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Abstract

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

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

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

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

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

and re-oxygenation (Vaupel et al., 2004). While the physiological pressure of O_2 (pO_2) in normal tissues is between 1 and 11%, the mean tumor pO_2 is below 2% (Li Petri et al., 2020; Mckeown, 2014; Muz & Azab, 2015; Raz et al., 2014). Depending on pO_2, hypoxic oscillations, concomitant shortage of other nutrients such as glucose and amino acids, cancer cells growing in hypoxic areas can either slow their proliferation rate, hence undergoing necro-apoptosis, or adapt to the hypoxic conditions. This adaptation selects certain phenotypic features – increased cell cycling, migration, stemness, epithelial mesenchimal transition (EMT), resistance to stress – that confer a selective advantage over the less adaptable clones (Erin et al, 2020; Santoro et al, 2017). This natural selection renders the tumor more aggressive and difficult to be eradicated by radiotherapy and chemotherapy (Gacche & Assaraf, 2018; Huijbers et al., 2016; Kleibeuker et al., 2012; Suh et al., 2014).

The adaptation to hypoxia is coordinated by the up-regulation of the hypoxia-inducible factor (HIF) proteins, a family of transcription factors sensing intra-tissue pO_2 and controlling more than 200 genes (Gacche & Assaraf, 2018; Godet et al., 2019; Raz et al., 2014; Semenza, 2013b). HIF proteins are heterodimers, composed of the O_2-sensitive α subunits (namely HIF-1α, HIF-2α and HIF-3α), which are degraded under normoxia conditions, and the stable, O_2-insensitive β subunit (Kaelin & Ratcliffe, 2008). Most of the transcriptional programs driven by hypoxia in tumors are controlled by HIF-1α and HIF-2α, while the role of HIF-3α is still poorly known (Duan, 2020). Under normoxic conditions, α subunits are hydroxylated on proline 402 and 564 by the O_2-depending prolyl hydroxylase dioxygenases (PHDs) (Semenza, 2001). This process creates a binding site for the von Hippel Lindau tumor suppressor protein (pVHL), which pomotes the ubiquitination and proteasome degradation of α subunits (Kaelin, 2008: Shen and Kaelin, 2013). Conversely, under hypoxia conditions, the activity of PHDs is low and α subunits are stabilized up to one hour: they hence heteropdimerize with β subunits and translocate as active transcription factors to the nucleus.

Also miRNAs (Pugh & Ratcliffe, 2017), oncogenic pathways active in tumors - as the Ras/phosphatidylinositol 3'-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway (Semenza, 2013) -, inactvating mutations in the oncosuppressor TP53 (Sethi, 2019), BRCA1 DNA repair associated (BRCA1) or tensin homolog deleted on chromosome 10 (PTEN) (Li et al., 2018) proteins, intratumor reactive oxygen species (ROS), which inactivate PHDs (Kaelin & Ratcliffe, 2008), metabolites produced by the cancer associated fibroblasts (CAFs) (Assaraf et al., 2019; Raz et al., 2014) and diffused paracrinely within the tumor microenvironment (TME) such as glutamate (Briggs et al., 2016), stabilize HIFα in an O_2-independent manner. Hence, multiple cross-talks can finely tune – either enforcing or attenuating HIF-driven programs – the response of cancer cells to hypoxia.

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

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

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

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

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

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

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

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

2. The imprinting of hypoxia on tumor microenvironment reduces the efficacy of immune checkpoint inhibitors

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

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

2a. Hypoxia induces an immunosuppressive environment

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

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

Part of the hypoxic effect is mediated by the metabolic reprogramming characterized by increased intratumor acidosis, production of kynurenine and adenosine (Pietrobon & Marincola, 2021). The acidification produced by surface carbonic anhydrase (CA) IX and XII, Na^+/HCO_3^- antiporter or Na^+/H^+ exchanger, under the transcriptional control of HIF-1α

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

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

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

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

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

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

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

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

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

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

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

2b. Hypoxia renders cancer cell more immunoresistant

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

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

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

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

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

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

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

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

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

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

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

2c. Hypoxia limits the efficacy of immune checkpoint inhibitors

Since HIF-1α up-regulates PD-L1 on tumor and stromal cells, PD-1, CTLA-4 LAG-3 on immune cells (Chen et al., 2015; Cubillos-Zapata et al., 2017; Doedens et al., 2013; Koh et al., 2016; Noman et al., 2014), it is not surprising that it attenuates the efficacy of ICPIs. ICPIs are more active in well-oxygenated areas than in hypoxic areas. For instance, in murine melanoma models the efficacy of anti PD-1 treatment, in terms of increasing activity of cytotoxic TILs and tumor regression, is greater at higher pO_2 (Scharping et al., 2017). Similar

