluable patients, 18.5% patients  
490 achieved a clinical benefit and 3.7% an objective response (Terranova-Barberio et al., 2020).

491 The phase II open label trial NCT02538510 enrolled patients with recurrent metastatic  
492 HNSCC and salivary gland cancer receiving vorinostat and pembrolizumab. In the HNSCC  
493 group, the combination therapy showed PFS and OS superior to pembrolizumab alone, but  
494 also a 36% grade >3 toxicity, that was higher than that reported with the ICPI alone  
495 (Rodriguez et al., 2020). A phase I/Ib study (NCT02638090) evaluating the combination of  
496 vorinostat with pembrolizumab in patients with advanced/metastatic NSCLC, either ICPI  
497 naïve or pre-treated with pembrolizumab, reported a 33% of patients with progressive  
498 disease, 53% with stable disease and 13% achieving partial response, with good tolerability.  
499 Notably the percentages were similar in pembrolizumab pre-treated patients (Gray et al.,  
500 2019), suggesting the ability of vorinostat to overcome the acquired resistance eventually

501 developed toward pembrolizumab treatment. In the phase II of this ongoing trial, it was  
502 confirmed that the combination of vorinostat and pembrolizumab had a considerably higher  
503 ORR (66.7% vs 33.3 %) compared to ICPI monotherapy (Saltos et al., 2020).

504 Although it is arduous to clarify by which mechanism - e.g. dependent or independent from  
505 HIF-1 $\alpha$  inhibition - vorinostat affects the response to immunotherapy, the association of  
506 vorinostat and ICPIs has proved to be a promising treatment option for patients with different  
507 cancer types and warrants further investigation.

508 Other approaches have been studied in order to relieve the impact of hypoxia, with the aim of  
509 using less toxic and more effective strategies. One physical approach to reverse hypoxia has  
510 been the exposure of patients to a hyper-oxygenated atmosphere. However, in a phase III  
511 trial, the use of a hyperbaric chamber in patients with central nervous system tumors did not  
512 improve the outcome compared with the current standard treatment (Stępień et al., 2016).

513 Among the pharmacological agents, OXPHOS inhibitors have been proposed as O<sub>2</sub>-sparing  
514 drugs. In this respect, metformin, an anti-diabetic drug that inhibits the complex I of the  
515 electron transport chain, has been repurposed as an immune-sensitizer: by reducing the  
516 mitochondrial O<sub>2</sub> consumption, it synergized with anti-PD-1 antibody in immunocompetent  
517 mice bearing melanomas, where the combination improved the cytolytic activity of TILs and  
518 achieved tumor regression (Scharping et al., 2017).

519 Another approach is based on hypoxia-activated prodrugs (HAPs) including evofosfamide  
520 (TH-302), PR-104, tarloxotinib and CP-506 (Hegde et al., 2021). HAPs are biologically  
521 inactive prodrugs in oxygenated tissues whereas under hypoxic conditions prevalent in  
522 tumors, they undergo enzymatic reduction, becoming biologically active compounds which  
523 exert a cytotoxic effect (Fu et al., 2021). Evofosfamide is the best studied compound of this  
524 family and it has been designed to release the alkylating agent bromo-isophosphoramidate  
525 mustard in the hypoxic TME (Weiss et al., 2011). The combination of evofosfamide with

526 anti-CTLA-4 and anti-PD-1 agents effectively reduced the mass of prostate tumors in  
527 syngeneic mice models, increased T-cell infiltration (Ai et al., 2015) and reduced MDSCs  
528 recruitment (Jayaprakash et al., 2018). The synergism between evofosfamide and anti-CTLA-  
529 4 antibody is not tumor-specific, since a similar mechanism has been reported in HNSCC  
530 models (Jamieson et al., 2018). In a phase III trial, the doxorubicin-evofosfamide  
531 combination did not increase the OS of patients with disseminated sarcomas (Tap et al.,  
532 2017), blunting the enthusiasm for the association between HAPs and chemotherapy. Very  
533 recently, the results of a phase I study (NCT03098160) on the safety and tolerability of the  
534 combination between evofosfamide and the anti-CTLA4 ipilimumab in advanced solid  
535 malignancies have been published (Hegde et al., 2021). Twenty-two patients with castration-  
536 resistant prostate cancer, immunotherapy-resistant melanoma, HNSCC and pancreatic cancer  
537 received evofosfamide on days 1 and 8 of the cycles 1-2, and ipilimumab on day 8 of cycles  
538 1-4. Of 18 patients with measurable disease at baseline, 12 achieved stable disease and 3  
539 partial responses. Additionally, an improved peripheral T-cell proliferation and an increased  
540 intratumoral T-cell infiltration into hypoxic tumors was observed. The combination was well  
541 tolerated and drug-related hematologic toxicities, fever, rash, nausea, and elevation of liver  
542 enzymes were observed in < 10% of the patients (Hegde et al., 2021).

543 A very recent approach designed to overcome hypoxia is based on hypoxia-relieving  
544 nanoparticles (NPs). One of these formulations, i.e. NPs coated with melanoma cell  
545 membrane (mZCD), carrying catalase (CAT) enzyme and doxorubicin, has proven to relieve  
546 hypoxia and enhance the therapeutic efficacy of chemotherapy and immunotherapy. The NPs  
547 were targeted to melanoma, where CAT transformed the H<sub>2</sub>O<sub>2</sub> present within the tumor into  
548 O<sub>2</sub>. The decrease in ROS, reduced the expression of HIF-1 $\alpha$  and PD-L1, facilitating the  
549 cytotoxic activity of doxorubicin (Zou et al., 2018). The combination of mZCD-CAT-NPs  
550 and the anti-PD-1 antibody achieved synergistic effects reflected in prevention of tumor

551 recurrence and metastasis (Zou et al., 2018). The same goals of relieving hypoxia and  
552 restoring a proper immune landscape were achieved by the combination of CAT-NPs and  
553 anti-CTLA-4 treatment that reduced the ratio between tumor-infiltrating Treg and CD8<sup>+</sup> T-  
554 cells (Song et al., 2018). In a further development, an anti-PDL-1 antibody was directly  
555 conjugated to CAT-NPs, in order to increase the controlled release of the ICPI within the  
556 hypoxic tumor site, minimizing off-target effects, enhancing the activation of cytotoxic TILs  
557 and the therapeutic benefits (Hei et al., 2020). We believe that nanomedicine may represent  
558 the future of oncological therapy, because nanocarriers increase the biocompatibility and  
559 solubility of the reagents, prolong their circulation time and allow a better targeting of the  
560 anticancer drugs, reducing peripheral toxicity and side effects. At the present time, however,  
561 no immuno-formulations entered clinical trials. Therefore, a definitive evaluation of their  
562 relative efficacy is yet to come. Indeed, the clinical results obtained with HAPs or  
563 reoxygenation strategies as single agents or in combination with chemotherapy, were  
564 disappointing and none of these therapeutic approaches have been approved by regulatory  
565 agencies. On the other hand, the promising preclinical studies and the very recent phase I  
566 NCT03098160 trials suggested the possible use of these agents in combination with ICPIs.  
567 The use of ICPIs in tumor treatment and the emergence of resistant patients are relatively  
568 recent. Therefore, the studies aiming to reverse the resistance to ICPIs by combining other  
569 agents are still an open field.

570

#### 571 **4. The cross talk between hypoxia and angiogenesis: another piece of the puzzle** 572 **determining the activity of immune checkpoint inhibitors**

573 When the tumors grow, new blood vessels form to provide nutrients and O<sub>2</sub>. However, the  
574 newly formed blood vessels are often structurally and morphologically aberrant, and create a



575 TME with persistent or cycling hypoxia, acidosis and high interstitial fluid pressure (Lugano  
576 et al., 2020). These conditions impair the extravasation of immune cells and create an  
577 immunosuppressive landscape (Pietrobon & Marincola, 2021), but also offer new therapeutic  
578 opportunities to combine anti-angiogenic therapies with ICPIs to enhance the efficacy of the  
579 latter (Figure 4).

