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ON THE HYPOXIA REGULATION OF CD137 AND CD69 EXPRESSION

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SOBRE LA REGULACIÓN DE CD137 Y CD69 MEDIADA POR HIPOXIA

Memoria presentada por Dª Sara Labiano Almiñana para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Inmunología e Inmunoterapia del Centro de Investigación Médica Aplicada y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, Julio de 2016

Dr. Ignacio Melero Bermejo

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A mis padres: Maite y Francisco

A Jesús

“El motor de la ciencia es la curiosidad con las preguntas constantes: ¿Y eso cómo es? ¿En qué consiste? ¿Cómo funciona? Y lo más fascinante es que cada respuesta trae consigo nuevas preguntas. En eso los científicos le llevamos ventajas a los exploradores, cuando creemos haber llegado a la meta anhelada, nos damos cuenta de que lo más interesante es que hemos planteado nuevos problemas para explorar”.

César Milstein, Premio Nobel de Medicina en 1984

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Cuando uno empieza la tesis ve muy lejos el día en que tenga que enfrentarse a esta página en blanco. Pues bien, ya ha llegado y mirando hacia atrás son muchas las personas que de forma laboral, personal o ambas, han hecho posible esta tesis.

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ABBREVIATIONS

A

ADCC: antibody-dependent cell-mediated cytotoxicity
AICD: Activation-induced cell death
AIM: Activation Inducer Molecule
AP-1: Activator protein 1
APCs: Antigen presenting cells
ATF: activating transcription factor-1

C

cAMP: cyclic adenosin monophosphate
CART: chimeric-antigen receptor T cells
CD137L: CD137 Ligand
ChIP: Chromatin Immunoprecipitation Assay
CIA: collagen-induced arthritis
cIAP1/2: Cellular inhibitors of apoptosis protein 1/2
CNS: conserved non-coding sequences
CREB: cAMP response element-binding
CTL: Cytotoxic T lymphocytes
CTLD: C-lectin-like domain

D

DAPK: Death-associated protein kinase
DCs: Dendritic cells
DMOG: Dimethyloxaloylglycine

E

EAM: experimental autoimmune myocarditis
EGR: Early growth response
EPO: Erythropoietin
ETC: Electron transport chain

F

FACS: Fluorescence-activated cell sorting
FIH: Factor inhibiting HIF-1

G

Gal-1: Galectin 1
Gal-9: Galectin 9
GAPDH: gliceraldheide-3-P-deshydrogenase
GLUT1: Glucose transporter 1

H

H: Hypoxia

HIF-1α: Hypoxia inducible factor 1 alpha
HIF-1β: Hypoxia inducible factor 1 beta
HIF-2α: Hypoxia inducible factor 2 alpha
HIFs: Hypoxia inducible factors
HK: Hexokinase
HREs: Hypoxia response elements

I

IDO: Indoleamine 2 3-dioxygenase
IFN-γ: Interferon-gamma
IL1β: Interleukin 1 beta
IL2: Interleukin

J

JAK3: Janus kinase 3
JNK: c-Jun NH(2)-terminal kinase

L

Lck: Lymphocyte specific protein kinase
LDHA: Lactate dehydrogenase A
LPS: Lipopolysaccharide

M

mAb: Monoclonal antibodies
MAPK: Mitogen-Activated Protein Kinases
MDSC: Myeloid-derived suppressor cells
MHC: Major histocompatibility complex
mTOR: Mechanistic target of rapamycin
Mθ: Macrophage

N

N: Normoxia
NF-κB: Nuclear factor-kappa B
NIK: NF-κB-inducing kinase
NK: Natural Killer lymphocyte

O

OXPHOS: Oxidative phosphorylation

P

PBMCs: Peripheral blood mononuclear cells
pDC: Plasmacytoid DC
PDH1-3: Prolyl hydroxylases 1-3

PDK1: Pyruvate dehydrogenase kinase 1
PFK: Pphosphofructokinase
PKM1/2: Pyruvate kinase M1/M2
PMA: Phorbol myristate acetate

R

ROS: Reactive oxygen species

S

S1P: Sphingosine-1-phospate
$S1P_1$: Sphingosine-1-phospate receptor 1
sCD137: Soluble CD137
SN: supernatant
SP-1: Specific protein 1
STAT5: Signal transducers and activators of transcription 5

T

TAM: Tumor-associated macrophages
TAN: Tumor-associated neutrophils
TCA: Tricarboxylic acid cycle
TCR: T-cell receptor
TGFβ: transforming growth factor beta
Th1: T helper type 1
Th2: T helper type 2
Th17:T helper type 17
TILs: Tumor infiltrating T cells
TLR: Toll-like receptor
TM: transmembrane
TNFRSF: TNF Receptor Superfamily
TNFα: Tumor necrosis factor alpha
TRAF: TNFR-associated factor
Treg: Regulatory T lymphocyte

V

VEGF: Vascular endothelial growth factor
VHL: von Hippel–Lindau tumor-suppressor protein

GENERAL INTRODUCTION

1. Immune response regulation in the tumor microenvironment by hypoxia

1.1. Hypoxia sensing and signaling.

Oxygen tension at physiological levels varies in the different tissues. Primary lymphoid organs are known to have low oxygen availability (Caldwell et al. 2001). The bone marrow microenvironment, where hematopoietic stem cells and immune progenitors develop, is known to be hypoxic (Nombela-Arrieta et al. 2013). The thymus is also hypoxic under physiological conditions (Hale et al. 2002), and importantly, mechanisms dependent on oxygen tension in thymocytes are critical for their survival and development (Biju et al. 2004).

Oxygen availability can dramatically decrease in tissues under pathological insults such as inflammation, infection, necrosis and autoimmunity. This is especially true and well documented in solid tumors, where aberrant vascularization and an imbalanced blood supply shape a hostile microenvironment for stromal and malignant cells (Kandalaft et al. 2011; Berraondo et al. 2012). Cancer cells quickly adapt due to rapid selection and genetic/epigenetic instability. Immune cells infiltrating tumors also undergo metabolic reprogramming (Wang et al. 2011) to attain an adequate supply of energy to support the immune response (Frauwirth et al. 2002).

Hypoxia inducible factors (HIFs) are heterodimeric helix-loop-helix proteins which are the main transcriptional mechanism sensing and responding to hypoxia. HIF-1α and HIF-2α are the most widely studied proteins in this system (Semenza 2014). Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases (PHD1, PHD2 and PHD3) exquisitely sensitive to O_2 concentrations at the physiologic levels on two proline residues (Pro-402 and Pro-564). The hydroxylated forms are recognized by the von Hippel–Lindau tumor-suppressor protein (VHL), which in turn recruits a K48

E3 ubiquitin ligase. Ubiquitination in lysine 48 catalyzed by this complex targets HIF-1α for swift proteasomal degradation. Another important regulatory layer is provided by the hydroxylation of the highly conserved asparagine 803 (Asn 803) residue by the Factor inhibiting HIF-1 (FIH) (Lando et al. 2002). This modification impedes the binding of the transcriptional co-activator p300/CBP to the HIF transcriptional complex (Arany et al. 1996). Figure 1 schematically summarizes such mechanisms. In addition to Fe (II) and 2-oxoglutarate (2-OG), both hydroxylation reaction types require oxygen to be catalyzed.

Under hypoxic conditions, PHDs and FIH functions are inhibited, leading to HIF stabilization and nuclear translocation. The HIF transcriptional complex is comprised of the constitutively expressed subunit HIF-1β (also known as ARNT) and one of the HIF subunits: HIF-1α or HIF-2α (EPAS-1). The heterodimer binds to genomic DNA in regions called hypoxia response elements (HREs), which are five-nucleotide sequences (5′-RCGTG-3′) located mostly in the promoters of target genes (Wenger et al. 2005). Targets include genes related to anaerobic metabolism, such as glucose transporters and rate-limiting glycolytic enzymes (Semenza et al. 1996), erythropoiesis (EPO) (Semenza and Wang 1992) and pro-angiogenic factors such as VEGF (Forsythe et al. 1996) and adrenomedullin (Garayoa et al. 2000).

Immune cells can also stabilize HIF by oxygen-independent mechanisms. For instance, inflammation and bacterial products such as LPS can lead to HIF stabilization in macrophages (Blouin et al. 2004; Peyssonnaux et al. 2005; Kandalaft et al. 2011; Tannahill et al. 2013), a process mediated by nuclear factor-kappa B (NF-κB) (Rius et al. 2008). It has also been reported that upon TCR ligation, T cells stabilize HIF-1α even in the presence of oxygen (Nakamura et al. 2005). It has been reported that hypoxia can be sensed by other mechanisms dependent on NF-κB activation by free

radicals (Chandel et al. 2000) and increased levels of adenosine acting on purinergic receptors (Sitkovsky et al. 2014)

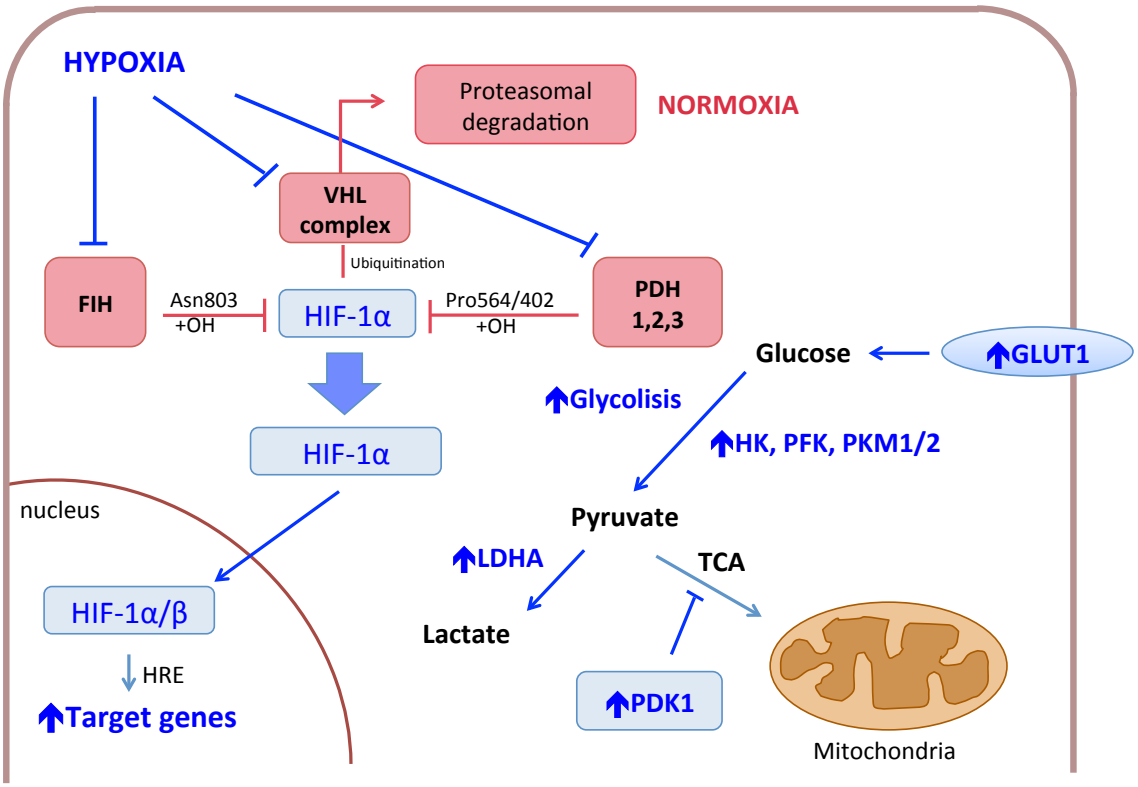


FIGURE 1. Schematic representation of the intracellular mechanisms sensing hypoxia through the HIF pathway and its role in metabolism regulation. GLUT1, Glucose transporter 1; HK, Hexokinase; HRE, Hypoxia response elements; LDHA, Lactate dehydrogenase A; PKM1/2, Pyruvate kinase M1/M2; PDK1, Pyruvate dehydrogenase kinase 1, PFK, phosphofructokinase; TCA, Tricarboxylic acid cycle.

1.2. Innate immunity: Dendritic Cells, macrophages and neutrophils

Innate immunity mechanisms play a crucial a role in pro-tumoral inflammation but also can be recruited by adaptive immunity in tumor destructive reactions. We will consider separately the effects of hypoxia on the different leukocyte subtypes. The main mechanisms are highlighted in Figure 2.

Dendritic cells

Antigen presenting cells (APCs) perform key roles in both the induction and maintenance of antitumor immunity, and provide a link between the innate and immune system (Steinman 2012). Dendritic cells (DCs) are crucial in cancer immunosurveillance by initiating the immune response in a process by which cancer antigens are uptaken, processed and presented by MHC molecules to tumor-specific CD4 and CD8 T lymphocytes (Palucka and Banchereau 2012). Recent evidence highlights the interplay of different DC subsets transiently located in the tumor microenvironment in successful anti-tumor immunity (Fuertes et al. 2011). Due to this capacity to regulate anti-tumor immunity, DCs are used in different immunotherapy strategies as cancer vaccine adjuvants (Wimmers et al. 2014). Given that DCs uptake tumor antigens in malignant tissues often subjected to hypoxia, HIF-1α could play a role in the processes of antigen uptake, maturation, migration and antigen processing in addition to its actions on T and NK cells. Indeed, both hypoxia and toll-like receptor (TLR) ligation clearly induce HIF-1α accumulation in DCs (Jantsch et al. 2011).