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

The findings obtained in animal models are corroborated by few clinical studies. A retrospective study in squamous cell carcinoma of the head and neck (HNSCC) patients treated with anti PD-1 ICPIs as second-line treatment after chemotherapy showed that the less hypoxic and acidic tumors, measured as tumors with lower expression of CAIX, had a better response to the ICPI in terms of overall survival (OS). In this model, the acidic TME typical of hypoxic areas seems the only factor predicting a dismal response to ICPIs, because no correlations were found between ICPI efficacy, intratumor pO_2, PD-L1 levels, amount of infiltrating $CD8^+$ T-lymphocytes or Treg cells (Zandberg et al., 2020). Conversely, another work on HNSCC demonstrated that higher intratumor pO_2 was directly correlated with the amount and activity of infiltrating $CD8^+$T-lymphocytes and with a better response to anti PD-1 treatments, evaluated as progression-free survival (PFS) and OS (Zandberg et al., 2021). HIF-1α is not the only factor reducing the ICPIs efficacy. In hepatocellular (HCC) patients, both HIF-1α and CXCL12 levels were associated with tumor areas characterized by high PD-L1 expression. Since HIF-1α, CXCL12 and PD-L1 levels all correlated with a worse prognosis, this study provides a rational basis to adopt a triple combination therapy based on sorafenib, ICPIs and anti C-X-C motif chemokine receptor 4 (CXCR4)/CXCL12 agents against resistant HCCs (Semaan et al., 2017).

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

3. Mitigating intratumor hypoxia to overcome resistance to immune checkpoint inhibitors: a versatile and open therapeutic field

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

Although pharmacological inhibitors of HIF are apparently the easiest category of drugs to be tested, they did not reach the expected therapeutic success in clinical trials (https://clinicaltrials.gov/), because of the lack of tumor specificity and the inhibition of physiological processes controlled by HIF. As a result, most inhibitors have produced predicted toxicities and only a few of them are now under clinical evaluation to improve ICPIs efficacy. Belzutifan (PT2977, MK-6482) is one of the latest, potent and selective second-generation HIF-2α inhibitor that allosterically disrupts the heterodimerization of HIF-2α and HIF-β subunits, blocking the transcription of HIF2α-responsive genes (Choueiri & Kaelin, 2020; Xu et al., 2019). This small molecule is currently under investigation in 10 trials (https://clinicaltrials.gov/) and on March 16, 2021 it received a Priority Review from the FDA for VHL disease-associated ccRCC not requiring immediate surgery. The review was based on the objective response rate (ORR) obtained in the open label phase 2, NCT03401788 trial (Iliopoulos et al., 2021; Srinivasan et al., 2021). After the evaluation of pharmacodynamics, pharmacokinetics, anti-tumor activity and safety in the first-in-human phase 1 NCT02974738 study (Choueiri et al., 2021c) (Choueiri et al, 2021a), belzutifan was evaluated as single agent (NCT02974738) or in combination with the tyrosine kinase receptor inhibitor cabozantinib (NCT03634540) for metastatic ccRCC previously treated with PD-

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

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

Among the FDA-approved HIF inhibitors under evaluation for the possible combination with ICPs is vorinostat (suberoylanilide hydroxamic acid, SAHA), a well-known histone deacetylase (HDAC) inhibitor used for the treatment of cutaneous T-cell lymphoma, capable of decreasing both HIF-1α expression (Hutt et al., 2014) and nuclear translocation (Zhang et al., 2017). Therefore, it represents a multi-target drug endowed with an additional antitumor mechanism of action beyond his epigenetic effect. Recently, in a randomized phase II study (NCT02395627), 34 estrogen receptor (ER)-positive breast cancer women who have progressed on a median of five prior therapeutic regimens, received vorinostat, the anti-ER tamoxifen and pembrolizumab. Although the study was terminated because of the low efficacy in the whole population enrolled, among the 27 evaluable patients, 18.5% patients achieved a clinical benefit and 3.7% an objective response (Terranova-Barberio et al., 2020). The phase II open label trial NCT02538510 enrolled patients with recurrent metastatic HNSCC and salivary gland cancer receiving vorinostat and pembrolizumab. In the HNSCC group, the combination therapy showed PFS and OS superior to pembrolizumab alone, but also a 36% grade >3 toxicity, that was higher than that reported with the ICPI alone (Rodriguez et al., 2020). A phase I/Ib study (NCT02638090) evaluating the combination of vorinostat with pembrolizumab in patients with advanced/metastatic NSCLC, either ICPI naïve or pre-treated with pembrolizumab, reported a 33% of patients with progressive disease, 53% with stable disease and 13% achieving partial response, with good tolerability. Notably the percentages were similar in pembrolizumab pre-treated patients (Gray et al., 2019), suggesting the ability of vorinostat to overcome the acquired resistance eventually