580 HIF-1 $\alpha$  is a transcriptional activator of pro-angiogenic factors produced by tumor- or TME-  
581 associated cells; these pro-angiogenic factors include VEGF, PDGF- $\beta$ , placental growth  
582 factor (PGF), angiopoietin-2 (ANGPT2), and CXCL12/SDF-1 (Lugano et al., 2020). Most of  
583 which mediate the recruitment of immunosuppressive cell populations such as Treg cells  
584 (Pietrobon & Marincola, 2021) and MDSCs (Du et al., 2008; Lin et al., 2012) that induce the  
585 anergy of cytotoxic CD8<sup>+</sup>T-lymphocytes and favor the up-regulation of ICPIs on TILs  
586 (Pietrobon & Marincola, 2021). Moreover, VEGF also inhibits lymphocyte extravasation  
587 (Schaaf et al., 2018), the proliferation and effector functions of CD8<sup>+</sup> T-lymphocytes, by  
588 inhibiting DC maturation and antigen presentation, and recruiting Treg cells, M2-TAMs and  
589 MDSCs in the tumor site (Tamura et al., 2020).

590 VEGF increases ICP expression, either directly or by triggering the release of specific soluble  
591 mediators in the hypoxic TME. For instance, VEGF increases the amount of PD-1 on CD8<sup>+</sup>T-  
592 lymphocytes by activating the VEGFR-2/phospholipase C $\gamma$  (PLC $\gamma$ )/calcineurin/NFAT-  
593 dependent pathway that leads to T-cell exhaustion (Voron et al., 2015). In a side-pathway,  
594 VEGF induced the differentiation of monocytes into TAMs which are rich in PD-L1 that  
595 repressed the activity of CD8<sup>+</sup> T-lymphocytes, NK cells and DCs (Ramos et al., 2020).

596 Several soluble factors downstream of VEGF also increase ICPIs in the hypoxic TME. Indeed,  
597 VEGF induced the secretion of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by activating cyclo-oxygenase 2  
598 (COX2) present in the endothelial cells (Tamura et al., 2020). PGE<sub>2</sub> suppressed DC  
599 maturation and NK activity (Tamura et al., 2020). This triggers a vicious cycle: NK cells are

600 endogenous inhibitors of neo-angiogenesis, because they secrete a soluble VEGFR that  
601 scavenges VEGF in response to hypoxic conditions (Krzywinska et al., 2017). Conversely,  
602 the low activity of NK cells fuels neo-angiogenesis. Moreover, PGE<sub>2</sub> directly up-regulates  
603 PD-L1 on MDSCs and TAMs: indeed, PD-L1 levels are increased when PGE<sub>2</sub> synthesizing  
604 enzymes (COX2 and microsomal PGE<sub>2</sub> synthase 1) are high, and reduced when the PGE<sub>2</sub>  
605 degrading enzyme (15- hydroxyprostaglandin dehydrogenase) is high (Tamura et al., 2020).  
606 By cooperating with IL-10 and PGE<sub>3</sub>, VEGF also increased the Fas ligand (FasL) on  
607 endothelial cell surface: the binding of T-cells to FasL selectively killed CD8<sup>+</sup> T-  
608 lymphocytes, but it spared Treg cells that are protected by the high levels of the anti-  
609 apoptotic protein cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP)  
610 (Motz et al., 2014). This mechanism leads to the progressive enrichment of Treg cells and to  
611 the deprivation of CD8<sup>+</sup> TILs.

612 If neo-angiogenesis creates the proper conditions for CD8<sup>+</sup> T-lymphocyte anergy, the  
613 opposite scenario, with cytotoxic TILs normalizing tumor vasculature, occurs too. Indeed,  
614 during their activation, CD8<sup>+</sup> T-lymphocytes secrete IFN-γ which following binding to its  
615 receptor on pericytes and endothelial cells, normalized the tumor vasculature in murine  
616 models of lung, breast and colon cancers. Vasculature normalization mediated by IFN-γ is  
617 paralleled by the increased accumulation of eosinophils and decreased infiltration of Treg  
618 cells, a condition that restores CD8<sup>+</sup> T-lymphocyte activity (Roberts et al., 2021; Zheng et al.,  
619 2018). Interestingly, normalization of blood vessels is achieved by treating CD8<sup>+</sup> T-  
620 lymphocytes with anti-PD-1 (Roberts et al., 2021; Zheng et al., 2018) or anti-CTLA-4 (Zheng  
621 et al., 2020) antibodies that likely restore the secretion of IFNγ, relieving T-cell exhaustion.

622 **4a. Exploiting anti-angiogenic therapy to restore normoxia and immune checkpoint**  
623 **inhibitors efficacy: preclinical evidence**

624 Anti-angiogenic therapy was born with the idea of inhibiting new blood vessel formation and  
625 preventing tumor cell starvation. However, a complete blockade of intra-tumor blood flow  
626 also prevented the delivery of drugs and the infiltration of immune cells, resulting in extreme  
627 hypoxia and severe immunosuppression within the TME. In contrast, mild anti-angiogenic  
628 treatments could be more advantageous to establish an equilibrium between anti-angiogenic  
629 and pro-angiogenic signals within the TME (Lugano et al., 2020), relieving the  
630 immunosuppression induced by hypoxia and enhancing the efficacy of ICPIs.

631 Indeed, emerging preclinical evidence demonstrate the potential of combining  
632 immunotherapy with vascular-targeting treatment. Blocking VEGFR2 with sorafenib or  
633 monoclonal DC101 antibody enhanced the efficacy of anti-PD-L1 antibody in refractory  
634 pancreatic, breast and brain tumor models in mice. This treatment induced the stabilization of  
635 venules and at the same time promoted the infiltration of cytotoxic lymphocytes, increases  
636 M1/M2 ratio and reduced the amount of Treg cells (Allen et al., 2017). Similarly, the anti-  
637 VEGFR fruquintinib or apatinib, combined with anti-PD-1 treatment, decreased  
638 angiogenesis, normalized the vascular structure, alleviated tumor hypoxia, restoring the anti-  
639 PD-1 efficacy in cancers resistant to ICPIs (Cai et al., 2020; Wang et al., 2020). Blocking  
640 VEGF instead of its receptors also sensitized tumors to ICPIs. In small cell lung cancer  
641 murine models, the association of anti-VEGF and anti PD-L1 antibodies is superior to  
642 monotherapy. Indeed, mice treated with anti-PD-L1 alone relapsed after 3 weeks and their  
643 tumors were rich in PD-1/TIM-3 exhausted T-lymphocytes. This phenotype was promoted by  
644 high levels of VEGF within the TME and was counteracted by the anti-VEGF/anti PD-L1  
645 combined treatment (Meder et al., 2018).

646 Another important angiogenic pathway is mediated by ANGPT2. A bispecific antibody  
647 blocking both ANGPT2 and VEGF (A2V), combined with anti-PD-1 treatment, was superior  
648 to the single agents in metastatic melanoma, breast, pancreatic and neuroendocrine tumors.

649 A2V increased tumor antigen presentation by DCs and the intratumor accumulation of  
650 cytotoxic TILs. When used alone, AV2 up-regulated PD-L1 expression on tumor blood  
651 vessels via IFN- $\gamma$  signalling, but the association with an anti-PD-1 antibody overcame this  
652 negative effect (Schmittnaegel et al., 2017). Recently, the stimulator of interferon genes  
653 (STING)-dependent pathway was reported to normalize the tumor vasculature, synergizing  
654 with the anti-VEGFR2 DC10 antibody and ICPIs. Indeed, STING agonists combined with  
655 anti-VEGFR2 and/or ICPIs promoted the regression of tumors resistant to either anti-  
656 angiogenic or ICPIs monotherapy (Yang et al., 2019), paving the way to a new triple  
657 combination therapy.