Oxygen deprivation has been shown to inhibit the surface expression of the co-stimulatory molecules CD80 and CD86 by LPS-treated monocyte-derived human DCs in vitro (Mancino et al. 2008). However, conditional deletion of HIF-1α under the Tie-2-cre promoter in myeloid cells resulted in lower expression of MHC-II and CD80 and CD86 by DCs in mice (Bhandari et al. 2013). Upon culture with a PHD inhibitor (AKB-4924), DCs stabilized HIF-1α, increased their co-stimulation capacity resulting in more robust T cell proliferation and activation (Bhandari et al. 2013).

A number of experimental studies have focused on DC migration in the context of decreased oxygen availability. Hypoxia has been shown to alter the homing of DCs to lymph nodes via CCR7 as a result of an HIF-1α-dependent mechanism (Mancino et

al. 2008; Kohler et al. 2012). Moreover, hypoxia decreases the chemotaxis of human monocyte-derived DC to CCR5 and CCR4 ligands (Zhao et al. 2005; Mancino et al. 2008). In line with this, hypoxic DCs have been shown to have an altered chemokine receptor (CCR3, CCR2, CXCR4) expression profile (Ricciardi et al. 2008) and enhanced expression of the proinflammatory cytokines TNFα and IL1β (Mancino et al. 2008).

DC differentiation into variety of subsets can also shape the immune response (Shortman and Liu 2002). For instance, hypoxia skews dendritic cells to a T helper type 2-stimulating phenotype and reportedly results in impaired T cell proliferation (Yang et al. 2009). Importantly, HIF-1α prevents the differentiation of bone marrow precursors into plasmacytoid DC (pDC) (Weigert et al. 2012), thereby potentially altering antiviral defenses. Given the importance of DCs and pDC in anti-cancer immunity, further studies on the relevance of HIF-1α in these leukocyte populations are warranted for the design of more effective DC-based immunotherapies or to understand immune dysfunction in cancer-bearing hosts.

Monocytes, Macrophages and Myeloid-derived suppressor cells

Among myeloid cells, tumor-associated macrophages (TAM) are the most widely studied immune population in the context of hypoxia (Noy and Pollard 2014). Although monocytes and macrophages are phagocytes that can destroy tumor cells, they constitute a heterogeneous population that is usually classified as M1 or M2-like based on their expression of different receptors, chemokines and growth factors (Noy and Pollard 2014). TAM polarization is greatly affected by microenvironmental cues in cancer, and TAM are usually defined as M2-like, which constitutes a pro-inflammatory phenotype that contributes to tumor progression (Mantovani et al. 2002). Levels of

macrophage infiltration can vary greatly depending on the tumor type, and usually higher numbers correlate with a worse prognosis (Noy and Pollard 2014; Leblond et al. 2016).

Hypoxic areas in tumors are enriched in macrophages, as a result of secretion of chemo-attractants by tumor cells such as VEGF and endothelins (Murdoch et al. 2004). This effect leads to enhanced migration of TAMs into less well-vascularized areas of the tumor. Different tumor-expressed molecules play a role in the recruitment of TAM to hypoxic tumor areas. Semaphorin 3A, for instance, contributes to this phenomenon by binding to Neuropilin-1 (Casazza et al. 2013). Gene deletion of Neuropilin-1 in macrophages favored TAM localization in normoxic areas, preventing entry into hypoxic niches and inhibiting tumor growth and metastasis. Tumor-derived Semaphorin 3A also played a role in hypoxic retention of macrophages by binding to PlexinA1/PlexinA4 (Casazza et al. 2013). Other chemokines such as CCL2 (Li et al. 2016) and CCL26 (Chiu et al. 2016) are involved in TAM recruitment. Apart from these mechanisms, tumor cells under hypoxia avoid the phagocytosis by up-regulating CD47 in a HIF-1α dependent manner. CD47 as a 'don't-eat-me' signal favors the maintenance of cancer stem cells (Zhang et al. 2015).

When TAM are deprived of oxygen, they stabilize HIF, which results in an increase in VEGF transcription and secretion, thus playing a crucial role in pro-tumoral angiogenesis (Lewis et al. 2000; Cramer et al. 2003). Among the different monocyte populations, Angiopoietin-2-expressing (Tie2+) TAM have been identified as the most pro-angiogenic subsets (De Palma et al. 2005). Importantly, Tie2 expression itself is also known to be induced by hypoxia in monocytes (Lewis et al. 2007).

Apart from inducing angiogenesis, HIF-1 expression by TAM can dampen adaptive immunity by suppressing T cell responses (Doedens et al. 2010). Myeloid-

derived suppressor cells are immature myeloid cells that also more efficiently suppress tumor-specific CD8 T cells when they express HIF-1α (Corzo et al. 2010). This is probably due in part to the fact that HIF-1 directly up-regulates PD-L1 on MDSC and other myeloid tumor infiltrating populations (Noman et al. 2014). Indeed, PD-L1 is a direct HIF transcriptional target both in tumor and stromal cells (Noman et al. 2014).

HIF-2 can also be stabilized in hypoxic macrophages, where it regulates migration, invasion and chemotactic receptor expression (Imtiyaz et al. 2010) (Talks et al. 2000). How the balance between HIF-1 and HIF-2 affects TAM polarization is still unclear. In this regard, HIF-2 expression has been linked to M2-like polarization (Takeda et al. 2010) and it is interesting that M2-like TAM are the main subset found in hypoxic regions of tumors (Movahedi et al. 2010; Laoui et al. 2014).

Neutrophils

Tumor-associated neutrophils (TAN) are short-lived cells that can also influence the tumor microenvironment by the production and release of secreted proinflammatory factors (Gregory and Houghton 2011). The presence of TAN is commonly associated with poor prognosis (Shen et al. 2014). Hypoxic neutrophils also stabilize HIF-1α, which increases their survival depending on NF-κB-mediated mechanisms (Hannah et al. 1995; Walmsley et al. 2005). PHD3 activity in neutrophils subjected to hypoxia contributes to extend survival of these leukocytes (Walmsley et al. 2011). Hence, selective ablation of PHD3 in neutrophils reduces inflammation in a colitis mouse model (Walmsley et al. 2011). The role of HIF-1 in TAN and its influence on tumor progression remains almost totally unexplored, but could be potentially relevant given the pro-inflammatory phenotype of hypoxic neutrophils. TAN are also commonly classified according to type 1 or type 2 polarization (Fridlender et al. 2009), giving rise

to distinct phenotypes likely to have a different impact on cancer progression. The potential role of HIF-1/HIF-2 in neutrophil polarization has yet to be established, and this is also applicable to the granulocytic subsets of myeloid-derived suppressor cells.

1.3. Lymphocyte-mediated adaptive immunity: T lymphocytes and NK cells

Specific tumor antigen recognition can be mediated by T lymphocytes. NK cells recognizing antibody coated tumor cells also participate in adaptive anti-tumor immune responses while also mediating spontaneous cytotoxicity. Lymphocyte physiology is clearly modified under hypoxia and the main mechanisms are highlighted in figure 2.

T lymphocytes

Tumor infiltrating lymphocyte subsets are localized in different areas in the tumor microenvironment. Regulatory T cells (T regs) are mostly localized in the hypoxic regions of the tumor, whereas effector T cells are usually present around the blood vessels. In this context, the study of how hypoxia modulates T cell activation, function and differentiation is essential to elucidate T cell behavior in the tumor microenvironment.

HIF-1α levels are increased in T cells as a result of TCR activation in an mTOR-dependent manner under both normoxic and hypoxic conditions (Nakamura et al. 2005). Previous studies have suggested that HIF-1α acts as a negative regulator of T-cell effector response. Indeed, it has been reported that activated HIF-1α-deficient T lymphocytes showed an increase in proliferation (Thiel et al. 2007) and secretion of pro-inflammatory cytokines such as IFN-γ (Roman et al. 2010). Moreover, it has been described that HIF-1α promotes the development of regulatory CD4 T cells under inflammatory hypoxia. TCR activation under hypoxic conditions results in a FoxP3 increase in CD4 T cells. Such an increase of FoxP3 takes places in a HIF-1α and TGFβ-

dependent manner, thereby impacting the differentiation towards regulatory T cells. The inhibitory functions of the differentiated Tregs are thus partially prevented in the absence of HIF-1α (Clambey et al. 2012).

In contrast, another study has shown that HIF-1α can promote FOXP3 degradation in the proteasome (Dang et al. 2011), highlighting that different microenvironments containing diverse sets of cytokines could be determinant for the effects of HIF-1 on FoxP3 and Treg function. This could be especially relevant in cancer immunobiology, where the influence of hypoxia on Treg remains largely unexplored.

In addition to these direct mechanisms on T cells, HIF-1α and hypoxia play different indirect roles by contributing to develop an immunosuppressive context in the tumor. For instance, the production of certain cytokines and chemokines by hypoxic tumor cells, such as TGFβ and CCL28 (Facciabene et al. 2011), favors the entrance of Tregs into the tumor microenvironment and their function. These regulatory T lymphocytes in turn promote angiogenesis and tumor immune tolerance because of inhibiting T-cell cytotoxic effects (Hasmim et al. 2011; Deng et al. 2013; Hasmim et al. 2013).

Another layer of immunosuppression consists of the release of ATP by dying cells in the tumor microenvironment. The CD73 and CD39 ectoenzymes, which are up regulated in a hypoxic context, are able to metabolize extracellular ATP to adenosine. Adenosine binds to its receptor on the T cell membrane (A2R) promoting an increase in intracellular cAMP, which is known to negatively regulate T-cell effector functions (Ohta et al. 2006; Sitkovsky et al. 2008; Zhang 2010).

All these studies provide evidence for the immunosuppressive role of HIF-1α in the tumor microenvironment. Nevertheless, several publications suggest that this

transcription factor is also involved in promoting an effector phenotype in T lymphocytes acting in a lymphocyte intrinsic fashion. It has recently been reported that HIFs promote T effector functions in a context of antigen persistence, such as tumor-bearing mice or chronic lymphocytic choriomeningitis virus (LCMV) infection. In these settings, HIFs prevent the attenuation/exhaustion of antigen-specific CD8 T lymphocytes (Doedens et al. 2013). Activated CD8 T lymphocytes cultured under hypoxic conditions, as well as VHL-deficient lymphocytes, overexpress some pro-inflammatory (TNFα and IFNγ) and cytotoxic (granzyme B) mediators (Doedens et al. 2013).

In this regard, it has been reported that HIF-1 is involved in the up-regulation of co-inhibitory and co-stimulatory receptors, such as CTLA-4, LAG3, CD137 and OX40, on the surface of hypoxic or VHL-deficient lymphocytes in comparison to lymphocytes under normoxia and control wild-type counterparts (Doedens et al. 2013). We have previously reported that tumor-infiltrating lymphocytes express high levels of CD137 in an HIF-1α-dependent manner (Palazon et al. 2012). These observations have profound implications for the immunotherapeutic effects of monoclonal antibodies directed at CD137, OX40, CTLA-4 and LAG-3. Such effects are currently being tested in clinical trials and warrant further investigation. It may well be the case that T cells cultured under hypoxia might perform better for the purposes of adoptive transfer therapy.

In addition to the HIFs, some microRNAs have been described to be involved in hypoxia-elicited regulatory mechanisms. Among these, miR-210 has attracted most attention and in particular its role in limiting the availability of HIF-1 translation under hypoxia. This new negative feedback loop has been reported as a route to control Th17 differentiation (Wang et al. 2014). The prevention of Th17 differentiation was also shown in a mouse model of autoimmunity as a result of Death-associated protein kinase

(DAPK)-mediated degradation of cytoplasmic HIF-1α (Chou et al. 2016).