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

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

Other approaches have been studied in order to relieve the impact of hypoxia, with the aim of using less toxic and more effective strategies. One physical approach to reverse hypoxia has been the exposure of patients to a hyper-oxygenated atmosphere. However, in a phase III trial, the use of a hyperbaric chamber in patients with central nervous system tumors did not improve the outcome compared with the current standard treatment (Stępień et al., 2016). Among the pharmacological agents, OXPHOS inhibitors have been proposed as O_2-sparing drugs. In this respect, metformin, an anti-diabetic drug that inhibits the complex I of the electron transport chain, has been repurposed as an immune-sensitizer: by reducing the mitochondrial O_2 consumption, it synergized with anti-PD-1 antibody in immunocompetent mice bearing melanomas, where the combination improved the cytolytic activity of TILs and achieved tumor regression (Scharping et al., 2017).

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

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

A very recent approach designed to overcome hypoxia is based on hypoxia-relieving nanoparticles (NPs). One of these formulations, i.e. NPs coated with melanoma cell membrane (mZCD), carrying catalase (CAT) enzyme and doxorubicin, has proven to relieve hypoxia and enhance the therapeutic efficacy of chemotherapy and immunotherapy. The NPs were targeted to melanoma, where CAT transformed the H_2O_2 present within the tumor into O_2. The decrease in ROS, reduced the expression of HIF-1α and PD-L1, facilitating the cytotoxic activity of doxorubicin (Zou et al., 2018). The combination of mZCD-CAT-NPs and the anti-PD-1 antibody achieved synergistic effects reflected in prevention of tumor

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

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

When the tumors grow, new blood vessels form to provide nutrients and O_2. However, the newly formed blood vessels are often structurally and morphologically aberrant, and create a

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

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

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

Several soluble factors downstream of VEGF also increase ICPs in the hypoxic TME. Indeed, VEGF induced the secretion of prostaglandin E_2 (PGE_2) by activating cyclo-oxygenase 2 (COX2) present in the endothelial cells (Tamura et al., 2020). PGE_2 suppressed DC maturation and NK activity (Tamura et al., 2020). This triggers a vicious cycle: NK cells are

endogenous inhibitors of neo-angiogenesis, because they secrete a soluble VEGFR that scavenges VEGF in response to hypoxic conditions (Krzywinska et al., 2017). Conversely, the low activity of NK cells fuels neo-angiogenesis. Moreover, PGE_2 directly up-regulates PD-L1 on MDSCs and TAMs: indeed, PD-L1 levels are increased when PGE_2 synthesizing enzymes (COX2 and microsomal PGE_2 synthase 1) are high, and reduced when the PGE_2 degrading enzyme (15- hydroxyprostaglandin dehydrogenase) is high (Tamura et al., 2020). By cooperating with IL-10 and PGE_3, VEGF also increased the Fas ligand (FasL) on endothelial cell surface: the binding of T-cells to FasL selectively killed $CD8^+$ T-lymphocytes, but it spared Treg cells that are protected by the high levels of the anti-apoptotic protein cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) (Motz et al., 2014). This mechanism leads to the progressive enrichment of Treg cells and to the deprivation of $CD8^+$ TILs.

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

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

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

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

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

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

4b. Combining anti-angiogenic therapy and immune checkpoint inhibitors in clinical practice

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

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

Since 2019 the advanced RCC treatment landscape includes another combination regimen based on pembrolizumab and the anti-panVEGFR axitinib, after publishing the results of the multicenter, open-label phase III KEYNOTE-426 (NCT02853331) trial enrolling 861 naïve patients. The combination arm displayed a statistically significant improvement in OS and in PFS compared to patients treated with the standard-of-care anti-VEGF sunitinib, regardless of 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

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

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

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

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

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

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

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

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

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

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

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

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

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

Since the immunosuppressive TME is the major obstacle for CAR-T-cells therapy in solid tumors, several strategies directed to regulate TME plasticity and reverse the TME-dependent immunosuppression are being explored. Armored CAR T-cells expressing pro-inflammatory cytokines, combination of CAR T-cells with oncolytic viruses, new generation of CAR T-cells targeting CAFs, T-reg cells, M2 TAMs or MDSCs are under development (Rodriguez-Garcia et al., 2020). It is known that an ECM rich in collagen and poorly vascularized provides a physical barrier, preventing the efficient homing and infiltration of CAR T-cells. Moreover, the hypoxic environment up-regulates ICPs and respective ligands, expands immunosuppressive cells, triggers the releases of immunosuppressive soluble factors (adenosine, PGE_2), induces a metabolic pressure on effector T-cells by subtracting key nutrients (Glover et al., 2021). All these factors, which are common to the resistance mechanisms toward ICPIs, impair the efficacy of CAR T-cells as well.

