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THESE

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Présentée par

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Influence of hypoxia on tumor cell susceptibility to cytotoxic T lymphocyte mediated lysis

Influence de l'hypoxie sur la susceptibilité des cellules tumorales à la lyse induite par les lymphocytes T cytotoxiques

Soutenue le 28 septembre 2012 devant le jury composé des :

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ABSTRACT

Hypoxia is a common feature of solid tumors and one of the hallmarks of tumor microenvironment. Tumor hypoxia plays an important role in angiogenesis, malignant progression, metastatic development, chemo-radio resistance and favours immune evasion by the emergence of tumor variants with increased survival and anti-apoptotic potential. There is very little work done on the impact of tumor hypoxia on the regulation of tumor susceptibility to the lysis induced by cytotoxic antitumor response. Therefore, we asked whether hypoxia confers tumor resistance to cytotoxic T lymphocyte (CTL)-mediated killing.

We demonstrated that exposure of target cells to hypoxia has an inhibitory effect on the CTL-mediated autologous target cell lysis. Such inhibition was not associated with an alteration of CTL reactivity and tumor target recognition. We also showed that the concomitant hypoxic induction of Signal transducer and activator of transcription 3 (STAT3) phosphorylation on tyrosine 705 residue (pSTAT3) and hypoxia inducible factor 1 alpha (HIF-1α) is functionally linked to the alteration of Non small cell lung carcinoma (NSCLC) target susceptibility to CTL-mediated killing.

We also showed that hypoxia-induced resistance of lung tumor to CTL-mediated lysis was associated with autophagy induction in target cells. Inhibition of autophagy resulted in impairment of pSTAT3 (via inhibition Src kinase) and restoration of hypoxic tumor cell susceptibility to CTL-mediated lysis. Moreover, *in vivo* inhibition of autophagy by hydroxychloroquine (HCQ) in B16F10 tumor bearing mice and mice vaccinated with TRP2 peptide dramatically increased tumor growth inhibition. Collectively, the current study establishes a novel functional link between hypoxia-induced autophagy and the regulation of antigen specific T cell lysis and points to a major role of autophagy in the control of in vivo tumor growth.

Finally, as resistance of tumor targets to killer cells is likely to be regulated by multiple factors, we further aimed to identify the microRNA's regulated by hypoxia in NSCLC and melanoma and their putative involvement in the regulation of tumor susceptibility to antigen-specific CTL-mediated killing. MicroRNA-210 (miR-210) was significantly induced in a HIF-1α dependent manner in NSCLC and melanoma cells and miR-210 was expressed in hypoxic zones of human NSCLC tissues. Moreover, we demonstrated that hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated lysis in part by suppressing PTPN, HOXA1 and TP53I11 expression indicating that miR-210 plays a potential role in the regulation of anti-tumor immune response.

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ANGPTL4	Angiopoietin-like protein 4
APC	Antigen Presenting Cell
ATP	Adenosine triphosphate
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2 associated X protein
Bcl-2	B Cell Lymphoma-2
bHLH	Basic helix-loop-helix
BID	BH3-interacting domain death agonist
CAD	Caspase activated desoxyribonuclease
CTL	Cytotoxic T lymphocyte
CTLA-4	CTL Associated Antigen-4
CXCR 4	C-X-C chemokine receptor type 4
DISC	Death-inducing signaling complex
DNA	Desoxyribonucleic acid
EFNA-1	Ephrin A1
EGF	Epidermal Growth Factor
EPAS-1	Endothelial PAS domain protein
EPO	Erythropoietin
EMT	Epithelial-Mesenchymal Transition
FADD	Fas associated death domain
FH	Factor inhibiting hypoxia-inducible factor
FIH	Fumarate Hydratase
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Fator
GrB	Granzyme B
HCQ	Hydroxychloroquine
HRE	Hypoxia Responsive Element
HER2/neu	Human Epidermal Growth Factor Receptor-2/neu
hsp 90	Heat Shock Protein 90
HAF	Hypoxia associated factor
HIF	Hypoxia inducible factor
HLA A/G	Human leucocyte antigen A/G
ICAM	Intercellular adhesion molecules
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IS	Immunological synapse

LC-3	Light chain 3
LFA-1	Lymphocyte function-assosiated antigen I
MART-1	Melanoma-associated antigen recognized by T cells
MDM2	murine double minute
MDSC	Myeloid-derived suppressor cells
MDR	Multidrug Resistance protein 1
MHC-I	Major histocompatibility complex I
MiR	Micro RNA
MMP-7	Matrix metalloproteinase-7
MTOC	MicroTubules Organizing Center
NF-KB	Nuclear Factor- κB
NK	Natural killer cell
NKT	Natural killer T cell
NO	Ntiric Oxide
NSCLC	Non small cell lung carcinoma
OCT-4	Octamer-binding transcription factor 4
PETSCAN	Positron emission tomography
PD-1	Programmed Death 1
PI3Kinase	Phosphatidyl-Inositol-3-Kinase
PKC	Protein kinase C
P300	E1A binding protein p300
ROS	Reactive oxygen species
Serpin I9	Serine protease inhibitor I9
SDF-1	Stromal cell-derived factor-1
STAT3	Signal Transducers and Activators of Transduction 3
TAA	Tumor associated antigen
TAP	Transporter activated peptide
TAM	Tumor associated Macrophages
TCR	T Cell Receptor
TGF β	Transforming growth factor β
TIL	Tumor Infiltrating Lymphocyte
TNF α	Tumor Necrosis Factor α
TRP-2	Tyrosine-related protein-2
T reg	T regulatory cells
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

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Your unconditional support, unlimited love and countless prayers have always helped me to fulfill my dreams!!!!

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PREFACE

Cancer Immunotherapy has developed a lot during the 90's. The discovery of tumor associated antigen (TAA) has brought a new revolution in this field. However the results of the clinical studies remained in the background because of the existing conflict/interactions between the immune system of the host, its tumoral system and its microenvironment, which is able to neutralize the antitumor cytotoxic effectors of various types. It has become clear that by analysing the interactions between the immune system and the tumoral system could facilitate the development and emergence of new approaches in antitumor immunotherapy. Several reports indicated that tumor cells evade adaptive immunity by a variety of mechanisms including development of hypoxia (low oxygen tension) [1]. Tumor hypoxia plays an important role in angiogenesis, malignant progression, metastatic development, chemo-radio resistance [2] and favours the emergence of tumor variants with increased survival and anti-apoptotic potential [3]. There is very little work done on the impact of tumor hypoxia on the regulation of tumor susceptibility to the lysis induced by cytotoxic antitumor response.

The first section of this manuscript introduces the topic by providing an overview of our actual knowledge about tumor hypoxia and hypoxia inducible factors (HIF) with special focus on their contribution in the field of tumor development and progression. It also deals about how hypoxia by modulating tumor and immune system, favours immune evasion. It outlines how hypoxic stress confers resistance to tumor cells, and describes the molecular aspects of the cross-talk between hypoxia induced factors (HIF-1-α, p-stat3 and miR-210 etc) and resistance to cytotoxic treatments. Furthermore, how cells of the immune system (both innate and adaptive) respond to hypoxia in terms of their survival and effector functions and the ways in which hypoxia promotes their recruitment, activation, and survival are also discussed. Finally, anti-tumor immune response and different mechanisms of immune evasion are also explained in detail.

In the second section, the various results obtained during this thesis are presented and discussed. The published articles are included in this section.

INTRODUCTION

I. HYPOXIA

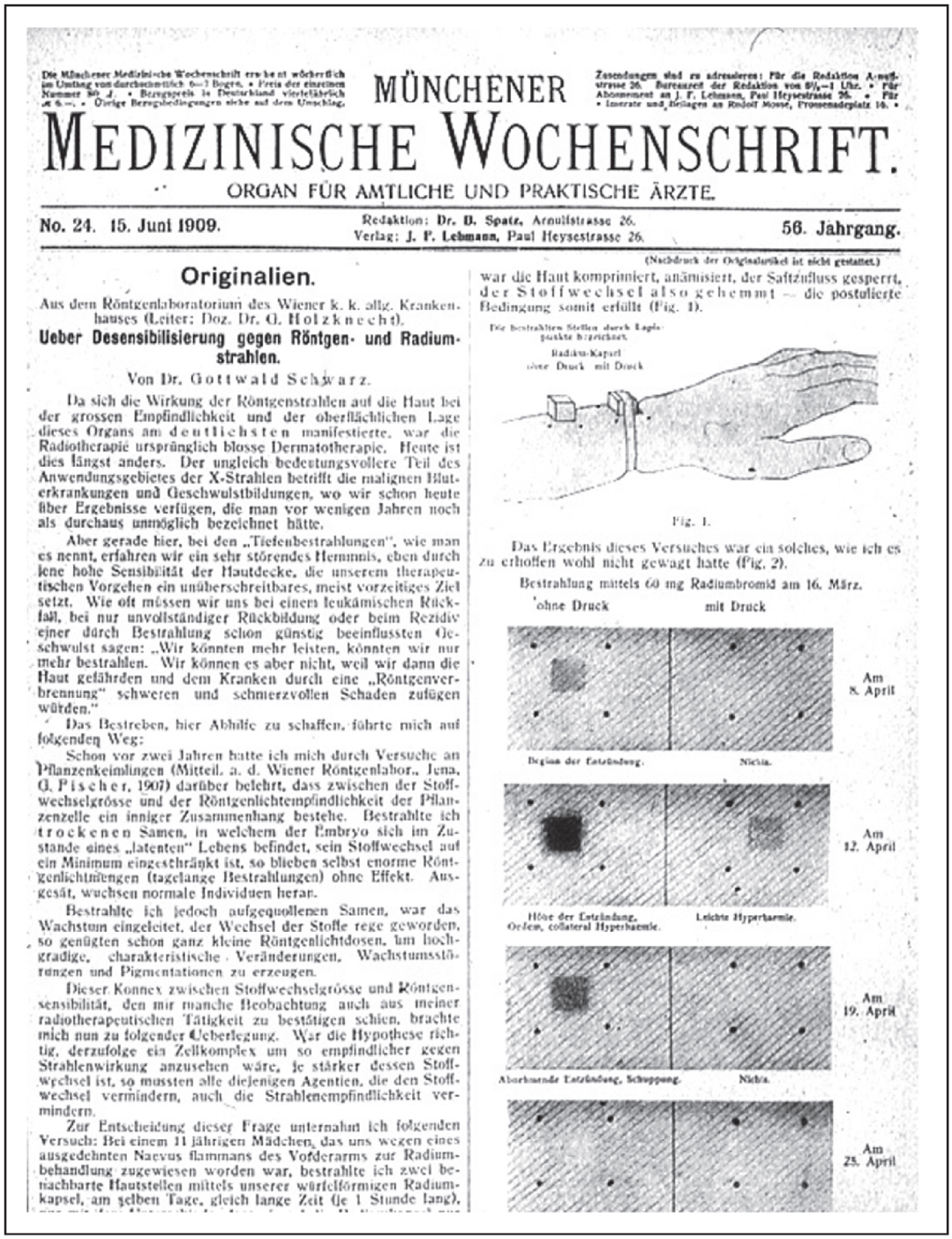
Hypoxia is defined as a reduction in the normal level of tissue oxygen tension [4]. It occurs in a variety of conditions such as stroke (acute and chronic vascular disease), tissue ischaemia, inflammation, pulmonary disease and cancer. Severe or prolonged hypoxia can lead to cell death in both normal and cancer cells [5]. Hypoxia differs from anoxia which is the total absence of oxygen within a body tissue. Normal ambient air is 21% oxygen (partial pressure of oxygen (pO_2) 150 mm Hg) and most of the mammalian tissues exist at 2% to 9% oxygen (on average 40 mm Hg). Hypoxia is defined as ≤ 2% oxygen (10-15mmHg), and severe hypoxia or anoxia is defined as ≤ 0.02% oxygen (< 10mmHg) [6] [2].

A. History of research on hypoxia

Joseph Priestley was the first person to demonstrate the importance of molecular oxygen for animal life in 1774 when he placed a burning candle in a bell jar alongside a mouse. The burning candle consumed oxygen leading to low levels of oxygen in the jar and the ultimate death of the unfortunate rodent [7].

The foundations of research on hypoxia in tumor biology were laid down by radiobiologist Gottwald Schwarz and colleagues in the early 20th century. As early as 1909, Schwarz and colleagues observed that normal mammalian cells irradiated in the presence of oxygen were more sensitive to irradiation than those irradiated under conditions of hypoxia or anoxia [8] [9] (Figure 1).

Pioneering work by many scientists exposed important effects of tumor hypoxia on tumor biology. The research on hypoxia has provided significant advancement in our understanding of the molecular pathways by which oxygen influences tumour proliferation, angiogenesis and resistance to radiation, chemotherapy and immune system. A timeline of the history of research on tumor hypoxia is summarized in Figure 2.



MÜNCHENER
MEDIZINISCHE WOCHENSCHRIFT.
ORGAN FÜR AMTLICHE UND PRAKTISCHE ÄRZTE.

No. 24. 15. Juni 1909. Redaktion: Dr. B. Spatz, Arnulfstrasse 26. Verlag: J. F. Lehmann, Paul Heysestrasse 26. 56. Jahrgang.

(Nachdruck der Originalartikel ist nicht gestattet.)

Originalien.

Aus dem Röntgenlaboratorium des Wiener k. k. allg. Krankenhauses (Leiter: Doz. Dr. G. Holzknecht).

Ueber Desensibilisierung gegen Röntgen- und Radiumstrahlen.

Von Dr. Gottwald Schwarz.

Da sich die Wirkung der Röntgenstrahlen auf die Haut bei der grossen Empfindlichkeit und der oberflächlichen Lage dieses Organs am deutlichsten manifestierte, war die Radiotherapie ursprünglich blosse Dermatotherapie. Heute ist dies längst anders. Der ungleich bedeutungsvollere Teil des Anwendungsgebietes der X-Strahlen betrifft die malignen Bluterkrankungen und Geschwulstbildungen, wo wir schon heute über Ergebnisse verfügen, die man vor wenigen Jahren noch als durchaus unmöglich bezeichnet hätte.

Aber gerade hier, bei den „Tiefenbestrahlungen", wie man es nennt, erfahren wir ein sehr störendes Hemmnis, eben durch jene hohe Sensibilität der Hautdecke, die unserem therapeutischen Vorgehen ein unüberschreitbares, meist vorzeitiges Ziel setzt. Wie oft müssen wir uns bei einem leukämischen Rückfall, bei nur unvollständiger Rückbildung oder beim Rezidiv einer durch Bestrahlung schon günstig beeinflussten Geschwulst sagen: „Wir könnten mehr leisten, könnten wir nur mehr bestrahlen. Wir können es aber nicht, weil wir dann die Haut gefährden und dem Kranken durch eine „Röntgenverbrennung" schweren und schmerzvollen Schaden zufügen würden."

Das Bestreben, hier Abhilfe zu schaffen, führte mich auf folgenden Weg:

Schon vor zwei Jahren hatte ich mich durch Versuche an Pflanzenkeimlingen (Mitteil. a. d. Wiener Röntgenlabor., Jena, G. Fischer, 1907) darüber belehrt, dass zwischen der Stoffwechselgrösse und der Röntgenlichtempfindlichkeit der Pflanzenzelle ein inniger Zusammenhang bestehe. Bestrahlte ich trockenen Samen, in welchem der Embryo sich im Zustande eines „latenten" Lebens befindet, sein Stoffwechsel auf ein Minimum eingeschränkt ist, so blieben selbst enorme Röntgenlichtmengen (tagelange Bestrahlungen) ohne Effekt. Ausgesät, wuchsen normale Individuen heran.

Bestrahlte ich jedoch aufgequollenen Samen, war das Wachstum eingeleitet, der Wechsel der Stoffe rege geworden, so genügten schon ganz kleine Röntgenlichtdosen, um hochgradige, charakteristische Veränderungen, Wachstumsstörungen und Pigmentationen zu erzeugen.

Dieser Konnex zwischen Stoffwechselgrösse und Röntgensensibilität, den mir manche Beobachtung auch aus meiner radiotherapeutischen Tätigkeit zu bestätigen schien, brachte mich nun zu folgender Ueberlegung. War die Hypothese richtig, derzufolge ein Zellkomplex um so empfindlicher gegen Strahlenwirkung anzusehen wäre, je stärker dessen Stoffwechsel ist, so mussten alle diejenigen Agentien, die den Stoffwechsel vermindern, auch die Strahlenempfindlichkeit vermindern.

Zur Entscheidung dieser Frage unternahm ich folgenden Versuch: Bei einem 11 jährigen Mädchen, das uns wegen eines ausgedehnten Naevus flammans des Vorderarms zur Radiumbehandlung zugewiesen worden war, bestrahlte ich zwei benachbarte Hautstellen mittels unserer würfelförmigen Radiumkapsel, am selben Tage, gleich lange Zeit (je 1 Stunde lang), war die Haut komprimiert, anämisiert, der Saftzufluss gesperrt, der Stoffwechsel also gehemmt — die postulierte Bedingung somit erfüllt (Fig. 1).

Die bestrahlten Stellen durch Kegel punktiert bezeichnet.

Radium-Kapsel ohne Druck mit Druck

Fig. 1.

Das Ergebnis dieses Versuches war ein solches, wie ich es zu erhoffen wohl nicht gewagt hatte (Fig. 2).

Bestrahlung mittels 60 mg Radiumbromid am 16. März.

ohne Druck mit Druck

Am 8. April

Beginn der Entzündung. Nichts.

Am 12. April

Höhe der Entzündung, Oedem, collateral Hyperhaemie. Leichte Hyperhaemie.

Am 19. April

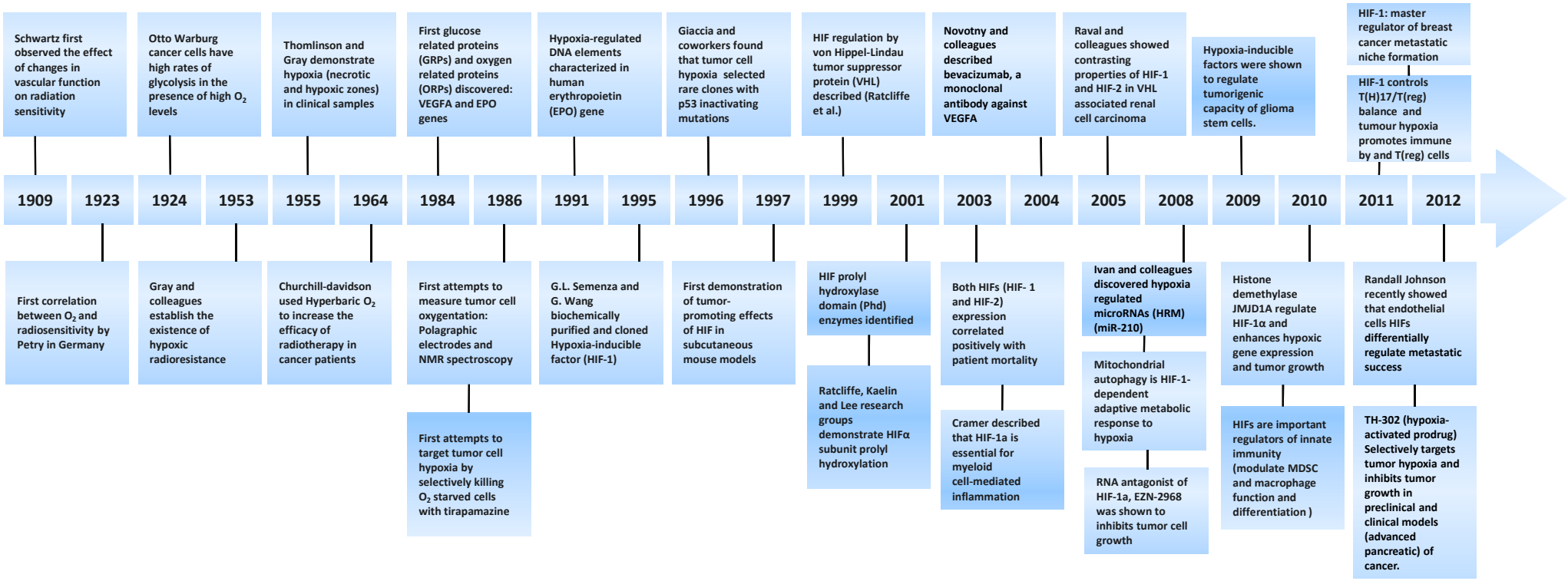
Abortierende Entzündung, Schuppung. Nichts.

Am 25. April

Figure 1: Article describing the first clinical observation of the importance of hypoxia.

Gottwald Schwarz (1880-1959) findings were used to introduce the concept of "kompressionsana¨mie" by which the skin was made anemic, thereby allowing a higher dose de given to deeply situated tumors, a biologic concept in "Röngtentiefenterapie". In 1914, he also observed the magnitude of the oxygen enhancement ratio 2: "Kompressionsana¨mie der Haut setzt die Röntgenempfindlichkeit derselben auf ein Drittel herab," but without realizing that oxygen was the causal factor.

Figure 2. Timeline: The history of research on tumor hypoxia



B. Tumor Hypoxia

Tumor hypoxia commonly refers to a condition in tumors where the oxygen pressure is less than 5 to 10 mmHg [4]. Hypoxia is a common feature of almost all of the solid tumors and mainly occurs due to a mismatch between tumor growth and angiogenesis [5]. Tumor Hypoxia is one of the hallmarks of cancer [10].

1. Normoxic, hypoxic and necrotic zones

Nearly 55 years ago, in 1955, Thomlinson and Gray reported that cancer cells grow in cords around blood vessels. They noticed the presence of a central necrotic core surrounded by a region of viable cells near a blood vessel. They also proposed that the edges of such necrotic cores harbour viable hypoxic tumor cells [11]. It is now well established that solid tumors contain areas of variable oxygen concentrations [5] (Figure 3A). Tumor cells require oxygen and nutrients for their survival and therefore should be located within 100 to 200 μm of blood vessels which is the diffusion limit for oxygen. Tumor cells closest to a perfused blood vessel have relatively high O_2 concentrations, which decline as the distance from the vessel increases [12]. Hence, in solid tumors there are areas which are well oxygenated, poorly oxygenated and finally necrotic in which cancer cells have died due to inadequate oxygenation [5] [13] (Figure 3B and 3C).

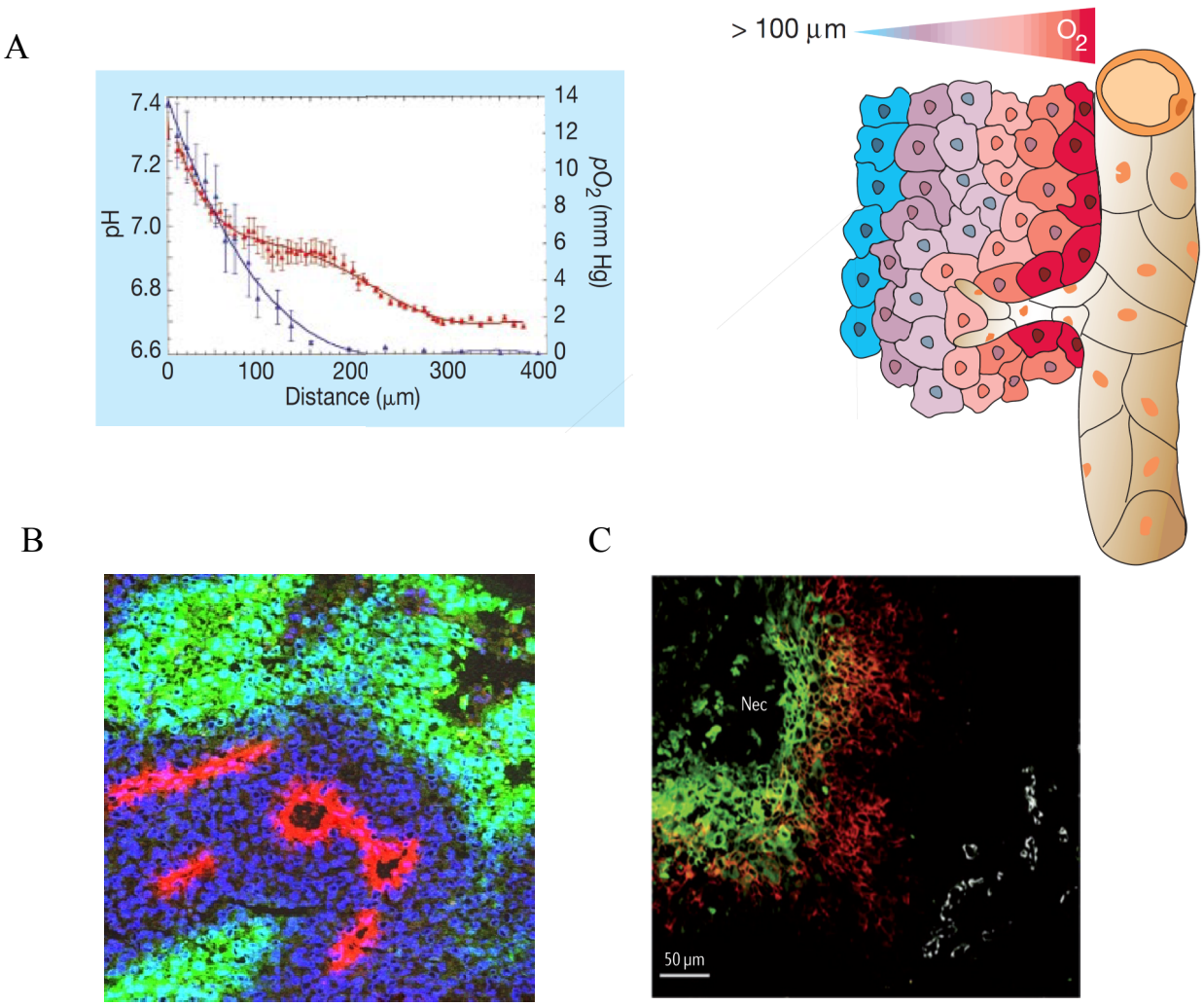


Figure 3: Tumor hypoxia (normoxic, hypoxic and necrotic zones)

A) Graph showing the relationship between the distance of tumor cells from nearby vessels and partial pressure of oxygen (blue line) and acidosis (red line) (Helmlinger et al., 1997 Nature Med.) **B)** Pseudocolour immunoflourescense showing the difference in distribution of covalently bound pimonidazole (green), a 2-nitroimidazole hypoxia marker, and the endothelial cell marker, CD31 (red) in B16-F10 melanoma xenograft. **C)** Immunoflourescense staning of a human squamous cell carcinoma of larynx showing the difference in distribution of pimonidazole (green) and hypoxia-inducible factor 1 (HIF1)-regulated carbonic anhydrase IX (CAIX; red), an endogenous marker of hypoxia (William et al., 2011 *NATURE REVIEWS CANCER*).

2. Types of tumor hypoxia

The tumor microenvironment is highly dynamic and consists of a heterogeneous population of cancer cells. These subpopulations of cancer cells have highly dynamic gradients of oxygen levels, genomic content, genomic stability, cellular pH and cellular metabolism. Hence solid tumors may contain group of cells that have been exposed to varying levels of oxygen for minutes to hours or to even days and then reoxygenated. This phenomenon is called 'cycling hypoxia' and occurs frequently in solid tumors. Tumor cells in hypoxic microenvironment can be exposed to fluctuating levels of cycling hypoxia at different oxygen gradients for different periods of time [14]. These fluctuations determine tumor cell fate depending on the severity, duration and outcome of hypoxia (whether ended by cell death or reoxygenation). Similarly, in tumors, the abnormal vascular architecture and blood flow that arises owing to unregulated angiogenesis is also an important factor in the development of hypoxia [15].

Depending on these two factors (cycling hypoxia and abnormal vasculature); tumor hypoxia can be broadly divided into two types: acute/transient or perfusion-limited hypoxia and chronic or diffusion-limited hypoxia

a. Acute/transient or perfusion-limited hypoxia

In acute hypoxia, tumor cells have been differentially exposed to low intracellular oxygen levels for minutes to hours and then reoxygenated.

Acute hypoxia occurs when abnormal/aberrant blood vessels suddenly shut down leading to the development of severe hypoxia at the site innervated by it. This can also cause blood flow to be reversed [16]. This shut down is normally transient and closed blood vessels can be reopened, leading to reperfusion of acute hypoxic tissue with oxygenated blood. Reperfusion with oxygenated blood leads to a process known as 'reoxygenation injury' characterized by a sudden and sharp increase in free radicals, tissue damage and activation of stress-response genes [17]. Free radicals contain oxygen and have unpaired electrons in their outer orbit which makes them a source of damage to cells and tissues. Free radicals can react with and modify the molecular structures of lipids, carbohydrates, proteins and DNA [18]. They are produced during the process of cellular metabolism but their rate is significantly increased under acute hypoxia (or ischaemia followed by reperfusion) [19]. Acute hypoxia can also be caused by increases in the surrounding interstitial fluid pressure [16] [20].

b. Chronic or diffusion-limited hypoxia

In chronic hypoxia, tumor cells (at a distance of >100–200 μm from nearby vessel) are exposed to a more prolonged hypoxia for hours to days and ultimately undergo cell death or are reoxygenated. These perinecrotic regions of a tumor are located at a median distance of 130 μm from blood vessels, reflecting the limit in oxygen diffusion [21].

In solid tumors tumor angiogenesis lags behind tumor growth. This uncontrolled proliferation causes tumors to outgrow their blood vessels which results in the formation of large hypoxic zones with few or none blood vessels. The diffusion of oxygen and nutrients is limited in these zones and the tumor cells are under continuous severe hypoxia named as chronic hypoxia [22].

c. Acute versus chronic hypoxia

One of the major limitations of the studies performed to describe the effects of hypoxia on tumor cell biology (angiogenesis, chemo-radioresistance, cell survival and apoptosis etc) is that *in vitro* assays usually mimic short term acute hypoxia followed by rapid reoxygenation. However tumor cell biology (especially survival) may be entirely different when cells are exposed to (greater than 24h) and maintained under chronic hypoxia.

In this regard, it was shown that acutely hypoxic tumor cells irradiated immediately after reoxygenation are more radiosensitive as compared to cells irradiated under acute hypoxia alone [23]. Similarly, irradiated chronically hypoxic cells (maintained under hypoxia for up to 72 h) acquire increased radiosensitivity when compared with irradiated cells exposed to acute hypoxia (4–24 h) [24]. These studies also showed that radiosensitization with drugs (misonidazole or SR2508) were increased in chronically hypoxic cells when compared with the toxicity in acutely hypoxic cells (despite the same level of oxygen at the time of irradiation). Later on, it was proposed that this differential sensitization could be partially explained by relative ability of DNA repair among acutely hypoxic and chronically hypoxic tumor cells [22].

Another interesting question is that whether exposure to acute or chronic hypoxia will differentially regulate the hypoxia-induced increased potential of tumor cells (for example metastasis potential) and whether it also depends upon the severity of hypoxia. Rofstad et al., using human melanoma xenografts demonstrated that acute hypoxic exposure might have a more important role than chronic hypoxia in metastasis induction [25]. Similar results showed that acute hypoxia in vivo could increase the metastasis in both rodent and human xenograft models [26] [27].

The outcome of acute or chronic hypoxia was shown to depend on the relative tumor cell proliferation, distance from nearby vasculature, and transient time through oxygen gradient and tumor cell adaptation to the microenvironment [22]. Comparisons of differential gene expression of acute versus chronic hypoxia are needed to address this issue.

3. Hypoxia inducible factors (HIF's) as master regulators of adaptation to hypoxia

Tumor hypoxia has long been correlated with poor prognosis, resistance to radiotherapy and chemotherapy and increased malignancy [28], fuelling exhaustive research into tumor cell response to limited oxygen concentrations. Over the years, a particular interest has been taken in studying the mechanisms by which hypoxic tumor cells alter their transcriptional profiles to modulate proliferation, survival, glycolysis and invasion to persist under conditions of hypoxic stress [2]. Pioneering work by Peter Vaupel and colleagues showed convincingly for the first time the direct evidence of hypoxia in human cancers. They studied tumor oxygen supply using oxygen electrodes [29] and also showed that low oxygen tension in tumors was associated with increased metastasis and poor survival in patients suffering from squamous tumors of the head and neck, cervical or breast cancers. Tumor cells undergo a variety of biological response in response to hypoxia. In a hypoxic cell, one of the earliest responses is the shift from aerobic to anaerobic metabolism [30]. Hypoxia also induces erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which induces neo-angiogenesis (new blood vessel formation) [28]. One important question is how cells sense oxygen and responds to it and what are the signalling pathways with mediate cellular oxygen responses?

4. Molecular Biology of HIF family

Scientist began looking for one or more factors that cells might use to respond to reduced oxygen levels and would be oxygen sensors. These investigations led to the discovery of one of the most striking responses to hypoxia-the induction of the hematopoietic growth hormone erythropoietin (EPO) [31]. At high altitude or in anemia, the blood oxygen content is reduced and the EPO production in renal interstitial fibroblasts is rapidly activated. This induction (more than several 100 folds) of EPO mRNA and protein induces erythropoietic responses that directly increase blood oxygen transport [31].

In early 1990's, several groups identified a cis-acting hypoxia response element (HRE) (5′-TACGTGCT-3′) in the 3′-flanking region of this locus that confers oxygen regulation of EPO expression [32] [33] [34] [35]. Gregg Semenza and Guang Wang in 1992 discovered a nuclear factor that is induced by hypoxia, binds to the EPO HRE and promotes transcriptional activation of EPO in oxygen-starved cells [36] [37]. It was designated hypoxia inducible

factor 1 (HIF-1) by Semenza and Wang. Later on, HIF-1 binding site within the EPO HRE was used for purification of HIF through DNA affinity chromatography and the cloning of cDNAs encoding the HIF-1α and HIF-1β subunits was done in 1995 [38].

Three isoforms of HIFα have been identified: HIF-1α, HIF-2α, and HIF3α. HIF-1α and HIF-2α (also known as EPAS1) have the same structure and are very well characterized. HIF3α mostly acts as a negative regulator of HIF-1α and HIF-2α [39]. HIF-1α is ubiquitously expressed in all mammalian cells, whereas HIF-2α and HIF3α are selectively expressed in certain tissues such as vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage [40].

It has become clear now that HIF family of transcriptional factors is the key regulators of hypoxic response in all mammalian cells. HIF family has a critical role in development, physiology, cancer and other diseases. In tumor biology, HIF's mediate pathways like angiogenesis, metabolic adaptation, cell growth, cell survival and apoptosis etc. [41] [2].

5. Structure of Hypoxia inducible factors

Hypoxia inducible factors (HIF's) are the main mediators of primary transcriptional responses to hypoxic stress in normal and transformed cells. They are DNA binding basic helix-loop-helix proteins of the PAS family (PER, AHR, ARNT and SIM family) [38]. HIF are obligate heterodimers consisting of oxygen-labile α-subunit (HIF-1α, HIF-2α (encoded by EPAS1) or HIF3α) and a stable β-subunit (HIF-1β; also known as ARNT) [42]. HIFα contains two oxygen dependent degradation domains (ODDD): a N-terminal (N-ODDD) and a C-terminal portion (C-ODDD), [33, 43]. HIF-1α and HIF-2α have two transactivation domains (TADs): an N-terminal TAD (which overlaps with the C-ODDD) and a C-terminal TAD [44] (Figure 4).

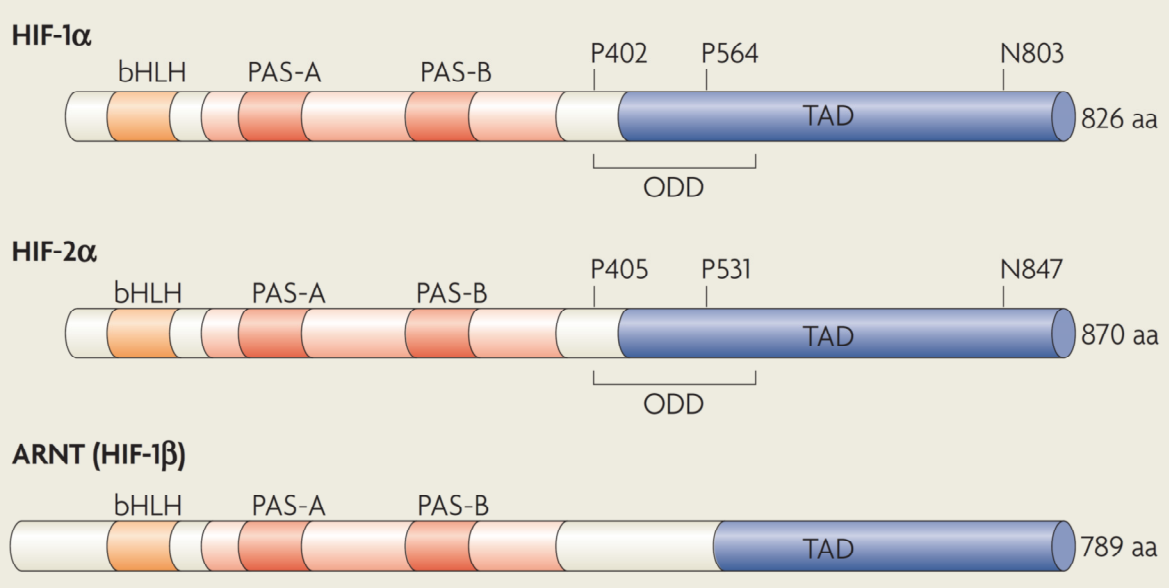


Figure 4: Structure of Hypoxia inducible factors.

Schematic structure of two HIFα and one HIFβ isoforms. The PAS and bHLH domains are dedicated to dimerization and recognition of target DNA sequences. bHLH, basic helix-loop helix-domain; PAS, per arnt sim domain subdivided into PAS A and PAS B; ODD, oxygen-dependent degradation domain; TAD, transactivation domain. HIF-1α and HIF-2α have two distinct TAD, in the C- (C-TAD) and N- (N-TAD) terminal domains. (From Simon et al. 2008 *Nature Reviews Molecular Cell Biology*).

6. Oxygen dependent regulation of HIFα

HIFα subunits under normoxic conditions have a short half life of less than 5 minutes [45]. Normoxic cells constantly synthesize HIFα proteins and degrade them. However, the degradation of HIFα is retarded under low levels of oxygen conditions [46].

a. Role of prolyl hydroxylase domain in the regulation of HIFα protein degradation

HIFα in the presence of oxygen is hydroxylated on proline residue 402 and/or 564 by prolyl hydroxylase domain protein 2 (PHD2) in the ODDD [47] [43]. This oxygen dependent hydroxylation results in the binding of HIFα with von Hippel–Lindau tumor suppressor protein (pVHL). pVHL is the recognition component of an E3 ubiquitin-protein ligase complex that eventually targets HIFα for proteolysis by the ubiquitin–proteasome pathway [48]. Enzymes regulating HIFα proteasomal degradation were first identified to be related to egl-9 in Caenorhabditis elegans and termed prolyl hydroxylase domain (PHD) enzymes (PHD1–PHD3) [49] [50]. PHDs are non-heme Fe (II)- and 2-oxoglutarate-dependent dioxygenases that split molecular oxygen. PHD2 uses oxygen as a substrate, and thus, its activity is inhibited under hypoxic conditions [50]. In the reaction, one oxygen atom is inserted into the prolyl residue, and the other atom is inserted into the co substrate α-ketoglutarate, splitting it into CO2 and succinate [47]. Other components of HIFα-pVHL complex are elongin B, elongin C, and Cul2, which also participate in other E3 ubiquitin ligase complexes [51] (Figure 5A).

Under hypoxic conditions, prolyl hydroxylation is inhibited and HIFα protein proteasomal degradation is inhibited. HIFα rapidly accumulates, translocates to the nucleus and dimerizes with HIFβ. HIFα HIFβ heteromeric dimer HIF binds to the HRE in target genes, recruits co-activators and activates transcription [2] (Figure 5B). PHD's are the main oxygen sensors and hypoxia inhibits PHD activity through various mechanisms, including substrate limitation [52].

Similar to HIF-1α, HIF-2α is also regulated by oxygen-dependent hydroxylation [53]. HIF-1α and HIF-2α are structurally similar in DNA binding and dimerization domains but differ in their transactivation domains [54]. Consistently, they share overlapping target genes, whereas each also regulates a set of unique targets [55]. By using genome wide chromatin immunoprecipitation combined with DNA microarray (ChIP-chip) or DNA sequencing (ChIP-seq), it was shown that more than 800 genes are direct targets of HIF (one in 30 of all human genes) [56] [57]. HIFs are also shown to regulate a number of hypoxia-regulated microRNAs (HRM) [58] and chromatin modifying enzymes [59].

HIF-3α lacks the transactivation domain and may function as an inhibitor of HIF-1α and HIF-2α and its expression is transcriptionally regulated by HIF-1 [39].

b. Role of factor-inhibiting HIF (FIH) in the regulation of HIFα transactivation

Oxygen regulates the stability as well as the transcriptional activity of HIFα subunits [2].The transcriptional activity of HIFα is regulated by the hydroxylation of a C-terminal asparagine residue (Asn 803 in human HIF-1α) [60]. This hydroxylation reaction is carried out by an asparaginyl hydroxylase termed FIH. This enzyme is oxygen dependent and represents another component of the oxygen-sensing machinery. FIH represses HIF-1α transactivation function [61] by using O2 and a-ketoglutarate as substrates, thereby blocking the association of HIF-1α C-TAD with the CH-1 domain of the transcriptional coactivator protein p300 [60] [62].Thus, in contrast to the prolyl hydroxylation that enables protein–protein interaction (HIFα stability), the asparaginyl hydroxylation prevents protein recruitment (HIFα transactivation).

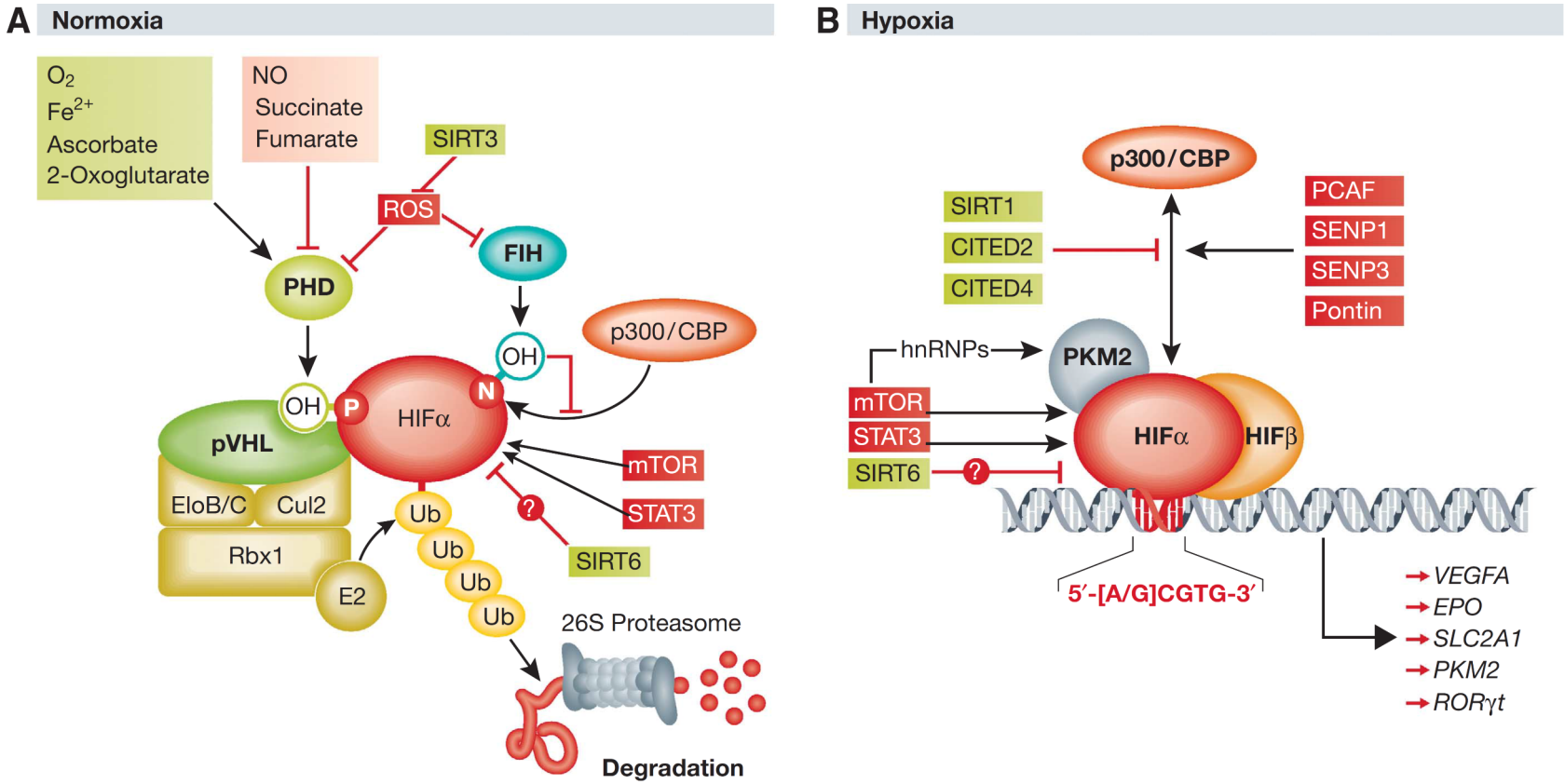


Figure 5: Oxygen dependent regulation of hypoxia-inducible factor

A) Under normoxia, HIFα is subject to oxygen-dependent PHDs mediated degradation by the 26S proteasome. STAT3 and mTOR upregulate HIFα at the mRNA level. **B)** Under hypoxia, HIFα is stabilized and dimerizes with nuclear HIFβ. The HIF-α protein binds to HRE elements in the promoters of HIF-responsive genes. PHD, prolyl-hydroxylase domain; NO, nitric oxide; SIRT1/3/6, sirtuin 1/3/6; FIH, factor inhibiting HIF; OH, hydroxyl group; mTOR, mammalian target of rapamycin; STAT3, signal transducer and activator of transcription 3; pVHL, von Hippel-Lindau protein; ROS, reactive oxygen species; HIF, hypoxia-inducible factor; (Taken from Greer et al. 2012 *The EMBO Journal*).

7. Oxygen independent regulation of HIFα (pseudo hypoxia or hypoxia like normoxia)

Apart from strict oxygen dependent regulation of HIF's, HIF-1α and HIF-2α are also regulated by several oxygen independent mechanisms [63] (Figure 6). In addition to intratumoral hypoxia, loss-of-function (LOF) for tumor suppressor genes (most notably, pVHL), and gain-of-function (GOF) for oncogenes and viral transforming genes increases HIFα protein levels and their activity in tumor cells. These constitutively active HIF-1α and HIF-2α in normoxic cells seems to be cell specific [28].

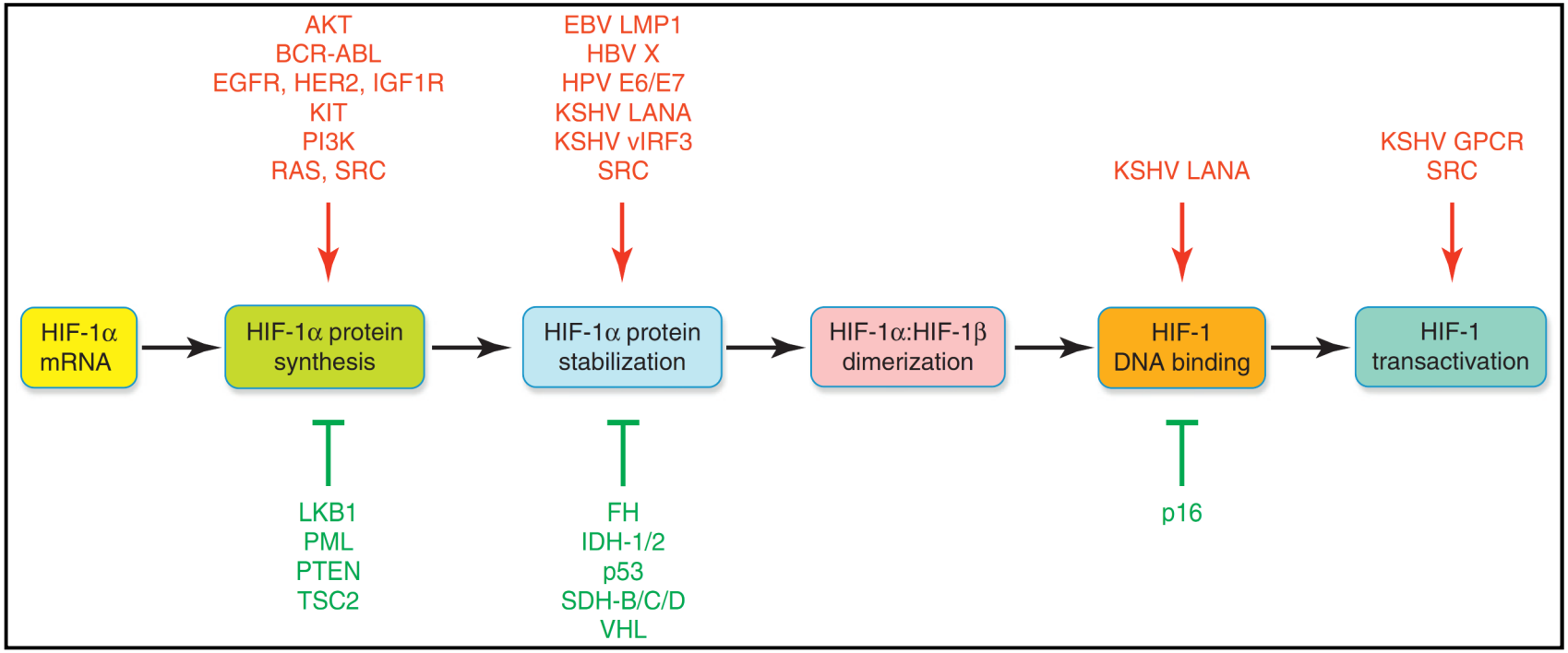


Figure 6: Regulation of HIF-1 activity by oncoproteins and tumor suppressors

HIF-1 activity is stimulated by oncoproteins (red) gain-of-function. HIF-1 activity is inhibited by tumor suppressors (green) and their loss-of-function therefore stimulates HIF-1 activity. Transforming proteins encoded by Epstein-Barr virus (EBV), hepatitis B virus (HBV), human papilloma virus (HPV), and Kaposi sarcoma herpesvirus (KSHV) also activate HIF-1. (From Semenza et al. 2012 *TRENDS in pharmacological science*).

a. The tumor suppressor gene LOF contributes to increased levels of HIFα

Both HIF-1α and HIF-2α protein levels are increased in tumor cells due to LOF of different tumor suppressors. These LOF may result in either increased HIF-1α synthesis or decreased HIF-1α degradation. Mutations of *pVHL, PTEN, B-RAF, SDH, FH* and *MITF* are able to regulate HIF-1α and HIF-2α under normal level of O_2 (hypoxia like normoxia) resulting in HIFα stabilized and activated (Table 1). This stability of HIFα under normoxia leads to its increased transcriptional activity, promotes tumor growth and may have a huge impact on cell biology and cancer development [2, 63].

Table 1: Tumor suppressor gene LOF contributes to increased HIF-1α in human cancers.

TSG	Tumor(s) with TSG loss of function	Effect on HIF-1α
PVHL	RCC, hemangioblastoma	↓ ubiquitination
SDHB	Paraganglioma	↓ hydroxylation
SDHC	Paraganglioma	↓ hydroxylation
SDHD	Paraganglioma	↓ hydroxylation
FH	Leiomyoma, RCC	↓ hydroxylation
IDH1	Glioblastoma	↓ hydroxylation
P53	Colon adenocarcinoma	↓ ubiquitination
TSC2	Tuberous sclerosis	↑ synthesis
PTEN	Glioblastoma, others	↑ synthesis
LKB1	Gastrointestinal hamartoma	↑ synthesis

Abbreviations: ↓ decreased; ↑ increased; TSG, tumor suppressor gene, RCC, Renal cell carcinoma
Modified from Semenza et al. 2010 *oncogene*.

b. Regulation of HIF-1 activity by oncoproteins

Apart from intratumoral hypoxia, GOF for oncogenes and viral transforming genes also regulates HIFα protein levels and their activity in cancer cells (Table 2). Interestingly, a large number of oncoproteins (proteins encoded by transforming viruses that form tumors) in human have been shown to induce HIF-1α activity [2] [64]. Most of these oncoproteins regulate HIF-1α activity by either decreasing HIF-1α protein degradation [65] [66] or by increasing HIF-1α protein [67] [68]. Most extraordinary is the case of Kaposi's sarcoma herpes virus (KSHV) which completely mimics the effects of hypoxia [69]. KSHV infection

results in the formation of a highly vascularized tumor. KSHV encodes 03 different oncoproteins which together increases HIF-1α protein half-life, nuclear localization and transactivation under normoxic conditions [70] [71].

Table 2: Proteins of oncogenic viruses increase HIF-1 activity.

Viral oncoprotein	Effect on HIF-1α
EBV latent membrane protein 1	↓ degradation
Hepatitis B virus X protein	↓ degradation
Human papillomavirus E6/E7 proteins	↑ HIF-1α protein
Human T-cell leukemia virus tat protein	↑ HIF-1α protein
KSHV G-protein-coupled receptor	↑ transactivation
KSHV latency-associated nuclear antigen	↑ nuclear localization
KSHV latency-associated nuclear antigen	↓ degradation
KSHV viral interferon regulatory factor 3	↓ degradation

Abbreviations: EBV, Epstein–Barr virus; KSHV, Kaposi's sarcoma herpesvirus, Adapted from Semenza et al. 2010 *oncogene*.

8. Diverse factors which regulate HIFα

Several other factors which modulate the HIF pathway including metabolites, Redox, inflammatory mediators, microRNAs and oncogenes/tumor suppressors have been summarized in (Table 3).

Table 3: Diverse regulators of HIF activity.

HIF regulators	Effect on HIF-1α and HIF-2α
Siah1a/2	↑ HIF-1α stability; promotes degradation of PHD1/3 in hypoxia
RSUME	↑ HIF-1α stability; enhances SUMOylation
SENP1	↑ HIF-1α stability; removes SUMO moieties
HSP90	↑ HIF-1α stability
COMMD1	↓ HIF-1α stability and disrupts HIFα/β dimerization
HSP70/CHIP	↓ HIF-1α stability but not HIF-2α stability
CITED2	↓ HIF-1α activity
Metabolites/Related	
2-oxoglutarate, Ascorbate and Fe^{2+}	↓ HIFα stability as PHD cofactor
IRP	↓ HIF-2α mRNA translation in response to high intracellular iron
NO	Modulates HIFα expression
Intermittent hypoxia	↑ HIF-1α stability but ↓ HIF-2α stability
Redox	
ROS	↑ HIFα stability; observed in inflammatory cells
Sirt1	↓ HIF-1α and ↑ HIF-2α transcriptional activity
Sirt6	Binds to and ↓ HIF-1α stability/activity
MicroRNAs	
miR-107	microRNA leads to ↓ ARNT expression
miR-17-92	miRNA cluster; microRNAs lead to ↓ HIF-1α expression
Oncogenes	
PI3K/Akt	↑ HIF-1α expression
mTORC1	↑ HIF-1α mRNA translation
GSK3β	↓ HIF-1α stability
p53	↓ HIF-1α/ARNT expression
β-catenin	Binds to HIF-1α; ↑ HIF-1α transcriptional activity
Ras	↑ HIF-1α expression by ROS generation
ERβ	↓ HIF-1α stability
Inflammation	
NF- κB	↑ HIF-1α transcription
p44/42 MAPK	↑ HIF-1α expression downstream of LPS
IFN-γ and IL-4	↑ HIF-1α and HIF-2α(?) expression

Abbreviations: ↓, decreased; ↑, increased, adapted from Simon et al. 2010 Molecular Cell.

C. Hypoxia (HIF-1α and HIF-2α) and cancer

1. Clinical data linking HIF-1α and HIF-2α levels to patient mortality

A large number of clinical data shows a strong positive relation between patient mortality and hypoxia-induced HIF-1α and HIF-2α [2] summarized in Table 4.

It has been shown that early stages of breast, cervical, and endometrial cancers are associated with good prognosis but as the tumor grows, tumor hypoxia increases (HIF-1α or HIF-2α) and it significantly increases patient mortality [2].

Table 4: Correlation of prognosis and HIF-1α or HIF-2α expression in human cancers.

Cancer type	Prognosis	Prognosis
	HIF-1α expression	**HIF-2α expression**
Astrocytoma	Poor	Poor
Bladder	Poor	ND
Breast	Poor	Poor
Cervical	Poor	Poor
Colorectal	Poor	Poor
Gastric	Poor	NC
	Poor	ND
GIST	Poor	ND
Glioblastoma	ND	Poor
Glioma	NC	Poor
Head and neck	Poor	Poor
Hepatocellular	ND	Poor
NSCLC	Poor	Poor
	Poor	ND
	NC	Poor
Melanoma	Poor	Poor
Neuroblastoma	Favourable	Poor
Ovarian	Poor	ND
	Poor	Poor
Pancreatic	Poor	ND
Prostate	Poor	Poor
RCC	Favourable	ND
	Poor	ND

GIST, gastrointestinal stromal tumor; HIF, hypoxia-inducible factor; NC, no correlation; ND, not determined; NSCLC, non-small-cell lung cancer; RCC, renal cell carcinoma. Modified from Simon et al. 2012 *Nature Reviews Cancer*.

2. Role of HIF-1α and HIF-2α in cancer progression

In accordance with the clinical data, a large set of experimental data clearly demonstrates that both HIF-1α and HIF-2α promotes tumor progression by regulating shared and unique genes. HIF-1α and HIF-2α LOF or HIF-1α and HIF-2α GOF have opposite effects on tumor growth, vascularization and metastasis [63] as summarized in Table 5.

Table 5: Mouse models testing altered expression of HIFα proteins in tumor growth.

Tumor or cell type	HIF-1α status	HIF-2α status	Phenotypes
Xenograft tumors			
Teratoma	Loss-of-function knockout	Wild-type	↓ growth and angiogenesis
Teratoma	Wild-type	Loss-of-function knockout	↑ growth
Fibrosarcoma	Loss-of-function knockout	Wild-type	↓ growth
RCC	Gain of function	Wild-type	↓ growth
RCC	Wild-type	Gain of function	↑ growth
Autochthonous tumors			
MMTV-PyMT mammary tumors	Conditional knockout	Wild-type	↓ metastasis
KRAS-driven NSCLC	Conditional knockout	Wild-type	No effect
KRAS-driven NSCLC	Wild-type	Conditional knockout	↑ tumor burden and progression
p53-driven thymic lymphoma	Heterozygous germline knockout	Wild-type	↓ tumor incidence
Tumor-associated stromal cells			
Tumor-associated macrophages	Conditional knockout	Wild-type	↓ NO, ↑ T cell-mediated immunosurveillance and ↓ autochthonous mammary tumor growth
Tumor-associated macrophages	Wild-type	Conditional knockout	↓ macrophage infiltration into autochthonous liver and colon tumors and tumor growth
Vascular ECs	Conditional knockout	Wild-type	↓ xenograft tumor angiogenesis and growth
Vascular ECs	Wild-type	Conditional knockout	Non-productive angiogenic sprouting and impaired vessel remodelling

EC, endothelial cell; MMTV, mouse mammary tumor virus; NO, nitric oxide; PyMT, polyoma middle T antigen; RCC, renal cell carcinoma. Modified from Simon et al. 2012 *Nature Reviews Cancer*.

3. Effects of genes regulated by HIFs on different aspects of cancer biology

HIFs play a major role in many critical aspects of cancer biology including angiogenesis [72], metabolic reprogramming [73], epithelial-mesenchymal transition [74] [75], invasion [75] [76], metastasis [77, 78] [79], resistance to radiation therapy [80] and chemotherapy [81], selection of genotypes favouring survival under hypoxia–re-oxygenation injury (such as TP53 mutations) [82] [64], pro-survival changes in gene expression that suppress apoptosis (down-regulation of Bid and Bax) [83], supports autophagy [84, 85], autocrine growth factor signaling [86] [87] and stem cell maintenance [88] [89]. In addition, hypoxia also contributes to loss of genomic stability through the increased generation of reactive oxygen species (ROS) [90] and the downregulation of DNA repair pathways [22] as well as suppressing immune reactivity [91] [3].Several specific HIF-regulated genes that play key roles in crucial aspects of cancer biology have been summarized in Figure 7.

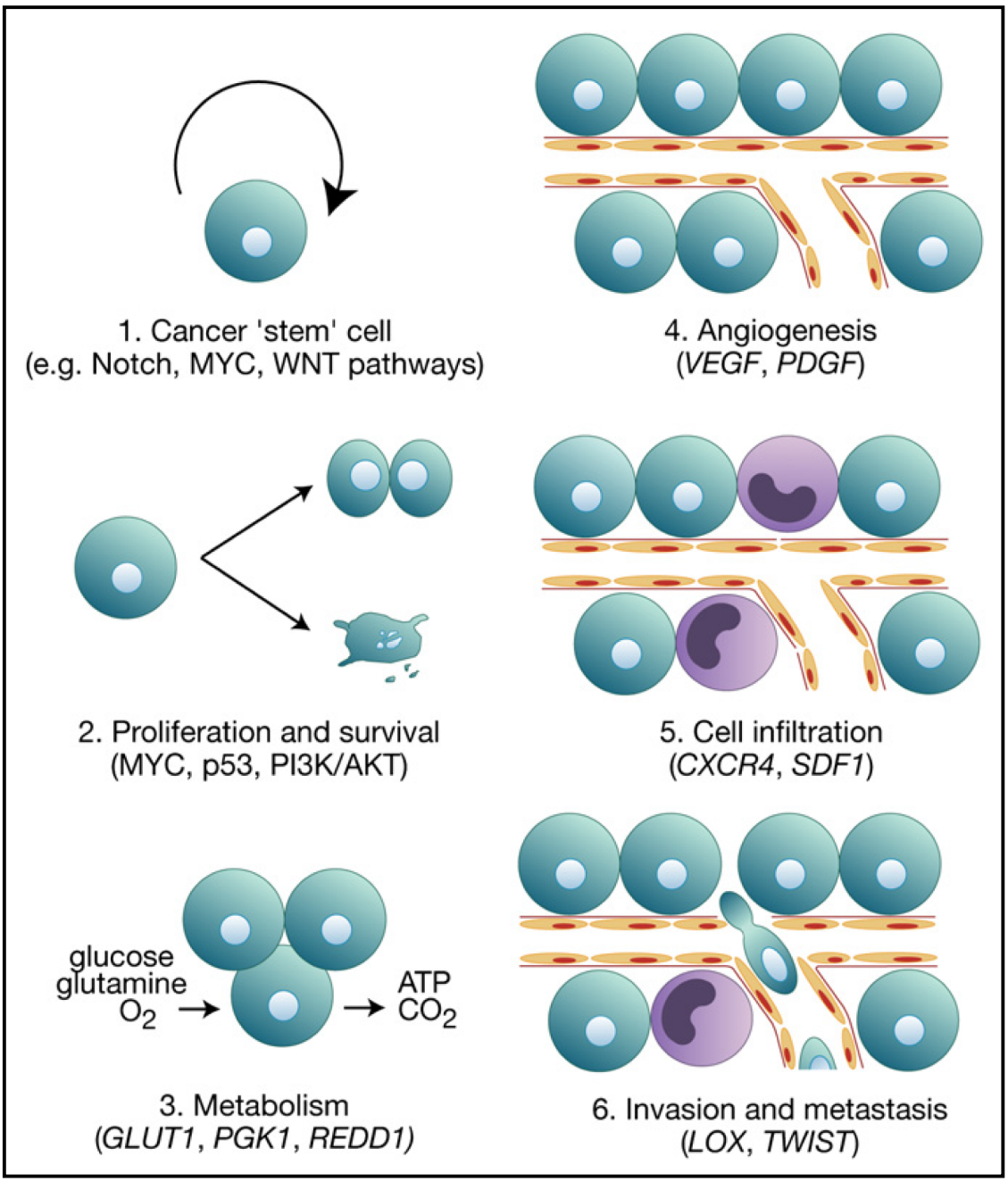


Figure 7: Effect of HIF on multiple steps of tumor cell development and biology

HIF activity in cancer has been associated with (1) cancer "stem" cell maintenance and increased expression of genes involved in (2) proliferation and survival, (3) metabolism, (4) angiogenesis, (5) recruitment of infiltrating cells such as tumor associated macrophages (TAMs) and bone marrow-derived cells, and (6) tumor cell invasion and metastasis. Some examples of HIF-regulated genes and oncogenic pathways are given in parentheses (From Simon et al. 2010 *Molecular Cell*).

4. Gene expression regulation by HIF-1α and HIF-2α

Work from many laboratories has revealed that HIF-1α or HIF-2α could regulate the expression of same hypoxia induced targets and each HIFα can have its unique target genes [92] [93] (Table 6).

Interestingly, it was proposed that HIF-1α and HIF-2α transcriptional specificity is regulated by post-DNA binding mechanisms [55] and resides in the N-TAD suggesting that differential interactions with transcriptional cofactors probably determine differential gene activation [54] [55].

In MCF-7 breast cancer cells, a direct comparison of HIF-1α and HIF-2α binding sites demonstrated that some sites bind HIF-1α exclusively, many others bind both HIF-1α and HIF-2α with equal affinity, despite the fact that HIF-2α contributes to the hypoxic expression of relatively few genes in these cells [94]. It is surprising to note that HIF-1α and HIF-2α were shown to bind preferentially to specific genes that each factor is known to preferentially regulate. HIF-1α has a relatively higher level of binding with glycolytic pathway genes [57] and HIF-2α for stemness gene like OCT4 [95].

Interestingly, HIF-1α and HIF-2α can have opposing effects on the cell cycle and on cell proliferation. The best example of this is in clear cell renal carcinoma, in which HIF-2α is described to drive the tumor growth while HIF-1α may restrict proliferation [96]. This opposite effect of HIF-1-α and HIF-2-α could be related to the mechanism involving the regulation of c-Myc. Indeed, it has been suggested that HIF-1α antagonizes the activity of Myc while HIF-2α enhances Myc activity [97].

Gene (protein)	Function	HIF-1α target gene	HIF-2α target gene	Cell type
SLC2A1 (GLUT1)	Glucose transport	+	+	RCC and mouse ESCs
PLIN2 (ADRP)	Lipid metabolism	+	+	RCC
CA12 (CAXII)	pH homeostasis	+	+	RCC
FLG (filaggrin)	Cytoskeletal structure	+	+	RCC
IL6 (IL-6)	Immune cytokine	+	+	RCC
ADM (adrenomedullin)	Angiogenesis	+	+	RCC
VEGFA (VEGFA)	Angiogenesis	+	+	RCC and Hep3B cells
	Angiogenesis	+	–	Mouse ECs and mouse ESCs
BNIP3 (BNIP3)	Autophagy and apoptosis	+	–	RCC
HK1 (hexokinase 1)	Glycolysis	+	–	Mouse ESCs
HK2 (hexokinase 2)	Glycolysis	+	–	RCC and mouse ESCs
PFK (phosphofructokinase)	Glycolysis	+	–	RCC and mouse ESCs
ALDOA (ALDA)	Glycolysis	+	–	RCC and mouse ESCs
PGK1 (PGK1)	Glycolysis	+	–	RCC and mouse ESCs
LDHA (LDHA)	Glycolysis	+	–	RCC and mouse ESCs
NOS2 (iNOS)	NO production	+	–	Macrophages
ABL2 (ARG)	Inhibitor of NO production	–	+	Macrophages
EPO (erythropoietin)	Erythropoiesis	–	+	Kidney and liver
POU5F1 (OCT4)	Stem cell identity	–	+	Mouse ESCs
SCGB3A1 (secretoglobin 3A1)	Growth-inhibitory cytokine?	–	+	NSCLC
TGFA (TGFα)	Growth factor	–	+	RCC
CCND1 (cyclin D1)	Cell cycle progression	–	+	RCC
DLL4 (DLL4)	NOTCH signalling and EC branching	–	+	Mouse ECs
ANGPT2 (angiopoietin 2)	Blood vessel remodelling	–	+	Mouse ECs

Table 6: Gene expression regulation by HIF-1α and HIF-2α.

ADRP, adipose differentiation-related protein; ALDA, fructose-bisphosphate aldolase A; ARG, Abelson-related gene protein; CA, carbonic anhydrase; DLL4, delta-like 4; EC, endothelial cell; ESC, embryonic stem cell; GLUT1, glucose transporter 1; IL, interleukin; iNOS, inducible nitric oxide synthase; LDHA, lactate dehydrogenase A; NO, nitric oxide; NOS, nitric oxide synthase; NSCLC, non-small-cell lung cancer; PGK1, phosphoglycerate kinase 1; PLIN2, perilipin 2; RCC, renal cell carcinoma, TGF, transforming growth factor; VEGFA, vascular endothelial growth factor A. . Modified from Simon et al. 2012 Nature Reviews Cancer.

