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

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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 O₂ (pO₂) in
53 normal tissues is between 1 and 11%, the mean tumor pO₂ is below 2% (Li Petri et al., 2020;
54 Mckeown, 2014; Muz & Azab, 2015; Raz et al., 2014). Depending on pO₂, 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 pO₂ 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 O₂-sensitive α subunits (namely
67 HIF-1 α , HIF-2 α and HIF-3 α), which are degraded under normoxia conditions, and the stable,
68 O₂-insensitive β subunit (Kaelin & Ratcliffe, 2008). Most of the transcriptional programs
69 driven by hypoxia in tumors are controlled by HIF-1 α and HIF-2 α , while the role of HIF-3 α
70 is still poorly known (Duan, 2020). Under normoxic conditions, α subunits are hydroxylated
71 on proline 402 and 564 by the O₂-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 α subunits (Kaelin, 2008; Shen and Kaelin, 2013). Conversely, under hypoxia conditions, the
75 activity of PHDs is low and α subunits are stabilized up to one hour: they hence
76 heterodimerize with β 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 α in an O₂-
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⁺ 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 α and HIF-2 α
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 α , 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 α and HIF-2 α . The destabilization of TP53,
134 coupled with the HIF-1 α -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 α 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 α 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⁺ 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⁺ 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⁺ T-lymphocytes. Moreover, hypoxia decreases
190 cytokines, such as interferon- γ (IFN- γ) 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 α 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⁺/HCO₃⁻ antiporter or Na⁺/H⁺ exchanger, under the transcriptional control of HIF-1 α

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⁺ 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⁺ 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⁺ 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⁺ 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⁺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 α 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⁺ 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 β -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 1α 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⁺ T-lymphocytes. Moreover, HIF- 1α and HIF- 2α , 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 α 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 α 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 α 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- β (TGF- β) and IL-10, inhibits CD8⁺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- β 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- β (Pietrobon & Marincola, 2021; Wang et al., 2021).

270 MDSCs are other group of immunosuppressive cells abundant in the hypoxic TME which
271 reduce CD8⁺ T-lymphocyte activation by releasing the inhibitory cytokines IL-10 and IL-6.
272 In the same time, HIF-1 α 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 α and HIF-2 α 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 α (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- β , 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⁺ 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 α
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 α , as proved by the down-

301 regulation of PD-L1 in oral squamous cell carcinoma (OSCC) and adenocarcinoma cells
302 treated with HIF-1 α inhibitors (Chen et al., 2015; Koh et al., 2016; Noman et al., 2014). In
303 addition, the activation of NF- κ B (Antonangeli et al., 2020), elicited by inflammatory
304 cytokines as tumor necrosis factor- α (TNF- α) or IFN- γ (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 α (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 α and/or reduced EMT program.

315 PI3K/mTOR is another point of intersection between HIF-1 α and PD-L1: indeed, PI3K
316 increases the transcription of HIF-1 α 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 α 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 α 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 α -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 α 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 α -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 α 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 pO₂ (Scharping et al., 2017). Similar

401 results were obtained in murine glioma models where the increase in HIF-1 α 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⁺ 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 pO₂, PD-L1 levels, amount of
412 infiltrating CD8⁺ T-lymphocytes or Treg cells (Zandberg et al., 2020). Conversely, another
413 work on HNSCC demonstrated that higher intratumor pO₂ was directly correlated with the
414 amount and activity of infiltrating CD8⁺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 α is not the only factor reducing the ICPIs efficacy. In hepatocellular (HCC) patients,
417 both HIF-1 α and CXCL12 levels were associated with tumor areas characterized by high PD-
418 L1 expression. Since HIF-1 α , 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 α 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 α ,
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 α inhibitor that allosterically disrupts the heterodimerization of HIF-
441 2 α and HIF- β subunits, blocking the transcription of HIF2 α -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 α 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 α 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 α 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 O₂-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 O₂ 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 H₂O₂ present within the tumor into
548 O₂. The decrease in ROS, reduced the expression of HIF-1 α 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⁺ 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 O₂. 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 α is a transcriptional activator of pro-angiogenic factors produced by tumor- or TME-
581 associated cells; these pro-angiogenic factors include VEGF, PDGF- β , 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⁺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⁺ 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⁺T-
592 lymphocytes by activating the VEGFR-2/phospholipase C γ (PLC γ)/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⁺ 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 E₂ (PGE₂) by activating cyclo-oxygenase 2
598 (COX2) present in the endothelial cells (Tamura et al., 2020). PGE₂ 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, PGE₂ directly up-regulates
603 PD-L1 on MDSCs and TAMs: indeed, PD-L1 levels are increased when PGE₂ synthesizing
604 enzymes (COX2 and microsomal PGE₂ synthase 1) are high, and reduced when the PGE₂
605 degrading enzyme (15- hydroxyprostaglandin dehydrogenase) is high (Tamura et al., 2020).
606 By cooperating with IL-10 and PGE₃, VEGF also increased the Fas ligand (FasL) on
607 endothelial cell surface: the binding of T-cells to FasL selectively killed CD8⁺ 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-1 β -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⁺ TILs.