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

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

5b. Linkage between CAR T-cells and hypoxia

A very common mechanism of drug resistance in solid tumors is hypoxia, a hallmark of the TME in solid cancers (Berahovich et al., 2019) that also impairs the efficacy of adoptive immunotherapy. The O_2- and glucose-deficient TME deprives T-lymphocytes, including CAR T-cells, of the main energy source, pushing them to exhaustion (Schurich et al., 2019). This is one of the first mechanisms explaining the lower efficacy of CAR- T-cells in the treatment of solid tumors. Indeed, both activated T-lymphocytes and cancer cells preferentially use glucose. The strongly energy demand of cancer cells renders the TME poor in glucose for T-cells. At the same time, the hypoxic TME impairs the mitochondrial OXPHOS in T-lymphocytes, leading to a metabolic and functional exhaustion (Schurich et al., 2019). While tumor cells grow well in hypoxic niches, T-cell fitness and survival is limited in these niches, where an efficient trafficking and penetration of CAR T-cells is not achieved (Wagner et al., 2020) (Figure 5).

These events make hypoxia an inducer of resistance to CAR T-cell therapy. Several research groups recently began to address the "hypoxia problem" by generating O_2-sensitive self-decision making engineered CAR T-cells, (Juillerat et al., 2017; Kosti et al., 2021). The hypoxia-sensing CAR T-cell system (called HypoxiCAR T or HiCAR T) is designed to express a CAR under the control of a stringent hypoxia-sensing safety switch, avoiding off-tumor activation of CAR T-cells and delivering efficient anti-tumor killing in hypoxic TME (Kosti et al., 2021). This approach may represent a good modality to improve the efficacy of CAR T-cells againts hypoxic solid tumors, a challenge that remains open at the present time.

6. Conclusions and future perspectives

Hypoxia is a driver of multiple aggressive features in tumors, inducing metabolic rewiring, apoptosis inhibition, cell migration and increased adaptability to unfavorable conditions. The first consequence of these transformations is the higher resistance of hypoxic tumors to chemotherapy and radiotherapy, as well as to other stressful conditions which usually kill normoxic cells including nutrient deprivation, calcium oscillation, endoplasmic reticulum stress) (Akman et al., 2021; Belisario et al., 2020). The effects of hypoxia alter not only the cancer cell, but also tumor-associated cells, such as CAFs, endothelial cells and immune-infiltrating cells. The response of each component is strictly interconnected and synergizes to generate more aggressive and chemoresistant tumors. In response to hypoxia, CAFs secrete soluble factors favoring the EMT program, lactate and building blocks for cancer cells, neo-angiogenesis factors, chemokines and cytokines attracting immune cells with immunosuppressive potential. Endothelial cells respond with the formation of an irregular and leaky vasculature that does not compensate for the low pO_2 and impairs the delivery of drugs, as well as the extravasation of anti-tumor immune cells. Immune-infiltrating cells are characterized by low levels of anti-tumor cytotoxic populations with functional anergy and high expression of ICPs, and high levels of immunotolerant/immunosuppressive cells, low activity of CAR T-cells. By directly affecting the proliferation and differentiation of effectors cells, or by triggering the secretion of immunosuppressive cytokines by TME cells, hypoxia generates an immune disaster.

The recent introduction of ICPIs was a revolution for the therapeutic outcome of specific tumors, particularly immunologically “hot” tumors as melanoma and NSCLC. On the other hand, the increasing use of ICPIs has been paralleled by the first cases of resistance. 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-1α-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.