5. Hypoxia (HIF-1α, HIF-2α) and p53

P53 is accumulated within hypoxic regions of solid tumors and is correlated with apoptosis [98]. Transcriptionally active wild-type p53 is stabilized through a physical association with HIF-1α [82]. Murine double minute (MDM2) oncogene acts as bridge and mediates the interaction between p53 and HIF-1α [99]. Whereas HIF-1α fails to bind p53 *in vitro*, it directly binds MDM2, which suppresses the MDM2-dependent ubiquitylation of p53 in vivo and p53 nuclear export. Surprisingly, MDM2 overexpression promotes p53 accumulation and the transcription of p53 target genes when HIF-1α is activated in hypoxic cells [99]. HIF-1α seems to enhance the activation of p53 by ionizing radiation, which results in increased phosphorylation of p53 and increased p53-mediated apoptosis [100]. Ionizing radiation increases HIF-1α activity in tumors by increasing production of ROS and reactive nitrogen species. This relationship between HIF-1α and p53 provides a potential negative feedback loop for HIF-1α activity. Ravi et al. [101] suggested that p53 can induce HIF-1α turnover by promoting MDM2-mediated ubiquitylation and proteasomal degradation of HIF-1α.

In contrast to HIF-1α, HIF-2α does not bind MDM2 [102], and seems to inhibit p53 indirectly by multiple mechanisms [103]. Increased HIF-2α expression inhibits p53 phosphorylation and stabilization in RCC cell lines, whereas knocking down HIF-2α expression increased p53 transcriptional activity and target gene expression. HIF-2α expression in RCC tumor samples correlated with decreases in p53 phosphorylation and p53 target gene expression, and may contribute to radioresistance in HIF-2α-expressing RCCs [102]. One very interesting question is why these two HIFα subunits have opposite effects on certain pathways (MYC, p53 and mTORC1) in the same cell?

D. Detection and measurement of tumor hypoxia

In the past few decades a lot of progress has been made in the development of imaging approaches for the detection and quantification of tumor oxygenation levels in vivo. Especially the field of nuclear medicine has contributed a lot by the development of several radiolabeled hypoxia markers. Although hypoxia also occurs in other pathologic conditions including myocardial ischemia and stroke, hypoxia imaging is much more advanced in oncology applications such as prediction of response to therapy and overall prognosis.

Imaging hypoxia is now routinely used in the clinics and has developed from a mere detection method to a potential tool for monitoring personalized chemo-radiotherapy. Since its first observation by Tomlinson and Gray in 1955, tumor hypoxia has become a central issue in cancer treatment [63]. Therefore, the ability to detect hypoxia within tumors has significant implications for cancer management and therapy. The extent of hypoxia in tumor levels depends on the methods used to detect it (Table 7). Tumor hypoxia can be detected by direct and indirect methods.

***Direct measurement of oxygen concentration*:** Eppendorf oxygen electrode

***Indirect measurement of oxygen concentration*:** Methods based on the measurement of endogenous markers (such as HIF-1α, HIF-2α, CAIX, Glut-1 and measurement of levels of plasma osteopontin) and Exogenous probes (Pimonidazole and EF5).

More recently, several non-invasive imaging techniques for hypoxia include the use of radiolabelled 2-nitroimidazoles and other probes imaged with positron emission tomography (PET), single photon emission computed tomography (SPECT), functional computed tomography (CT SCAN) and blood oxygenation level dependent magnetic resonance imaging (BOLD MRI) [104] [22] [105]. Apart from Eppendorf oxygen electrode, all other methods are non-invasive techniques. Functional imaging provides anatomical information in addition to tumor perfusion and vascular permeability [16] [106].

The current gold standard for *in vivo* measurement of tumor oxygenation is the Eppendorf needle electrode system, which allows for direct measurement of pO_2 in tumors [29]. However, it is an invasive procedure that often requires ultrasound-guided placement of the electrode, and its use is limited to easily accessible tumors [107]. Therefore, evaluation of hypoxia in clinic is rapidly shifting to monitoring of endogenous markers, especially HIFs and

their transcriptional targets (CAIX, VEGF, Glut-1 etc) and exogenous 2-nitroimidazole probes (pimonidazole: binds covalently to SH-containing molecules (thiols) and forms protein adducts in hypoxic tissues- pO2 levels below 10 mm Hg) [16] [108]. It was shown that although both pimonidazole and CAIX staining correlated significantly with the stage of patients with larynx carcinomas, CAIX had only limited value for measuring hypoxia and was not as robust as pimonidazole [109].

In summary, *in vivo* measurement of tumor hypoxia using these methods could be helpful in identifying patients with worse prognosis or patients that could benefit from appropriate treatments. Such strategies have now been applied in the treatment of several cancer types, for example NSCLC and head and neck cancers [110] [111] [112] [113] [114, 115]. Although the search for an 'ideal' hypoxia marker in not yet over, current PET hypoxia imaging methods have already shown promise for the selection of patients who are likely to benefit from therapies targeting hypoxia.

Table 7: Different methods of detection and measurements of tumor hypoxia.

Measure of hypoxia	Probe
Oxygen concentration	Eppendorf oxygen electrode
Endogenous markers	HIF-1α
	HIF-2α
	CAIX
	Osteopontin
	Lysyl oxidase
	Hypoxic gene signature
Exogenous probes	Pimonidazole
	EF5

CAIX, carbonic anhydrase IX; EF5, etanidazole pentafluoride and HIF, hypoxia-inducible factor.
Modified from William R. Wilson & Michael P. Hay 2011 *Nature Reviews Cancer*

II. HYPOXIA: A KEY PLAYER IN TUMOR MICROENVIRONMENT

Tumor microenvironment is a complex and highly dynamic environment, providing very important clues to tumor development and progression [116]. It is now acknowledged that tumor cells and their stroma co-evolve during tumorigenesis and tumor progression [117]. Therefore, tumor growth and spread depend as much on the host response as on the biologic characteristics of the tumor itself and on the influence of the tumor microenvironment [116].

Hypoxia through HIF's plays a key role in radioresistance, chemoresistance and tumor stemness. HIF-1α overexpression is associated with poor treatment response in many human tumors [2] [105]. To some extent, because of these effects on tumor cell development and biology, hypoxia has been shown to be implicated in resistance to therapy through multiple mechanisms (Table 8).

Table 8: Mechanisms of resistance (and sensitivity) of hypoxic cells to cytotoxic therapy.

Effect of hypoxia	Resistance or sensitivity?	Mechanism	Agents affected	Example
Lack of oxidation of DNA free radicals by O_2	Resistance	Failure to induce DNA breaks	Ionizing radiation	2–3-fold increase in ionizing radiation
			Antibiotics that induce DNA breaks	Bleomycin
Cell cycle arrest in G1 or G2 phase	Resistance	Repair before progression to S or M	Cycle-selective chemotherapy drugs	5-Fluorouracil
Cell cycle arrest in S phase	Sensitivity	Collapse of stalled replication forks	PARP inhibitors[‡]	Veliparib (ABT-888)
Distance from vasculature (indirect)	Resistance	Compromised drug exposure	Drugs extensively bound in tumor cells	Taxanes
Extracellular acidification (indirect)	Resistance	Decreased uptake	Basic drugs	Doxorubicin
	Sensitivity	Increased uptake	Acidic drugs	Chlorambucil
Resistance to apoptosis	Resistance	Genetic selection of *TP53*mutants	Multiple	
		Downregulation of BID and BAX	Multiple	Etoposide
Genomic instability	Resistance	Mutagenesis	Multiple	*DHFR* amplification and methotrexate
Suppression of DNA repair	Resistance	Downregulation of MMR	DNA methylating agents	
	Sensitivity	Downregulation of NER	Bulky DNA monoalkylating and crosslinking agents	
		Downregulation of HR	DNA crosslinking agents	Cisplatin
HIF-1 stabilization	Resistance	Expression of ABC transporters	ABC transporter substrates	MDR1 and doxorubicin
		Downregulation of NHEJ	Agents that induce DSBs	Etoposide

BAX, BCL2-associated X protein; BID, BH3 interacting domain death agonist; *DHFR*, dihydrofolate reductase; DSB, double strand break; HR, homologous recombination; MDR1, multidrug resistance protein 1; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PARP, poly(ADP-ribose) polymerase. Taken from William R. Wilson & Michael P. Hay 2011 *Nature Reviews Cancer.*

A. Hypoxia increases resistance to cytotoxic drugs

During tumor expansion, tumor angiogenesis lags behind growth resulting in malformed and malfunctioning tumor vessels leading to severe hypoxia [28]. Although some cells die because of extremely low oxygen levels, a small fraction of tumor cells survive the hypoxic stress and adapt to the hypoxic microenvironment. These hypoxic cells become more aggressive and resistant to cell death during cancer therapy [106] [5] (Table 9). Hypoxic tumor microenvironment favours the emergence of tumor variants with increased metastatic and invasive potential [75].

Table 9: Overview of HIF-1-mediated drug resistance mechanisms.

(Cancer) Cell model	Drug/molecule	Resistance phenotype	Molecular basis (if known)
Glioma cells	Etoposide, doxorubicin	Drug efflux	MRP1
HeLa cells	4-HPR	Autophagy induction	Beclin1
Gastric cancer cells	5-Fluorouracil	Apoptosis inhibition	p53, NF-κB
Breast cancer cells	Paclitaxel	Apoptosis inhibition	Caspases 3, 8, 10, Bak, TNFRSF10A, Mcl-1
Prostate cancer cells	Flutamide	Apoptosis inhibition	Bcl-xL
Glioblastoma cells, colon cancer cells	Adriamycin	Apoptosis inhibition	Bcl-2
HCC cells	Etoposide	Apoptosis inhibition	Bak
Fibrosarcoma cells	Cisplatin	Apoptosis inhibition	Bid
Gastric cancer cells	Multiple drugs	Apoptosis inhibition	Bcl-2, Bax
Breast cancer cells	Docetaxel	Apoptosis inhibition	Survivin
Pancreatic cancer cells	5-Fluorouracil, doxorubicin, gemcitabine	Apoptosis inhibition	Survivin
Fibrosarcoma cells, colon cancer cells	Etoposide	Apoptosis inhibition	Bid
HNSCC cells	Paclitaxel	Apoptosis inhibition	Bid
Colon cancer cells	Etoposide, oxaliplatin	Apoptosis inhibition	Bid
Breast cancer cells, prostate cancer cells	Etoposide	DNA damage inhibition	Topoisomerase II alpha
OSCC cells	5-Fluorouracil, cisplatin	ROS decrease	HO-1, MnSOD, Ceruplasmin

4-HPR, N-(4-Hydroxyphenyl)retinamide; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma. Adapted from Nadine Rohwera and Thorsten Cramer 2011 Drug Resistance Updates.

B. Hypoxia increases cell proliferation and survival

One of the first differences between cancer cells and normal cells is the increased rate of cell proliferation and decreased rate of cell death as a result of increased expression of survival/growth factors. Cancer cells express cognate membrane receptors for growth factors resulting in increased autocrine signalling and thus increasing the cell survival/growth of its neighbors. HIFs are well known to promote cell immortalization, cell survival, autocrine growth signals and stem cell renewal by regulating genes like erythropoietin (EPO), vascular endothelial growth factor (VEGF), endothelin 1 (EDN1), transforming growth factor α (TGFα), adrenomedullin (ADM), insulin-like growth factor 2 (IGF2) and octamer binding protein 4 (OCT4 [POU5F1]) etc [63].

One of the well known HIF target, EPO and its receptor has been shown to increase the growth and survival of human melanoma, breast, prostate and renal cancers [118]. Similarly, VEGF has been shown to mediate tumor cell function via activation of its receptors on tumor cells [119] [120] in colorectal, gastric, and pancreatic cancer. Other HIF regulated genes which are also to participate in autocrine signalling are: TGFα in clear-cell renal carcinoma [121], IGF2 in colorectal carcinoma [122], EDN1 in breast, prostate, and ovarian cancer [123] and ADM in pancreatic and prostate cancer [124]. Human telomerase (hTERT) promoter activity was shown to be regulated by HIF-1 and represents a mechanism for trophoblast growth under hypoxia [125], thereby suggesting a novel mechanism of telomerase activation in cancers and hypoxia-induced tumor progression. Hypoxia can also favor increased survival of tumor cells by inducing human embryonic stem cells (hESC) like transcriptional program. HIF have been shown to induce hESC markers NANOG (NANOG) and OCT4 in primary glioma cells [126]. Factors which block cellular senescence such as the glycolytic enzymes glucosephosphate isomerase (GPI) and phosphoglycerate mutase (PGM) [127] are also regulated by HIF.

C. Hypoxia: pro-survival versus pro-cell death?

In cancer cells, hypoxia can induce multiple cellular responses directing them towards survival or cell death. These cellular responses might be HIF-1 dependent and/or independent [128] [5]. The precise roles of HIF-1 in the regulation of apoptosis are highly complex and are shown to be dependent upon the biological background of cells. Most of the primary cells respond to decreased oxygen levels by inducing cell cycle arrest and ultimately apoptosis in a HIF-1 dependent manner [129]. But already primary cells display great differences in response to hypoxia. Inflammatory cells such as neutrophils and macrophages must survive in highly inflamed, infected or wounded areas to function properly. So these cells have evolved strategies to resist and survive in these hypoxic inflamed areas. Neutrophils are key effector cells of the innate immune response and are required to migrate and function within adverse microenvironmental conditions. In this regard, it has been shown that HIF-1 can regulate neutrophils survival under hypoxia by stabilization of NF-κB and subsequent expression of anti-apoptotic NF-κB target genes [130].

Tumor cells have developed many mechanisms to evade HIF-1-mediated cell death under hypoxic conditions. One of the first examples of HIF-1 as a promoter of cell survival and drug resistance is the direct evidence that hypoxia via HIF-1 downregulated Bid and Bax in colon cancer [83], in HNSSC [131] and fibrosarcoma cells [132] [133]. Similarly hypoxia had a direct protective effect on apoptotic cell death by HIF-1 mediated down regulation of proapoptotic mitochondrial proteins like Bak [134] and death caspases (CASP3, CASP8, CASP10) [135].

On the other hand, HIF-1 induces expression of several well established anti-apoptotic proteins like survivin in pancreatic cancer [136] and in breast cancer cells [137] and inhibitor of apoptosis-2 gene (IAP2) [138]. More interestingly, HIF-1 regulates the expression of Bcl-xL in prostate cancer cells [139] and Bcl-2 in gastric cancer [140] and other cancer cells [141].

Hypoxia via HIF-1α acts as robust suppressor of apoptosis and the down regulation of HIF-1α results in enhanced cell death upon treatment with chemotherapeutic agents in different cancer models [81].

III. HIF INHIBITORS FOR CANCER THERAPY

Given its central role in tumor progression and resistance to therapy, tumor hypoxia has always been considered as a high priority target for cancer therapy.

In early 1960s, several attempts were made to exploit oxygen to promote the efficiency of radiation treatment of cancer patients. Churchill-Davidson and colleagues were the first to use hyperbaric oxygen and they made some encouraging observations in cancer patients treated with radiation in combination with hyperbaric oxygen [142] [143]. Hyperbaric oxygen, RBC transfusion and erythropoietin administration were attempted to increase tumor oxygenation but these approaches gave conflicting reports of their clinical efficacy [144].

Over 20 years ago, Tirapazamine was described as a hypoxia-activated prodrug in combination with radiation but clinical trials showed limited benefit [145] [146]. Later on, clinical trials with this agent in combination with radiation or chemotherapy have demonstrated benefits in patients with lung cancer or head and neck cancer [147] [148].

Over the years, many new strategies have been developed to identify novel inhibitors of HIFs. Some of these are presently being tested in clinical trials. The different therapeutic agents can be divided into three different groups depending on how they alter the hypoxia pathway (Figure 8).

1) Upstream targets of HIF
2) Direct HIFs (HIF-1α and HIF-2α) targets
3) Downstream targets of HIF transcriptional activity

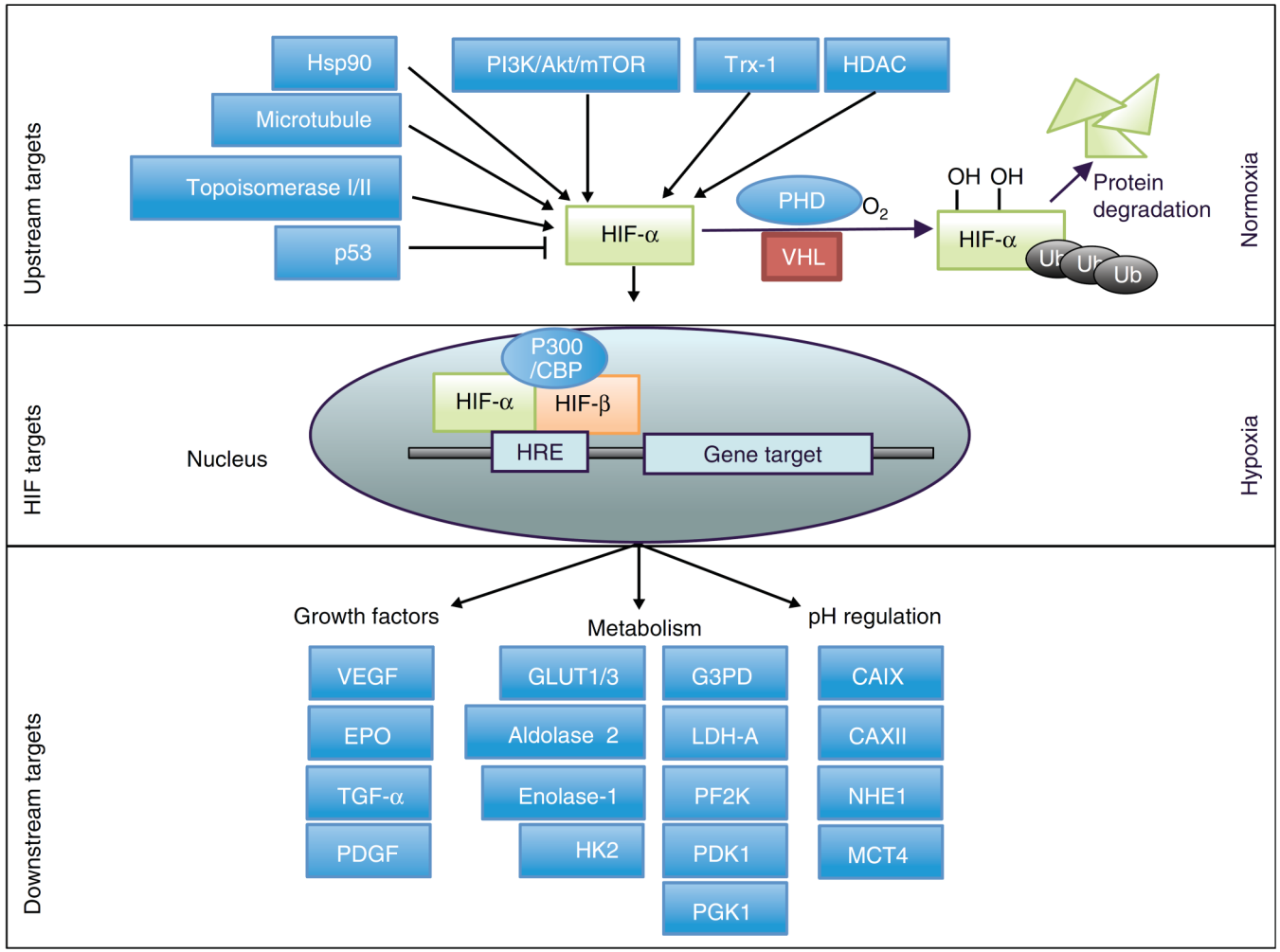


Figure 8: Diverse strategies for targeting the hypoxia response pathway.

HIF-α pathway can be targeted at multiple levels; upstream, downstream of HIF's and by inhibiting HIF-α binding to hypoxia responsive genes. VEGF, glucose transporter type 1 (GLUT1) and carbonic anhydrase IX (CAIX); EPO: Erythropoietin; G3PD: Glyceraldehyde-3-phosphate dehydrogenase; HK2: Hexokinase 2; LDH-A: Lactate dehydrogenase A; MCT4: Monocarboxylate transporter 4; PDGF: Platelet-derived growth factor; PDK1: Pyruvate dehydrogenase kinase, isozyme 1; PF2K: Phosphofructo-2-kinase; PGK1: Phosphoglycerate kinase 1; TGF-α: Transforming growth factor-α. (Taken from Harris et al. 2012 *EXPERT OPINION*)

A. Upstream targets of HIF

Upstream targets of HIFα modulate HIFs by regulating different hypoxia signalling pathways. HIFs expression and activity can be regulated by many factors. Apart from PHDs, these factors include PI3K/Akt/mTOR pathway, Trx, microtubules, topoisomerases, Hsp90, p53 and HDAC.

B. Direct targets of HIFs (HIF-1α and HIF-2α)

The HIF signalling pathway regulators modulate multiple pathways, their inhibition may have off target effects. On the contrary, specific and direct targeting of HIFs will result in a more precise and direct response with a fewer on none off target effects.

A growing number of chemical compounds have been shown to block tumor xenograft growth and inhibit HIF activity through a wide variety of molecular mechanisms, including decreased HIF-1α mRNA levels, decreased HIF-1α protein synthesis, increased HIF-1α degradation, decreased HIF subunit hetero-dimerization, decreased HIF binding to DNA, and decreased HIF transcriptional activity (Figure 9). Many of these are drugs that are in clinical cancer trials or are already approved for the treatment of cancer or other diseases [63].

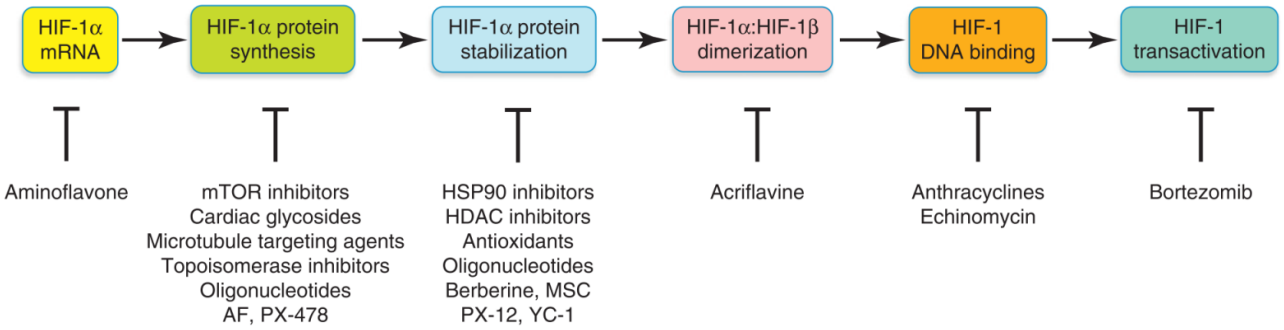


Figure 9: Molecular mechanism of action of drugs that inhibit HIF-1.

The steps required for the transactivation of target genes by HIF-1 are shown in the colored ovals and the drugs that inhibit each step are shown below (From Semenza et al. 2012 *Trends in Pharmacological Sciences*).

1. HIF-α antisense, dominant negative HIF-α and viral vectors

EZN-2968 is an antagonist of HIF-1α mRNA, selectively reduces HIF-1α mRNA expression, and causes a reduction in expression of HIF-1α regulated genes in vitro and in vivo [149]. It is composed of 16 nucleotide residues and is completely complementary to mouse and human HIF-1α mRNA. EZN-2968 in vitro results in the inhibition of tumor cell growth and

downregulation of HIF-1α-regulated genes and in vivo has been shown to decrease tumor growth in models of human prostate cancer [149]. Currently, EZN-2968 is being tested in Phase I clinical trials in patients with advanced solid tumors and potential activity has been observed in one patient with metastatic renal cell carcinoma and one patient with hepatocellular carcinoma (http://enzon.com/files/HIF-1α-2.pdf). Very recently, EZN-2208 has shown superior antitumor activity in glioma xenografts [150]. Another potent approach is to use adenovirus with siRNA against HIF-1α or dominant negative HIF-1α [151] [152] [153].

2. Small molecules that inhibit HIF at multiple sites

Of the small-molecule inhibitors of HIF, YC-1 and PX-478 have been widely studied and are presently in clinical trials [154] [155]. YC-1 inhibits HIF-1α synthesis, blocks HIF-1α–p300 interaction, promotes HIF-1α degradation [154] [156] [157] and sensitizes tumor xenografts to radiation therapy [158]. PX-478 can also inhibit HIF-1α by inhibiting HIF-1α transactivation and translation, by decreasing HIF-1α protein and mRNA levels [155] and by inhibiting HIF-1α deubiquitination leading to increase in the levels of polyubiquitinated HIF-1α [159]. Both YC-1 and PX-478 target both HIFs [160] and have shown impressive activity in tumor xenograft models. PX-478 is presently in Phase I clinical trial against advanced solid tumors and lymphoma [155]. The results so far have been promising, where a high proportion of patients achieved stable disease and a dose-dependent inhibition of HIF-1α following treatment with PX-478.

C. Downstream targets of HIF

It has been shown that more than 800 genes are direct targets of HIF's (one in 30 of all human genes) [56] [57].The number of inhibitors presently under development, being tested or in use in clinical settings is extensive. Hypoxia-regulated processes can be targeted by targeting angiogenic growth factors, metabolism and pH regulation [161].

D. HIF-1α vs HIF-2α targeting?

Many of the HIF inhibitors effectively downregulate HIF expression and activity but fail to distinguish between HIF-1α and HIF-2α subunits that are expressed in different cell types. For example, drug like THS-044 was specifically designed to bind to the PAS-B domain of HIF-2α and not HIF-1α, and inhibit its binding to HIF-2β [162]. Hence it is important to assess whether targeting both HIF-1α and HIF-2α or either variant selectively will provide better therapeutic effect in specific tumor types.

E. Can HIF inhibitors improve current therapies?

HIF inhibitors could improve the efficacy of classic chemotherapy regimens. A number of molecular mechanisms underlie this effect in a cell type and chemotherapy specific manner.

1) HIFs have been shown to regulate the expression of genes encoding ATP-binding cassette multidrug transporters, including MDR1 (ABCB1) and BCRP (ABCG2), which efflux chemotherapy drugs from cancer cells [163] [164].
2) HIF-1 inhibits expression of proapoptotic mitochondrial proteins (BAX, BID) and caspases (CASP3, CASP8 and CASP10) and induces expression of antiapoptotic proteins (BCL2, BIRC5) [83] [137] [132] [140] [135].
3) HIF-1 inhibits chemotherapy-induced cancer cell senescence [165].
4) HIF-1 prevents chemotherapy-induced DNA damage by inhibiting the expression of topoisomerase-IIa protein [166] or the DNA dependent protein kinase complex [167].
5) HIF-1-dependent metabolic reprogramming [2] [168] [169] [170] may decrease ROS levels and thereby inhibit chemotherapy-induced cell death [171].

Only a few HIF inhibitors have been shown to work in vivo and to have significant anti-tumor activity, so understanding the mechanism of action of these inhibitors on the HIF pathway is of importance when used in combination with other drugs or as a single therapy against a particular type of tumor.

IV. ANTI-TUMOR IMMUNE RESPONSE

The major feature of the immune system is its ability to recognize "self" from "foreign" and to destroy the latter, potentially dangerous for host homeostasis. In this context, the recognition and elimination of tumor cells, different from their normal counterparts nevertheless self-derived, was controversial for almost a century. Initially proposed by Paul Ehrlich in 1909, who suggested that the immune system could control tumor development [172], the concept of tumor immunosurveillance is now well established [173] [174], and immune response is considered as an important biomarker in cancer [175]. Despite early skepticism, major progress in tumor immunology has ensued since the 1980s, together with growing understanding of the immune system in general and development of new methodologies e.g. hybridoma technology [176], propagation of human DC [177] and T cells [178], discovery of critical reagents e.g. interleukine-2 [179] and establishment of relevant animal models. One of the major breakthroughs came with the identification by Thierry Boon's group of a tumor Ag recognized by CTL in humans [180], the finding of which stimulated a productive effort to identify numerous tumor-specific and tumor-associated antigens [181]. Soon, the concept of tumor immunogenicity was supported by growing evidence demonstrating that an effective immune response can be elicited against the development of spontaneous and chemically induced tumors [182] [173]. These outcomes revived enthusiasm in the field of tumor immunology and gave rise to discussion about beneficial versus deleterious consequences of immune response to cancer. This led to the re-formulation of the tumor immunosurveillance hypothesis into "immunoediting" theory, which recognized the selective pressure of the immune system on malignant cells resulting in the emergence of resistant variants and tumor escape [173].

A. Cancer Immuno-surveillance theory

The cancer immunosurveillance theory was formulated nearly 50 years ago by Burnet and Thomas [183] [184-186]. They proposed that nascent transformed cells, arising could be recognized and eliminated by lymphocytes before they became clinically detectable. This would represent an extrinsic tumor suppression mechanism acting when the intrinsic ones, like DNA repair or apoptosis, failed. However, convincing evidence supporting this hypothesis was only obtained much later, with the development of transgenic immunodeficient mouse models. Original experiments based on athymic nude mice, had not

provided satisfactory results [187] [188-191]. It was due to some degree of immunity, mediated by NK cells, although reduced in number, still functional in these mice [192, 193].

1. Experimental studies

The highest incidence of spontaneous or carcinogen (such as methylcholanthrene (MCA))-induced tumors, was clearly demonstrated in mice in which immune effector mechanisms were systematically eliminated by gene deletion, compared to wild-type counterparts. Thus, mice insensitive to IFN-γ [194-196], lacking perforin [197-199] or deprived of functional T, B and NKT cells resulting from a deficiency in the recombinase-activating gene (RAG)-1 and 2, responsible for the rearrangement of lymphocyte antigen receptors, [200-202], provided incontestable evidence that tumor development in mice was controlled by components of the immune system.

2. Clinical evidence

Further evidence in favor of cancer immunosurveillance theory came from clinical studies. Much more difficult to obtain because of the requirement for a large number of patients with long-term follow-up, these data are often indirect or correlative and can be problematic in interpretation. Nevertheless a few lines of evidence emerge from accumulating studies:

a. Patients with congenital or acquired immunodeficiency's display higher incidence of virally induced malignancies [203, 204].

b. Incidence of non-viral cancer is more prevalent in Immuno-suppressed transplant recipients than in matched immunocompetent control groups [205].

c. Tumor-specific lymphocytes and antibodies (Ab) have been detected in cancer patients, suggesting the generation of spontaneous immune response [180, 206, 207].

d. The presence of tumor-infiltrating lymphocytes (TIL) is a positive prognostic factor for patient survival [208, 209].

e. The presence of T cells in the resected tumor has an impact on cancer recurrence and was shown to be predictive for the clinical outcome after the surgery [210, 211].

B. The cancer Immuno-editing hypothesis

Evidence was provided that the immune system controlled not only tumor occurrence but also tumor quality (i.e., immunogenicity) [182]. Experimental data from a study done in 2001 show that a significant portion (40%) of Methylchloranthen (MCA)-induced sarcomas derived from immunodeficient Rag2−/− mice was spontaneously rejected when transplanted into naive syngeneic wild-type mice, whereas all MCA sarcomas derived from immunocompetent

wild-type mice grew progressively when transplanted into naive syngeneic wild-type hosts [182]. Thus, tumors formed in the absence of an intact immune system are more immunogenic than tumors that arise in immunocompetent hosts. These results show that the immune system not only protects the host against tumor formation, but also edits tumor immunogenicity. These data caused a refinement of the cancer immunosurveillance concept and led to the formulation of the cancer immunoediting hypothesis, which heightens the dual host-protective and tumor-sculpting effects of immunity on developing tumors.

Cancer immunoediting is now viewed as a dynamic process comprising three phases: 1) elimination, 2) equilibrium, and 3) escape. During elimination, innate and adapative immunity works together to detect and destroy nascent tumors. However, tumor cell variants may not be completely eliminated, leading to an equilibrium phase where the immune system controls net tumor outgrowth. A dynamic balance between the immune system and the tumor cells characterize the equilibrium phase, where antitumor immunity contains, but does not fully eradicate, a heterogeneous population of tumor cells, some of which have acquired means of evading immune-mediated recognition and destruction. This phase may correspond to a latency period clinically silent, that may remain for an indefinite time. Equilibrium may be the immunoediting phase where immunity selects for the tumor cells acquiring the most immunoevasive mutations, potentially leading to clinically detectable disease. Finally, due tumor-induced immunosuppression or immune system breakdown, tumor cell variants begin to grow in an immune unrestricted manner leading to the escape phase and emergence of cancer [173] (Figure 10).

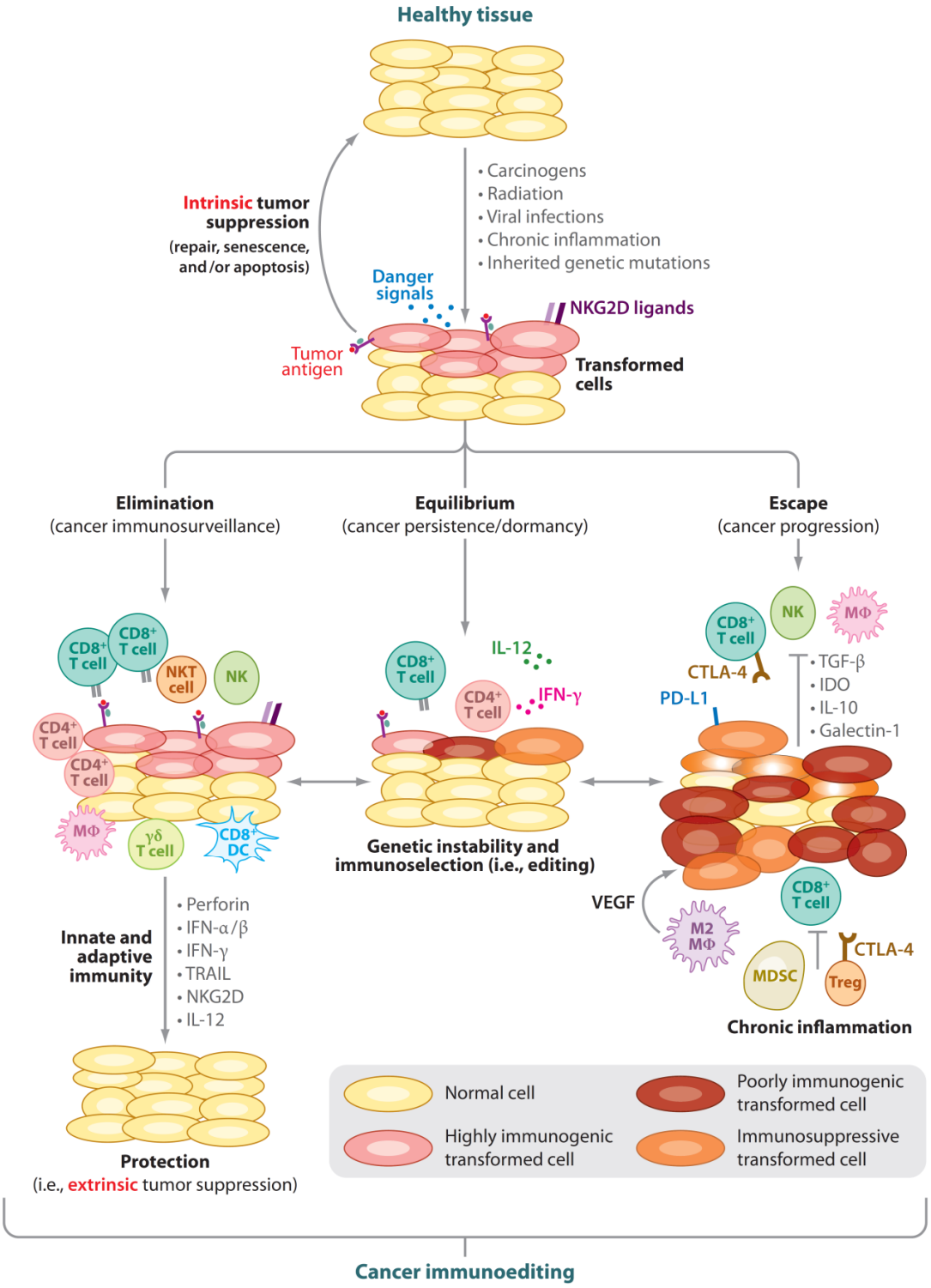


Figure 10: The three phases of cancer immunoediting.

Cancer immunoediting is the result of three processes that function either independently or in sequence to control and shape cancer. CTLA-4, cytotoxic T lymphocyte associated protein-4; IDO, indoleamine 2,3-deoxygenase; IFN, interferon; IL, interleukin; M, macrophage; MDSC, myeloid-derived suppressor cells; NK, natural killer; NKG2D, NK group 2, member D; PD-L1, programmed cell death 1 ligand 1; TGF-β, transforming growth factor-β; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cell. (Figure from Smyth et al. 2011 The *Annual Review of Immunology*).

C. The antitumor immune response

The generation of an effective immune against a developing tumor response results from coordinated interactions between innate and adaptive immunity [212] (Figure 11).

The innate immune response represents the first line of defense against tumors and provides the biological context for the adaptive immune response. Innate immunity is mediated by cells and soluble factors naturally present in tissues and body fluids, and can interfere with tumor cell target growth and survival. Cellular effectors of innate immunity includes macrophages, granulocytes, NK cells (CD3-CD56+), non-MHC restricted T cells (CD3+CD56-), and γδ T cells [213]. Serum factors, including complement proteins, C-reactive protein, mannose-binding protein and serum amyloid protein also play a role in the innate immune response [214]. Other serum factors such as natural antibodies with specificities for surface component of tumor cells might be present in the sera of patients with cancer [215]. The adaptive immune response is mediated by CD3+ T-cell receptor+ (TCR+) T cells recognizing tumor cell-derived peptide bound to self MHC-molecules present at the surface of antigen-presenting cells (APC).

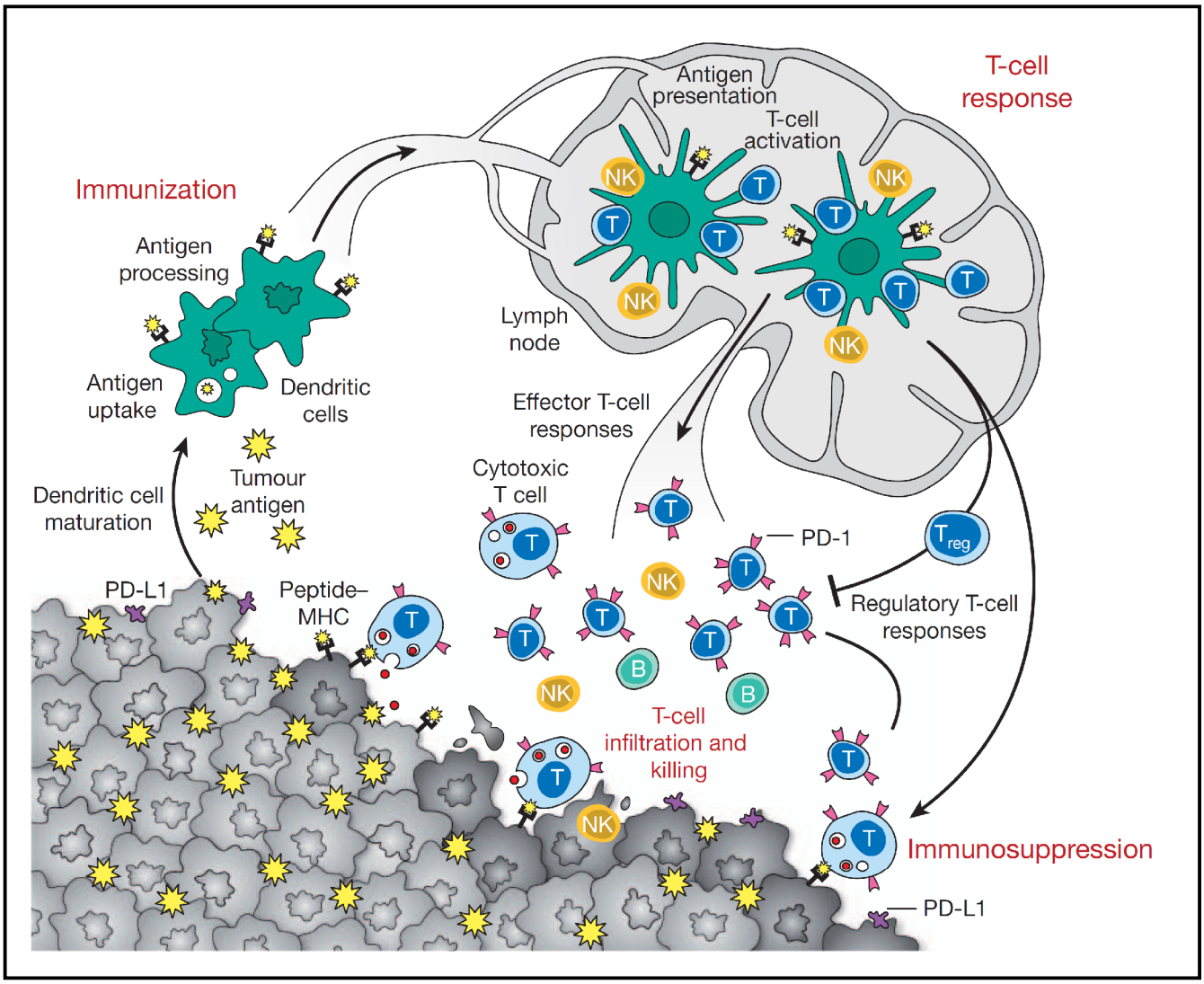


Figure 11: Generation and regulation of antitumor immunity.

Antitumor immune responses begin by the capture of tumor associated antigens, processing and cross-presentation on MHC class II and class I molecules by dendritic cells (DC) which then migrate to draining lymph nodes. Depending on the presence of an immunogenic maturation stimulus, DC will either induce or inhibit antitumor effector T-cell response. An effective antitumor T-cell response also depends on the interaction of T-cell co-stimulatory molecules with their surface receptors on dendritic cells. Interaction of CD28 with CD80/86 will promote potentially protective T-cell responses, while interaction of CTLA4 with CD80/86 or PD-1 with PD-L1/PD-L2 will suppress T-cell responses, and possibly promote Treg formation. These antigen-educated T cells (along with B cells and NK cells) then exit the lymph node and enter the tumor bed where they encounter various immunosuppressive mechanisms. (Taken from Glenn Dranoff et al. 2011 Nature).

1. The adaptive immune response

The adaptive immune response is mediated by lymphocytes. It is initiated in secondary lymphoid organs, and is mainly based on the recognition by $CD4^+$ (helper or Th) and $CD8^+$ (cytotoxic or CTL) T cells of peptide-MHC complexes expressed on APCs.

a. $CD4^+$ T cells

The $CD4^+$ T cells are primed following recognition of a cognate peptide presented via MHC class II molecules and play a major role in anti-tumor immunity. Injection of either a $CD4^+$ T-cell-activating cancer vaccine [216] or in vitro generated tumor-antigen-specific $CD4^+$ T cells [217] has been shown to clear established tumors from mice. In addition, $CD4^+$ T cells are critically involved in the initiation and/or maintenance of the $CD8^+$ CTL response [218]. As well, a more efficient antitumor response is observed when $CD4^+$ and $CD8^+$ T cells act together [219]. The auxiliary signal delivered by activated $CD4^+$ T cells is largely based on the activation of the CD40 receptor of DC (Figure 12). Besides, following activation, $CD4^+$ T cells can differentiate into several subpopulations with potentially oppposing functions. The most studied are Th1 and Th2 $CD4^+$ T cells. Th1 cells secrete mainly IFN-γ, IL-2, TNF-α and TNF-β and are involved in inhibition of tumor growth. In this respect, the production of IFN-γ [220] seems to be one important mechanism by which $CD4^+$ Th1 cells kill tumor cells and prevent or suppress the development of cancers. IFN-γ, indeed, is reported to increase the infiltration of $CD8^+$ cytotoxic T cells into the tumor [217]. Th2 cells preferentially produce IL-4, IL-5, IL-10, IL-13 and TGF-β (Transforming Growth Factor β) and are proposed to promote tumor growth. This pro-tumoral effect is largely attributed to IL-10-mediated inhibition of DC antigen processing and presentation, and activation of an immunosuppressive $CD4^+$ subset called regulatory T cells [221]. However, evidence show that $CD4^+$ Th2 cells can decrease tumor growth and induce anti-tumor immune activity via production of IL-4 [222] and recruitment of eosinophils [223]. Recently, another subset of $CD4^+$ T cells has been indentified in patients with ovarian carcinoma [224] [225]. $CD4^+$ Th17 T cells produce IL-17, IL-21, and IL-22, which promote tissue inflammation. Despite their presence has been found to correlate with a favorable prognosis [224], their role in anti-tumor immunity remains controversial (Figure 12).

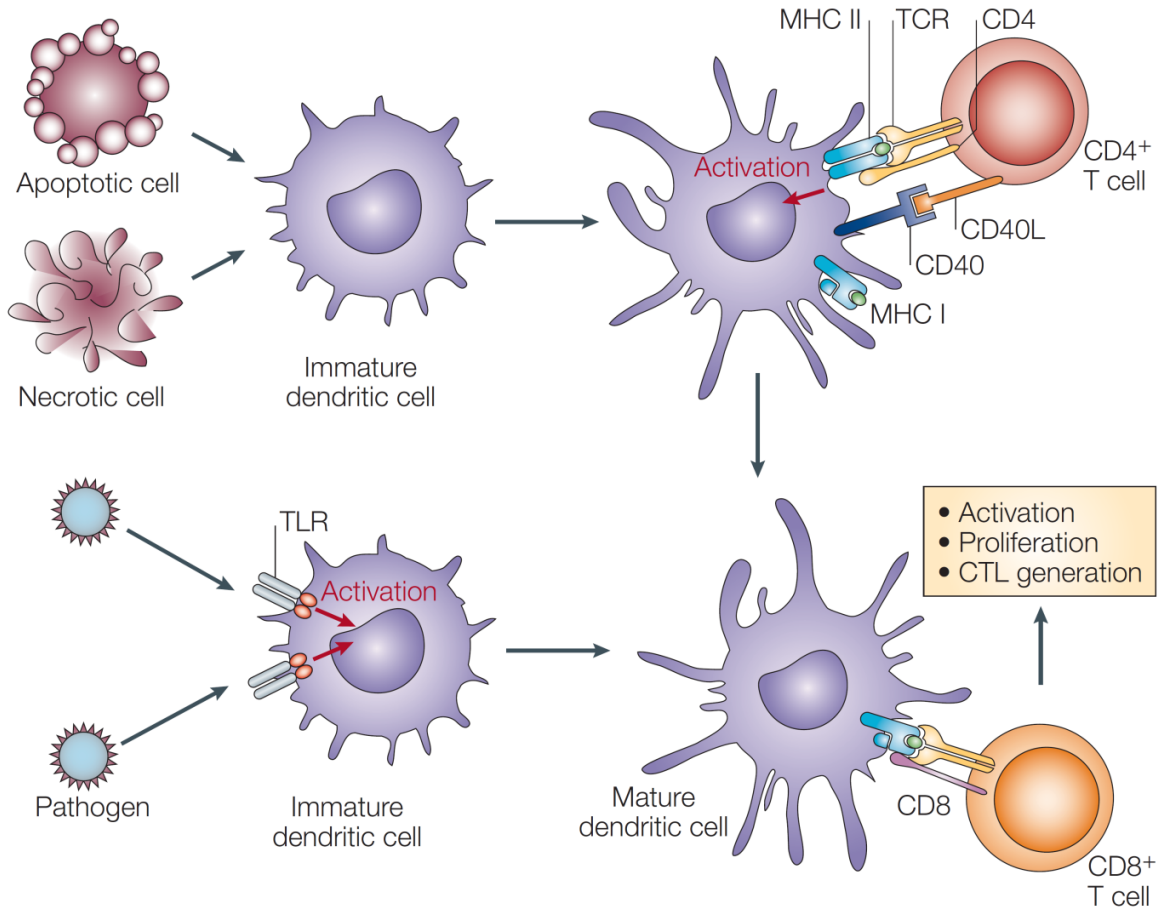


Figure 12: CD4+ T cells are required for certain cytotoxic T lymphocyte (CTL) responses. An antigen-presenting cell (APC), often a dendritic cell (DC), might acquire cellular antigen by phagocytosis of an apoptotic or necrotic cell that carries helper (that is, MHC class-II-restricted) and killer (that is, MHC class-I-restricted) antigens. Phagocytosed antigen is presented to CD4+ T cells, which activate the APC through CD40–CD40-ligand (CD40L) interactions. The activated DC can then promote the CD8+ T-cell response, generating cytotoxic effector T cells and memory cells. (From Bevan et al. 2004 *Nature Reviews Immunology*).

b. CD8+ T cells (Cytotoxic T lymphocyte)

The principal cellular effector mechanisms of anti-tumor immunity are mediated by tumor-specific CD8+ CTL, relying on their ability to recognize and directly (by cytolytic factor release) or indirectly (by effector cytokine secretion) kill tumor cells that present antigenic peptides in the context of MHC class I molecules (MHC-I) [226]. Anti-tumor CD8+ T lymphocytes were first identified in mice through their specificity to peptides derived from mutated antigens not detected on healthy tissues [227]. In humans, the presence of anti-tumor CD8+ T cells was demonstrated in peripheral blood and tumor tissues of patients suffering from different types of cancer, including lung carcinoma [228-230]. The identification of TAA and the presence of antigen-specific CD8+ TIL in spontaneously regressing tumors provided irrefutable evidence for the existence and the importance of CTL-mediated anti-tumor immune response [231]. Moreover, a positive correlation between patient survival and important tumor infiltration by $CD8^+$ lymphocytes, despite some controversy, has been established [173, 232]. It is actually acknowledged that this prognostic factor depends highly on the environmental context of the infiltrate and is associated with better outcomes in the absence of immunosuppressive mechanisms abrogating CTL effector functions. To further emphasize the major role of CD8+ T cells, it has been proposed that tumor infiltration by antigen-specific CTLs triggers the invasion by other immune effectors, including neutrophils, macrophages and NK cells, resulting in amplification of anti-tumor immune responses [233].

c. CTL killing mechanisms

TCR-mediated recognition of antigenic peptides [234] leads to the generation of tumor-reactive CTLs equipped with lytic granules and able to secrete effector cytokines upon re-activation [235]. CTL effector mechanisms proceed by:

Direct cell contact-mediated cytotoxicity inducing an apoptotic response in the target cell triggered by:

- calcium-dependent pore-forming perforin and several serine esterases called granzymes from specialized secretory lysosomes into the synaptic cleft between T cells and target cell (perforin/granzyme-mediated pathway) (Figure 13).
- Calcium-independent exposition of transmembrane proteins, such as FasL in the T cell-target cell contact area (FasL-mediated pathway) (Figure 13).

The secretion of effector cytokines and chemokines, including IFN-γ or TNF, CCL3 and CCL5, which may have a direct effect on tumor cells or indirectly change the tumor microenvironment by angiogenesis inhibition or immune cell recruitment and activation

To exert their function, CD8+ T cells have to physically engage their target in an Ag-dependent manner. The specific TCR-mediated recognition of peptide-MHC complexe on tumor cells is required either for killing by perforin and FasL-dependent pathways as well as for cytokine secretion. Nevertheless, while lytic granule release takes place within minutes after specific Ag recognition, cytokine secretion occurs hours after initial recognition and requires sustained TCR signaling [236].

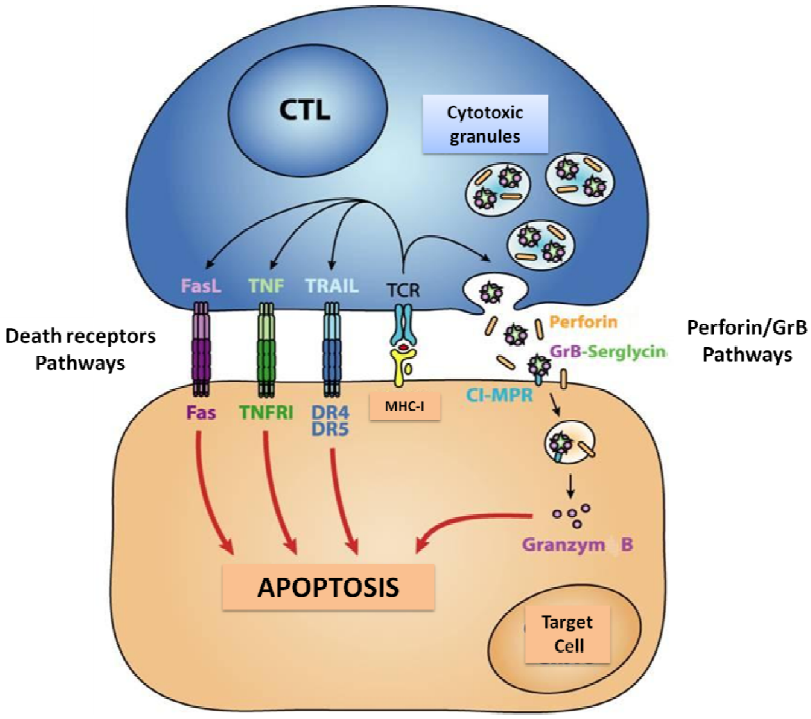


Figure 13: CTL-mediated cytotoxic pathways.

The CTL uses two different types of cytotoxic mechanisms for killing specific target cells. Death receptor expressed on tumor cells (Fas, TNFRI, DR4, DR5) and exocytosis of cytotoxic granules (Perforin and GranzymeB B).

d. The immunological synapse (IS)

i. Formation of the immunological synapse

Naïve $CD8^+$ T cells continuously traffic though secondary lymphoid organs where they systematically scan the surface of DCs searching for TAAs. TA are peptides produced by degradation of cytosolic proteins via the immunoproteasome and are transported to the endoplasmic reticulum through the transporter TAP (Transporter Associated with Antigen Processing) where they bind to MHC class I or class II molecules. The encounter of naïve T cells with DC that present cognate TCR ligand results in the formation of stable, long-lasting conjugates [237]. This involves the organization of a specialized adhesive cell-cell junction, termed an immunological synapse (IS), considered crucial for full-fledged T-cell priming [236, 238]. At the molecular level, IS consists in cognate interaction-dependent, ordered segregation of TCRs, as well as co-stimulatory and adhesion molecules, in the interface between the T cell and APC (Figure 14). Upon initial TCR engagement by an antigenic peptide-MHC complexe (pMHC), highly dynamic receptor microclustering and early signaling indicate an immature IS organization. Subsequently, centripetal transport of peripherally engaged TCR, with a concurrent process of LFA-1 and ICAM-1 exclusion, results in the formation of supramolecular activation clusters (SMAC) and establishment of mature IS. The central region of the SMAC, named “central SMAC” (cSMAC), is thus enriched in TCR-pMHC complexes accompanied by costimulatory receptors, like CD28 and CD2 on T cells, interacting with their respective ligands CD80/86 and CD48/CD58 on DCs. A ring of adhesion molecules with high density of LFA-1 and ICAM-1 complexes, referred to as peripheral SMAC (pSMAC), surrounds the cSMAC.

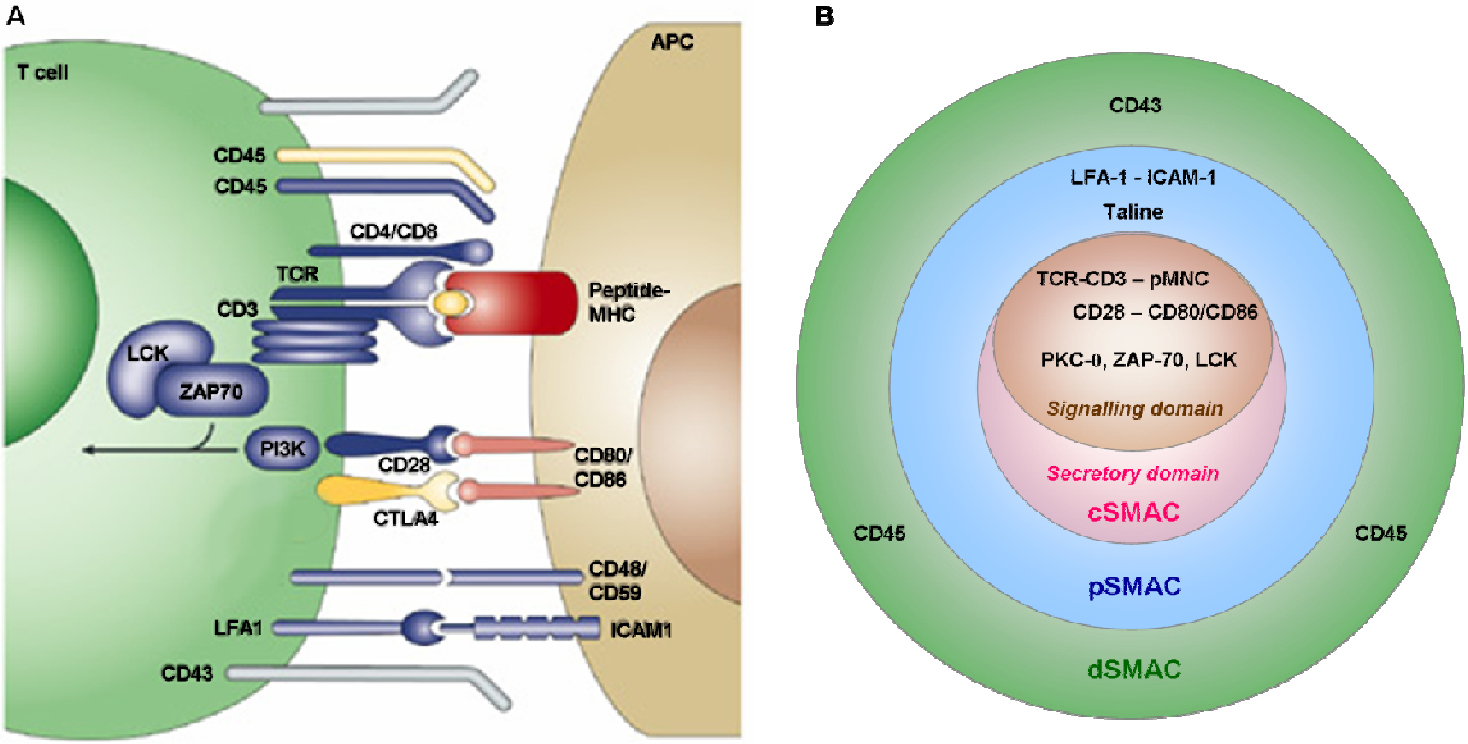


Figure 14: Schematic model of mature immunological synapse.

A) A profile view of key molecules involved in T cell-APC interactions. **B**) The face on view of the synapse with the concentrically organized zones including the central region of the cSMAC surrounded by peripheral domain, the pSMAC, and the distal region of the dSMAC. Signaling (TCR/CD3 - pMHC) and co-stimulation (CD28 - CD80/CD86) molecules enriched in the cSMAC are depicted as well as some of downstream signaling effectors (PKC-θ, ZAP-70, LCK). Adhesion molecules (LFA-1 - ICAM-1) accumulate in the pSMAC and large proteins (CD43 and CD45) are excluded. Secretory domain within the cSMAC for polarized exocytosis of effector molecules such as some cytokines or lytic granules in the case of CTLs, is specified. According to (Huppa and Davis, 2003)

ii. The lytic synapse (polarization of lytic granules)

The release of cytotoxic granules, containing preformed soluble cytotoxic mediators, such as perforin and granzymes, represents one of the major target cell killing strategies used by CTLs [239]. At the subcellular level, it has been demonstrated that upon even a brief contact with a cognate Ag-expressing target cells, CTLs rapidly reorganize their adhesion and signaling proteins into an immunological synapse (IS), termed lytic synapse. A notable difference between IS formed by naive and effector T cells is the discrete signaling reflected by a spike-like pattern of calcium mobilization and the formation of secretory domains within the cSMAC of CTLs. The secretory domain localizes immediately opposite the target cell and appears to be involved in the polarization of the lytic granules to the contact site, culminating in the exocytosis of cytolytic contents in the cleft formed between the CTL and target cell [240, 241]. The polarization of the MTOC associated with the Golgi apparatus toward the contact site represents an important step in lytic granule reassembly for exocytosis [242]. Indeed, the granules move along microtubules towards the polarized MTOC, however, their directional secretion does not require actin or microtubule motors, but centrosome-mediated delivery of these organelles to the contact site [243]. In the final step of secretion, cytotoxic granules fuse with exocytic vesicles, which are endosomal in nature, directly at the plasma membrane [244]. The entire process is completed within minutes succeeding TCR stimulation and does not exhaust the entire complement of lytic granules. Thus, the CTL can undergo repeated cycles of Ag recognition, polarization and cytolysis, a process known as serial killing [245] (Figure 15).

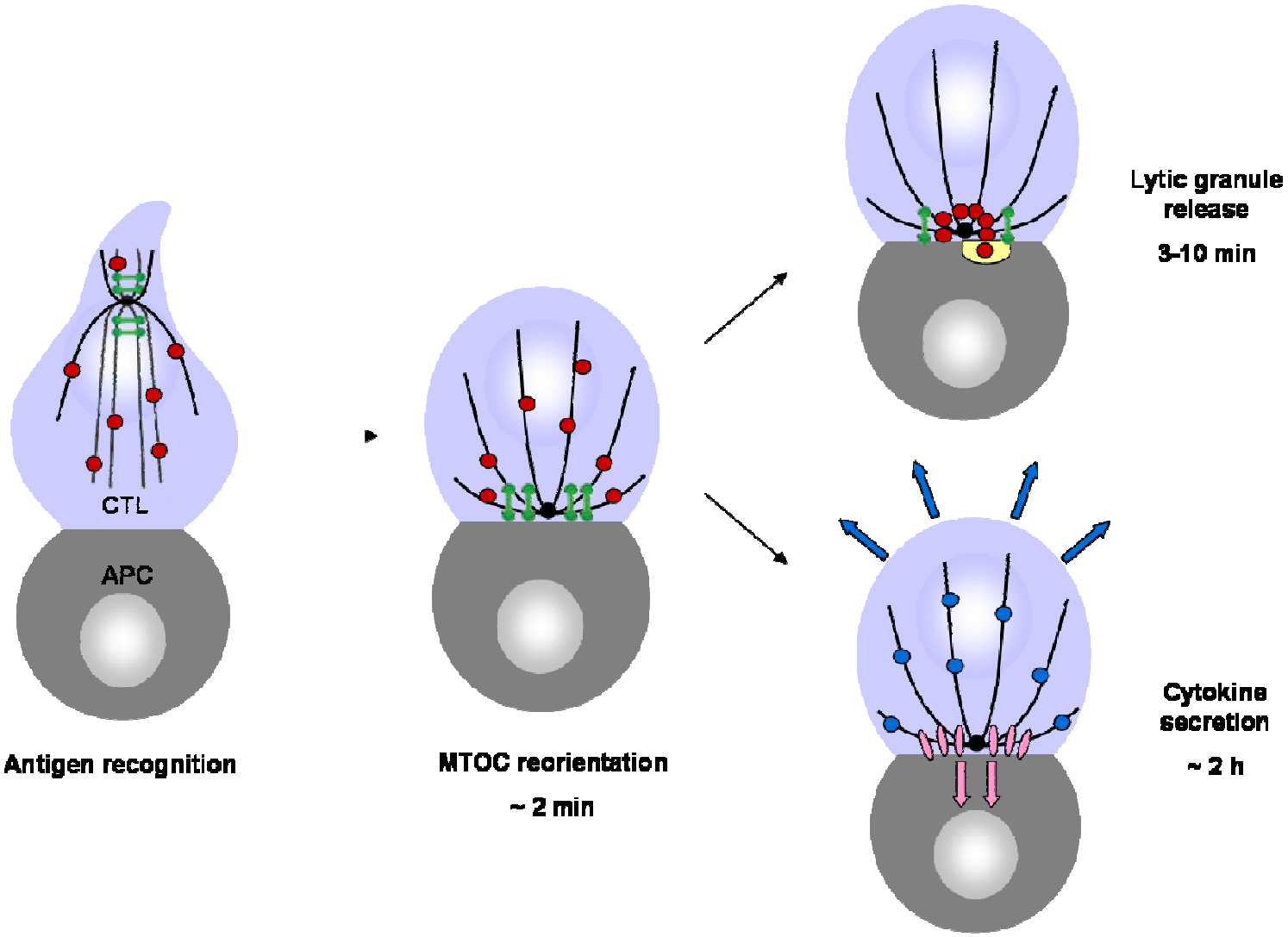


Figure 15:CD8$^+$ T cell secretory response

Ag recognition triggers microtubule network reorientation toward the target (grey) and synapse formation. The granules move in a minus-end direction toward the polarized MTOC (black dot) and accumulate at cell:cell contact site. Within less than 10 min after Ag encounter, the granules content is release into the secretory cleft (yellow). Cytokine secretion typically occurs hours after initial Ag recognition and can be synaptic (pink arrows) or multidirectional (blue arrows). Synaptic secretion is facilitated by Golgi apparatus (green) polarization at the IS. Cytokines destined for multidirectional secretion are actively transported. According to (Stinchcombe and Griffiths, 2007) and (Huse et al., 2008)

It has further been speculated that certain adhesion molecules, organized in pSMAC, can also play an important function in granule polarization. In this context, LFA-1 interactions with its cognate ligand ICAM-1, initially considered to provide an adhesive barrier that protect bystander cells from cytotoxic damage [241], were found to be necessary for polarization of lytic machinery and subsequent directional exocytosis required for target cell lysis. In the absence of LFA-1–ICAM-1 interactions, another adhesion couple $\alpha_E\beta_7$–E-cadherin has been proposed to substitute the LFA-1-mediated role in promoting anti-tumor CTL activity [246].
Although it has been clearly demonstrated that IS can indeed be formed between CTLs and tumor cells [245], its importance for cytotoxicity remains controversial. This relies on observations suggesting that efficient target cell killing, at least in vitro, can occur with only rudimentary or without IS formation at all [247]. These contradictory results indicate the necessity of further investigation of the IS requirement in target cell killing, particularly in in vivo studies.

e. Mechanism of cytotoxic granule killing

CTL use the granule exocytosis pathway to induce target cell death. The major constituents of cytotoxic granules are perforin and granzymes, which combine to promote rapid cell death when delivered to the target cell cytosol. The pore-forming protein, perforin, was originally thought to facilitate granzyme entry into target cells by physically forming holes in the cell membrane through which granzymes may pass [248]. Studies using knockout mice have unequivocally demonstrated the crucial role that this protein has in granzyme-mediated cytotoxicity [249]. As effector cell lacking perforin cannot deliver granzymes to target cells, perforin deficiency translates into a complete loss of cytotoxic granzyme function, with perforin-deficient CTLs defective for target cell killing [249].
Granzymes are a distinct family of serine proteases, with different members harbored by humans and mice. Granzyme B is one of the most abundant granzymes and, consequently, granzyme B-mediated cytotoxicity has been intensively studied [250]. Effector cells lacking granzyme B kill targets at a much slower rate than do wild-type cells, which demonstrates the important role that this protease has in executing the timely demise of infected or tumorigenic cells [251]. The efficiency of granzyme B-dependent killing is largely due to the ability of this protease to activate in target cells the intrinsic cell death proteases or caspases, either directly or indirectly (Figure 16). Direct proteolysis and activation of caspases 3 and -7 by granzyme B leads to the caspase mediated degradation of hundreds of cellular protein substrates, which promotes fast and efficient apoptosis [252].

Granzyme B can also promote caspase activation through the well-defined cytochrome c/Apaf-1 pathway in which proteolysis and activation of the BH3-only protein BID by granzyme B promotes BID-mediated opening of the BAX/BAK channel in the mitochondrial outer membrane (Figure 16). This important event leads to BAX/BAK-mediated release of cytochrome c from the mitochondrial intermembrane space into the cytosol, where it binds to and activates a caspase-activating platform, called the apoptosome. In turn, the apoptosome activates downstream caspase leading to cell death [253]. Importantly, experimental inactivation of caspases does not inhibit granzyme B mediated cell death, which still takes place although with much slower kinetics. This results from BID-mediated mitochondrial dysfunction, together with proteolysis of other substrates by granzyme B such as ICAD and b-tubulin (Figure 16) [254] [255]. Effector cells lacking granzyme B retain cytotoxic activity through the actions of the remaining granzymes, of which granzyme A is the most abundant and promotes target cell death through multiple pathways [256].

Granzyme K shows tryptase-like activity and has been shown to process similar substrates than granzyme A [257]. Evidence of a role for granzyme C in promoting cell death is scarce, although it may be cytotoxic in some contexts [258]. The Metase, granzyme M, has been shown to promote a caspase- and mitochondria independent mechanism of cell death [259].

Problems associated with studies relating to the cytotoxic activity of various granzymes are whether the concentrations of granzymes used to induce cytotoxicity are achievable under physiological settings. Whereas granzyme B displays cytotoxic activity at low nanomolar concentrations, many studies have used granzymes at micromolar concentrations that may not be attainable in vivo. Because the concentration of granzymes that are delivered at the immunological synapse is unknown, it remains to be determined whether all granzymes are truly cytotoxic at physiological levels or have other roles in T cell mediated processes.