#### 658 **4b. Combining anti-angiogenic therapy and immune checkpoint inhibitors in clinical** 659 **practice**

660 Intrinsic and acquired resistance to monotherapy with ICPIs remains a challenge. Many  
661 ongoing trials started to evaluate combination therapies with TKIs endowed with anti-  
662 angiogenic properties and ICPIs, in tumors with an unfavourable immune environment as  
663 unresectable RCC or HCC. In the last years, these combinations have been evaluated in a  
664 plethora of other tumors ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). In the KEYNOTE-146 study  
665 (NCT02501096), an active non recruiting multinational, open-label, single-arm study, the  
666 combination of the anti-panVEGFR lenvatinib and anti-PD1 pembrolizumab is being  
667 evaluated for malignancies with currently limited available therapies, as NSCLC, RCC,  
668 endometrial carcinoma, urothelial carcinoma, HNSCC, melanoma (Taylor et al., 2020). The  
669 preliminary results in patients with endometrial cancer indicated a positive outcome in terms  
670 of ORR, duration of response (DOR), PFS and OS, particularly in tumors with microsatellite  
671 instability (Makker et al., 2020) which are more responsive to ICPIs (Ackroyd et al., 2021).  
672 Interestingly, tumors characterized by high microsatellite stability, which are usually poorly  
673 responsive to ICPIs, displayed a significant ORR of 33% (Makker et al., 2020). Based on

674 these findings, the FDA granted the accelerated approval to pembrolizumab plus lenvatinib  
675 for the treatment of women with advanced endometrial carcinoma that is not microsatellite  
676 instability-high or mismatch repair-deficient, characterized by disease progression following  
677 prior systemic therapy and not candidates for curative surgery or radiation ([www.fda.gov](http://www.fda.gov)).  
678 The same combination achieved positive results in ICPIs-naïve and ICPIs-pre-treated patients  
679 with gastric cancer (EPOC1706 phase II trial) (Kawazoe et al., 2020), advanced melanoma  
680 progressed after a previous anti-PD-1/anti-PD-L1 treatment (NCT03776136) (Arance et al.,  
681 2021), unresectable HCC (Finn, Ryoo, et al., 2020), advanced endometrial carcinoma and  
682 metastatic ccRCC (NCT03713593, NCT02811861, NCT03517449), one of the most  
683 unresponsive to chemotherapy and ICPIs (Lee et al., 2021; Makker et al., 2020; Motzer et al.,  
684 2021). Although an important ORR was achieved in 69% of the patients, the increase in PFS  
685 and OS was not always reached and grade  $\geq 3$  treatment-related adverse events were  
686 registered in 67% of patients (Finn, et al., 2020a), mitigating the enthusiasm and denying the  
687 accelerated FDA approval of the pembrolizumab plus lenvatinib combination for  
688 unresectable HCC. Recently, however, the FDA has granted priority review to the latter  
689 combination for both advanced RCC and endometrial carcinoma, based on results from the  
690 pivotal phase 3 CLEAR study (KEYNOTE-581; NCT02811861) (Motzer et al, 2021) and  
691 confirmatory phase 3 KEYNOTE-775 trial (NCT03517449) (Makker et al, 2021),  
692 respectively.

693 Since 2019 the advanced RCC treatment landscape includes another combination regimen  
694 based on pembrolizumab and the anti-panVEGFR axitinib, after publishing the results of the  
695 multicenter, open-label phase III KEYNOTE-426 (NCT02853331) trial enrolling 861 naïve  
696 patients. The combination arm displayed a statistically significant improvement in OS and in  
697 PFS compared to patients treated with the standard-of-care anti-VEGF sunitinib, regardless of  
698 other prognostic indices and PD-L1 expression (Rini et al., 2019a). Liver toxicities were

699 equally distributed between the two arms of the study (Rini et al., 2019a), and the extended  
700 follow up of this trial up to 42.8 months confirmed the efficacy of this association (Plimack et  
701 al., 2021; Powles et al., 2020; Rini et al., 2021), supporting its application as the standard of  
702 care in RCC. Very similar results were obtained with the combination of axitinib and another  
703 ICP, the anti-PD-L1 avelumab in the multicenter, open-label phase III JAVELIN Renal 101  
704 trial (NCT02684006) on RCC (Motzer et al., 2019), reporting a preliminary improvement in  
705 PFS versus patients treated with sunitinib (Choueiri & Kaelin, 2020; Tomita et al., 2021).  
706 These promising results led to both the approval of the axitinib plus avelumab combination as  
707 first line therapy for RCC and to the design of the phase II open label, single arm NEOAVAX  
708 trial (NCT03341845) that evaluates the efficacy of this association as neo-adjuvant treatment  
709 in high-risk non-metastatic RCC patients (Bex et al., 2019). On the other hand, the results  
710 were not so brilliant in the NCT02636725 study, focused on patients with advanced or  
711 metastatic sarcomas, where only the subgroup of patient with alveolar soft-part sarcoma had  
712 benefits from the combination of axitinib and pembrolizumab compared to patients treated  
713 with axitinib in monotherapy or chemotherapy regimens including TKIs (Wilky et al., 2019).  
714 This discrepancy suggests that a better molecular annotation of the tumor and of the immune  
715 environment is required to stratify patients who may have a real benefit from the combination  
716 of ICPIs and anti-angiogenic drugs.

717 In May 2020, the FDA approved the use of the anti-PDL-1 atezolizumab in combination with  
718 the anti-VEGF bevacizumab for the treatment of patients with unresectable or metastatic  
719 HCC who have not received prior therapy for the advanced disease. The approval was based  
720 on the positive results from the open-label, multicenter, phase III IMbrave150 trial  
721 (NCT03434379), showing better OS and median PFS with this association than with  
722 sorafenib (Finn et al., 2020b; Finn et al., 2021). The results were of particular relevance  
723 because previous studies on ICPIs as single agents failed to show a survival benefit in

724 patients with HCC (Yau et al., 2019). Serious adverse reactions were noted in 38% of patients  
725 who received the combination therapy; however, no unexpected toxic side effects were  
726 observed. The phase II IMmotion150 trial (McDermott et al., 2018) and the subsequent phase  
727 III IMmotion151 (NCT02420821) trial (Rini et al., 2019b), focused on metastatic RCC, were  
728 in line with these results and confirmed the superior efficacy, measured as PFS and OS, of the  
729 atezolizumab plus bevacizumab combination versus the monotherapy. Additionally, patient-  
730 reported outcomes from IMmotion151 suggested that the combination does not significantly  
731 increase treatment burden compared with sunitinib (Atkins et al., 2020). The combination  
732 was further studied in patients with advanced variant histology RCC or any RCC with at least  
733 20% sarcomatoid differentiation, characterized by worse prognosis and lower response rates  
734 to targeted therapies than their counterparts with clear cell RCC, in an active phase II, single  
735 arm, open label trial (NCT02724878). ORR was 26% for variant histology RCC and 50% for  
736 RCC with sarcomatoid differentiation, with treatment-related grade 3 toxicities in 13%  
737 patients (Mcgregor et al., 2019). These encouraging results prompted the expansion of the  
738 study of atezolizumab and bevacizumab combination to unresectable/metastatic anal cancer  
739 (NCT03074513) (Morris et al., 2020), advanced mucosal melanoma (NCT04091217) (Si et  
740 al., 2021), NSCLC (NCT03836066, NCT03896074), HNSCC (NCT03818061), and  
741 metastatic/unresectable urothelial cancer (NCT03272217), leading to 57 recruitments, and 14  
742 still active trials (<https://clinicaltrials.gov/>), whose results will be likely disclosed in the near  
743 future.

744 The last combination approved by the FDA for metastatic RCC has been the anti-PD-1  
745 nivolumab and the anti-VEGFR cabozatinib, after the results of the randomized, phase III  
746 open-label trial CHECKMATE-9ER (NCT03141177), showing a two-fold increase in PFS  
747 and ORR in patient treated with this combination, compared to patients receiving the single  
748 agent or sunitinib, with no additional incidence of grade  $\geq 3$  toxicities (Choueiri et al., 2021c).