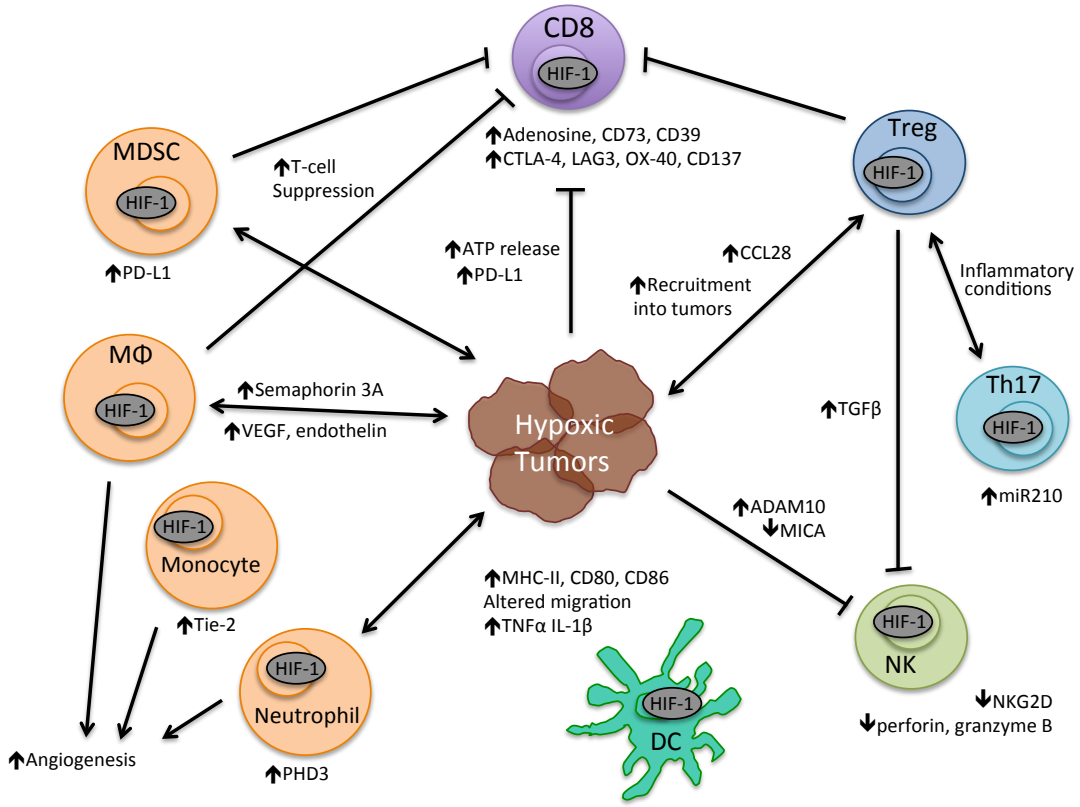


FIGURE 2. Mechanisms governed by hypoxia in tumor tissue that affect the different cells and functions of the immune system. The different activating or inhibitory mechanisms are shown with reference to the literature.

Natural killer cells

Natural killer cells (NK) have been demonstrated to be effective in eradicating solid and hematopoietic tumors, and a high number of tumor infiltrating NKs is a good prognosis factor in some types of cancer (Senovilla et al. 2012). The tumor microenvironment, and even more so under hypoxia, impairs NK cytotoxic effects at different levels. As in the case of T lymphocytes, Treg infiltration promoted by hypoxia hinders NK functions through the activity of TGFβ. Indeed, high amounts of this cytokine are present in hypoxic tumor-derived microvesicles that regulate NK cells in a negative manner (Berchem et al. 2016).

Hypoxic tumor cells increase the expression of the metalloproteinase ADAM10 in an HIF-1α-dependent fashion. This enzyme is responsible for MICA shedding from the surface of malignant cells. Soluble MICA is a ligand for the NKG2D activating receptor on NK and T cells and this mechanism contributes to tumor escape from the immune system, since soluble MICA results in down-regulation of NKG2D expression (Barsoum et al. 2011).

Moreover, the hypoxic microenvironment in some solid tumors is known to down-regulate other activating NK cell receptors such as NKp46, NKp30 and NKp44 involved in target cell recognition and killing, without affecting CD16 that is the trigger of antibody-dependent cellular cytotoxicity (Balsamo et al. 2013). This impairment of NK cell function is not an exclusive feature of solid tumors since it has also been described in hematopoietic tumors resulting in reductions of the NK granular content of perforin and granzymes (Sarkar et al. 2013).

1.4. Immune metabolism and migration in the tumor microenvironment.

As a result of genomic and epigenetic instability and tumor-associated inflammation, cancer cells undergo major adaptive changes to support growth and proliferation (Hanahan and Weinberg 2011). One of the main features is a shift in metabolism, which allows efficient nutrient uptake, biosynthesis and energy expenditure to support cell division. While healthy cells rely on oxidative phosphorylation (OXPHOS) to produce ATP, cancer cells obtain energy from aerobic glycolysis, which meets the critical biosynthetic demands for proliferation but is less efficient than OXPHOS in terms of ATP production. Several molecular players have a role in this metabolic adaptation, including oncogenes (MYC) and aberrantly-expressed or mutated

metabolic enzymes (PKM2, IDH1/2). All these functions are fine-tuned by hypoxia-sensing pathways (Cairns et al. 2011), as highlighted in figure 1.

Tumor infiltrating immune cells share the same habitat with cancer cells, thus it is not surprising that they must undergo similar metabolic adaptation to mount effective immune responses. This is more evident in the case of T lymphocytes, which upon activation proliferate at very high rates and have much increased nutrient and energy demands. Naive T cells have low biosynthetic requirements, resulting in low basal nutrient uptake and glycolytic rates. TCR ligation and co-stimulation leads to a metabolic activation characterized by an increase in nutrient uptake and glycolytic rate (Frauwirth et al. 2002). HIF-1α plays a central role in the shift to aerobic glycolysis by regulating the expression of genes encoding glycolytic enzymes and inhibiting the entry of pyruvate into the tricarboxylic acid cycle (TCA) in immune cells (Figure 1). Although OXPHOS activity is decreased upon antigen recognition, it has been recently shown that mitochondrial ATP production and reactive oxygen species (ROS) are necessary for T cell activation (Chang et al. 2013; Sena et al. 2013). Interestingly, ROS can inhibit PHD function, and since activated T cells produce high levels of ROS, this could be an additional mechanism by which T cells stabilize HIF-1α in the presence of oxygen.

Among HIF-1α metabolic target genes, Glucose transporter GLUT-1, glycolysis rate-limiting enzymes and pyruvate dehydrogenase kinase 1 (PDK-1) are crucial for the T cell metabolic adaptive switch. Importantly, the PDK-1 kinase controls carbon entry into the TCA, limiting mitochondrial activity, ROS production and oxygen consumption by the electron transport chain (ETC) (Kim et al. 2006) (Figure1). Coordinated HIF-1, c-myc and mTOR functions are required for optimal T cell metabolism and activation (Wang et al. 2011; Finlay et al. 2012)

Tumor infiltrating T cells (TILs) are deprived of glucose and oxygen in malignant tissue. Upon antigen presentation, CD28 co-stimulation increases the glycolytic rate (Frauwirth et al. 2002), and glycolysis is required for optimal IFNγ production while OXPHOS is not. This is mediated by a post-transcriptional mechanism consisting of enhanced glyceraldehyde 3-phosphate dehydrogenase (GAPDH) binding to IFNγ mRNA (Chang et al. 2013). If present in TILs, this mechanism could be another layer of tumor-induced immunosuppression suggesting that decreased glucose availability limits cytotoxic functions (Chang et al. 2015). In this regard, it has recently been reported that availability of GAPDH in a glycolytically inactive cell, such as naïve or memory T cell, is important to avoid the translation of HIF-1α by binding to the 3'UTR region of its mRNA (Xu et al. 2016). The fact that mitochondrial activity, via ATP and ROS production, is also required for full T-cell activation makes limited oxygen availability in tumors another complex layer of metabolic immunosuppression.

Importantly, HIF-1-dependent metabolic adaptation in CD8 T cells can alter the expression of chemokines and chemokine receptors involved in CTL migration and extravasation. Loss of HIF-1β resulted in sustained CD62L expression and increased T cell homing to secondary lymphoid organs. Activated T cells with low glucose availability also fail to down-regulate CD62L. This study (Finlay et al. 2012) also shows that loss of HIF-1β up-regulates CXCR3, CCR5, S1PR1 and CCR7 mRNA in CD8 T cells. Of note, high CCR7 expression on TILs has been correlated with increased overall survival in colorectal carcinoma patients (Correale et al. 2012).

Apart from its effector function, T cell metabolism affects CD8 memory differentiation. Long-lived antigen-specific T cells are postulated to be essential for long-term anti-tumor control. Memory T cell nutrient uptake is lower than that in effector T cells, due to the difference in proliferative rate. Thus, memory T cells have

low glycolytic rates but rely on OXPHOS to obtain energy. Interestingly, IL-15 promotes mitochondrial biogenesis (van der Windt and Pearce 2012), inhibits glycolysis and enhances memory formation (Sukumar et al. 2013). It is of much interest that adoptively transferred pmel TCR-transgenic CD8 T cells generated in vitro in the presence of 2-deoxyglucose showed improved anti-tumor activity in gp100-vaccinated B16 melanoma tumor-bearing animals (Sukumar et al. 2013). How HIF-1α controls memory formation and its implications in anti-tumor immunity is a field that remains largely unexplored.

T-cell metabolism has been also linked to T cell differentiation. While pro-inflammatory Th1, Th2 and Th17 populations have increased glycolysis in detriment of OXPHOS, Treg cells depend on lipid oxidation and OXPHOS (Michalek et al. 2011). Among different CD4 subpopulations, HIF-1α is selectively overexpressed in Th17 cells, a phenomenon that drives increased glycolysis. Accordingly, HIF-1α-deficient CD4 T cells showed delayed MOG/CFA-induced experimental autoimmune encephalomyelitis, which is mostly a Th17-mediated autoimmune disease model (Shi et al. 2011).

Among innate cells, macrophages have been the focus on most studies in terms of their metabolism and anti-tumor immunity. During macrophage polarization, metabolic reprogramming could play a role in acquiring a pro-inflammatory M1 vs M2 fate. M1 are characterized by high glycolytic rates and low oxygen consumption and OXPHOS, whereas M2 are less glycolytic and have higher oxygen consumption rates (Haschemi et al. 2012). It is postulated that HIF transcription factors control macrophage-mediated inflammation by controlling their glycolytic capacity (Cramer et al. 2003) with a key role of mitochondria postulated for these phenomena (Garaude et al. 2016)

1.5. Taking hypoxic microenvironments into consideration for immunotherapy

Many unknowns remain in the relationship between tumor hypoxia and immunotherapy. Many mechanisms are set in motion by hypoxia that exert functional effects on the immune system. Aside from their function, O_2 tissue levels also determine lymphocyte and myeloid cell differentiation. Therefore, it is increasingly clear that the hypoxia response modulates immunity by multiple direct and indirect mechanisms. Hypoxia controls the expression of co-stimulatory (CD137, CD134) and co-inhibitory (PD-L1) molecules for T and NK cell activation. The discovery that PD-L1 is a direct transcriptional target of HIF can be of utmost importance since this key pathway of resistance to immunity is not only under the control of inflammatory cytokines but also obeys to other environmental cues.

These mechanisms potentially have profound implications not only regarding the targets for immunotherapeutic interventions but also for co-developments of anti-angiogenic agents and radiotherapy both of which are known to exacerbate hypoxia in the tumor microenvironment.

Regulation of migration, differentiation and effector functions of immune cells by hypoxia and its molecular mechanisms deserve much more attention from the immunotherapy community. The current perspective is that, while some of the mechanisms are notoriously immunosuppressive, others can be exploited to improve cancer immunotherapies.

2. CD137

2.1. CD137: a costimulatory TNF Receptor Superfamily (TNFRSF) member

CD137, also known as 4-1BB, TNFRSF9 and ILA, is a 30kDa type I glycoprotein that belongs to the TNF Receptor Superfamily (TNFRSF). This family that exerts crucial roles in both innate and adaptive immunity includes 29 members that can be classified in three different groups: death domain–containing TNFRs, TNFR-associated factor (TRAF)-binding TNFRs and decoy receptors (Kwon et al. 1999; Locksley et al. 2001). The TRAF-binding TNFRs group contains surface proteins involved in cellular activation, proliferation and survival (Ha et al. 2009). Because of these functions on immune cells, these targets are being tested in immunotherapy for agonist stimulation. These moieties include CD137, CD134 (OX40, NFRSF4), GITR (CD357, TNFRSF18) and CD27 (TNFRSF7) (Figure 3A)

CD137 is usually induced in the surface of immune cells upon activation. Like other TNFRSF members, in the absence of a ligand, CD137 is present as a monomer, although partially dimerized complex are detected in steady state and galectin 9 (Gal-9) is known to cause some degree of trimerization (Madireddi et al. 2014). After the binding to a trimeric ligand or multivalent antibodies, these monomers interact to each other through extracellular cysteine-rich domains, and form a trimeric structure (Rabu et al. 2005; Melero et al. 2008). This molecular change, necessary to initiate the intracellular signaling of CD137, is also maintained by the binding of Gal-9 to the extracellular domains (Madireddi et al. 2014). A short transmembrane region connects the extracellular domain with the cytoplasmic tail that is known to mediate signaling through its association with the TRAF1, TRAF2 and potentially TRAF5 (TNF receptor-associated factors 1 and 2) (Wortzman et al. 2013). TRAFs are adaptor molecules that

recruit other proteins needed in a signaling cascade. Once CD137 trimerization occurs, TRAF2 and TRAF1 are recruited to the cytoplasmic tail or drawn to close molecular proximity, where they constitute a complex with the cIAP1/2 (cellular inhibitors of apoptosis protein). The polyubiquitination reactions carried out in substrate proteins by cIAP1/2 and the E3 ubiquitin-ligase domain of TRAF2 (RING domain), lead to the activation of the classical NF-κB, P38, JNK, and ERK MAPK signaling pathways [103] (Wortzman et al. 2013). TRAF1, which lacks the RING domain associated with the start of the classical NF-κB pathway, has been related to the activation of the alternative NF-κB pathway via NIK (NF-κB-inducing kinase) (Sanchez-Paulete et al. 2016). However, it has also shown that its binding to the complex is necessary for the recruitment of cIAP1 resulting in a better induction of the canonical NF-κB pathway (McPherson et al. 2012).