612 If neo-angiogenesis creates the proper conditions for CD8⁺ T-lymphocyte anergy, the
613 opposite scenario, with cytotoxic TILs normalizing tumor vasculature, occurs too. Indeed,
614 during their activation, CD8⁺ 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⁺ T-lymphocyte activity (Roberts et al., 2021; Zheng et al.,
619 2018). Interestingly, normalization of blood vessels is achieved by treating CD8⁺ 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 IFN γ , 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- γ 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). 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).
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 ≥ 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 ≥ 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 ζ -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⁺ 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⁺/CD8⁺ 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 (Fraietta 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, PGE₂), 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 O₂- 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 O₂-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 pO₂ 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 α -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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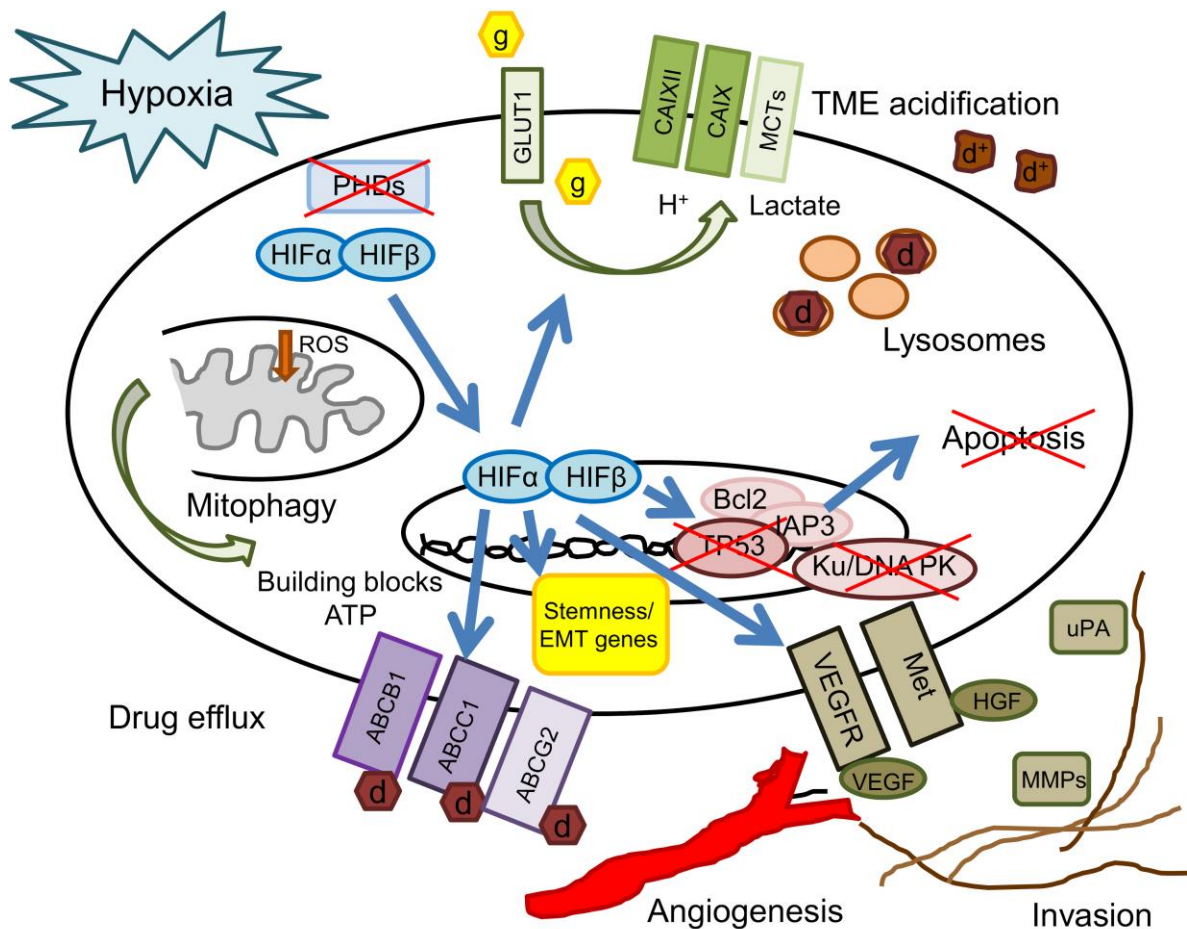
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Figure 1