749 A phase I, still recruiting study (NCT02496208) is evaluating the triple combination of  
750 cabozantinib, nivolumab and anti-CTLA-4 ipilimumab in patients with genitourinary tumors  
751 including metastatic urothelial carcinoma. The triple combination did not show a superior  
752 ORR or OS in this case, and was characterized by slightly higher grade 3 or 4 toxicities  
753 (Apolo et al., 2020). One bias of the study was that patients treated with the triple  
754 combination had more aggressive tumors and rarer histologies. The tumor heterogeneity and  
755 the small sample size do not allow to draw clear conclusion on the benefits of anti-angiogenic  
756 agents with two different ICPIs.

757 Overall, the clinical studies carried out to date have demonstrated that the combination of an  
758 ICPI with a TKI endowed with anti-angiogenic activity broadens the antitumor activity of  
759 immunotherapy, even in those tumors that become immunoresistant. Therefore, the toolbox  
760 of these associations is constantly expanding, as the number of studies testing their efficacy  
761 and safety in different cancers. However, caution should be exerted when interpreting data  
762 from single-arm trials, making cross-trial comparisons with studies on monotherapy.  
763 Moreover, larger randomized trials are needed to confirm the efficacy and safety observed.  
764 The future research should aim to discover predictive biomarkers of drug response, in order  
765 to better identify the patients with the best response upon the treatment with ICPIs and anti-  
766 angiogenic agents.

767

## 768 **5. Implication of hypoxia-driven changes in the efficacy of CAR T-cells**

769 CAR T-cells represent an effective form of adoptive T-cell therapy (ATC), developed to  
770 circumvent the immunotolerance of the T-cell repertoire and the MHC restriction, and to  
771 direct specific cytotoxicity to a target molecule on malignant cells. In this approach, T-cells  
772 isolated from the patient (or from an allogeneic donor) are genetically modified to express a  
773 tailored CAR toward a specific tumor antigen. Then, they are expanded and infused into the



774 patient. The first generation of CAR T-cells used in clinical trials did not show high efficacy,  
775 as they were based on the CD3  $\zeta$ -chain to simulate TCR signaling. New generation of CAR  
776 T-cells have been designed to include domains from CD28, CD40L and other positive  
777 regulators of T-cell, activation in order to potentiate their cytotoxicity in vivo (Waldman et  
778 al., 2020). The high expression of the CD19 antigen in specific B cell malignancies and its  
779 specificity for the B cell lineage, make this antigen an ideal candidate to be targeted. Indeed,  
780 anti-CD19 CAR T-cells therapy obtained the first clinical successes in 2010, achieving high  
781 remission rates in adults with follicular lymphoma (FL) (Kochenderfer et al., 2010) and  
782 chronic lymphocytic leukemia (CLL) (Porter et al., 2011), and later in children with B cell  
783 acute lymphoblastic leukemia (B-ALL) (Grupp et al., 2013). In patients with relapsed or  
784 refractory ALL, a 90% of complete response (CR) rate has been reported, while >50% CR  
785 rates have been reported in CLL and B-cell lymphoma (Cai et al., 2020). These results lead  
786 the FDA to approve in 2017 the first CAR T-cell treatment (Axicabtagene ciloleucel) for  
787 adult patients with large B-cell lymphoma, relapsed or refractory after two or more lines of  
788 systemic therapy. Other three CAR T-cells have been approved for B-cell malignancies,  
789 namely tisagenlecleucel for ALL, brexucabtagene autoleucel for mantle cell lymphoma, and  
790 more recently lisocabtagene maraleucel for relapsed or refractory large B cell lymphoma. In  
791 2021, the FDA approved the first CAR T-cell (idecabtagene vicleucel) directed towards  
792 another antigen, the B-cell maturation antigen (BCMA), present on plasmocytes (Mullard,  
793 2021b). This CAR T-cell has been approved to treat adult patients with multiple myeloma  
794 who have not responded to, or whose disease has relapsed after, at least four prior different  
795 lines of therapy. In 2020, 191 active preclinical and clinical CAR-T programs were directed  
796 to CD19, demonstrating that CD19 remains the most attractive target for cell therapy.

797 Other top targets include CD20, CD22 and HER2 (Mullard, 2021a). Furthermore, many  
798 emerging alternative targets under active research had being proposed, such as CD22,

799 CD123, CD38, CD133, CD20, chondroitin sulfate proteoglycan 4 (CSPG4), thymic stromal  
800 lymphopoietin receptor (TSLPR) (X. Xu et al., 2020) or B7-H3 (also known as CD276), a  
801 pan cancer target present in multiple paediatric solid tumors (Waldman et al., 2020). In  
802 addition, to act as cytolytic agents, CAR T-cells can also target the TME. For instance, a new  
803 generation of ‘armored’ CAR T-cells engineered to produce IL-12 overcome Treg- and  
804 MDSCs-induced immunosuppression, promoting the cytolytic activity of CD8<sup>+</sup> T-  
805 lymphocytes, enhancing the recruitment of anti-tumor myeloid cells and the antigen  
806 presentation by DCs (Luo et al., 2019).

807 These achievements show that CAR T-cell-based therapy is among the most promising  
808 anticancer therapies of all times (Shah et al., 2019) because it generates a durable and  
809 effective anti-tumor immune response. However, significant challenges remain, as  
810 oncologists strive to obtain durable remissions for all patients. Both antigen-positive and  
811 antigen-negative relapses have been documented in patients (Cai et al., 2020). For instance,  
812 the loss or down-regulation of CD19 or CD22, the epitope masking due to acquired mutations  
813 and alternatively spliced alleles, enable malignant B-cells to acquire resistance to CAR T-cell  
814 killing (Cheng et al., 2019; Shah et al., 2019). A long-term follow-up study demonstrated that  
815 disease relapse after anti-CD19 CAR T-cells therapy occurs in up to 50% of patients with  
816 pre-B cell ALL by 12 months after infusion (Park et al., 2018). Since patients who relapse  
817 following CAR T-cell therapy have very poor prognosis, novel approaches to overcome  
818 therapy resistance are urgently required.

### 819 **5a. Mechanisms of resistance to CAR T-cells therapy**

820 Despite the impressive responses in patients with hematologic malignancies, early clinical  
821 trials using CAR T-cells in patients with solid tumors have reported a limited antitumor  
822 activity. The lack of tumor-specific CAR targets (Kosti et al., 2021), the limited array of  
823 targetable antigens and the heterogeneous antigen expression (Wagner et al., 2020), the loss

824 of antigen expression, the T-cell dysfunction driven by CAR or chronic antigen exposure, and  
825 the immunosuppressive TME, characterized by severe hypoxia and abundant deposition of  
826 ECM (Labani-Motlagh et al., 2020), limit the applicability of CAR T-cells in solid tumors.  
827 Other important mechanisms of resistance to CAR T-cell immunotherapy are correlated with  
828 the CD4<sup>+</sup>/CD8<sup>+</sup> ratio of the T-lymphocytes infused or with the poor persistence of the CAR  
829 T-cells, which might be patient-dependent and therapy-dependent, because T-cells can be  
830 anergic or less reactive after intensive chemotherapy (Shah et al., 2019; Roselli et al., 2021).  
831 More specific mechanisms of resistance have been associated with the blockade of IL-  
832 6/STAT3 axis that diminishes CAR T-cell proliferation (Fraiatta et al., 2018), or with the  
833 transduction of a single leukemic B cell (Ruella et al., 2018).

834 Since the immunosuppressive TME is the major obstacle for CAR-T-cells therapy in solid  
835 tumors, several strategies directed to regulate TME plasticity and reverse the TME-dependent  
836 immunosuppression are being explored. Armored CAR T-cells expressing pro-inflammatory  
837 cytokines, combination of CAR T-cells with oncolytic viruses, new generation of CAR T-  
838 cells targeting CAFs, T-reg cells, M2 TAMs or MDSCs are under development (Rodriguez-  
839 Garcia et al., 2020). It is known that an ECM rich in collagen and poorly vascularized  
840 provides a physical barrier, preventing the efficient homing and infiltration of CAR T-cells.  
841 Moreover, the hypoxic environment up-regulates ICPs and respective ligands, expands  
842 immunosuppressive cells, triggers the releases of immunosuppressive soluble factors  
843 (adenosine, PGE<sub>2</sub>), induces a metabolic pressure on effector T-cells by subtracting key  
844 nutrients (Glover et al., 2021). All these factors, which are common to the resistance  
845 mechanisms toward ICPIs, impair the efficacy of CAR T-cells as well.