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

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

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

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Figures and figure legends

Figure 1

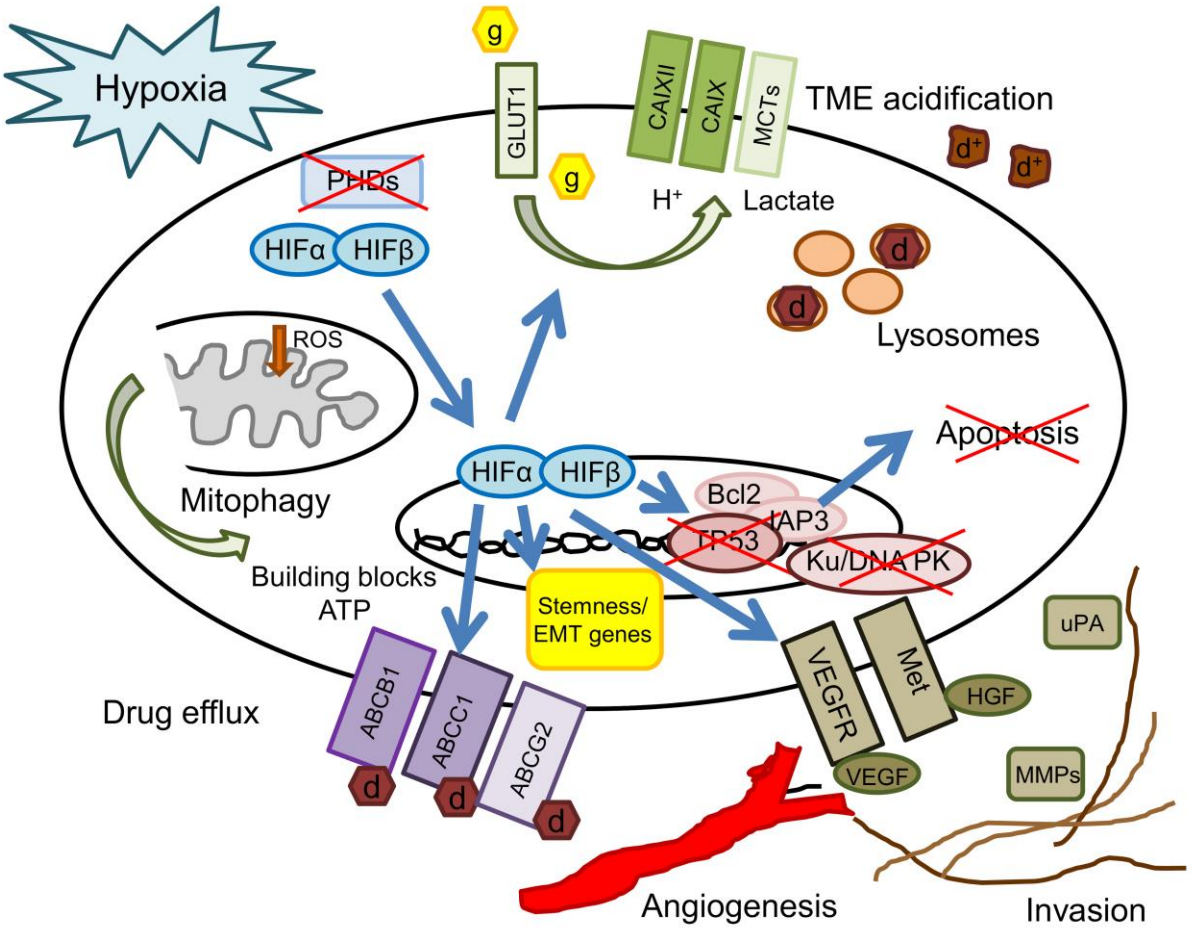

Figure 1. Hypoxia induces chemoresistance by pleiotropic mechanisms. Low pO_2 within the tumor microenvironment (TME) inhibits the enzymatic activity of prolyl hydroxylase dioxygenases (PHDs) that hydroxylate the O_2-sensitive, hypoxia-inducible factor subunit α (HIFα) and prime it for ubiquitination and degradation. Forming a heterodimer with the constitutive and O_2-independent HIFβ subunit, HIF transcriptionally up-regulates several genes mediating resistance. Glucose transporter 1 (GLUT1) and glycolytic enzymes are induced, promoting anaerobic glycolysis, intracellular acidification and TME acidification, regulated by the coordinated expression of the lactate/H^+ symporters (as monocarboxylate transporters, MCTs) and carbonic anydrase (CA) IX and XII. Acidosis favors the protonation of chemotherapeutic drugs (d) and the increased sequestration within lysosomes, away from