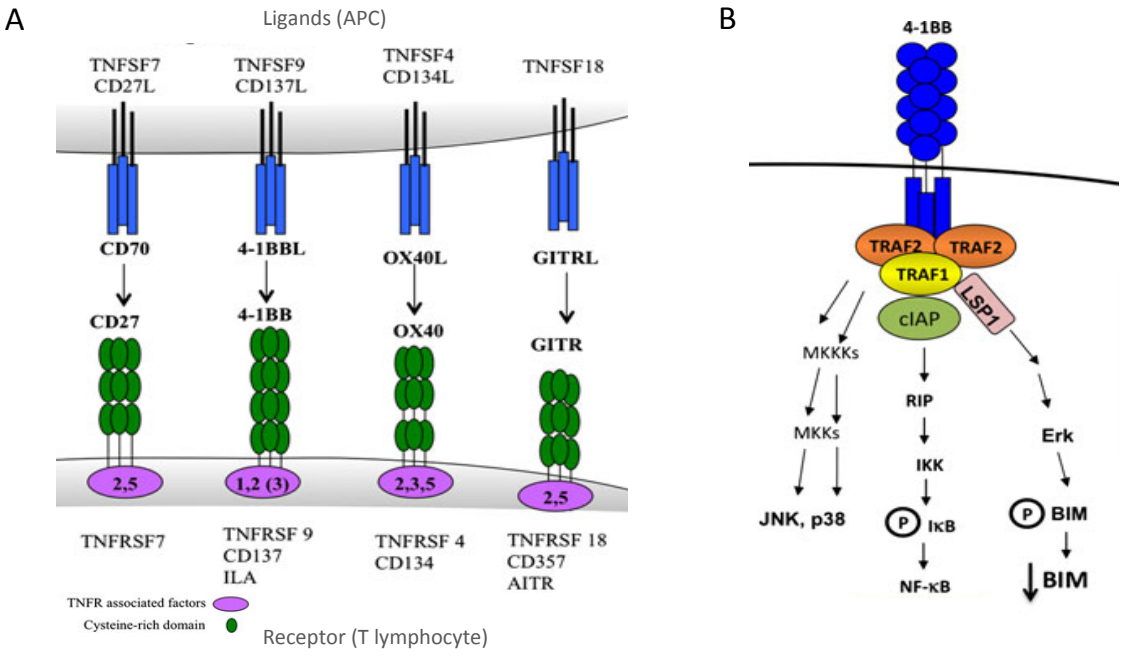


FIGURE 3. TNF receptor (TNFR) family members and ligands and the CD137 signaling pathway. (A) The coestimulatory TNF receptor (TNFR) family members (with ther implicated TRAF proteins) and ligands. (B) CD137 recruits TRAF2/TRAF1/cIAP complex that activates the classical and the alternate NF-κB pathway as well as the P38, JNK, and ERK MAPK pathways. Adapted from Wortzman et al. 2013.

2.2. Regulation of CD137 expression

The gene that encodes CD137 is localized in the human chromosome 1 and in the mouse chromosome 4. It contains eight exons, a short 5'UTR region and an unusually long 3'UTR region (Kwon and Weissman 1989). There are three alternative transcripts described in mouse: the variants 1 and 3 that correspond to a transmembrane isoform, and the variant 2 that lacks the exon seven, which codifies the transmembrane domain resulting in a soluble isoform (Shao et al. 2008). Both transcripts have also been described in humans as a consequence of an alternative splicing (Michel et al. 1998). In the 5'UTR sequences it have been found TATA box-related elements and binding sites for different transcription factor like NF-ϰB, AP-1 and SP-1 (Kim et al. 2011).

CD137 is an inducible receptor, not detected on resting or naive T cells, that is present on a variety of immune cells including activated T and B lymphocytes (Zhang et al. 2010; Vinay and Kwon 2011), Natural Killer cells (NKs) (Melero et al. 1998), regulatory T lymphocytes (T regs) (McHugh et al. 2002), dendritic cells (DCs)(Teijeira et al. 2012), monocytes (Kienzle and von Kempis 2000), neutrophils (Heinisch et al. 2000) and eosinophils (Heinisch et al. 2001), resulting to be functional in most of these cell subsets. *In vitro* activation of T-lymphocytes with anti-CD3, PMA or interleukin 2 (IL2) induces CD137 (Schwarz et al. 1995). Likewise, ligation of the FcRgIII receptor (CD16) with the Fc portion of mAbs that bind to target cells upregulates CD137 on NK cells (Kohrt et al. 2011).

In addition to immune system, CD137 expression has been found in cells from different linages, such as adipocytes (Tu et al. 2014) and endothelial cells (Palazon et al. 2011). Indeed, ectopic CD137 expression has also been reported in Hodgkin Lymphoma and Reed-Sternberg cells favoring escape of from immunosurveillance (Pang et al. 2013).

2.3. CD137 ligand and functions

The only natural ligand known for human and mouse CD137 is CD137L (4-1BBL, TNFSF9)(Kwon 2015). This is expressed on mature dendritic cells (Harfuddin et al. 2016), macrophages (Bae et al. 2011), activated B cells (Zhao et al. 2013) and in some T and B-cell lines (Palma et al. 2004). Its ligation with CD137 mediates diverse effects in the cell that expresses the receptor, but in addition there is reverse signaling that affects the cell expressing the ligand (Lippert et al. 2008; Shao and Schwarz 2011). When CD137L is artificially expressed on tumor cells it enhances immunogenicity (Melero et al. 1998).

CD137 is considered as a potent coestimulatory receptor that upon ligation with agonistic mAb or with the natural ligand, it mediates effector T-cell expansion and cytokine induction (IFNγ or IL-2) (Snell et al. 2011); prevents activation-induced cell death (AICD) (Mittler et al. 2004) and has antiapoptotic effects inducing Bcl-XL (Lee et al. 2002). Moreover, it is important in the differentiation and maintenance of CD8 T memory cells (Hendriks et al. 2005; Myers et al. 2006). In NK cells, costimulation of CD137 promotes cell proliferation, IFNγ production and enhances the antibody-dependent cell-mediated cytotoxicity (ADCC) (Kohrt et al. 2011; Kohrt et al. 2014). In regulatory T cells, CD137 stimulation results in their proliferation and enhancement of their immunosuppressive effects (So et al. 2008), but the role of CD137 in Tregs is poorly understood yet.

Based on CD137 immunostimulatory effects and the fact that tumor infiltrating T-lymphocytes express this receptor (Palazon et al. 2012; Ye et al. 2014), it is an interestingly target for immunotherapy of cancer (Vinay and Kwon 2014; Makkouk et al. 2016). There are many strategies to agonistically mimic the effects that produced the binding of the natural ligand, including monoclonal antibodies (mAb) (Vinay and Kwon

2016), biespecific antibodies (Makkouk et al. 2016), RNA aptamers (Pastor et al. 2011), chimeric-antigen receptor T cells (CART) (Song et al. 2011) and recombinant CD137L proteins (Wang et al. 2012).

In 1997, promising results were obtained on mouse transplantable syngeneic models of sarcoma and mastocytoma treated with anti-CD137 monoclonal antibodies (Melero et al. 1997). Since then, other preclinical studies have demonstrated that the therapeutic effect of monoclonal antibodies was essentially dependent on CD8+ T cells with the contribution by NK cells in some tumor models (Melero et al. 1998; Miller et al. 2002; Xu et al. 2004; Palazon et al. 2011; Morales-Kastresana et al. 2013). The mechanism of action of these agonist antibodies on CD8 T cells resides mainly in the potentiation of cytotoxic effector functions, production of cytotoxic molecules and inhibition of apoptosis (Vinay and Kwon 2016).

Nowadays, the therapeutic use of CD137 agonist monoclonal antibodies is a reality. Two fully human IgG4 anti-CD137 mAbs (Urelumab and PF- 05082566) are currently being developed in phase I/II trials in the clinic, either as monotherapies or in combination with mAbs blocking PD-1 (NCT02253992, NCT02534506, NCT02179918, NCT01307267) (Sanchez-Paulete et al. 2016). In fact, the combination of this CD137-based immunotherapy with other anticancer therapeutics used in the clinic such as chemotherapy, adoptive T-cell therapy or radiotherapy, is becoming a rising and hopeful strategy (Figure 4).

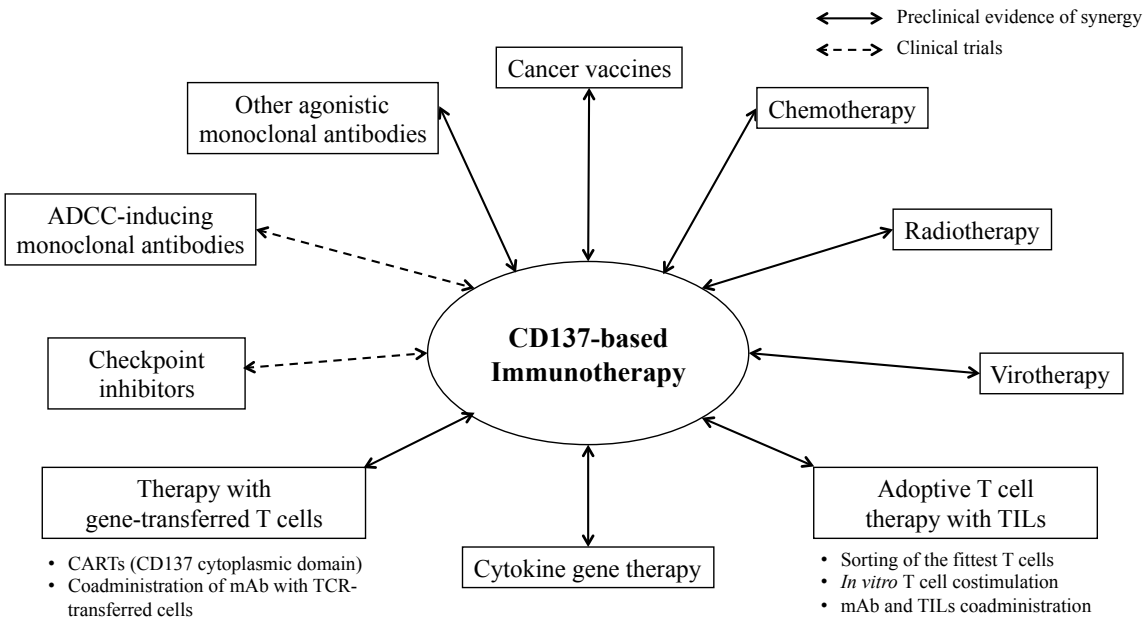


FIGURE 4. Landscape of synergistic interactions of immunotherapies based on the combination of CD137-based and other anticancer therapeutics. Arrows represent described combinations with main references to the literature provided. Adapted from Sanchez-Paulete et al. 2016.

2.4. Soluble CD137

In contrast to the transmembrane receptor, the expression of the soluble isoform is restricted to T and B lymphocytes (Michel and Schwarz 2000; Shao et al. 2008) (Setareh et al. 1995). It is secreted by stimulated lymphocytes in an attempt to control an excess of activation (Shao et al. 2008). It can be postulated to act as a decoy blocking CD137L. Indeed, it is present in the sera of patients with autoimmune disease such as multiple sclerosis (Sharief 2002) and rheumatoid arthritis (Jung et al. 2004), where it is considered a marker of the severity of the disease. Moreover, this soluble isoform has been detected in the sera of obese people (Tu et al. 2014) and patients with colorectal tumors (Dimberg et al. 2006) and lymphoma (Furtner et al. 2005)

2.5. CD137 and hypoxia

Recent studies have reported that the expression of CD137 is upregulated under hypoxia in tumor infiltrating T-lymphocytes (TILs), regulatory T cells and endothelial cells (Palazon et al. 2012) (Palazon et al. 2011). Experiments with inducible HIF-1α knockout mice, showed that although HIF-1α does not bind to the CD137 promoter, it controls the expression of this receptor in an indirect manner that is not yet elucidated (Palazon et al. 2012).

3. CD69

3.1. CD69: a member of C-type lectin protein family

CD69, also know as AIM (*Activation Inducer Molecule*) or Leu23, is a type II glycoprotein that belongs to the C-type lectin-like receptors superfamily that encompasses 17 different groups of lectins (sugar-binding proteins) with diverse functions including cell adhesion, migration, antigen recognition, complement activation, platelet activation, endocytosis, phagocytosis, and activation/inhibition of innate immunity (Weis et al. 1998; Zelensky and Gready 2005). Among these groups, CD69 belongs to the Natural Killer-cell receptors group that, despite the name, most of the members are not in the least exclusively expressed on NK cells.