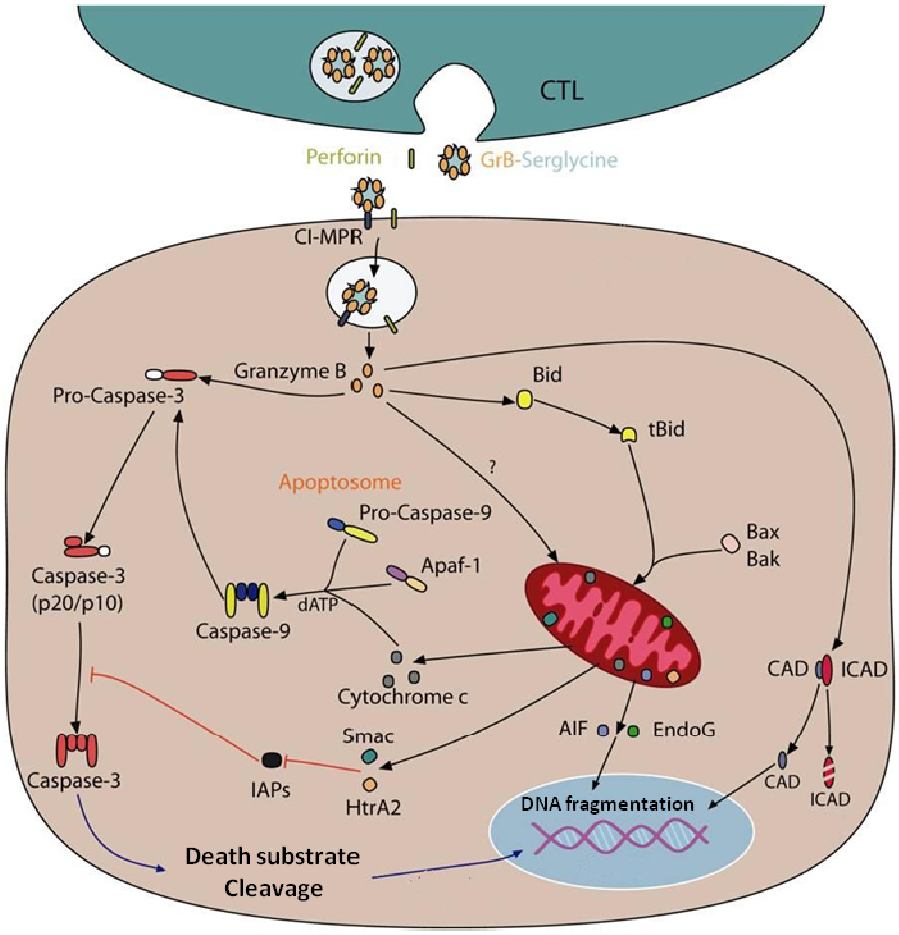


Figure 16: Pathways of granzyme B-mediated cell death.
Granzyme B, together with other granzymes, enters the target cell by a perforin-dependent mechanism. Upon entry into the target cell cytosol, granzyme B promotes apoptosis through two main pathways, either through BID-dependent mitochondrial permeabilization or through direct caspase processing and activation. Granzyme B-mediated proteolysis of the BH3-only protein BID exposes a myristolyation signal in this protein, targeting it to mitochondria, in which it induces oligomerization of BAX and/ or BAK in the mitochondrial outer membrane. The latter event facilitates cytochrome c release into the cytosol, assembly of the apoptosome, with subsequent caspase-9 activation and the ensuing caspase cascade. Note that antiapoptotic BCL-2 family members can inhibit cytochrome c release and block this pathway. Granzyme B can also directly process effector caspases 3 and -7 to promote apoptosis. Direct activation of the latter effector caspases leads to a caspase activation cascade and proteolysis of numerous caspase substrates, resulting in the efficient death of the target cell. Granzyme B can also directly cleave ICAD, the inhibitor of a DNase (CAD), which can promote internucleosomal DNA hydrolysis that is synonymous with this mode of killing.

f. Death domain receptor-mediated killing

CTLs use ligands of TNF superfamily, such as FasL, on their cell surface to bind and eliminate target cells expressing the corresponding receptors [260]. FasL is present in the granules of CTL [261]. FasL is a type II transmembrane protein present in two froms: a membrane form and a soluble form resulting from cleavage of the membrane form by metalloproteases [262] (Figure 17). The expression of FasL is limited to activated lymphocytes and NK cells. Expression on tumor cells remains controversial [263]. FasL mediates its cytotoxic action by binding its cognate receptor Fas on the surface of target cells. Fas is a type I transmembrane receptor ubiquitously expressed in normal cells, tumor cells, and on activated lymphocytes where it mediates activation-indcued cell death (AICD). Stimulation of Fas by FasL homotrimers leads to the rapid formation of receptor microaggregates (Figure 17). It results in the clustering of cytoplasmic death-domain of Fas, creating a plate-forme for the recruitment of the adapter protein FADD (Fas Associated Death Domain) through homolog interactions between the death domain of Fas and the death domain of FADD located in C-terminal [264]. The death-effector domain (DED) of FADD, in its N-terminal, enables then the recruitment of pro-initiator caspases, including pro-caspase-8 [265]. The multiprotein complex thus formed is called "death inducing signaling complex" or DISC. In the DISC, pro-caspases 8 undergo trans-proteolytic cleavage after aspartate residues to generate active caspase-8. In type I cells, sufficient amounts of active caspase-8 are produced to directly cleave and activate effector caspases such as caspase-3,-6 and -7 [266]. Effector caspases in turn cleave a series of cellular substrates, of which ICAD (Inhibitor of CAD (capsase-activated DNAse)), releasing CAD that translocates to the nucleus and degrade DNA in apoptosis-specific oligonucleosomic fragments. Type II cells produce very little active caspase-8 at the DISC and rely completely on the mitochondrial amplification of the Fas signal, which is proposed to be mediated by the pro-apoptotic BH3 domain only containing Bcl-2 family member Bid [267] [268]. Bid cleavage by caspase-8 results in its translocation to the mitochondria where it induces the release of the mitochondrial factors, ultimately enhancing the apoptotic signal. As these cells rely on the apoptotic functions of mitochondria, expression of Bcl-2/Bcl-x_L protects from Fas-mediated apoptosis in type II cells expressing these proteins display a significant blockade in cleavage of proteins associated with apoptosis [266].

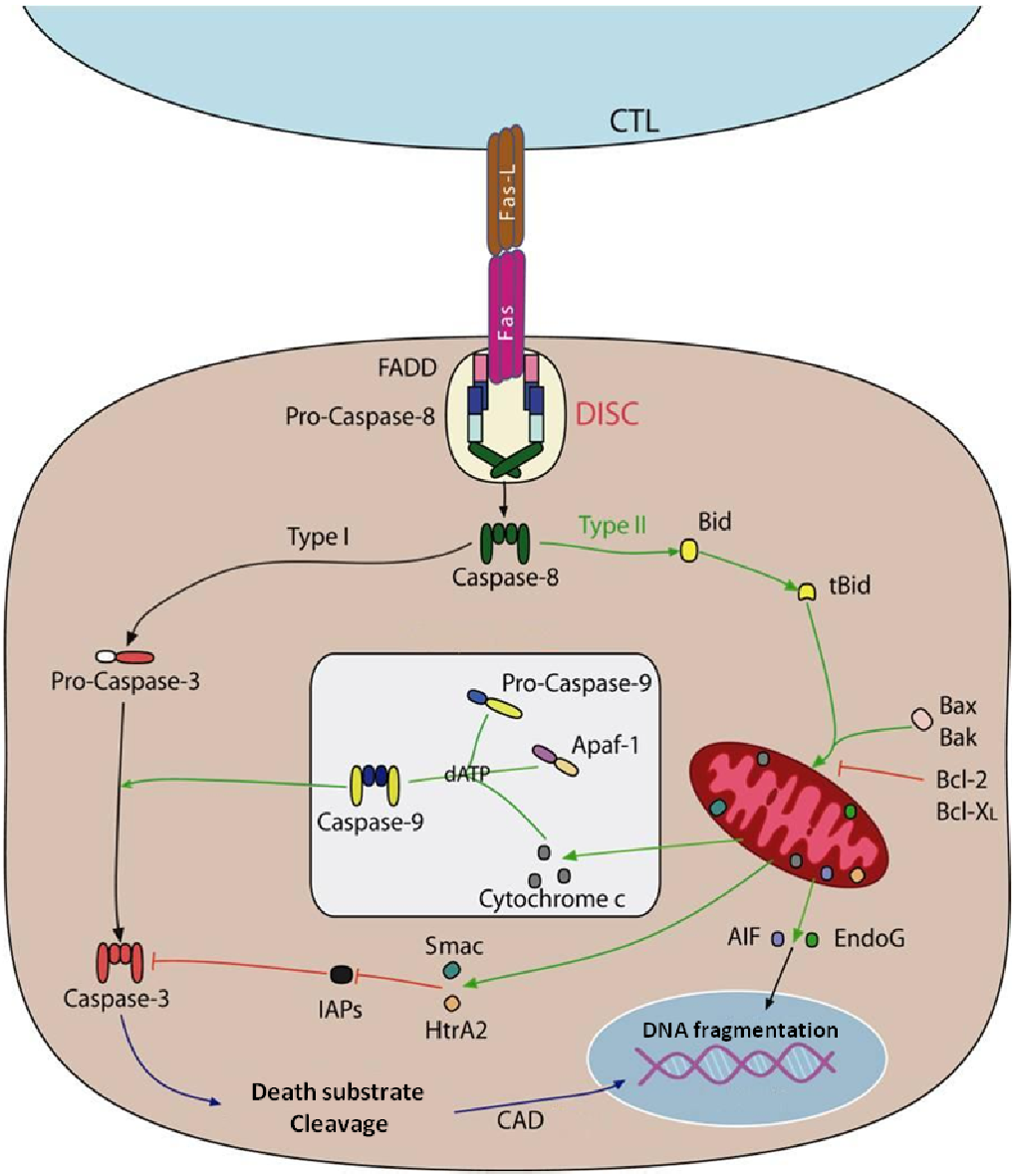


Figure 17: Fas/FasL Pathways.

FasL mediates its cytotoxic action by binding its cognate receptor Fas on the surface of target cells. See text for details.

2. Mechanisms of tumor escape to the immune system

The genetic instability of tumor cells [269] combined with the selection pressure exerted by the immune system lead to the emergence of tumors resistant variants to the immune response. Avoiding immune destruction is one of the hallmarks of cancer [10] and represents a significant barrier to effective immunotherapy protocols (Figure 18).

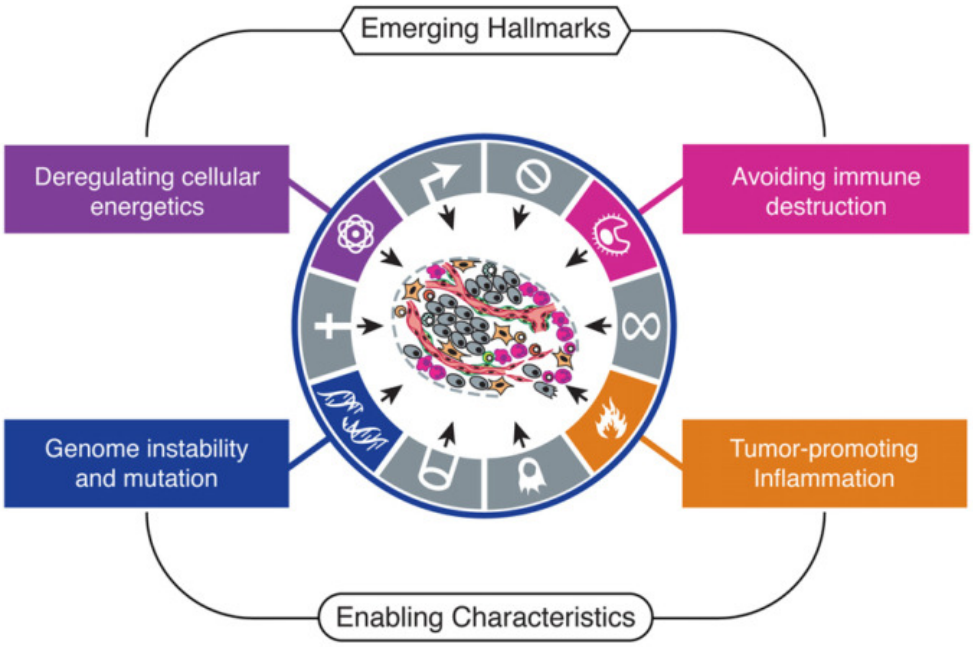


Figure 18: Emerging hallmarks of cancer.

An increasing body of research suggests that an additional emerging hallmark of cancer is involved in the pathogenesis of some and perhaps all cancers. It allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells (From Douglas Hanahan and Robert A. Weinberg 2011 Cell).

a. Tumor escape mechanisms inherent to tumor cells

Many studies have evidenced different mechanisms allowing tumors to escape or to interfere with the antitumor immune response [270] [271]. The escape mechanisms can be classified into several categories, depending whether they are inherent to the tumor cells, the immune cells, or present in the tumor microenvironment (Figure 19).

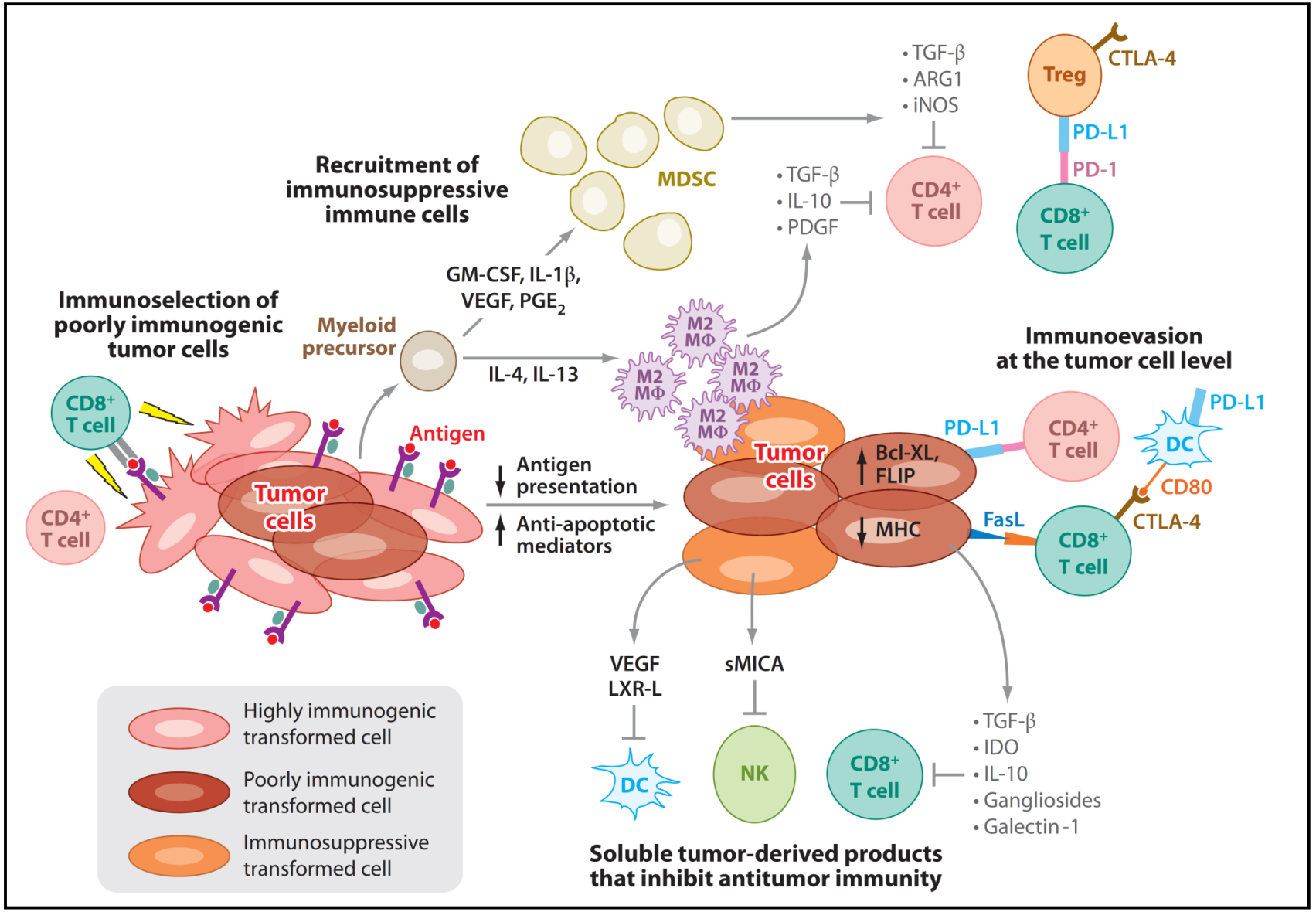

Recruitment of immunosuppressive immune cells
MDSC
• TGF-β
• ARG1
• iNOS
• TGF-β
• IL-10
• PDGF
CD4+ T cell
Treg
CTLA-4
PD-L1
PD-1
CD8+ T cell
GM-CSF, IL-1β, VEGF, PGE2
Immunoselection of poorly immunogenic tumor cells
Myeloid precursor
IL-4, IL-13
M2 MΦ
Immunoevasion at the tumor cell level
CD8+ T cell
Antigen
Tumor cells
CD4+ T cell
Antigen presentation
Anti-apoptotic mediators
Bcl-XL, FLIP
MHC
PD-L1
CD4+ T cell
DC
CD80
CTLA-4
FasL
CD8+ T cell
VEGF
LXR-L
sMICA
DC
NK
CD8+ T cell
• TGF-β
• IDO
• IL-10
• Gangliosides
• Galectin-1
Soluble tumor-derived products that inhibit antitumor immunity
Highly immunogenic transformed cell
Poorly immunogenic transformed cell
Immunosuppressive transformed cell

Figure 19: Tumor escape mechanisms.

Tumors have evolved different mechanisms to elude or inhibit immunity by both intrinsic and extrinsic means. Intrinsic alterations within tumor cells evade immunity by down regulating antigen presentation (MHC), up regulating inhibitors of apoptosis (Bcl-XL, FLIP), or expressing inhibitory cell surface molecules that directly kill cytotoxic T cells (PD-L1, FasL). In addition, tumor cells secrete factors that inhibit effector immune cell functions (TGF-β, IL-10, VEGF, LXR-L, IDO, gangliosides, or soluble MICA) or recruit regulatory cells to generate an immunosuppressive microenvironment (IL-4, IL-13, GM-CSF, IL-1β, VEGF, or PGE2). Once recruited, regulatory cells attenuate antitumor immunity through the liberation of immunosuppressive cytokines and alterations in the nutrient content of the microenvironment. (Abbreviations: ARG1, arginase 1; Bcl-XL, B cell lymphoma extra long; CTLA-4, cytotoxic T lymphocyte associated protein-4; DC, dendritic cell; FasL, Fas ligand; FLIP, apoptosis-stimulating fragment-associated protein with death domain-like interleukin-1 converting enzyme-like inhibitory protein; GM-CSF, granulocyte macrophage colony–stimulating factor; IDO, indoleamine 2,3-deoxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; LXR-L, liver X receptor ligand; MDSC, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MICA, MHC class I polypeptide-related sequence A; PDGF, platelet-derived growth factor; PD-L1, programmed cell death 1 ligand 1; PGE2, prostaglandin-E2; TGF-β, transforming growth factor-β; Treg, regulatory T cell; VEGF, vascular endothelial growth factor (Figure from Smyth et al. 2011 The *Annual Review of Immunology*).

i. Escape to recognition by immune system

Antigen presentation by MHC molecules is crucial both for the initiation of the T cell response and the maintenance of this response. Alterations in processing and/or presentation of the antigenic peptide were reported frequently in many tumors. Indeed, antigen presentation in tumor cells may be partially or totally altered due to mutations and/or deletions of one or more genes coding for the peptide processing machinery. Complete loss of MHC molecules was observed in many types of cancers such as breast, prostate or lung cancers [272]. The most common mechanism leading to loss of expression of MHC class I molecules is mutation or deletion of genes coding the β_2 microglobulin (β_{2M}) [273]. Selective loss of haplotype, locus or HLA allel has also been described. The decreased expression of TAP1 and TAP2 genes, involved in the transport of the peptide from the cytoplasm to the endoplasmic reticulum, and decreased expression of components of the immunoproteasome such as subunits LMP2 and LMP7, involved in the production of antigenic peptide [274], are also frequent mechanisms of tumor escape [275], causing alterations of antigen presentation and processing. Finally, the loss of expression of tumor antigens is an important mechanism of escape to T cell-mediated immune response. This phenomenon was particularly well demonstrated in a study realized in melanoma patients with recurrent metastases treated by adoptive transfer of MART-1-specific T cells. In three out of five patients, metastatic cells have lost the expression of MART-1, whereas the expression of other antigens such as gp100 or tyrosinase remained normal [276].

ii. Resistance to T cell mediated-lysis

Tumor cell susceptibility to lysis by the immune system remains ineluctably a determining element of the effectiveness of anti-tumor immune responses. Several mechanisms of resistance to cytotoxic functions of CTLs and NK cells, involving especially the perforin-granzyme and the death receptor pathways have been described [277].

Resistance to the perforin-granzyme pathway

Several mechanisms of resistance to lysis induced by the perforin-granzyme pathway have been described in different tumor models. These mechanisms interfere with the pro-apoptotic signaling of this cytotoxic pathway. For example, some tumors escape death induced through perforin-granzyme by expressing the Serpin PI9/SPI6, a cellular inhibitor of granzyme B [278]. In humans, in vivo expression of serpin was found in 39% of T-cell lymphomas, 27% of B-cell lymphomas and 10% of Hodgkin lymphoma [279]. Various alterations in mitochondrial signaling pathways (overexpression of Bcl-2, Bcl-XL, IAPs, and mutations of

Bax, Bak, or Apaf-1) are frequently observed in tumor cells and may alter the response to the perforin-granzyme pathway, mediating resistance to lysis.

Resistance to death receptor pathway

Resistance to apoptosis induced by FasL-Fas induced pathway has been demonstrated in many tumors. Several mechanisms have been described, including a lack of Fas expression. Indeed, it was shown that its expression is frequently reduced or even absent in many tumor types [280] [281]. In sera of patients with T-cell lymphoma or solid tumors, high levels of soluble Fas are found to interfere with membrane Fas [282]. Finally, overexpression of the molecule cFLIP in tumor cells, which acts as a dominant negative inhibitor of caspase8, prevents apoptosis induced by Fas.

iii. Expression of inhibitory molecules

B7-H1 and RCAS1 molecules

The expression by tumor cells of the B7-H1 molecule (also called programmed death ligand 1 (PD-L1)) that targets PD-1 on the surface of antigen-specific T cells has been described in many types of cancers [283] [284]. The engagement of B7-H1 with its receptor induces the apoptosis of activated T cells [285]. Also, triggering by PD-L1 positive tumor cells of PD-1 on T cells increases tumor cell resistance to immune-induced death [286], demonstrating that cancer cells can use receptors on immune cells to induce resistance. In addition, the presence of B7-H1 protein has been demonstrated by immunohistochemistry in a wide range of human cancers [287]. Another ligand expressed by tumor cells, RCAS1 (Receptor Binding Cancer Antigen-Face value on Siso cells), could also be involved in the inhibition of the cytotoxic response. The expression of RCAS1 has been demonstrated in cancers of the ovary and uterus. This molecule has recently been described as capable of inducing apoptosis of T cells and NK cells, especially in a glioma model [288].

The non-classic MHC molecules

In vitro studies have shown that HLA-G molecule, a non-classical MHC-I molecule belonging to the MHC- class Ib sub-group, is expressed in cell lines derived from various types of cancers (gliomas, melanomas, renal cell carcinoma). Through binding to many receptors such as ILT-4 (Immunoglobulin-Like Transcript 4) (expressed by macrophages, monocytes and DC) or KIR2DL4/p49 (expressed by NK), HLA-G plays a key role in the establishment and maintenance of immune tolerance by inhibiting the functions of immunocompetent cells (differentiation, proliferation, cytotoxicity, cytokine secretion) [289]. Exosomes secreted by melanoma cells expressing HLA-G also exhibit membrane expression of HLA-G [290] and

negatively modulate the antitumor immune response, particularly by inducing apoptosis of activated $CD8^+$T cells through Fas/FasL. The expression of HLA-G in ovarian carcinoma cells induces resistance to lysis mediated by $CD8^+$ T cells [291]. Similarly, the soluble and membrane expression of another non-classical MHC molecule, HLA-E, by melanoma cells, decreases their susceptibility to lysis induced by CTL [292].

iv. Absence of co-stimulatory signals

Lymphocyte anergy results in functional impairment of naive T cells activated by a cognate antigen to proliferate when re-stimulated with the same antigen [293]. It is now recognized that full activation of T cells requires two signals. The first is the antigen presentation by MHC molecules and is responsible for the specificity of the immune response. The second is called « co-stimulatory signal ». Blocking this co-stimulatory signal inhibits in vitro and in vivo activation of T lymphocytes. Therefore, a lack of expression of co-stimulatory molecules such as B7-1 and B7-2 by cancer cells could lead to T cell anergy.

v. Constitutive activation of signaling pathways

Activated STAT3 has anti-apoptotic functions and promotes tumor cell proliferation. Its functions in negative regulation of inflammation, DC and T cell activities are also described [294] [295]. NF-KB (nuclear factor-κ B) is constitutively active and promotes the expression of many target genes involved in cell cycle progression, survival, cell adhesion, angiogenesis, immune responses and inflammation [296] [297]. NF-κB interferes with CTL-induced death pathways by inducting the expression of anti-apoptotic genes such as Flip [298], Bcl-xL [299] and IAP [300] [301].

vi. Tumor cytoskeleton disorganization

Tumor cells can shift their cytoskeletal organization as a strategy to promote resistance after CTL selection pressure [302]. This phenomenon is associated with an overexpression of the Ephrin-A1 and Scinderin, and an inactivation of the protein kinase FAK, involved in focal adhesion and stress fiber formation [303].

vii. Metastatic tumoral resistance

Metastatic tumoral resistance involves multiple factors against apoptosis mediated by cytotoxic inducers (TRAIL, FasL, TNF, and CTL) [304]. Downregulation of ICAM-1 on metastatic melanoma cells is reported to decrease their sensitivity to CTL- and granzyme B-mediated lysis [305]. This down-regulation of ICAM-1 is correlated to a decreased PTEN

activity and subsequent activation of PI-3K/Akt pathway suggesting that PI3K/AKT pathway also plays an important role in the control of tumor susceptibility to CTL-mediated tumor cell killing.

viii. Epithelial-to-mesenchymal transition (EMT)

The hallmark of EMT is the downregulation of epithelial markers (E-cadherin) and the upregulation of mesenchymal markers (Vimentine, Fibronectine, the transcription factors Snail, Slug, Twist) leading to an organized disassembly of epithelial cell-cell contacts and an acquisition of mesenchymal motile phenotype [306]. A study by Kudo-Saito and colleagues demonstrate that Snail-induced EMT accelerates cancer metastasis through not only enhanced invasion but also induction of immunosuppression by inducing Treg and impairing DC maturation. In addition, although Snail expressing melanoma did not respond to immunotherapy, intratumoral injection with snail-specific siRNA significantly inhibited tumor growth and metastasis following increase of tumor-specific tumor-infiltrating lymphocytes and systemic immune responses [307].

b. Immune escape mechanisms inherent to immune system or tolerance

i. Ignorance or lack of presentation by DC

Naive T cells can not recognize a cognate antigen if it is not presented by DC. Indeed, DCs infiltrating tumors have an immature phenotype characterized by low production of proinflammmatory cytokines and low expression of co-stimulatory and MHC molecules. The major pathway responsible for this immature phenotype is the STAT3 pathway. Indeed, constitutively active STAT3 in DCs induces the production of factors such as VEGF and IL-10, which inhibit DC maturation [308]. Inhibition of STAT3 activity in DC progenitor cells reduces the accumulation of immature DC in the tumor microenvironment [309].

ii. Dysfunctions of $CD8^+$ T cells

The expression by $CD8^+$ T cells of inhibitory receptors similar to those of NK cells alters their activation leading to tumor escape. Indeed, the expression of CD94/NKG2A receptors belonging to the family of KIR (p58.1/KIR2DL1 and p58.2/KIR2DL2) inhibits the lytic activity of tumor-specific T cells [310, 311]. Tumor cells secreting soluble form of MICA resulted in the endocytosis and degradation of NKG2D in T cells leading to impaired activation of CD8+ T cells [312].

iii. Deletion of T cells

The deletion of tumor-specific T cells results in a state called "peripheral tolerance". Two main mechanisms are involved. First model is the "counter-attack model" when FasL expressed on tumor cells induced apoptosis of tumor-infiltrating lymphocytes expressing the Fas receptor [263]. Similarly, the expression of FasL on the surface of microvesicles secreted by melanoma cells induces apoptosis of Jurkat cells in vitro [313]. Nevertheless, the counter-attack model is controversial due to conflicting reports casting doubts on its relevance *in vivo* [263] and rather proinflammatory role of FasL leading to a massive infiltration of neutrophils by tumor and subsequent tumor rejection [263]. Activation-induced cell death (AICD) is the second mechanism inducing antitumor T cell deletion and involves FasL/Fas. Precisely, activated T cells strongly express FasL causing their own death (suicide) or the death of surrounding T cells (Fratricide) [314]. Moreover, continuous stimulation of T cells by tumor cells over-expressing the cognate antigens can induce AICD of these effectors, leading to their deletion [315].

iv. Regulatory T cells

In 1995, the group of Sakaguchi identified a population of $CD4^+$ $CD25^+$ immunoregulatory T cells (Treg) involved in preventing physiological autoimmune diseases and inducing self-tolerance [316]. CD4+CD25+ forkhead box P3 (FOXP3) + are thought to be the major Tregs population [317].Treg cells are characterized by the secretion of TGF-β and IL-10. It results in inhibition of activation and proliferation of $CD4^+$ and $CD8^+$ and specific production of IL-2 [318]. In addition, some Treg cells express PD-1, which by binding to its ligang B7-H1 on DC suppresses the production of IL-12 by the latters, decreasing their ability to induce the immune response [319]. The presence of Treg in human cancers correlates with decreased survival in patients with ovarian cancer [320]. On the other hand, in some hematological malignancies, especially B-cell lymphoma, elevated number of $FOXP3^+$ cells was shown to correlate with improved survival [321]. Other studies consider the ratio of $CD8^+$ T cells to $CD4^+CD25^+FOXP3^+$ T cells and propose that a reduced ratio, as well as Tregs numbers in tumors, correlates with poor prognosis in patients with breast, gastric, ovarian, and colon cancer [317].

c. Tumor microenvironment

Diverse immunosuppressive factors present into the tumor microenvironment and derived from tumor cells as well as stromal cells contribute to immune suppression [322].

i. Immunosuppressive cytokine

Several immunosuppressive cytokines such as TGF-β, IL-10, IL-6, VEGF and M-CSF are secreted by many tumors. In a in vivo mouse model, selective inhibition of TGF-β signaling pathway in T cells has been reported to be necessary for the eradication of tumors secreting this cytokine [323]. IL-10 inhibits antitumor response by altering antigen presentation, T cell proliferation, cytokine production by Th1 $CD4^+$ T cells and secretion of IL-12. IL-10 also blocks the recruitment of DC in response to a vaccine based on the use of GM-CSF [324]. IL-6 and M-CSF (Macrophage Colony Stimulating Factor) produced by tumor cells, are capable of inhibiting the differentiation of myeloid progenitor cells into DC [325]. IL-6, by binding to its receptor on tumor cells, activates STAT3 [295].

ii. Metabolites

Indolemine 2, 3-dioxygenase (IDO) can suppress effector T-cell functions and may cause T cell apoptosis [326] [327] [328]. Some mucins (MUC1 and MUC2) have the ablity to inhibit T cell proliferation and to increase metastatic properties of tumor cells [329]. Similarly, some gangliosides including GM3 and GD3 can inhibit NK cytotoxicity [330] and DC differentiation [331]. Inducible nitric oxide synthase (iNOS) is one of the three enzymes responsible for synthesis of nitric oxide (NO) by arginine oxidation. NO plays an important role in tumor growth and metastasis formation [332] but is also able to strongly inhibit T cell-mediated cytoxicity at the tumor site [333].

iii. Hypoxia affecting the immune system

It is well known that immune system can effectively inhibit tumor growth [334]. However, tumors are known to establish diverse potent mechanisms, which help them to escape the immune system [270, 319, 335, 336]. Emerging evidence indicates a link between hypoxia and tolerance to immune system [3] [337].

Hypoxic zones in tumors attract a variety of immune cells in which HIF stabilisation is associated with the acquisition of both pro-angiogenic and immunosuppressive phenotype [3] [338]. In addition, a direct link between tumor hypoxia and tolerance through the recruitment of regulatory cells has been established [339]. The effects of hypoxia on immune cells are proposed to be critical factors for the development of tumor immune escape. The innate and adaptive immune systems respond differentially to decreased oxygen levels with a global amplification of the innate responses and a direct or indirect (via the innate system) shortcoming of the adaptive ones (Figure 20).

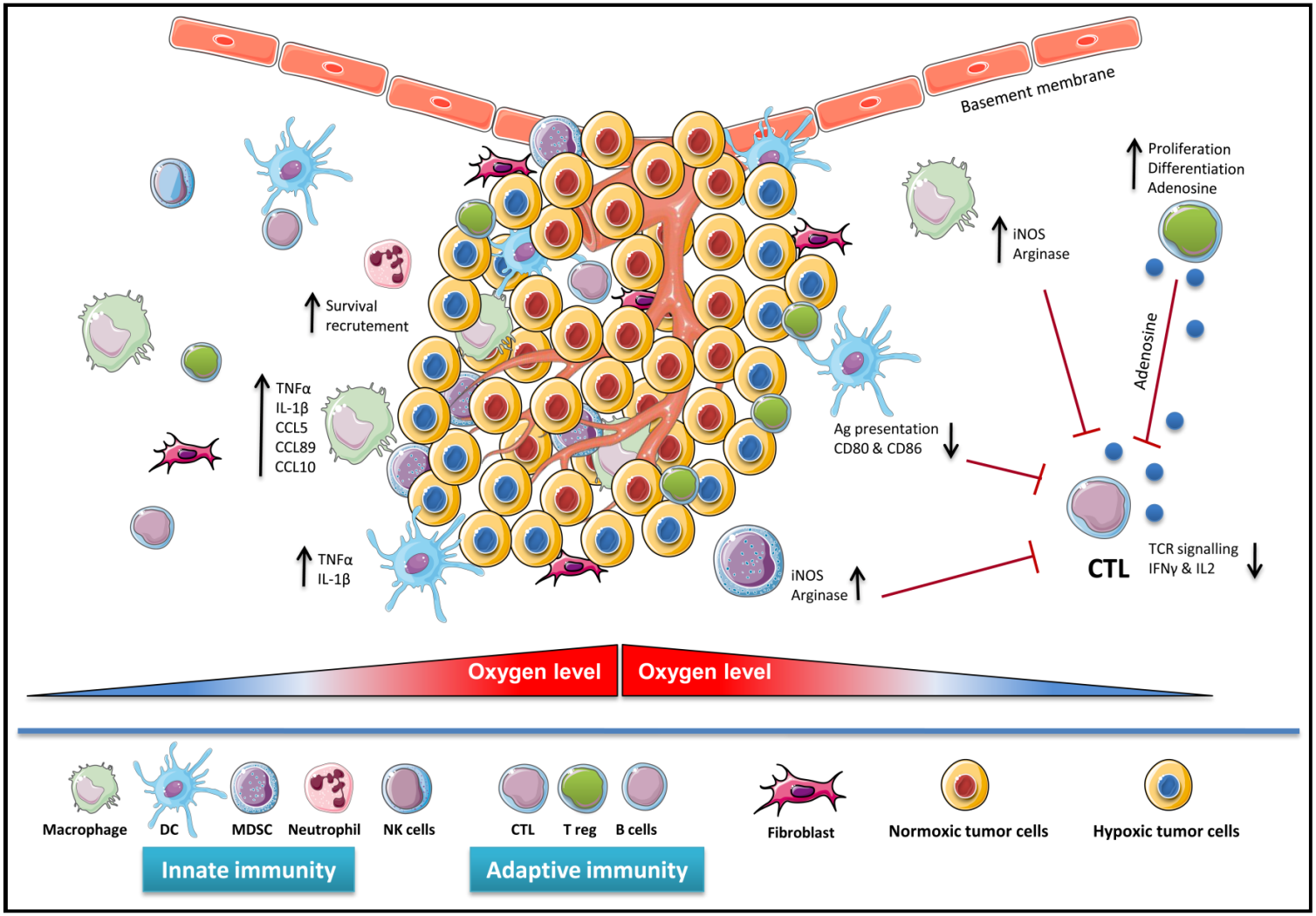


Figure 20: Influence of hypoxia on the innate and adaptive immune systems.
Diverse effects of hypoxia on innate immune system (Macrophage, DC, MDSC, NK and neutrophil) and adaptive immune system (CTL, T reg and B cells). In general, hypoxia amplifies the activity of innate immune cells while suppressing the response of the adaptive immune system. DC: dendritic cells, MDSC: myeloid derived suppressor cells, NK: Natural killer cells, CTL: cytotoxic T lymphocyte and T reg: T regulatory cells.

AIMS OF STUDY

Despite the impressive progress over the past decade, in the field of tumor immunology, such as the identification of tumor antigens and antigenic peptides, there are still many obstacles in eliciting an effective immune response to eradicate cancer. Cytotoxic T lymphocytes (CTLs) are important effector cells in tumor rejection [340]. Accumulating evidence indicated that tumor cells play a crucial role in the control of immune protection [341] [342] and tumor cell growth in vivo is not only influenced by CTL- tumor cell recognition, but also by tumor susceptibility to cell-mediated death [343]. The efficacy of antitumor CTL critically depends on functional processing and presentation of tumor antigen by the malignant cells but also on their susceptibility to CTL-induced lysis.

Cancer Immunotherapy has developed a lot during the 90's. The discovery of TAA has brought a new revolution in this field. However the results of the clinical studies remained in the back ground because of the existing conflict/interactions between the immune system of the host, its tumoral system and its micro environment, which is able to neutralize the antitumor cytotoxic effectors of various types. It has become clear that by analysing the interactions between the immune system and the tumoral system could facilitate the development and emergence of new approaches in antitumor immunotherapy. Several reports indicated that tumor cells evade adaptive immunity by a variety of mechanisms including development of hypoxia (low oxygen tension) [1]. Tumor hypoxia plays an important role in angiogenesis, malignant progression, metastatic development and chemo-radio resistance [2]. It is now well established that hypoxic tumor microenvironment favours the emergence of tumor variants with increased survival and anti-apoptotic potential [3].

There is very little work done on the impact of tumor hypoxia on the regulation of tumor susceptibility to the lysis induced/carried out by cytotoxic antitumor response. Since hypoxia is a common feature of solid tumors and one of the hallmarks of tumor microenvironment [63], we asked whether hypoxia confers tumor resistance to CTL-mediated killing. This work was carried out *in vitro* starting from tumor lines and their autologous cytotoxic T lymphocyte clones (NSCLC and melanoma cancer model) and eventually *in vivo* in mouse models (Figure 30).

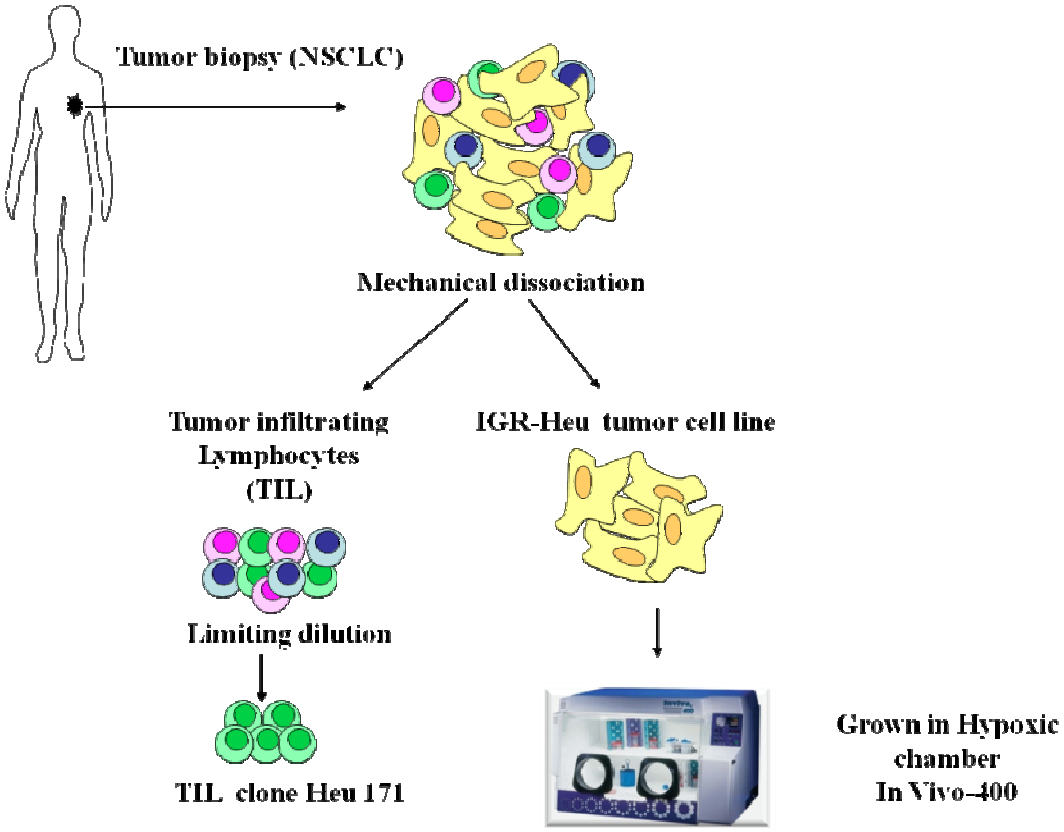


Figure 21: Experimental model.

A tumor cell line was established from a tumor biopsy of a lung carcinoma patient and $CD8^+$ TIL clones were generated by limiting dilution. Only tumor cell line (IGR-Heu) was kept under hypoxia.

The primary objectives of this project are:

1. To study the functional consequences of inductions of hypoxia and HIFs on the tumor cell susceptibility to CTL-mediated lysis
2. To dissect the potential involvement of HIF-1-α and P-STAT3 in the acquisition of a tumoral resistance to CTL-mediated lysis under hypoxia
3. To examine the role of hypoxia induced autophagy on the tumor cell susceptibility to CTL-mediated lysis and to describe the underlying mechanisms involved
4. As resistance of tumor targets to killer cells is likely to be regulated by multiple factors, we will further explore whether hypoxia (as inducer of HRM) is an important determinant involved in the control of target sensitivity to CTL-mediated lysis.

Hence the project of this doctorate was to study the effect/role of tumor hypoxic stress on antitumor cytotoxic response in vitro and in vivo. During this project, we focused on the impact of hypoxia on the regulation of the anti-tumor response and the subsequent tumor progression. We strongly believe that by manipulating hypoxia, we can modulate effector function and behavior of tumor cells.

RESULTS

Article 1: "The cooperative induction of hypoxia-inducible factor-1 alpha and STAT3 during hypoxia induced an impairment of tumor susceptibility to CTL-mediated cell lysis"

Noman, Muhammad Zaeem, S. Buart, J. Van Pelt, C. Richon, M. Hasmim, N. Mazure, P. Romero, F. Mami-Chouaib, and S. Chouaib.

Journal of Immunology, 2009, 182:3510-3521.

Article 2: "Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression"

Noman, Muhammad Zaeem, B. Janji, B. Kaminska, K. Van Moer, S. Pierson, P. Przanowski, S. Buart, G. Berchem, P. Romero, F. Mami-Chouaib, and S. Chouaib.

Cancer Research, 2011, 71:5976-5986.

Article 3: "Hypoxia-inducible miR-210 regulates the susceptibility of tumor cells to lysis by cytotoxic T cells"

Noman, Muhammad Zaeem, Stéphanie BUART, Pedro ROMERO, Sami KETARI, Bassam JANJI, Bernard MARI, Fathia MAMI-CHOUAIB and Salem CHOUAIB.

Cancer Research, In Press

A. Article 1: "The cooperative induction of hypoxia-inducible factor-1 alpha and STAT3 during hypoxia induced an impairment of tumor susceptibility to CTL-mediated cell lysis"

Cytotoxic T lymphocytes (CTLs) are important effector cells in tumor rejection [340]. Accumulating evidence indicated that tumor cells play a crucial role in the control of immune protection [344] [342] and tumor cell growth in vivo is not only influenced by CTL- tumor cell recognition, but also by tumor susceptibility to cell-mediated death [343]. Several reports indicated that tumor cells evade adaptive immunity by a variety of mechanisms including development of hypoxia (low oxygen tension) [1].

Since hypoxia is a common feature of solid tumors and one of the hallmarks of tumor microenvironment, we asked whether hypoxia confers tumor resistance to CTL-mediated killing. We demonstrate that exposure of target cells to hypoxia has an inhibitory effect on the CTL clone (H171) induced autologous target cell lysis. Such inhibition correlates with HIF-1α induction. While hypoxia had no effect on p53 accumulation, it induced the phosphorylation of STAT3 in tumor cells by a mechanism at-least in part involving VEGF secretion. While numerous findings provided compelling evidence that a causal relationship exists between signal transducer and activator of transcription (STAT3) activation and HIF-1α dependent angiogenesis [345], their relationship in regulating tumor cell susceptibility to CTL mediated specific lysis under hypoxic conditions is not yet known. STAT3 contributes to malignant transformation and progression by regulating genes involved in proliferation, survival, self-renewal, invasion, angiogenesis and immune evasion [295]. Interestingly, gene silencing of STAT3 by siRNA resulted in HIF-1α inhibition and a significant restoration of target cell susceptibility to CTL-induced killing under hypoxic conditions by a mechanism involving at least in part down-regulation of AKT phosphorylation. Moreover knock down of HIF-1α resulted in the restoration of target cell lysis under hypoxic conditions. This was further supported by DNA microarray analysis where STAT3 inhibition resulted in a partly reversal of the hypoxia-induced gene expression profile. The present study demonstrates that the concomitant hypoxic induction of pSTAT3 and HIF-1α are functionally linked to the alteration of NSCLC target susceptibility to CTL-mediated killing. Considering the eminent functions of STAT3 and HIF-1α in the tumor microenvironment, their targeting may represent novel targets for immunotherapeutic intervention.

The Cooperative Induction of Hypoxia-Inducible Factor-1α and STAT3 during Hypoxia Induced an Impairment of Tumor Susceptibility to CTL-Mediated Cell Lysis[1,2]

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Hypoxia is an essential component of tumor microenvironment. In this study, we investigated the influence of hypoxia (1% PO_2) on CTL-mediated tumor cell lysis. We demonstrate that exposure of target tumor cells to hypoxia has an inhibitory effect on the CTL clone (Heu171)-induced autologous target cell lysis. Such inhibition correlates with hypoxia-inducible factor-1α (HIF-1α) induction but is not associated with an alteration of CTL reactivity as revealed by granzyme B polarization or morphological change. Western blot analysis indicates that although hypoxia had no effect on p53 accumulation, it induced the phosphorylation of STAT3 in tumor cells by a mechanism at least in part involving vascular endothelial growth factor secretion. We additionally show that a simultaneous nuclear translocation of HIF-1α and phospho-STAT3 was observed. Interestingly, gene silencing of STAT3 by small interfering RNA resulted in HIF-1α inhibition and a significant restoration of target cell susceptibility to CTL-induced killing under hypoxic conditions by a mechanism involving at least in part down-regulation of AKT phosphorylation. Moreover, knockdown of HIF-1α resulted in the restoration of target cell lysis under hypoxic conditions. This was further supported by DNA microarray analysis where STAT3 inhibition resulted in a partly reversal of the hypoxia-induced gene expression profile. The present study demonstrates that the concomitant hypoxic induction of phopho-STAT3 and HIF-1α are functionally linked to the alteration of non-small cell lung carcinoma target susceptibility to CTL-mediated killing. Considering the eminent functions of STAT3 and HIF-1α in the tumor microenvironment, their targeting may represent novel strategies for immunotherapeutic intervention. *The Journal of Immunology,* **2009, 182: 3510–3521.**

Cytotoxic T lymphocytes are important effector cells in tumor rejection and have been described to play a crucial role in host defense against malignancies in both mouse and human (1). The major effector function of CTL is mediated through directional exocytosis of cytotoxic granules, primarily containing perforin and granzymes, into the target leading to cell death (2, 3). It is established that their antitumor response is regulated at several effector-target interaction levels involving both intracellular and extracellular stimuli (3). Accumulating evidence indicated that tumor cells play a crucial role in the control of immune protection (4) and contain many overlapping mechanisms to maintain their functional disorder and evasion. In this regard, it has been suggested that tumor cell growth in vivo is not only influenced by CTL-tumor cell interaction but also by tumor susceptibility to cell-mediated death (5). Even though the resistance of tumor cells to cell-mediated cytotoxicity remains a drawback in the immunotherapy of cancer, its molecular basis is poorly understood. A great deal of effort has been focused on trying to understand the tumor escape to immune surveillance and to understand the molecular basis of tumor tolerance.

Several reports indicated that tumor cells evade adaptive immunity by a variety of mechanisms, including development of hypoxia (low oxygen tension) (6), and that the adaptation of these cells to an hypoxic environment results in an aggressive and metastatic cancer phenotype associated with a poor treatment outcome (7). Hypoxic microenvironments are frequent characteristics of solid tumors and are the consequences of morphologically and functionally inappropriate neovascularization, irregular blood flow, anemia, and high oxygen consumption of rapidly proliferating malignant cells. Solid tumors characteristically contain areas of hypoxia, which is a powerful stimulus for the expression of genes involved in proliferation, glycolysis, and angiogenesis (8). The molecular signaling pathways mediating gene induction by hypoxia have been elucidated and extensively reviewed (9). Hypoxia-inducible

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[2] Microarray Data deposition: The detailed microarray data related to this paper have been submitted to the Array Express data repository at the European Bioinformatics Institute (www.ebi.ac.uk/arrayexpress; accession no. E-TABM-611).

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factor-1 (HIF-1)[4] is an $\alpha_1\beta_1$ heterodimer specifically recognizing hypoxia-response elements of oxygen-regulated genes. Several studies have shown that under hypoxic conditions, HIF-1α becomes constitutively up-regulated in many cancer types and plays a major role in tumor progression, mostly as a result of inhibition of protein degradation. In response to physiological hypoxia, HIF-1α becomes rapidly stabilized and is localized to the nucleus, where it specifically binds to a short DNA sequence (HREs, hypoxia response elements), thereby controlling the transcription of many genes that are critical for continued cellular function under hypoxic conditions (10, 11). Whereas the potential involvement of this factor in mediating resistance to radiation and drug therapy (12, 13) has been reported, the mechanisms underlying this resistance still need deeper understanding. Although the resistance of hypoxic cells to killing and the aggressiveness of highly hypoxic tumors are, in part, due to the overexpression of HIF-1α (14, 15), the consequences of HIF-1α induction on specific lysis of human tumor cells by CTL remain unknown. Therefore, since hypoxia is a common feature of solid tumors and one of the hallmarks of tumor microenvironment, we asked whether hypoxia confers tumor resistance to CTL-mediated killing.

While numerous findings provided compelling evidence that a causal relationship exists between STAT3 activation and HIF-1α-dependent angiogenesis (16), their relationship in regulating tumor cell susceptibility to CTL-mediated specific lysis under hypoxic conditions is not yet known. There is strong evidence that STAT3 contributes to malignant transformation and progression by transactivation of host target genes involved in fundamental events of tumor development, including proliferation, survival, self-renewal, invasion, and angiogenesis (17–19). STAT3 is critical for these processes, because its inhibition by a variety of means can exert an anticancer progression (20). In this context, STAT3 signaling has been shown to inhibit apoptosis and to induce a more aggressive phenotype through the activation of specific signaling pathways (21).

The primary focus of the current study was to examine the impact of hypoxia on specific lysis of tumor cells by CTL. Our results show that the cooperative induction of STAT3 and HIF-1α is functionally linked to the acquisition of tumor cell resistance to CTL-mediated killing under hypoxic conditions by autologous CTL. Targeting STAT3 resulted in a significant attenuation of this resistance, thus providing a lead for therapeutic modulation of hypoxia-mediated immunoresistance.

Materials and Methods

Reagents and Abs

Protease inhibitors were purchased from Roche Applied Science. Bicinchoninic acid protein assay reagent was obtained from Pierce. CHAPS was obtained from Sigma-Aldrich. For the detection of HIF-1α protein, two different Abs were used. For confocal microscopy, anti-HIF-1α (antiserum 2087) as previously described (22) was used. For Western blots, HIF-1α Ab (BD Transduction Laboratories) was purchased. Abs against STAT3 and phospho-STAT3 (p-STAT3) were purchased from Cell Signaling Technology. Actin (clone c-11) and Abs against p53, AKT and p-AKT were purchased from Santa Cruz Biotechnology. Cucurbitacin I (CCB-I) (JSI-124; National Cancer Institute NCI identifier: NSC 521777) was purchased from Indofine Chemical and kept as a stock solution of 50 mM in DMSO. Avastin (Bevacizumab) was purchased from Genentech.

[4] Abbreviations used in this paper: HIF-1, hypoxia-inducible factor-1; CCB-I, cucurbitacin-I; MHC-I, MHC class I; NSCLC, non-small cell lung cancer; p-STAT3, phospho-STAT3; PKB (also called AKT), protein kinase B; RNAi, RNA interference; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

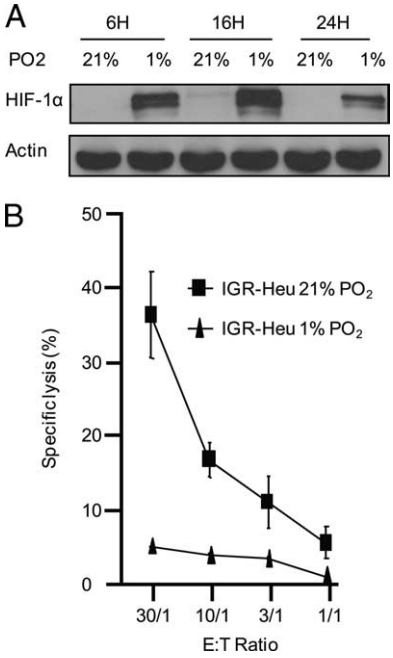

FIGURE 1. Hypoxic stress on IGR-Heu tumor cells induces HIF-1α and decreases susceptibility to CTL (Heu171)-mediated lysis. *A*, IGR-Heu tumor cells were incubated in normoxia (21% PO_2) and hypoxia (1% PO_2) for different time intervals. After hypoxic exposure, whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as the loading control. *B*, The consequence of hypoxic stress on IGR-Heu tumor cells to CTL-mediated lysis was studied. IGR-Heu tumor cells were incubated in normoxia (21% PO_2) and hypoxia (1% PO_2) for 16 h. Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at different ratios. Heu171 (TIL-derived T cell clone) was used as effectors. Bars, SD.

Culture of tumor cells and CTL

The IGR-Heu lung carcinoma cell line was derived and maintained in culture as described previously (23). Heu171 cell clone was derived from autologous TIL (24, 25). IGR-Heu lung carcinoma cell line was grown in DMEM/F12 medium supplemented with 10% FCS (Seromed), 1% Ultroser G (Life Technologies), 1% penicillin-streptomycin, and 1 mM sodium pyruvate.

Hypoxia treatment and HIF-1α induction

For the induction of HIF-1α, cell cultures were incubated in a hypoxia chamber ($InVivo_2$ 400 Hypoxia Workstation; Ruskinn) in a humidified atmosphere containing 5% CO_2 and 1% O_2 at 37°C (26). Once hypoxic conditions were optimized, cells were always exposed to the same optimized hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) at 37°C (16 h) for the optimal induction of HIF-1α. Cell culture medium and cells for protein analysis were harvested while in the hypoxia workstation and were not reoxygenated before harvesting.

Cytotoxicity assay

The cytotoxic activity of the CTL clone (Heu171) was measured by a conventional 4-h ^{51}Cr release assay by using triplicate cultures in round-bottom 96-well plates. Different E:T ratios were used. Briefly, E:T ratios 30:1, 10:1, 3:1, and 1:1 were used on 1000 target cells per well, and after 4 h of coculture at 37°C, the supernatants were transferred to LumaPlate 96 wells (PerkinElmer), dried down, and counted on a Packard's TopCount NXT. Percent-specific cytotoxicity was calculated conventionally as described earlier (27). All cytotoxicity experiments with ^{51}Cr were performed under normoxic conditions.

TNF production assay

TNF-β release was measured as described previously (28); briefly, TNF-β was detected by measuring the cytotoxicity of the culture medium on the TNF-sensitive WEHI-164c13 cells, with an MTT colorimetric assay.

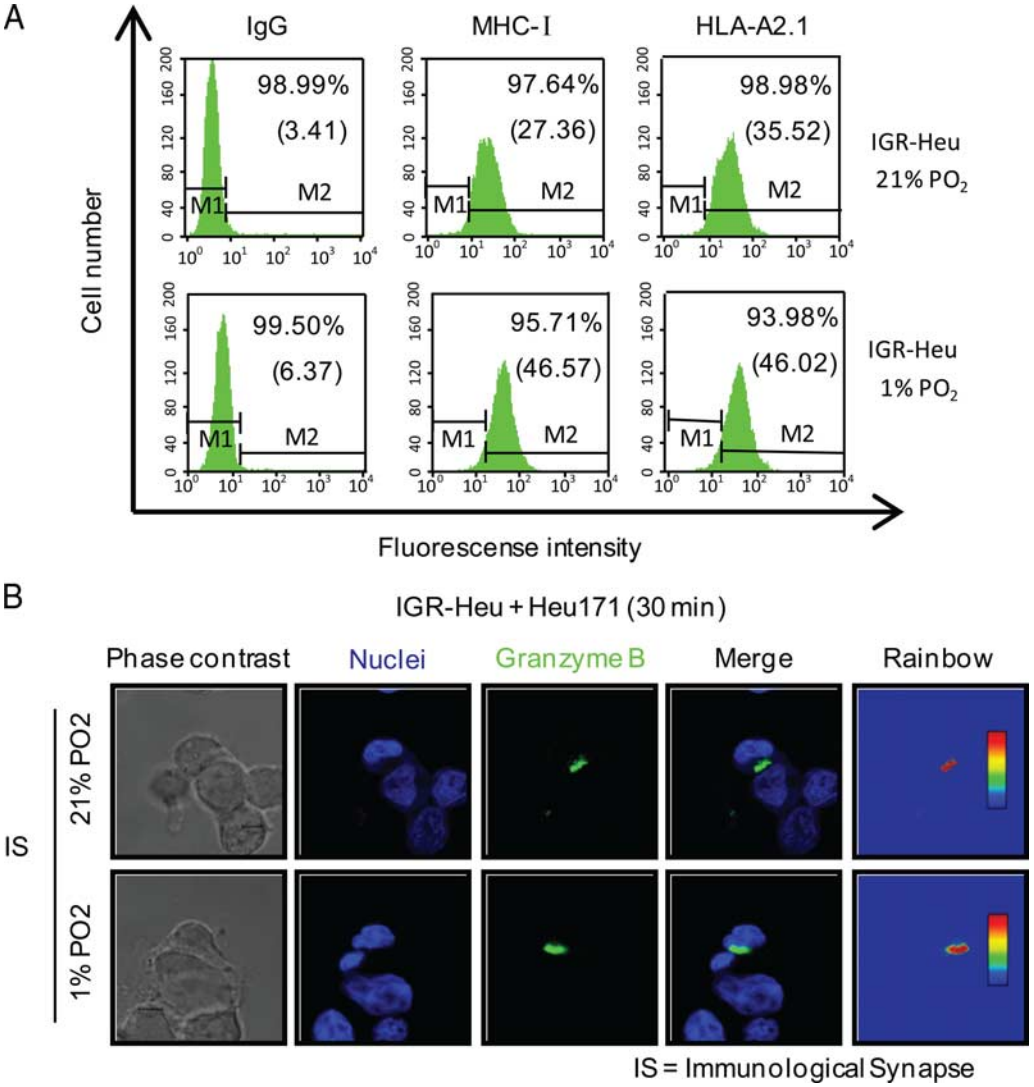

FIGURE 2. Hypoxic stress on IGR-Heu tumor cells does not affect CTL priming and cell morphology. *A*, Analysis of surface expression of HLA class I (MHC-I) and HLA-A2.1 was conducted on IGR-Heu tumor cells kept in normoxia (21% PO_2) and hypoxia (1% PO_2) for 16 h. Isotypic control mAb was included (IgG). *B*, Confocal microscopy analysis of granzyme B polarization in the contact area between tumor cells and CTL clone. IGR-Heu tumor cells were placed in normoxia (21% PO_2) and hypoxia (1% PO_2), followed by immunofluorescence staining with Abs recognizing granzyme B. Nuclei were counterstained with To-Pro-3 iodide. The confocal scanning fluorescence micrographs shown are representative of most of the cells analyzed (blue, nucleus; green, granzyme B). *C*, Hypoxic stress had no effect on tumor cell morphology. IGR-Heu tumor cells were placed in normoxia (21% PO_2) and hypoxia (1% PO_2) for 16 h. Immunofluorescence staining for phalloidin was done with appropriate Ab. Nuclei were counterstained with To-Pro-3 iodide. The confocal scanning fluorescence micrographs shown are representative of most of the cells analyzed (green, phalloidin). *D*, TNF-β production by the autologous T cell clone in response to IGR-Heu stimulation. CTL clone Heu171 was cocultured in the presence of IGR-Heu cells placed in normoxia (21% PO_2) and hypoxia (1% PO_2) for 24 h. The amount of TNF-β produced by the CTL clone was measured using the TNF-sensitive WEHI-164c13 cells. Bars, SD. *E*, Viability of IGR-Heu tumor cells under hypoxic and normoxic conditions. IGR-Heu tumor cells were placed in normoxia (21% PO_2) and hypoxia (1% PO_2) for 16 h. Apoptosis was assessed by Annexin V^{FITC}/propidium iodide staining.

RNA interference (RNAi)

Gene silencing of STAT3 and HIF-1α expression by the IGR-Heu cell line was performed by using chemically synthesized, double-stranded small interfering RNA (siRNA). STAT3 siRNA was obtained from Santa Cruz Biotechnology (sc-29493), which is a pool of three target-specific 20–25 nt siRNA designed to knock down gene expression (29). HIF-1α siRNA was purchased from Invitrogen Life Technologies (Validated stealth RNAi DuoPak ref 1299003). Luciferase siRNA (siRNA duplex, C G U A C G C G G A A U A C UU C G A dTdT, and U C G A A G U A U U C C G C G U A C G dTdT), included as a negative control, was purchased from Sigma-Proligo. Briefly, cells were transfected by electroporation with 50 nM siRNA in a gene Pulser Xcell electroporation system (Bio-Rad) at 300 V and 500 μF, using electroporation cuvettes (Eurogentec) and then allowed to grow for 72 h (30).

Confocal microscopy

For confocal microscopy analysis of localization of HIF-1α and p-STAT3, IGR-Heu tumor cells were plated on poly-L-lysine-coated coverslips (Sigma-Aldrich) in 6-well plates at a density of 5×10^4 cells/well and incubated for 16 h at 37°C in two different conditions: normoxia (21% PO_2) and hypoxia (1% PO_2). Afterward, cells were washed once with PBS, fixed with 4% paraformaldehyde for 1 h, and permeabilized with ice-cold 100% methanol at −20°C for 10 min. Cells were then blocked with 10% FBS for 20 min. Immediately after blocking, fixed cells were stained first with either anti-p-STAT3 or anti-HIF-1α primary Ab and afterward with a secondary Ab coupled to biotin (GAR IgG H+L biotin-xx; Molecular Probes) for 1 h. At last, cells were incubated with streptavidin Alexa Flour 488 (Molecular Probes) for 1 h. All Abs were diluted in PBS containing 1 mg/ml BSA. Nuclei were stained with TO-PRO-3 iodide (Invitrogen).

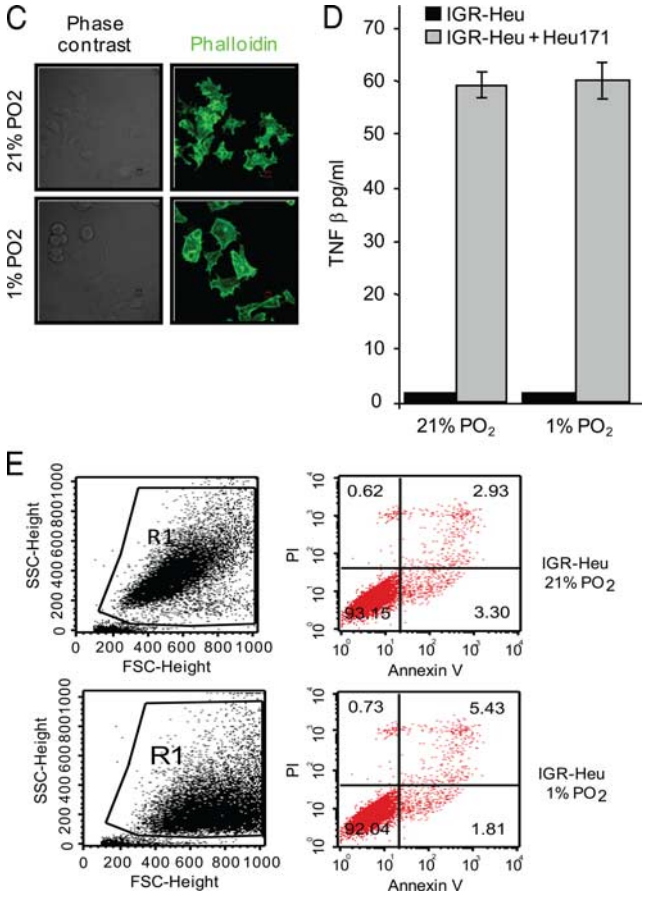

FIGURE 2. (*continued*)

Coverslips were mounted with Vectashield (Vector Laboratories) and analyzed using a fluorescence microscope (LSM-510; Carl Zeiss Micro Imaging) as described previously (31, 32).

IL-6, IL-10, and vascular endothelial growth factor (VEGF) ELISA

IL-6, IL-10, and VEGF protein levels were assessed using ELISA. IGR-Heu tumor cells were grown in 6-well plates (DMEM supplemented with 10% FBS) until they had reached 50–60% confluency. The media were then replaced with DMEM supplemented with 0.5% FBS. These cells were then kept under normoxia (21% PO_2) and hypoxia (1% PO_2) for an additional 24 h, whereupon their media were collected, filter sterilized, aliquoted, and stored at −80°C. Secretion of IL-6, IL-10, and VEGF was quantified using ELISA kits obtained from R&D Systems (catalog nos. D6050, D1000B, and DVE00, respectively). Each assay was performed per the manufacturer's instructions. Samples were assayed in triplicates. Error bars represent ± SD.

Flow cytometry analysis

Flow cytometry analysis was performed by using a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences). Anti-HLA-A2.1 (MA2.1), anti-HLA class I, or MHC class I (MHC-I) (W6/32) were used as reported previously (28). For apoptosis evaluation, the Annexin V^{FITC}/propidium iodide assay kit (BD Biosciences) was used according to the standard protocol.

Western blotting

Western blotting was performed as reported previously (32, 33). Briefly cells were grown in two different conditions normoxia (21% PO_2) and hypoxia (1% PO_2) for 16 h at 37°C. Cells were scrapped off, lysed in an appropriate buffer containing 20 mM Tris-HCl (pH 7.5), 1% CHAPS, 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, and a commercial protease inhibitor mixture (Complete Protease Inhibitor Mixture; Roche Molecular Biochemicals). Cells were then placed on ice for 30 min, centrifuged at 10,000 × *g* for 30 min at 4°C, and their supernatants were collected. Thirty micrograms of total protein extracts were resolved on 8 or 12% SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membrane by wet method, and unspecific binding sites were blocked with 3% milk/Tween 20 Tris-buffered saline (TTBS; 50 mM Tris HCl, 140 mM NaCl, and 0.05% Tween 20 (pH 7.2)) for 1 h. The primary Abs were added at a dilution of 1/1000 and incubated overnight at 4°C. Afterward, nitrocellulose membranes were washed three times for 5 min each with TTBS. For protein detection, blots were incubated with a HRP-labeled anti-mouse or anti-rabbit secondary Abs for 1 h at a dilution of 1/2000 and washed three times for 5 min each with TTBS, followed by ECL detection analysis. Correction for background was performed with NIH Image software.

Microarray analysis

DNA Microarray was performed using (Agilent Human Whole Genome Microarray: 44,000 spots) as previously reported (30) under two different conditions, hypoxia vs normoxia (Hypo Normo) and siRNA STAT3 vs siRNA-Luc (STAT3 CT) by using (Agilent Human Whole Genome Microarray: 44,000 spots). In both conditions, total RNA was extracted and compared by using Agilent oligonucleotide dual-color technology, running dye swap and duplicate experiments. Probe synthesis and labeling were performed by Agilent's Low Fluorescent Low Input Linear Amplification Kit. Hybridization was performed on human whole-genome 44,000 oligonucleotide microarrays (Agilent) by using reagents and protocols provided by the manufacturer. Feature extraction software provided by Agilent (version 7.5) was used to quantify the intensity of fluorescent images and to normalize results using the linear and lowess subtraction method. Primary analysis was performed by using Resolver software (Rosetta Laboratories) to identify genes differentially expressed between resistant and sensitive cell lines (IGR-Heu/IGR HeuR8) with a fold change > 2 and a value of $p < 10^{-5}$. For additional analysis, we used the GenMapp/Mapfinder software package (www.genmapp.org; Gladstone Institute, University of California San Francisco).