846 An increased understanding of the mechanisms underlying resistance to CAR T-cells and a  
847 more precise identification of patients with the highest likelihood of relapse is crucial to  
848 optimize CAR T-cell therapy. Novel strategies, such as the targeting more than one antigen

849 receptor with dual-targeting CAR T-cells, the use of fully human CAR T-cells, CAR NK-  
850 cells or combination therapies with ICPIs are being explored to surmount the resistance to  
851 CAR T cells and improve clinical outcomes in patients with relapsed and refractory  
852 malignancies (Song et al., 2019; Cai et al., 2020).

### 853 **5b. Linkage between CAR T-cells and hypoxia**

854 A very common mechanism of drug resistance in solid tumors is hypoxia, a hallmark of the  
855 TME in solid cancers (Berahovich et al., 2019) that also impairs the efficacy of adoptive  
856 immunotherapy. The O<sub>2</sub>- and glucose-deficient TME deprives T-lymphocytes, including  
857 CAR T-cells, of the main energy source, pushing them to exhaustion (Schurich et al., 2019).  
858 This is one of the first mechanisms explaining the lower efficacy of CAR- T-cells in the  
859 treatment of solid tumors. Indeed, both activated T-lymphocytes and cancer cells  
860 preferentially use glucose. The strongly energy demand of cancer cells renders the TME poor  
861 in glucose for T-cells. At the same time, the hypoxic TME impairs the mitochondrial  
862 OXPHOS in T-lymphocytes, leading to a metabolic and functional exhaustion (Schurich et  
863 al., 2019). While tumor cells grow well in hypoxic niches, T-cell fitness and survival is  
864 limited in these niches, where an efficient trafficking and penetration of CAR T-cells is not  
865 achieved (Wagner et al., 2020) (Figure 5).

866 These events make hypoxia an inducer of resistance to CAR T-cell therapy. Several research  
867 groups recently began to address the “hypoxia problem” by generating O<sub>2</sub>-sensitive self-  
868 decision making engineered CAR T-cells, (Juillerat et al., 2017; Kosti et al., 2021). The  
869 hypoxia-sensing CAR T-cell system (called HypoxiCAR T or HiCAR T) is designed to  
870 express a CAR under the control of a stringent hypoxia-sensing safety switch, avoiding off-  
871 tumor activation of CAR T-cells and delivering efficient anti-tumor killing in hypoxic TME  
872 (Kosti et al., 2021). This approach may represent a good modality to improve the efficacy of  
873 CAR T-cells against hypoxic solid tumors, a challenge that remains open at the present time.

874

875 **6. Conclusions and future perspectives**

876 Hypoxia is a driver of multiple aggressive features in tumors, inducing metabolic rewiring,  
877 apoptosis inhibition, cell migration and increased adaptability to unfavorable conditions. The  
878 first consequence of these transformations is the higher resistance of hypoxic tumors to  
879 chemotherapy and radiotherapy, as well as to other stressful conditions which usually kill  
880 normoxic cells including nutrient deprivation, calcium oscillation, endoplasmic reticulum  
881 stress) (Akman et al., 2021; Belisario et al., 2020). The effects of hypoxia alter not only the  
882 cancer cell, but also tumor-associated cells, such as CAFs, endothelial cells and immune-  
883 infiltrating cells. The response of each component is strictly interconnected and synergizes to  
884 generate more aggressive and chemoresistant tumors. In response to hypoxia, CAFs secrete  
885 soluble factors favoring the EMT program, lactate and building blocks for cancer cells, neo-  
886 angiogenesis factors, chemokines and cytokines attracting immune cells with  
887 immunosuppressive potential. Endothelial cells respond with the formation of an irregular  
888 and leaky vasculature that does not compensate for the low pO<sub>2</sub> and impairs the delivery of  
889 drugs, as well as the extravasation of anti-tumor immune cells. Immune-infiltrating cells are  
890 characterized by low levels of anti-tumor cytotoxic populations with functional anergy and  
891 high expression of ICPs, and high levels of immunotolerant/immunosuppressive cells, low  
892 activity of CAR T-cells. By directly affecting the proliferation and differentiation of effectors  
893 cells, or by triggering the secretion of immunosuppressive cytokines by TME cells, hypoxia  
894 generates an immune disaster.

895 The recent introduction of ICPIs was a revolution for the therapeutic outcome of specific  
896 tumors, particularly immunologically “hot” tumors as melanoma and NSCLC. On the other  
897 hand, the increasing use of ICPIs has been paralleled by the first cases of resistance.  
898 Remarkably, the introduction of CAR T-cells has obtained impressive improvements in the

899 treatment of hematological tumors, but the rate of success was significantly lower in solid  
900 tumors.

901 While resistance to conventional chemotherapeutic drugs or targeted therapies is often due to  
902 tumor intrinsic factors (e.g. mutations in the drug target, decreased drug entry, increased drug  
903 sequestration or efflux, increased metabolic inactivation of the drug and anti-apoptotic  
904 mechanisms), resistance to ICPI and CAR T-cells is more related to TME-dependent factors.  
905 One culprit is the hypoxic TME that acts at least at three levels. First, hypoxia expands  
906 immunosuppressive populations and anergic, ICP-rich effector cells that are difficult to be re-  
907 activated by ICPIs, while it prevents the activation of cytolytic functions of effector  
908 populations as CAR T-cells. Second, hypoxia up-regulates ICPLs on tumor cells and their  
909 downstream pathways, that have intensive cross-talks with HIF-1 $\alpha$ -dependent pathways in  
910 increasing cell survival, migration and resistance. Finally, hypoxia triggers a neo-angiogenic  
911 environment that further impairs the extravasation and activity of effector cells, and allows  
912 immunosuppressor cells to populate the TME.

913 Accordingly, ICPIs and CAR T-cells are less effective in hypoxic tumors. On the other hand,  
914 a good knowledge of the circuitries activated by hypoxia, also offers a tremendous  
915 opportunity for new combination therapies that could enhance the efficacy of ICPIs and CAR  
916 T-cells also in hypoxia. In this respect, the increasing number of clinical trials combining  
917 hypoxia correctors or anti-angiogenic agents with ICPIs indicates that such combination  
918 therapies are highly attractive, particularly for advanced tumors, poorly responsive to  
919 chemotherapy or targeted therapies. Notably, combination treatments were effective also in  
920 tumors that progressed when treated with ICPI as monotherapy, indicating that targeting  
921 hypoxia-dependent pathways may reverse the secondary resistance to ICPIs.

922 The main limitations of the current approaches are the low specificity and high toxicity, due  
923 to the inhibition of physiological processes triggered by hypoxia or requiring angiogenesis. A  
924 higher specificity, that could be achieved using tumor-specific, hypoxia-activated  
925 nanocarriers, may help to limit the undesired effects and maximize the therapeutic benefits. A  
926 second limitation emerging from the first studies using ICPIs combined with anti-angiogenic  
927 agents is that the efficacy of such a combination is highly dependent on tumor histology and  
928 subtype. A more precise molecular characterization than the simple histology is mandatory to  
929 dissect the circuitries that induce resistance to ICPIs and to move towards precision  
930 immunotherapy. Last but not least, it cannot be excluded that the blockade of a specific ICP  
931 results in a compensatory up-regulation of other ICPs (Huang et al., 2017). To avoid the onset  
932 of resistance, triple combinations – based on at least two ICPIs and one hypoxia  
933 corrector/anti-angiogenic drug – may provide a solution, with the disadvantage of increased  
934 untoward toxicities. At the present time, no clinical trials are based on CAR T-cells and  
935 hypoxia correctors or anti-angiogenic drugs, but they will likely be designed with the  
936 increasing diffusion on this adoptive immunotherapy in the treatment of solid tumors.