It is present on the surface of most of hematopoietic cells as a disulfide-linked homodimer with two differentially glycosylated subunits (28 and 32 KDa). The structure of this protein was determined by crystallography analyses carried out in 2000 and 2001. Like other members of C-type lectin-like receptors, the subunits of the homodimer consist of an extracellular conserved C-lectin-like domain (CTLD) but with the particularity of lacking the Ca^{2+}-binding site that is known to be the responsible for carbohydrate recognition. The CTLD is followed by a 20 amino acids neck (where the disulfide bridge is located) and by a transmembrane domain and a short cytoplasmic tail (Natarajan et al. 2000; Llera et al. 2001)

3.2. Regulation of CD69 expression

CD69 is encoded in a genomic region called the natural killer (NK) gene complex located in the human chromosome 12 and in the mouse chromosome 6. This region is characterized by encoding a cluster of receptors originally discovered on NK cells such as other C-type lectin-like receptors like NKG2D and CD94 (Yokoyama and

Plougastel 2003) (Figure 5A). The CD69 gene contains five exons that encode a single transmembrane isoform, a short 5'UTR region (81bp in human and 84bp in mouse) and a long 3'UTR region (1015bp in human and 936bp in mouse). The transcriptional control of CD69 has been studied in depth (Ziegler et al. 1994; Lopez-Cabrera et al. 1995). The characterization of the proximal promoter of CD69 revealed the presence of a region (-78pb to +16pb) that contains *cis*-acting elements with target sequences for AP-1, EGR and ATF/CREB transcription factor families that are crucial in the upregulation of CD69 expression in response to phorbol esters such as PMA (Castellanos et al. 1997; Castellanos Mdel et al. 2002). Furthermore, a region with two NF-κB motifs was discovered at positions −160 and −223 of the proximal promoter. The second binding site for NF-κB was reported to be necessary in the induction of CD69 in a TNFα-dependent manner (Lopez-Cabrera et al. 1995). Apart from the promoter region, four conserved non-coding sequences (CNS) have been identified in a 45-kb region upstream of the CD69 gene. These distal regulatory elements are likely to be favoring an accessible chromatin conformation to permit a rapid transcriptional induction upon stimulation (Vazquez et al. 2009).

The post-transcriptional regulation of CD69 was reported in 1995 when Santís et al. demonstrated that the mRNA levels were controlled by a rapid degradation pathway associated with AU-rich sequence motifs located in the 3'UTR region (Santis et al. 1995). CD69 mRNA is also a direct target for many microRNAs such as miR-17 and miR-20 (members of the miR-17-92 cluster) (Blevins et al. 2015), miR-130/301 (Zhang and Bevan 2010) and miR-181a (Blevins et al. 2015) that regulate its translation by binding to the 3'UTR region.

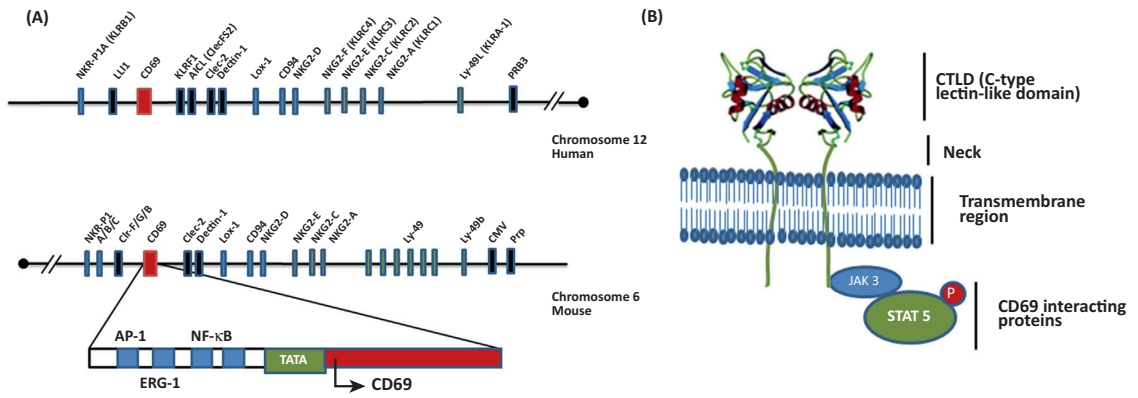


FIGURE 5. Localization and structure of CD69. (A) A representation of NK gene complex where CD69 is encoded and a schematic picture of the CD69 promoter region with the binding sites for AP-1, NF-κB and EGR-1 transcription factors. (B) CD69 homodimer structure at the plasma membrane and the intracellular interaction with Jak3/Stat5 signaling pathway. (Figure adapted from González-Amaro et al, 2013)

CD69 is the first activation-induced protein expressed on the membrane of T-lymphocytes and NK cells, being detectable as early as 3 hours after stimulation (Cebrian et al. 1988). It has also been reported to be upregulated under stimulation in other immune cell types such as B-lymphocytes (Sanchez-Mateos et al. 1989), macrophages (Marzio et al. 1997), neutrophils (Atzeni et al. 2002), eosinophiles (Matsumoto et al. 1998) and dendritic cells (Lamana et al. 2011). For this reason, it is commonly used as a marker of recent cell activation in many studies (Wieland and Shipkova 2016). There are many different activation stimuli that induce CD69 on the surface of these cell types. In T lymphocytes, for instance, the transcription of CD69 is enhanced upon stimulation through the TCR/CD3 (Cebrian et al. 1988) or with stimuli that mimic TCR/CD3 triggering such as PMA/Ionomicyn (Sanchez-Mateos et al. 1989), both activating the intracellular PKC signaling pathway and the release of calcium to the cytosol. Furthermore, a rapid induction of CD69 in the cell membrane was observed to be independent of mRNA and protein synthesis following costimulation of PBMCs with anti-CD3 and anti-CD28 antibodies, due to the association of CD69 to a GTP

binding protein in the cytoplasm of resting cells (Risso et al. 1991). In addition to being inducible, CD69 is constitutively expressed in many other bone marrow-derived cell subsets. This is the case of platelets (Testi et al. 1990), Langerhans dendritic cells (Bieber et al. 1992), monocytes (De Maria et al. 1994), regulatory T lymphocytes (Cortes et al. 2014) and tissue resident lymphocytes (Mackay et al. 2013). The persistency of CD69 expression on T cells can be mediated by certain conditions in tissues like chronic inflammation (Radulovic and Niess 2015). In this context, for instance, a late and stable expression of CD69 on regulatory T cells is reportedly promoted by the non-canonical NF-κB pathway (Saldanha-Araujo et al. 2012).

3.3. CD69 ligands and functions

The functions of CD69 have been difficult to study for many years due to the absence of any known ligand. While this situation has changed with the recent discovery of galectin-1 (de la Fuente et al. 2014) and the calcium-binding S100A8/S100A9 complex (Lin et al. 2015) as two ligands for CD69. Such interactions take place in a glycosylation-dependent manner but their functional significance is unclear to this point of time.

Some degree of functional characterization of CD69 was possible using agonistic monoclonal antibodies (mAbs) and CD69 knockout mouse models. The first *in vitro* studies that employed agonistic monoclonal antibodies against CD69 on T cells, in combination with PMA, showed more sustained intracellular Ca^{2+} concentration increases and an elevation of the synthesis of cytokines such as interleukin-2 (IL-2) (Cebrian et al. 1988), interferon-gamma (IFNγ) (Testi et al. 1989; Ziegler et al. 1993) and tumor necrosis factor-alpha (TNFα) (Santis et al. 1992). Therefore, monoclonal antibodies anti-CD69 promoted T-cell activation and proliferation (Cebrian et al. 1988),

suggesting that this receptor could be acting as a T cell costimulatory receptor. Indeed, the use of agonistic antibodies in other cell types like platelets (Testi et al. 1990) and monocytes (De Maria et al. 1994), extends this motion of CD69 as an activating molecule. In spite of all of these data, an induction of apoptosis mediated by CD69 engagement with monoclonal antibodies in monocytes (Ramirez et al. 1996) and eosinophils (Walsh et al. 1996) has been reported.

The development of CD69 knockout mice allowed researchers to approach the function of this surface molecule *in vivo*. In fact, these studies have decisively contributed to unravel that far from being a costimulatory receptor, as *in vitro* assays indicated, CD69 acts an immunoregulatory molecule. Indeed, CD69 plays different roles in autoimmunity, inflammation, migration or cell differentiation. However, its function is dispensable for thymic T-cell development, NK development and hematopoiesis (Lauzurica et al. 2000)

CD69 in autoimmunity, inflammation and Th17 and Treg differentiation

As mentioned, CD69 knockout mice showed that the phenotype is quite unaltered baseline (Lauzurica et al. 2000), suggesting that this molecule does not have a crucial role in steady state animals. However, when these knockout mice were employed to study different autoimmune and inflammatory diseases, CD69 was found to be an important immunoregulatory receptor (Gonzalez-Amaro et al. 2013). In a mouse model of collagen-induced arthritis (CIA), the blockade of CD69 with monoclonal antibodies (which mimic the phenotype of the CD69KO mice) exacerbates the disease. Moreover, the used of agonistic antibodies for CD69 promoted a decrease in proinflamatory cytokines and T cell proliferation, suggesting that CD69 signaling has an immunoregulatory role in controlling this autoimmune disease model of rheumatoid

arthritis (Sancho et al. 2006). Such an effect was mechanistically explained later, when it was reported that the cytoplasmic tail of CD69 was able to associate with the Jak3/Stat5 signaling pathway that negatively regulates the transcription factor RORγt resulting a CD4 T-cell differentiation towards Th1 and Th2 phenotypes instead of Th17 lineage (Martin et al. 2010) (Figure 5B). Likewise, this mechanism acts in other autoimmune diseases such as experimental autoimmune myocarditis (EAM) (Cruz-Adalia et al. 2010) and in inflammatory diseases like experimental asthma or contact dermatitis (Martin et al. 2010). Apparently, the binding of CD69 to one of the recently discovered ligands, galectin-1, could be responsible for the regulation of CD4 T cell differentiation (de la Fuente et al. 2014).

Despite of deciphering one of the possible mechanisms of CD69 stimulation, other authors have reported opposite effects for this molecule in similar experimental inflammatory contexts (Murata et al. 2003; Miki-Hosokawa et al. 2009) but using a different CD69 knockout mouse model (Murata et al. 2003). Indeed, CD69 has been very recently found associated to the amino acid transporter LAT1-CD98, regulating tryptophan uptake, AhR activation and IL-22 secretion in skin T lymphocytes, contributing to the development of psoriasis in an IL-23-induced mouse model (Cibrian et al. 2016). A contribution of γδ T cells was key in this model.

There are also studies that describe a role of CD69 in the differentiation of regulatory T cells (Tregs). This effect is mediated by the induction of TGFβ production upon CD69 crosslinking. In addition, the engagement of CD69 in a newly described T-cell population that lacks Foxp3 ($CD69^+CD4^+CD25^-Foxp3^-$) was sufficient to induce a suppressive phenotype mediated by membrane-bound TGF-β1, as shown in human and mice tumors (Han et al. 2009; Han et al. 2014).

CD69 in migration and tissue resident memory cells (T_{RM})

A role in cell trafficking was suggested by experiments of Shiow et al. showing that after stimulation, CD69 interacts on the plasma membrane with the sphingosine-1-phospate receptor 1 ($S1P_1$) that as a result is internalized. Loss of $S1P_1$ surface expression is behind the retention of the lymphocytes in lymphoid organs during antigen-driven activation since this mechanism prevents their exit in response to sphingosine-1-phospate (S1P) chemotactic gradients (Shiow et al. 2006). Such an effect has also been reported for skin dendritic cells (DC), which migrated more effectively to draining lymph nodes in response to S1P in the absence of CD69 (Lamana et al. 2011). In the same line, it has been described that the internalization of $S1P_1$ by CD69 is crucial for a prolonged retention of skin-infiltrating T lymphocytes and the resulting local memory formation (Mackay et al. 2015).

Notably, CD69 is considered as a tissue residency marker since tissue-resident memory T (T_{RM}) cells express this protein. T_{RM} cells are a long-lived memory subset that instead of recirculating through the body remains in lymphoid (spleen, thymus) and non-lymphoid tissues (lung, skin, gut). T_{RM} are characterized by constitutively expressed CD69 and the integrin CD103, and both molecules functionally ensure tissue permanence/residence (Mackay et al. 2013). Although the inhibition of the transcription of $S1P_1$ is a key factor in the development of this T cell subset (Skon et al. 2013), the crosstalk between CD69 and $S1P_1$ can further prevent recirculation. According to this, a very recent study showed the relevance of CD69 in the establishment of T_{RM} cells in lymphoid organs such as thymus, but pointing out that CD69 expression alone, without CD103, does not imply tissue residency (Park et al. 2016).

Apart from T_{RM} cells, CD69 is also involved in the generation and persistence of long-lived memory T cells in the bone marrow microenvironment (Shinoda et al. 2012)

(Okhrimenko et al. 2014) located in the vicinity of IL-7-expressing stromal cells (Sercan Alp et al. 2015).

CD69 in tumor immunity

There is little information regarding CD69 in tumor immunology or cancer immunotherapy. It is therefore impossible to interpret yet the function of this molecule in the context of cancer. An inhibitory role of CD69 on NK and T cells was described in RMA-S lymphoma-engrafted mice, where the use of monoclonal antibodies that *in vivo* internalize CD69 in all the lymphocytes has antitumoral effects mediated by NK cells (Esplugues et al. 2003; Esplugues et al. 2005).