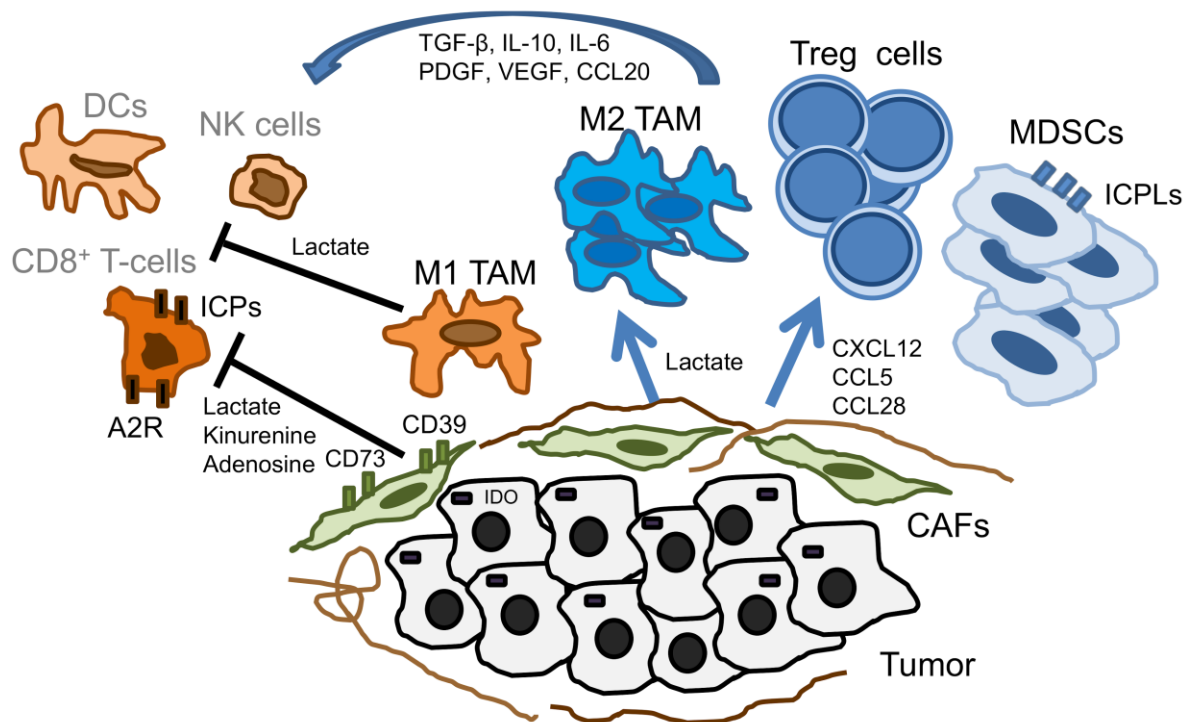
2056

2057 **Figure 1. Hypoxia induces chemoresistance by pleiotropic mechanisms.** Low pO₂ within
 2058 the tumor microenvironment (TME) inhibits the enzymatic activity of prolyl hydroxylase
 2059 dioxygenases (PHDs) that hydroxylate the O₂-sensitive, hypoxia-inducible factor subunit α
 2060 (HIFα) and prime it for ubiquitination and degradation. Forming a heterodimer with the
 2061 constitutive and O₂-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⁺ 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⁺ 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