937 In conclusion, if the combinatorial approaches associating immunotherapy with agents  
938 targeting hypoxia or hypoxia-induced angiogenesis may offer significant improvements in the  
939 treatment of tumors unresponsive to conventional therapies, the specificity, the efficacy and  
940 the safety of the combinations must be improved. These improvements require coordinated  
941 efforts of nanotechnology to realize more effective hypoxia-attenuating nanocarriers, cell  
942 biology to realize more accurate models reproducing the patient tumor, as immune-organoid  
943 and humanized mice bearing patient-derived tumors, drug discovery to develop engineered  
944 CAR or small molecules as ICPIs (Liu et al., 2021), characterized by a more favorable  
945 pharmacokinetic profile than monoclonal antibodies. The parallel advance in these branches

946 should readily improve the efficacy of immunotherapy in hypoxic tumors that are currently  
947 poorly responsive to the standard of care, bringing the future closer.

948

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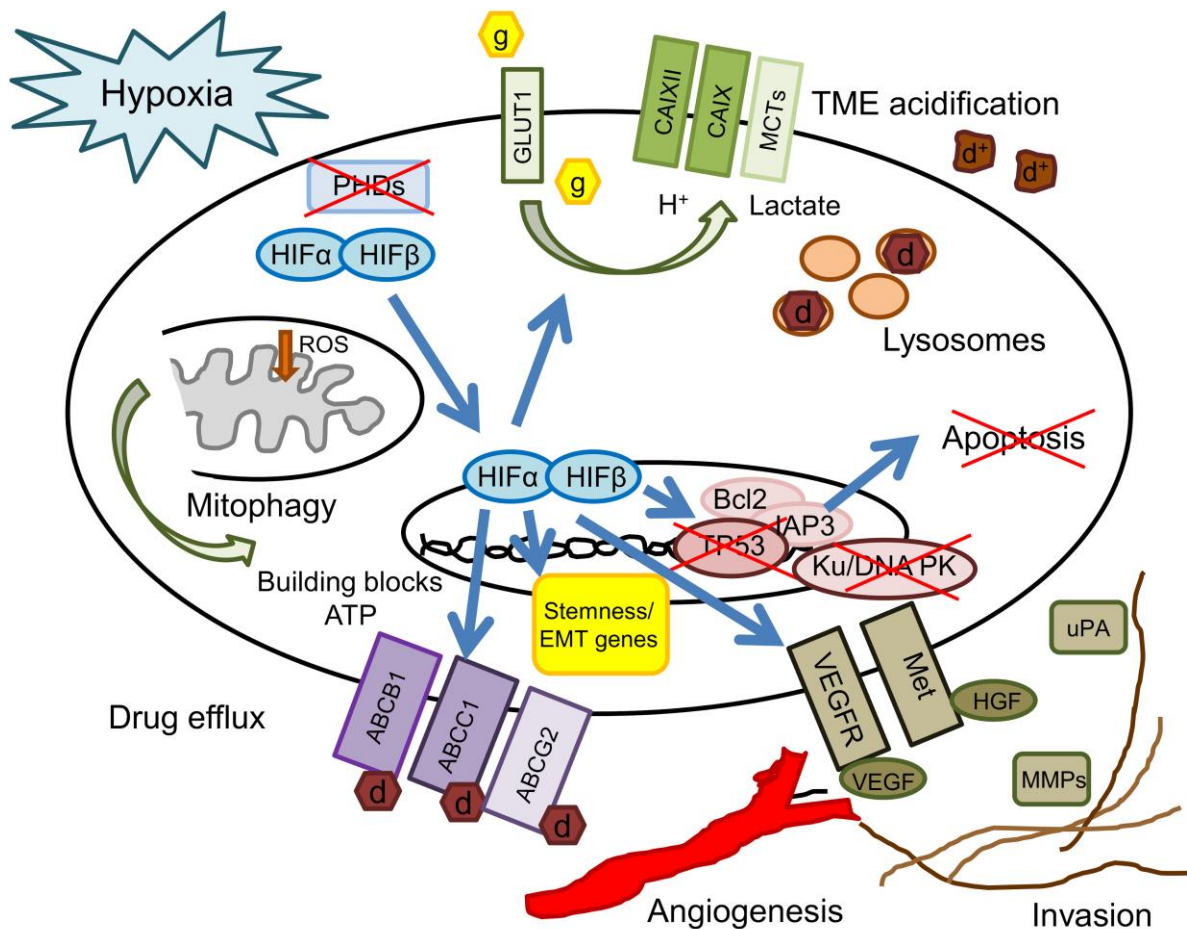
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Figure 1



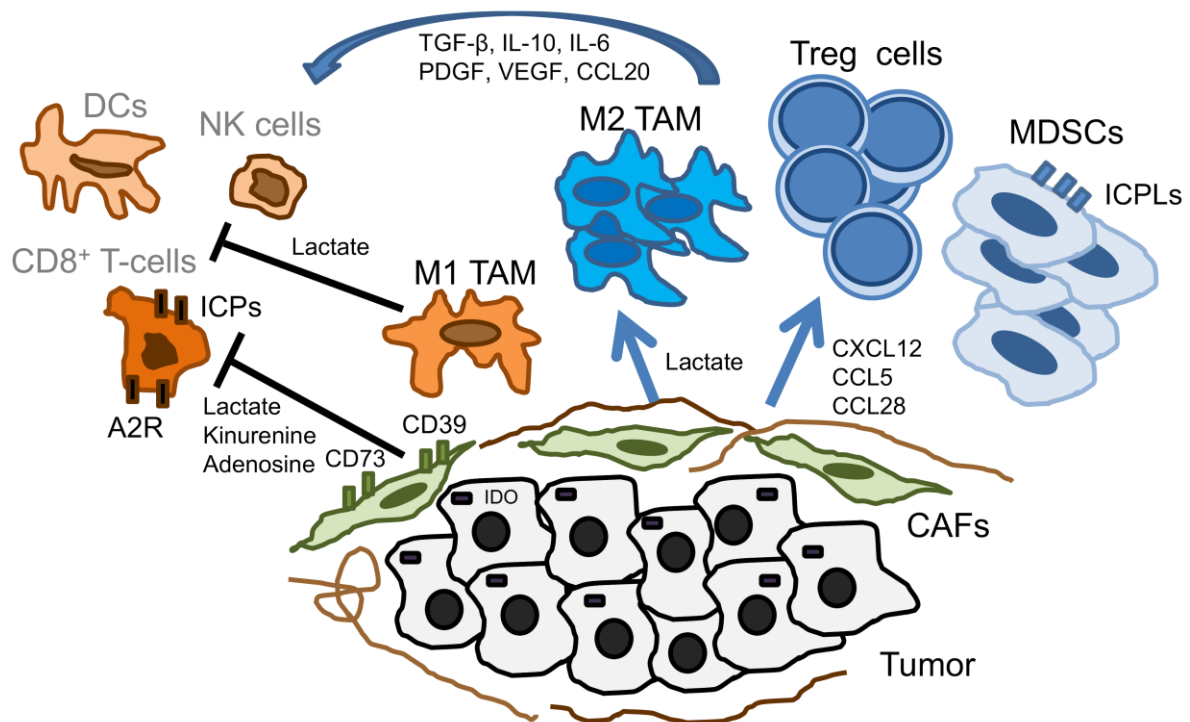
2056

2057 **Figure 1. Hypoxia induces chemoresistance by pleiotropic mechanisms.** Low pO<sub>2</sub> within  
 2058 the tumor microenvironment (TME) inhibits the enzymatic activity of prolyl hydroxylase  
 2059 dioxygenases (PHDs) that hydroxylate the O<sub>2</sub>-sensitive, hypoxia-inducible factor subunit α  
 2060 (HIFα) and prime it for ubiquitination and degradation. Forming a heterodimer with the  
 2061 constitutive and O<sub>2</sub>-independent HIFβ subunit, HIF transcriptionally up-regulates several  
 2062 genes mediating resistance. Glucose transporter 1 (GLUT1) and glycolytic enzymes are  
 2063 induced, promoting anaerobic glycolysis, intracellular acidification and TME acidification,  
 2064 regulated by the coordinated expression of the lactate/H<sup>+</sup> symporters (as monocarboxylate  
 2065 transporters, MCTs) and carbonic anhydrase (CA) IX and XII. Acidosis favors the protonation  
 2066 of chemotherapeutic drugs (d) and the increased sequestration within lysosomes, away from