OBJECTIVES

Hypoxia is a prominent feature of solid tumors that is likely to regulate many important functions in the biology of cancer. Based on previous research lines of our laboratory, we focused on the following objectives:

1. To analyze the expression and inducibility of CD137 (4-1BB) on hypoxic tumor cells and its effects on T-lymphocyte costimulation.

2. To study the influence of hypoxia in the expression of CD69 by tumor-infiltrating lymphocytes (TILs) and characterize the control of this receptor by the HIF-1α transcription factor.

ARTICLES

Hypoxia-induced soluble CD137 in malignant cells blocks CD137L-costimulation as an immune escape mechanism

Oncoimmunology. 2015 Jun 24;5(1)

ABSTRACT

Hypoxia is a common feature in solid tumors that has been implicated in immune-evasion. Previous studies from our group have shown that hypoxia up-regulates the co-stimulatory receptor CD137 on activated T lymphocytes and on vascular endothelial cells. In this study, we show that exposure of mouse and human tumor cell lines to hypoxic conditions (1% O_2) promotes CD137 transcription. However, the resulting mRNA is predominantly an alternatively spliced form that encodes for a soluble variant, lacking the transmembrane domain. Accordingly, soluble CD137 (sCD137) is detectable by ELISA in the supernatant of hypoxia-exposed cell lines and in the serum of tumor-bearing mice. sCD137, as secreted by tumor cells, is able to bind to CD137-Ligand (CD137L). Our studies on primed T lymphocytes in co-culture with stable transfectants for CD137L demonstrate that tumor-secreted sCD137 prevents co-stimulation of T lymphocytes. Such an effect results from preventing the interaction of CD137L with the transmembrane forms of CD137 expressed on T lymphocytes undergoing activation. Indeed, silencing CD137 with shRNA renders more immunogenic tumor-cell variants upon inoculation to immunocompetent mice but which readily grafted on immunodeficient or CD8 T-cell-depleted mice. These mechanisms are interpreted as a molecular strategy deployed by tumors to repress lymphocyte co-stimulation via CD137/CD137L.

INTRODUCTION

Malignant tissues deploy an array of mechanisms that interfere with potentially tumoricidal immune responses (1, 2). For these purposes cancer cells exploit the natural mechanisms that prevent autoimmunity and excessive inflammation in the organism. Some of the immunosuppressive factors are soluble and reach distant hematopoietic and lymphoid organs, while others act locally in the malignant tissue. A link between hypoxia and immunomodulatory factors has become apparent over recent years (3) with important implications for cancer immune escape.

Co-stimulation and co-inhibition of T-cell activation (4) involves a set of functions that critically determines the outcome of T-cell mediated antitumor responses. Immunomodulation with a therapeutic purpose can be achieved by disrupting the function of co-inhibitory receptors (5, 6) or by turning on the activity of co-stimulatory receptors (7). Gene transfer of a co-stimulatory ligand to tumor cells renders such a cell line more immunogenic (8-11). On the contrary, tumor cells frequently escape cancer by providing ligands for co-inhibitory receptors (5, 12).

CD137 (4-1BB, Tnfsfr9) is a co-stimulatory member of the TNFR family discovered on T-cells undergoing activation (13). On lymphocytes, CD137 ligation contributes to enhance proliferation and effector functions, while importantly it prevents apoptosis (14). Its expression was also found on activated NK cells (15), where it enhances antibody-dependent cellular cytotoxicity (16-18). Other leukocytes subsets gain CD137 expression upon activation (19, 20), but the functional significance of this finding is as yet unclear. Surprisingly, CD137 is also expressed on endothelial cells in the microcirculation of tumors (21-23) and atherosclerotic lesions (24) where it is instigated by hypoxia. Apart from the membrane attached form of

CD137, a soluble form generated by alternative splicing has been identified (25, 26). Circulating sCD137 has been detected by ELISA in the serum and tumor homogenates of colorectal cancer patients undergoing surgery. The significance of this finding has not been established (27).

Agonist anti-CD137 antibodies frequently mediate tumor eradication in mice (28) and are being tested in humans with encouraging results in phase I/II trials (*Clinical trial.gov* NCT 01307267, NTC 0147121). Agonist antibodies engineered to be displayed on the membrane of tumor cells also dramatically enhance the immunogenicity of tumors (11, 29), as has also been observed with anti-CD137 agonist RNA aptamers targeted to the tumor cell surface (30). The mechanism of action is mainly and ultimately dependent on the enhancement of cytotoxic T-lymphocytes that destroy malignant lesions by direct cytotoxicity (28, 31, 32).

Interestingly, CD137 expression is upregulated by hypoxia through HIF-1α indirectly mediated effects (33) and as a result, CD137 is more prominently expressed on endothelial cells in tumor vasculature cells (21) and on tumor infiltrating T lymphocytes (33). While on lymphocytes agonist anti-CD137 mAb provide co-stimulation, on endothelial cells ligation results in an enhancement of adhesion and chemotactic functions for T-cell homing (21).

The only natural ligand known for CD137 is CD137L (4-1BBL, Tnfsf9)(34). This is expressed on activated dendritic cells, macrophages and B cells (35). Upon ligation it also mediates reverse signaling thus promoting inflammation (34) and when it is artificially expressed on tumor cells it enhances immunogenicity (10, 36).

In this study we report that hypoxia up-regulates CD137 in a panel of mouse and human tumor cell lines. However, the predominant splicing form is soluble and able to bind to CD137L, thereby blocking its ability to provide co-stimulation to

primed T lymphocytes. Accordingly, CD137-silenced tumor cells become more immunogenic upon grafting onto immunocompetent mice. These results contribute a novel and mischievous immunosuppressive mechanism cunningly deployed by tumors under hypoxia to counteract a pathway of T-cell co-stimulation.

MATERIAL AND METHODS

Mice and Cell lines

BALB/c wild type mice (6-7 weeks old) were purchased form Harlan Laboratories. OT-1 and Rag2IL2Rγ$^{-/-}$ mice were purchased from the Jackson Laboratories and bred in our facilities. CD137$^{-/-}$ mice in BALB/c background have been previously described (21). OT1 CD137$^{-/-}$ mice were bred in our laboratory by crossing OT-1 mice with CD137$^{-/-}$ mice in C57BL/6 background. All animal procedures were approved and conducted under institutional ethical committee guidelines (study number 137/12).

Mouse CT26 colon carcinoma, RENCA renal carcinoma, EL-4 thymoma and B16F10 melanoma cell lines were obtained from American Type Culture Collection (ATCC). AXBI human melanoma cells were derived at the clinical facility Erlangen from primary surgical samples and were used at early culture passages (kindly gift from Dr. Kaempgen, Erlangen. Germany). A549 human lung cancer and HEPG2 hepatocellular carcinoma cells were obtained from ATCC. RCC10 renal cell carcinoma was kindly provided by Dr Luis del Peso (CSIC-UAM, Madrid, Spain).

Mouse cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Sigma-Aldrich), 100IU/mL penicillin and 100ug/mL streptomycin (Gibco) and 5 x 10^{-5}M 2-mercaptoethanol (Gibco). Human cell lines were cultured in the same medium without 2-mercaptoethanol. To study hypoxia conditions, cell lines were cultured for 48h under 1% O_2 atmosphere in the H35 Hypoxystation (Don Whitley) incubator.

Generation of stable transfectants

CD137 stable transfectants in 293T cells have been previously described (37). To generate CD137L transfectants, plasmids pcDNA3-CD137L, was provided by Dr C. Smerdou (CIMA, Universidad de Navarra); pEZ-M02-CD137 (purchased from GeneCopoeia) were transfected using Lipofectamine (Invitrogen) into 293T cells cultured in p100 culture dishes (Corning) at a concentration of 2.5μg/dish. Following a 7-day culture, $CD137L^+$ cells were sorted (FACS Aria II, BD Pharmigen) and those with the highest stable expression of the transgene were cloned and further expanded.

pGFP-C-shLenti vectors encoding CD137 and scramble siRNAs (purchased from Origene) were transiently transfected into 293T cell line with the lentiviral packaging plasmids pCMV-dR8.2 dvpr and pCMV-VSVG. The CT26 cell line was incubated 72h with the supernatant of packaging 293T containing lentiviral particles and brightly GFP-expressing cells were sorted by FACS and expanded in selection media containing puromycin (5μg/mL).

Tumor growth studies

Female BALB/c, $CD137^{-/-}$ or $Rag2IL2R\gamma^{-/-}$ mice were inoculated subcutaneously in the flank with 5 x 10^5 CT26 wild type cells or transfected variants in 50 μL of PBS. For experiments with shCD137 or shControl CT26, tumor cells were cultured prior to inoculation for 36h under hypoxic conditions. Mice and tumor size were monitored twice a week and mice were sacrificed when tumor areas reached $300mm^2$. For CD8 depletion experiments 200μg per dose of anti-CD8β mAb (clone H35-17-2) were given to mice on days -2, 0 and +3 with respect to the day of tumor cell inoculation. Completeness of depletion was checked by FACS on day +11 on peripheral blood lymphocytes.

CD137 expression

Total RNA was extracted from cell lines or tumors using a Maxwell 16LEV simplyRNA tissue kit (Promega). Reverse transcriptions were performed with M-MLV reverse transcriptase (Invitrogen) in the presence of RNAse OUT (Invitrogen). Real time PCR was carried out with iQ SYBR green supermix in a iQ5 real time PCR detection system (Biorad). PCRs included primers for mouse CD137 cDNA (fw: 5'-AACATCTGCAGAGTGTGTGC-3', rev: 5'-AGACCTTCCGTCTAGAGAGC-3'), mouse CD137 transmembrane cDNA (fw: 5'- AGAAGGACGTGGTGTGTGG-3', rev: 5'-TAAGGACCTGCAAGGAGTGC-3'), human CD137 cDNA (fw: 5'-CACTCTGTTGCTGGTCCTCA-3', rev: 5'-CACAGGTCCTTTGTCCACCT-3') and human CD137 transmembrane cDNA (fw: 5'-GAAGGAGAGGGACGTGGTCT-3', rev: 5'-GCGCAAGAAAGAAGGAGATG-3'). Data were normalized by comparison with levels of β-actin (mouse: fw: 5'-CGCGTCCACCCGCGAG-3', rev: 5'-CCTGGTGCCTAGGGCG-3'; human: fw: 5'-AGCCTCGCCTTTGCCGA-3', rev: 5'-CTGGTGCCTGGGGCG-3'). The expression of each transcript was represented according to this formula $2^{\Delta Ct\ (Ct\ \beta\text{-actin-}\ Ct\ CD137)}$.

We also carried out a semi-quantitative RT-PCR to amplify in the same assay the transmembrane and the soluble transcripts of mouse CD137 (fw: 5'-AACATCTGCAGAGTGTGTGC-3', rev: 5'-GAGCTGCTCCAGTGGTCTTC-3'). The following program was used for the PCR: 94ºC for 5 min, then 40 cycles: 94ºC for 45 sec, Tm 64 ºC for 45 sec, 72ºC 45 sec in a 2720 Thermal Cycler (Applied Biosystems) with BioTaq DNA Polymerase (Bioline). Transmembrane isoform product length: 504 bp; soluble isoform product length: 369 bp.

Surface levels of CD137 in the cell lines were determined by direct immunofluorescence and flow cytometry. In every case positive staining of activated

T cells or a CD137 stable transfectant was used as a positive control. Antibodies used included anti-mouse CD137 PE and biotinylated (clone 17B5, Biolegend) and anti-human CD137 PE (clone 4B4-1, Biolegend), and Syrian Hamster IgG PE and biotinylated (Biolegend) and mouse IgG1 PE (Biolegend) as isotype-matched negative controls.

ELISA quantitation of soluble CD137

Protein levels of soluble CD137 were measured by employing a homemade sandwich ELISA. As capture antibody, plates were coated overnight with a monoclonal rat anti-mCD137 antibody (clone 2A, kindly provided by Dr Lieping Chen, Yale University New Haven, CT) at a concentration of 10μg/mL. After blocking with PBS supplemented with 10% FBS, samples were incubated for 2h at RT and plates were extensively washed with PBS 1% Tween. As detection antibody, a biotinylated monoclonal hamster anti-mCD137 (clone 17B5, Biolegend) was incubated at a concentration of 0.5 μg/mL for 1h before washing and adding streptavidin-HRPO (at 1/250 dilution, DB Biosciences). Serial dilutions of mouse recombinant 4-1BB-Fc chimeric protein (R&D Systems) were used for the standard curve.

sCD137binding assays to CD137L

mCD137L stable transfectants in 293T cells were incubated with the serum of CT26-bearing Rag2IL2R$\gamma^{-/-}$ mice or the culture supernatants of the indicated cell lines treated under hypoxia or normoxia conditions. The binding of the sCD137 present in the samples was detected on the cell surface by FACS analysis using an anti-CD137

monoclonal antibody that does not compete for the CD137L binding site (anti-CD137 PE clone 17B5, Biolegend).