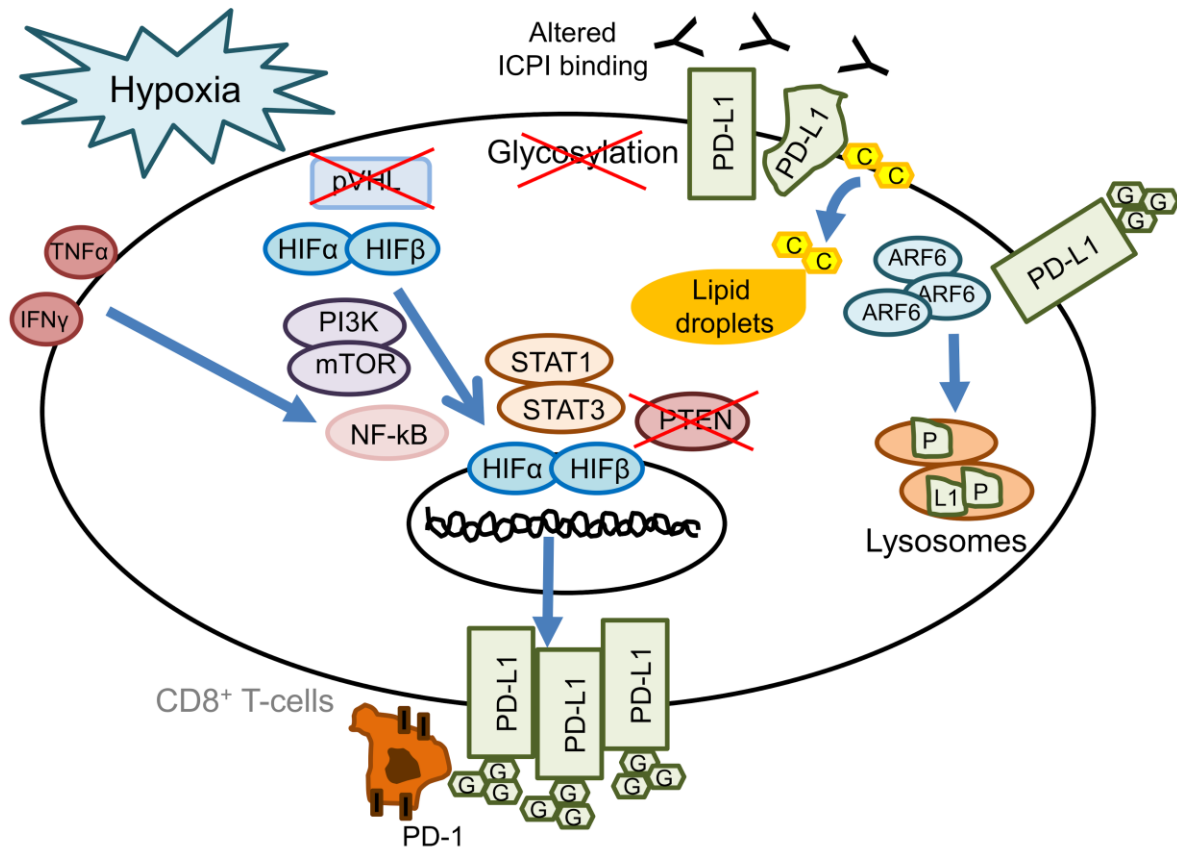
2092 immunosuppressive cells, such as T-regulatory (Treg) cells and myeloid-derived suppressor

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 (HIF α), inactivation of the von Hippel Lindau

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

2103 kinase(PI3K)/mammalian target of rapamycin (mTOR), NF- κ B 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⁺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 O₂-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