Results

Hypoxic stress-induced HIF1α is associated with a decrease in CTL-mediated tumor cell lysis

Hypoxia is a specific property of solid tumors and has been widely documented to contribute to low apoptotic potential. To investigate the influence of hypoxic stress on human tumor cell susceptibility to CTL-mediated killing, we used the human non-small cell lung carcinoma (NSCLC) cell line IGR-Heu and the autologous CTL clone (Heu171), recognizing a mutated α-actinin-4 peptide in an HLA-A2.1 context. As previously described, IGR-Heu cells were lysed by the CTL clone through the perforin/granzymes pathway (23, 34, 35). Because cancer cells can adapt to hypoxia primarily through a transcriptional response pathway mediated by the HIF-1-α, its induction in IGR-Heu cells under hypoxic conditions (1% PO_2) was first determined. As depicted in Fig. 1*A*, Western blot analysis shows a HIF-1α induction upon culture of IGR-Heu under hypoxic conditions. Kinetic analysis indicated that optimal induction was obtained after 16 h of cell culture under hypoxic conditions. Such an induction was associated with a dramatic decrease (80% inhibition at E:T ratio of 30:1) in CTL-mediated IGR-Heu killing (Fig. 1*B*). These results indicate that hypoxic tumor cells are less susceptible to lysis induced by the autologous CTL clone.

Hypoxic stress does not affect CTL reactivity and tumor cell morphology

To determine whether hypoxic stress-induced alteration of target susceptibility to CTL-mediated lysis involves an alteration in CTL reactivity or a change in tumor cell morphology, we have examined the influence of hypoxia on MHC-I molecule expression using cytometry analysis and specific Abs. As shown in Fig. 2*A*, no difference in staining with W6/32 (HLA class I specific) and MA2.1 (specific for HLA-A2) Abs was observed on hypoxic cells (1% PO_2) as compared with cells

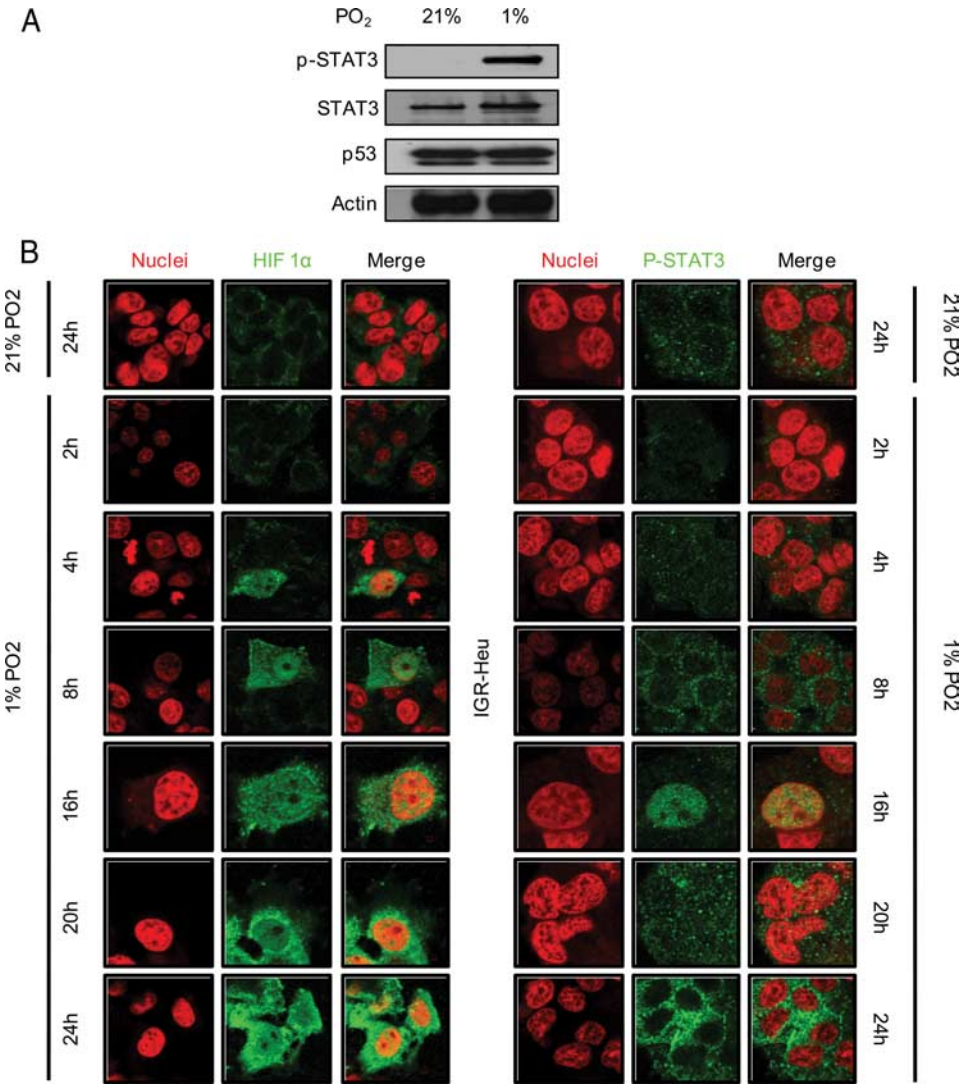

FIGURE 3. Hypoxic stress on IGR-Heu tumor cells induced phosphorylation of STAT3 and simultaneous nuclear translocation of HIF-1α and p-STAT3. *A*, IGR-Heu tumor cells were kept in normoxia (21% PO_2) and hypoxia (1% PO_2). After 16 h of incubation, whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as the loading control. *B*, Confocal microscopy analysis for nuclear translocation of HIF-1α and p-STAT3. IGR-Heu tumor cells were placed in normoxia (21% PO_2) and hypoxia (1% PO_2), followed by immunofluorescence staining with Abs recognizing HIF-1α and p-STAT3. A time course study was done to follow the cytoplasmic localization and, eventually, the hypoxia-mediated nuclear translocation of HIF-1α and p-STAT3. It was observed that both HIF-1α and p-STAT3 translocated in the nucleus after 16 h of hypoxia. Nuclei were counterstained with To-Pro-3 iodide. The confocal scanning fluorescence micrographs shown are representative of most of the cells analyzed (blue, nucleus; green, HIF-1α and p-STAT3).

cultured under normoxic conditions (21% PO_2). To examine the recognition of hypoxic cells by the CTL clone, we examined the reactivity of the CTL clone to hypoxic autologous target. Confocal microscopy analysis shown in Fig. 2*B* indicates the polarization of cytotoxic granules as defined by granzyme B accumulation in the contact area between the CTL clone and hypoxic or normoxic IGR-Heu tumor cells. This indicates that hypoxic tumor cells are efficient in triggering the reactivity of the specific CTL clone that was further confirmed by TNF secretion assay (Fig. 2*D*).

Because we have recently provided evidence that a change in tumor cell morphology could lead to a decrease in their susceptibility to CTL-induced tumor cell death (30), we asked whether the inhibitory effect of hypoxia involves a tumor cell morphological change that would be associated with the observed alteration of their killing by Heu171 CTL clone. Results of confocal microscopy analysis shown in Fig. 2*C* indicate that the acquisition of resistance to CTL following treatment of target cells with hypoxic stress did not result in tumor cell morphological changes since F-actin content, as revealed by Alexa Fluor 568-phalloidin staining, was similar in hypoxic and normoxic cells. This rules out that the acquisition of hypoxic tumor resistance to CTL-mediated killing might result from a shift in the level of actin polymerization. Furthermore, as depicted in Fig. 2*E*, the hypoxic stress had no effect on IGR-Heu viability as revealed by annexin V/propidium iodide staining.

STAT3 phosphorylation in tumor cells under hypoxic conditions correlates with the acquisition of resistance to CTL-induced lysis

The interaction of STAT3 with HIF-1α was identified in hypoxic tumor cells (16). Therefore, we wished to delineate the respective involvement of these factors in the regulation of target cell susceptibility to specific lysis. For this purpose, total extracts of IGR-Heu cultured under hypoxic or normoxic conditions were subjected to Western blot analysis. While under hypoxic conditions, a dramatic increase in STAT3 phosphorylation was observed; there was no effect on the tumor suppressor protein p53 (Fig. 3*A*) and survivin (data not shown). Next, we examined the temporal kinetics of HIF-1α and STAT3 nuclear translocation in IGR-Heu cultured under hypoxic conditions from 2 to 20 h using confocal microscopy. Data shown in Fig. 3*B* indicate that at least 16 h of hypoxic treatment are required for nuclear translocation of both HIF-1α and STAT3. In fact, before 16 h both HIF-1α and STAT3 are predominantly present in the cytosol. At 16 h, the hypoxic cells exhibited an optimal HIF-1α and STAT3 accumulation in the nucleus.

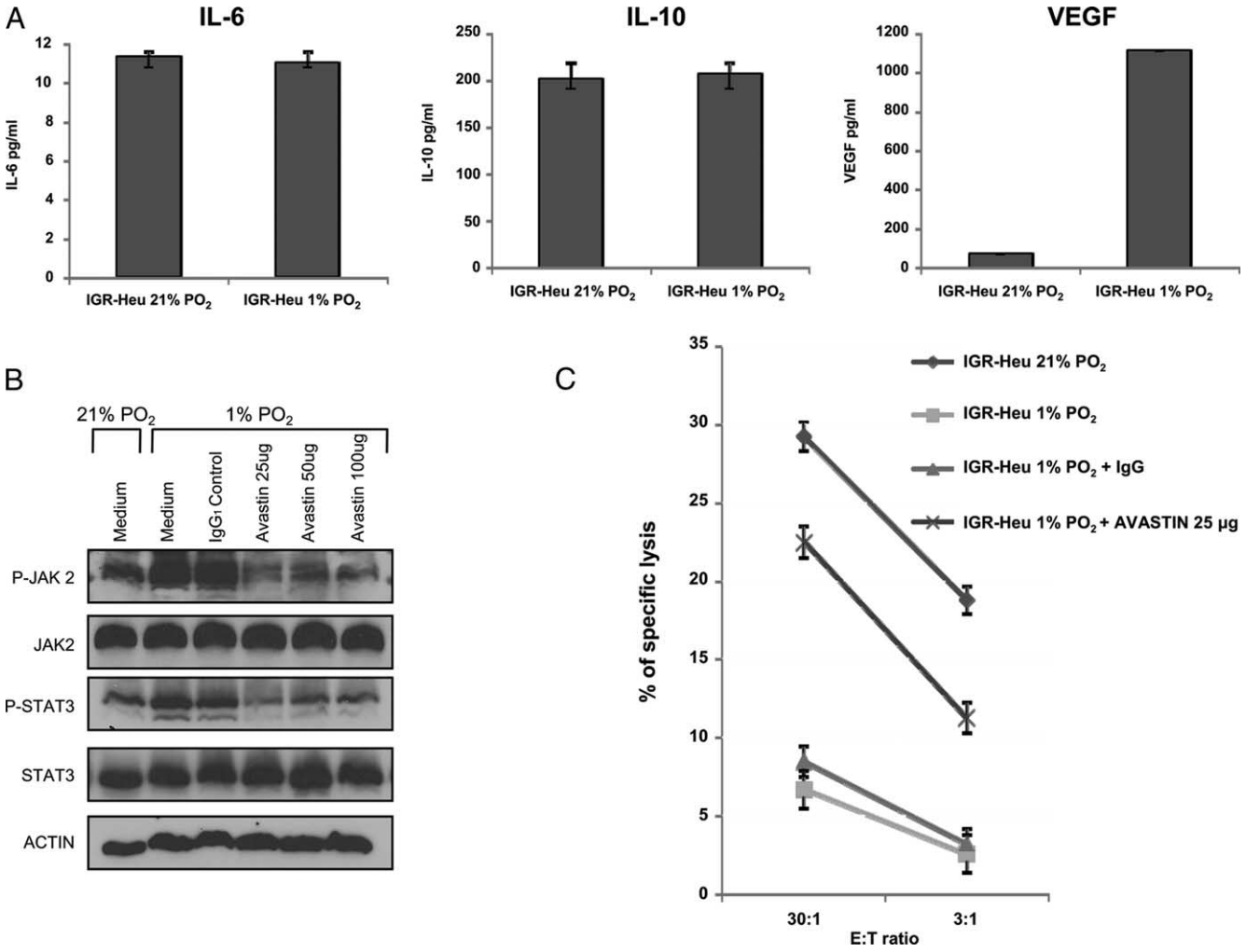

FIGURE 4. Involvement of VEGF in STAT3 activation under hypoxia and relationship with JAK/STAT3 pathway. *A*, IGR-Heu tumor cells were kept under normoxia (21% PO_2) and hypoxia (1% PO_2) for 24 h. ELISA was performed for quantification of IL-6, IL-10, and VEGF by Quantikine Human immunoassay R&D Systems. Bars, SD. The results shown are representative of two independent experiments. *B*, IGR-Heu tumor cells were incubated for 24 h with 25, 50, and 100 μg/ml Avastin (Genentech) or an isotype control Ab (human IgG1; Sigma-Aldrich) in reduced serum conditions (0.5% FBS) under (21% PO_2) and hypoxia (1% PO_2). Whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as the loading control. *C*, Attenuation of IGR-Heu tumor cells resistance to CTL (Heu171)-mediated lysis under hypoxic conditions following treatment with anti-VEGF (Avastin). IGR-Heu tumor cells were incubated for 24 h with 25 μg/ml Avastin or an isotype control Ab (human IgG1; Sigma-Aldric h) under normoxia (21% PO_2) and hypoxia (1% PO_2). Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay. Heu171 (TIL-derived T cell clone) was used as effectors. Bars, SD.

Hypoxia-induced phosphorylation of STAT3 is associated with induction of VEGF

The role of hypoxia in controlling the activation of STAT3 in tumor cells is not fully understood. Soluble factors like IL-6, IL-10, and VEGF are known to induce activation of STAT3 through JAK/STAT3 pathway (36), and furthermore, autocrine and paracrine IL-6 has been shown to activate STAT3 via IL-6/JAK pathway (37). Therefore, we investigated the role of these mediators in activating JAK/STAT3 pathway under hypoxic conditions. As depicted in Fig. 4*A*, 24-h hypoxic conditioning (1% PO_2) of IGR-Heu tumor cells does not alter their production of IL-6 and IL-10. However, a 16-fold increase in the production of VEGF under hypoxia was observed.

To determine the role of VEGF in modulating the hypoxic activation of STAT3, we incubated IGR-Heu tumor cells with different concentrations of anti-VEGF (Avastin) under normoxia (21% PO_2) and hypoxia (1% PO_2) for 24 h. Using Western blot analysis, we found that the neutralization of VEGF by Avastin under hypoxic stress resulted in a dramatic inhibition of p-STAT3. This VEGF-mediated p-STAT3 inhibition was also associated with an inhibition of p-JAK2 (Fig. 4*B*). These observations indicate that VEGF is involved in the control of hypoxic induction of p-STAT3 through an autocrine mechanism involving JAK/STAT3 pathway.

To find out the potential involvement of hypoxia-induced VEGF in modulating the tumor cell susceptibility to CTL-mediated lysis via p-STAT3, we incubated IGR-Heu tumor cells for 24 h with 25 μg/ml Avastin or an isotype control Ab human IgG1 under normoxia (21% PO_2) and hypoxia (1% PO_2). Data depicted in Fig. 4*C* indicate a remarkable increase in CTL-mediated killing of anti-VEGF (Avastin)-treated cells as compared with control cells. This finding further strengthens the notion that the activation of STAT3 via VEGF-JAK/STAT3 autocrine loop plays an essential part in the acquisition of tumor cell resistance to CTL-induced killing under hypoxic conditions.

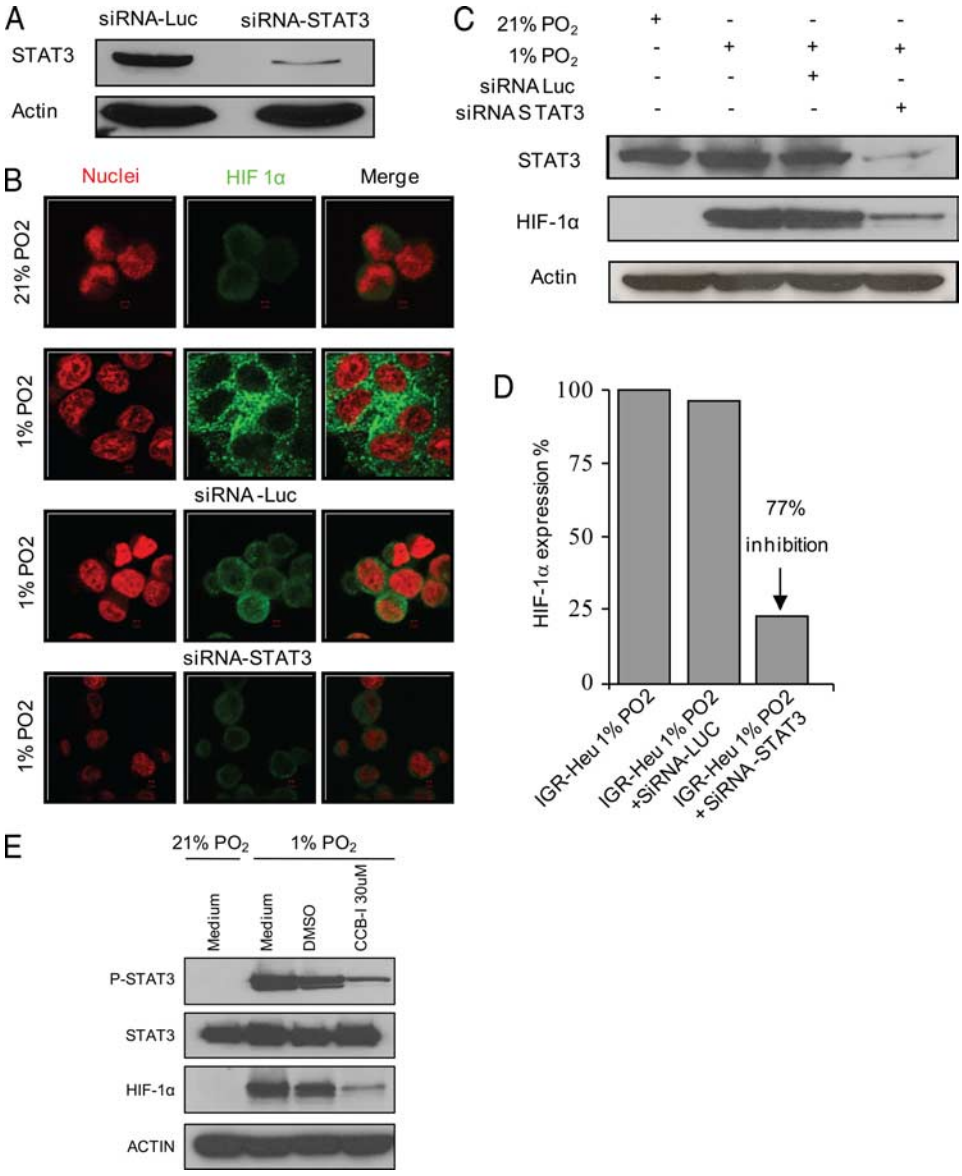

FIGURE 5. Gene silencing of STAT3 and pharmacological inhibition of p-STAT3 resulted in HIF-1α inhibition. *A*, Knockdown of STAT3 expression. IGR-Heu tumor cells were transfected with siRNA STAT3 or control siRNA-luciferase. Seventy-two hours after transfection, whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as the loading control. *B*, Confocal microscopy analysis of localization of HIF-1α. IGR-Heu tumor cells were placed in normoxia (21% PO_2) and hypoxia (1% PO_2), followed by immunofluorescence staining with Abs recognizing HIF-1α. Nuclei were counterstained with To-Pro-3 iodide. The confocal scanning fluorescence micrographs shown are representative of most of the cells analyzed (blue, nucleus; green, HIF-1α). *C*, Western blots analysis of HIF-1α following the knockdown of STAT3 by siRNA. Actin was used as a loading control. *D*, Densitometry analysis of HIF-1α inhibition using Image J. *E*, CCB-I-mediated inhibition of p-STAT3 under hypoxic stress is associated with inhibition of HIF-1α. IGR-Heu tumor cells were incubated under normoxia (21% PO_2) and hypoxia (1% PO_2) (medium, a volume equivalent of DMSO and along with 30 μM CCB-I) for 6 h. Afterward, whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as a loading control.

Silencing of STAT3 in hypoxic tumor cells resulted in HIF-1α inhibition

To further assess the putative hypoxia-induced functional interaction between HIF-1α and STAT3 in the regulation of IGR-Heu susceptibility to CTL-mediated killing, transfection of hypoxic IGR-Heu with siRNA-targeting STAT3 was performed. The inhibition of STAT3 in hypoxic tumor cells was confirmed by Western blot analysis, showing that an efficient and specific knockdown of the levels of STAT3 protein was obtained. In contrast, luciferase siRNA (siRNA-Luc), used as a control, had no effect on STAT3 protein levels (Fig. 5*A*).

Because STAT3 has been reported to modulate the stability and the activity of HIF-1α in tumor cells (16), we assessed whether such an interplay exists in NSCLC. Results of confocal microscopy depicted in Fig. 5*B* indicate that the induction of HIF-1α in NSCLC hypoxic cells was dramatically inhibited in STAT3 siRNA-treated cells as compared with the control cells transfected with siRNA-Luc. These results were further confirmed by Western blot analysis (Fig. 5*C*), indicating a significant inhibition (77%) of HIF-1α after gene silencing of STAT3 as revealed by densitometry (Fig. 5*D*).

Pharmacological inhibition of STAT3 phosphorylation in hypoxic tumor cells resulted in the inhibition of HIF-1α induction under hypoxic stress

Although STAT3 has been reported to modulate the stability and activity of HIF-1α in tumor cells (16), the relationship between hypoxic induction of HIF-1α and STAT3 activation remains to be elucidated. For this purpose, we used CCB-I, known to specifically inhibit STAT3 activation in various human cancer cell lines and, more importantly, to inhibit the tumor growth in mice models (38).

Western blot analysis indicate that the CCB-I treatment of IGR-Heu tumor cells resulted in an inhibition of p-STAT3 in a dose-dependent manner under hypoxic conditions (Fig. 5*E*). As depicted in the figure, the inhibition of P-STAT3 was accompanied by an inhibition of HIF-1α induction by the hypoxic stress

RNAi-mediated knockdown of STAT3 increased hypoxic target susceptibility to CTL-induced killing

To gain insight into the potential role of STAT3 in the regulation of hypoxic target cell susceptibility to CTL-mediated lysis, the ability of CTL to kill STAT3-siRNA-transfected IGR-Heu cells treated with hypoxia was evaluated. Interestingly, as shown in

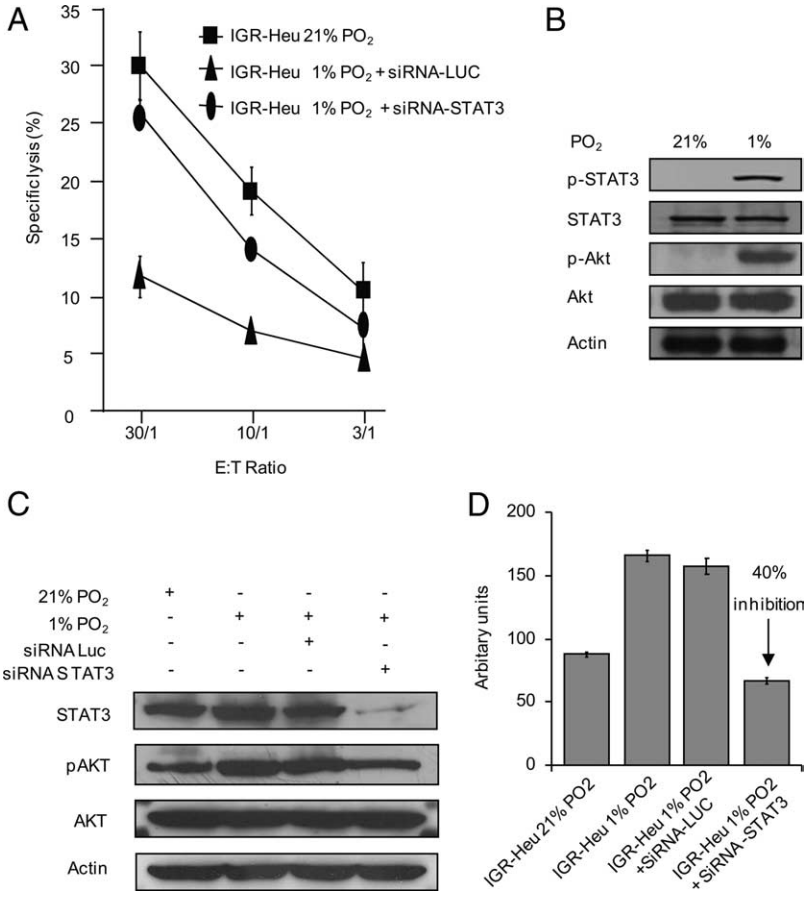

FIGURE 6. Gene silencing of STAT3 results in restoration of IGR-Heu tumor cells susceptibility to CTL (Heu171)-mediated lysis. *A*, Role of STAT3 in Heu171 TIL clone-mediated lysis toward autologous IGR-Heu tumor cells. IGR-Heu tumor cells electroporated with siRNA STAT3 or siRNA-luciferase in normoxia (21% PO_2) and hypoxia (1% PO_2) for 16 h. Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at different ratios. Heu171 (TIL-derived T cell clone) was used as effectors. Bars, SD. *B*, IGR-Heu tumor cells were kept in normoxia (21% PO_2) and hypoxia (1% PO_2). After 16 h of incubation, whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as the loading control. *C*, Western blots analysis after the knockdown of STAT3 by SiRNA. *D*, Densitometry analysis of P-AKT inhibition after gene silencing of STAT3. The images were analyzed by Image J.

Fig. 6*A*, a remarkable increase at all the E:T ratios tested in CTL-induced killing of STAT3-siRNA IGR-Heu-transfected cells as compared with control cells was observed. These data strengthen the notion that activation of STAT3 plays an essential part in the acquisition of tumor cell resistance to CTL-induced killing under hypoxic conditions. Because hypoxia is known to induce AKT phosphorylation, we wished to examine its status in hypoxic IGR-Heu cells as well as its putative involvement in STAT3-induced inhibition of tumor cell susceptibility to CTL-induced lysis. Results shown in Fig. 6*B* indicate that hypoxic treatment resulted in the simultaneous phosphorylation of STAT3 and AKT in IGR-Heu cells, suggesting a role of AKT in the resistance of hypoxic IGR-Heu to CTL. As shown in Fig. 6*C*, knocking down of STAT3 in hypoxic cells resulted in down-regulation of p-AKT phosphorylation. As shown in Fig. 6*D*, a significant decrease (40%) in the induction of p-AKT following STAT3 knockdown was observed.

Gene silencing of HIF-1α resulted in restoration of IGR-Heu tumor cells susceptibility to CTL-mediated killing under hypoxic stress.

To delineate the role of HIF-1α in the regulation of tumor cell susceptibility to CTL mediated lysis under hypoxic conditions, siRNA mediated knock down of HIF-1α under hypoxic conditions was performed. As shown in Fig. 7*A*, a strong inhibition of HIF-1α induction (>90%) under hypoxic stress was observed. Using three different siRNA sequences, when these cells were tested for their susceptibility to CTL induced killing, a remarkable restoration of cell lysis was obtained (Fig. 7*B*). This point to a role of HIF-1α in the negative regulation of hypoxic tumor targets susceptibility to lysis by the autologous CTL.

Microarray expression profiles of tumor cells in response to hypoxia: relationship with STAT3

To further investigate the role of HIF-1α and STAT3 in controlling tumor cell susceptibility to lysis by CTL, we performed a global analysis using DNA Microarray (Agilent Human Whole Genome Microarray: 44,000 spots) under two different conditions, hypoxia vs normoxia (Hypo Normo) and siRNA STAT3 vs siRNA-Luc (STAT3 CT). Of 44,000 probe sets, for Hypo Normo, 4138 probe sets were differentially expressed with a 2-fold change: 1582 probe sets were down-regulated and 2556 up-regulated. For (STAT3 CT), we found 354 probe sets that were differentially expressed with a 2-fold change: 88 probe sets were down-regulated and 266 up-regulated. These results are summarized in Fig. 8*A* and point to a potential role of both hypoxia-induced HIF-1α and hypoxia-activated STAT3 in modulating the tumor behavior.

To identify the molecular processes/potential clusters associated with the modulation of tumor cell susceptibility to CTL-mediated lysis, the differentially expressed probe sets were analyzed by Rosetta resolver software. This analysis indicates that the majority of genes differentially expressed under hypoxic conditions belong to five major groups, namely metabolism, apoptosis, cell cycle, cell adhesion, and transcriptional regulation. Similarly, under siRNA STAT3 inhibition condition, the overall profile showed a similar gene ontology distribution. These results further strengthen the role of STAT3 in modulating the hypoxic response (Fig. 8*B*). In addition, as shown in Fig. 8*A*, under (STAT3 CT), the number of differentially expressed genes was reduced and using pathway analysis softwares Rosetta and GenMapp/Mapfinder; these genes were found to be dispersed among a number of pathways and not

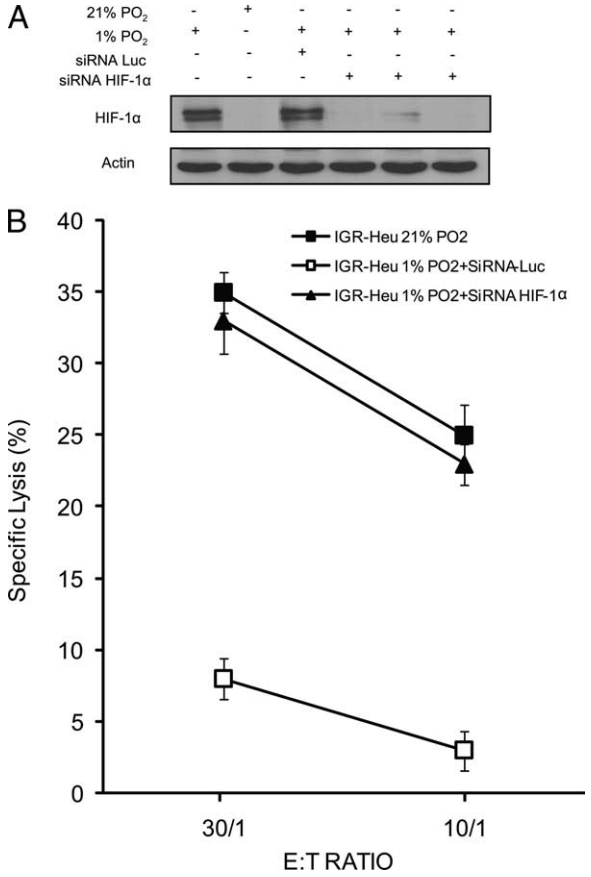

FIGURE 7. Restoration of IGR-Heu tumor cells susceptibility to CTL (Heu171)-mediated lysis following RNAi-mediated gene silencing of HIF-1α under hypoxic conditions. *A*, Gene silencing of HIF-1α under hypoxic conditions. IGR-Heu tumor cells were transfected with siRNA HIF-1α or control siRNA-luciferase. Seventy-two hours after transfection, whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with Abs, as indicated. Actin was used as the loading control. *B*, Role of HIF-1α in modulating tumor cell susceptibility to Heu171-CTL clone-mediated lysis. IGR-Heu tumor cells electroporated with three different siRNA HIF-1α or siRNA-luciferase in hypoxia (1% PO_2) for 16 h. Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at different ratios. Heu171 (CTL clone) was used as effectors. Bars SD.

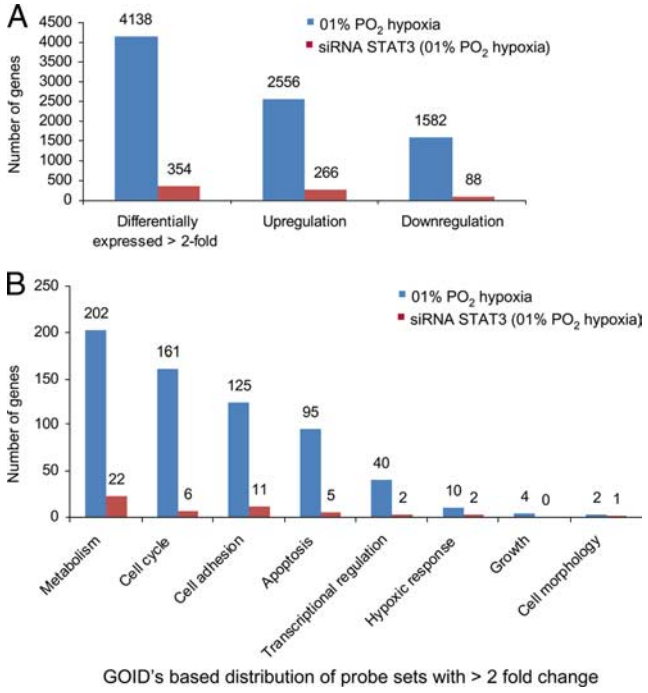

FIGURE 8. Differential gene expression under 1% PO_2 hypoxic and siRNA STAT3 conditions. *A*, IGR-Heu tumor cells were kept under normoxia (21% PO_2) and hypoxia (1% PO_2) for 24 h. Total RNA was isolated, and DNA microarray analysis was performed using an Agilent Human Whole Genome Microarray. *B*, Gene ontology identification (GOID) classification based distribution of probe sets >2-fold change under hypoxic and siRNA STAT3 conditions. Rosetta resolver software was used for analysis.

linked to a single pathway. We further proceeded by making a list of genes that had changed significantly under hypoxia (up or down) and linked it with the expression of the same genes under STAT3 inhibition. These combined results (mHypxmSTAT) indicate that there is a reversal (partial or full) of the expression when STAT3 is inhibited under hypoxia and returning to a normoxic profile. The most prominent changes are listed in Table I with some of the genes in this list related to cell death: AATK, MAF, MAPK4, and MYCT1. Further analysis of the molecular pathways resulted in the identification of an additional list of genes that are known to play a role in regulation of resistance/apoptosis: DFFB, TRIB3, ERN1, BCLAF1, and NALP1. It is interesting to mention here that in the list of differential expressed genes there were also several genes linked to NF-κB signaling.

Taken together, the microarray analysis suggests that hypoxia-induced HIF-1α and p-STAT3 may act coordinately to mediate hypoxia-induced alteration of tumor susceptibility to CTL-mediated lysis.

Discussion

The efficacy of antitumor CTL critically depends on functional processing and presentation of tumor Ags by the malignant cells but also on their susceptibility to CTL-induced lysis. The identification of tumor-associated Ags and their epitopes recognized by autologous T cells has lead to their broad use as immunogens to induce or augment tumor-associated Ag-specific immune responses in vaccination strategies (23, 39–42). However, the understanding of tumor-host interactions remains elusive despite this identification. In fact, tumor rejection in patients does not always follow successful induction of tumor-specific immune responses by cancer vaccine immunotherapy (4). Even if a strong and sustained cytotoxic response is induced, complex issues such as tumor evasion and selection of tumor-resistant variants remain. In this respect, solid tumors with disorganized, insufficient blood supply contain hypoxic cells that are resistant to classical cytotoxic treatments (43). The results of the present studies provide the first demonstration that tumor cells, through their adaptation to hypoxic stress, impede CTL cytotoxic activity independently of their potential to trigger CTL reactivity. However, our data differ from earlier report of MacDonald and Koch (44). This discrepancy may lie in the fact that the reported data were collected using mice system, CTL generated in mixed leukocyte cultures, and hypoxic chambers, whereas we respectively used human Ag-specific CTL clone and hypoxic station. These observations are consistent with the notion that hypoxia-induced adaptive changes contribute to alterations that lend toward a surviving phenotype. Thus, it is conceivable to imagine that tumors frequently develop this specific strategy to shift the balance from immune surveillance to tolerance. In this context, the various strategies aimed at the induction of antitumor cytotoxic responses should consider the crucial role of tumor hypoxia as an additional antitumor mechanism of tumor escape that is partly involved in resistance of tumor cells to Ag-specific cytotoxicity.

Table I. *List of genes that gave a reversal (partial or full) of expression under STAT3 inhibition*[a]

Primary Sequence Name	Accession No.	MeanHYP	MeanSTAT	mHypxmSTAT
AATK[b]	NM_001080395	8.085192508[c]	0.419208371[d]	3.389380384
ABLIM3	NM_014945	6.649144582	0.426638997	2.836784373
ADH1A	NM_000667	0.167966415	2.019030276	0.339129278
ADH1A	NM_000667	0.231080138	2.226025826	0.514390355
ANGPTL4	NM_139314	31.45983155	0.307092850	9.661089326
BNC2	NM_017637	2.349535188	0.479137192	1.125749692
C16orf78	NM_144602	0.501471398	2.068901367	1.037494861
CALB2	NM_001740	33.55977682	0.412261363	13.83539933
CD72	NM_001782	4.753225996	0.403382591	1.917368619
CNN1	NM_001299	2.143496155	0.405801693	0.869834369
EBI3	NM_005755	17.33144061	0.393332038	6.817010850
EFNA1	NM_004428	12.93814775	0.481075561	6.224226688
FGF21	NM_019113	3.191536311	0.328079978	1.047079163
FGG	NM_000509	4.171329662	0.275914383	1.150929851
GLT1D1	NM_144669	7.621011279	0.399096116	3.041515999
HCG4	NR_002139	3.418579404	0.387191733	1.323645684
HMGCLL1	NM_019036	2.456706141	0.408455845	1.003455983
HTR3A	NM_213621	4.450853706	0.434079395	1.932023882
IL21R	NM_181078	7.608334185	0.180035020	1.369766595
JAK3	BC028068	2.418664759	0.444393028	1.074837756
KCNMB4	NM_014505	4.107457716	0.493705933	2.027876243
LCN2	NM_005564	2.432430479	0.317610668	0.772565868
LY96	NM_015364	8.183074746	0.382420550	3.129375943
MAF[b]	AF055376	3.373622120[c]	0.488526477[d]	1.648103730
MAPK4[b]	X59727	60.05014398[c]	0.414925830[d]	24.91635583
MYCT1[b]	NM_025107	3.398289596[c]	0.483077899[d]	1.641638598
NNMT	NM_006169	28.66610466	0.342793814	9.826563336
OR51E2	NM_030774	0.073586722	2.456032870	0.180731407
PECAM1	NM_000442	10.68675702	0.437580990	4.676321721
PLA2G3	NM_015715	3.960428765	0.449291227	1.779385898
PTGS1	NM_000962	5.496247103	0.435281377	2.392414008
SOCS3	NM_003955	30.13365930	0.354334745	10.67740248
SRPX2	NM_014467	4.621833783	0.416867169	1.926690763
STAT3	NM_213662	2.128718329	0.248762483	0.529545258
TNFRSF11A	AB209762	2.400841610	0.487085993	1.169416319
TNS1	NM_022648	9.331115713	0.482862705	4.505647770
TNXB	NM_032470	11.10529609	0.429757704	4.772586553
TSKS	NM_021733	5.177481996	0.324157290	1.678318533
BCLAF1[b]	NM_014739	0.503805042[d]	1.283749270[c]	0.632957814
DFFB[b]	NM_004402	0.430407334[d]	1.201755655[c]	0.518365789
ERN1[b]	AK055561	2.047796458[c]	0.965524773[c]	1.908652475
NALP1=NLRP1[b]	NM_033004	0.557328993[d]	1.288832253[c]	0.708904005
TRIB3[b]	NM_021158	2.175000632[c]	0.783258470[d]	1.704318640

[a] Using GenMAPP2 analysis, a list of genes was created that gave a reversal under STAT3 inhibition. MeanHYP, mean hypoxia in absolute numbers; MeanSTAT, mean STAT inhibition; and meanHYPXSTAT, product meanHYP × meanSTAT should give indication on the expression between normoxic cells and hypoxic cells with STAT inhibition.
[b] Genes related to cell death.
[c] Genes up-regulated.
[d] Genes down-regulated.

To get more insights into the mechanism associated with alteration of tumor susceptibility to CTL-mediated lysis under hypoxic conditions, we asked whether hypoxia resulted in reshaping immunogenicity of tumor cells that may contribute to lose or avoid recognition. We found that hypoxic tumor treatment did not result in alteration of CTL reactivity as measured by TNF secretion and granzyme B polarization, indicating that tumor-induced priming of the autologous CTL clone was not most likely affected after exposure to hypoxia. We further investigated the influence of hypoxia on cell morphology since disruption of the cytoskeletal network by hypoxia has been reported in diverse tissues (45). We did not detect any influence of hypoxia on tumor cell morphological change.

It is well established that hypoxia-induced gene transcription promotes characteristic tumor adaptations, including resistance to cytotoxic treatments. However, the mechanisms underlying this resistance still need a deeper understanding. Although increasing evidence supports a link between hypoxia and resistance to apoptosis (46), the mechanism by which the hypoxic stress inhibits apoptosis is not well understood and remains unclear. In this regard, it has been suggested that alterations in the Bcl-2 family of proapoptotic and antiapoptotic proteins may play a role in this process (47). In addition, HIF-1α-mediated increase in glucose uptake has been also reported to play an important role in conferring apoptosis resistance and that its effect is mediated in part via Mcl-1 gene expression (48). To gain a better understanding of the influence of hypoxia on non-small lung cancer cells' resistance to CTL, we asked whether it interferes with the expression of p53, survivin, AKT, and STAT3 pathways. Under our experimental conditions, we failed to observe any p53 and survivin induction in hypoxic NSCLC, suggesting that the expression of HIF-1α and these proteins may be coupled during the hypoxia response depending on the cell type tested and confirming the existence of a cell-specific effect of hypoxia (49). In the present study, we

demonstrate that culture of IGR-Heu cells under hypoxic conditions resulted in a dramatic induction of STAT3 tyrosine phosphorylation. Moreover, confocal microscopy analysis revealed that the hypoxic cells exhibited an optimal and simultaneous accumulation of HIF-1α and STAT3 in the nucleus. To further delineate the putative interplay between STAT3 and HIF-1α in hypoxia-induced alteration of tumor susceptibility to CTL-mediated cell lysis, we performed experiments to block STAT3 under hypoxic conditions using RNAi. We found that such knockdown resulted in an inhibition of HIF-1α induction. This suggests that STAT3 is an essential component of HIF-1 pathway under hypoxic conditions and fits with a recent report indicating that hypoxia-phosphorylated STAT3 up-regulates HIF-1α stability through delaying protein degradation and accelerating protein synthesis in human renal cell carcinoma (16, 50).

To further elaborate the existing relationship between hypoxia-induced HIF-1α and activated STAT3, using CCB-I, a known inhibitor of STAT3 phosphorylation, we observed that both STAT3 phosphorylation and HIF-1α induction was inhibited in hypoxic tumor cells. These results shown in Fig. 5 strengthen the existing of an interplay between HIF-1α and p-STAT3 during the regulation of specific tumor lysis under hypoxic conditions.

It is also important to note that STAT3 activation has been associated with cytokine-induced proliferation, antiapoptosis, and transformation. We also showed that knockdown of STAT3 in hypoxic IGR-Heu cells resulted in a significant inhibition of AKT phosphorylation (40% inhibition) and significant restoration of tumor susceptibility to CTL, indicating that the attenuation of the resistance of hypoxic cells to CTL-induced cell death may be associated at least in part with inhibition of AKT activation in these cells (Fig. 6*D*). This is also in agreement with our unpublished data indicating that wortmannin, an AKT inhibitor; sensitized tumor cells to CTL-mediated lysis under hypoxic conditions. It should be noted that activated STAT3 in cancer cells does not just function as a mediator of intracellular signaling but also affects cell-cell interaction. In this respect, it is now well established that that STAT3 modulates the cross-talk between tumor and immune cells (36, 51). Very recently, a novel small molecule inhibitor of STAT3 has been reported to reverse immune tolerance in malignant glioma patients (52). It would be of interest to investigate the use of such an inhibitor to attenuate hypoxic tumor resistance to CTL-mediated cytotoxicity.

More interestingly, we have shown that VEGF neutralization resulted in the attenuation of hypoxic tumor target resistance to CTL-mediated killing. We have also demonstrated that STAT3 phosphorylation can be stimulated by autocrine signaling through VEGF, suggesting that tumor microenvironment through hypoxia-induced VEGF may play a key role in the induction of active form of stat3. In this regard, it is very likely that stat3 activation is associated with the regulation of target gene expression potentially involved in the alteration of hypoxic tumor target-specific killing. Therefore, understanding how VEGF and other soluble factors may lead to STAT3 activation via the tumor microenvironment may provide a more effective cancer treatment strategy for hypoxic tumors with elevated p-STAT3 levels. This also suggests that reduction of VEGF release, a main immunosuppressive factor, in tumor microenvironment may favor induction of a stronger anti-tumor CTL response against tumors expressing VEGFR. Our studies are in agreement with reports suggesting that inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer (53) and indicating a synergy between tumor immunotherapy and antiangiogenic therapy (54).

The results presented here provide evidence that hypoxia inhibits tumor-specific lysis and suggest that it induces cellular adaptation that compromise the effectiveness of killer cells. Using microarray analysis, we further demonstrate that hypoxia induces transcriptional changes that may promote cell survival and resistance to specific lysis. Cluster analysis of data reveals several possible pathways affected by hypoxia, including apoptosis, cell cycle, metabolism, cell adhesion and transcriptional regulation. Additionally, STAT3 inhibition under hypoxia affected the expression of genes belonging to the same or related pathways and resulted in a partial reversal of the differentially expressed genes under hypoxia. This point to the potential role of STAT3 in tumor adaptation induced by hypoxia. This emphasizes that a better understanding of the tumor behavior and its interplay with the killer cells in the context of the complexity and plasticity of a hypoxic microenvironment will be a critical determinant in a rational approach to tumor immunotherapy. Although resistance of tumor targets to killer cells is likely to be regulated by multiple factors (5), the data we present herein suggest that hypoxic microenvironment is an important determinant involved in the control of target sensitivity to CTL-mediated lysis. Therefore, the possibility that novel approaches targeting HIF-1α and STAT3 with potent small molecule drugs, being actively developed, may provide an exciting novel approach for cancer immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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B. Article 2: "Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression"

Tumor growth and spread depends as much on the host response as on the biological characteristics of the tumor itself and on the influence of the tumor microenvironment. Evidences indicate that cancer cells which remain viable in hypoxia often possess an increased survival potential and an aggressive growth [346]. Cytolytic T lymphocytes (CTLs) are important effector cells during tumor rejection [340]. Tumor cells develop resistance to CTL-mediated cytotoxis (resistance to perforin, granzyme-B or defect in death receptor expression or signalling) [343]. Hypoxia has been shown to render tumor targets resistant to CTL mediated lysis by signaling pathways involving HIF-1α and activation of STAT3 [347]. Autophagy is a protective mechanism involved in cell homeostasis and protection [348] under stress conditions. Moreover, autophagy is involved in regulating adaptive immune responses and tolerance induction [349]. Autophagy is also associated with tumor cell resistance to different apoptotic inducers [350] [351]. Recently, a relationship between tumor hypoxia and autophagy has been described [84] to promote cell survival [85]. The relationship between hypoxic stress, autophagy and specific cell-mediated cytotoxicity remains unknown. The present study demonstrates that hypoxia-induced resistance of lung tumor to CTL-mediated lysis is associated with autophagy induction in target cells. In turn this correlates with STAT3 phosphorylation on tyrosine 705 residue (pSTAT3) and HIF-1α accumulation. Inhibition of autophagy by siRNA targeting of either beclin1 or Atg5 resulted in impairment of pSTAT3 (via inhibition Src kinase) and restoration of hypoxic tumor cell susceptibility to CTL-mediated lysis. Autophagy-induced pSTAT3 and pSrc regulation appeared to involve the Ubiquitin Proteasome System (UPS) and p62/SQSTM1. In vivo experiments using B16F10 melanoma tumor cells indicated that depletion of beclin1 resulted in an inhibition of B16F10 tumor growth and increased tumor apoptosis. Moreover, in vivo inhibition of autophagy by hydroxychloroquine (HCQ) in B16F10 tumor bearing mice and mice vaccinated with TRP2 peptide dramatically increased tumor growth inhibition. Collectively, the current study establishes a novel functional link between hypoxia-induced autophagy and the regulation of antigen specific T cell lysis and points to a major role of autophagy in the control of in vivo tumor growth.

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Microenvironment and Immunology

Cancer Research

Blocking Hypoxia-Induced Autophagy in Tumors Restores Cytotoxic T-Cell Activity and Promotes Regression

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Abstract

The relationship between hypoxic stress, autophagy, and specific cell-mediated cytotoxicity remains unknown. This study shows that hypoxia-induced resistance of lung tumor to cytolytic T lymphocyte (CTL)–mediated lysis is associated with autophagy induction in target cells. In turn, this correlates with STAT3 phosphorylation on tyrosine 705 residue (pSTAT3) and HIF-1α accumulation. Inhibition of autophagy by siRNA targeting of either beclin1 or Atg5 resulted in impairment of pSTAT3 and restoration of hypoxic tumor cell susceptibility to CTL-mediated lysis. Furthermore, inhibition of pSTAT3 in hypoxic Atg5 or beclin1-targeted tumor cells was found to be associated with the inhibition Src kinase (pSrc). Autophagy-induced pSTAT3 and pSrc regulation seemed to involve the ubiquitin proteasome system and p62/SQSTM1. *In vivo* experiments using B16-F10 melanoma tumor cells indicated that depletion of beclin1 resulted in an inhibition of B16-F10 tumor growth and increased tumor apoptosis. Moreover, *in vivo* inhibition of autophagy by hydroxychloroquine in B16-F10 tumor-bearing mice and mice vaccinated with tyrosinase-related protein-2 peptide dramatically increased tumor growth inhibition. Collectively, this study establishes a novel functional link between hypoxia-induced autophagy and the regulation of antigen-specific T-cell lysis and points to a major role of autophagy in the control of *in vivo* tumor growth. *Cancer Res; 71(18); 5976–86. ©2011 AACR.*

Introduction

Tumor growth and spread depend as much on the host response as on the biologic characteristics of the tumor itself and on the influence of the tumor microenvironment. Hypoxic microenvironment plays a role in shifting the normal balance toward malignancy. Evidence indicates that cancer cells which remain viable in hypoxia often possess an increased survival potential and an aggressive growth (1). A better understanding of the hypoxic tumor context could improve the prospect of developing effective cancer immunotherapy.

Cytolytic T lymphocytes (CTL) are important effector cells during tumor rejection (2). Currently, most cancer immunotherapy approaches involve the generation of CTLs against tumor-associated antigens (TAA) through vaccination strategies that induce or optimize TAA-specific immune responses. However, tumor rejection does not always follow successful induction of tumor-specific immune responses (3). Numerous studies have shown a paradoxical coexistence of cancer cells with TAA-specific T cells in an immune-competent host. Moreover, tumor cells themselves play a crucial role in controlling the antitumor immune response (4) allowing them to maintain their functional disorder and evade destruction by CTLs. Clearly, a strong and sustained CTL response is insufficient for successful elimination of tumors. Tumor growth is influenced by tumor cell recognition by specific CTLs and tumor susceptibility to T cell–mediated target cell death. In addition to chemo- and radioresistance, tumor cells also develop resistance to CTL-mediated cytotoxicity (resistance to perforin, granzyme-B, or defect in death receptor expression or signaling) leading to tumor cell survival and proliferation (5). In addition, resistance of tumor cells to cell-mediated cytotoxicity involves several strategies including cross-talk with the tumor microenvironment (6). Therefore, a better understanding of how the tumor microenvironment contributes to the resistance of tumors to killer cells is critically important for improved immune intervention. In this regard, hypoxic areas within tumors results from morphologically and functionally inappropriate neovascularization leading to the expression of genes involved in proliferation, glycolysis, and angiogenesis (7). Tumor cells evade adaptive immunity under hypoxia (8), and the adaptation to hypoxia results in an aggressive and metastatic cancer phenotype associated

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with a poor treatment outcome (9). In addition, hypoxia favors the emergence of tumor phenotypes that are resistant to therapy approaches (10). Signaling pathways involving hypoxia-inducible factor HIF-1α (11) and hypoxia-dependent activation of STAT3 (12) have been well elucidated (13). These hypoxia-dependent signaling pathways contribute to malignant transformation (14) and progression by transactivating host target genes involved in tumor proliferation, survival, self-renewal, invasion, and angiogenesis (15).

Autophagy, initially described as a mechanism involved in cell homeostasis and protection (16), has been also proposed to mediate tumor progression and promotion of cancer cell death (17). Moreover, autophagy has been involved in regulating adaptive immune responses and tolerance induction (18). Autophagy is also associated with tumor cell resistance to different apoptotic inducers (19, 20) and can modulate different stages of cancer development. Recently, a relationship between tumor hypoxia and autophagy has been described (21) to promote cell survival (22).

This study aims to examine the involvement of hypoxia-induced autophagy in the regulation of tumor cell lysis by specific CTLs. We show that hypoxia-induced autophagy promotes tumor cell resistance to specific lysis by a mechanism dependent on STAT3 phosphorylation. We also show that autophagy and the proteasome pathway cooperate to regulate STAT3 activation in hypoxic tumor cells. Simultaneous stimulation of immune system and inhibition of autophagy *in vivo* significantly inhibited tumor growth. These data uncover a key role of autophagy in the regulation of antitumor immunity.

Materials and Methods

Tumor cells and CTLs culture

IGR-Heu lung carcinoma cell line and Heu171 cell clone were derived and maintained in culture as described (23). B16-F10 melanoma cell line was purchased from American Type Culture Collection. Cells were transfected with LC3 cDNA fused with GFP (provided by Dr. Mizushima, Tokyo, Japan). To detect ubiquitination, cells were transfected with Hemagglutinin (HA)-tagged ubiquitin vector (pMT123), provided by Dr. Matthias Treier (EMBL, Germany) using Lipofectamine 2000 (Invitrogen). Unless otherwise indicated the induction of HIF-1α in cells was done in a hypoxia workstation (Invivo$_2$ 400, Ruskinn), as previously described (23).

Reagents and antibodies

Reagents include protease inhibitors and anti-HA (12CA5; Roche); bicinchoninic acid protein assay reagent (Pierce); CHAPS, E64d, pepstatin, PP2, 3-methyladenine (3-MA), cobalt chloride (CoCl2), and hydroxychloroquine (HCQ) sulfate (Sigma); and bortezomib (Velcade; Millennium Pharmaceuticals). Antibodies include the following: mouse anti-HIF-1α and -p62 (BD-Transduction Laboratories); rabbit anti-LC3B, -pSTAT3, -Src, -pSrc, -Beclin1, -Atg5, and mouse anti-STAT3 (Cell signaling, Bioké); mouse anti-actin (clone c-11) and -Beclin1 (clone G-11; Santa Cruz Biotechnology); mouse anti-Bcl2 (clone 124), and rabbit anti-ubiquitin (Dako).

Cytotoxicity assay

Cytotoxic activity of CTL clone (Heu171) was measured by a conventional 4-hour Cr^{51} release assay and percent-specific cytotoxicity was calculated as described (23).

RNA interference and generation of short hairpin RNA Atg5 and Beclin tumor cells

Cells were transfected by electroporation with 50 nmol/L siRNA of human and mouse *BECN1* (beclin1), p62 or Atg5 (Qiagen), or Luciferase (Invitrogen) as previously described (23). Atg5 short hairpin RNA (shRNA) expressing cells were generated by infection with commercial human *APG5* shRNA lentiviral particles (Santa Cruz Biotechnology) according to manufacturer's protocol. Details for the generation of shRNA Beclin B16-F10 cells are available upon request.

Western blot and immunoprecipitation

Western blotting was done as previously reported (23). Immunoprecipitation was done on 1 mg of total protein using Dynabeads protein G (Invitrogen) according to the manufacture's protocol.

Live cell imaging and confocal microscopy

Time-lapse video microscopy was done using Axiovert 200 M microscope (Carl Zeiss MicroImaging). Cells were cultured with 150 μmol/L $CoCl_2$ to mimic hypoxia condition over a period of 24 hours. Frames were taken with ×40 oil objective lense in intervals of 1 frame/3 minutes. Cells were analyzed with a Zeiss laser scanning confocal microscope LSM-510 Meta (Carl Zeiss).

In vivo experiments

C57BL/6 mice (Charles River Laboratories) were housed at the Institut Gustave Roussy animal facility and treated in accordance with institutional animal guidelines. Six to 7-week-old mice ($n = 10$ per group) were inoculated s.c. with 3×10^5 B16-F10 cells. Tumor volume was measured using a caliper every other day and estimated as follows: Volume (cm^3) = $(\text{width})^2 \times \text{length} \times 0.5$.

TRP-2 $_{180-188}$ peptide vaccination and HCQ treatment

Lyophilized TRP-2(180-188) peptide (SVYDFFVWL; Proteogenix) was diluted in dimethyl sulfoxide and stored at −20°C. Synthetic CpG ODN 1826 (TCCATGACGTTCCTGACGTT; Oligofactory) was dissolved in sterile PBS. TRP-2(180-188) peptide vaccination was done by s.c. injection of 50 μg CpG ODN and 50 μg TRP-2(180-188) emulsified in 100 μL of IFA on the left side of back. The vaccination was boosted at day 4 and day 12. Daily HCQ treatment [30 mg/kg administered intraperitoneally (i.p.)] was started at day 8 for 10 days.

Immunohistochemical analysis

B16-F10–engrafted tumors were fixed in 4% PFA, and paraffin sections were stained with hematoxylin-eosin-saffranin. Apoptotic cells were quantified using *in situ* cell death detection kit (Roche) based on the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method as previously described (24). Hypoxic tumor areas were detected after injection of mice with pimonidazole (60 mg/kg i.p.

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hypoxyprobe-1; Chemicon) 60 minutes before killing. Frozen, acetone-fixed sections were stained using anti-cleaved LC3B (Abgent), anti-pimonidazole (Chemicon) and Mouse CD31 (BD biosciences) antibodies.

Statistics

Data were analyzed with GraphPad Prism. Student t test was used for single comparisons. Data were considered statistically significant when $P < 0.005$.

Results

Hypoxic stress-induced impairment of tumor susceptibility to CTL-mediated lysis is associated with the induction of autophagy

We have previously reported (23) that hypoxic stress-induced HIF-1α is associated with a decrease in CTL-mediated tumor cell lysis (Fig. 1A) and correlated with an increase in pSTAT3 (Fig. 1B). To investigate whether hypoxia-dependent impairment of target cell susceptibility to CTL-mediated lysis is associated with autophagy activation, we analyzed the expression of p62/SQSTM1 and the lipidated form of microtubule-associated protein light chain 3 (LC3-II). Figure 1C shows a time-dependent decrease in p62/SQSTM1 expression in hypoxic cells which correlated with an accumulation of LC3-II only in hypoxic cells treated with lysosomal protease inhibitors E64 and pepstatin (Fig. 1D) reflecting the activation of autophagy under hypoxia. This was further confirmed by time-lapse video microscopy (Supplementary Data S1A and B) using GFP-LC3 expressing IGR-Heu cells (Supplementary Data S2). Representative images from time-lapse experiments showed a time-dependent increase of autophagosomes per cell under hypoxia (Fig. 1E, top and bottom). The molecular mechanism

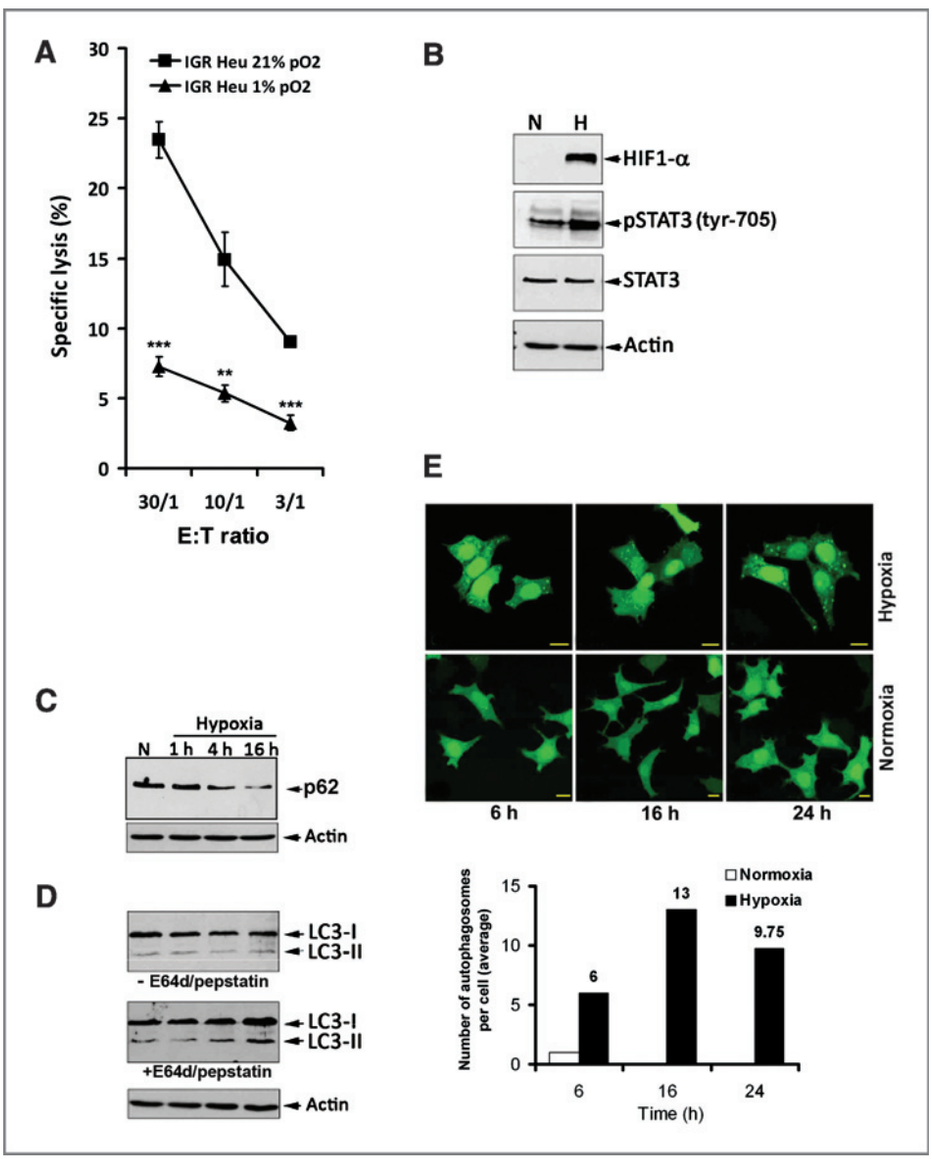

Figure 1. Hypoxia decreases susceptibility of IGR-Heu cells to CTL-mediated lysis and induces autophagy. A, cells were incubated in normoxia (21% pO_2) or hypoxia (1% pO_2) for 16 hours. Cytotoxicity was determined at different effector–tumor (E:T) cell ratios. Tumor-infiltrating lymphocyte (TIL)–derived T-cell clone Heu 171 cells were used as effectors. A statistically significant difference (indicated by asterisks) in specific lysis was observed between normoxic and hypoxic tumor cells. (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$). B, cells were cultured under normoxia (N) and 16 hours of hypoxia (H). Immunoblot analysis was done on 40 μg of total protein extracts using HIF1-α, pSTAT3, STAT3 antibodies. β-Actin was used as a loading control. C, time-dependent expression of p62/SQSMT1 under normoxia (N) and hypoxia at 1, 4, and 16 hours by immunoblot using specific antibody. Hypoxia was generated by adding 150 μmol/L $CoCl_2$ to the culture medium. D, time-dependent expression of LC3 by immunoblot. Cells were grown as described in C in the absence (top) or presence (middle) of lysosomal protease inhibitors E64d/pepstatin. E, top, representative images from time-lapse video microscopy experiments showing the presence of autophagosomes in hypoxic as compared with normoxic GFP-LC3 expressing IGR-Heu cells. Bar, 10 μm. Bottom, quantification of the number of autophagosomes of the top images. Hypoxia was generated as described in C.

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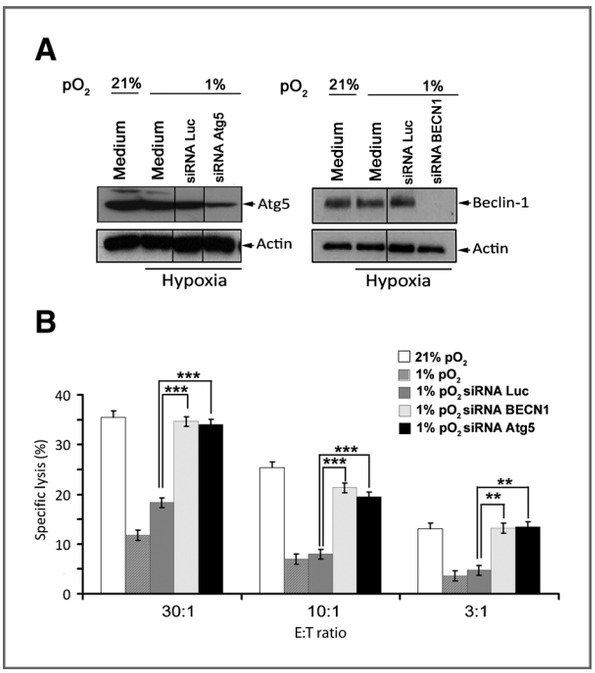

Figure 2. Targeting autophagy restores IGR-Heu tumor cell susceptibility to CTL-mediated lysis. A, expression of Atg5 and Beclin1 by immunoblot in normoxic (pO_2 21%) and hypoxic (pO_2 1%) cells transfected with Atg5, Beclin1 (*BECN1*), or luciferase (Luc) siRNA. B, CTL-mediated cytotoxicity of IGR-Heu tumor cells at different E:T ratios. Data represent 3 independent experiments with SD. Statistically significant differences (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$).

involved in the activation of autophagy was next investigated. Results shown in Supplementary Data S3A reveal a lower amount of Bcl-2 coimmunoprecipitated with Beclin1 in hypoxic as compared with normoxic cells suggesting that the activation of autophagy involves a disruption of the autophagy inhibitory complex of Beclin 1/Bcl-2. BNIP3 and BNIP3L seem to be involved in displacing Beclin1 from Bcl-2 under hypoxia (21) because the expression of BNIP3 and BNIP3L was increased in hypoxic as compared with normoxic cells (Supplementary Data S3B and C). Silencing *BNIP3* and *BNIP3L* inhibited autophagosome formation in hypoxic IGR-Heu cells (Supplementary Data S3D). Collectively, our results show that hypoxic stress–induced impairment of tumor susceptibility to CTL-mediated lysis is associated with the induction of autophagy in IGR-Heu cells by a mechanism involving BNIP3/BNIP3L.

Targeting autophagy restores IGR-Heu tumor cell susceptibility to CTL-mediated lysis

To investigate whether the inhibition of hypoxia-induced autophagy is able to restore tumor cell susceptibility to CTL-mediated lysis, we targeted autophagy genes Atg5 and Beclin1 by siRNA (Fig. 2A). As shown in Fig. 2B, there was a remarkable reversal of hypoxia-induced inhibition of CTL-mediated killing in autophagy defective cells. Interestingly, CTL-induced killing of autophagy defective cells was observed at all effector–target (E:T) ratios tested, compared with control cells. These data suggest that hypoxia-induced autophagy plays an important role in the acquisition of tumor cell resistance to T cell receptor–dependent killing.

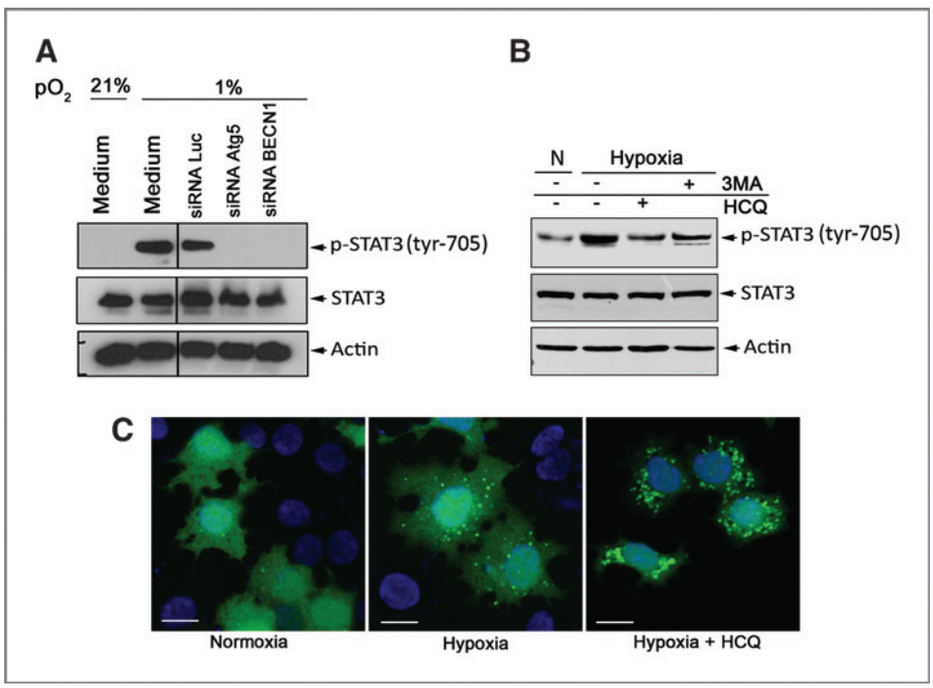

Figure 3. Targeting autophagy inhibits hypoxia-induced pSTAT3. A, expression of pSTAT3 by immunoblot in IGR-Heu cells transfected with siRNA targeting autophagy genes and cultured under normoxia (pO_2 21%) and hypoxia (pO_2 1%). B, expression of pSTAT3 in IGR-Heu cells untreated (−) or treated (+) with autophagy inhibitors HCQ (60 μmol/L) or 3 MA (1 mmol/L) and cultured under normoxia (N) or hypoxia for 16 hours. Hypoxia was generated as described in Fig. 1C. C, accumulation of hypoxia-dependent autophagosomes shown by confocal microscopy in hypoxic GFP-LC3 expressing IGR-Heu cells treated with HCQ (60 μmol/L). Nuclei were stained with 4′,6-diamidino-2-phenylindole. Bar, 10 μm.