Quantitation of anti-CD137 antibodies

Serial dilutions of the serum of CT26-bearing CD137$^{-/-}$ mice were incubated in ELISA plates coated with mouse recombinant 4-1BB-Fc chimeric protein (R&D Systems) and the antibodies against CD137 were detected and tittered using a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) according to the manufacturer's instructions.

These sera were also incubated with mouse CD137-293T transfectants and an anti-mouse IgG FITC secondary antibody (DakoCytomation) was employed to detect IgG antibodies captured at the cell surface CD137. Non-transfected 293T cells and pre-immune sera were used as controls for these indirect immunofluorescence assays developed by FACS.

Co-stimulation assays

CD8$^+$ T lymphocytes were isolated from spleen of OT-1 and OT-1 CD137$^{-/-}$ transgenic mice using a magnetic isolation kit (mouse CD8$^+$ T cells isolation kit, Miltenyi). CD8$^+$ T cells were loaded with 0.5μM of Violet 450 fluorescent dye (Violate proliferation dye 450, BD Horizon) and activated with plate-bound anti-CD3ε (clone 145-2C11, Biolegend) for 48h. For co-culture assays, 1 x 10^5 irradiated (30000 Rads) mCD137L-293T or non-transfected 293T cells were cultured in 96 well plates with 2 x 10^5 pre-activated CD8$^+$ OT-1 T cells for 72h adding tissue culture supernatants from the indicated cell lines as a source of tumor-derived sCD137. Relative fluorescence to assess fluorescent dye dilution resulting from proliferative activity was analyzed by FACS.

Statistics

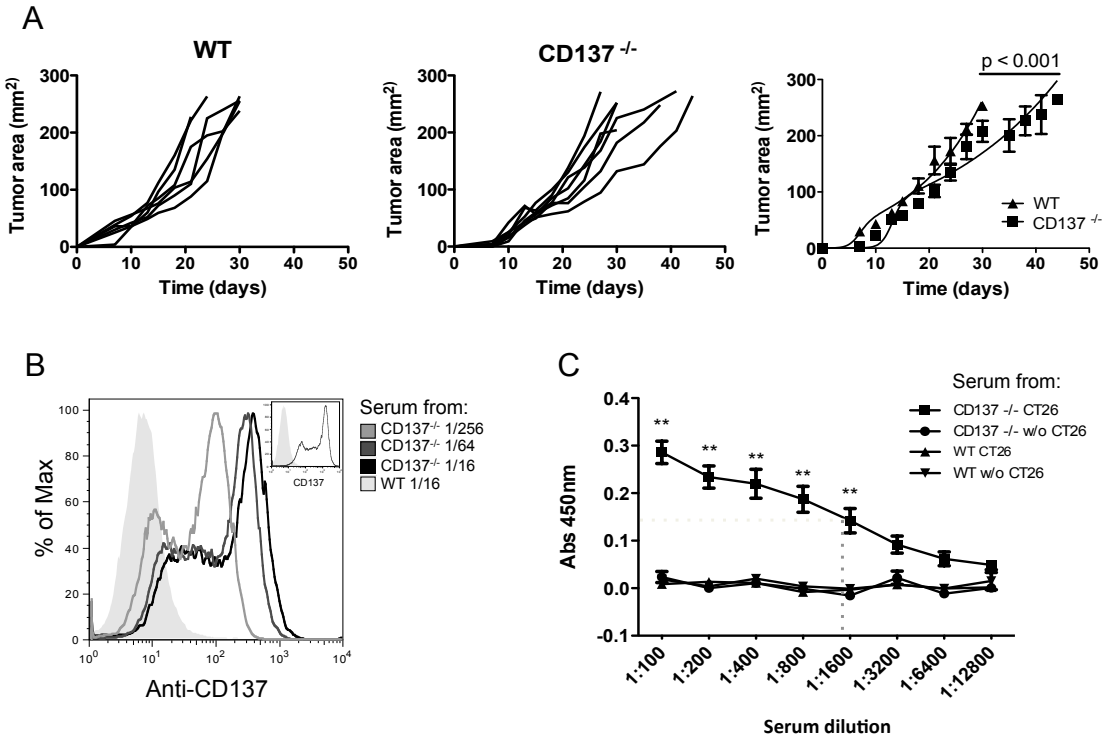
Prism software (GraphPad Software) was used to analyze IgG titter, CD137 mRNA and protein expression by applying unpaired Mann Whitney test. For tumor growth studies, mean diameters of tumors over time were fitted using the following model: $y = \frac{A \times e^{(t-t0)}}{1+e^{\frac{(t-t0)}{B}}}$: $*p < 0.05, **p < 0.01, ***p < 0.001$

RESULTS

Retarded growth of CT26 colon cancer-derived tumors in CD137$^{-/-}$ mice.

Reportedly, CD137$^{-/-}$ and CD137L$^{-/-}$ mice show a relative deficiency in the control of viral infections by CTL responses (38, 39). While performing experiments to examine if transplantable tumors would progress more aggressively and rapidly in CD137$^{-/-}$ mice, we noted a tendency toward the opposite outcome, since growth tended to be delayed several days and there were cases of spontaneous rejection.

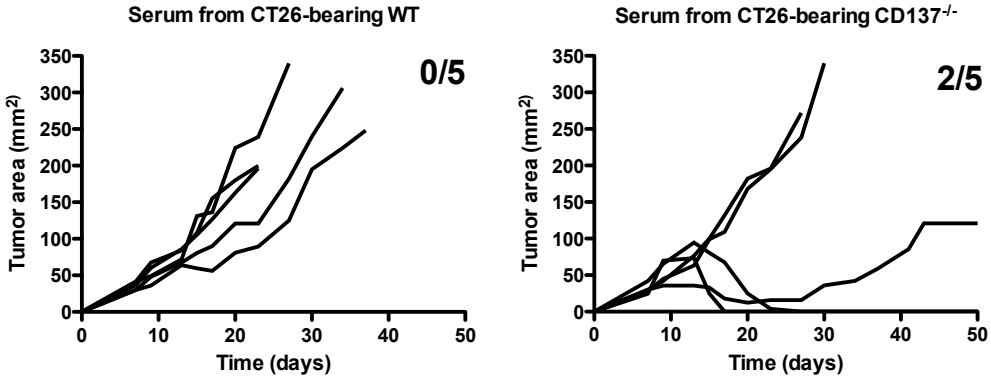
As can be seen in Supplementary figure 1A, the growth of transplanted syngeneic CT26 cells showed a surprising delay in CD137$^{-/-}$ mice. The sera of such tumor-bearing mice contained IgG antibodies directed to native CD137 as detected by indirect immunofluorescence on CD137-transfected 293T cells (Supplementary Figure 1B) and by ELISA on plate-absorbed recombinant CD137 (Supplementary Figure 1C).



SUPPLEMENTARY FIGURE 1. Retarded tumor progression and induction of anti-CD137 antibodies in CD137$^{-/-}$ mice. (A) Comparative progression in size of individual tumors in BALB/c and CD137$^{-/-}$ mice following subcutaneous inoculation of CT26 cells (colon carcinoma) on day 0. Mice

were hosted under identical conditions and the experiment is representative of at least three repetitions. The right graph shows the statistical comparison of tumor progression in the indicated mouse stains. (B) Serum IgG from CD137$^{-/-}$ bearing CT26 tumors selectively binds to mCD137-transfected 293T cells as revealed by indirect immunofluorescence and flow cytometry using the serum samples at the indicated dilutions in PBS. The inset histogram shows the expression profile of CD137 on 293T stable transfectant detected with a specific mAb. (C) Representative ELISA assay analyzing the binding of IgG in pooled sera from CT26-bearing CD137$^{-/-}$ and WT mice to mCD137 coated plates. The experiment is representative of at least three similarly performed. $^{**}p < 0.01$

Since CD137$^{-/-}$ mice are not immunologically tolerant to CD137, they can be readily immunized by this antigen. Therefore we serendipitously reached the conclusion that CT26 tumor cells had to somehow express CD137, resulting in tumor growth retardation and induction of anti-CD137 antibodies in CD137$^{-/-}$ mice. Indeed, transfer of sera containing CD137 antibodies from these CD137$^{-/-}$ tumor-bearing mice to WT mice grafted with CT26 tumors, retarded tumor growth and caused some complete rejections (Supplementary Figure 2).



SUPPLEMENTARY FIGURE 2. Tumor progression in mice treated with the sera of CT26-bearing CD137$^{-/-}$ mice which contain anti-CD137 antibodies. Comparative progression in size of individual tumors in BALB/c mice following subcutaneous inoculation of CT26 cells on day 0. Mice were treated at day 5, 7 and 9 with 100 μl of sera from either CT26-bearing CD137$^{-/-}$ or WT mice. Sera from CD137$^{-/-}$ mice were confirmed to contain antibodies against CD137 by ELISA.

Transplantable mouse tumor cell lines express CD137 under hypoxia

In previous studies we have documented that hypoxia promoted CD137 expression in the case of both T lymphocytes (33) and endothelial cells in a HIF-1α-

dependent fashion (21). Therefore, we performed experiments to determine if hypoxia could induce CD137 on tumor cell lines raised from mouse tumors of different tissue origins.

Figure 1 shows that CD137 was upregulated at the mRNA level not only in CT26 cells, but also in EL-4, RENCA and B16 tumor cells. In this vein, a similar phenomenon has been observed in short-term passaged cell lines derived in our laboratory from spontaneous lung tumors indicating a more general trend (40).

However, when analyzing the cell surface for the presence of CD137 by a sensitive flow cytometry assay, we were surprised by the weak surface levels detected in the case of CT26 cells and by the undetectable levels on the other cell lines tested (Figure 1).

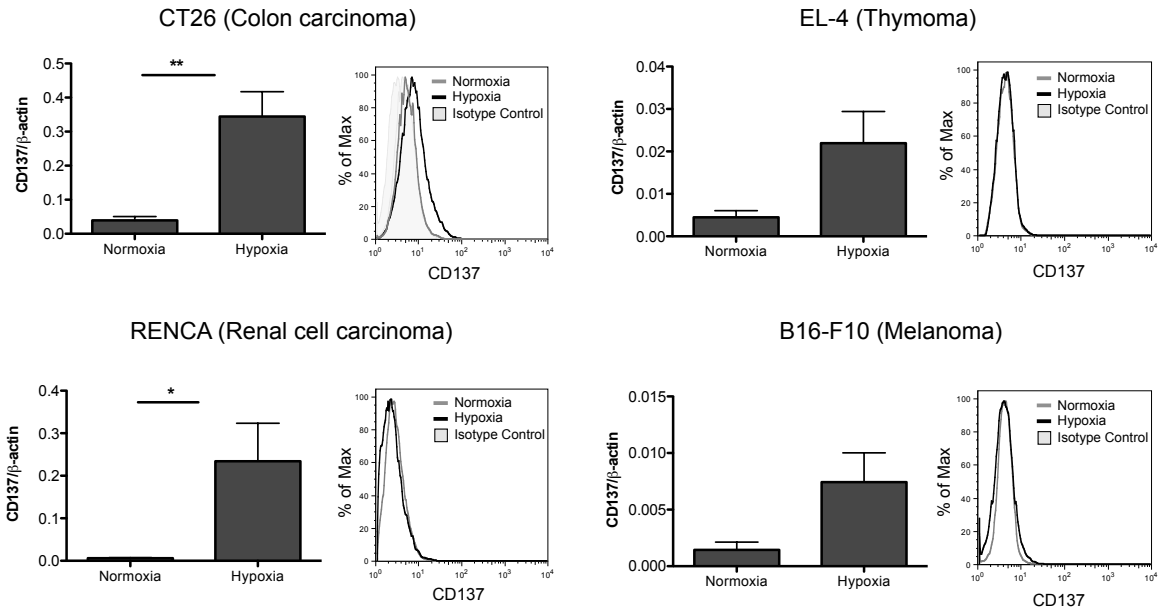


FIGURE 1. CD137 mRNA expression is upregulated by hypoxia in mouse tumor cell lines. Quantitative RT-PCR assessment of CD137 mRNA expression under 21% and 1% O_2 (hypoxia) in the indicated cell lines cultured under these conditions for 48h. A representative direct immunofluorescence staining for CD137 surface expression analyzed flow cytometry is presented in a corresponding histogram showing the discrepancy between the mRNA levels and the weak or dim surface protein staining. *$p < 0.05$, **$p < 0.01$

Hypoxia induces a soluble CD137 spliced form in tumor cells

A possible explanation for the absence of membrane staining was a predominance of the soluble alternative splicing form of CD137 (26). To address this issue, combinations of primers detecting the soluble and transmembrane CD137 isoforms were used (Figures 2A and B) in quantitative RT-PCRs performed on the mRNA from the cell lines. Figure 2B shows that the transmembrane form is clearly in the minority as is shown by the electrophoresis migration of the amplified PCR products corresponding to the soluble and transmembrane forms (Figure 2C).