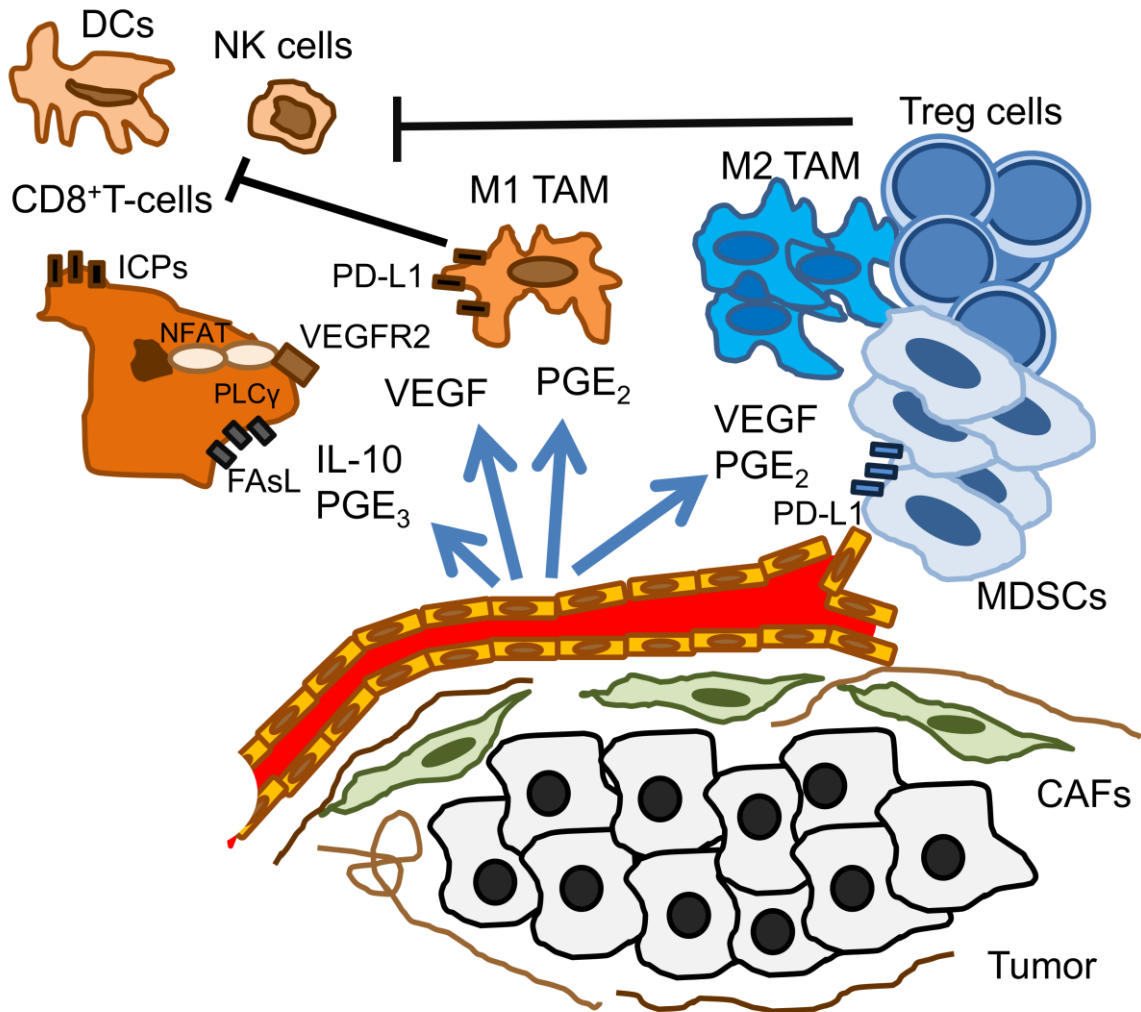
2111 PD-L1 and ICPIs binding. ARF6 also blocks PD-L1 recycling and degradation in the

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⁺ T-lymphocytes, natural killer (NK) cells and

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

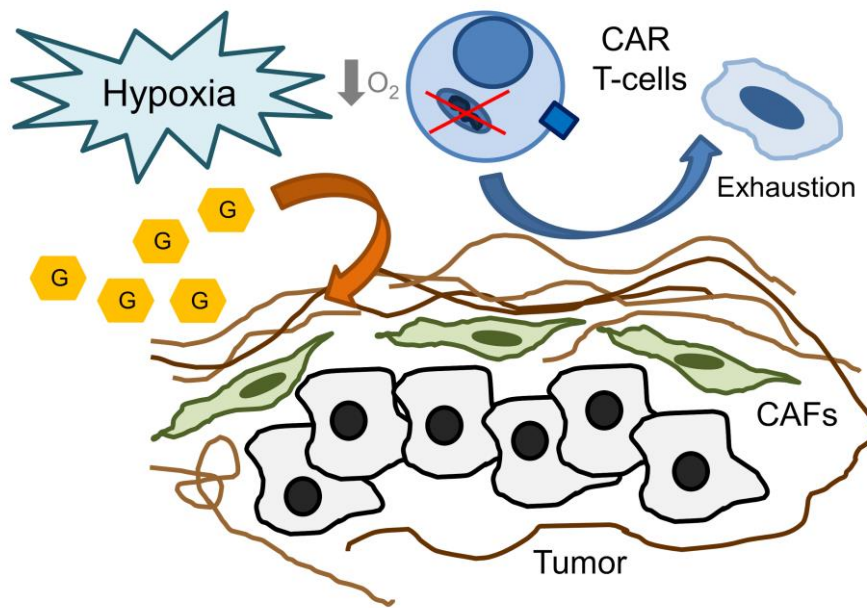
2124 CD8⁺T-lymphocytes, VEGF activates the phospholipase C_γ (PLC_γ)/calcineurin/nuclear

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 E₂ (PGE₂): the crosstalk of VEGF and PGE₂
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⁺ T-lymphocytes and NK
2130 cells. Moreover, VEGF cooperates with IL-10 and PGE₃ in increasing the expression of the
2131 apoptotic executer Fas ligand (FasL) on CD8⁺ 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 pO₂
2140 characteristic of hypoxic tumors impairs an alternative, oxidative-based phosphorylation
2141 metabolism, leading to CAR T-cell metabolic and functional exhaustion.