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Hypoxia-induced pSTAT3 is regulated by autophagy in IGR-Heu cells

Because hypoxia-dependent impairment of IGR-Heu cell susceptibility to CTL-mediated lysis correlated with HIF-1α and pSTAT3 induction (23), we investigated whether the autophagic process operates upstream of hypoxia-dependent induction of pSTAT3. Targeting Beclin1 or Atg5 under hypoxia abrogates pSTAT3 expression without affecting STAT3 expression (Fig. 3A). Similar results were obtained using autophagy inhibitors 3-MA and HCQ, which block the early and late events of autophagy, respectively (Fig. 3B). Figure 3C shows a strong accumulation of autophagosomes in hypoxic cells treated with HCQ, thus showing the effectiveness of the HCQ concentration used in blocking the autophagic flux. These results show that hypoxia-induced autophagy regulates the induction of pSTAT3.

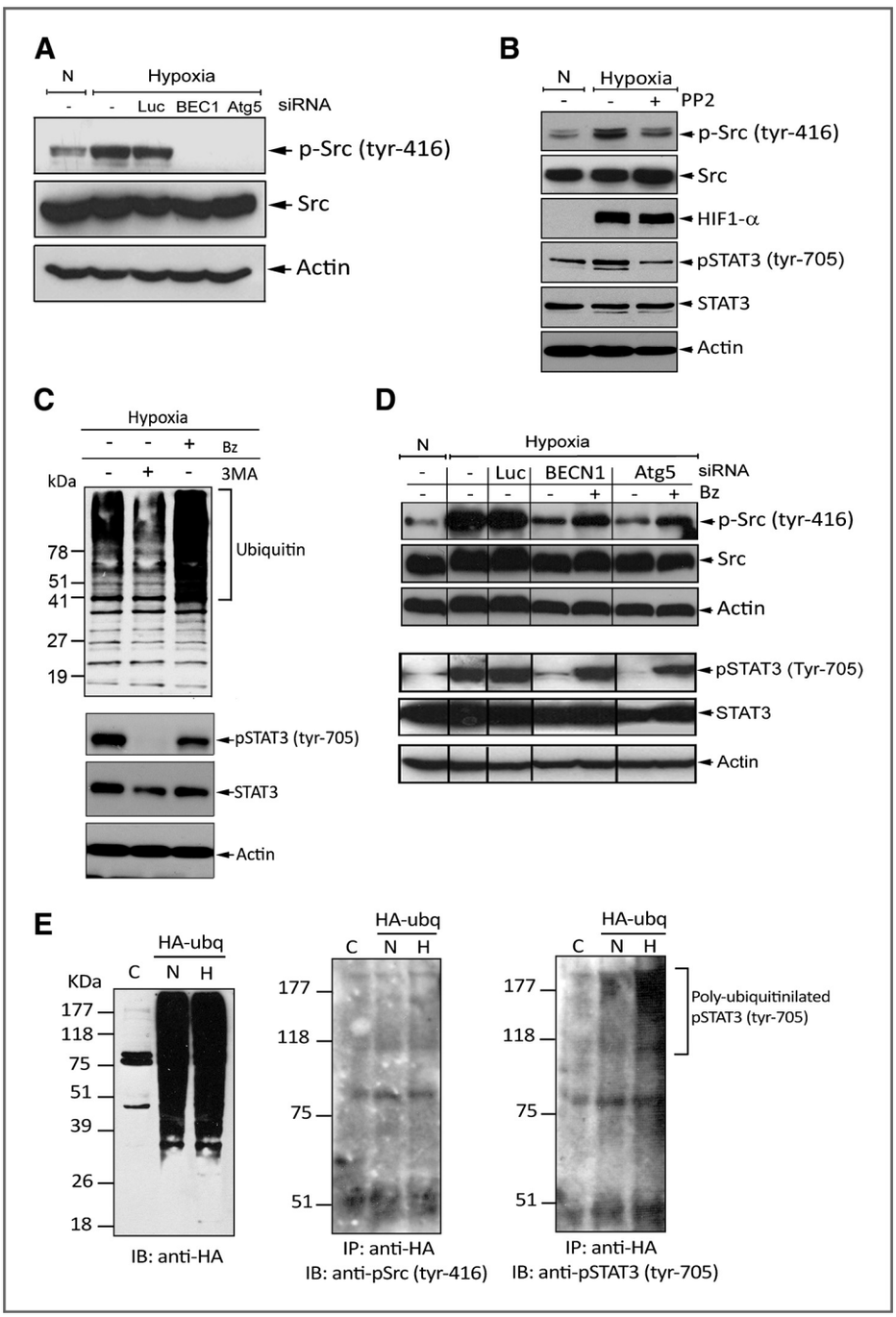

Figure 4. Autophagy and ubiquitin proteasome system cooperate to regulate pSTAT3 in hypoxic IGR-Heu cells. A, expression of pSrc by immunoblot in normoxic (N) and hypoxic cells transfected with Luciferase (Luc), Beclin1 (*BECN1*) or Atg5 siRNA. B, expression of pSrc and pSTAT3 by immunoblot in normoxic (N) and hypoxic cells untreated (–) or treated with Src kinase inhibitor (PP2, 10 μmol/L). C, immunoblot analysis of ubiquitinated protein in hypoxic cells untreated (–) or treated (+) with 3MA or Bz. D, expression of pSrc and pSTAT3 in normoxic (N) and hypoxic cells untreated (–) or treated (+) with Bz. Hypoxic cells were transfected with Luc, *BECN1*, or Atg5 siRNA. E, immunoblot analysis of pSrc and pSTAT3 polyubiquitination. Left, immunoblot analysis of the expression of HA-ubiquitin in control (C) untransfected and transfected cells cultured under normoxia (N) or hypoxia (H). Middle and right panels represent immunoprecipitation experiments using anti-HA antibody followed by immunoblot using anti-pSrc (middle) or anti-pSTAT3 (right).

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Impairment of pSTAT3 in autophagy defective cells is related to Src kinase activity and involves the ubiquitin proteasome system

We next investigated the involvement of the receptor-associated–specific Janus kinases (JAK) and Src in the phosphorylation of STAT3 to get more insights into the mechanisms associated with hypoxia-induced pSTAT3 and to elucidate the role of autophagy in this process. Our data show that inhibition of autophagy in hypoxic cells does not affect the phosphorylation of JAK2 (data not shown) but dramatically decreased the tyrosine-416 phosphorylation of Src (pSrc). It is noteworthy that pSrc expression is highly increased under hypoxia (Fig. 4A). Figure 4B shows that inhibition of Src activity in hypoxic cells by PP2 significantly inhibits hypoxia-induced pSTAT3. Together our data show that targeting autophagy inhibits hypoxia-induced pSrc.

Analysis of the ubiquitination profile of proteins in autophagy-defective cells cultured under hypoxia shows that high-molecular-weight proteins (up to 40 KDa) are highly ubiquitinilated (Fig. 4C). The ubiquitination level is decreased in autophagy-defective cells and dramatically reaccumulated in cells treated with the proteasome inhibitor bortezomib, highlighting the role of the ubiquitin proteasome system (UPS) in the clearance of pSTAT3 and/or pSrc in autophagy defective cells under hypoxia. This was supported by our data (Fig. 4D) showing that inhibition of UPS by bortezomib in autophagy-defective cells completely restores the expression of pSTAT3 and partially that of pSrc.

Because UPS-dependent protein clearance implies their ubiquitination, we analyzed the ubiquitination profile of pSTAT3 and pSrc in autophagy defective cells expressing HA-tag ubiquitin. As shown in Fig. 4E left panel, no HA-ubiquitin was detected in untransfected cells, but equal and high expression level of HA-ubiquitin was detected in transfected cells cultured under normoxia and hypoxia. Immunoprecipitation results (Fig. 4E, middle) clearly show the absence of pSrc ubiquitination. However, a strong high-molecular-weight smear was detected with anti-pSTAT3 antibody in hypoxic cells most likely corresponding to a polyubiquitinated form of pSTAT3 (Fig. 4E, right). Together, these results indicate that autophagy and UPS cooperate to regulate pSTAT3 in hypoxic tumor cells.

P62/SQSTM1 is required for autophagy-dependent pSTAT3 degradation

P62/SQSTM1 (p62) is a multifunctional protein acting as an adaptor between ubiquitylated protein aggregates and autophagy (25). p62 degradation by autophagy represents another way for cancer cells to survive under hypoxia (26). We targeted p62 in autophagy-competent and -defective cells and analyzed the expression of pSTAT3 and pSrc. Figure 5A shows that targeting p62 in autophagy-defective cells induces a reaccumulation of pSTAT3 and pSrc under hypoxia. siRNA p62 alone did not affect the expression of pSTAT3 and pSrc in autophagy-competent cells (Fig. 5A). These results suggest that p62 is required for autophagy-dependent pSTAT3 degradation and could play a role as a cargo for pSTAT3 or pSrc degradation. Immunoprecipitation experiments on normoxic and hypoxic

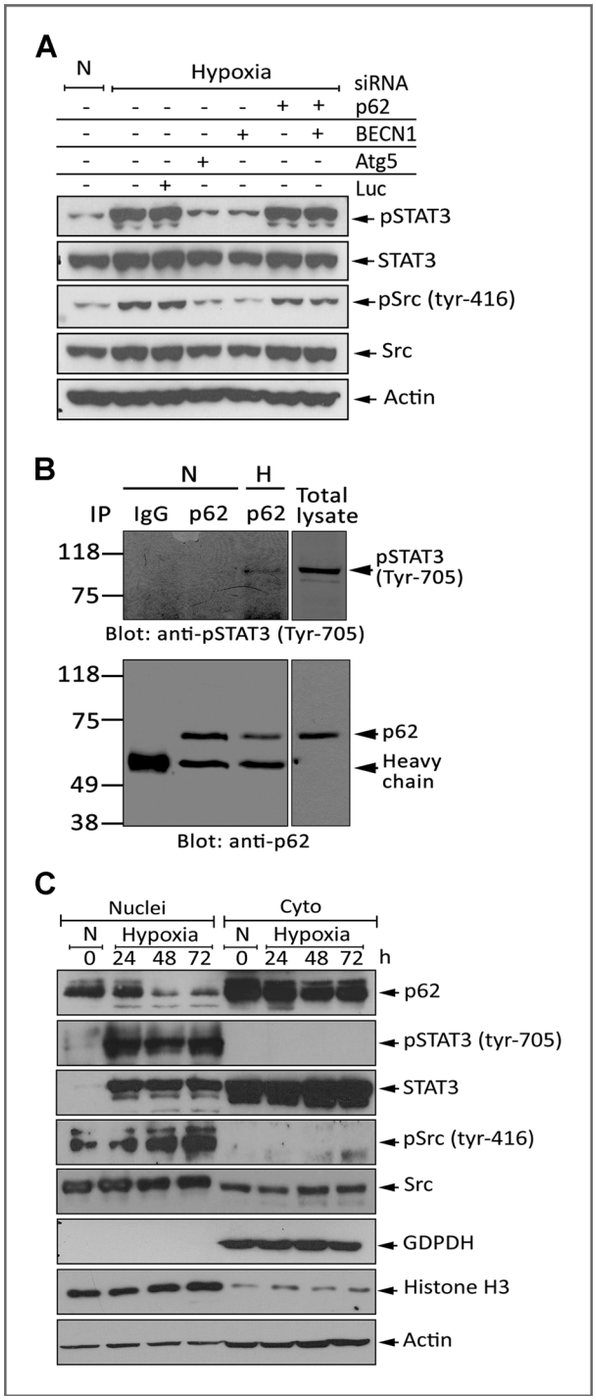

Figure 5. Involvement of p62 in autophagy-dependent degradation of hypoxia-induced pSTAT3. A, expression of pSTAT3 and pSrc by immunoblot in normoxic (N) and hypoxic cells untransfected (–) or transfected with Atg5 siRNA and Beclin1 and/or p62 siRNAs. Luc siRNA was used as control. B, immunoprecipitation (IP) of pSTAT3 and p62 in normoxic (N) or hypoxic (H) cells using p62 antibody on whole-cell lysate (1 mg). C, nuclear and cytoplasmic (cyto) expression of pSTAT3, pSrc, and p62 by immunoblot in cells under normoxia (N) and different duration of hypoxia (H).

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cells using anti-p62–specific antibody (Fig. 5B) provided compelling evidence for a direct interaction between p62 and pSTAT3 under hypoxia, suggesting that p62 interacts with pSTAT3 to trigger its degradation. These results are supported by data in Fig. 5C showing that under hypoxia pSTAT3, pSrc, and a fraction of p62 are present in the nuclear compartment.

Autophagy is activated in B16-F10 hypoxic tumor areas

To determine whether autophagy was induced in hypoxic zones of tumors, we analyzed the colocalization of LC3 (autophagy marker) in the hypoxic zones (stained with pimonidazole) of the B16-F10 tumors established in C57BL/6 mice. B16-F10 melanoma cells are able to activate autophagy under hypoxia (Supplementary Data S4A and B). Figure 6A (I) shows the presence of hypoxic areas (green), and Fig. 6A (II and III) show a colocalization of LC3 (blue staining in II and red staining in III) in hypoxic areas (green), suggesting that autophagy was strongly induced in hypoxic zones of the tumor. Moreover, Fig. 6A (IV) shows a cytoplasmic expression of LC3 in the entire hypoxic zone with strongest expression seen in extreme hypoxic areas. The impact of Beclin1 silencing on B16-F10–engrafted tumor progression was next evaluated. Figure 6B shows that the inhibition of autophagy in beclin1 silenced B16-F10–engrafted tumors resulted in a significant decrease in tumor growth. TUNEL staining clearly shows the presence of apoptotic foci and an increase of the number of TUNEL-positive nuclei in autophagy-defective tumors as compared with control (Fig. 6C). These data strongly suggest a role for autophagy in mediating hypoxia tolerance.

***In vivo* inhibition of autophagy potentiates the antitumor effect of tyrosinase-related protein-2 peptide vaccination**

Vaccination with peptide from the melanocyte differentiation antigen tyrosinase-related protein-2 (TRP2) in combination with oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) as adjuvant has been reported to be efficient in the induction of tumor cell–specific CTLs (27).

We hypothesized that autophagy blockade in combination with TRP2 peptide vaccination would result in a potent antitumor effect. B16-F10–engrafted tumors were treated with different doses of HCQ (30 mg/kg) and vaccinated with TRP2 peptide antigen. A significant decrease in tumor

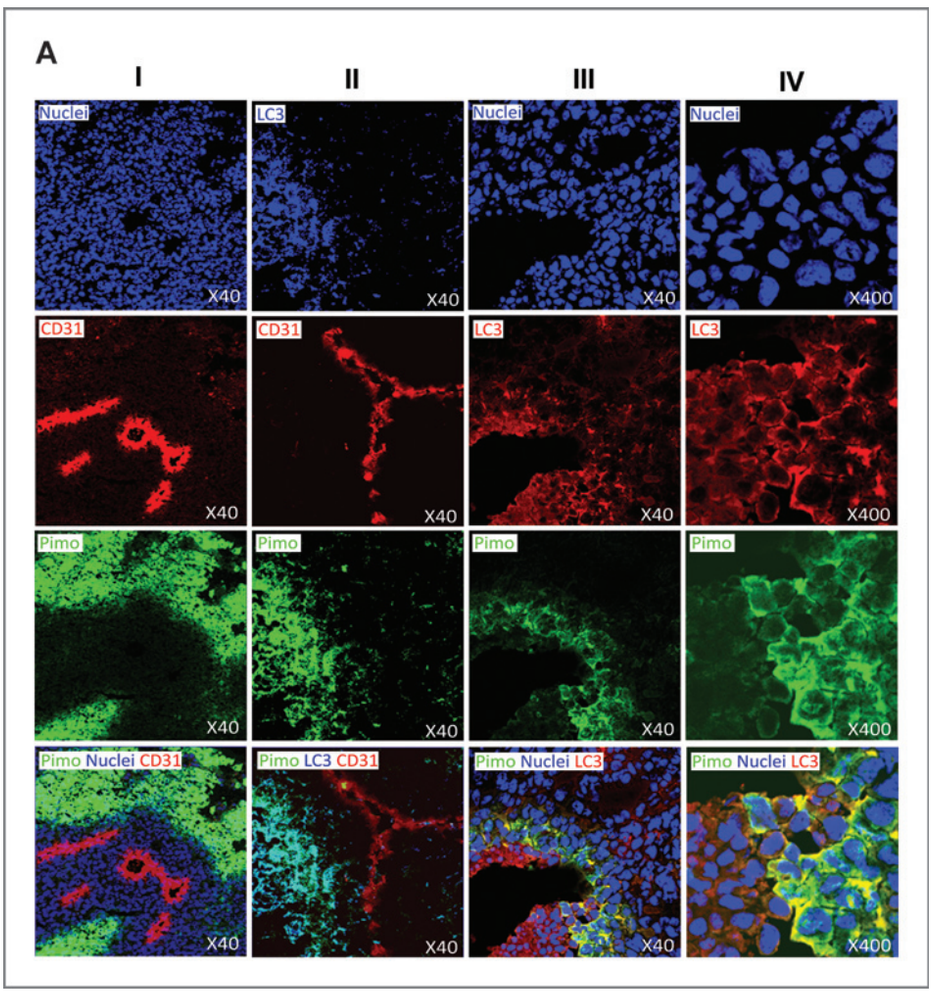

Figure 6. Autophagy is primarily detected in hypoxic areas of the tumor and its blockade decreases tumor growth. A, confocal microscopy analysis of LC3, hypoxic area, blood vessels, and nuclei in B16-F10 melanoma-engrafted tumor sections. Merged images show hypoxic area of tumor (I) and colocalization of LC3 in hypoxic area (II and III). IV (higher magnification ×400) shows a strong cytoplasmic LC3 staining within severe hypoxic regions.

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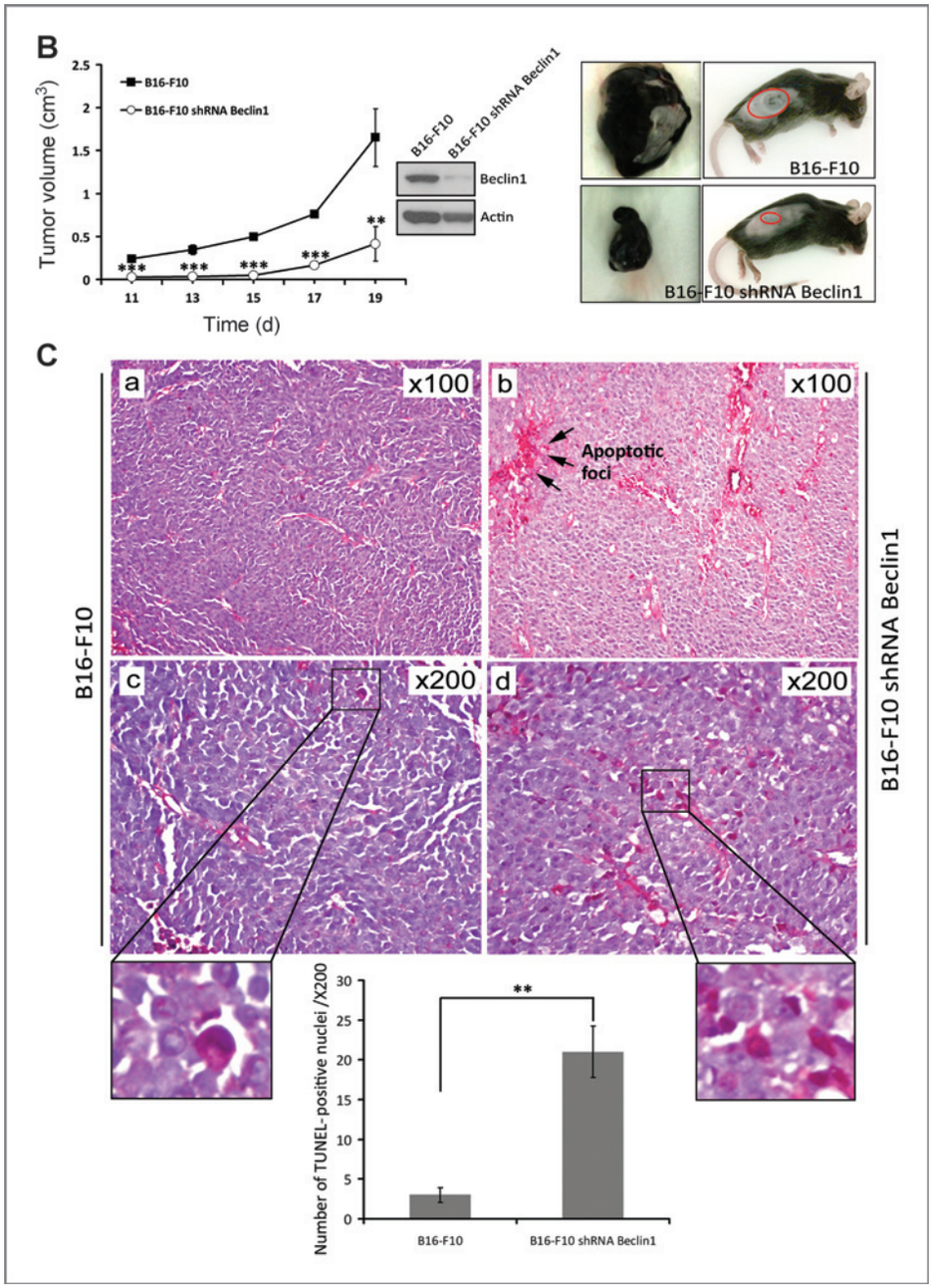

Figure 6. (*Continued*) B, growth curve of control (B16-F10) or Beclin1-deficient (B16-F10 shRNA-Beclin1) engrafted tumor in C57BL/6 mice ($n = 10$). Data (mean ± SEM) represent 3 independent experiments. Statistically significant differences (indicated by asterisks) are shown. (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$). C, TUNEL staining of apoptotic cell death in tumors from mice shown in B. (a) and (b) show the apoptotic foci, and (c) and (d) show TUNEL-positive nuclei in indicated tumors. Boxes represent an enlarged region of apoptotic cells. Images represent 2 independent experiments using samples isolated from 3 individual mice. Statistically significant differences (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$).

growth was observed as compared with control mice (Fig. 7A). A similar effect on the inhibition of tumor growth with 60 mg/kg HCQ was observed (data not shown). Strikingly, the combination of TRP2 peptide vaccination and HCQ treatment resulted in complete abrogation of tumor growth (Fig. 7A). These results were further confirmed by TUNEL staining, which showed an increase in the number of TUNEL-stained positive nuclei following HCQ treatment and vaccination (Fig. 7B and C). Moreover, a dramatic increase (20-fold) in TUNEL-positive nuclei was observed in tumors after the combined treatment.

Discussion

Hypoxia is a common feature of solid tumors and a major limiting factor in successful cancer treatment (7). Previously, we showed that hypoxia impairs tumor susceptibility to CTL-mediated cell lysis by a mechanism involving cooperation between HIF-1α and pSTAT3 (23). Given the role of autophagy in induction of the adaptive immune system (18) and cellular survival under hypoxia (28), we sought to determine the molecular basis of the hypoxia/autophagy interaction and its interference with tumor cell survival and growth. Here,

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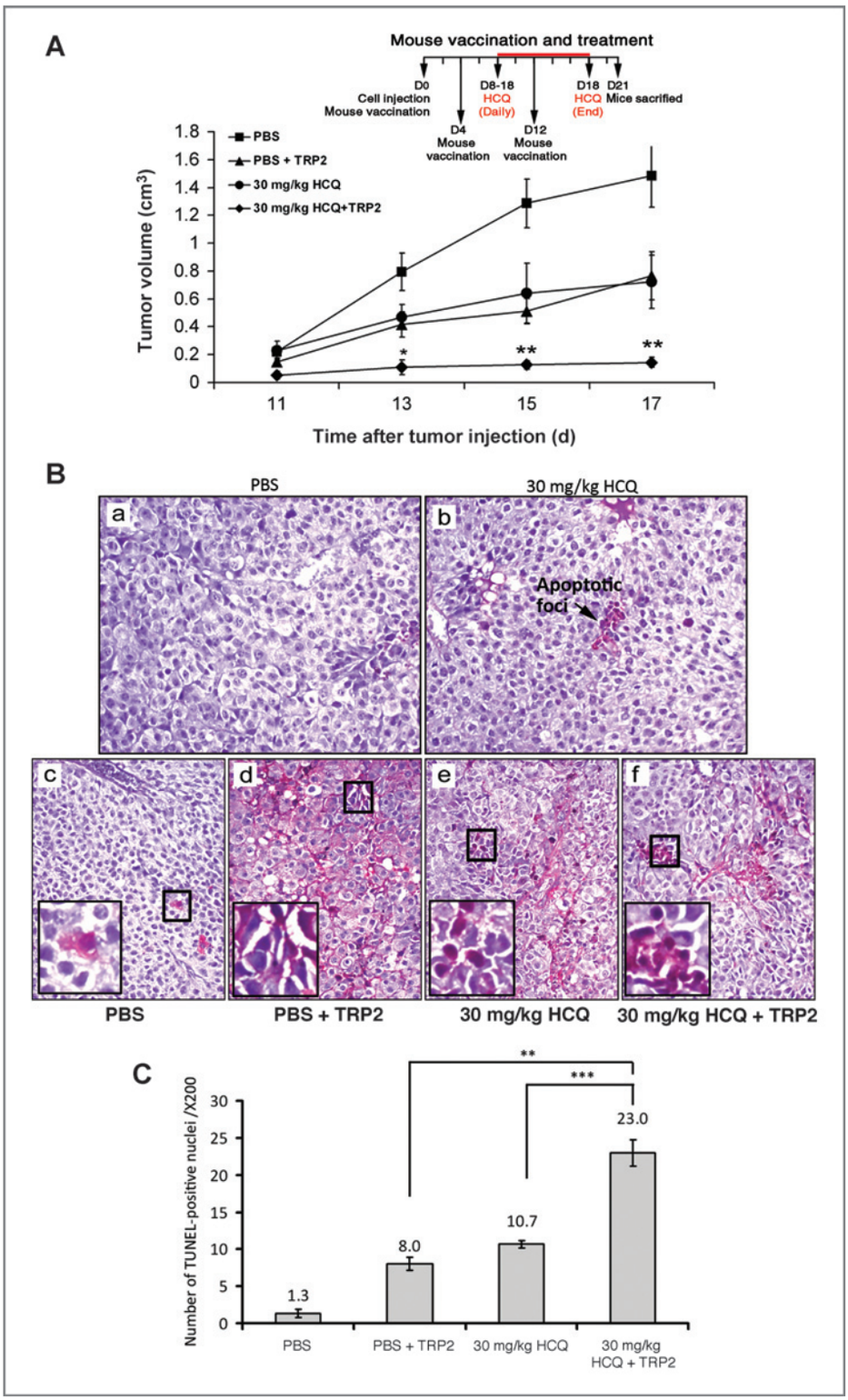

Figure 7. Autophagy inhibition by HCQ potentiates the antitumor effects of TRP-2(180–188) peptide vaccination. A, growth curve of melanoma-engrafted tumor in the following group of C57BL/6 mice ($n = 10$): control unvaccinated mice treated with PBS; vaccinated mice treated with PBS (PBS + TRP2); unvaccinated mice treated with 30 mg/kg HCQ (30 mg/kg HCQ) and vaccinated mice treated with 30 mg/kg HCQ (30 mg/kg HCQ + TRP2). Data (mean ± SEM) are representative of 3 independent experiments. Statistically significant differences (indicated by asterisks) are shown (*, $P < 0.05$, **, $P < 0.005$; and ***, $P < 0.0005$). B, TUNEL staining of apoptotic cell death in tumor from mice shown in A. (a) and (b) show apoptotic foci (×100) and (c) to (f) show, with enlarged region, TUNEL-positive nuclei (×200) within the indicated tumors. Images are representative of 2 independent experiments using samples isolated from 3 individual mice. C, representative results of TUNEL-positive nuclei quantification from 2 independent experiments using samples isolated from 3 individual mice. Statistically significant differences (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$).

we show that hypoxia-induced autophagy acts as a tumor cell resistance mechanism to specific T cell–mediated lysis. Targeting autophagy was sufficient to significantly restore tumor cell susceptibility to CTL-mediated lysis. We show for the first time that autophagy is a protective cell survival mechanism against CTL-mediated lysis under hypoxia.

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It has been suggested that the unfolded protein response enhances the capacity of hypoxic tumor cells to activate autophagy by PKR-like endoplasmic reticulum kinase (PERK) and ATF4-dependent upregulation of Atg5 and LC3 mRNA (20). This mechanism seems unlikely to be involved in the induction of autophagy in hypoxic IGR-Heu cells because there is no evidence for the activation of PERK and ATF4 and for the upregulation of Atg5 and LC3 mRNA (data not shown). However, we show (Supplementary Data S1) that hypoxia induces autophagy by BNIP3/BNIP3L-dependent displacement of the autophagy-inhibitory complex Beclin1/Bcl-2 (21).

We attempted to investigate the role of autophagy in the regulation of tumor susceptibility to CTL-mediated lysis by blocking autophagy. Although much still remains to be learned mechanistically, our data show that autophagy inhibition compromised the hypoxia-dependent induction of pSTAT3. Consistent with the clearance function of autophagy, it is difficult to reconcile the idea that the autophagy machinery plays a role in a specific stabilization of pSTAT3 and/or pSrc. Indeed, it is now well documented that there is cross-talk between the UPS and autophagy (25, 29). On the basis of our results, we believe that the decreased level of pSTAT3 in hypoxic and autophagy-defective IGR-Heu cells is related to its degradation by UPS. This was supported by our data showing that hypoxia induces a polyubiquitination of pSTAT3 and our results showing that inhibition of UPS by bortezomib accumulates pSTAT3 in hypoxic and autophagy-defective IGR-Heu cells. It is noteworthy that a UPS-dependent degradation of phospho-p53 (30) and phospho-PKC-δ (31) has been previously described. The interplay between autophagy and UPS is mainly mediated by the adaptor protein p62/SQSTM1, which plays a role as a cargo for targeting proteins to proteasomes degradation and autophagy (32). Because p62 is an autophagic substrate, an accumulation of this protein was observed in autophagy-deficient cells. In this regard, it has been recently reported that an excess of p62 inhibits the clearance of ubiquitinated proteins destined for proteasomal degradation by delaying their delivery (32). Our results show discrepancy with this mechanism as we show that the accumulation of p62 in autophagy-defective cells correlates with a decrease, rather than an accumulation, of pSTAT3 in hypoxic IGR-Heu cells, and targeting p62 in autophagy-defective cells reaccumulates pSTAT3. Although additional work needs to be done to clarify this discrepancy, one possible reason is that degradation of p62 by autophagy in IGR-Heu cells under hypoxia could represent a feedback mechanism to restrict further autophagy and excessive destruction of proteins under hypoxia. Such a feedback mechanism has been recently described (26).

Vaccine approaches have proven effective in enhancing antitumor immunity against tumor cells expressing targeted antigens *in vitro* (33). However, clinical and animal studies have shown little success in the inhibition of tumor growth by vaccines (34). One of the most important factors that could be responsible for this failure is the hypoxia-dependent activation of prosurvival pathways. Inhibition of autophagy has been reported to sensitize tumor cells to cytotoxic treatments including antitumoral agents and irradiation (35). In light of our *in vitro* observations, we asked whether targeting of hypoxia-induced autophagy could influence *in vivo* tumor growth. To address this issue, we used the transplantable murine melanoma B16-F10 cell line that expresses different TAA, including TRP2 (36). Our data showed that autophagy is primarily localized to tumor hypoxic regions as previously reported in other tumor models (20). We also show that targeting beclin1 reduced tumor growth, which correlated with an increase in TUNEL staining. In addition, we observed a significant decrease of tumor growth in mice treated with the autophagy inhibitor HCQ, used at a concentration that had no effect on cell proliferation (data not shown). These observations are in agreement with recent reports indicating that inhibition of autophagy by HCQ inhibits *in vitro* cell growth and *in vivo* tumor growth via induction of apoptosis (37, 38). Our results are also supported by several studies showing that inhibition of autophagy promotes cancer cell death (39) and potentiates anticancer treatments (19, 40, 41). HCQ has been used *in vitro* for the inhibition of autophagy in several tumor models including melanoma (42) and is currently being tested in different ongoing phase II studies (43, 44)

Finally, with regard to potential therapeutic treatment, we show that the combination of TRP2 peptide vaccination with HCQ treatment results in a strong inhibition of tumor growth. These observations further establish the significance of hypoxia in inducing autophagy, demonstrate that it is an adverse prognostic factor, and confirm its critical role not only in radio- and chemoresistance but also in immune effector cell resistance. A better understanding of the hypoxic tumor context is likely to improve the prospect of developing effective cancer immunotherapy. Together, our results show that targeting autophagy could have significant therapeutical implications for tumor progression and extend the notion that simultaneously boosting the immune system and targeting of autophagy could enhance the therapeutic efficacy of cancer vaccines and may prove beneficial in cancer immunotherapy.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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Hypoxia-induced autophagy

A new player in cancer immunotherapy?

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A major challenge in formulating an effective immunotherapy is to overcome the mechanisms of tumor escape from immunosurveillance. We showed that hypoxia-induced autophagy impairs cytotoxic T-lymphocyte (CTL)-mediated tumor cell lysis by regulating phospho-STAT3 in target cells. Autophagy inhibition in hypoxic cells decreases phospho-STAT3 and restores CTL-mediated tumor cell killing by a mechanism involving the ubiquitin proteasome system and p62/SQSTM1. Simultaneously boosting the CTL-response, using a TRP-peptide vaccination strategy, and targeting autophagy in hypoxic tumors, improves the efficacy of cancer vaccines and promotes tumor regression in vivo. Overall, in addition to its immunosuppressive effect, the hypoxic microenvironment also contributes to immunoresistance and can be detrimental to antitumor effector cell functions.

Keywords: hypoxia, autophagy, adaptive immunity, cancer vaccines

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Despite considerable progress made in the identification of new strategies based on boosting the antigen-specific antitumor responses, the immunotherapy approaches that are currently used in the clinic have only a limited success. The tumor escape from immunosurveillance represents the last series of hurdles to be overcome in formulating a more effective cancer immunotherapy.

In this regard, the hypoxic tumor microenvironment has attracted much attention from immunologists as a major contributor in tumor escape from immunosurveillance. Indeed, several lines of evidence documented that immune effector cells recruited to the hypoxic tumor site exhibit distinct functions compared with those present in normoxic areas. Subsequently, their antitumor activities are largely attenuated. In addition, immune cells in the tumor microenvironment not only fail to exert their antitumor effector functions, but they are co-opted to promote tumor growth. In this report, we discuss a new insight into how tumor cells escape from (CTL) specific lysis under the conditions of the hypoxic tumor microenvironment.

Using a human non-small cell lung carcinoma (NSCLC) cell line (IGR-Heu) and the autologous CTL clones, we have previously shown that hypoxic tumor cells are less susceptible to specific T cell receptor (TCR)-dependent lysis. Consistent with the fact that hypoxia can inhibit T cell-mediated immune response, we showed that impairment of tumor cell susceptibility to CTL-mediated lysis under conditions of hypoxia is neither related to an alteration of the effector immune response, nor to a defect in the interaction between effector cells and target cells. However, this impairment was associated with the hypoxia-dependent phosphorylation of STAT3 (pSTAT3) at the Tyr705 residue in the target cells by a mechanism involving, at least in part, vascular endothelial growth factor (VEGF). This study predicts that, despite an apparently intact cytolytic potential of CTL, tumor cells escape CTL-mediated lysis under hypoxic conditions by activating a hypoxia inducible factor (HIF)-1α-dependent intrinsic signaling pathway leading to

STAT3 phosphorylation. Overall, this study highlights pSTAT3 in tumor targets as a key determinant in the regulation of CTL-mediated tumor cell lysis under hypoxia. We have extended our previous findings by showing that STAT3 phosphorylation depends on Src kinase phosphorylation at the Tyr416 residue (pSrc). Indeed, pSrc inhibition by PP2 dramatically decreases pSTAT3 without affecting total STAT3 expression. Consistent with the role of autophagy as a cell protective mechanism in response to oxidative stress, we showed that hypoxia leads to autophagy induction in tumor cells and that the pSTAT3 level is tightly regulated by autophagy. Targeting autophagy with *BECN1* or *ATG5* siRNA significantly decreases hypoxia-dependent induction of pSTAT3 and restores CTL-mediated lysis of tumor cells. This could allow the prediction that autophagy induction under hypoxia plays a role in pSTAT3 stabilization in tumor cells. Given the role of autophagy as a protein degradation system, however, it is difficult to reconcile the idea that the autophagy machinery plays a role in the stabilization of pSTAT3 under hypoxia. The mechanism responsible for the decrease in pSTAT3 level in autophagy-defective hypoxic tumor cells was, therefore, extensively addressed by focusing on the crosstalk mediated through the cargo receptor/adaptor protein p62/SQSTM1 between the ubiquitin-proteasome system (UPS) and autophagy. Using genetic and pharmacological approaches to inhibit p62/SQSTM1 and the UPS in either autophagy competent or defective tumor cells, our results unraveled the mechanism by which hypoxia-induced autophagy regulates the level of pSTAT3 (**Fig. 1**). Indeed, hypoxia-dependent induction of HIF-1α has two effects in target cells: by an as yet undefined mechanism, but still under investigation, HIF-1α induces pSrc, which subsequently phosphorylates STAT3 at the Tyr705 residue. HIF-1α also induces autophagy by a mechanism involving the upregulated expression of BNIP3/BNIP3L and the dissociation of the BECN1-BCL2 complex. Autophagy activation results in degradation of the p62/SQSTM1 protein, responsible for the targeting of pSTAT3 to the UPS. In keeping with the fact that p62/SQSTM1 is the receptor/adaptor protein for pSTAT3 targeting to the UPS, its degradation by autophagy leads to pSTAT3 accumulation. In autophagy-defective cells, p62/SQSTM1 is no longer degraded and its accumulation accelerates the process of pSTAT3 targeting for degradation by the UPS.

In light of our in vitro observation, we investigated whether targeting hypoxia-induced autophagy could affect CTL-mediated tumor cell lysis in vivo. To address this issue, we used the autophagy inhibitor hydroxychloroquine (HCQ) in combination with a tyrosinase-related protein-2 (TRP2) peptide-based vaccination strategy. TRP2 combined with oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) vaccination has been previously reported to be efficient in the induction of tumor cell-specific CTLs. Using a transplantable murine melanoma B16-F10 cell line we first provided evidence that autophagy is primarily detected in hypoxic areas of the tumor. Inhibition of autophagy in B16-F10 engrafted tumors results in a significant decrease in tumor growth by inducing apoptosis, as revealed by TUNEL staining. These results strongly argue for a role of autophagy in mediating hypoxia tolerance to the immune system. More interestingly, we showed a significant decrease in tumor growth of the vaccinated and HCQ-treated group of mice as compared with control and to either treatment alone. Although subcutaneous tumor implantation models have their limits, it would be interesting to perform

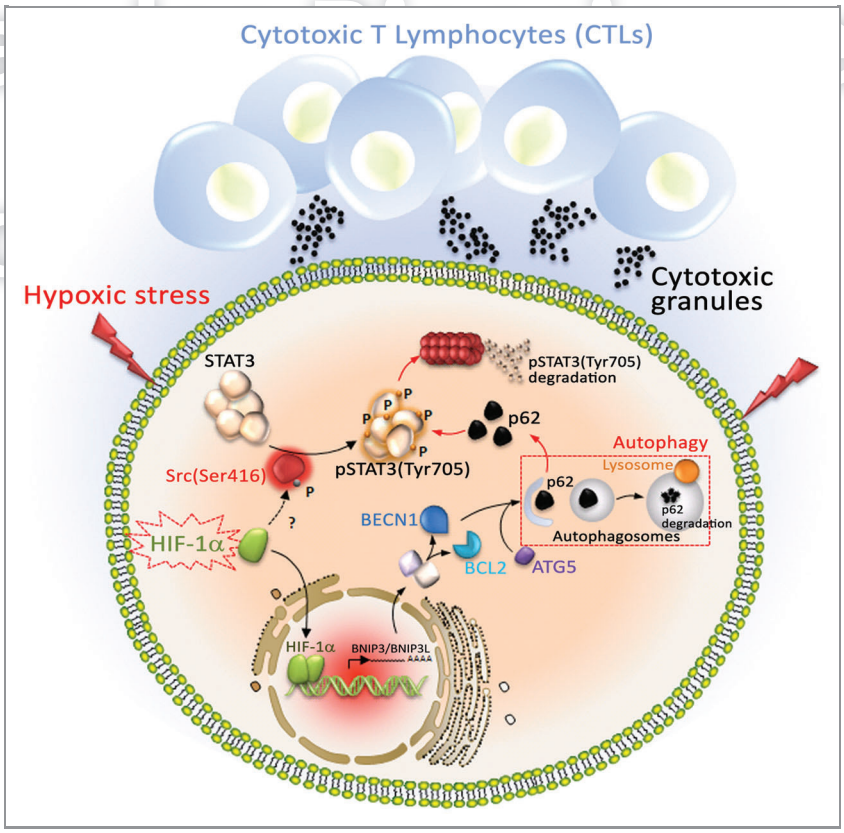

Figure 1. Model of pSTAT3 regulation by hypoxia-induced autophagy in tumor cells. Hypoxic stress leads to the accumulation of HIF-1α. By an as yet undefined mechanism, HIF-1α increases the level of phospho-Src, which subsequently phosphorylates STAT3 at the Tyr705 residue. As HIF-target gene products, BNIP3 and BNIP3L are transcriptionally upregulated and compete with the BECN1-BCL2 complex. This competition releases BECN1 from the complex and then activates the autophagic machinery by recruiting several autophagic proteins including ATG5. As an autophagic substrate, p62/SQSTM1 is degraded in the autophagosomes following their fusion with lysosomes. In view of the fact that p62/SQSTM1 is involved in targeting pSTAT3 to the UPS, its degradation leads to the accumulation of pSTAT3 in cells. In autophagy-defective cells, p62/SQSTM1 is no longer degraded, and its accumulation accelerates the UPS-dependent degradation of pSTAT3.

simultaneous boosting of the immune system and inhibition of autophagy in spontaneous animal tumor models. Transgenic mouse models of spontaneous oncogene-driven tumors that are deficient in autophagy could be useful to explore the impact of the hypoxic tumor microenvironment and autophagy inhibition on the antitumor immune responses. Nevertheless, our results strongly argue that in vivo inhibition of autophagy potentiates the antitumor effect of a TRP2-based vaccine. It would be interesting to investigate whether the inhibition of autophagy will affect other parameters of the antitumor T cell activity in vivo and in vitro (i.e., cytokine production, proliferation, activation, etc.). Several other important mediators of immune tolerance such as the number of the myeloid-derived suppressor cells (MDSCs) and the tumor-specific T regulatory cells (Tregs) should also be studied.

It has become increasingly clear that the tumor microenvironment plays a crucial role in the control of immune protection and contains many overlapping mechanisms, which ultimately lead to tumor evasion of antigen-specific immunotherapy. Obviously, tumors have evolved to utilize hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important for progression, namely survival and metastasis. In this regard, we provided evidence that hypoxia-induced autophagy plays a determinant role in tumor evasion of specific CTL-mediated lysis. Furthermore, our findings establish, for the first time, that simultaneously boosting the immune system and inhibiting autophagy can enhance the therapeutic efficacy of antigen-based cancer vaccines. This study highlights autophagy inhibition in hypoxic tumors as a cutting-edge approach to improve cancer immunotherapy and paves the way to formulate more effective cancer vaccine-based therapy. While several clinical trials using the autophagy inhibitor hydroxychloroquine in combination with several chemotherapy drugs are currently in progress, trials combining peptide vaccination with autophagy inhibitors could be an innovative strategy in cancer immunotherapy. Integrating clinical and lab findings will be crucial to understand the complex role of hypoxia-induced autophagy in modulating host tumor immunity.

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C. Article 3: "Hypoxia-inducible miR-210 regulates the susceptibility of tumor cells to lysis by cytotoxic T cells"

Although hypoxia has been reported to play a major role in the acquisition of tumor resistance to cell death [83, 346, 352], the molecular mechanisms that enable the survival of hypoxic cancer cells have not been fully elucidated. Recently, evidence has shown that aberrantly expressed microRNAs (miRNAs or miRs) are highly associated with tumour development, progression, and specific clinical phenotypes such as disease progression or recurrence [353]. Furthermore, a set of hypoxia-regulated microRNAs (HRMs) were identified that suggest a link between a tumor-specific stress factor and control of gene expression [354]. One particular miRNA, miR-210, has been frequently reported as the master regulator of tumor hypoxic response [355]; however, a significant number of additional miRNAs have also been linked to the cellular response to hypoxia [356]. Although the role of miR-210 in tumorigenesis, angiogenesis, mitochondrial metabolism, cell survival and DNA repair has been well characterized [355], its role in the immune response remains unknown. Of particular interest is its role in the regulation of tumor susceptibility to antigen specific killer cells.

Hypoxia in the tumor microenvironment plays a central role in the evolution of immune escape mechanisms by tumor cells. In this study, we report the definition of miR-210 as a microRNA regulated by hypoxia in lung cancer and melanoma, documenting its involvement in blunting the susceptibility of tumor cells to lysis by antigen-specific cytotoxic T lymphocytes (CTL). miR-210 was induced in hypoxic zones of human tumor tissues. Its attenuation in hypoxic cells significantly restored susceptibility to autologous CTL-mediated lysis, independent of tumor cell recognition and CTL reactivity. A comprehensive approach using transcriptome analysis, argonaute protein immunoprecipitation and luciferase reporter assay revealed that the genes PTPN1, HOXA1and TP53I11 were miR-210 target genes regulated in hypoxic cells. In support of their primary importance in mediating the immunosuppressive effects of miR-210, coordinate silencing of PTPN1, HOXA1 and TP53I11 dramatically decreased tumor cell susceptibility to CTL-mediated lysis. Our findings show how miR-210 induction links hypoxia to immune escape from CTL-mediated lysis, by providing a mechanistic understanding of how this miRNA mediates immunosuppression in oxygen-deprived regions of tumors where cancer stem-like cells and metastatic cellular behaviors are known to evolve.

Microenvironment and Immunology

Cancer Research

Hypoxia-Inducible miR-210 Regulates the Susceptibility of Tumor Cells to Lysis by Cytotoxic T Cells

Muhammad Zaeem Noman[1], Stéphanie Buart[1], Pedro Romero[2], Sami Ketari[1], Bassam Janji[3], Bernard Mari[4], Fathia Mami-Chouaib[1], and Salem Chouaib[1]

Abstract

Hypoxia in the tumor microenvironment plays a central role in the evolution of immune escape mechanisms by tumor cells. In this study, we report the definition of miR-210 as a miRNA regulated by hypoxia in lung cancer and melanoma, documenting its involvement in blunting the susceptibility of tumor cells to lysis by antigen-specific cytotoxic T lymphocytes (CTL). miR-210 was induced in hypoxic zones of human tumor tissues. Its attenuation in hypoxic cells significantly restored susceptibility to autologous CTL-mediated lysis, independent of tumor cell recognition and CTL reactivity. A comprehensive approach using transcriptome analysis, argonaute protein immunoprecipitation, and luciferase reporter assay revealed that the genes PTPN1, HOXA1, and TP53I11 were miR-210 target genes regulated in hypoxic cells. In support of their primary importance in mediating the immunosuppressive effects of miR-210, coordinate silencing of PTPN1, HOXA1, and TP53I11 dramatically decreased tumor cell susceptibility to CTL-mediated lysis. Our findings show how miR-210 induction links hypoxia to immune escape from CTL-mediated lysis, by providing a mechanistic understanding of how this miRNA mediates immunosuppression in oxygen-deprived regions of tumors where cancer stem-like cells and metastatic cellular behaviors are known to evolve. *Cancer Res; 1–13. ©2012 AACR.*

Introduction

Cytotoxic T lymphocytes (CTL) are important effector cells in tumor rejection and play a crucial role in host defense against malignancies in both mouse and human (1, 2). Currently, most cancer immunotherapy approaches involve the generation of CTLs against tumor-associated antigens (TAA) through vaccination strategies that induce or optimize TAA-specific immune responses (3). However, tumor rejection does not always follow successful induction of tumor-specific immune responses (4). Numerous studies have shown a paradoxical coexistence of cancer cells with TAA-specific T cells in immune-competent hosts. Moreover, tumor cells themselves play a crucial role in controlling the antitumor immune response (5), allowing them to maintain their functional disorder and evade destruction by CTLs. In this regard, it has been suggested that tumor cell growth *in vivo* is not only influenced by CTL-tumor cell recognition (6) and tumor susceptibility to cell-mediated death, but also by the complex and highly dynamic tumor microenvironment, providing very important clues to tumor development and progression (7).

Hypoxia, a common feature of solid tumors and one of the hallmarks of the tumor microenvironment, is known to favor tumor survival and progression (8, 9). Although hypoxia has been reported to play a major role in the acquisition of tumor resistance to cell death (10, 11), the molecular mechanisms that enable the survival of hypoxic cancer cells have not been fully elucidated. Recently, attention has been focused on the mechanisms by which hypoxic tumor cells alter their transcriptional profiles to modulate glycolysis, proliferation, survival, and invasion, allowing them to persist under the conditions of hypoxic stress (12, 13).

Emerging evidence has shown that aberrantly expressed miRNAs are highly associated with tumor development, progression, and specific clinical phenotypes such as disease progression or recurrence (14). Recently, a set of hypoxia-regulated miRNAs (HRM) were identified that suggest a link between a tumor-specific stress factor and control of gene expression (15, 16). One particular miRNA, miR-210, has been frequently reported as the master regulator of tumor hypoxic response (17); however, a significant number of additional miRNAs have also been linked to the cellular response to hypoxia (18). Although the role of miR-210 in tumorigenesis, angiogenesis, mitochondrial metabolism, cell survival, and DNA repair has been well characterized (17), its role in the immune response remains unknown. Of particular interest is its role in the regulation of tumor susceptibility to antigen-specific killer cells.

We have previously reported that hypoxic induction of HIF1α and pSTAT3 (19) and autophagy (20) modulates tumor

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cell susceptibility to CTL-mediated lysis. In this study, we conducted miRNA profiling to improve our understanding of tumor cell resistance to CTL-mediated cell lysis under hypoxic stress. We provide evidence that hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated cell lysis by a mechanism involving its downstream targets PTPN1, HOXA1, and TP53I11.

Materials and Methods

Culture of tumor cells and CTLs

The human IGR-Heu NSCLC (non–small cell lung carcinoma) cell line and its autologous TIL clone Heu171 were derived and maintained in culture as described (21). The human melanoma cell line NA-8 and CTL clones R11, R18P1, and R2C9 were provided by Dr. Pedro Romero (Ludwig Center for Cancer Research, Lausanne, Switzerland). Human NSCLC tissues were obtained from Dr Fathia Mami-Chouaib (INSERM U753, IGR, France).

Reagents and antibodies

SDS was obtained from Sigma. Mouse anti-HIF-1α from BD Transduction Laboratories; rabbit anti-PTPN1, anti-pSTAT3, anti-PIM1, and mouse anti-STAT3 from Cell signaling; and rabbit anti-TP53I11, mouse anti-Actin-HRP, and goat-anti-HOXA1 from Abcam.

Hypoxic conditioning of tumor cells

Hypoxic treatment was conducted in a hypoxia chamber as previously described (19).

^{51}Cr cytotoxicity assay

The cytotoxic activity of the CTL clone (Heu171) was measured by a conventional 4-hour ^{51}Cr release assay (22).

Tumor necrosis factor and interferon production assay

TNF-β and IFN-γ production by the CTL clones Heu 171 (cocultured with IGR-Heu) and R2C9 (cocultured with NA-8) were measured as described (19).

Flow cytometry analysis

Flow cytometry analysis was conducted by using a FACS-Calibur flow cytometer (19).

Gene silencing by RNA interference

Predesigned siRNA (HIF-1α, PTPN1, HOXA1, and TP53I11) were obtained from Ambion and transfected as described earlier (19).

miR-210 blockade and overexpression

Transfections were conducted with anti-miR-210 (miR inhibitor, Ambion) and pre-miR-210 (miR precursor, Ambion) under hypoxic and normoxic conditions, respectively. siPORT NeoFx Transfection Agent (NeoFx; Ambion) was used for transfection according to manufacturer's instructions. Anti-miR-CT (miR negative control, Ambion) and pre-miR-CT (miR positive control, Ambion) were used as controls under hypoxic and normoxic conditions, respectively.

RNA isolation and SYBR-GREEN qRT-PCR

Total RNA was extracted from the samples with TRIzol solution (Invitrogen). DNase I-treated 1 μg of total RNA was converted into cDNA by using TaqMan Reverse transcription reagent (Applied Biosystems) and mRNA levels were quantified by SYBR-GREEN qPCR method (Applied Biosystems). Relative expression was calculated by using the comparative C_t method (2-ΔC_t). Primer sequences are available upon request.

MicroRNA (miR) isolation and detection and microRNA microarray experiment

For extraction of miRs, TRIzol (Invitrogen) was used. DNase I-treated total RNA (8 ng) was subjected to qRT-PCR analysis using TaqMan miR Reverse Transcription Kit (Applied Biosystems). MiR-210 was detected and quantified by using specific miRNA primers from Ambion. Expression levels of mature miRNAs were evaluated using comparative C_t method (2-ΔC_t). Transcript levels of RNU44 were used as endogenous control. miRNA microarray analysis was conducted using Agilent human miRNA microarray. Rosetta resolver software was used for analysis. The microarray data related to this paper have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-1157.

Western blot analysis

Western blotting was conducted as previously reported (20).

Immunoprecipitation of FLAG/HA Ago2-containing RISC—argonaute 2 ribonucleoprotein immunoprecipitation

IGR-Heu cells were transfected with pIRESneo-FLAG/HA Ago2 (Addgene plasmid 10821; ref. 23) by using Lipofectamine 2000 Transfection Reagent (Invitrogen). Immunoprecipitation was conducted as described (24). B2M, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and 18S (shown to be RISC-associated but not miR-210 targets) were used for normalization.

Luciferase reporter assay for miR-210 target gene validation

The 3′ UTRs of PTPN1, HOXA1, and TP53I11 were cloned into a pSI-CHECK-2 vector by PCR amplification of genomic DNA. Primer sequences are available upon request. IGR-Heu cells were cotransfected with 800 ng pSI-CHECK-2 and 10 nmol/L of pre-miR in 24-well plates with Lipofectamine 2000 (Invitrogen) in OPTIMEM (Invitrogen) medium. After 48 hours, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter assay (Promega).

Immunohistochemical staining for CA-IX expression

Hypoxic zones were detected by CA-IX staining on human NSCLC sections. Immunohistochemistry was conducted as previously described (25).

Locked nucleic acid-based *in situ* detection of miR-210 in human NSCLC tissues

Human NSCLC sections were fixed in 4% paraformaldehyde. miRNA ISH Optimization Kit 2 (FFPE; Exiqon) was used for miR-210 staining as per manufacturer's protocol.

Statistics

Data were analyzed with GraphPad Prism. Student *t* test was used for single comparisons. Data were considered statistically significant when *P* was less than 0.005.

Results

miR-210 is the miRNA predominantly induced by hypoxia in NSLCC and melanoma cells

To identify the miRs that are induced by hypoxia in non–small cell lung cancer (NSCLC) and melanoma, we conducted miRNA expression profiling using human NSCLC (IGR-Heu) and human melanoma (NA-8) cell lines following incubation of these cells under conditions of normoxia (21% O_2) or hypoxia (1% O_2) for 6, 16, or 24 hours (Fig. 1A–D). Several miRs that exhibited more than a 2-fold change under hypoxia were identified in both cell lines. Twenty-six miRs were upregulated in IGR-Heu cells and 9 miRs were upregulated in NA-8 cells at one or more time points under hypoxia as compared with normoxia (Supplementary data S1). Among these putative hypoxia-induced miRs, miR-210 was highly upregulated in IGR-Heu cells (Fig. 1A) and NA-8 cells (Fig. 1C). Volcano plot analysis further confirmed that miR-210 was the most highly (more than 10-fold change) and significantly (adjusted *P* value < 0.05) upregulated miR under hypoxia in IGR-Heu cells (Fig. 1B) and NA-8 cells (Fig. 1D).

Expression of miR-210 in hypoxic zones of human NSCLC tissues

To determine whether miR-210 is selectively expressed in the hypoxic zones of human NSCLC tissues, we first selected human NSCLC tissues on the basis of a well-established hypoxia marker (CA-IX; carbonic anhydrase-9; ref. 13) expression by immunohistochemistry (data not shown). We identified 12 CA-IX–positive tissues out of 31 NSCLC specimens. We next conducted miR-210 staining by *in situ* hybridization on serial sections. As shown in Fig. 2A and B, miR-210–positive staining was observed in the hypoxic zones (CA-IX–positive staining) of tumor areas. While 23% of the tumor areas were positively stained for CA-IX, 19% of the tumor areas were positive for miR-210. As depicted in Fig. 2C, an overlap of CA-IX and miR-210 staining was observed: 68% of the CA-IX–positive cells were also positive for miR-210. Moreover, using ImageJ JACoP analysis, a positive Pearson correlation coefficient between miR-210 expression ($r = 0.685$) and CA-IX positivity was found. These findings clearly indicate *in vivo* miR-210 expression in the hypoxic zones of NSCLC tissues.

miR-210 upregulation in melanoma and ~~NSCLCC~~ NSCLC under hypoxia is HIF-1α–dependent

We next measured the expression levels of miR-210 in hypoxic IGR-Heu and NA-8 tumor cells. As shown in Fig. 3A, hypoxia resulted in the significant induction of miR-210 after 6 hours (4-fold), 16 hours (6-fold), and 24 hours (10-fold) in IGR-Heu cells. Similarly, Fig. 3B shows that miR-210 was upregulated in NA-8 cells as early as 6 hours (2-fold), 16 hours (4-fold) and 24 hours (6-fold) of hypoxia induction. Similar results were obtained by using a panel of 5 human NSCLC and 5 human melanoma cell lines (Supplementary data S2). We further show that siRNA-mediated knockdown of HIF-1α with 3 different siRNAs efficiently silenced HIF-1α and completely inhibited miR-210 induction in IGR-Heu cells under hypoxia (Fig. 3C). Same results were obtained using NA-8 cells (Fig. 3D). Collectively, these results indicate that hypoxia-induced miR-210 is HIF-1α–dependent.

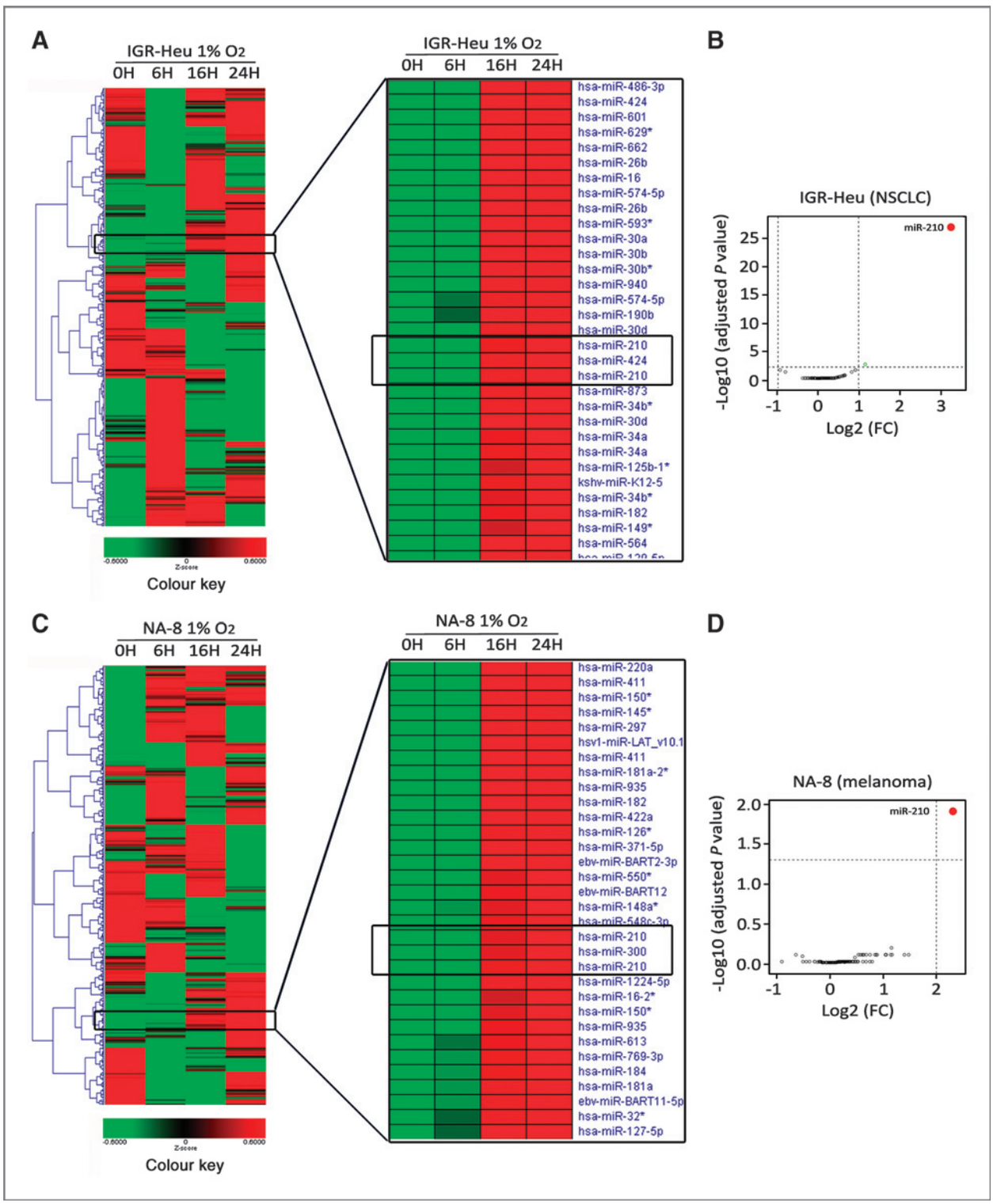
Hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated lysis

To investigate the potential role of hypoxia-induced miR-210 in regulating the hypoxic tumor cell susceptibility to CTL-mediated lysis, IGR-Heu and NA-8 tumor cells were transfected with anti-miR-210 (miR-210 inhibitor) or anti-miR-CT (miR-control inhibitor) and cultured under hypoxia. Transfection of these cells with anti-miR-210 resulted in the abrogation of miR-210 expression in both IGR-Heu (Fig. 3E) and NA-8 (Fig. 3F) cell lines as compared with anti-miR-CT (miR-control inhibitor).

We next examined the susceptibility of IGR-Heu and NA-8 tumor cells in which miR-210 expression had been abrogated to CTL-mediated lysis under hypoxic conditions. Interestingly, as shown in Fig. 3G, there was a significant and remarkable restoration of tumor cell susceptibility to CTL-mediated lysis in hypoxic IGR-Heu cells transfected with anti-miR-210 as compared with anti-miR-CT. More interestingly, this restoration of susceptibility to CTL-induced killing in miR-210 abrogated IGR-Heu tumor cells (anti-miR-210–transfected cells) was observed at all effector:target ratios (E:T) tested in comparison with control cells. Similarly, data depicted in Fig. 3H clearly show that hypoxic NA-8 cells transfected with anti-miR-210 were more susceptible to CTL-mediated lysis as compared with cells transfected with anti-miR-CT. Similar results were obtained for NA-8 cells with 2 different CTL clones (Supplementary data S3). These data strongly indicate that hypoxia-induced miR-210 plays an important role in the acquisition of tumor cell resistance to CTL-mediated lysis.

miR-210 in hypoxic cells does not alter tumor cell recognition and CTL reactivity

We next examined the influence of miR-210 on MHC class I molecule expression. As shown in Table 1, no difference in staining with HLA class I-specific antibody and HLA-A2–specific antibody was observed in hypoxic IGR-Heu cells transfected with miR-210 inhibitor (anti-miR-210) as compared with cells transfected with anti-miR-CT. Similarly, no difference was observed in normoxic IGR-Heu cells transfected with miR-210 precursor (pre-miR-210) as compared with miR-control precursor (pre-miR-CT). Similar results were obtained for NA-8 cells (Table 1). Next, we analyzed the reactivity of the



Q4 **Figure 1.** Hypoxic stress on tumor cells results in the upregulation of miRNA-210 (miR-210). A, heat map of the hypoxia regulated miRNAs in IGR-Heu cells. Red signifies upregulation, whereas green signifies downregulation. B, volcano plot of miRNA expression (Log2 fold change) and adjusted *P* values for IGR-Heu under hypoxia 1% O_2. C, miR-210 is significantly upregulated in NA-8 tumor cells. Heat map of the hypoxia regulated miRNAs in NA-8 cells. Red signifies upregulation, whereas green signifies downregulation. D, volcano plot of miRNA expression (Log2 fold change) and adjusted *P* values for NA-8 under hypoxia (1% O_2). Data shown are representative of 2 independent experiments.

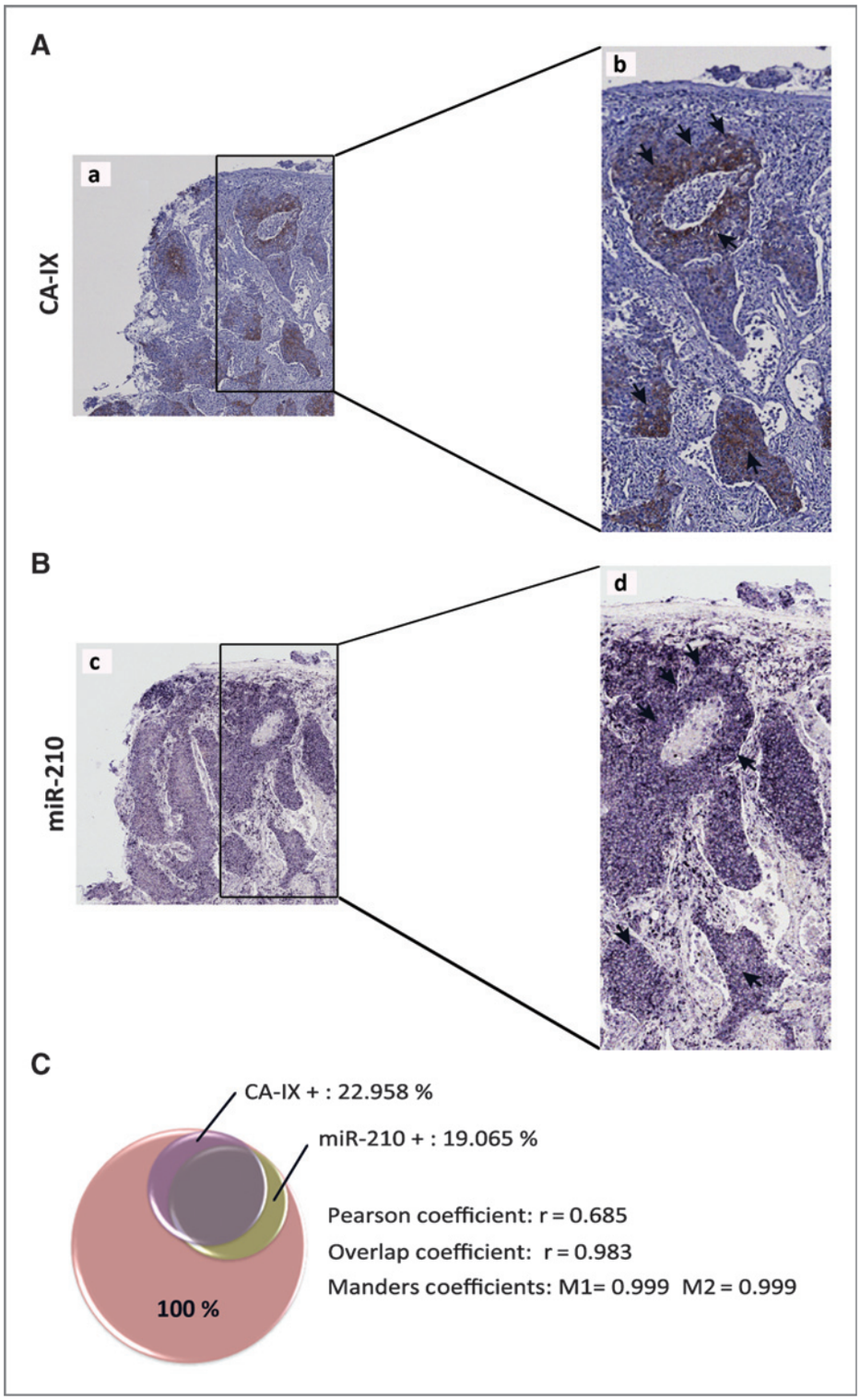


Figure 2. Hypoxia induced miR-210 staining in human NSCLC tissues colocalizes with CA-IX. Human NSCLC biopsies were stained for CA-IX expression by immunohistochemistry and miR-210 expression by *in situ* hybridization. (A) and (B) are representative images of CA-IX/miR-210 staining on serial sections of specimen (IGR-2220). C, Venn diagram representation of the staining analysis of CA-IX and miR-210 showing overlap on serial sections of specimen (IGR 2220). Colocalization analysis was conducted by using JACoP Plug-in in ImageJ software. Arrows represent areas positively stained for CA-IX and miR-210. Magnification: ×50.