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

Figure 2

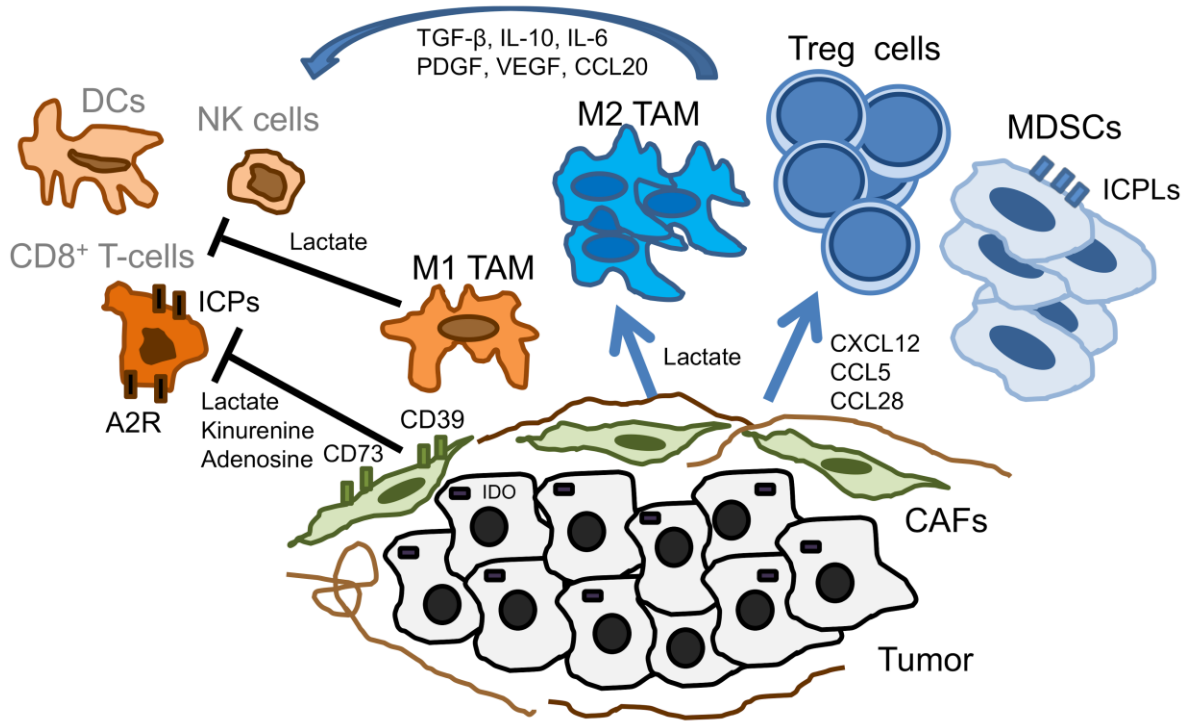

Figure 2. Hypoxia increases the ratio between immunosuppressive and effector cells. Hypoxic cancer cells and cancer-associated fibroblasts (CAFs) produce lactate via anaeobic glycolysis, kynurenine via the indoleamine dioxygenase (IDO) enzyme that catabolizes tryptophan, and adenosine through the ectonucleotidase CD73 and CD39, abundant on CAFs. All these molecules reduce survival, proliferation and cytolytic funtions of anti-tumor cells, such as $CD8^+$ T-lymphocytes, natural killer (NK) cells and dendritic cells (DCs). The presence of immune checkpoints (ICPs) on effectors cells contributes to their anergy. Lactate, also produced by macrophages infiltrating the hypoxic environment, increases the ratio between M2-polarized and M1-polarized tumor-associated macrophages (TAMs). C-C motif chemokine ligand 5 (CCL5), CCL28 and C-X-C motif chemokine ligand 12/stromal cell-derived factor (CXCL12/SDF-1) produced by hypoxic tumor cells recruit immunosuppressive cells, such as T-regulatory (Treg) cells and myeloid-derived suppressor cells (MDSCs), rich in ICP ligands (ICPLs). These cells reduce the activity of effector cells by secreting immunosuppressive factors, such as transforming growth factor-β (TGF-β),