2067 drug targets. The decreased oxidative-phosphorylation-based metabolism and the increased  
2068 mitophagy occurring in hypoxia reduce the levels of harmful reactive oxygen species (ROS)  
2069 and increase the rescue of building blocks and ATP, necessary for cell proliferation,  
2070 migration and drug efflux via ATP binding cassette transporters ABCB1, ABCC1 and  
2071 ABCG2, also up-regulated by HIF. The reduced apoptosis caused by the up-regulation of B-  
2072 cell lymphoma 2 (Bcl2) and inhibitor of apoptosis protein 3 (IAP-3) gene, and/or by the  
2073 inactivation of TP53 and DNA repair genes (Ku70, Ku80, DNA-PK), the increased stemness  
2074 and invasive nature driven by the epithelial mesenchymal transition (EMT) genes, hepatocyte  
2075 growth factor (HGF) Met receptor, metalloproteinases (MMPs) and urokinase-type  
2076 plasminogen activator (uPA), the neo-angiogenesis promoted by the increased expression of  
2077 vascular endothelial growth factor (VEGF) and its receptor (VEGFR) all contribute to the  
2078 dominant chemoresistance characteristic of hypoxic tumors.

2079

Figure 2



2080

2081 **Figure 2. Hypoxia increases the ratio between immunosuppressive and effector cells.**

2082 Hypoxic cancer cells and cancer-associated fibroblasts (CAFs) produce lactate via anaerobic

2083 glycolysis, kynurenine via the indoleamine dioxygenase (IDO) enzyme that catabolizes

2084 tryptophan, and adenosine through the ectonucleotidase CD73 and CD39, abundant on CAFs.

2085 All these molecules reduce survival, proliferation and cytolytic functions of anti-tumor cells,

2086 such as CD8<sup>+</sup> T-lymphocytes, natural killer (NK) cells and dendritic cells (DCs). The

2087 presence of immune checkpoints (ICPs) on effector cells contributes to their anergy. Lactate,

2088 also produced by macrophages infiltrating the hypoxic environment, increases the ratio

2089 between M2-polarized and M1-polarized tumor-associated macrophages (TAMs). C-C motif

2090 chemokine ligand 5 (CCL5), CCL28 and C-X-C motif chemokine ligand 12/stromal cell-

2091 derived factor (CXCL12/SDF-1) produced by hypoxic tumor cells recruit

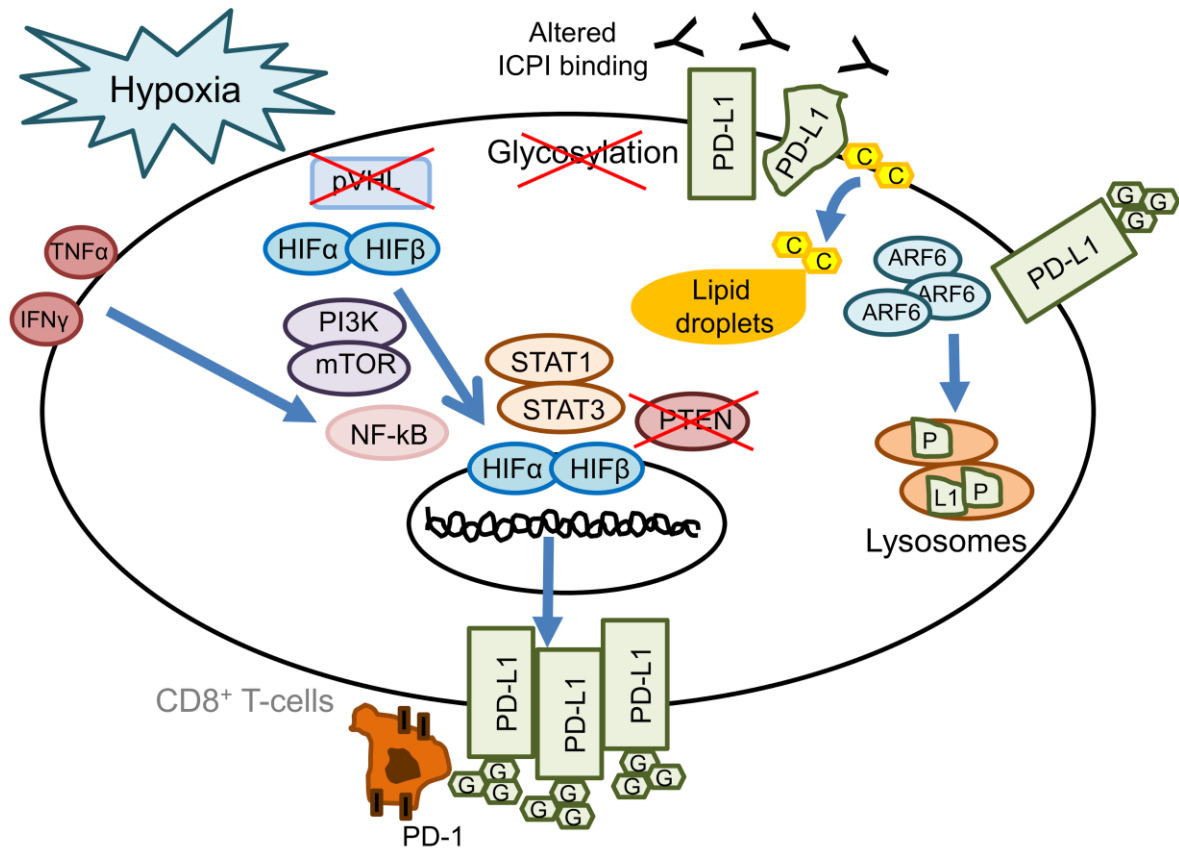
2092 immunosuppressive cells, such as T-regulatory (Treg) cells and myeloid-derived suppressor

2093 cells (MDSCs), rich in ICP ligands (ICPLs). These cells reduce the activity of effector cells

2094 by secreting immunosuppressive factors, such as transforming growth factor-β (TGF-β),

2095 interlekin-10 (IL-10), IL-6, vascular endothelial growth factor (VEGF), platelet-derived  
2096 growth factor (PDGF), CCL20. The result is the prevalence of immunosuppressive cells  
2097 associated with an immune desert in terms of effector cells. A2R: adenosine 2 receptor.  
2098

Figure 3



2099

2100 **Figure 3. Hypoxia triggers tumor-induced immunosuppression.** Hypoxic tumors with

2101 activated hypoxia-inducible factor subunit α (HIFα), inactivation of the von Hippel Lindau

2102 tumor suppressor protein (pVHL), activation of phosphatidylinositol 3'-

2103 kinase(PI3K)/mammalian target of rapamycin (mTOR), NF-κB or STAT1/STAT3 axes, loss

2104 of tensin homolog deleted on chromosome 10 (PTEN), have an increased transcription of the

2105 immune checkpoint ligand (ICPL) programmed death-ligand 1 (PD-L1) that triggers the

2106 anergy of CD8<sup>+</sup>T-lymphocytes expressing the cognate ICP PD-1. At least other three

2107 mechanisms impair the efficacy of ICP inhibitors (ICPIs) in hypoxic cells. Indeed, the low

2108 activity of O<sub>2</sub>-dependent glycosyltransferase reduces PD-L1 glycosylation (G), altering the

2109 ICPIs binding. The increased activity of ADP ribosylation factor 6 (ARF6) that controls

2110 cholesterol (C) retrograde trafficking and membrane fluidity, alters the 3D conformation of