CTL clone to hypoxic autologous targets with abrogated miR-210. We observed no difference in TNF-α and IFN-γ production by the autologous T-cell clone in response to stimulation with IGR-Heu cells (Fig. 4A and C) and NA-8 cells (Fig. 4B and D) with abrogated (anti-miR-210) or overexpressed miR-210 (pre-miR-210). These results clearly show that miR-210 does not alter tumor cell recognition and CTL priming in IGR-Heu and NA-8 tumor cells.

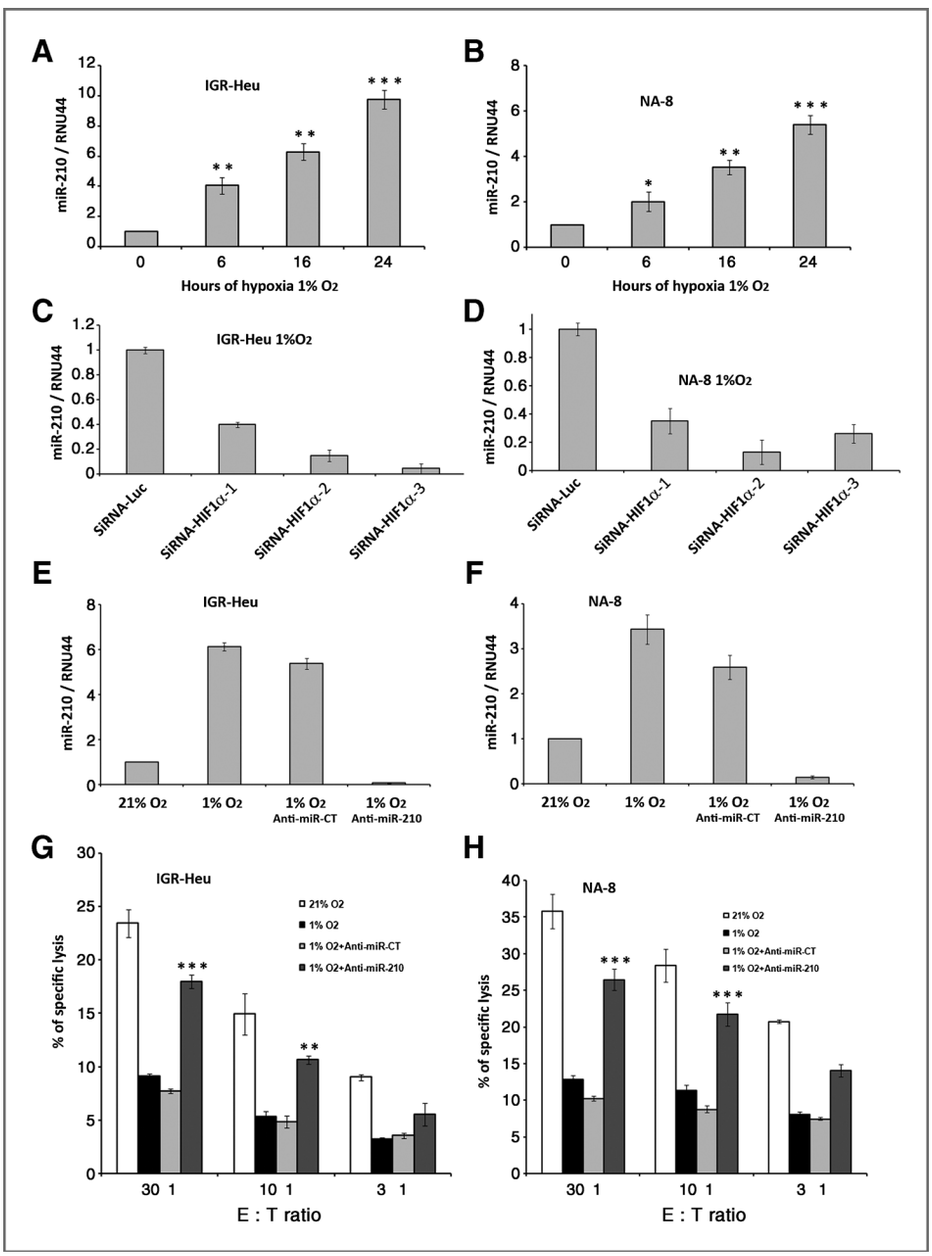


Figure 3. Hypoxia induced miR-210 is HIF-1α–dependent in IGR-Heu cells and targeting miR-210 by anti-miR-210 under hypoxia resulted in the restoration of tumor cell susceptibility to CTL-mediated lysis. A, miR-210 expression was monitored by TaqMan qRT-PCR in IGR-Heu with or without exposure to 1% O_2 hypoxia at indicated times. B, miR-210 expression was monitored by TaqMan qRT-PCR in NA-8 with or without exposure to 1% O_2 hypoxia at indicated times. Expression levels of RNU44 were used as endogenous control. C, expression of miR-210 in IGR-Heu tumor cells transfected with different siRNAs targeting either HIF-1α or Luciferase (Luc) and cultured under hypoxic conditions (1% O_2) for 24 hours. D, expression of miR-210 in NA-8 cells transfected with different siRNAs targeting either HIF-1α or Luciferase (Luc) and cultured under hypoxic conditions (1% O_2) for 24 hours. E, TaqMan qRT-PCR showing successful knockdown of hypoxia-induced miR-210 in IGR-Heu tumor cells and in (F) NA-8 cells. G, CTL-mediated lysis of IGR-Heu tumor cells at different E:T ratios. Heu 171 cells were used as effectors. Data represents 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$). H, CTL-mediated lysis of NA-8 cells at different E:T ratios. R2C9 TIL clone was used as effector.

Table 1. Anti-miR-210 and pre-miR-210 does not affect tumor cell recognition in IGR-Heu and NA-8 tumor cells

	(NSCLC) IGR-Heu				(Melanoma) NA-8			
	HLA Class I		HLA-A2		HLA Class I		HLA-A2	
Conditions	21%O_2	1%O_2	21%O_2	1%O_2	21%O_2	1%O_2	21%O_2	1%O_2
Medium	99.9% (8444)	99.8% (9083)	99.5% (6151)	99.8% (6150)	99.91% (635.62)	99.96% (590.09)	99.92% (113.19)	99.91% (84.71)
Pre-miR-CT	99.8% (8222)		99.4% (6121)		99.90% (625.25)		99.90% (109.85)	
Pre-miR-210	97.5% (7745)		99.5% (5609)		99.87% (580.02)		99.91% (105.78)	
Anti-miR-CT		99.9% (7560)		99.7% (5365)		99.97% (580.12)		99.89% (87.89)
Anti-miR-210		99.9% (7599)		99.3% (5988)		99.98% (595.56)		99.90% (90.55)

NOTE: Analysis of surface expression of MHC class-I in IGR-Heu and NA-8 tumor cells under different conditions. HLA class I and HLA-A2 surface expression was detected by using W632 and MA2.1 antibodies respectively. Isotypic control mAb (IgG) was used.

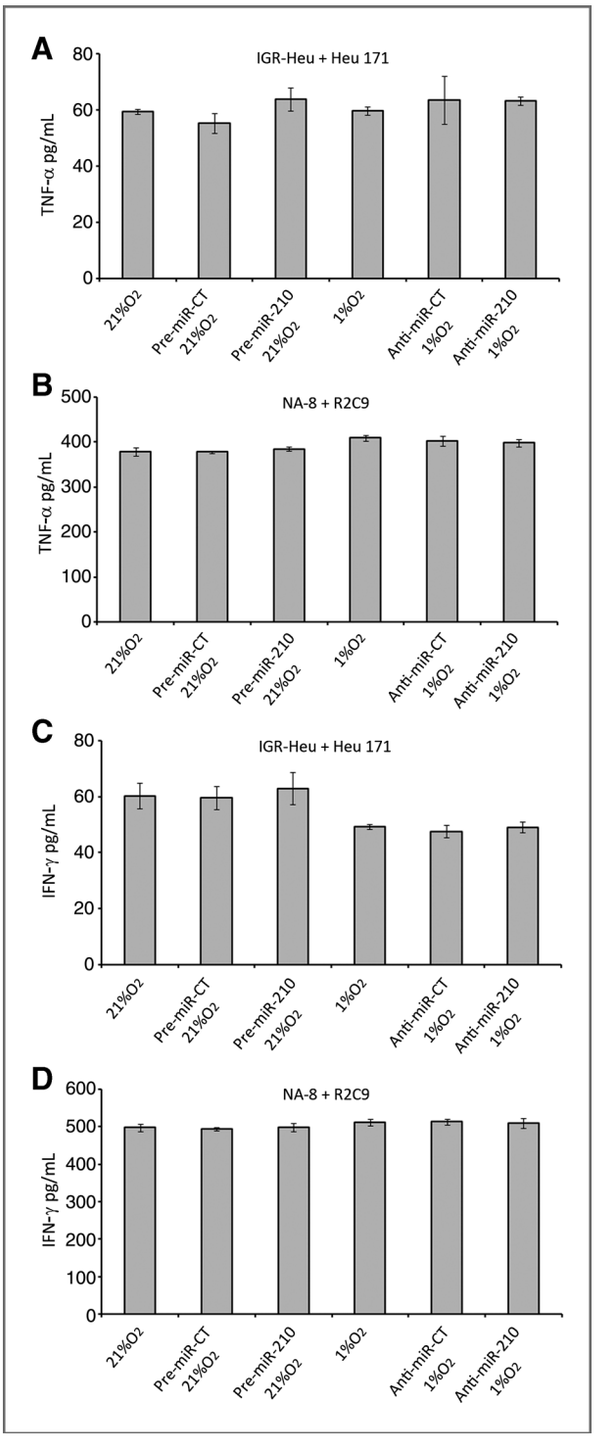


Figure 4. Anti-miR-210 and pre-miR-210 does not affect CTL priming in IGR-Heu and NA-8 tumor cells. A–D, TNF-α and IFN-γ production by the CTL clones Heu 171 (cocultured with IGR-Heu) and R2C9 (cocultured with NA-8) for 24 hours under different conditions. Data represents 3 independent experiments with SD.

miR-210 has no effect on hypoxia-induced transcription factors (HIF1α, HIF2α, and pSTAT3) in IGR-Heu cells

It has been well established that HIF1α, HIF2α, and pSTAT3 are the main mediators of hypoxia-induced responses in tumors (19, 20). We next wondered whether miR-210 is able to regulate these hypoxia-induced factors (HIF1α, HIF2α, and pSTAT3) in IGR-Heu cells. We observed that both HIF1α and pSTAT3 were increased under hypoxic conditions (6, 16, 24, 48, and 72 hours of 1% O_2). However, abrogation or overexpression of miR-210 (anti-miR-210 in hypoxic IGR-Heu cells and pre-miR-210 in normoxic cells) had no effect on HIF-1α and pSTAT3 protein levels. We also quantified, using the same experimental conditions, changes in mRNA expression levels of HIF1α, HIF2α, VEGF, and GLUT, no difference was observed (Supplementary data S4).

Identification of miR-210 candidate target genes in IGR-Heu tumor cells

We next conducted a comprehensive transcriptome analysis using RNA from IGR-Heu cells transfected with anti-miR-210 or anti-miR-CT under hypoxia. We selected a panel of 44 genes (validated miR-210 targets) from a miRNA database, miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/), which has the largest number of validated miRNA-target interactions (MTI).

We selected 11 genes that meet the following criteria:

1. Significantly downregulated under hypoxia as compared to normoxia.
2. Significantly upregulated when hypoxic IGR-Heu cells were transfected with anti-miR-210 as compared to transfected with anti-miR-CT.
3. Significantly downregulated when transfected with pre-miR-210 under normoxic conditions (Supplementary data S5).

As shown in Fig. 5A and B, the expression levels of ISCU, E2F3, FGFRL1, NPTX1, PTPN1, HOXA1, TP53I11, RAD52, and EFNA3 were, respectively, increased or downregulated following anti-miR-210 and pre-miR-210 transfection in IGR-Heu cells.

We selected 3 of these 11 identified genes (PTPN1, HOXA1, and TP53I11) based on their involvement in tumor cell death and apoptosis. These identified target genes were further validated by Western blot analysis. Figure 5C clearly shows that the protein level of PTPN1 was significantly decreased by pre-miR-210 as compared with pre-miR-CT under normoxia, and significantly increased when IGR-Heu cells were transfected by anti-miR-210 as compared with anti-miR-CT under hypoxia. Similar results were obtained for HOXA1 (Fig. 5D) and TP53I11 (Fig. 5E). PIM1 was used as control (Fig. 5F). Taken together, these results point to the regulation of PTPN1, HOXA1, and TP53I11 at both mRNA and protein levels by miR-210.

Validation of PTPN1, HOXA1, and TP53I11 as miR-210 target genes

To validate the identified targets, we inserted miR-210 binding sites sequence from the 3′ UTR of PTPN1, HOXA1, and TP53I11 mRNA (Fig. 6A) into the 3′ UTR of pSI-check2

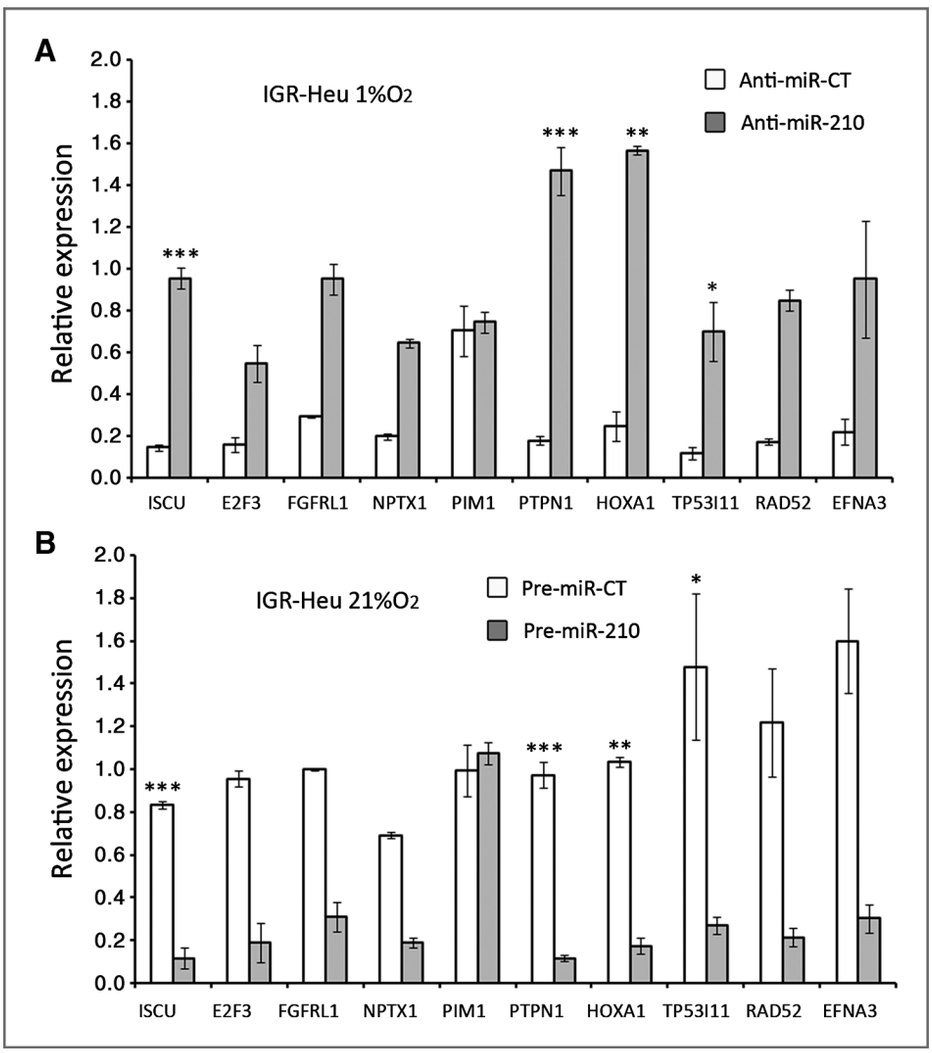


Figure 5. Confirmation of a panel of mirtarbase (MTI-miRNA target identification) database-identified targets in IGR-Heu tumor cells. A, IGR-Heu tumor cells were transfected with anti-miR-210 or anti-miR-CT used as a control and cultured under hypoxia (1% O_2) for 24 hours. ISCU, E2F3, FGFRL1, NPTX1, PIM1, PTPN1, HOXA1, TP53I11, RAD52, and EFNA3 expression was monitored by SYBR-GREEN qRT-PCR. B, IGR-Heu tumor cells were transfected with pre-miR-210 or pre-miR-CT used as a control and cultured under normoxia (21% O_2) for 24 hours. ISCU, E2F3, FGFRL1, NPTX1, PIM1, PTPN1, HOXA1, TP53I11, RAD52, and EFNA3 expression was monitored by SYBR-GREEN RT-qPCR. Expression level of 18S was used as endogenous control.

vector and conducted luciferase reporter assays. We show that pre-miR-210 significantly reduced the luciferase activities of PTPN1, HOXA1, and TP53I11 reporters by 65% to 40% and 25%, respectively, as compared with pre-miR-CT (Fig. 6B). In contrast, pSI-check2 (empty vector control) and PIM1 reporter luciferase activities were not repressed by pre-miR-210, confirming that PTPN1, HOXA1, and TP53I11 target sites directly mediates repression of the luciferase activity through seed-specific binding. In addition, we used a biochemical assay based on the immunoprecipitation of RNA-induced silencing complexes (RISC) enriched for miR-210 and its targets. To accomplish this, IGR-Heu cells were stably transfected with pIRESneo-FLAG/HA Ago2 and cultured under normoxic or hypoxic conditions for 24 hours. miR-210 was selectively enriched in Ago2-HA immunoprecipitates under hypoxia as compared with normoxia, suggesting that the argonaute protein immunoprecipitation (miRNP-IP) worked efficiently (Supplementary data S6). To further validate this, IGR-Heu cells stably expressing pIRESneo-FLAG/HA Ago2 were transfected with pre-miR-210 or pre-miR-CT and cultured under normoxia. First, we showed that ISCU and EFNA3, 2 well-established miR-210 targets (24), were significantly enriched in normoxic pre-miR-210-Ago2-HA immunoprecipitates (Fig. 6C). Furthermore, as shown in Fig. 6C, PTPN1, HOXA1, and TP53I11 were selectively and significantly increased in the immunoprecipitates of the IGR-Heu cells transfected with pre-miR-210 as compared with pre-miR-CT. It is worth noting that B2M, GAPDH, and PIM1 remain unchanged (Fig. 6D). These results clearly show that PTPN1, HOXA1, and TP53I11 are validated targets of miR-210 in IGR-Heu cells.

miR-210 modulates IGR-Heu tumor target cell susceptibility to CTL-mediated lysis by targeting PTPN1, HOXA1, and TP53I11

We next asked whether miR-210 confers resistance in hypoxic tumor targets to CTL-mediated lysis by degrading its target genes (PTPN1, HOXA1, and TP53I11). Using a ^{51}Cr cytotoxicity assay and target gene silencing, we show that siRNA-mediated silencing of PTPN1 resulted in a significant decrease in the IGR-Heu tumor cell susceptibility to CTL-

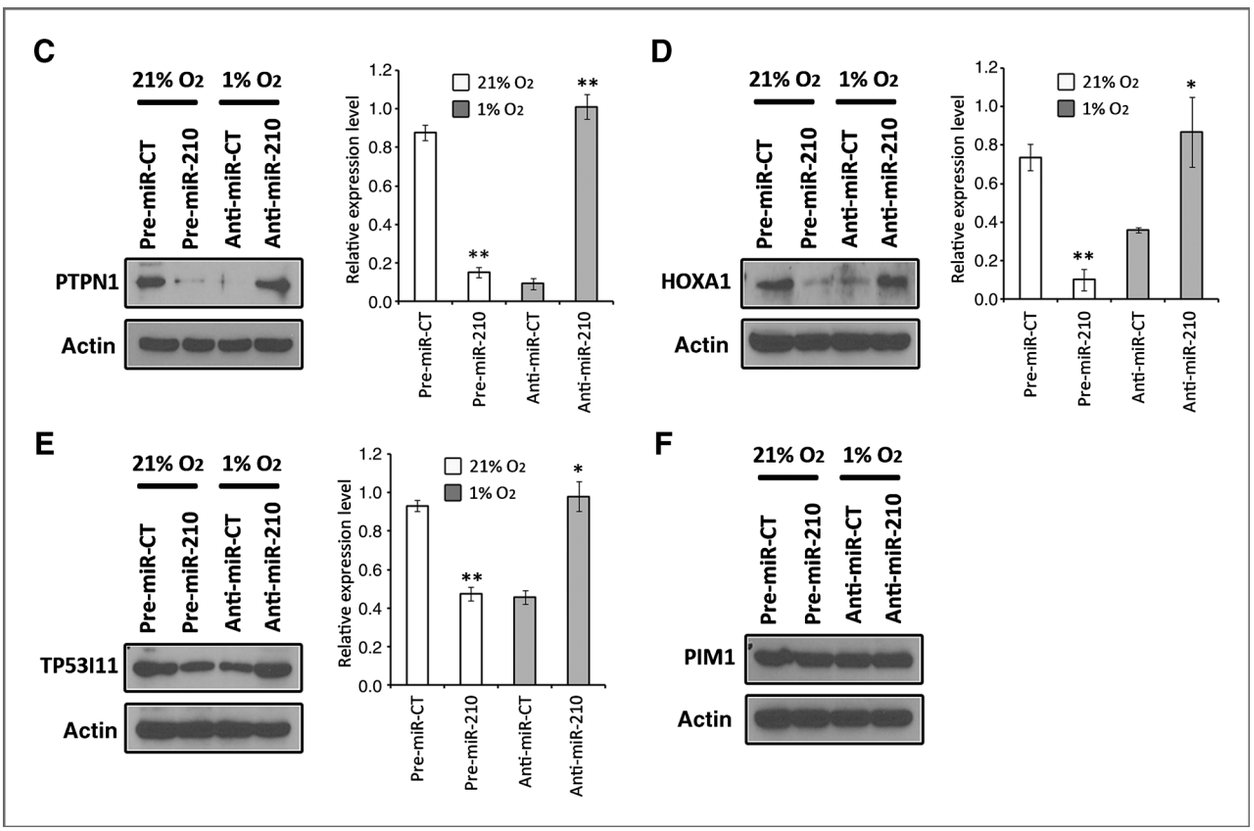


Figure 5. (*Continued*) C–F, IGR-Heu tumor cells were transfected with either anti-miR-210 or anti-miR-CT and cultured under hypoxia (1% O_2) or transfected with pre-miR-210 or pre-miR-CT and cultured under normoxia (21% O_2) for 24 hours. Western blot analysis and densitometry analysis were conducted to show PTPN1 (C), HOXA1 (D), and TP53I11 (E) protein levels, which were significantly regulated by miR-210. F, PIM1 is used as a control. Data represents 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$, and ***, $P < 0.0005$).

mediated lysis (Fig. 7A). However, siRNA silencing of HOXA1 (Fig. 7B) or TP53I11 (Fig. 7C) had no effect on tumor cell susceptibility to CTL-mediated lysis. Surprisingly, as depicted in Fig. 7D, siRNA silencing of all 3 genes (PTPN1, HOXA1, and TP53I11) resulted in a dramatic decrease in target susceptibility to CTL-mediated lysis. Taken together, these results strongly show that miR-210 is able to confer resistance in hypoxic target cells by degrading PTPN1, HOXA1, and TP53I11.

Discussion

Tumors have evolved to use hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important in angiogenesis, cell-cycle regulation, cell survival, progression, survival, metastasis, and resistance to apoptosis (9–11, 19). Tumor resistance remains a crucial issue in immunotherapy, as it limits the effectiveness of cancer immunotherapy (5, 6). It has become clear that a hypoxic tumor microenvironment plays a determining role in neutralizing the immune system of the host and negatively impacting the outcome of therapy (26). We observed a significant miRNA dysregulation between normoxic and hypoxic melanoma and NSCLC cells (Supplementary data S1). This is in agreement with the concept that tumors may adapt to hypoxic stress in different ways depending on their genetic background and phenotypic characteristics (10).

To our knowledge, this is the first time we showed that miR-210 is expressed in the hypoxic zones of NSCLC tissues and in hypoxic melanoma tumor cells. Our results support the claim that induction of miR-210 is the most important hypoxia-induced miR in different cancer types (17, 27). miR-210 is also generally recognized as a robust HIF1 target (16, 28) and an *in vivo* marker of tumor hypoxia (29, 30). The increased expression of miR-210 correlates with a poor prognosis in patients with breast and pancreatic cancer (31, 32). Zhang and colleagues provided evidence indicating that miR-210 levels are high in tumors and correlate with their metastatic behavior, suggesting a potential oncogenic role for miR-210 (33, 34).

Although the role of miR-210 in tumorigenesis, angiogenesis, mitochondrial metabolism, cell survival, and DNA repair has been well characterized (17), its role in the immune response is less well understood, in particular, its role in the regulation of tumor cell susceptibility to antigen-specific killer T cells. We showed for the first time that expression of miR-210 under hypoxic conditions correlates with the alteration of tumor susceptibility to CTLs by a mechanism independent of target recognition and alteration of CTL reactivity. Mandelboim and colleagues have shown that several human miRNAs are able to

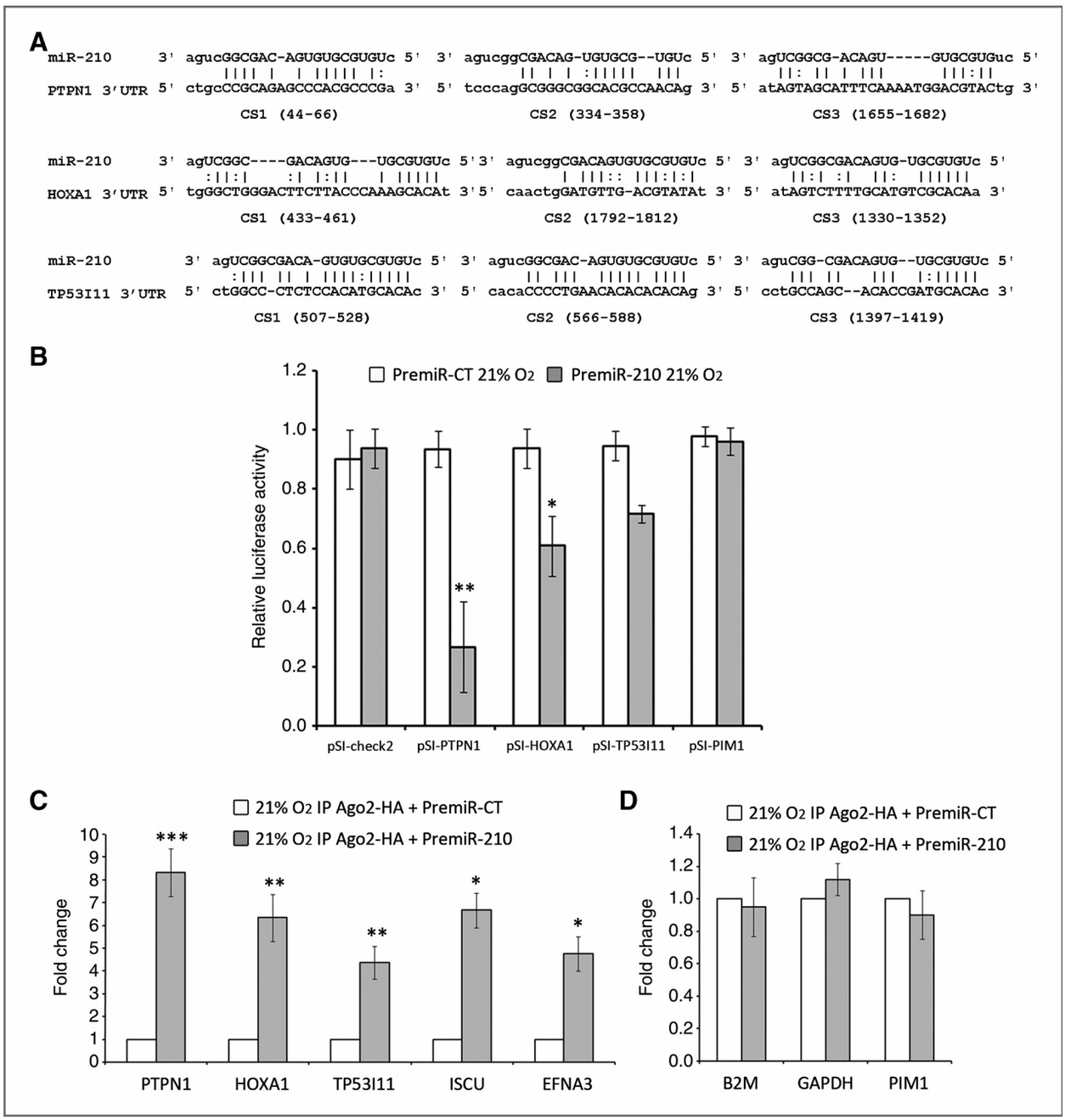


Figure 6. Confirmation of the identified miR-210 target genes. A, schematic view of miR-210 complementary sites (CS) in the 3′ UTR of PTPN1, HOXA1, and TP53I11 genes (predicted by miRanda). B, IGR-Heu cells were cotransfected with 10 nmol/L Pre-miR-CT or Pre-miR-210 and different pSI-Check-2 constructs. After 48 hours, cells were harvested and luciferase activities analyzed. All *Renilla* luciferase activities were normalized to the firefly luciferase activity. pSI-PTPN1, pSI-HOXA1, pSI-TP53I11, and pSI-PIM1 correspond to distinct fragments of PTPN1, HOXA1, TP53I11, and PIM1 3′UTR containing miR-210 putative binding sites, respectively. pSI-check2 was used as an empty vector control. C, IGR-Heu cells stably expressing pIRESneo-FLAG/HA Ago2 were cotransfected with pre-miR-210 or pre-miR-CT and cultured under normoxia (21% O_2) for 24 hours. Anti-HA antibody was used to immunoprecipitate the miR-210/HA-Ago2–containing complexes. Anti-IgG antibody was used as IP-CT (immunoprecipitation control). RNA was purified and specific mRNAs were measured by SYBR GREEN qRT-PCR. D, B2M, GAPDH, and PIM1 did not display any significant differences and thus were used for normalization. Data represents 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$).

regulate stress-induced immune responses mediated by the NKG2D receptor (35). It has been recently reported that frequent loss of miR-34a/miR-34c expression helps metastatic melanoma cells to escape the tumor immune surveillance by enhancing ULBP2 expression (36). Similarly, a report of Udea and colleagues indicate that dicer-regulated miR-222 and miR-

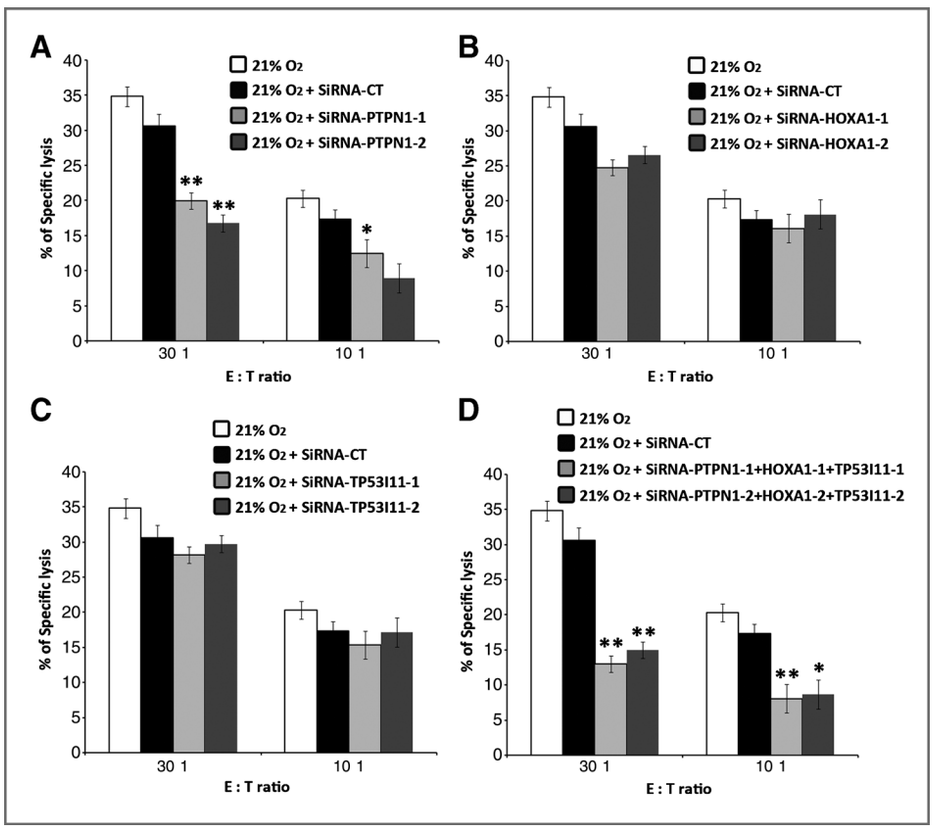


Figure 7. miR-210 targets PTPN1, HOXA1, and TP53I11 modulate IGR-Heu tumor target cell susceptibility to CTL-mediated lysis. IGR-Heu tumor cells were transfected with 2 different siRNA targeting either PTPN1 (A), HOXA1 (B), or TP53I11 (C), or siRNA targeting PTPN1 + HOXA1 + TP53I11 (D), or Luciferase (Luc) used as a control and cultured under normoxic conditions (21% O_2) for 24 hours. Heu 171 cells were used as effectors. Data represents 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$, **, $P < 0.005$; and ***, $P < 0.0005$).

339 promote resistance of cancer cells to CTLs by down regulating ICAM and subsequently affecting immune regulation (37).

To get more insight into how miR-210 confers resistance to hypoxic tumor targets to CTL-mediated lysis, we conducted a comprehensive transcriptome analysis to identify miR-210 target genes. Among these genes, E2F3, FGFRL1, and RAD52 have already been reported as validated targets of miR-210 involved in cell-cycle regulation and DNA repair (17, 38). However, we observed no effect on cell-cycle regulation when IGR-Heu cells were transfected with either anti-miR-210 or pre-miR-210 (data not shown). We showed that the expression levels of PTPN1, HOXA1, and TP53I11, were respectively upregulated or downregulated following anti-miR-210 or pre-miR-210 transfection in IGR-Heu cells. We also found that simultaneously silencing PTPN1, HOXA1, and TP53I11 resulted in a dramatic decrease in target susceptibility to CTL-mediated lysis. Among these 3 genes, PTPN1 has been shown to play a role in immune regulation. PTPN1 is a negative regulator of cytokine receptors and receptor tyrosine kinases in lymphohematopoietic cells (39). Knockdown of endogenous PTPN1 expression increases production of TNF-α, IL-6, and IFN-β in TLR-triggered macrophages (40). PTPN1 has been shown to function as a critical negative regulator of inflammatory responses (41). In addition, PTPN1 and TP53I11 have been reported to be involved in the regulation of cell survival and apoptosis (42–46). HOXA1 has been shown to be involved in cell proliferation and tumor initiation (30). It would be of major interest to determine how these genes interfere with CTL-induced hypoxic tumor cell death.

In conclusion, we provide evidence that hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated lysis by a mechanism involving its downstream targets PTPN1, HOXA1, and TP53I11. Taken together, our studies show that miR-210 is mechanistically linked to the regulation of the antigen-specific tumor cell lysis and suggest that in addition to its potential as a prognostic biomarker, miR-210 may have therapeutic applications in the field of cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.Z. Noman, S. Buart, B. Janji, S. Chouaib
Development of methodology: M.Z. Noman, S. Buart, B. Janji, S. Chouaib
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.Z. Noman, P. Romero, S. Ketari, F. Mami-Chouaib, S. Chouaib
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.Z. Noman, S. Buart, S. Ketari, B. Mari, S. Chouaib
Writing, review, and/or revision of the manuscript: M.Z. Noman, S. Buart, P. Romero, B. Janji, F. Mami-Chouaib, S. Chouaib
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.Z. Noman, S. Chouaib
Study supervision: M.Z. Noman, B. Janji, F. Mami-Chouaib, S. Chouaib

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DISCUSSION AND CONCLUSIONS

A. Hypoxia-mediated inhibition of specific tumor cell lysis

The efficacy of antitumor CTL critically depends on functional processing and presentation of tumor antigens by the malignant cells but also on their susceptibility to CTL-induced lysis. Tumor rejection in patients does not always follow successful induction of tumor-specific immune responses by cancer vaccine immunotherapy [342]. Even, if a strong and sustained cytotoxic response is induced, complex issues such as tumor evasion and selection of tumor resistant variants remain. In this respect solid tumor with disorganized, insufficient blood supply contains hypoxic cells that are resistant to classical cytotoxic treatments [357].

Hypoxia-induced HIF-1α is associated with a decrease in CTL-mediated tumor cell lysis

Our results provided the first demonstration that tumor cells, through their adaptation to hypoxic stress, impede CTL cytotoxic activity independently of their potential to trigger CTL reactivity. However, our data differ from earlier report of MacDonald [358]. This discrepancy may lie in the fact that the reported data were collected using mice system, CTLs generated in mixed leukocyte cultures and hypoxic chambers whereas we respectively used human antigen specific CTL clone and hypoxic station. These observations are consistent with the notion that hypoxia-induced adaptive changes contribute to alterations that lend towards a surviving phenotype. Thus, it is conceivable to imagine that tumors frequently develop this specific strategy to shift the balance from immune surveillance to tolerance. In this context, the various strategies aimed at the induction of antitumor cytotoxic responses should consider the crucial role of tumor hypoxia as an additional antitumor mechanism of tumor escape that is partly involved in resistance of tumor cells to antigen specific cytotoxicity.

Hypoxia confers resistance to CTL-mediated lysis by STAT3 and AKT phosphorylation

It is well established that hypoxia-induced gene transcription promotes characteristic tumor adaptations, including resistance to cytotoxic treatments [2]. However, the mechanisms underlying this resistance still need deeper understanding. Although increasing evidence supports a link between hypoxia and resistance to apoptosis [359], the mechanism by which the hypoxic stress inhibits apoptosis is not well understood and remains unclear. In this regard it has been suggested that alterations in the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins may play a role in this process [83]. In addition HIF-1α mediated increase in glucose

uptake has been also reported to play an important role in conferring apoptosis resistance and that its effect is mediated in part via Mcl-1 gene expression [360]. To gain a better understanding of the influence of hypoxia on non-small lung cancer cells resistance to CTLs, we asked whether it interferes with the expression of p53, survivin, AKT, and STAT3 pathways. Under our experimental conditions, we failed to observe any p53 and survivin induction in hypoxic NSCLC suggesting that the expression of HIF-1α and these proteins may be coupled during the hypoxia response depending on the cell type tested and confirming the existence of a cell specific effect of hypoxia [361]. We observed that culture of IGR-Heu cells under hypoxic conditions resulted in a dramatic induction of STAT3 tyrosine phosphorylation and that SiRNA knock down of STAT3 resulted in an inhibition of HIF-1α induction. This suggests that STAT3 is an essential component of HIF-1 pathway under hypoxic conditions and fits with a recent report indicating that hypoxia-phosphorylated STAT3 up-regulates HIF-1α stability through delaying protein degradation and accelerating protein synthesis in human renal cell carcinoma [345, 362].

It is also important to note that STAT3 activation has been associated with cytokine-induced proliferation, anti-apoptosis, and transformation [363]. It should be noted that activated STAT3 in cancer cells does not just function as a mediator of intracellular signalling but also affects cell-cell interaction. In this respect it is now well established that that STAT3 modulates the cross talk between tumor and immune cells [295, 364]. Very recently a novel small molecule inhibitor of STAT3 has been reported to reverse immune tolerance in malignant glioma patients [365]. It would be of interest to investigate the use of such an inhibitor to attenuate hypoxic tumor resistance to CTL-mediated cytotoxicity.

VEGF is involved in STAT3 phosphorylation under hypoxia in tumor cells

More interestingly, we have shown that VEGF neutralization resulted in the attenuation of hypoxic tumor target resistance to CTL-mediated killing and that hypoxia induced STAT3 phosphorylation can be stimulated by autocrine signalling through VEGF. In this regard, it is very likely that stat3 activation is associated with the regulation of target gene expression potentially involved in the alteration of hypoxic tumor target specific killing. Therefore, understanding how VEGF and other soluble factors may lead to STAT3 activation via the tumor microenvironment may provide a more effective cancer treatment strategy for hypoxic tumors with elevated p-STAT3 levels. This also suggests that reduction of VEGF release, a main immunosuppressive factor, in tumor microenvironment may favour induction of a stronger anti-tumor CTL response against tumors expressing VEGF receptors. Our studies are

in agreement with reports suggesting that inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer [366] and indicating a synergy between tumor immunotherapy and anti-angiogenic therapy [367].

Our data provide evidence that hypoxia inhibits tumor specific lysis and suggest that it induces cellular adaptation that compromise the effectiveness of killer cells and point to the potential role of STAT3 in tumor adaptation induced by hypoxia. In this regard, better understanding of the tumor behaviour and its interplay with the killer cells in the context of the complexity and plasticity of a hypoxic microenvironment will be a critical determinant in a rational approach to tumor immunotherapy. Although resistance of tumor targets to killer cells is likely to be regulated by multiple factors [343], the data we present herein suggest that hypoxic microenvironment is an important determinant involved in the control of target sensitivity to CTL-mediated lysis. Therefore the possibility that novel approaches targeting HIF-1α and STAT3 with potent small molecule drugs, being actively developed, may provide an exciting novel approach for cancer immunotherapy.

B. Autophagy and hypoxia-induced immune resistance

Hypoxia is a common feature of solid tumors and a major limiting factor in successful cancer treatment [160]. Given the role of autophagy in induction of the adaptive immune system [349] and cellular survival under hypoxia [368], we sought to determine the molecular basis of the hypoxia/autophagy interaction and its interference with tumor cell survival and growth.

Targeting hypoxia induced autophagy restores IGR-Heu tumor cell susceptibility to CTL-mediated lysis

We demonstrate that hypoxia-induced autophagy acts as a tumor cell resistance mechanism to specific T-cell-mediated lysis. Targeting autophagy was sufficient to significantly restore tumor cell susceptibility to CTL-mediated lysis. It has been suggested that the unfolded protein response (UPR) enhances the capacity of hypoxic tumor cells to activate autophagy by PERK and ATF4-dependent upregulation of Atg5 and LC3 mRNA [351]. This mechanism seems to be unlikely involved in the induction of autophagy in hypoxic IGR-Heu cells because there is no evidence for the activation of PERK and ATF4 and for the upregulation of Atg5 and LC3 mRNA. However, we demonstrate that hypoxia induces autophagy by BNIP3/BNIP3L-dependent displacement of the autophagy inhibitory complex Beclin1/Bcl-2 [84].

Autophagy and ubiquitin proteasome system cooperate to regulate STAT3 phosphorylation in hypoxic IGR Heu cells.

We attempted to investigate the role of autophagy in the regulation of tumor susceptibility to CTL-mediated lysis by blocking autophagy. While much still remains to be learned mechanistically, our data demonstrate that autophagy inhibition compromised the hypoxia-dependent induction of pSTAT3. Consistent with the clearance function of autophagy, it is difficult to reconcile the idea that the autophagy machinery plays a role in a specific stabilization of pSTAT3 and/or pSrc. Indeed, it is now well documented that there is a crosstalk between the UPS and autophagy [369]. Based on our results we believe that the decreased level of pSTAT3 in hypoxic and autophagy defective IGR-Heu cells is related to its degradation by UPS. This was supported by our data demonstrating that hypoxia induces a polyubiquitination of pSTAT3 and our results showing that inhibition of UPS by bortezomib accumulates pSTAT3 in hypoxic and autophagy-defective IGR-Heu cells. It is noteworthy that a UPS-dependent degradation of phospho-p53 [370] and phospho-PKC-delta [371] has been previously described. The interplay between autophagy and UPS is mainly mediated by the adaptor protein p62/SQSTM1 which plays a role as a cargo for targeting proteins to proteasomes degradation and autophagy [372]. Because p62 is an autophagic substrate, an accumulation of this protein was observed in autophagy deficient cells. In this regard, it has been recently reported that an excess of p62 inhibits the clearance of ubiquitinated proteins destined for proteasomal degradation by delaying their delivery [372]. Our results show discrepancy with this mechanism as we show that the accumulation of p62 in autophagy defective cells correlates with a decrease, rather an accumulation, of pSTAT3 in hypoxic IGR-Heu cells, and targeting p62 in autophagy-defective cells reaccumulates pSTAT3. While additional work needs to be performed to clarify this discrepancy, one possible reason is that, degradation of p62 by autophagy in IGR-Heu cells under hypoxia could represent a feedback mechanism to restrict further autophagy and excessive destruction of proteins under hypoxia. Such a feedback mechanism has been recently described [373].

In vivo inhibition of autophagy potentiates the antitumor effect of tyrosinase-related protein-2 peptide vaccination

Vaccine approaches have proven effective in enhancing antitumor immunity against tumor cells expressing targeted antigens *in vitro* [374]. However, clinical and animal studies have shown little success in the inhibition of tumor growth by vaccines [375]. One of the most important factors that could be responsible for this failure is the hypoxia-dependent activation

of prosurvival pathways. Inhibition of autophagy has been reported to sensitize tumor cells to cytotoxic treatments including anti-tumoral agents and irradiation [376]. In light of our *in vitro* observations, we asked whether targeting of hypoxia-induced autophagy could influence *in vivo* tumor growth. To address this issue, we used the transplantable murine melanoma B16-F10 cell line that expresses different tumor-associated antigens TAA, including TRP2. Our data showed that autophagy is primarily localized to tumor hypoxic regions as previously reported in other tumor models [351]. We also show that targeting autophagy reduced tumor growth which correlated with an increase in apoptosis. These observations are in agreement with recent reports indicating that inhibition of autophagy by HCQ inhibits *in vitro* cell growth and *in vivo* tumor growth via induction of apoptosis [377, 378]. Our results are also supported by several studies showing that inhibition of autophagy promotes cancer cell death [379] and potentiates anti-cancer treatments [350] [380]. HCQ has been used *in vitro* for the inhibition of autophagy in several tumor models including melanoma [381] and is currently being tested in different ongoing Phase II studies [382].

Finally with regards to potential therapeutic treatment, we show that the combination of TRP2 peptide vaccination with HCQ treatment results in a strong inhibition of tumor growth. These observations further establish the significance of hypoxia in inducing autophagy, demonstrate that it is an adverse prognostic factor and confirm its critical role not only in radio- and chemoresistance but also in immune effector cell-resistance. A better understanding of the hypoxic tumor context is likely to improve the prospect of developing effective cancer immunotherapy. Together, our results show that targeting autophagy could have significant therapeutical implications for tumor progression and extend the notion that simultaneously boosting the immune system and targeting of autophagy could enhance the therapeutic efficacy of cancer vaccines and may prove beneficial in cancer immunotherapy.

PERSPECTIVES

Cancer vaccines are expected to augment already established anti-tumor immune responses and to induce de novo immunity or reverse tolerance. Even, if a strong and sustained cytotoxic response is induced, complex issues remain such as tumor evasion, tolerance and selection of tumor resistant variants [374, 375] [270]. The hypoxia's critical role in radioresistance, chemoresistance, tumor stemness and its significance as an adverse prognosis factor have been well established [2]. In addition, a direct link between tumor hypoxia and tolerance through the recruitment of regulatory cells has been established [339]. Hypoxia is therefore attracting a particular attention in the field of tumor immune biology since hypoxic stress impact angiogenesis, tumor progression and immune tolerance. Important questions remain to answer as how hypoxia favors the emergence of a resistant phenotype? Whether such phenotype is resistant to anti-tumor immune effectors? And how peripheral immune tolerance and hypoxic programs are closely connected and cooperate under hypoxic conditions to sustain tumor growth?

Hypoxia-mediated inhibition of specific tumor cell lysis

Although we have clearly shown that the concomitant hypoxic induction of pSTAT3 and HIF-1α are functionally linked to the alteration of NSCLC target susceptibility to CTL-mediated killing, the mechanisms underlying this resistance still need a deeper understanding and a number of issues need to be further investigated.

We have already observed that hypoxic tumor targets are resistant to Granzyme B, it would be interesting to investigate the molecular mechanisms underlying this resistance, more importantly the activation of caspases (caspase3 and caspase8), p53 accumulation, the pro-apoptotic genes (IAPs, survivin, Mcl-1, Bcl-xL and Bcl2 family members) and various regulators of mitochondrial apoptotic pathway? It would be of great interest to investigate the effect of in-vivo inhibition of tumor hypoxia on the antitumor cytotoxic response (quality and quantity of tumor infiltra-CD8, Treg and MDSC) after vaccination under the conditions of hypoxia inhibition.

Considering the eminent functions of STAT3 and HIF-1α in the tumor microenvironment, their targeting by potent small molecules, being actively developed, may provide an exciting novel approach for cancer immunotherapy.

Autophagy and hypoxia-induced immune resistance

We have shown that hypoxia-induced autophagy impairs CTL-mediated tumor cell lysis by regulating phospho-STAT3 in target cells. More interestingly, simultaneous boosting of the CTL-response (TRP2-peptide vaccination) and targeting autophagy in hypoxic tumors, improved the efficacy of cancer vaccines by promoting tumor regression in vivo.

Transgenic mouse models of spontaneous oncogene-driven tumors that are deficient in autophagy could be useful to explore the impact of the hypoxic tumor microenvironment and autophagy inhibition on the antitumor immune responses. Very recently, Michaud et al. have shown that in mice, chemotherapy-induced autophagy causes can stimulate antitumor immune responses [383]. It would be interesting to study whether the inhibition of autophagy directly by autophagy inhibitors like hydroxychloroquine (HCQ) or indirectly by targeting hypoxia (HIF-1α inhibitors) will affect other parameters of the anti-tumor immunity like CD8 T lymphocyte activity in vivo and in vitro (i.e., cytokine production, proliferation, activation, etc.). Several other important mediators of immune tolerance like MDSC and T regulatory cells (recruitment, function and differentiation) should also be studied. Autophagy inhibition in hypoxic tumors can be used as a cutting-edge approach to improve cancer immunotherapy and to formulate more effective cancer vaccine-based therapy.

micoRNA-210 (miR-210) and hypoxia-induced immune resistance

Although we provide evidence that hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated lysis by a mechanism involving its downstream targets PTPN, HOXA1, and TP53I11, a number of important questions remains unanswered. It is still unclear how the simultaneous inhibition of these PTPN, HOXA1, and TP53I11 results in resistance to CTL-mediated killing. Do these genes differentially regulate the activation of caspases (caspase3 and caspase8), p53 accumulation, the pro-apoptotic genes (IAPs, survivin, Mcl-1, Bcl-xL and Bcl-2 family members) and various regulators of mitochondrial apoptotic pathway?

We strongly believe that by modulating tumor hypoxia (inhibition of HIF-1α, P-STAT3 and autophagy or miR-210), we will be able to inhibit tumor growth, reverse immunosuppressive microenvironment (by decreasing T reg, MDSC and favouring CD8 T lymphocyte infiltration) and modulate anti-tumor immune response (by increasing anti-tumor CD8 effector response) to achieve their killing activity.

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ANNEXE

Hypoxia-Dependent Inhibition of Tumor Cell Susceptibility to CTL-Mediated Lysis Involves NANOG Induction in Target Cells

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Hypoxia is a major feature of the solid tumor microenvironment and is known to be associated with tumor progression and poor clinical outcome. Recently, we reported that hypoxia protects human non-small cell lung tumor cells from specific lysis by stabilizing hypoxia-inducible factor-1α and inducing STAT3 phosphorylation. In this study, we show that NANOG, a transcription factor associated with stem cell self renewal, is a new mediator of hypoxia-induced resistance to specific lysis. Our data indicate that under hypoxic conditions, NANOG is induced at both transcriptional and translational levels. Knockdown of the NANOG gene in hypoxic tumor cells is able to significantly attenuate hypoxia-induced tumor resistance to CTL-dependent killing. Such knockdown correlates with an increase of target cell death and an inhibition of hypoxia-induced delay of DNA replication in these cells. Interestingly, NANOG depletion results in inhibition of STAT3 phosphorylation and nuclear translocation. To our knowledge, this study is the first to show that hypoxia-induced NANOG plays a critical role in tumor cell response to hypoxia and promotes tumor cell resistance to Ag-specific lysis. ***The Journal of Immunology*****, 2011, 187: 4031–4039.**

The tumor microenvironment is an integral part of tumor physiology, structure, and function, playing a critical role in tumor cell survival and growth and contributing to cell transformation and tumor development. Indeed, it is well established that a disrupted relationship between tumoral and stromal cells is essential for tumor cell growth, progression, and development (1), suggesting that an improved understanding of this interaction may provide new and valuable clinical targets for controlling tumor progression.

Tissue oxygenation is an important component of the microenvironment and can acutely alter cell behavior through the direct regulation of genes involved in cell survival, apoptosis, glucose metabolism, and angiogenesis (2). Evidence indicates that hypoxia, defined as oxygen deprivation, is an important feature of the microenvironment of a wide range of solid tumors. Its critical role in radio- and chemoresistance and its significance as an adverse prognosis factor have been well established during the last decades (2). Hypoxia has been shown to induce the loss of differentiation markers of several tumor types while increasing the expression of embryonic markers such as the transcription factors NANOG, octamer-binding transcription factor (OCT) 4, SOX2, and the Notch ligand (3, 4). This reprogramming of non-stem cancer cells toward a cancer stem phenotype is associated with increased tumorigenic capacity (4).

At a cellular level, hypoxia evokes a complex molecular response mainly characterized by changes in gene expression that are mediated by hypoxia-inducible factors (HIFs) (2). The HIFs are heterodimers that consist of α and β subunits. Three isoforms of the α subunit are described: HIF-1α, HIF-2α, and HIF-3α (2). Under hypoxic conditions, the α subunit is stabilized and activated and binds the HIFβ isoform, which is not regulated by oxygen levels and is constitutively present in the nucleus (2). A broad range of tumors respond to hypoxia by stabilizing the HIF-1α subunit (5).

In a previous study, we reported that tumor hypoxia decreases tumor susceptibility to CTL-mediated lysis via HIF-1α stabilization and STAT3 phosphorylation (6). Although it has been reported that tumor stem cells were intrinsically susceptible to immune effector cell cytotoxicity (7), the effects of hypoxia-driven tumor dedifferentiation on the response to CTL-mediated lysis have not been reported.

NANOG, a homeodomain-containing protein expressed in embryonic cells, germ cells, and pluripotent stem cells, has the ability when overexpressed to inhibit in vitro embryonic stem cell differentiation and maintain their self-renewal capability independently of the LIF/gp130/STAT3 pathway (8). In human cancers, NANOG expression has been found in germ cell tumors where NANOG functions are required, and it has been reported to increase in several tumoral tissues (breast, prostate, kidney) compared with matched benign tissues (9). Although accumulating evidence suggests that the protumoral functions of NANOG rely on promoting tumor cell proliferation (9), stimulation of STAT3 transcriptional activity has also been reported, leading to tumor resistance to chemotherapy and improved cell survival (10). In line with these reports, NANOG expression in head and neck cancers has recently been associated with unfavorable clinical

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Abbreviations used in this article: ALDH, aldehyde dehydrogenase; EMT, epithelial to mesenchymal transition; HES, hairy and enhancer of split; HIF, hypoxia-inducible factor; OCT, octamer-binding transcription factor; pO_2, partial pressure of O_2; RT-qPCR, quantitative real-time PCR; siRNA, small interfering RNA.

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outcome (11). However, whether NANOG interferes with tumor cell susceptibility to CTL-mediated cytotoxicity is not yet known.

The present study shows that hypoxia increases NANOG expression in non-small cell lung carcinoma cells and that this induction contributes to hypoxia-induced tumor target resistance to CTL-mediated lysis. Such induction is also associated with the regulation of cell replication and of nuclear translocation of STAT3. Our findings provide new insights into understanding the role of hypoxia-induced NANOG in regulating the cellular response to hypoxia and tumor target susceptibility to CTL-dependent killing.

Materials and Methods

Culture of tumor cells and CTL

The IGR-Heu lung carcinoma cell line was derived and maintained in culture as described previously (12). Briefly, the cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FCS, 1% Ultroser G, 1% penicillin-streptomycin, and 1 mM sodium pyruvate (Life Technologies, Cergy Pontoise, France). The Heu161 cytotoxic T cell clone was derived from autologous tumor-infiltrating lymphocytes (13).

Reagents and Abs

Protease inhibitors and sodium orthovanadate were purchased from Roche Molecular Biochemicals. Abs against CD133 and OCT4 were from Abcam. Abs against NANOG were purchased from Santa Cruz Biotechnology (for Western blotting) and R&D Systems (for confocal microscopy). Ab against HIF-1α was from BD Transduction Laboratories. Abs against total STAT3, phospho-STAT3 (STAT3Y705), total Src, and phosphor-Src (Y416) were from Cell Signaling Technology. Abs against β-actin and GAPDH were from Sigma-Aldrich.

Hypoxia treatment

For hypoxia treatment, cell cultures were incubated in a hypoxia chamber (Invivo$_2$ 400 hypoxia workstation; Ruskinn) with a humidified atmosphere containing 5% CO_2, 01% O_2, and 94% N_2 at 37˚C (14). Cells were exposed to these hypoxic conditions for 24, 48, and 72 h. Cells for protein analysis were harvested while inside the hypoxia workstation and were not reoxygenated before harvesting.

Flow cytometry analysis

Flow cytometry analysis was performed using a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences). Anti-HLA class I (W6/32) Ab was used as reported previously (15).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol (Sigma-Aldrich) following the manufacturer's instructions. For quantification of OCT4, NANOG, and hairy and enhancer of split (HES) 1 expression, cDNA was synthesized from 1 μg mRNA using an Applied Biosystems kit (N808-0234). Real-time PCR analysis was performed using a LightCycler (Roche Applied Science, Meylan, France) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Applied Science) according to the manufacturer's instructions with an initial denaturation step at 95˚C for 10 min, followed by 45 cycles at 95˚C for 10 s and annealing at specific temperature for 10 s and at 72˚C for 10s. Primers used for NANOG, OCT4, and HES1 amplification were: NANOG forward, 5′-CAGCTGTGTGTACTCAATGATAGA-3′, reverse, 5′-ACACCATTGCTATTCTTCGGCCAG-3′; OCT4 forward, 5′-ACATCAAAGCTCTGCAGAAAGAACT-3′, reverse, 5′-CTGAATACCTTCCCAAATAGAACCC-3′; HES1 forward, 5′-AGGCGGCACTTCTGGAAATG-3′, reverse, 5′-CGGTACTTCCCCAGCACACTT-3′ (at a 63˚C annealing temperature). Specificity of PCR amplicons was confirmed by melting curve analysis. Expressions of target genes were normalized to that of RPL13. Relative expression of tested genes was calculated using the target threshold cycle value (Ct) and the $2^{-\Delta Ct}$ method. For each gene, values were averaged over three independent measurements and the relative transcript level was calculated.

For quantification of apoptosis-related genes, reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Ambion). Real-time PCR was done using Platinum SYBR Green qPCR SuperMix-UDG w/ROX (Invitrogen) and an Applied Biosystems 7900HT Fast real-time PCR system. Apoptosis-dedicated arrays were elaborated by the PETC platform of INSERM Unité 576.

Western blotting

Adherent tumor cells were washed in 1× PBS and lysed in plates with lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% w/v SDS, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 25 μM leupeptin, 5 mM benzamidine, 1 μM pepstatin, 25 μM aprotinin). Lysates were sonicated on ice, resolved by SDS-PAGE electrophoresis (60 μg/lane), and transferred onto nitrocellulose membranes. After incubation in blocking buffer, the membranes were probed overnight at 4˚C with the indicated Abs. The labeling was visualized using peroxidase-conjugated secondary Abs and with an ECL kit (Amersham International). Blots were scanned and processed by Adobe Photoshop 7.0 software.

Cytotoxicity assay

Four-hour chromium release assays were performed as described previously (6). Briefly, different E:T ratios were used on 1000 target cells per well (round-bottom 96-well plates). After 4 h coculture, the supernatants were transferred to LumaPlate-96 wells (PerkinElmer), dried down, and counted on a Packard Instrument TopCount NXT. Percentage-specific cytotoxicity was calculated conventionally as described earlier (6). All cytotoxicity experiments with [^{51}Cr] were performed under normoxic conditions.

TNF-β production assay

TNF-β release was measured as described previously (16). Briefly, TNF-β was detected by measuring the cytotoxicity of the culture medium on the TNF-sensitive WEHI-164c13 cells with an MTT colorimetric assay.

Confocal microscopy

Cells were plated in 8 well μ-slide (Ibidi) and incubated for 24, 48, and 72 h at 37˚C and 01% partial pressure of O_2 (pO_2). Afterwards, cells were washed twice in 1× PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked for 45 min with 0.1% Triton X-100, 10% FCS, and 1% BSA. After blocking, cells were incubated overnight with diluted anti-STAT3Y705 and anti-NANOG primary Abs, followed by incubation with Alexa 488 and Alexa 546 secondary Abs (Molecular Probes), respectively, in the dark for 1 h at room temperature. Wells were covered with Fluoromount-G (SouthernBiotech, Birmingham, AL) and analyzed the following day by a Zeiss laser scanning confocal microscope (LSM-510; Zeiss, Jena, Germany). Z-projection of slices was performed using LSM Image Examiner software (Zeiss).

Gene silencing

Gene silencing of *NANOG* was performed using sequence-specific small interfering RNA (siRNA; Santa Cruz Biotechnology). Briefly, 8×10^6 cells were electroporated twice in 48 h in serum-free medium with 20 μM siRNA in an EasyJect Plus electroporation system (EquiBio; 260 V, 450 μF) and then allowed to grow for 24, 48, and 72 h in hypoxia. siRNA targeting luciferase was used as a negative control (5′-GCAAGCUGACCCUGAAGUUCAU-3′). Gene-specific targeting was evaluated by quantitative real-time PCR (RT-qPCR) or Western blot.

Cell fractionation

For isolation of cytoplasmic and nuclear proteins, the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas) was used following the manufacturer's instructions. Briefly, hypoxic or normoxic cells were harvested after trypsinization and solubilized in lysis buffer with protease inhibitors and DTT. After centrifugation, the cytoplasmic fraction was isolated. Nuclear pellets were washed twice and then lysed in nuclear lysis buffer added with protease inhibitors and DTT. Nuclear lysates were obtained after centrifugation.

Cell cycle

Electroporated IGR-Heu cells were incubated in normoxia or hypoxia (01% pO_2) for 72 h. In the last 10 min, BrdU was added to the culture medium. Then, cells were harvested and fixed in 80% ice-cold ethanol. For BrdU detection, DNA was first denaturated in a pepsine buffer (0.5 mg/ml pepsine, 2 N 30 mM HCl) for 20 min at 37˚C. Cell pellets were then resuspended in 2 N HCl for 20 min at room temperature. After washing in ice-cold 1× PBS, detection of replicating cells was performed by BrdU staining. Cells were washed in BrdU detection buffer (0.5% FCS, 0.5% Tween 20, 20 mM HEPES in 1× PBS) and BrdU was detected in the dark using a specific primary Ab for 45 min followed by a FITC-conjugated secondary Ab. To detect total DNA, cell pellets were resuspended in a propidium iodide solution (25 μg/ml propidium iodide, 50 μg/ml RNase A in 1× PBS) and incubated in the dark at room temperature for 30 min. Cells were then analyzed by flow cytometry.

Statistical analyses

Data were analyzed with GraphPad Prism. A Student *t* test was used for single comparisons. A p value of <0.05 was considered statistically significant.

Results

The resistance of hypoxic tumor cells to CTL is not associated with the acquisition of a stem cell-like phenotype

As previously reported, data depicted in Fig. 1*A* show that exposure of the lung tumor cell line IGR-Heu to hypoxic treatment resulted in an inhibition of its susceptibility to autologous CTL-mediated killing. We next examined the relationship between hypoxia-induced resistance to specific lysis and the adoption of a stem cell-like phenotype. Indeed, cancer stem cells have been shown to resist cell death induced by apoptotic and chemo- or radiotherapeutic agents (17). For this purpose, we first investigated the influence of hypoxic stress on the expression of the cell surface protein CD133 and the cytosolic enzyme aldehyde dehydrogenase (ALDH) 1. These two markers are respectively associated with lung cancer stem cell proliferation and chemoresistance (18–20).

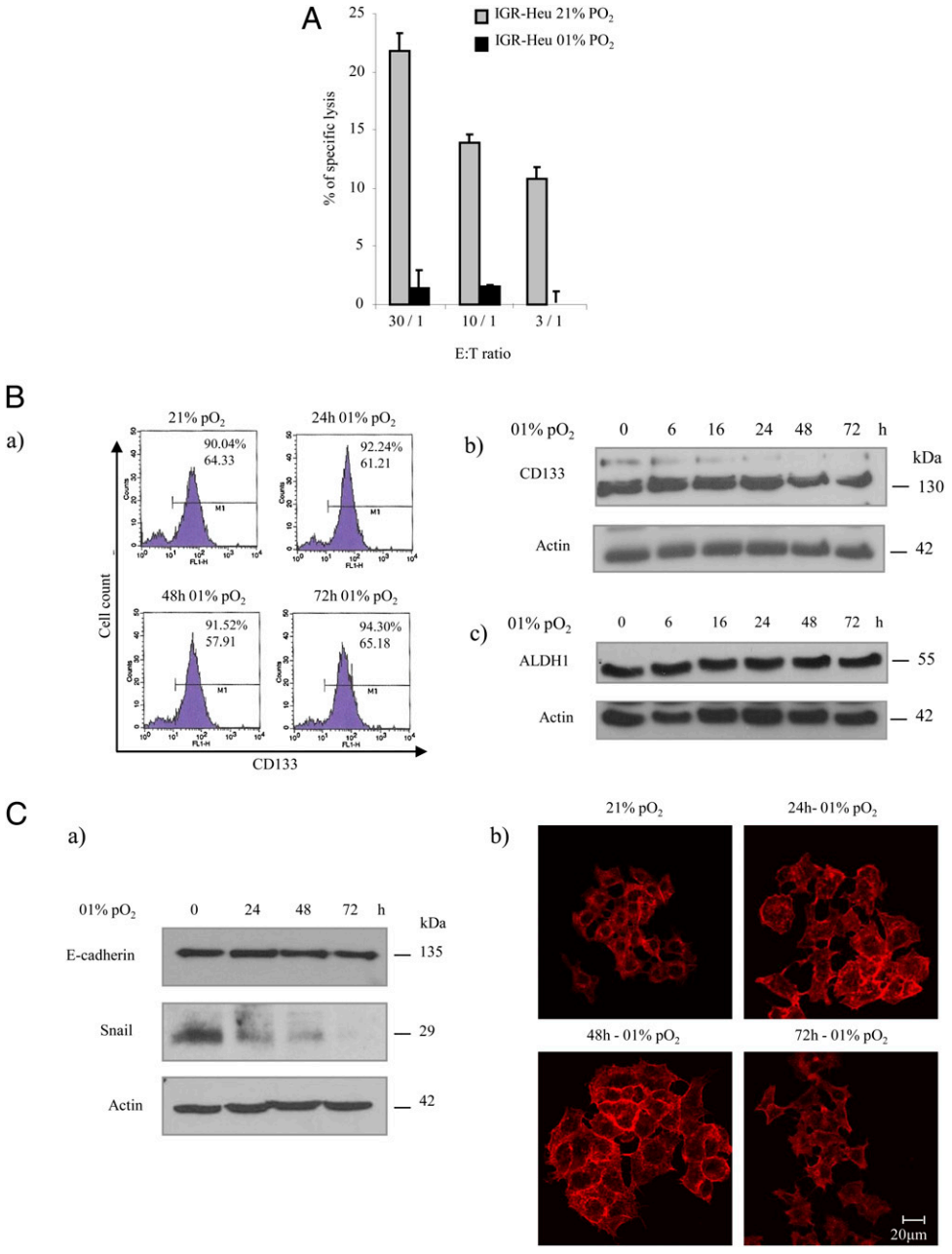

FIGURE 1. Hypoxia-induced resistance to specific lysis is not associated with the acquisition of a dedifferentiated phenotype. *A*, IGR-Heu cells were incubated in hypoxia (01% pO_2) or normoxia for 48 h and CTL-mediated lysis was measured by a conventional 4-h ^{51}Cr-release assay at different ratios. Cells from the Heu161 tumor-infiltrating lymphocyte-derived T cell clone were used as effectors. Data show the percentage of IGR-Heu–specific lysis of three independent experiments ± SD. *B*, Expression level of CD133 was compared between IGR-Heu cells incubated in normoxia or hypoxia for the indicated times. *a*, Cell surface expression was analyzed by cytometry and (*b*) total expression was evaluated by Western blot using a specific Ab. *c*, Influence of hypoxia on ALDH1 expression in IGR-Heu cells was analyzed by Western blot in total cellular extracts. β-actin was used as a loading control. Results are representative of three experiments with similar results. *C*, Influence of hypoxic stress on EMT induction in IGR-Heu cells. *a*, E-cadherin and Snail expression levels following exposure to hypoxia for 24, 48, and 72 h were evaluated by Western blot using specific Abs. Equal protein loading was assessed using β-actin. *b*, IGR-Heu cells incubated in hypoxia for the indicated times were stained by rhodamine-phalloidin to visualize cell morphology. Images shown are representative of most of the analyzed cells. Experiments were carried out three times.