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

Figure 3

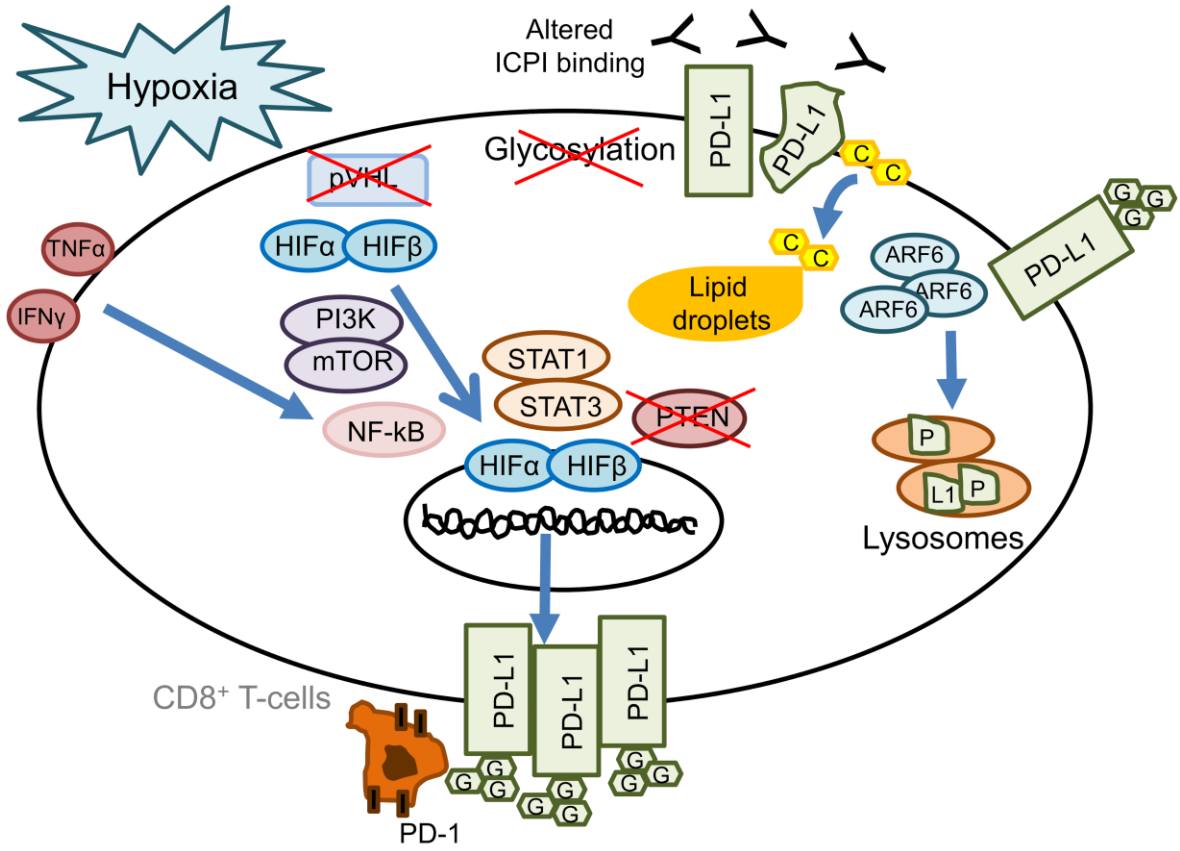

Figure 3. Hypoxia triggers tumor-induced immunosuppression. Hypoxic tumors with activated hypoxia-inducible factor subunit α (HIFα), inactivation of the von Hippel Lindau tumor suppressor protein (pVHL), activation of phosphatidylinositol 3'-kinase(PI3K)/mammalian target of rapamycin (mTOR), NF-kB or STAT1/STAT3 axes, loss of tensin homolog deleted on chromosome 10 (PTEN), have an increased transcription of the immune checkpoint ligand (ICPL) programmed death-ligand 1 (PD-L1) that triggers the anergy of $CD8^+$T-lymphocytes expressing the cognate ICP PD-1. At least other three mechanisms impair the efficacy of ICP inhibitors (ICPIs) in hypoxic cells. Indeed, the low activity of O_2-dependent glycosyltransferase reduces PD-L1 glycosylation (G), altering the ICPIs binding. The increased activity of ADP ribosylation factor 6 (ARF6) that controls cholesterol (C) retrograde trafficking and membrane fluidity, alters the 3D conformation of PD-L1 and ICPIs binding. ARF6 also blocks PD-L1 recycling and degradation in the

lysosomal compartment. The qualitative and quantitative alterations of PD-L1 render hypoxic cells more resistant to ICPIs.