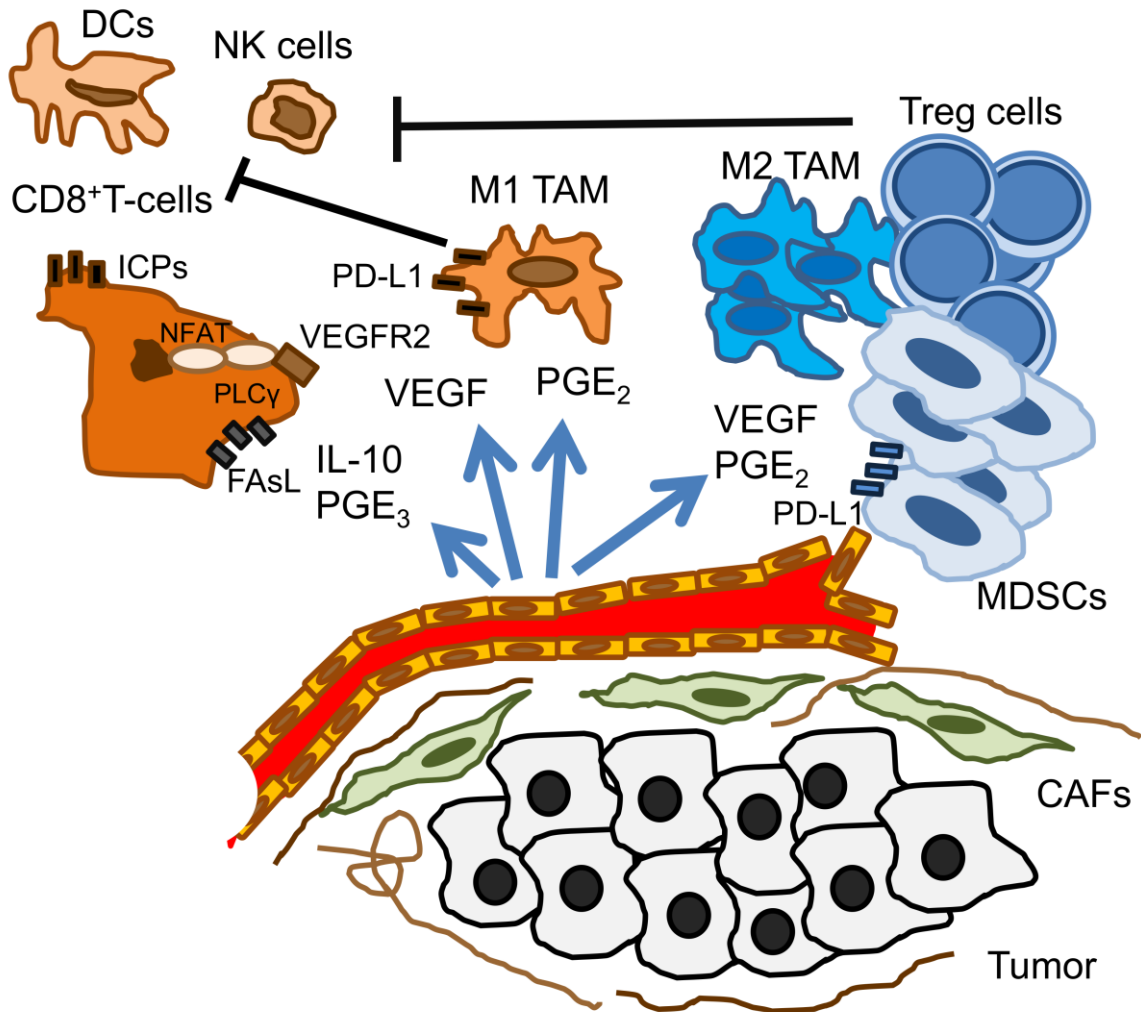
2111 PD-L1 and ICPIs binding. ARF6 also blocks PD-L1 recycling and degradation in the

2112 lysosomal compartment. The qualitative and quantitative alterations of PD-L1 render hypoxic

2113 cells more resistant to ICPIs.

2114

Figure 4



2115

2116 **Figure 4. Contribution of neo-angiogenesis to the resistance towards immune**

2117 **checkpoint inhibitors.** Endothelial cells, tumor cells and cancer associated fibroblasts

2118 (CAFs) growing in an hypoxic tumor microenvironment release several mediators inducing

2119 immunosuppression. Vascular endothelial growth factor (VEGF), a target gene of hypoxia-

2120 inducible factor (HIF), increases the expansion of T-regulatory (Treg) cells, M2-polarized

2121 tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) that

2122 repress the activities of the effector cells, CD8<sup>+</sup> T-lymphocytes, natural killer (NK) cells and

2123 dendritic cells (DCs). By interacting with the VEGF receptor 2 (VEGFR2) present on

2124 CD8<sup>+</sup>T-lymphocytes, VEGF activates the phospholipase C<sub>γ</sub> (PLC<sub>γ</sub>)/calcineurin/nuclear

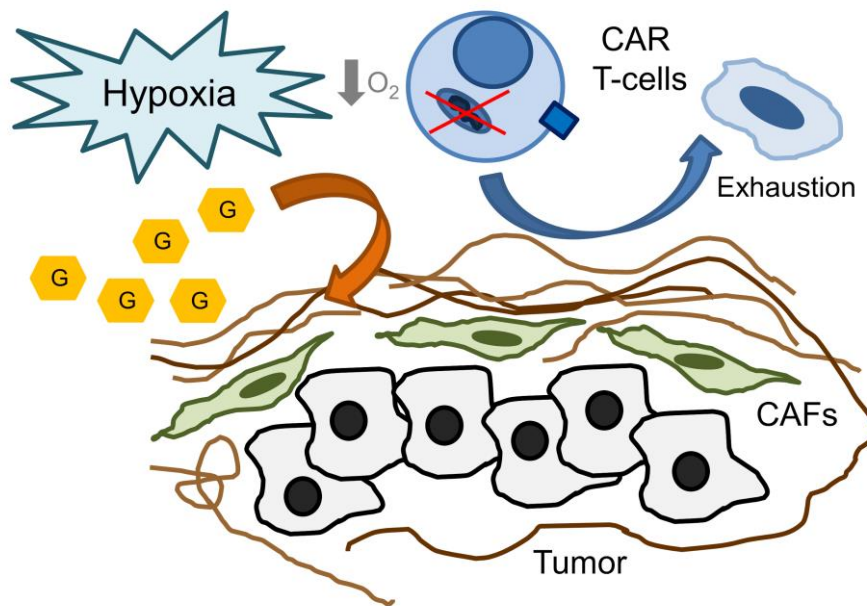
2125 factor of activated T-cell (NFAT) axis that up-regulates immune checkpoints (ICPs) and



2126 leads to T-lymphocyte anergy. VEGF also acts in an indirect manner by increasing the  
2127 endothelial production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>): the crosstalk of VEGF and PGE<sub>2</sub>  
2128 signalling increases the levels of programmed death ligand 1 (PD-L1) on M1 TAMs and  
2129 MDSCs, making these cells strong inducers of the anergy of CD8<sup>+</sup> T-lymphocytes and NK  
2130 cells. Moreover, VEGF cooperates with IL-10 and PGE<sub>3</sub> in increasing the expression of the  
2131 apoptotic executer Fas ligand (FasL) on CD8<sup>+</sup> T-lymphocytes, further worsening their anti-  
2132 tumor potential.

2133

Figure 5



2134

2135 **Figure 5. Hypoxia impairs the activity of CAR T-cells.** Rapidly proliferating tumors  
2136 growing in hypoxic niches are characterized by abundant deposition of extracellular matrix  
2137 by cancer associated fibroblasts (CAFs) that constitutes a physical barrier to the penetration  
2138 of chimeric antigen receptor (CAR) T-cells. Moreover, the extensive consumption of glucose  
2139 by cancer cells deprives CAR T-cells of their preferential fuel. At the same time, the low  $pO_2$   
2140 characteristic of hypoxic tumors impairs an alternative, oxidative-based phosphorylation  
2141 metabolism, leading to CAR T-cell metabolic and functional exhaustion.