Flow cytometry analysis indicated that hypoxia did not affect the cell surface expression of CD133 (Fig. 1*Ba*). Most of the IGR-Heu cells express CD133 constitutively, suggesting that CD133 may not be an exhaustive marker for detection of stem cell-like tumor cells. Total cellular extracts were analyzed by Western blot, and expression levels of CD133 (Fig. 1*Bb*) and ALDH1 (Fig. 1*Bc*) were not influenced by exposure to hypoxic stress. Because epithelial to mesenchymal transition (EMT) has been reported to be associated with both acquisition of an immature phenotype (21) and hypoxic stress (22), we investigated the induction of EMT in hypoxic IGR-Heu cells. The expression of the epithelial marker E-cadherin and of the EMT activator Snail, which is also an important regulator of E-cadherin expression (23), was analyzed. Fig. 1*Ca* shows that the total expression of E-cadherin, as well as surface E-cadherin expression (data not shown), remained unchanged after 72 h exposure to hypoxia. In contrast, Snail expression was significantly decreased after 24 h hypoxia (Fig. 1*Ca*). Because EMT is associated with morphological changes resulting in a distinct elongated phenotype, cell morphology of hypoxic IGR-Heu cells was therefore visualized by phalloidin staining. Data shown in Fig. 1*Cb* indicate that hypoxia had no effect on IGR-Heu morphology for up to 72 h. Phalloidin staining also showed the absence of cell rounding, which is a morphological feature associated with the acquisition of immature phenotypes (24). These results indicate that hypoxia-induced tumor resistance was not associated with the acquisition of a stem cell-like phenotype in IGR-Heu cells.

NANOG is selectively induced in hypoxic tumor cells

Although hypoxic stress did not result in phenotypic changes in IGR-Heu cells, hypoxia-induced dedifferentiation may be associated with altered expression of specific genes. Thus, to evaluate the potential role of dedifferentiation genes in hypoxic tumor cells, we selected OCT4 and NANOG genes, as well as HES1, a Notch target gene, based on their increased expression under hypoxia (4, 25, 26) as well as their reported role in the control of stem cell self-renewal and multipotency (27–29). Using RT-qPCR, we measured their respective mRNA levels under hypoxic conditions and found that OCT4 and NANOG mRNAs but not HES1 mRNA markedly increased following exposure to hypoxia for 24, 48, and 72 h (Fig. 2*A*). Whereas Western blot analysis did not reveal OCT4 expression (data not shown), fitting with its marginal mRNA levels under hypoxia, NANOG protein was induced up to 72 h hypoxia (Fig. 2*B*). This was confirmed by confocal

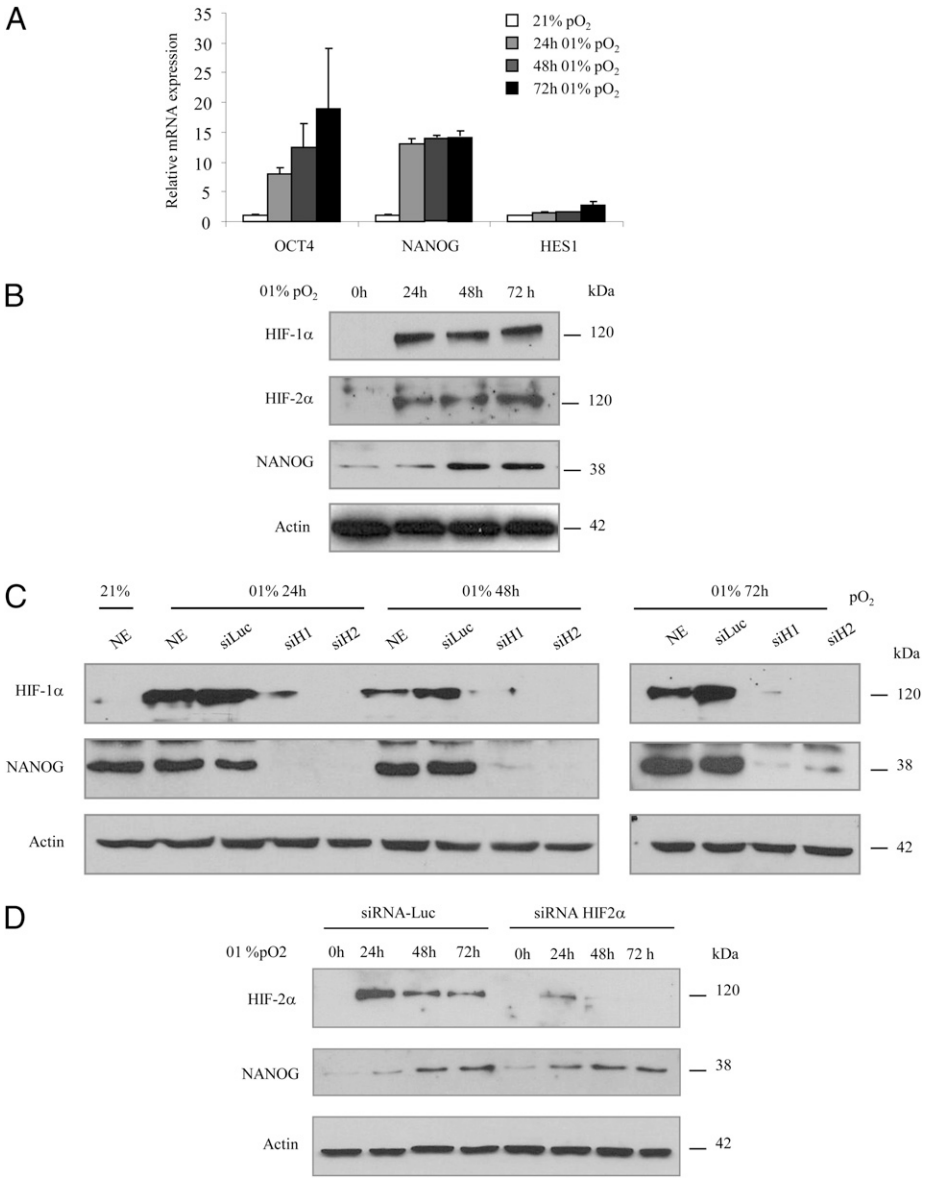

FIGURE 2. Hypoxic stress leads to selective induction of NANOG expression via HIF-1α. *A*, Hypoxia effects on mRNA expression levels of *OCT4*, *NANOG*, and *HES-1* genes measured by RT-qPCR. *B*, IGR-Heu tumor cells were incubated in normoxia or hypoxia for different intervals. After hypoxia exposure, total cell lysates (60 μg) were subjected to SDS-PAGE, blotted, and probed with specific Abs against HIF-1α, HIF-2α, and NANOG, as indicated. β-actin was used as the loading control. *C* and *D*, Western blot analysis of NANOG expression levels under hypoxia following specific inhibition of *HIF-1α* (*C*) or *HIF-2α* (*D*) expression by siRNA. Each experiment was repeated at least three times. NE, non-electroporated; siH1, anti–HIF1-α siRNA1; siH2, anti–HIF1-α siRNA2; siLuc, anti-luciferase siRNA.

microscopy analysis, which revealed increased staining of NANOG along with the time of exposure to hypoxia (data not shown). Fig. 2*B* also showed that the induction of NANOG under hypoxia correlated with HIF-1α and HIF-2α stabilization. To delineate the potential link between hypoxia-induced NANOG and HIF-1α or HIF-2α, NANOG protein expression was analyzed following HIF-1α or HIF-2α knockdown using specific siRNAs. As shown in Fig. 2*C*, specific silencing of HIF-1α resulted in the inhibition of hypoxia-induced NANOG after 24, 48, and 72 h of hypoxic stress whereas HIF-2α silencing had no effect (Fig. 2*D*). These results indicate that hypoxia-induced NANOG was dependent on HIF-1α and raise the possibility that NANOG may play a role in the acquisition of tumor cell resistance to CTL lysis under hypoxic conditions.

Attenuation of hypoxia-induced tumor resistance to CTL-mediated killing following NANOG knockdown

To delineate the role of NANOG induction in hypoxia-mediated tumor cell resistance to specific lysis, we investigated the susceptibility of NANOG-targeted IGR-Heu cells using siRNA under hypoxic conditions. The inhibition of NANOG in hypoxic tumor cells was performed using specific siRNA and confirmed by RT-qPCR, showing an efficient and specific inhibition of NANOG mRNA expression after 72 h hypoxia, whereas luciferase siRNA used as a control had no effect on NANOG mRNA level (Fig. 3*A*). As shown in Fig. 3*B*, *NANOG* knockdown in hypoxic IGR-Heu cells was associated with sensitization of IGR-Heu cells to CTL-mediated lysis following 48 h exposure to hypoxia as compared with control hypoxic cells. We also show that the targeting of *NANOG* in hypoxic IGR-Heu cells was not associated with an alteration in autologous CTL reactivity, as TNF-β secretion by CTL was not affected (Fig. 3*C*). Moreover, such targeting did not alter MHC class I expression in IGR-Heu cells (Fig. 3*D*). This is consistent with the fact that attenuation of hypoxia-induced tumor cell resistance following NANOG targeting was not due to an alteration in CTL reactivity and target cell recognition.

NANOG targeting increases cell death and interferes with cell cycle transition in hypoxic tumor cells

To investigate the molecular mechanisms associated with the attenuation of target cell resistance to CTL following NANOG targeting under hypoxic conditions, we analyzed the expression of a series of antiapoptotic and proapoptotic genes using an apoptosis-related array of 94 genes. NANOG knockdown in hypoxic tumor

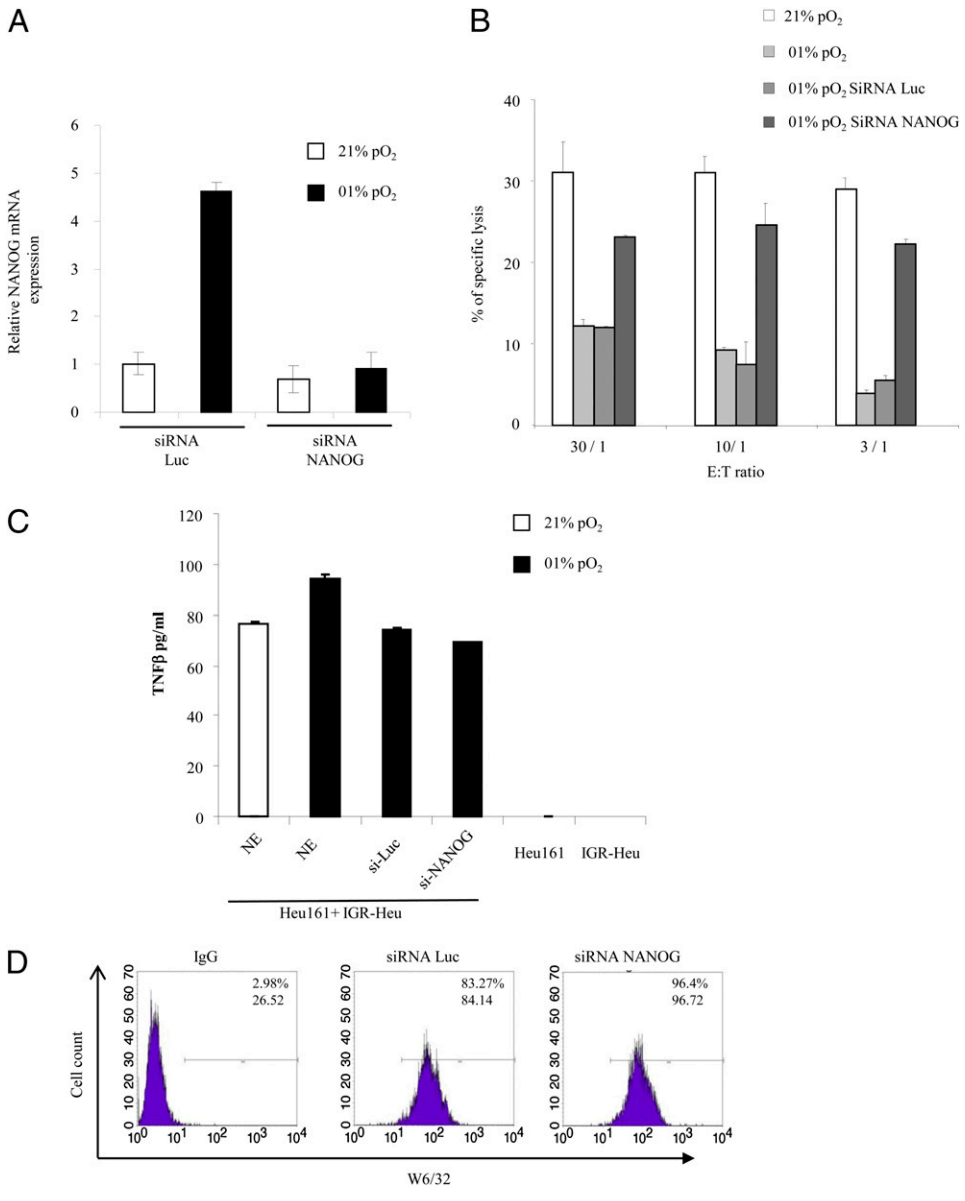

FIGURE 3. NANOG knockdown restores tumor cell susceptibility to lysis by CTL in hypoxia. *A*, NANOG expression was silenced using specific siRNA administered by electroporation. Silencing efficiency was assessed by RT-qPCR after 48 h hypoxia. *B*, Role of NANOG in CTL-mediated lysis toward autologous IGR-Heu tumor cells. IGR-Heu tumor cells were electroporated with NANOG or luciferase siRNAs and kept in normoxia (21% pO_2) and hypoxia (01% pO_2) for 48 h. Cytotoxicity was determined by a conventional 4-h ^{51}Cr-release assay at different ratios. Heu161 CTL clone cells were used as effectors. Data show the percentage of IGR-Heu–specific lysis of three independent experiments ± SD. *C*, TNF-β production by the autologous T cell clone in response to stimulation by nonelectroporated, control, and NANOG-deficient IGR-Heu cells. CTL clone Heu161 cells were cocultured in the presence of IGR-Heu cells placed in normoxia (21% pO_2) versus hypoxia (01% pO_2) for 48h. The amount of TNF-β produced by the CTL clone was measured using the TNF-sensitive WEHI-164c13 cells. Each bar is the mean of three independent experiments ± SD. *D*, Comparative analysis of surface expression of HLA class I (W6/32) conducted on control and NANOG-deficient IGR-Heu tumor cells kept in hypoxia (01% pO_2) for 48 h. Isotypic control of mAb was included (IgG). Results are representative of three independent experiments. NE, nonelectroporated; siRNA Luc, luciferase siRNA.

cells revealed an increase in the expression of proapoptotic and antiapoptotic genes (Fig. 4*A*). However, NANOG depletion in hypoxic cells resulted in a limited but consistent increase in both apoptotic (sub-G_1) and nonreplicating (S-BrdU-negative) cells, suggesting that one of the roles of NANOG in hypoxia is to sustain cell viability (Fig. 4*B*). Additionally, flow cytometry analysis of BrdU-labeled cells revealed that hypoxia modified the overall level of nucleotides incorporation (Fig. 4*Bb*). Indeed, we

A Genes differentially expressed in hypoxic IGR-Heu cells knocked-down or not for NANOG

Gene symbol	Gene bank ID	Fold change range
Pro-apoptotic genes		
CYCS	NM_08947	1,5-1,7
FAS	NM_000043	2,3-2,7
CASP10	NM_001230	0,5-0,3
CASP4	NM_001225	2,5-2,2
CASP8	NM_001228	1,5-2,4
SCYE1	NM_004757	2,6-2,4
TRAIL	NM_003810	1,8-1,9
NOXA	NM_021127	1,6-2,0
SIVA1	NM_006427	1,8-2,1
PAWR	NM_02583	2,1-1,9
DR5	NM_003842	2,9-1,2
BID	NM_197966	1,8-2,0
Anti-apoptotic genes		
TGFB1	NM_000660	0,4-0,4
BIRC2	NM_001166	1,5-1,9
BIRC3	NM_001165	2,5-2,5
BIRC4	NM_001167	1,5-1,9
FAIM	NM_001033030	1,8-1,7

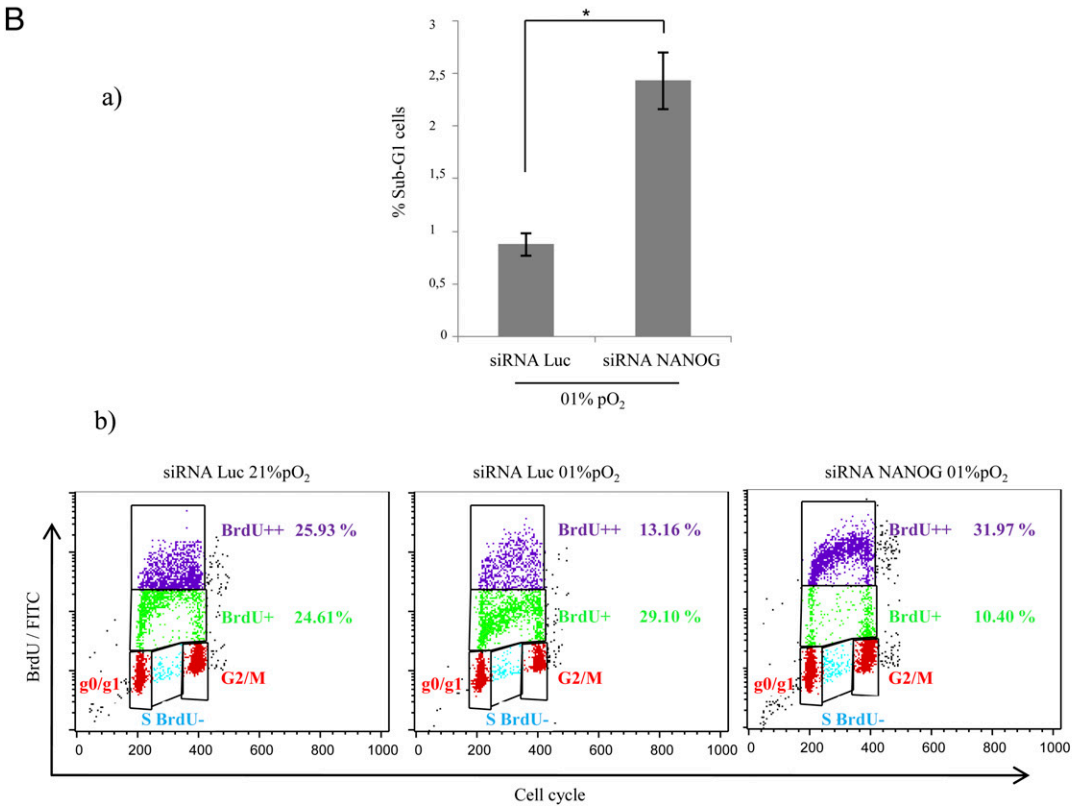

FIGURE 4. NANOG knockdown increases cell death and restores BrdU incorporation in hypoxic tumor cells. *A*, Pro- and antiapoptotic gene transcript quantification by qPCR using a 94 apoptosis dedicated array. *Ba,* Sub-G_1 cell quantification by flow cytometry based on propidium iodide incorporation in hypoxic control and NANOG knocked down IGR-Heu cells. A statistically significant difference in sub-G_1 cell percentage between hypoxic control and NANOG-targeted cells was observed. $*p < 0.05$. *Bb*, Cells were gated according to their DNA content, propidium iodide incorporation (*x*-axis), and their capability to incorporate BrdU during S phase (*y*-axis). Red indicates cells in G_0/G_1 (*lower left*) and G_2/M (*lower right*). Cells in blue indicate S-phase BrdU-negative cells, that is, cells showing an S-phase DNA content but unable to replicate their DNA and incorporate BrdU. Green and purple identify replicating S-phase cells. The gated BrdU-positive cells were arbitrarily separated in two fractions as a function of their capability to incoporate BrdU. Green indicates cells with low replication capabilities, as a consequence of the accumulation of slowing DNA replication forks, the decrease in the number of active DNA replication forks, or both. Purple represents the fraction of cells able to replicate their DNA at a normal rate. Numeric values correspond to the percentage of gated cells that have incorporated BrdU at a normal and a low level. They are indicated with the corresponding color. Results are representative of two independent experiments.

noticed the appearance of a cell subpopulation characterized by lower BrdU incorporation. This subpopulation represented the majority of S-phase cells in hypoxic conditions. Importantly, NANOG depletion restored a cell cycle profile similar to that observed in normoxic conditions. These results indicate that NANOG knockdown in hypoxic tumor cells correlates with increased replication levels, an event associated to and responsible for DNA damage and apoptosis (30).

Regulation of STAT3 phosphorylation and nuclear translocation by NANOG

We have recently shown that hypoxia-induced resistance of IGR-Heu cells to specific lysis was critically dependent on STAT3 phosphorylation (6). Additionally, a functional cooperation between STAT3 and NANOG for gene transcription has been reported in cancer drug resistance (10). Therefore, we asked whether a functional interaction exists between NANOG and

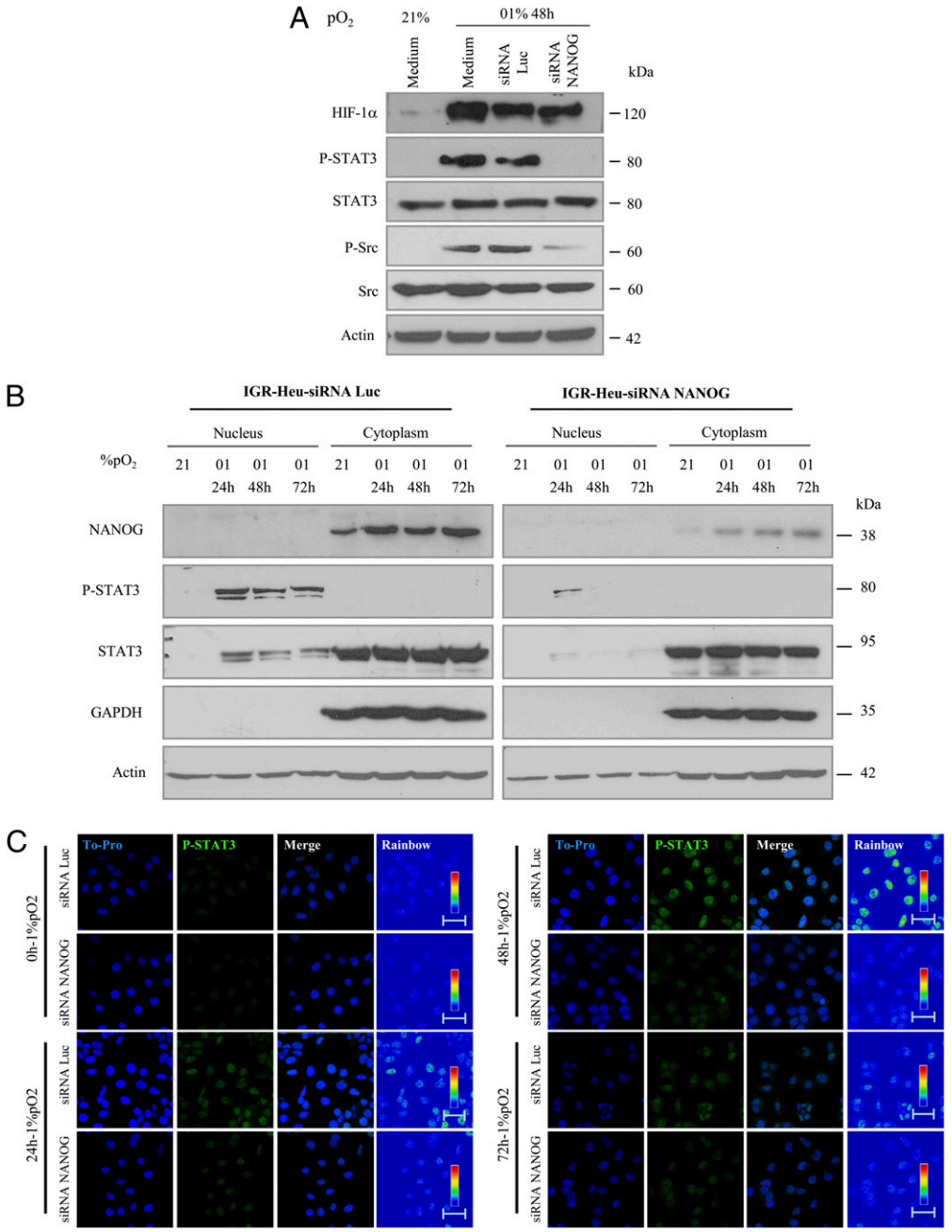

FIGURE 5. NANOG knock down inhibits hypoxia-induced STAT3 phoshorylation and its nuclear translocation. *A*, Western blot analysis of the influence of NANOG silencing on STAT3 and Src phosphorylation during hypoxic stress. After 48 h exposure to hypoxia, 60 μg total protein extracts were run with β-actin used as the loading control. *B*, Luciferase siRNA- and siRNA-NANOG–electroporated IGR-Heu cells were kept in hypoxia for 24, 48, and 72 h and cell lysates were fractionated in cytoplamsic and nuclear fractions following the manufacturer's instructions. GAPDH was used for technical control and, as expected, it was respectively found mainly in the cytosol for both luciferase siRNA- and siRNA-NANOG–electroporated IGR-Heu cells. Equal loading was confirmed by β-actin blotting. *C*, Confocal microscopy analysis of pSTAT3-Y705 staining in NANOG-deficient IGR-Heu tumor cells kept in normoxia (21% pO_2) versus hypoxia (01% pO_2), followed by immunofluorescence staining with pSTAT3-Y705–specific Ab. Nuclei were counterstained with To-Pro-3 iodide. Rainbow panel indicates staining intensity (blue to red correspond, respectively, from low to high intensity). The confocal scanning fluorescence micrographs shown are representative of most of the cells analyzed (blue, nucleus; green, pSTAT3-Y705). Experiments were performed at least three times. Scale bars, 20 μm. siRNA Luc, luciferase siRNA.

STAT3 upon hypoxic stress. Fig. 5*A* indicates that silencing of hypoxia-induced NANOG resulted in an inhibition of STAT3 phosphorylation on Tyr^{705}, a crucial event for STAT3 dimerization and translocation to the nucleus (31). We next examined in hypoxic NANOG-downregulated cells the phosphorylation state of JAK2 and Src, two kinases involved in STAT3 phosphorylation (32) (33). Results indicate that NANOG downregulation under 48 h hypoxia resulted in selective inhibition of Src (Fig. 5*A*) but had no effect on JAK phosphorylation (data not shown).

Phosphorylation of STAT3-Y705 is crucial for STAT3 translocation to the nucleus (31). However, unphosphorylated nuclear forms of STAT3 have already been described (34). Therefore we investigated the role of NANOG on STAT3 nuclear translocation in hypoxic IGR-Heu cells using siRNA-targeting NANOG. Fig. 5*B* indicates that knocking down NANOG in hypoxic NANOG-targeted IGR-Heu cells suppressed STAT3 phosphorylation and its subsequent translocation into the nucleus after 24, 48, and 72 h of hypoxic stress. In contrast, in control hypoxic cells, phosphorylated STAT3 was induced and located in nuclear fractions. It also shows that in NANOG-targeted and control cells, hypoxia-induced NANOG did not translocate to the nucleus but remained in the cytoplasm (Fig. 5*B*). Confocal microscopy analysis indicated a constant decrease of pSTAT3-Y705 nuclear staining in siRNA-NANOG–treated IGR-Heu cells under 24, 48, and 72 h hypoxia compared with control cells (Fig. 5*C*). This further points to the role of NANOG in STAT3 phosphorylation on Y705.

Discussion

It is well established that microenvironmental factors, in particular hypoxia, act to shift the normal balance toward malignancy. Accumulating evidence indicates that cancer cells that remain viable in hypoxic conditions often possess an increased survival potential and tend to grow particularly aggressively. Hypoxia induces tumor progression through multiple mechanisms, including several transcriptional programs (2). In this study we show, in human non-small cell lung carcinoma cells, that hypoxic stress selectively induces the stem cell marker NANOG, which is involved in the hypoxia-mediated resistance to specific lysis. Hypoxia-induced NANOG has the ability to control the expression of a series of proapoptotic genes, to regulate cell replication, and to contribute to STAT3 and Src phosphorylation.

The selective increase in NANOG expression under hypoxia was not associated with alteration of CD133 or ALDH1 expression, two reported markers for lung cancer stem cells (20). Additionally, IGR-Heu cells did not lose their epithelial features under hypoxia, as Snail expression was decreased and E-cadherin level remained unchanged in IGR-Heu hypoxic cells. Whether Snail decrease is due to transcriptional repression or increased degradation following hypoxic stress is currently under investigation. Nonetheless, this suggests that in our experimental system, tumor cells did not undergo a phenotypic dedifferentiation but indeed displayed increased expression of NANOG, a transcription factor associated with stemness. NANOG induction in hypoxia has been recently reported (4, 35, 36), suggesting its putative involvement in cell adaptation to hypoxic stress. In this regard, it has been shown that NANOG possesses proangiogenic activities by controlling vascular endothelial growth receptor-2 expression in endothelial cells (37). Although induction of this transcription factor under hypoxia is usually described to be dependent on HIF-2 in cancer stem cells (38, 39) and embryonic stem cells (35), our results clearly indicate that only HIF-1α selectively regulates NANOG induction in our experimental model.

To determine the functional relevance of NANOG in hypoxic tumor resistance to CTL-mediated lysis, we targeted NANOG and found that it resulted in the attenuation of target resistance to CTL-mediated killing. How this transcription factor regulates tumor susceptibility to lysis is not yet established. Nevertheless, our data considering the relevance of NANOG induction following hypoxic stress support the idea that NANOG is a significant mediator of the adaptive cellular response to hypoxia. NANOG has been reported to have antiapoptotic functions by inhibiting apoptosis in transformed human stem cells (40) and choriocarcinoma cells (41). NANOG targeting significantly increased the percentage of sub-G_1 dead cells, suggesting that hypoxia-induced NANOG has a protective role on tumor cells. By analyzing cell cycle transition, we show a decrease in BrdU incorporation under hypoxia. This could be related to the activation of DNA repair processes subsequent to DNA damage known to be induced by exposure to hypoxia (42). Additionally, a major event in shifting cells form normoxia to hypoxia is cell cycle arrest (43) (44). Interestingly, our data show that NANOG knockdown restored BrdU incorporation in hypoxic cells, suggesting that NANOG, under conditions of hypoxic stress, could be involved in the activation of checkpoint signaling resulting in slowing down S phase. This is supported by our finding that hypoxia induced p53 protein accumulation and that this event was inhibited following NANOG knocking down (data not shown). To further delineate the role of NANOG in hypoxia, we explored the effect of NANOG on hypoxia-induced STAT3 activation. Bourguignon et al. (10) demonstrated functional cooperation of NANOG with activated STAT3 in inducing expression of the chemoresistance gene *MDR1*. Previously, we provided evidence indicating that STAT3 phosphorylation was a major event in the acquisition of hypoxic tumor cell resistance to CTL-mediated killing (6). In the present report, our data point to the potential role of NANOG in regulating STAT3 phosphorylation and its subsequent nuclear translocation. To our knowledge, this is the first demonstration involving a functional interaction between NANOG and STAT3 in the regulation of tumor cell susceptibility to CTL-dependent cytotoxicity. More importantly, we showed that NANOG targeting resulted in the inhibition of hypoxia-induced Src phosphorylation. Because Src is known to be required for STAT3 phosphorylation (32, 33), it is tempting to speculate that NANOG controls STAT3 phosphorylation by a mechanism involving Src phosphorylation under hypoxia. This is in agreement with our recent observations showing that chemical inhibition of Src phosphorylation in hypoxic IGR-Heu cells was able to block hypoxia-induced STAT3 phosphorylation (45).

Along with inhibition of hypoxia-induced delay on cell replication levels, NANOG knockdown seems to allow hypoxic tumor cells to gain a "normoxia-like" state that may shift CTL-resistant hypoxic tumor cells to sensitive ones.

Collectively, these results point to a novel function of NANOG and suggest the existence of a new relationship between stem cell markers, hypoxia, and resistance to CTL-mediated lysis.

Disclosures

The authors have no financial conflicts of interest.

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Cytotoxic T cells – Stroma interactions

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Abstract. The tumor microenvironment is a complex system playing an important role in tumor development and progression. Besides tumor cells, the tumor microenvironment harbours a variety of host-derived cells, such as endothelial cells, fibroblasts, innate and adaptive immune cells, as well as extracellular matrix (ECM) fibers, cytokines, and other mediators. This review discusses the potential role of hypoxia and endothelial cells within tumor microenvironment and emphasizes their interaction with antigen specific killer cells. ▲

Key words: hypoxia, STAT3, HIF-1α, endothelial cells, tumor cells, CTLs

Introduction

Hypoxia is a common feature of solid tumors and one of the hallmarks of tumor microenvironment [1]. In a solid tumor microenvironment, a gradient of oxygen exists which extends from well-oxygenated areas to hypoxic areas and finally the necrotic areas where oxygen concentration falls to zero [2].

Tumor cells adapt to hypoxic microenvironment by the regulation of hypoxia inducible factor family of transcription factors (HIF's). This family is composed of three members namely HIF-1, HIF-2 and HIF-3. HIF-1 and HIF-2 has α and β subunits. HIF-1 is the major player in mediating tumor hypoxic response but HIF-2 is being implicated in participating in this response. HIF-3 is only a negative regulator of HIF-1 and HIF-2. It is well established that intratumoral hypoxia is the main inducer of HIF-1α but other factors like genetic alteration (VHL mutations, etc) may also contribute to the regulation of the stability of HIF-1α [2]. A large body of clinical data shows a positive correlation between increased hypoxic expression of HIF-1α and HIF-2α and patient's mortality [3]. Both HIF-1α and HIF-2α have common target genes as well as their respective target genes. The genes induced by hypoxia dependent HIF-1α and HIF-2α play important roles in regulating different aspects of tumor biology like angiogenesis, cell survival, chemo- and radioresistance, proliferation, invasion and metastasis, pH regulation and metabolism, resistance to immune system and maintenance of cancer stem cells [4, 5].

It is now well established that hypoxic tumor microenvironment favours the emergence of tumor variants with increased metastatic and invasive potential [6]. HIF-1α is believed to play a protective role under hypoxic conditions [7, 8]. Since these tumor variants are resistant to radiotherapy and chemotherapy, one might postulate that the exposure to low-levels of oxygen may lead to adaptive responses allowing tumor cells to escape from immune surveillance. Fink *et al.* reported inhibition of NK cytotoxicity toward liver cell lines under hypoxic conditions [9]. Siemens *et al.* have recently reported that hypoxia contributes to tumor cell shedding of MIC (MHC class I chain-related molecules) through a mechanism involving impaired nitric oxide (NO) signalling [10].

An active and bi-directional molecular cross talk between tumor cells and host cells has profound implications for immunological recognition of tumor cells and the formation of a microenvironment modulating tumor progression [11, 12]. Several reports [13] underscore the contribution of the microenvironment to tumor development and it has become clear that tumors are not merely masses of neoplastic cells, but instead, are complex tissues composed of both non-cellular (matrix proteins) and cellular components (tumor-associated fibroblasts, capillary-associated cells and inflammatory cells), in addition

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to the ever-evolving neoplastic cells. All of these components might be involved in shaping the interactive and migratory behavior of tumor-infiltrating T lymphocytes with and among tumor cells. In the context of microenvironment complexity and plasticity, tumor cells orchestrate the modification of the microenvironment by attracting or activating many non-tumoral cells, including blood and lymphatic endothelial cells, fibroblasts, bone marrow-derived cells, immune and inflammatory cells [13]. It is now acknowledged that tumor cells and their stroma co-evolve during tumorigenesis and progression. Besides, the critical importance of the recruitment of endothelial cells by a tumor to achieve tumor angiogenesis, leukocyte-endothelial interactions within tumor microvasculature are critical for mounting a host immune response against tumor tissue and for controlling tumor progression [14].

Recent evidences point at endothelial cell implication in the indirect rejection of the tumor by the immune system [15]. In fact, EC express the protein machinery for antigen processing, including proteasome subunits, TAP proteins, and both MHC classes I and II, and can present endogenous peptides to activated T cells. Several studies demonstrated the potential of endothelial cells to cross-present exogenous antigens from apoptotic tumor cells [16-18], or derived from proteins secreted by surrounding cells or donated by live cells interacting with the endothelium [19-21]. Recently, [22] it has been demonstrated that several natural epitopes from an endogenous protein constitutively expressed by endothelial cells, that is vascular endothelial growth factor receptor-2 (VEGFR-2), were presented in both HLA classes I and II [23].These observations clearly sustain the importance of endothelial cells as "target" for the immune system.

Results

Influence of hypoxic stress on antitumor cytotoxic response

Since hypoxia is a common feature of solid tumors and one of the hallmarks of tumor microenvironment, it is of interest to find out whether hypoxia confers tumor resistance to CTL-mediated killing. We have shown [24] that indeed hypoxic exposure of target tumor cells inhibits the CTL clone-induced autologous target cell lysis. Interestingly the observed lysis inhibition was not associated with an alteration of CTL reactivity and tumor cell recognition indicating that tumor-induced priming of the autologous CTL clone was not affected after exposure to hypoxia. We further demonstrated that HIF-1α induction and STAT3 activation are responsible for mediating hypoxia induced lysis inhibition [24]. Our results suggest a new role for hypoxia (HIF and STAT3) in tumor resistance to the immune system. Here, we discuss how these results add an important new facet to our traditional view of hypoxia and cancer.

Role of hypoxia activated STAT3 in modulating antitumor immune response

We show that hypoxia is directly implicated in the acquisition of tumor cell resistance to CTL-mediated lysis *via* HIF induction and STAT3 activation. Furthermore, STAT3 activation within tumor microenvironment is known to be associated with cytokine-induced proliferation, anti-apoptosis and transformation. Moreover, it is now well established that STAT3 modulates the cross talk between tumor and immune cells [25, 26]. More interestingly, we have shown that vascular endothelial growth factor (VEGF) neutralization resulted in the attenuation of hypoxic tumor target resistance to CTL-mediated killing [24]. We have also demonstrated that STAT3 phosphorylation can be stimulated by autocrine signalling through VEGF [24], suggesting that tumor microenvironment through hypoxia-induced VEGF may play a key role in the induction of active form of STAT3. In this regard, it is very likely that STAT3 activation is associated with the regulation of target gene expression potentially involved in the alteration of hypoxic tumor target-specific killing. Therefore, understanding how VEGF and other soluble factors may lead to STAT3 activation *via* the tumor microenvironment may provide a more effective cancer treatment strategy for hypoxic tumors with elevated P-STAT3 levels. This also suggests that reduction of VEGF release in tumor microenvironment may favour induction of a stronger antitumor CTL response against tumors expressing VEGFR. Our studies are in agreement with reports suggesting that inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer [27] and indicating a synergy between tumor immunotherapy and anti-angiogenic therapy [28].

The consequence of hypoxic activation of STAT3 extends beyond its critical role in controlling cell survival and apoptosis. This emphasizes that a better

understanding of the tumor behavior and its interplay with the killer cells inside a complex and plastic hypoxic microenvironment will be a critical determinant for a rational approach of tumor immunotherapy. Although resistance of tumor targets to killer cells is likely to be regulated by multiple factors [29], the data we present herein suggest that hypoxic microenvironment is an important determinant involved in the control of target sensitivity to CTL-mediated lysis *(figure 1)*. Therefore, the possibility that novel approaches targeting HIF-1α and STAT3 with potent small molecule drugs, being actively developed, may provide an exciting novel approach for cancer immunotherapy.

Endothelial cells targeted by melanoma-specific cytotoxic T lymphocytes

The interaction of melanoma-derived endothelial cells with autologous tumoral cells was investigated in the presence of an autologous melanoma-specific T cell clone. To better analyze these cellular interactions, we set up a three-dimensional culture system (3D-construct) based on collagen matrix containing these cells derived from the same biopsy. This construct presents many advantages in comparison to 2D-culture environment:

- cell morphology and signalling are often more physiological compared to routine 2D-cell culture;
- it permits rapid experimental manipulations and hypotheses confirmation;
- and permits a better real-time and/or fixed imaging by microscopy than in animals.

In fact, it allows CTL migration and killing of specific tumor cells and better reflects the cell-to-cell and cell-to-matrix interactions occurring in an *in vivo* tumor microenvironment. Thus, a 3D-construct containing endothelial, tumor cells and autologous CTL clone was prepared and cellular interactions were monitored by live confocal microscopy. Eighteen hours after co-culture, we observed that CTLs interacted and killed tumor cells (about 70% of melanoma cells were

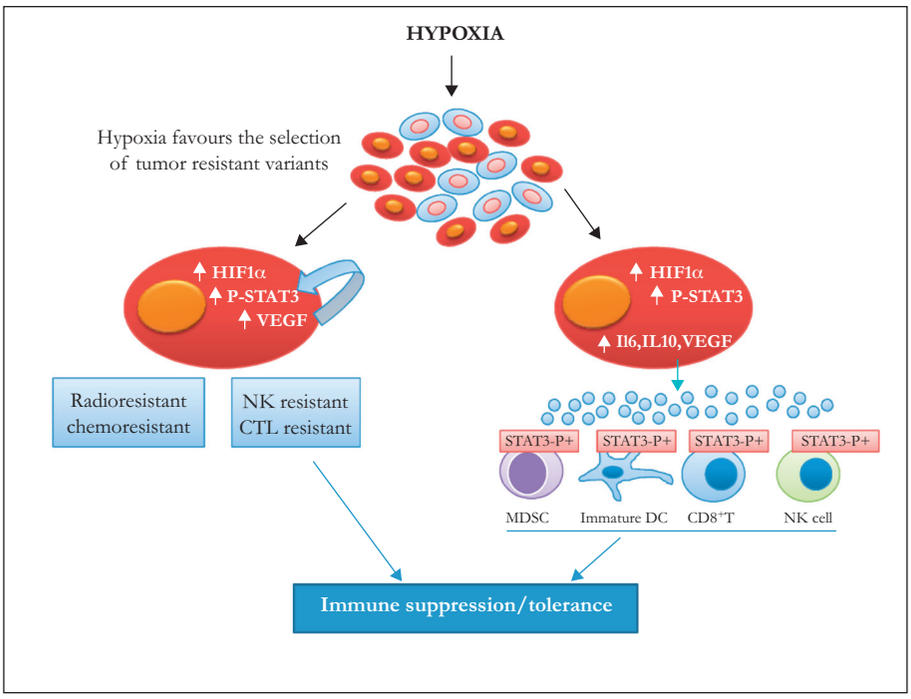

Figure 1. Role of hypoxia induced P-STAT3 in tumor immune suppression. Hypoxic tumor microenvironment is responsible for the emergence of resistant variants. These hypoxic variants have increased HIF-1-α and P-STAT3, which renders them resistant to chemo- and radiotherapy and to the cytotoxic immune effectors NK and CTL. The increased VEGF secreted by tumor variants contributes to the activation of STAT3. Hypoxia activated STAT3 in tumor variants promotes production of factors such as IL6, IL10 and most importantly VEGF. VEGF in turn activates STAT3 in various immune cells, such as MDSC, immature dendritic cells, CD8+ T lymphocyte and NK cells leading to the formation of an immunosuppressive network favouring tumor growth.

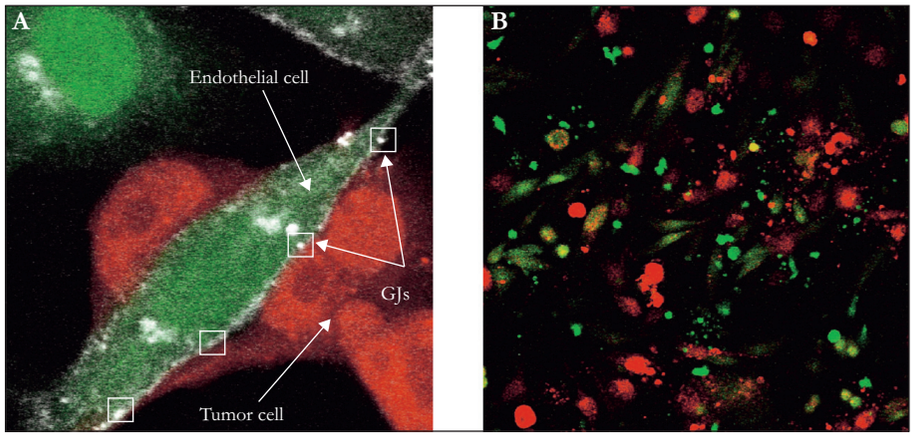

Figure 2. Intercellular communication between endothelial and autologous tumor cells. **A)** Gap-junction formation (white) between endothelial cell (green) and tumor cell (red). **B)** A three-dimensional collagen matrix containing a mix of endothelial cells (yellow), tumor cells (red), and CD8 T cell clone (green). Confocal microscopy analysis of the 3D-construct, after 18 hours of live monitoring, showed the lysis of tumor cells by the autologous CTL clone and that endothelial cells (yellow) became more rounded and lost their elongated shape as well, illustrating the initiation of their killing by CTL clone.

killed) but, interestingly, we also observed the lysis of endothelial cells (about 50%). To-Pro staining performed 48 hours after the beginning of the experiment indicated that only the CTL clone was alive whereas tumor and endothelial cells were lysed. Additional data indicated that the lysis of endothelial cells by the tumor specific clone required the presence of autologous M4T melanoma cells and that endothelial cell lysis was TCR/HLA peptide interaction dependent. To get more insight into the mechanisms associated with the EC recognition by CTLs, we investigated the classical antigen cross-presentation pathways in this process by testing the uptake of tumor secreted molecules and the phagocytosis of tumor cell fragments. We demonstrated that our endothelial cells did not undergo cell death subsequently to these two mechanisms, indicating that the mechanism of antigenic peptide cross-presentation by EC depended on another one [30]. Recently, a new antigen cross-presentation pathway *via* gap-junction (GJ) formation was published by the group of Jacques Neefjes [44]. GJ allows intercellular communications between adjacent cells permitting a non-specific exchange of small cytoplasmic molecules such as peptides. We then demonstrated that endothelial cells and tumor cells form connexin 43-based GJ plaques *(figure 2)*. These GJs are functional and allows the transfer of peptides from tumor to endothelial cells. In order to confirm that a peptide was indeed transferred from melanoma to endothelial cells, we treated melanoma cells with the proteasome inhibitor epoxomicin. When added in the 3D-construct, epoxomycin inhibited the lysis of melanoma cells but also of endothelial ones. These data indicate that a peptide transfer indeed occurred from melanoma to endothelial cells through GJs.

Conclusion

In this review, we addressed the recent advances in our understanding of the contributions of hypoxia and endothelial cells within tumor microenvironment, their ability to actively perturb antitumor cytotoxic effector cells. We also propose new therapeutic approach to disrupt the immunosuppressive network orchestered by the hypoxic tumor microenvironment.

We have shown that hypoxia is able to decrease tumor target susceptibility to antigen specific cytotoxic T cells. This resistance was mediated by the induction of HIF-1α and by the activation of STAT3 in tumor targets under hypoxic conditions [24]. STAT3 propagates several levels of cross talk between tumor and immune system [26]. This dual role of STAT3 in tumor cells and immune cells renders it a central and critical target for inhibiting tumor growth. Recently, a novel small molecule inhibitor of STAT3 has been reported to reverse immune tolerance in malignant glioma patients [31]. More recently sunitinib was shown to positively change the immunosuppressive phenotype in RCC tumors [32] and more interestingly Ozao-Choy *et al.* have shown that sunitinib malate, a receptor tyrosine kinase inhibitor, could reverse MDSC-mediated immune suppression and modulate the tumor microenvironment by increasing percentage and infiltration of CD8+ and CD4+ T cells, thereby improving the efficacy of immune-based

therapies [33]. These findings suggest that sunitinib can be used to reverse immune suppression and as a potentially useful adjunct for enhancing the efficacy of immune-based cancer therapy for advanced malignancies.

We have also shown that hypoxia activates STAT3 by autocrine signalling through HIF-1α induced VEGF *(figure 1)* and VEGF neutralization resulted in the attenuation of hypoxic tumor target resistance to CTL-mediated killing. Therefore, understanding how VEGF and other soluble factors may lead to STAT3 activation *via* the tumor microenvironment may provide a more effective cancer treatment strategy for hypoxic tumors with elevated P-STAT3 levels. This also suggests that reduction of VEGF release in tumor microenvironment might favour induction of a stronger antitumor CTL response against tumors expressing VEGFR. Our studies are in agreement with reports showing that inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer [27] and indicating a synergy between tumor immunotherapy and anti-angiogenic therapy [28].

Our results points to a potential role of HIF-1α and STAT3 in tumor adaptation to hypoxia. Therefore, modulating hypoxic microenvironment either by inhibiting HIF-1α or P-STAT3 would result in potentiating the antitumor cytotoxic effector response.

In this regard, the possibility that novel approaches targeting HIF-1α and STAT3 with potent small molecule drugs, being actively developed, may provide an exciting novel approach for cancer immunotherapy.

Recently, we provided evidence indicating that endothelial cells cross-present tumor antigens and are recognized and killed by melanoma-specific CTLs. It is assumed that human endothelial cells do not express CD80 and CD86 molecules [34], but they express constitutively the co-stimulatory molecule inducible T cell costimulator-ligand (ICOS-L) that mediate cytokines secretion by T cells [35] after interaction with ECs. In another hand, PD-L1 and PD-L2 expressed by ECs regulate negatively the cytokine secretion by CD4+ and CD8+ cells. The capacity of ECs to stimulate or activate T cell is discussed. In fact, it was reported that cross-presentation of exogenous or apoptotic antigens by ECs to CD8+ T lymphocytes conducted them to tolerance [17, 21, 36]. Furthermore, it was shown that ECs exclusively stimulate activated memory CD8+ T lymphocytes to differentiate into EC-selective CTL, and that any stimulation and proliferation was observed in the case of naive CD8+ T cells [37, 38]. It is to note that these observations were made in the context of murine ECs and little is known about human ECs.

Although it is assumed that ECs are not a good "APC", it appears clearly that they can be "the origin" of slower tumor progression. It has been previously suggested that the antigen-presenting functions of endothelial cells could contribute to lymphocyte homing [39, 40]. Also, it has been demonstrated that IFN-γ treated rat microvascular endothelial cells are able to stimulate the rejection of a rat heart that has been depleted of passenger leukocytes [41]. Recently, it was demonstrated that vaccination of endothelium inhibits angiogenesis of colon cancer [42] and that injection of dendritic cells pulsed with endothelium lysates was followed by induction of immune response against tumor angiogenesis [43]. In addition to these previous observations, we evidenced for the first time that human ECs can be recognized and killed by melanoma-specific cytotoxic lymphocytes. We can then speculate that ECs in contact with tumor cells can cross-present tumor-antigenic peptide to CTL and that a close ECs/CTL interaction occurs during tumor infiltration by T cells (following extravasation) leading to the recognition of ECs and to the destruction of microvascular vessels, thus suggesting a potential "anti-angiogenic activity for CTL".

Our results further strengthen the notion that many aspects of tumor biology can only be explained by a detailed understanding of the interaction between tumor and its microenvironment. The development of effective anticancer therapies must take into consideration not only the tumor cells but also different components of tumor microenvironment. As it has been clearly established that the outcome of immunotherapy is mostly dependant on the host tumor microenvironment, it would be interesting to design anticancer treatment that may facilitate antitumor effector response. It would be interesting to try to enhance the effects of immunotherapy by targeting tumor hypoxia (targeting HIF-1 or STAT3 or VEGF) while reverting the immunosuppressive hypoxic tumor microenvironment. Further studies on cellular and molecular mechanisms to contribute to antitumor immune responses will be needed to overcome immunological tolerance and promote tumor rejection. ▼

Conflict of interest : none.

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Microenvironmental Hypoxia Orchestrating the Cell Stroma Cross Talk, Tumor Progression, and Antitumor Response

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ABSTRACT: Hypoxia, a common feature of solid tumors and one of the hallmarks of tumor microenvironment, favors tumor survival and progression. Although hypoxia has been reported to play a major role in the acquisition of tumor resistance to cell death, the molecular mechanisms that control the survival of hypoxic cancer cells and the role of hypoxic stress in shaping the cross talk between immune cells and stroma components are not fully elucidated. Recently, several lines of investigation are pointing to yet another ominous outcome of hypoxia in the tumor microenvironment involving suppression of antitumor immune effector cells and enhancement of tumor escape from immune surveillance. Although the identification of tumor-associated antigens provided a new arsenal of approaches to enhance antigen-specific response, the immunotherapy approaches that are currently used in the clinic have only limited success. In fact, tumor stroma components including hypoxia are engaged in an active molecular cross talk that has serious implications for immunological recognition of tumor in shaping the microenvironment. In this review, we will focus on the impact of hypoxia on the regulation of the antitumor response and the subsequent tumor progression. We will also in particular discuss data that indicate that manipulation of hypoxic stress may represent an innovative strategy for a better immunotherapy of cancer.

KEY WORDS: hypoxia, HIF-1α, antitumor response and tumor progression

ABBREVIATIONS: CSC: cancer stem cells; **CTL:** cytotoxic T lymphocyte; **DC:** dendritic cell; **EMT:** epithelial to mesenchymal transition; **FIH-1:** factor inhibiting HIF-1; **HIF:** hypoxia inducible factor; **HRM:** hypoxia-regulated micro-RNA; **MDSC**: myeloid derived suppressive cell; **NF-κB:** nuclear factor kappa-light-chain-enhancer of activated B cells; **NK:** natural killer; **PHD2:** prolyl hydroxylase domain protein 2; **STAT-3:** signal transducer and activator of transcription-3; **TAM:** tumor-associated macrophage; **TGF-β:** transforming growth factor beta; **VHL:** von Hippel-Lindau tumor suppressor protein

I. INTRODUCTION

The term tumor hypoxia commonly refers to a condition in tumors where the oxygen pressure is less than 5–10 mm Hg.[1] Hypoxia is a common feature of almost all of the solid tumors and one of the hallmarks of tumor microenvironment.[2]

Solid tumors contain areas of variable oxygen concentrations. Tumor cells closest to a perfused blood vessel have relatively high O_2 concentrations, which decline as distance from the vessel increases. There are areas that are well oxygenated, poorly oxygenated, and finally necrotic in which cancer cells have died due to inadequate oxygenation[2,3] [Fig. 1(a)]. Accumulating evidence indicates that hypoxic zones in solid tumors have temporal fluctuations in oxygen concentration.[4]

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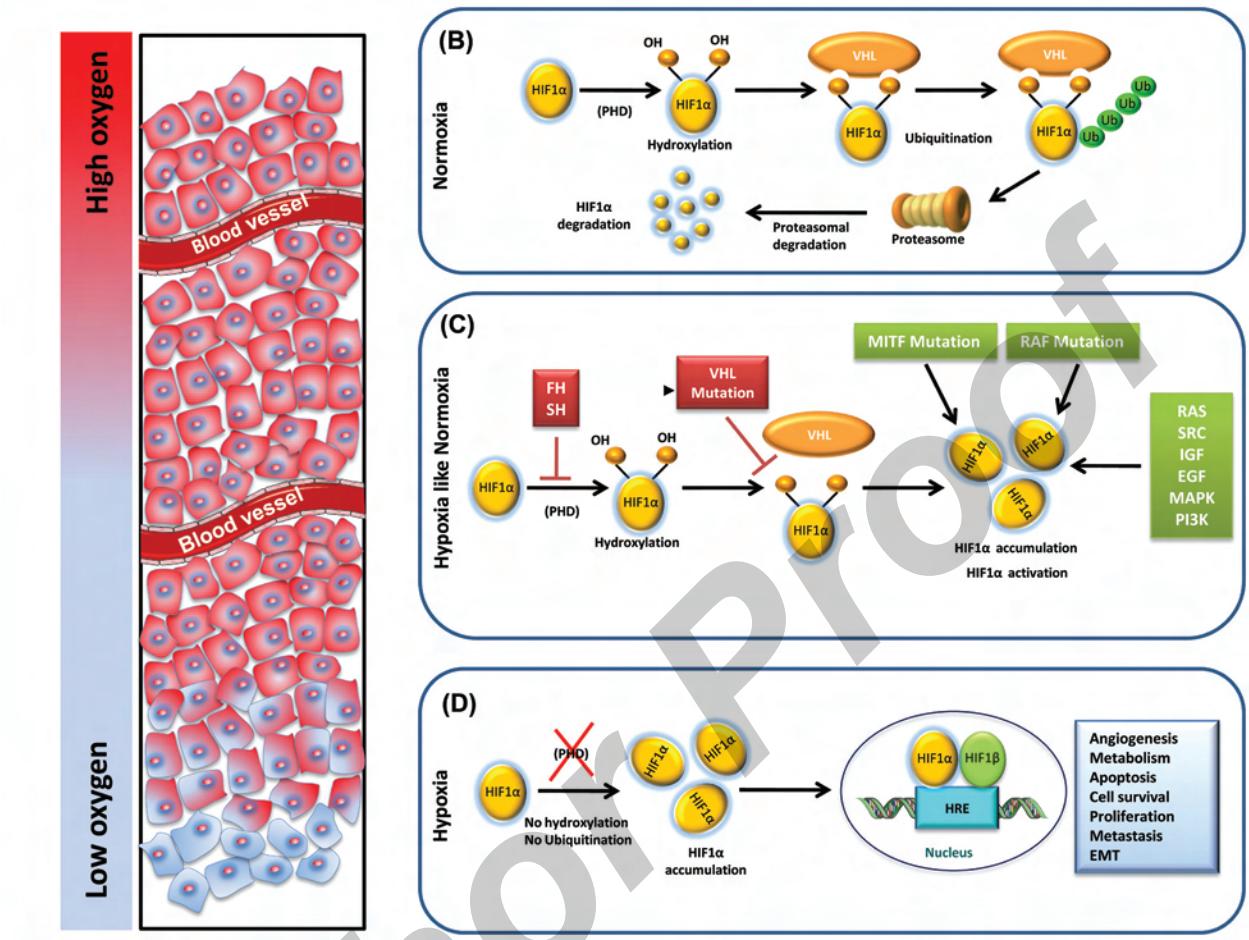

FIGURE 1. Schematic overview of normoxic and hypoxic regulation of HIF-1α. (A) Solid tumors contain areas of variable oxygen concentrations. Tumor cells closest to a perfused blood vessel have relatively high O_2 concentrations (normoxic cells, highlighted in red). The O_2 concentrations decline as distance from the vessel increases (hypoxic cells, highlighted in blue). (B) In normoxia, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs), resulting in its interaction with the von Hippel-Lindau tumor suppressor protein (VHL), which recruits an E3 ubiquitin-protein ligase that subsequently catalyzes polyubiquitination of HIF-1α, thereby targeting it for proteasomal degradation. (C) Apart from strict oxygen-dependant regulation, HIF-1α is also regulated by several oxygen-independent mechanisms (hypoxia such as normoxia). Mutations of fumarate hydratase (FH) and succinate hydratase (SH) inhibit the PHDs. VHL mutations lead to constitutive activation of HIFs. RAF and MITF mutations are able to regulate HIF-1α under a normal level of O_2. (D) Under hypoxic conditions, hydroxylation is inhibited and HIF-1α rapidly accumulates, dimerizes with HIF-1β, and binds to the HREs (hypoxia response elements) in target genes. HIF-1 thereby controls several important processes in tumor biology.

A. Hypoxia Inducible Factors (HIFs) and Tumor Cell Adaptation to Hypoxia

Tumor cells adapt to hypoxic microenvironment by the regulation of hypoxia inducible factor family of transcription factors (HIFs). This family is composed of three members, namely, HIF-1, HIF-2, and HIF-3. HIF-1 is a heterodimeric protein that is composed of a constitutively expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit.[5]

HIF-1α in the presence of O_2 is hydroxylated on proline residue 402 and/or 564 by prolyl

hydroxylase domain protein 2 (PHD2), resulting in its interaction with the von Hippel-Lindau (VHL) tumor suppressor protein, which recruits an E3 ubiquitin-protein ligase that eventually catalyzes polyubiquitination of HIF-1α, thereby targeting it for proteosomal degradation [6] [Fig. 1(b)]. Under hypoxic conditions, hydroxylation is inhibited and HIF-1α rapidly accumulates, dimerizes with HIF-1β, binds to the core DNA binding sequence 50-RCGTG-30 [R, purine (A or G)] in target genes, recruits coactivators, and activates transcription [Fig. 1(d)]. In addition, O_2-dependent hydroxylation of asparagine-803 by factor-inhibiting HIF-1 (FIH-1) blocks interaction of HIF-1α with the coactivators P300/CBP under normoxic conditions.[7] Both PHD2 and FIH-1 use O_2 and α-ketoglutarate as substrates and generate CO_2 and succinate as by-products of the hydroxylation reaction.[8] Similar to HIF-1α, HIF-2α is also regulated by oxygen-dependent hydroxylation.[9] HIF-1α and HIF-2α are structurally similar in DNA binding and dimerization domains but differ in their transactivation domains. Consistently, they share overlapping target genes, whereas each also regulates a set of unique targets.[10] HIF-3α lacks the transactivation domain and may function as an inhibitor of HIF-1α and HIF-2α and its expression is transcriptionally regulated by HIF-1.[11]

Apart from strict oxygen-dependant regulation of HIFs, HIF-1α and HIF-2α are regulated by several oxygen-independent mechanisms.[12] It has been reported that mutation of VHL, PTEN, B-RAF, SDH, FH, and MITF are able to regulate HIF-1α under normal level of O_2 (hypoxia like normoxia) [Fig. 1(c)]. This stability of HIF-1α under normoxia leads to its increased transcriptional activity and may have a huge impact on cell biology and cancer development.

A large body of clinical data shows a positive correlation between increased hypoxic expression of HIF-1α and HIF-2α and patient mortality.[8] Both HIF-1α and HIF-2α have common target genes as well as their respective target genes. The genes induced by hypoxia-dependent HIF-1α and HIF-2α play important roles in regulating different aspects of tumor biology such as angiogenesis, cell survival, chemo- and radioresistance, proliferation, invasion and metastasis, pH regulation and metabolism, resistance to immune system, and maintenance of cancer stem cells.[13,14]

Interestingly, HIF-1α and HIF-2α can have opposing effects on the cell cycle and on cell proliferation. The best example of this is in clear cell renal carcinoma, in which HIF-2α is described to drive the tumor growth while HIF-1α may restrict proliferation.[15] This opposite effect of HIF-1α and HIF-2α could be related to the mechanism involving the regulation of c-Myc. Indeed, it has been suggested that HIF-1α antagonizes the activity of Myc while HIF-2α enhances Myc activity.[16]

In this review, we will discuss how cells of the immune system (both innate and adaptive) respond to hypoxia in terms of their survival and effector functions. We will show the ways in which hypoxic tumor microenvironment promotes the recruitment, activation, and survival of innate immune cells while inhibiting the adaptive immunity. We will outline how hypoxic stress confers resistance to tumor cells, and describe the molecular aspects of the cross talk between hypoxia induced factors (HIF-1α, p-stat3 and mir- **[AQ: 1],** etc.) and resistance to cytotoxic treatments. We will also illustrate the relationship between hypoxia and cancer stem cells. Finally, we will discuss whether the inhibition of hypoxic signaling pathways in different compartments of the solid tumor microenvironment will open new therapeutic opportunities in cancer immunotherapy.

II. HYPOXIA AFFECTING THE IMMUNE SYSTEM

The effects of hypoxia on immune cells are proposed to be critical factors for the development of tumor immune escape. The innate and adaptive immune systems respond differentially to decreased oxygen

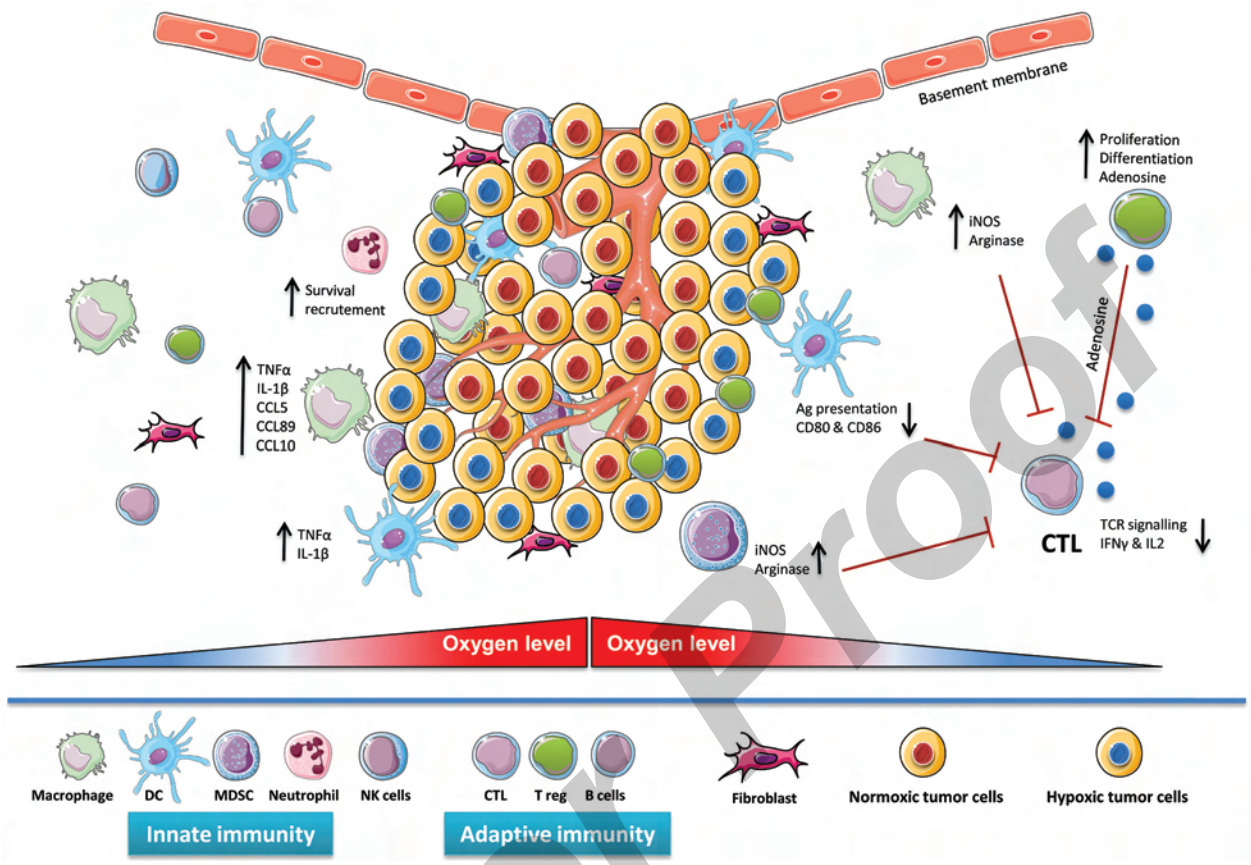

FIGURE 2. Influence of hypoxia on the innate and adaptive immune systems. Diverse effects of hypoxia on innate immune system (macrophage, DC, MDSC, NK, and neutrophil) and adaptive immune system (CTL, Treg, and B cells). In general, hypoxia amplifies the activity of innate immune cells while suppressing the response of the adaptive immune system. DC, dendritic cells; MDSC, myeloid-derived suppressor cells; NK, natural killer cells; CTL, cytotoxic T lymphocyte; Treg, T regulatory cells.

levels with a global amplification of the innate responses and a direct or indirect (via the innate system) shortcoming of the adaptive ones. These diverse effects are summarized in Fig. 2.

A. Hypoxia Interferes with Innate Immunity

Hypoxia promotes neutrophils survival by inhibiting apoptosis and inducing the release of the neutrophil survival factor MIP-1β.[17,18] It also increases neutrophil blood vessel extravasation by modulating β integrin expression[19] and neutrophil tumor infiltration through the secretion of hypoxia-induced chemokines by hypoxic epithelial and endothelial cells.[20] Hypoxic zones in tumors are also able to attract macrophages leading to macrophage accumulation.[21] The presence of HIF-1α in tumor-associated macrophages suppresses cytotoxic T-cell function, and loss of HIF-1α in macrophages is sufficient to relieve tumor-infiltrating lymphocytes (TILs) from immune suppression and decrease tumor growth.[22] TAM and tumor-infiltrating neutrophils play a key role in cancer development and metastasis.[23,24] TAM can also be issued from myeloid-derived suppressor cell (MDSC) differentiation following HIF1α stabilization in MDSC within the hypoxic tumor

microenvironment. HIF-1α in MDSC makes them able to suppress antigen-specific T-cell functions, which further supports the immunosuppressive environment observed within tumors.[25]

The presence of HIF-1α in dendritic cells (DCs) seems to have contradictory effects. Indeed, low oxygen levels are reported to inhibit DC maturation and differentiation but to enhance DC inflammatory functions. This leads to an inhibition of adaptive immune functions of DC but reinforcing their innate immune ones.[26] In contrast, other studies show that hypoxia induces human DC maturation and differentiation.[27,28] These mature DCs overexpress HIF-1α and their coculture with T cells induces T-cell maturation. These conflicting results could be explained by differences in the experimental approaches with regard to the source and purity of DC precursors, the protocols used for maturation and differentiation of DC, and the hypoxic stimulus (duration, level, hypoxic chambers versus $CoCl_2$). Future research is needed to further dissect the cross talk between hypoxia/HIFs **[AQ: 2]** and DCs, which represents a critical issue for our understanding of host responses to tumors.