Figure 4

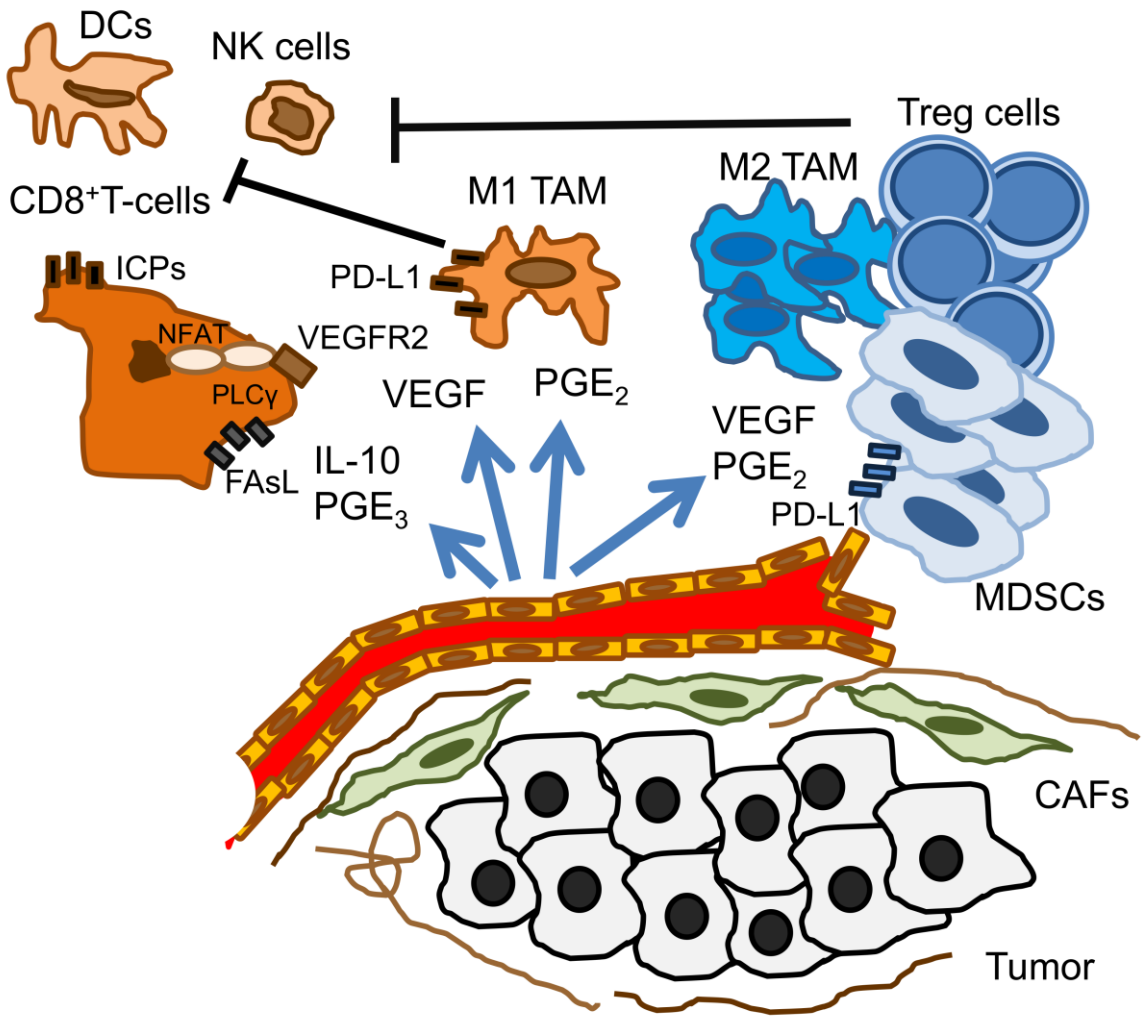

Figure 4. Contribution of neo-angiogenesis to the resistance towards immune checkpoint inhibitors. Endothelial cells, tumor cells and cancer associated fibroblasts (CAFs) growing in an hypoxic tumor microenvironment release several mediators inducing immunosuppression. Vascular endothelial growth factor (VEGF), a target gene of hypoxia-inducible factor (HIF), increases the expansion of T-regulatory (Treg) cells, M2-polarized tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) that repress the activities of the effector cells, $CD8^+$ T-lymphocytes, natural killer (NK) cells and dendritic cells (DCs). By interacting with the VEGF receptor 2 (VEGFR2) present on $CD8^+$T-lymphocytes, VEGF activates the phospholipase Cγ (PLCγ)/calcineurin/nuclear factor of activated T-cell (NFAT) axis that up-regulates immune checkpoints (ICPs) and

leads to T-lymphocyte anergy. VEGF also acts in an indirect manner by increasing the endothelial production of prostaglandin E_2 (PGE_2): the crosstalk of VEGF and PGE_2 signalling increases the levels of programmed death ligand 1 (PD-L1) on M1 TAMs and MDSCs, making these cells strong inducers of the anergy of $CD8^+$ T-lymphocytes and NK cells. Moreover, VEGF cooperates with IL-10 and PGE_3 in increasing the expression of the apoptotic executer Fas ligand (FasL) on $CD8^+$ T-lymphocytes, further worsening their anti-tumor potential.

Figure 5

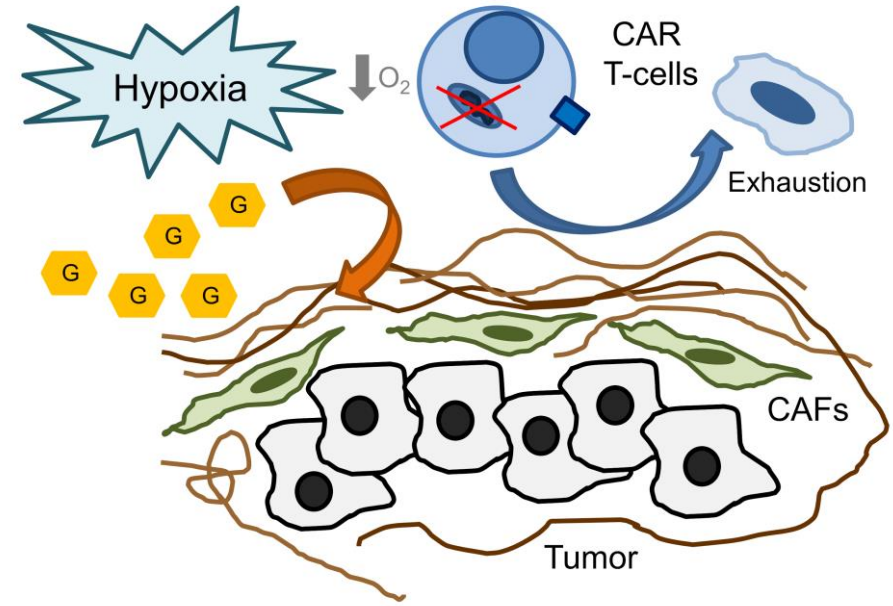

Figure 5. Hypoxia impairs the activity of CAR T-cells. Rapidly proliferating tumors growing in hypoxic niches are characterized by abundant deposition of extracellular matrix by cancer associated fibroblasts (CAFs) that constitutes a physical barrier to the penetration of chimeric antigen receptor (CAR)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