B. Influence of Hypoxia on Adaptive Immunity

Hypoxia leads to the development of a proinflammatory microenvironment that promotes innate immunity while it inhibits adaptive immunity.[18] Nonetheless, the role of hypoxia and HIFs on the adaptive immune system is not well characterized. It is reported that hypoxia promotes T-cell lymphocyte apoptosis.[29,30] Other studies show on the contrary that hypoxia prevents T-cell activation–induced cell death resulting in increased survival.[31] Deficiency for HIF-1α in thymocytes from mice also results in increased caspase-8–mediated apoptosis.[32] These conflicting results need to be reconciled. With regard to T-cell functions, hypoxia depresses CTL functions by inhibiting TCR signaling.[29] It also induces increased local concentration of extracellular adenosine[33,34] that acts on T-cell A2A adenosine receptors (A2AR) causing intracellular cAMP accumulation and subsequent inhibition of activated T cells. The use of A2AR antagonists or genetic depletion of A2AR weakens inhibition of antitumor T cells and inhibits or delays tumor growth.[35] These findings imply that hypoxia-mediated inhibition of T cells confers protection to the hypoxic tissue. Accordingly, in an inflammatory context, targeting of HIF-1α in activated T cells is reported to increase T-cell responses suggesting that HIF-1α, in addition to mediating physiological responses to hypoxia, also plays a negative regulatory role on T-cell functions.[36,37] Recently, HIF-1α has been shown to mediate Th17 differentiation and to inhibit regulatory T cells (Tregs) differentiation from naive T cells by activation of the glycolytic pathway.[38]

With regard to B cell lymphocytes, HIF-1α deficiency in chimeric mice causes lineage-specific defects in B-cell development with decreased proliferation of B-cell progenitors, appearance of abnormal B-cell population, and, similarly to the enhancement of T-cell functions in HIF-1α-targeted T cells, autoimmune disorders.[39] In this context, it has been shown that HIF-1α is required for efficient glycolysis in B cells in a stage-specific differentiation manner.[40] Thus, a potential role of HIF-1α in autoimmunity has been proposed through the regulation of the development, metabolism, and functions of B cells.

III. IMPACT OF HYPOXIA ON TUMOR CELLS

A. Influence of Hypoxia-Induced Regulation of Tumor Cell Susceptibility to Cell-Mediated Cytotoxicity

It is now well established that hypoxic tumor microenvironment favors the emergence of tumor

variants with increased metastatic and invasive potential.[41] Several other evidences suggest that HIF-1α may have a protective role under hypoxic conditions.[42,43] Since these tumor variants are resistant to radiotherapy and chemotherapy, one might postulate that the exposure to low levels of oxygen may lead to adaptive responses allowing tumor cells to escape from immune surveillance. Fink and colleagues reported the inhibition of natural killer (NK) cytotoxicity toward liver cell lines under hypoxic conditions.[44] **Siemens** et al. have recently reported that hypoxia contributes to tumor cell shedding of the MHC class I chain-related molecule (MIC) through a mechanism involving impaired nitric oxide (NO) signaling.[45] These findings suggest that hypoxia is able to confer tumoral cell resistance to effector cell cytotoxicity.

Recently, we have shown[46] that hypoxic exposure of target tumor cells inhibits the CTL clone-induced autologous target cell lysis. Interestingly, the observed lysis inhibition was not associated with an alteration of CTL reactivity and tumor cell recognition indicating that tumor-induced priming of the autologous CTL clone was not affected after exposure of tumor target cells to hypoxia. We further demonstrated that HIF-1α induction and STAT3 activation are associated with hypoxia-induced lysis inhibition. Our results suggest a new role for hypoxia-dependent induction of HIF and activation of STAT3 in tumor resistance to the immune system.[46]

B. Role of Hypoxia-Activated STAT3 in Modulating Antitumor Immune Response

STAT3 activation within tumor microenvironment is known to be associated with cytokine-induced proliferation, antiapoptosis, and transformation.[47,48] Moreover, it is now well established that STAT3 modulates the cross talk between tumor and immune cells.[49] A novel small-molecule inhibitor of STAT3 has been reported to reverse immune tolerance in malignant glioma patients.[50] Another inhibitor, sunitinib, was shown to positively change the immunosuppressive phenotype in RCC tumors,[51] and more interestingly, Ozao-Choy et al. have shown that sunitinib malate, a receptor tyrosine kinase inhibitor, could reverse MDSC-mediated immune suppression and modulate the tumor microenvironment by increasing higher percentage and infiltration of CD8 and CD4 cells, thereby improving the efficacy of immune-based therapies.[52] This points to the potential role of STAT3 in tumor adaptation induced by hypoxia. The proposed role for STAT3 suggests that the effect of hypoxic induction of STAT3 extends beyond its critically important role in controlling cell survival and apoptosis. This emphasizes that a better understanding of the tumor behavior and its interplay with the killer cells in the context of the complexity and plasticity of a hypoxic microenvironment will be a critical determinant in a rational approach to tumor immunotherapy.

Although resistance of tumor targets to killer cells is likely to be regulated by multiple factors,[53] we believe that hypoxic microenvironment is a key determinant involved in the control of target sensitivity to CTL-mediated lysis. Therefore, the possibility that novel approaches targeting HIF-1α and STAT3 with potent small-molecule drugs, being actively developed, may provide an exciting novel approach for cancer immunotherapy.

C. Role of Hypoxia-Induced Autophagy in the Adaptation of Tumor Cells to Anticancer Therapies

Autophagy is an evolutionarily conserved cellular process involving the formation of double-membrane vesicles called autophagosomes. These autophagosomes sequester organelles and cytoplasmic contents to deliver them to lysosomes for degradation.[54] The autophagic process allows cells to recycle damaged proteins and organelles to maintain nutrient and energy homeostasis during

sublethal stress including nutrient or growth factor deprivation, reactive oxygen species, and hypoxia.[55] It has been well documented that hypoxia activates the autophagy pathway in cancer cells [Fig. 3(a)]. The activation of autophagy under hypoxia depends on the induction of HIF-1α, which activates the transcription of the BH3-only proteins *BNIP3* and *BNIP3L* leading to the disruption of the inhibitory interaction between Beclin 1 and Bcl-2 [Fig. 3(b)].[56,57] In addition to *BNIP3* and *BNIP3L*, it has been reported that hypoxia, through the transcription factors ATF4 and CHOP, increases the transcription of *LC3* and *ATG5*,[58] two essential genes involved in the formation and maturation of autophagosomes. Several other pathways have been described to activate autophagy under hypoxia.[55] Autophagy has also recently been demonstrated as important for conferring resistance to different anticancer therapies including immunotherapy.[59] In several human cancer cell lines, hypoxia increased transcription of the essential autophagy genes such as LC3. This transcriptional induction replenished LC3 protein that was turned over during extensive hypoxia-induced autophagy in order to maintain the autophagic process during hypoxia and thereby promotes the survival of cancer cells.[58] Consistent with these data, autophagy induction occurred preferentially in hypoxic regions of human tumor xenografts. Furthermore, pharmacological inhibition of autophagy sensitized human tumor cells to hypoxia, reduced the fraction of viable hypoxic tumor cells, and sensitized xenografted human tumors to anticancer therapy.[58] Collectively, these studies strongly argue that autophagy is an important mediator of the hypoxic tumor microenvironment and that it contributes to resistance to treatment. Beside its function as a protein degradation process, recent evidence points to a novel role of autophagy in innate and adaptive immunity.[60] Indeed, the autophagy pathway can modulate key steps in the development of adaptive immunity. In this context, it has been proposed that autophagy regulates the development and survival of lymphocytes as well as the modulation of antigen processing and presentation.[61] Autophagy induction in target cells also increases their potential to serve as immunogens for dendritic cell cross-presentation to CD8+ T cells. Furthermore, the autophagy pathway can also modulate the selection and survival of some CD4+ T cells in the thymus.[62] However, much still remains to be learned about the relationship between hypoxia-induced autophagy and the tumor immunotherapy. Obviously, targeting autophagy in hypoxic tumor cells may have a major impact on cancer immunotherapy.

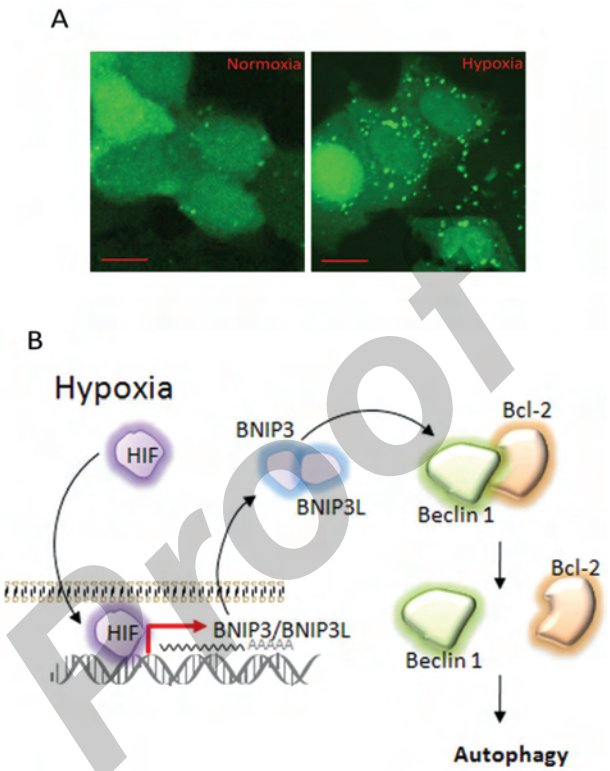

FIGURE 3. (A) Hypoxia induction of autophagy in MCF-7 breast adenocarcinoma cell line. Cells transfected with the autophagy marker LC3 fused with GFP were cultured under normoxia (pO_2 21%) or hypoxia (pO_2 1%). The induction of autophagy was monitored by the formation of green dot–like structures corresponding to autophagosomes; bar, 10 mm. (B) Involvement of HIF-1α in the induction of autophagy. Under hypoxia, HIF-1α activates the transcription of BNIP3/BNIP3L proteins. The overexpression of these proteins dissociates the autophagy inhibitory protein complex between Beclin 1 and Bcl-2, and consequently activates autophagy.

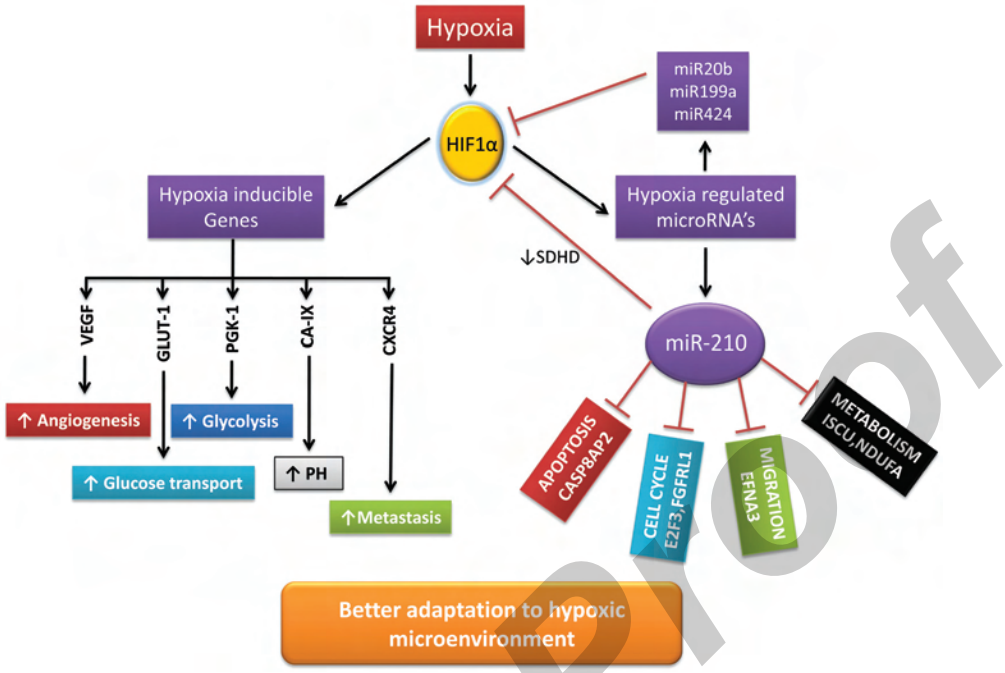

FIGURE 4. A proposed model of how hypoxia regulates hypoxia-inducible genes and hypoxia-regulated micro-RNAs (HRMs) leading to better adaptation to the hypoxic microenvironment. Hypoxia activates HIF-1α, which in turn regulates the hypoxia-inducible genes (left side) such as VEGF, GLUT-1, PGK-1, CA-IX, and CXCR4. On the other hand, hypoxia regulates several micro-RNAs called hypoxia-regulated micro-RNAs (HRMs). Once induced, HRMs such as miR20b, miR199a, and miR424 affect HIF-1α expression under hypoxia. One of the HRMs, miR-210, is able to repress several genes implicated in various physiopathological pathways.

D. Role of Hypoxia-Regulated Micro-RNAs in the Control of Tumor Behavior

Micro-RNAs (miRNAs) are about 18–24 nucleotides, and are small noncoding RNAs. They negatively regulate mRNA expression by repressing translation or directly cleaving the targeted mRNA.[63] Over the past few years, the role of miRNA has expanded from their functions in the development of roundworms to ubiquitous regulator implicated in several critical processes, including proliferation, cell death and differentiation, metabolism, and, importantly, tumorigenesis.[64]

1. Hypoxia-Regulated Micro-RNAs (HRMs)

Hypoxia as an essential component of tumor microenvironment is capable of stabilizing transcription factor HIF-1α, which in turn is capable of regulating its target genes (classical pathway of response to hypoxia), but also much of micro-RNAs (new pathway of response to hypoxia). These micro-RNAs regulated by hypoxia are known as hypoxia-regulated micro-RNAs (HRMs) (Fig. 4). These HRMs are capable of repressing the expression of different target genes, thereby influencing important processes in tumor development such as angiogenesis, cell survival and cell death, etc.[65,66] Recently, it has been shown that certain HRMs are capable of affecting HIF-1α expression.[67]

2. Mir-210: Mediator of Hypoxic Response

Among these HRMs, miR-210 is the only miRNA consistently upregulated in both normal and transformed hypoxic cells, and it is also generally

recognized as a robust HIF target.[68] Mir-210 has been considered as an *in vivo* marker of tumor hypoxia.[69] Mir-210 has been correlated positively to poor patient prognosis in head and neck cancers, and mir-210 has been detected in the serum of breast cancer patients.[70–72] Mir-210 also participates in the hypoxic response of endothelial and neuronal cells.[73]

More importantly, the increased expression of miR-210 also correlates with the improved survival of transplanted mesenchymal stem cells (MSCs) in a rat model. By downregulating caspase-8–associated protein 2 (CASP8-AP2), a proapoptotic regulator of Fas-mediated apoptosis,[74] miR-210 protects MSC from cell death. Finally, mir-210 is capable of regulating several cellular processes by regulating the expression of genes involved in angiogenesis, cell cycle, cell survival, and tumor initiation[68] (Fig. 4).

In summary, miR-210 plays a crucial role in mediating the cellular response to hypoxia resulting in a better adaptation of hypoxic cells to tumor microenvironment. Manipulating miR-210 within the tumor microenvironment may therefore lead to novel diagnostic and therapeutic approaches.

E. Hypoxia and Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is a complex molecular process during which epithelial cells lose apico-basal polarity and acquire mesenchymal motile phenotype. A hallmark of the EMT is the downregulation of epithelial markers such as cadherin and cytokeratin, and the upregulation of mesenchymal markers such as vimentin leading to an organized disassembly of epithelial cell-cell contact.[75–77] EMT has a central role in cancer progression and metastasis.[78,79] Several signaling pathways (TGF B, Wnt, Notch, etc.) and transcriptional factors (Snail, Slug, Twist, etc.) can induce EMT.[79] Recently, hypoxic signaling through HIF was implicated as an important trigger and modulator of EMT.[80,81]

1. Hypoxic Regulation of EMT Triggering Signaling Pathways

Under hypoxic microenvironment, different signaling pathways, directly involved in triggering EMT, are activated[80] (Fig. 5). Members of TGF β are major inducers of EMT during embryonic development, fibrotic diseases, and cancer progression.[82] TGF β, after binding to serine/threonine kinase receptors TβRI and II, recruits and phosphorylates TGF β cytoplasmic mediators SMADs. SMADs interact with EMT transcriptional factors (Snail, Slug, and SIP) and thereby inducing mesenchymal transition.[83] TGF β can also trigger EMT through cooperation with oncogenic pathways such as Ras, Wnt/β catenin, and NF-κB in order to maintain mesenchymal and invasive tumor cells' properties.[84] Several studies reported the existence of cooperation between hypoxia and TGF β in activating EMT. In fact, hypoxia induces an increase in TGF β production from mesenchymal and cancer cells.[85,86] In hepatocytes, it was demonstrated that TGF-β signaling pathway is downstream of HIF activation and that hypoxia-induced EMT depends on TGF β signaling.[87] On the other hand, HIF-1 and TGF β co-regulate some gene targets such as the cyclooxygenase-2, which is implicated in enhancing EMT.[88]

NF-κB induces expression of the master EMT regulators Snail, Slug, ZEB1, ZEB2, and Twist, which repress expression of genes encoding epithelial markers. NF-κB also promotes a mesenchymal phenotype, migration, and invasion of tumor cells.[89] Different studies highlight the implication of hypoxia in the induction of EMT via the NF-κB pathway most likely through cross talk between NF-κB and HIF signaling pathways.[90] For example, it was demonstrated that inhibition of oxygen-dependent hydroxylases under hypoxia

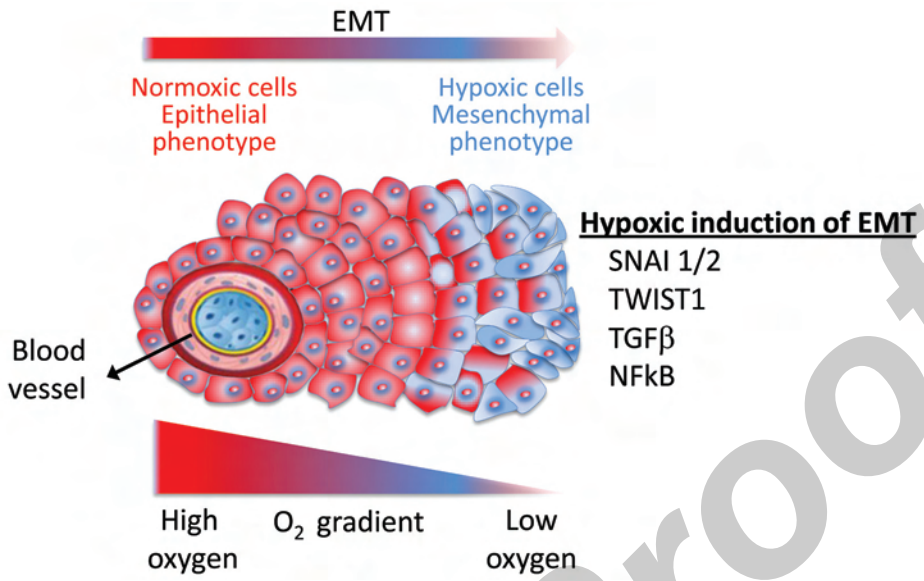

FIGURE 5. Schematic representation of the EMTs occurred under hypoxia. Cells located in the well oxygenated areas (normoxic areas) closest to blood vessel display an epithelial phenotype; however, cells located in poorly oxygenated areas lose their epithelial phenotype and gain mesenchymal properties following the activation of several transcription factors and pathways involved in the induction of the EMT.

leads to the activation of both HIF and NF-κB pathways.[91]

The Notch signaling pathway can induce EMT via increasing Snail expression or by interacting with TGF β/SMADs and NF-κB pathways.[92] It has been shown that HIF-1 binds the Notch intracellular domain and stabilizes its transcriptional activity leading to the regulation of the Notch pathway under hypoxia.[93] Recent findings demonstrated that Notch pathway inhibition abrogated the hypoxia-mediated increase in Slug and Snail expression, as well as decreased breast cancer cell migration and invasion.[94]

2. Hypoxic Regulation of EMT Transcription Factors

After hypoxia or constitutive HIF expression, epithelial cell lines regulate the expression of major EMT-associated transcription factors. Under hypoxic conditions, Twist **[AQ: 3]** protein level is significantly upregulated in different tumor cell lines. Reduced endogenous HIF-1 reverted the Twist level in cells, indicating the key role of HIF in Twist expression regulation.[95] Twist overexpression induced in hypoxia conditions leads to repression of E-cadherin and promotes EMT and metastatic ability. Additionally, coexpression of Twist, Snail, and HIF-1α was recently correlated with bad prognosis and metastasis.[96,97]

Snail, a zinc-finger transcription factor, is involved in the repression of many epithelial markers expression and plays a crucial role in carcinoma cells invasion. It has been associated with decreased recurrence-free survival.[98] Increased Snail expression, under hypoxia, accompanied by decreased E-cadherin expression, has been reported in ovarian cancer cell lines.[99] Exposure of human breast cell lines to hypoxia also induced EMT with elevated Snail and vimentin expression.[100] Additionally, some hypoxia targets [lysyl oxidase like 2, urokinase plasminogen activator (uPA) and its receptor **[AQ: 4]**] could regulate Snail and

trigger EMT.[101,102] Recently, Luo et al. identified a hypoxia-responsive element within close proximity of the minimal Snail promoter, which binds both HIF-1 and HIF-2 target genes and activates Snail transcription.[103] Hypoxia can also increase the expression of epithelial repressor Slug via HIF-1α promoting cancer cells invasiveness. Recently, Chen et al. reported that induced Slug and Snail expression by hypoxia-mediated Notch signaling is associated with increased breast cancer cell migration and invasion.[94]

Thus, it is well established that hypoxia-regulated EMT is directly implicated in tumor growth, invasion, and metastasis. In the future, we have to consider targeting both hypoxia and EMT in the design of new drugs.

F. Hypoxia and Cancer Stem Cells

Tumor growth is dependent on the presence of a subpopulation with stemlike properties called cancer stem cells (CSCs) within the tumor.[104] CSCs, which are in an undifferentiated state, undergo self-renewal and when implanted in immune-deficient mice are able to develop tumors and to reestablish the bulk tumoral heterogeneity.[105] CSCs also have the property to resist conventional antitumor therapies,[106] which makes them a probable cause of tumor recurrences after treatment. Therefore, their eradication in the tumor is a therapeutic challenge that justifies a better understanding of their emergence and persistence in the tumoral tissue.

In this regard, hypoxia and HIFs have been described to induce tumor cell dedifferentiation toward an immature phenotype and similarly to maintain tumor cells with stem cell properties.[13] Several reports show the role of hypoxia and HIFs in promoting a stemlike phenotype through the expression of genes such as OCT4, SOX2, and NANOG required for self-renewal maintenance in stem cells or the activation of the Notch signaling pathway that regulate cell self-renewal and differentiation.[13] A study from Jogi et al. has shown that culturing neuroblastoma cells under hypoxia led to an increase of neural crest gene expression and a decrease of neuron lineage marker expression.[107] In glioblastoma, Mc Cord et al. have reported that glioblastoma neurospheres under hypoxia show an increased proportion of $CD133^+$ stemlike cells and induction of embryonic markers such as OCT4 and SOX2. This was associated with a selective increase of HIF-2α.[108] Hypoxia was reported by Chen et al. to activate Notch signaling pathway in lung adenocarcinoma, which revealed to be essential since using a Notch signaling inhibitor under hypoxia induced cell death.[109] However, Notch pathway can also promotes cell differentiation in keratinocytes and certain neural stem cells.[110,111] This ability of hypoxia to increase the stem cell–like subset inside a tumor cell population reflects the plasticity of the CSC compartment and the role of microenvironmental stimuli in shaping this particular subset.

Some of the effects of hypoxia on tumor cell differentiation are directly mediated by the HIFs. Li et al. reported that targeting HIF-1α and HIF-2α in $CD133^+$ glioma stem cells decreased their survival and their tumorigenic and angiogenic potentials.[112] They also reported a preferential expression of HIF-2α in $CD133^+$ glioma stem cells, whereas HIF-1α was present in both stem and nonstem tumor cells and needed more severe hypoxia to be stabilized. Another study using human neuroblastoma cells also found a selective expression of HIF-2α in an immature cell subset, with induction of differentiation when targeting HIF-2α.[113] Overexpression of HIF-2α in non-glioma stem cells was sufficient to induce a stem cell–like phenotype (sphere-forming ability, larger tumors after mice engraftment).[114] At a clinical level, HIF-2α expression in patients correlated with poorer prognosis.[112,113] These findings support a preferential targeting of HIF-2α for selective eradication of CSCs without adverse effects on normal progenitor cells. HIF-1α is not outdone since a recent study by Wang et al. using human leukemia showed a selective activation of HIF-1α

in CSCs under normoxic conditions due to VHL deficiency, and that blocking HIF-1α activity was able to eliminate leukemia stem cells without affecting the normal hematopoietic stem cells.[115]

These studies and others describe the effects of hypoxia in converting differentiated cancer cells into stemlike cancer cells, and the possibility that stabilized HIF-1α or HIF-2α may represent critical targets for eradicating the tumoral stem cell compartment. Thereby, HIF protein functions in tumors go beyond mediating adaptation to hypoxia by modulating cell metabolism and survival and driving vessel development. Intriguingly, CSCs were proposed to localize more within a tumor and hypoxic niches than in perivascular and consequently oxygenated regions. In this regard, it has been proposed that both hypoxic and nonhypoxic mechanisms of CSC regulation **[AQ:5]**, although this hypothesis remain to be investigated.[116]

G. Hypoxia-like Normoxia: Oxygen-Independent Regulation of HIF1α and HIF2α

Apart from oxygen-dependent strict regulation of HIFs, HIF1α and HIF2α can also be regulated by oxygen-independent mechanisms, leading to increased HIF transcriptional program under normal level of O_2 and promoting tumor growth.[8] Here, we will discuss how genetic alterations such as gain of function mutations of oncogene or loss of function of tumor suppressor gene can be responsible for overexpression of HIFs in a cell type–specific manner. Recently, it has been shown that inducers of HIFα such as reactive oxygen[4] and nitrogen species[117] can also be found in the microenvironment.

1. Constitutive HIF Activation through Inactivation of VHL Gene

A constitutive HIF gene activation can be observed in VHL diseases (renal cell carcinomas patients and most of sporadic clear cell RCCs) characterized by an inactivation of VHL gene. The double allele loss of function (deletion, mutation, CpG island hypermethylation) $VHL^{-/-}$ gives rise to an increased level of HIF-1α and HIF-2α[118,119] or only of HIF-2α, which seems to be necessary and sufficient for tumor growth in $VHL^{-/-}$ RCC cell lines contrary to HIF1α.[119,120]

2. Constitutive HIF Activation by RAS/RAF/MITF Overactivation

Activating mutation of RAS[121] and RAF[122] were also linked to enhanced levels of HIFα protein, even in normoxic conditions. In colon cancer, it was described that RAS and RAF mutation enable a better response of cancer cells to hypoxia. This underlines their differential impact on HIF1α or HIF2α and justifies phenotypic differences in KRAS or BRAF colon cancer.[123] Furthermore, in melanoma cells, mutant BRAF can enhance HIF1α expression in normoxia[124] and is also described as a regulator of microphthalmia-associated transcription factor (MITF) through a "tricky" process implying an increase in ERK-induced MITF degradation in parallel with an increase of MITF transcription by BRN2.[125] MITF is itself described as a transcriptional activator of HIF1α in melanoma.[126]

3. Constitutive HIF Activation through Inactivation of Fumarate and Succinate Hydratase Gene

Inactivation by germ line mutations of fumarate and succinate hydratase genes in renal cancer cell predisposes to hereditary leiomyomatosis syndrome (HLRCC) and induces hereditary paraganglioma syndrome, respectively. It has been shown that accumulation of fumarate or succinate is accompanied by a high level of HIF under normoxia. This accumulation seems to be mediated by an inhibition of PHD.[127,128]

4. Growth Factor, Oncogenic Signaling Pathways, and Increase of HIF Level

HIF1α levels can be increased by growth factor stimulation. It is noteworthy that a hypoxia-dependent increase of HIF-1α in cell-type specific is not due to its reduced degradation, as observed under hypoxia, but rather to a stimulation of HIF1α synthesis through oncogenic signaling pathways.[12] For example, insulin, insulinlike growth factor, epidermal growth factor, and fibroblast growth factor can induce expression of HIF-1α in normoxia via mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways.[129] It is interesting to note that the Akt pathway can be overactivated in cells displaying either a loss of function of PTEN[130] or an overexpression of HER2.[131]

5. Constitutive HIF Activation by Src Overactivation

Hyperactivity of Src can be responsible for a high level of HIF-1α under normoxia. The high level of HIF-1α in cells displaying activated Src is related to the production of ROS, which reduces cellular vitamin C required for the activity of PHD2. A defect of PHD2 activation leads to HIF-1α accumulation by preventing its hydroxylation-dependent ubiquitinylation.[132]

Overall, constitutively active HIF-1α and HIF-2α play a major role in cancer by inducing adaptation of tumor cell to hypoxia microenvironment. However, activated HIFs in normoxic cells seems to be cell specific.[12]

IV. DOES TARGETING OF HYPOXIA RESULT IN A MORE EFFICIENT IMMUNOTHERAPY?

Clinical vaccination trials aimed at triggering or enhancing antitumor immunity are at present disappointing, given the different mechanisms of tumor escape from immunosurveillance that represent the last series of hurdles to be overcome in formulating truly effective cancer immunotherapy.[133,134] Clearly, tumor-induced alterations to the patient's immune system may subvert anticancer responses and even promote tumor growth. To provide a significant clinical advancement, new concepts have to be introduced to aid the design of new tools for therapy. In addition, there are increasing indications that tumor stroma components including hypoxia play a crucial role in the control of immune protection and contain many overlapping mechanisms to maintain tumor functional disorder and evasion of antigenic-specific immunotherapy. Therefore, in parallel to the efforts oriented toward the identification of potential candidate antigens for vaccination, closer attention should be paid to the complexity of the tumor ecosystem in deviating the functions of tumor infiltrating cells. It seems obvious that more could be achieved by combining therapies that tackle malignancies from multiple angles, with the tumor microenvironment conditioned to support a powerful effector arm generated by immunotherapy. Tumor immunotherapy in the clinic has not taken it into account the hypoxic microenvironment and its impact on the therapeutic outcome. Because the hypoxia-inducible factor (HIF) was recently shown to regulate the tumorigenic capacity of tumor and cancer stem cells under hypoxic conditions,[112] further investigation is required to demonstrate if HIF-1 is prevalent enough in human cancer to be a general target. One of the challenges of cancer immunotherapy is how to design combination therapies that modify the hypoxic tumor microenvironment so as to promote immunity and better design more adapted immunotherapy approaches. Whether the suppression of HIF-1α expression may be a promising strategy that is selective for facilitating immunotherapeutic efficacy in cancer patients is at present under investigation.

V. CONCLUSIONS

The growth and spread of cancer depends as much on the host response to tumor as on the biological characteristics of the tumor itself. This interaction is at its most intimate and dynamic within the tumor microenvironment. The role of the latter during the initiation and progression of carcinogenesis is now realized to be of critical importance for both enhanced understanding of fundamental cancer immunology and biology and exploiting this source of relatively new knowledge for improved molecular diagnostics, therapeutics, and immune intervention. It has become clear now that tumor stroma components including hypoxia are engaged in an active molecular cross talk that has serious implications for immunological recognition of tumor in shaping the microenvironment. Indeed, hypoxia incapacitates several different types of immune effector cells, enhances the activity of immunosuppressive cells, and provides new avenues that help "blind" immune cells to detect the presence of tumor cells. Obviously, understanding the tumor microenvironment and its targeting to awaken or reawaken immune cells, or to redirect it from a protumor to an antitumor state, will help to instruct clinical immunotherapy.

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REVIEW ARTICLE
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Hypoxia promotes tumor growth in linking angiogenesis to immune escape

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Despite the impressive progress over the past decade, in the field of tumor immunology, such as the identification of tumor antigens and antigenic peptides, there are still many obstacles in eliciting an effective immune response to eradicate cancer. It has become increasingly clear that tumor microenvironment plays a crucial role in the control of immune protection. Tumors have evolved to utilize hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important in progression, survival, and metastasis. Hypoxia-inducible factor (HIF-1) and vascular endothelial growth factor (VEGF) play a determinant role in promoting tumor cell growth and survival. Hypoxia contributes to immune suppression by activating HIF-1 and VEGF pathways. Accumulating evidence suggests a link between hypoxia and tumor tolerance to immune surveillance through the recruitment of regulatory cells (regulatory T cells and myeloid derived suppressor cells). In this regard, hypoxia (HIF-1α and VEGF) is emerging as an attractive target for cancer therapy. How the microenvironmental hypoxia poses both obstacles and opportunities for new therapeutic immune interventions will be discussed.

Keywords: hypoxia, HIFα, angiogenesis, immune tolerance, tumor progression

INTRODUCTION

Cancer vaccines are expected to augment already established anti-tumor immune responses and to induce *de novo* immunity or reverse tolerance. Major advances have led to several immunization strategies to boost immune responses against some tumor-associated antigens. In this regard, strategies involving various forms of peptides either alone or in combination with different cytokines, adjuvant, or dendritic cells have been used to enhance specific immune responses. Despite the enthusiasm for current vaccination approaches, it should be noted that tumor rejection in patients does not always follow successful induction of tumor-specific immune responses by cancer vaccines. Even, if a strong and sustained cytotoxic response is induced, complex issues remain such as tumor evasion, tolerance and selection of tumor resistant variants (Rosenberg et al., 1998, 2004; Hamai et al., 2010). Nevertheless, systemic administration of monoclonal antibodies targeting immune modulatory receptors: cytotoxic T-lymphocyte antigen 4 (CTLA-4; Eggermont et al., 2010) and Programmed Death 1 (PD1) on T reg and effector cells have been recently reported to increase anti-tumor immunity both experimentally and clinically (Alexandrescu et al., 2010; Callahan et al., 2010; Eggermont and Robert, 2011; Mansh, 2011; Mellman et al., 2011; Pandolfi et al., 2011). In early phase I trials, PD1 have shown good activity in a variety of cancer types and have a toxicity profile that seems safer than ipilimumab (Brahmer et al., 2010). A recent clinical trial with the longest follow-up of melanoma patients treated with ipilimumab has shown that ipilimumab can induce durable, potentially curative tumor regression in a small percentage of patients with metastatic melanoma (Prieto et al., 2012).

It has become increasingly clear that tumor microenvironment plays a crucial role in the control of immune protection and contains many overlapping mechanisms to evade antigenic specific immunotherapy (Zou, 2005; Nagaraj and Gabrilovich, 2007; Marigo et al., 2008). Several reports underscore the contribution of the microenvironment to tumor development and it is well admitted that tumors are not merely masses of neoplastic cells, but instead, are complex tissues composed of both non-cellular (matrix proteins) and cellular components, in addition to the ever-evolving neoplastic cells. Tumor microenvironment is a complex and highly dynamic environment, providing very important clues to tumor development and progression (Petrulio et al., 2006). In the context of microenvironment complexity and plasticity, tumor cells orchestrate the modification of the microenvironment by attracting or activating many non-tumoral cells, including fibroblasts, blood and lymphatic endothelial cells, bone marrow-derived cells, immune and inflammatory cells. Moreover, it is now acknowledged that tumor cells and their stroma co-evolve during tumorigenesis and tumor progression (Hiscox et al., 2011). Therefore, tumor growth and spread depend as much on the host response as on the biologic characteristics of the tumor itself and on the influence of the tumor microenvironment (Petrulio et al., 2006).

The hypoxia's critical role in radio-resistance, chemoresistance, tumor stemness, and its significance as an adverse prognosis factor have been well established and at present hypoxia-induced

angiogenesis has become an attractive target for cancer therapy (Semenza, 2010). Hypoxia-inducible factor (HIF-1α) overexpression and its association with poor treatment response and outcome has been demonstrated in an extensive range of human tumors (Semenza, 2010; Wilson and Hay, 2011). In addition, a direct link between tumor hypoxia and tolerance through the recruitment of regulatory cells has been established (Facciabene et al., 2011). Hypoxia-induced vascular endothelial growth factor (VEGF) produced by most tumors plays an important role in tumor angiogenesis (Takenaga, 2011). It also plays a key role in immune escape by licensing invasion, metastasis, impacting therapeutic response and favoring immune escape mechanisms.

Hypoxia is therefore attracting a particular attention in the field of tumor immune biology since hypoxic stress impact angiogenesis, tumor progression and immune tolerance. Modulating the stromal hypoxia may constitute in fact a very potent strategy for targeted therapeutic approaches. How peripheral immune tolerance and angiogenesis programs are closely connected and cooperate under hypoxic conditions to sustain tumor growth will be discussed in this article.

HYPOXIA-INDUCED ANGIOGENESIS AND RESISTANCE TO CYTOTOXIC TREATMENTS

HYPOXIA-INDUCIBLE FACTORS: DETERMINANT FACTORS IN TUMOR CELL ADAPTATION TO HYPOXIC STRESS

Tumor cells adapt to hypoxic microenvironment by the regulation of HIFs family of transcription factors. This family is composed of three members namely HIF-1, HIF-2, and HIF-3. HIFs are a heterodimeric proteins composed of a constitutively expressed HIF-β subunit and an O_2-regulated HIF-α subunit (Wang et al., 1995). Both HIF-1α and HIF-2α have common target genes as well as their respective target genes (Lau et al., 2007). The genes induced by hypoxia-dependent HIF-1α and HIF-2α play important roles in regulating different aspects of tumor biology especially angiogenesis, cell survival, chemo- and radio-resistance, proliferation, invasion and metastasis, pH regulation and metabolism, resistance to immune system, and maintenance of cancer stem cells (Keith and Simon, 2007; Lukashev et al., 2007; Keith et al., 2011).

In the presence of O_2, HIF-1α is hydroxylated on proline residue 402 and/or 564 by prolyl hydroxylase domain protein 2 (PHD2), resulting in its interaction with the *von Hippel–Lindau* (*VHL*) protein, which recruits an E3 ubiquitin-protein ligase that eventually catalyzes polyubiquitination of HIF-1α thereby targeting it for proteasomal degradation (Salceda and Caro, 1997). Under hypoxic conditions, hydroxylation is inhibited and HIF-1α rapidly accumulates, translocates to the nucleus, dimerizes with HIF-1β and activates transcription. Similar to HIF-1α, HIF-2α is also regulated by oxygen-dependent hydroxylation (Patel and Simon, 2008). HIF-1α and HIF-2α differ in their transactivation domains but are structurally similar in DNA binding and dimerization domains. HIF-3α lacks the transactivation domain and may function as an inhibitor of HIF-1α and HIF-2α and its expression is transcriptionally regulated by HIF-1 (Makino et al., 2007). HIF-1α and HIF-2α are also regulated by several oxygen-independent mechanisms involving mutation of VHL, PTEN, BRAF, SDH, FH, and MITF (Semenza, 2010; Noman et al., 2011c).

HYPOXIA-INDUCED VEGF AS A KEY EVENT IN PROMOTING ANGIOGENESIS

Growing tumor mass requires neo-vascularization to provide rapidly proliferating tumor cells with an adequate supply of oxygen and metabolites. Diffusion distances from the existing vasculature increases as tumor expands resulting in local hypoxia (Harris, 2002; Brahimi-Horn et al., 2007; Dewhirst et al., 2008). Increasing evidence shows that the hypoxic stress plays a key role in the regulation of angiogenesis that is required for invasive tumor growth and metastasis (Semenza, 2010). HIF-1α stabilization and nuclear accumulation results in the subsequent activation of a wide range of target genes belonging to angiogenesis and promoting endothelial cell proliferation, migration, permeability, and survival (Wang et al., 1995). HIF-2 is also able to promote tumor angiogenesis through mobilization of circulating progenitor endothelial cells (Kim et al., 2009) and through VEGF induction (Raval et al., 2005; Li et al., 2009). HIF-1α and HIF-2α can also be regulated by oxygen-independent mechanisms leading to increased HIF transcriptional program under normal level of O_2 and promoting tumor growth and angiogenesis (Semenza, 2010). In this regard, the double allele loss of function (deletion, mutation, CpG island hypermethylation) $VHL^{-/-}$ gives rise to increased constitutive level of HIF-1α and/or HIF-2α (Baldewijns et al., 2010). VHL can also directly regulate the stability of certain mRNAs such as that of VEGF (Datta et al., 2005) and mRNAs related to growth factor signaling, suggesting that mRNA changes observed after VHL loss are not solely due to the subsequent HIF accumulation (Kaelin, 2008).

Although vessel growth and maturation are complex and highly coordinated processes requiring the sequential activation of a multitude of factors, there is a consensus that VEGF signaling represents a crucial step in tumor angiogenesis (Liao and Johnson, 2007). It should also be noted that antiangiogenic drugs and vascular destructive agents may in turn promote tumor cell invasion and metastasis in association with drug-induced tumor hypoxia.

HYPOXIA-INDUCED ANGIOGENESIS LEADS TO INCREASED RESISTANCE TO CYTOTOXIC TREATMENTS

Tumor hypoxia-induced abnormal neo-angiogenesis leads to increased resistance to cytotoxic treatments. Indeed, tumor vessels are labyrinthine and branched in an irregular and chaotic manner with uneven diameters due to the compression of their walls by tumor or stromal cells (Jain, 1988, 2005; Nagy et al., 2010). These abnormalities in hypoxia-induced tumor vessels have been found in a wide range of tumor types, in mice both in transplantable tumors and in spontaneous tumors arising from exposure to carcinogens or expression of oncogenes (Baluk et al., 2005; Hamzah et al., 2008; Van de Veire et al., 2010) and in tumors from patients (Willett et al., 2004; Batchelor et al., 2007). Tumor vessel blood flow is compromised and stagnant due to increased vascular resistance and improper vasoregulation (Jain, 2005; Fukumura et al., 2010). Similarly hypoxia-induced tumor endothelial cells are abnormal. Normal vessels are lined with a monolayer of interconnected endothelial cells, whereas tumor endothelial cells are often leaky, with wide open junctions and many fenestrations resulting in limited tissue perfusion (Jain, 1988). These abnormal neo-vessels with leaky walls facilitate the escape of tumor

cells (Jain, 2005). All these abnormalities lead to a decrease in uniform and efficient delivery of nutrients and drugs (Jain, 2005; Jain and Stylianopoulos, 2010) as well as influx of immune cells (Hamzah et al., 2008). As irradiation and certain chemotherapeutics rely on the formation of reactive oxidative species to kill cancer cells, tumor hypoxia also reduces the efficacy of conventional anticancer treatments (Moeller et al., 2007). Thus, it is clear that hypoxia-induced angiogenesis is responsible for an abnormal tumor vasculature (Carmeliet and Jain, 2011), which can influence tumor response to anticancer treatments as well as tumor infiltration by immunocompetent cells.

ROLE OF HYPOXIA-INDUCED VEGF IN MODULATING TUMOR RESPONSE TO CYTOTOXIC T-LYMPHOCYTES

We have shown that hypoxic exposure of tumor cells inhibits autologous cytotoxic T-lymphocytes (CTL)-mediated lysis (Noman et al., 2009, 2011b). We provided evidence indicating that HIF-1α induction and signal transducer and activator of transcription-3 (STAT3) activation are associated with hypoxia-induced lysis inhibition. This suggests a new role for hypoxia-dependent induction of HIF-1α and activation of STAT3 in tumor resistance to the immune system.

Signal transducer and activator of transcription-3 activation within tumor microenvironment is known to be associated with cytokine-induced proliferation, anti-apoptosis, and transformation. Moreover, STAT3 modulates the cross-talk between tumor and immune cells (Wang et al., 2004; Yu et al., 2007). More interestingly, we have shown that VEGF neutralization resulted in the attenuation of hypoxic tumor target resistance to CTL-mediated killing (Noman et al., 2009). We have also demonstrated that STAT3 phosphorylation can be stimulated by autocrine signaling through VEGF, suggesting that tumor microenvironment through hypoxia-induced VEGF may play a key role in the induction of active form of STAT3. In this regard, it is very likely that STAT3 activation is associated with the regulation of gene expression potentially involved in the alteration of hypoxic tumor target-specific killing. Therefore, understanding how VEGF and other soluble factors lead to STAT3 activation via the tumor microenvironment may provide a more effective cancer treatment strategy for hypoxic tumors with elevated P-STAT3 levels. This also suggests that reduction of VEGF release in tumor microenvironment may favor induction of a stronger anti-tumor CTL response against tumors expressing VEGF receptor (VEGFR). In this regard, inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer (Gabrilovich et al., 1996) pointing to the existence of a synergy between tumor immunotherapy and antiangiogenic therapy (Nair et al., 2003).

HYPOXIA INDUCES EPITHELIAL-TO-MESENCHYMAL TRANSITION

Epithelial-to-mesenchymal transition (EMT) is a complex molecular process during which epithelial cells lose their apico-basal polarity and acquire a mesenchymal and motile phenotype. The hallmarks of EMT are the down regulation of epithelial markers (E-cadherin) and the up regulation of mesenchymal markers (Vimentin, Fibronectin, the transcriptional factors Snail, Slug, Twist, and Zeb1/2) leading to an organized disassembly of epithelial cell-to-cell contacts enabling motility and invasiveness (Thiery, 2002). EMT has therefore become a prominent program involved in carcinogenesis, metastasis, tumor recurrence and resistance to apoptosis and to chemotherapy (Barrallo-Gimeno and Nieto, 2005, p. 172; Thiery and Sleeman, 2006, p. 173). The activation of EMT in tumor cells has been reported to be associated with interactions of tumor cells with tumor-associated stromal cells, implicating neoplastic microenvironmental stimuli in EMT induction. In this regard, tumor hypoxia through HIF-1α has recently been proposed to be an important trigger and modulator of EMT (Lundgren et al., 2009; Sleeman and Thiery, 2011).

During hypoxic stress, different signaling pathways seem to be involved in triggering EMT (Jiang et al., 2011). In this regard, the existence of cooperation between hypoxia and TGFβ signaling resulting in EMT activation has been reported (Schaffer et al., 2003; Nishi et al., 2004). Recent findings also demonstrate that, in breast cancer cells, the Notch pathway plays a role in hypoxia-activated EMT since its inhibition abrogates hypoxia-mediated increase in Slug and Snail expression, and decreases cell migration and invasion (Chen et al., 2010). Lysyl oxidase (LOX) and the related LOX-like 2 (LOXL2) have recently been reported to be direct transcriptional targets of HIF-1α that are sufficient to repress E-cadherin and induce EMT under hypoxia (Schietke et al., 2010). Urokinase plasminogen activator receptor (uPAR), another direct transcriptional target of HIF-1α (Buchler et al., 2009), is described to be involved in E-cadherin repression and EMT activation during hypoxic stress in medulloblastoma cells (Gupta et al., 2011). Interestingly, Luo et al. (2011) have recently identified a hypoxia-response element in close proximity with the minimal promoter of human and mouse Snail, which binds both HIF-1 and HIF-2 proteins and which activates snail transcription. This provides new evidence of the direct involvement of HIFs in Snail activation and EMT-regulation.

It should be noted that EMT contributes to malignancy not only by enhancing motility, invasiveness, resistance to apoptosis and drugs, but also by inducing immune escape. In fact, Kudo-Saito et al. report that Snail expression in melanoma cells resulted in the impairment of dendritic cell maturation, expansion of T regulatory cells, and resistance to CTL-mediated lysis. Moreover, intratumoral targeting of Snail in melanoma significantly inhibited tumor growth and metastasis in correlation with an increase of tumor-specific tumor-infiltrating lymphocytes and of systemic immune responses (Kudo-Saito et al., 2009). It seems that EMT program in tumors is able to redirect the immune system toward immune tolerance. Since hypoxia has been also reported to mediate immune tolerance, the role of EMT factors in this process remains to be investigated.

TUMOR HYPOXIA PROMOTES ANGIOGENESIS AND IMMUNE TOLERANCE

It is well known that immune system can effectively inhibit tumor growth (Zhang et al., 2003; Koebel et al., 2007; Fridman et al., 2011). However, tumors are known to establish diverse potent mechanisms, which help them to escape the immune system (Zou, 2005; Nagaraj and Gabrilovich, 2007; Marigo et al., 2008; Hamai et al., 2010). Emerging evidence indicates a link between hypoxia-induced angiogenesis and tolerance to immune system (Manning et al., 2007; Buckanovich et al., 2008; Tartour et al., 2011). Tumor

hypoxia drives angiogenesis by inducing synthesis of VEGF and other pro-angiogenic factors in tumor cells via HIFs and promotes immunosuppression (Noman et al., 2009, 2011a). The association between angiogenesis and immunosuppression comes from the immunosuppressive activities of angiogenic factors such as VEGF, a cytokine secreted by most tumors (Toi et al., 1996a,b), making it a critical actor in tumor angiogenesis and immune tolerance (Motz and Coukos, 2011). Hypoxic zones in tumors attract a variety of immune cells in which HIF stabilization is associated with the acquisition of both pro-angiogenic and immunosuppressive phenotype (Noman et al., 2011c).

MYELOID CELLS

Myeloid cells are perhaps the best-studied cell types in terms of their ability to promote immunosuppression and angiogenesis in tumors (Gabrilovich and Nagaraj, 2009; Motz and Coukos, 2011).

Tumor-associated macrophages

Macrophages constitute a major component of the immune infiltrate seen in tumors (Bingle et al., 2002). In the tumor microenvironment, they differentiate into tumor-associated macrophages (TAM) with expression of TAM markers such as CD206 (Mantovani et al., 2002). High TAM numbers in tumors in majority of cases are correlated with reduced survival (Bingle et al., 2002). Exposure of TAM to tumor-derived cytokines such as IL-4 and IL-10 is able to convert them into polarized type II or M2 macrophages with immune-suppressive activities and pro-angiogenic effects, resulting in tumor progression (Mantovani et al., 2002). TAM are found to be preferentially located in tumor hypoxic areas, where they accumulate HIF-1 and HIF-2 and up regulate VEGF and other pro-angiogenic factors (Lewis et al., 2000). The relative contribution of HIF-1 and HIF-2 in the regulation of gene expression in TAM is not yet completely elucidated. In human macrophages, HIF-2 has been reported to be a crucial inducer of pro-angiogenic molecules (White et al., 2004). In another study, Werno et al. (2010) proposes that HIF-1 is indispensable for angiogenic-promoting properties of TAM, at least in murine macrophages. Besides this angiogenesis promoting activity, HIF-1α was also reported to be crucial for macrophage-mediated inhibition of T cells in hypoxic conditions (Doedens et al., 2010). In hypoxic areas of tumors, TAM also up-regulate the expression of MMP-7 protein in hypoxic areas of tumors (Burke et al., 2003). MMP-7 is known to cleave the Fas ligand from neighboring cells, making tumor cells less responsive to lysis by NK and T cells (Fingleton et al., 2001). MMP-7 is also known to stimulate endothelial cell proliferation and migration, which can support tumor angiogenesis (Nishizuka et al., 2001).

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) have also been demonstrated to directly promote angiogenesis (Yang et al., 2004) and immune tolerance (Gabrilovich and Nagaraj, 2009). In tumor bearing hosts, tumor-derived factors such as VEGF, GM-CSF, prostaglandins also restrain DC maturation and promote the accumulation of MDSCs in tumoral tissues and secondary lymphoid organs (Gabrilovich, 2004). In these sites, MDSCs induce T cell anergy, restrain the effector phase of the CD8+ T cell, and can promote antigen-specific T reg proliferation (Gabrilovich, 2004; Serafini et al., 2008). Moreover, HIF-1α has been directly shown to regulate the function and differentiation of MDSC within the hypoxic tumor microenvironment (Corzo et al., 2010). A cross-talk between MDSC and macrophages has been reported, proposing that MDSC down-regulate IL-12 production by macrophages and increase their own production of IL-10 in response to signals from macrophages. This interaction between MDSC and macrophages polarizes classically activated (M1) macrophages toward a type 2 and immunosuppressive phenotype and accentuates the M2 phenotype of M2 macrophages, which is likely to establish an environment that skew CD4+ and CD8+ T cell immunity toward a tumor-promoting type 2 response (Trinchieri, 2003). As a result, MDSC directly and through their cross-talk with macrophages, suppress both adaptive and innate anti-tumor immunity, which facilitate tumor growth. MDSC also have pro-angiogenic activities that can mediate tumor refractoriness to anti-VEGF treatment (Shojaei et al., 2007).

Dendritic cells

The dendritic cell subset is also diverted by VEGF from its highly specialized antigen-presenting and T cell activating functions. Indeed, production of VEGF by human tumors inhibits the functional maturation of dendritic cells and thereby promotes immune escape of tumor cells (Gabrilovich et al., 1996). Recombinant VEGF administered to tumor-free mice resulted in repressed dendritic cell development associated with accumulation of Gr-1+ immature myeloid-derived suppressor cells (MDSC) that inhibit T cell functions (Gabrilovich et al., 1998), illustrating the proper immune-suppressive functions of VEGF. Tumor-associated myeloid dendritic cells, in response to tumor-derived VEGF, increase the expression of PDL-1 which is a negative regulator of T cell function (Curiel et al., 2003). Moreover, anti-VEGF therapy was associated with a decrease in immature myeloid and dendritic cells in patients (Osada et al., 2008).

LYMPHOCYTES

Lymphocytes play a crucial role in immunosuppression and tolerance (Fridman et al., 2011). In an ovarian cancer model, very recently, it has been shown that hypoxia promotes the recruitment of the immunosuppressive CD4+ CD25+ FOXP3+ T regulatory cells (T reg) through the induction of CCL28 expression by hypoxic tumor cells. T reg in turn secrete VEGF, contributing to the VEGF pool in the tumor microenvironment and to immune tolerance (Facciabene et al., 2011). Moreover, CD4-deficient mice have an impaired angiogenesis response to hypoxia during ischemia (Stabile et al., 2003). Moreover VEGF targeting reduces intratumoral T regulatory cells and increases the efficacy of cancer immunotherapy in B16 melanoma and the CT26 colon carcinoma models (Li et al., 2006). In addition, an inverse correlation between angiogenesis and tumor-infiltrating T cells has already been reported (Zhang et al., 2003; Buckanovich et al., 2007, 2008; Kandalaft et al., 2009). Other lymphocyte cells (including regulatory B cells, NK cells, and NKT cells) with immunosuppressive functions have been reported to produce VEGFA (Motz and Coukos, 2011). However, the precise role of these cells in tumor angiogenesis remains largely unknown. Therefore, the exact

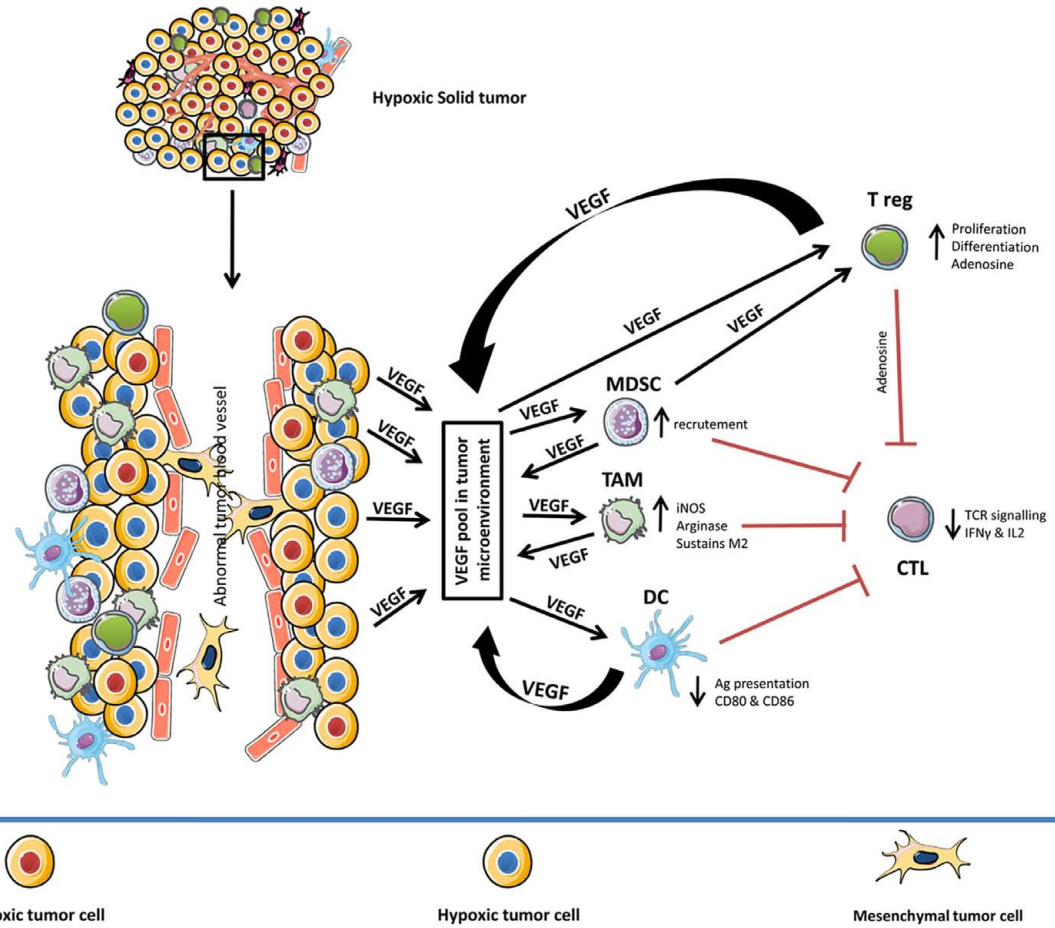

FIGURE 1 | Role of hypoxia-induced VEGF in mediating immune tolerance. Diverse effects of hypoxia-induced VEGF on different components of the immune system (TAM, MDSC, DC, CTL, and T reg). In general, hypoxia-induced VEGF pool in tumor microenvironment (VEGF secreted by hypoxic tumor cells, TAM, MDSC, DC, and T reg) amplifies the activity of TAM, MDSC, and T reg while suppressing the function of CTL and delaying the maturation of DC cells. TAM, tumor-associated macrophages; MDSC, myeloid derived suppressor cells; DC, dendritic cells; CTL, cytotoxic T-lymphocyte; T reg, T regulatory cells.

contribution of these lymphocytes in mediating VEGF-induced immune suppression requires further investigation.

Different facets of VEGF-induced immunosuppression in diverse cell types in hypoxic tumor microenvironment is summarized in **Figure 1**.

HYPOXIA, PRO-INFLAMMATORY MEDIATORS, AND IMMUNE SUPPRESSION

Hypoxia induces both angiogenesis and immunosuppression also by upregulating COX-2 in tumor cells (Greenhough et al., 2009). COX-2 is an inflammatory enzyme converting arachidonic acid into prostaglandin PGE2 which has angiogenic properties by promoting VEGF expression (Jain et al., 2008) and immunosuppressive functions by causing, in cooperation with hypoxia-induced increased extra-cellular adenosine, cAMP accumulation in effector T cells resulting in the suppression of their anti-tumor functions (Whiteside et al., 2011). PGE2 can also inhibit tumor immunity by inhibiting dendritic cell maturation, their expression of co-stimulatory molecules, and their production of IL-12. In addition, PGE2 enhances the suppressive activity of T reg and is involved in the conversion of CD4+ CD25− into T reg. Finally, PGE2 can stimulate the immunosuppressive functions of MDSC by binding the EP-4 receptor (Rodriguez et al., 2005; Sinha et al., 2007).

Thus hypoxia connects peripheral immune tolerance and angiogenic programs to sustain tumor growth. The counter-activation of tolerance mechanisms at the site of tumor hypoxia is therefore a crucial condition for maintaining the immunological escape of tumors.

THERAPEUTIC INTERVENTIONS TARGETING ANGIOGENESIS

Hypoxia-induced VEGF produced by most tumors plays an important role in tumor angiogenesis (Takenaga, 2011). Recognition of the VEGF pathway as a key regulator of angiogenesis has led to the development of several VEGF-targeted molecules (Porta et al., 2011). These molecules include neutralizing antibodies to VEGF (Escudier et al., 2008) or VEGFRs, mTOR kinase inhibitors (Azim et al., 2010), and tyrosine kinase inhibitors (TKIs; Porta et al., 2011) with selectivity for VEGFRs. The pioneers in the

field of angiogenesis inhibitors are bevacizumab (an anti-VEGF monoclonal antibody that binds specifically to the VEGF ligand and neutralizes its activity; Ferrara et al., 2004) and two multi-targeted kinase inhibitors (sorafenib and sunitinib; non-specific small molecules that inhibit multiple kinase receptors; Zhong and Bowen, 2011). Indirectly, the macrocyclic lactones temsirolimus and everolimus, inhibit also VEGF (Azim et al., 2010). These molecules inhibit the mTOR complex formation targeting mTORC1 that inhibits HIF-1α expression and consequently inhibits HIF-1α dependent VEGF transcription decreasing angiogenesis (Azim et al., 2010). These angiogenesis inhibitors have proven their therapeutic efficacy in several mouse models of cancer and in a large number of human cancers (Porta et al., 2011). However, clinical benefits are often followed by a restoration of tumor growth and progression (Burger et al., 2011; Perren et al., 2011). The Bevacizumab-chemotherapy combination seems to be successful in second-line settings in the treatment of patients with advanced colorectal cancer, compared to the disappointing efficacy of combining bevacizumab with chemotherapy in the first-line treatment of the patients (Jenab-Wolcott and Giantonio, 2010). Several intrinsic and adaptive mechanisms were shown to be involved in resistance to angiogenic therapy (Bergers and Hanahan, 2008).

Antiangiogenic therapy can lead to hypoxia as a compensatory mechanism (Casanovas et al., 2005; Bergers and Hanahan, 2008). It is now clear that hypoxia-induced by angiogenesis inhibitors might further elevate VEGF and other pro-angiogenic factors expression in tumors (Shaked et al., 2005; Mancuso et al., 2006). Sathornsumetee et al. (2008) have shown that hypoxic profiles can predict survival in malignant astrocytoma patients treated with bevacizumab. Another mechanism underlying resistance to anti-VEGF therapy involves infiltration of various pro-angiogenic bone marrow-derived cells, in part through increases in HIF-1α and its downstream effectors such as VEGF (Ceradini et al., 2004; Shaked et al., 2006). In a metastatic colorectal cancer model, bevacizumab therapy improved the impairment of lymphocyte subsets, especially for T cells. These effects correlated with more favorable clinical outcome (Manzoni et al., 2011). Interestingly, anti-VEGF agents can have contradictory effects on immune system (Seliger et al., 2010). Sorafenib appears to impair, while sunitinib stimulates terminal DC maturation (Hipp et al., 2008). Sunitinib induces Th-1 immune response (IFN-γ expression) while reducing T reg in renal cell carcinoma patients (Finke et al., 2008). Sunitinib, but not Sorafenib has been shown to inhibit MDSC immune-suppressive activity and reduces MDSC and T reg circulating numbers (Ko et al., 2009). Another study has shown that sunitinib administration in mouse renal cell carcinoma tumor bearing mice led to the inhibition of Stat3 in tumor-associated myeloid cells, including dendritic cells and MDSCs, which was accompanied by a reduction of tumor T reg (Xin et al., 2009). Du et al. (2008) have shown that impairment of VEGF signaling leads to an adaptive pro-invasive tumor phenotype.

A better understanding of the complex interaction between cancer cells, immune system and anti-VEGF agents is therefore needed for improving and sustaining the benefits of antiangiogenic therapy.

CONCLUSION

Advances in immunology and molecular biology have shown that cancers are potentially immunogenic and that host immune responses influence survival (Galon et al., 2006). However, immune surveillance and activation is frequently ineffective in preventing and/or controlling tumor growth. In fact, an active and bi-directional molecular cross-talk between tumor microenvironment and host cells has profound implications for immunological recognition and progression of tumor cells (Petrulio et al., 2006; Lorusso and Ruegg, 2008). The cross-talk between tumor cells and stromal cells within the tumor microenvironment mediates tumor initiation, progression, and response to anticancer therapy. In this regard, microenvironmental components including hypoxia appear to regulate gene expression in tumor cells thereby directing the tumor toward aggressiveness, angiogenesis, and metastasis. It is well documented that inflammation is an important part of hypoxic tumor microenvironment (Eltzschig and Carmeliet, 2011). Increasing evidence suggest the existence of a link between hypoxic stress and several inflammatory molecules (PEG2, COX-2, chemokines, and cytokines) responsible for tumor initiation and progression. These pro-inflammatory mediators not only support tumor survival and expansion but also the function of several immune cells notably dendritic and effector cells (Chow et al., 2011). Therefore, a well-integrated understanding of this intricate microenvironment may offer new opportunities for therapeutic intervention. Recent insights into cellular and molecular cross-talk suggest a model in which hypoxia, HIF, VEGF, and several other HIF target genes participate in the coordinated collaboration between tumor, endothelial, inflammatory/hematopoietic, and circulating endothelial precursor cells to enhance and promote tumor vascularization. Given its central role in tumor progression and resistance to therapy, tumor hypoxia might well be considered as one potential target that has yet to be exploited in oncology. Its targeting may be therefore an innovative approach to design new approaches in cancer therapy. Recently, a growing number of drugs that inhibit HIF-1 have been identified and validated as anticancer agents (Semenza, 2010; Wilson and Hay, 2011). It is also becoming evident that characterizing the tumor microenvironment can provide important prognostic and predictive information about tumors, independently of the tumor cell phenotype. Whether the inhibition of hypoxic signaling pathways in different compartments of the solid tumor microenvironment will open new therapeutic opportunities in cancer immunotherapy has to be established.

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Résumé

L'hypoxie est une caractéristique commune des tumeurs solides et l'une des spécificités du microenvironnement tumoral. L'hypoxie tumorale joue un rôle important dans l'angiogénèse, la progression maligne, le développement de métastases, la chimio/radio-résistance et favorise l'échappement au système immunitaire du fait de l'émergence de variant tumoraux avec un potentiel de survie et de résistance à l'apoptose augmenté. Cependant, très peu de travaux ont étudié l'impact de l'hypoxie tumorale sur la régulation de la susceptibilité des tumeurs à la lyse induite par la réponse immune cytotoxique. Nous nous sommes donc demandé si l'hypoxie pouvait conférer aux tumeurs une résistance à la lyse induite par les lymphocytes T cytotoxiques (CTL).

Nous avons démontré que l'exposition de cellules cibles tumorales à l'hypoxie possédait un effet inhibiteur sur la lyse de ces cellules tumorales par des CTL autologues. Cette inhibition n'est pas associée à des altérations de la réactivité de CTL ou de la reconnaissance des cellules cibles. Cependant, nous avons montré que l'induction hypoxique concomitante de la phosphorylation de STAT3 (pSTAT3) au niveau de la tyrosine 705 et du facteur HIF-1α (Hypoxia Inducible Factor-1 alpha) est liée fonctionnellement à l'altération de la susceptibilité de cellules tumorales bronchiques non à petites cellules (NSCLC) à la mort induite par les CTL.

Nous avons aussi montré que la résistance de cellules tumorales bronchiques à la lyse CTL induite par l'hypoxie était associée à une induction d'autophagie dans les cellules cibles. En effet, l'inhibition de l'autophagie empêche la phosphorylation de STAT3 (via l'inhibition de la kinase Src) et restaure la susceptibilité des cellules tumorales hypoxiques à la lyse induite par les CTL. De plus, l'inhibition *in vivo* de l'autophagie par l'hydroxychloroquine (HCQ) dans le modèle murin portant la tumeur B16F10 and chez les souris vaccinée avec le peptide TRP2 augmente de façon drastique l'inhibition de la croissance tumorale. Collectivement, cette étude établit un nouveau lien fonctionnel entre l'autophagie induite par l'hypoxie et la régulation de la lyse induite par les cellules T spécifique d'antigènes et souligne le rôle majeur de l'autophagie dans le contrôle de la croissance tumorale *in vivo*.

Finalement, étant donné que le la résistance tumorale à la lyse induite par les cellules tueuses est très probablement régulée par de multiples facteurs, nous avons aussi eu pour but d'identifier les micro-ARNs (miRs) régulés par l'hypoxie dans des modèles de NSCLC et de mélanome et leur implication putative dans la régulation de la susceptibilité tumorale à la lyse induite par les cellules T spécifique d'antigènes. Le micro-ARN 210 (miR-210) est ainsi significativement induit de manière dépendante de HIF-1α dans des cellules de NSCLC et de mélanome, et miR-210 est exprimé dans les zones hypoxiques de tissus issus de NSCLC. De plus, nous avons démontré que l'induction de miR-210 par l'hypoxie régule la susceptibilité tumorale à la lyse induite par les CTL en partie grâce à l'inhibition de l'expression de PTPN, HOXA1 et TP53I11, indiquant que miR-210 joue un rôle potentiel dans la régulation de la réponse immune antitumorale.

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