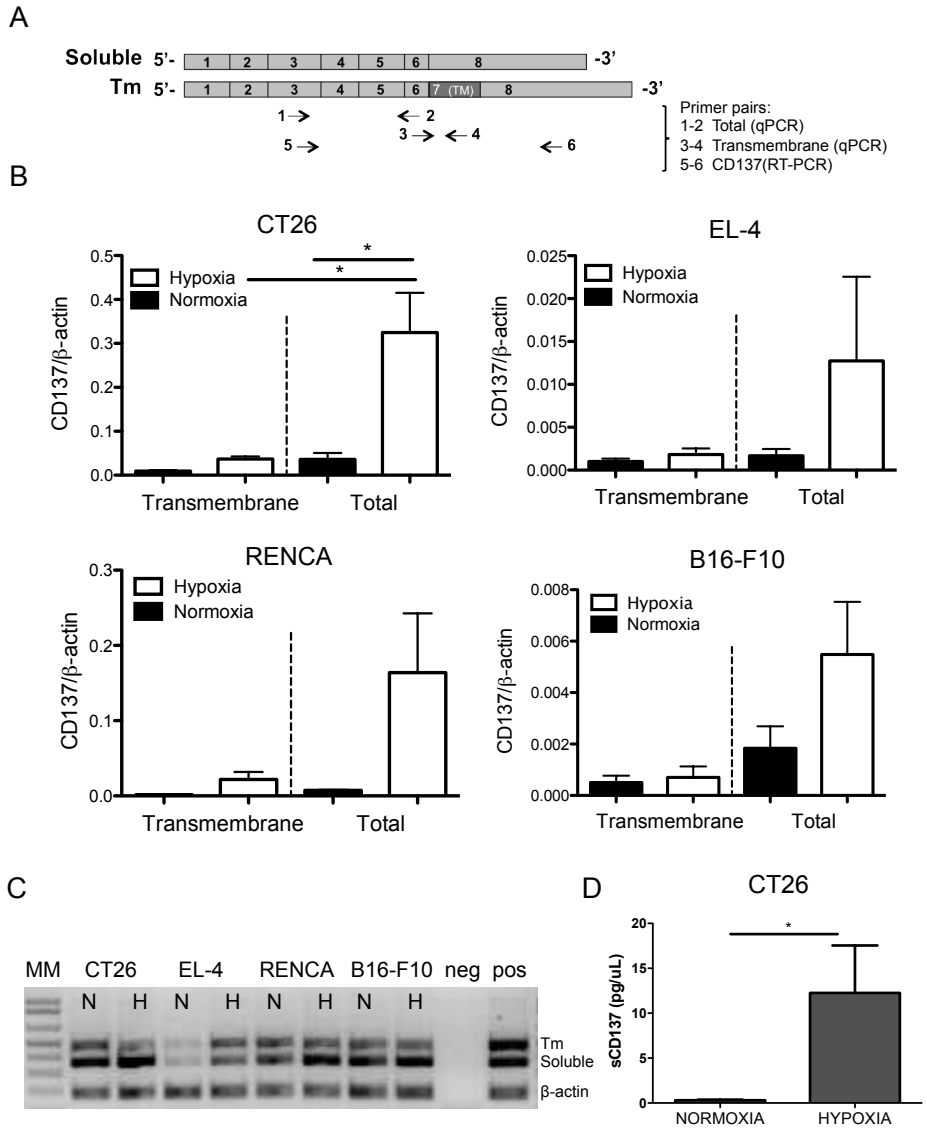
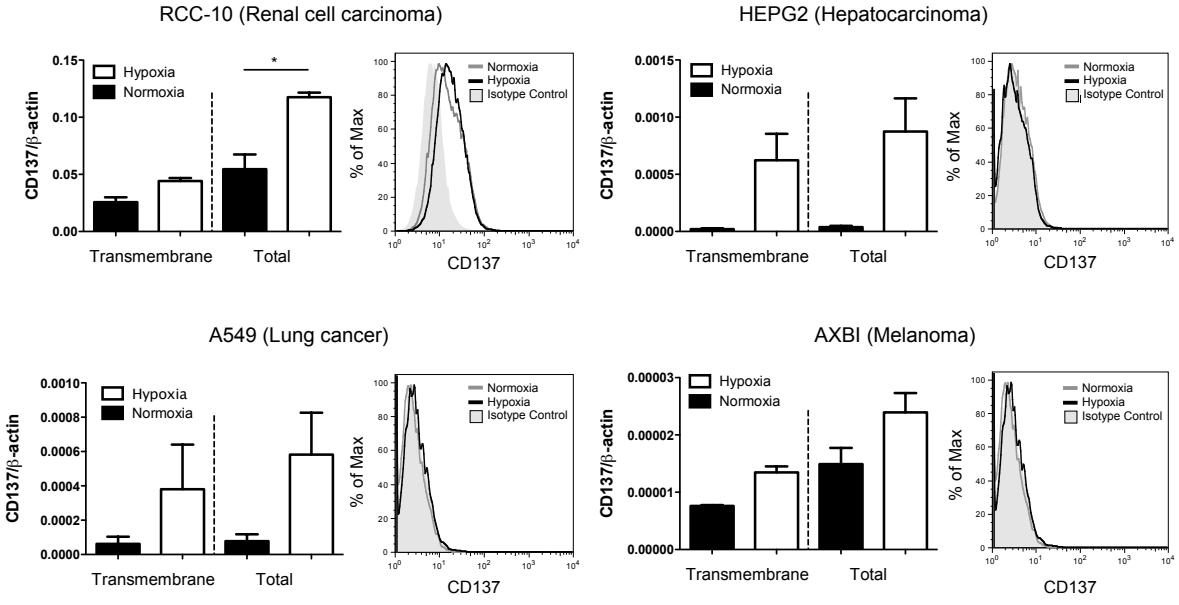


FIGURE 2. Soluble form of CD137 predominates over membrane-attached forms. (A) Diagram representing the mRNA with the exons of mouse CD137 and RT-PCR products amplified with the

indicated primer pairs. (B) Quantitative RT-PCR analyses using primers that amplify either transmembrane (primer pair 3-4) or total CD137 mRNAs (primer pair 1-2) in corresponding cell lines. (C) RT-PCR products of total CD137 mRNA (primer pair 5-6) amplification showing that the soluble CD137 (sCD137) predominates over the transmembrane CD137 (tmCD137) form. (D) Sandwich ELISA assessment of the concentration of sCD137 in the tissue culture supernatants of the CT26 cell line. TM: transmembrane domain; MM: molecular weight marker; N: normoxia; H: hypoxia.

To ascertain as to whether sCD137 was indeed produced at the protein level, we set up a quantitative ELISA assay that clearly shows that the supernatant of CT26 cells contained higher concentrations of sCD137 when these cells were cultured under hypoxic conditions (Figure 2D).

Our findings in human cell lines derived from renal, lung, melanoma and hepatocellular tumors support a similar pattern of induction of sCD137 by hypoxia, indicating a mechanism conserved across species (Supplementary Figure 3).



SUPPLEMENTARY FIGURE 3. Human tumor cell lines express CD137. Experiments as in Figure 1 with the indicated human tumor cell lines using the corresponding primer pairs to amplify the indicated human RT-PCR products.

Furthermore, we investigated if CD137 expression by tumor cells was taking place during in vivo tumor growth, given the fact that these tumors are hypoxic as we

have recently reported (21, 33). Figure 3A shows that CD137 mRNA is expressed by CT26 cells grafted as tumors in CD137$^{-/-}$ mice. Using CD137$^{-/-}$ mice excludes contaminating mRNA from infiltrating lymphocytes or stromal cells. Again, total CD137 mRNA predominates over the transmembrane form.

Consistent with these findings, the sera of immunoglobulin-deficient Rag2IL2Rγ$^{-/-}$ mice grafted with CT26 tumors contained readily detectable amounts of sCD137 by ELISA, that were undetectable prior to tumor grafting (Figure 3B).

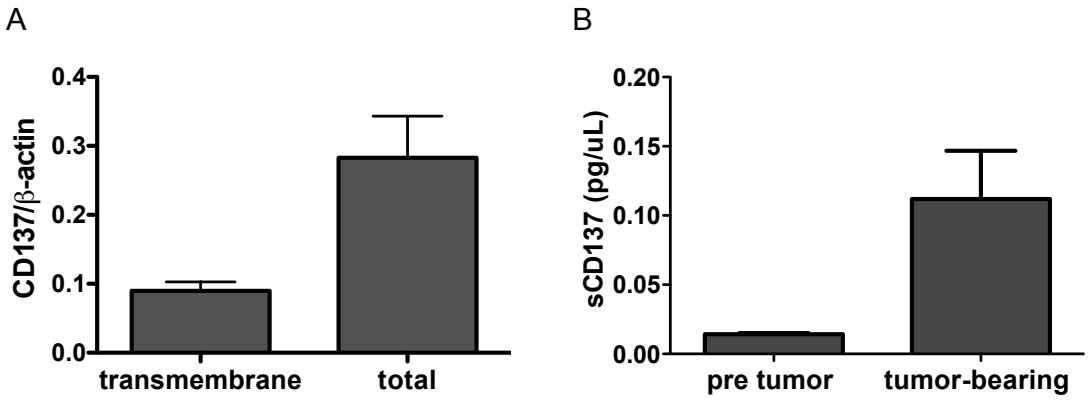


FIGURE 3. Soluble CD137 is produced by *in vivo* grafted tumors. (A) CT26 tumors (10x10 mm in diameters) excised from CD137$^{-/-}$ mice were analyzed by quantitative RT-PCR for mCD137 encoding the trans-membrane and the total CD137 isoforms. (B) The concentration of soluble CD137 (sCD137) assessed by ELISA in the sera of Rag$^{-/-}$ mice grafted with 5x10^5 CT26 cells for 21 days.

Soluble CD137 produced by tumor cells binds to CD137L and blocks its co-stimulatory function

The functional role of sCD137 was hypothesized to be the blockade of the interactions of CD137L with membrane-bound co-stimulatory CD137. Figure 4A shows that the supernatant of CT26 cells cultured under hypoxia contained a soluble CD137 moiety that can be absorbed onto 293T cells transfected to express CD137L, but not onto their untransfected counterparts. Moreover, the sera of Rag2IL2Rγ$^{-/-}$ mice

grafted with CT26 tumors contained a sCD137 form that was also able to bind to the CD137L transfectants, whereas it failed to bind to untransfected 293T cells. The sera from these mice prior to tumor grafting showed no signs of any such activity (Figure 4B).

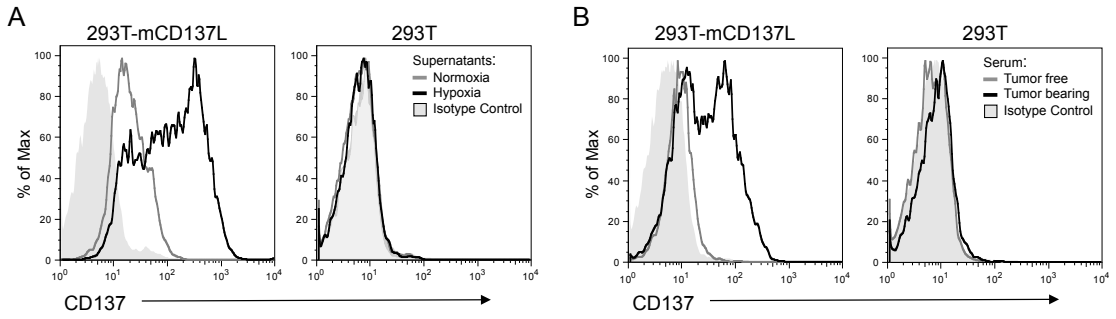


FIGURE 4. Soluble CD137 produced by tumor cells binds to CD137 ligand. (A) Binding of sCD137 present in the supernatant of CT26 cells cultured under hypoxia to CD137 ligand (CD137L) transfected to 293T cells. Untransfected 293T were used as a specificity control and supernatants from normoxia and hypoxia cultured CT26 cells were tested without dilution. Binding was revealed by an anti-CD137 mAb which does not interfere with ligand binding (1D8 clone) (B) Similar experiment as in A performed with the serum of CT26-bearing Rag2IL2Rγ$^{-/-}$ mice as a source of sCD137 or with pre-tumor serum as a control.

Binding of sCD137 to CD137L could functionally result in its blockade at the CD137 binding site, giving rise to a reduction in CD137L co-stimulatory activity. In assays of CD137L-293T cells co-cultured with primed CD137^{+}CD8^{+} T cells, proliferation was assessed by Violet-450 dye dilution. In these assays OT-1 TCR transgenic CD8 T cells were used to ensure homogeneity and permit comparisons with identical TCR-transgenic lymphocytes obtained from CD137$^{-/-}$ double-transgenic mice. OT-1 lymphocytes were pre-activated for 48h with agonist anti-CD3ε mAb and then co-cultured with non-transfected and CD137L-transfected 293T cells. In this setting, the conditioned supernatant of hypoxia treated CT26 cells inhibited proliferation while the supernatant of normoxic CT26 cells failed to inhibit proliferation (Figure 5 A and B). As a control, it was observed that such an effect was

not detectable when CD137$^{-/-}$ OT-1 lymphocytes were used, thus excluding CD137 unrelated effects of the supernatants in these co-cultures. Figure 5A shows representative histograms showing the degree of proliferation inhibition induced by conditioning these co-cultures with culture supernatants of hypoxic tumor cells. These T-cell proliferation experiments are summarized in Figure 5B.

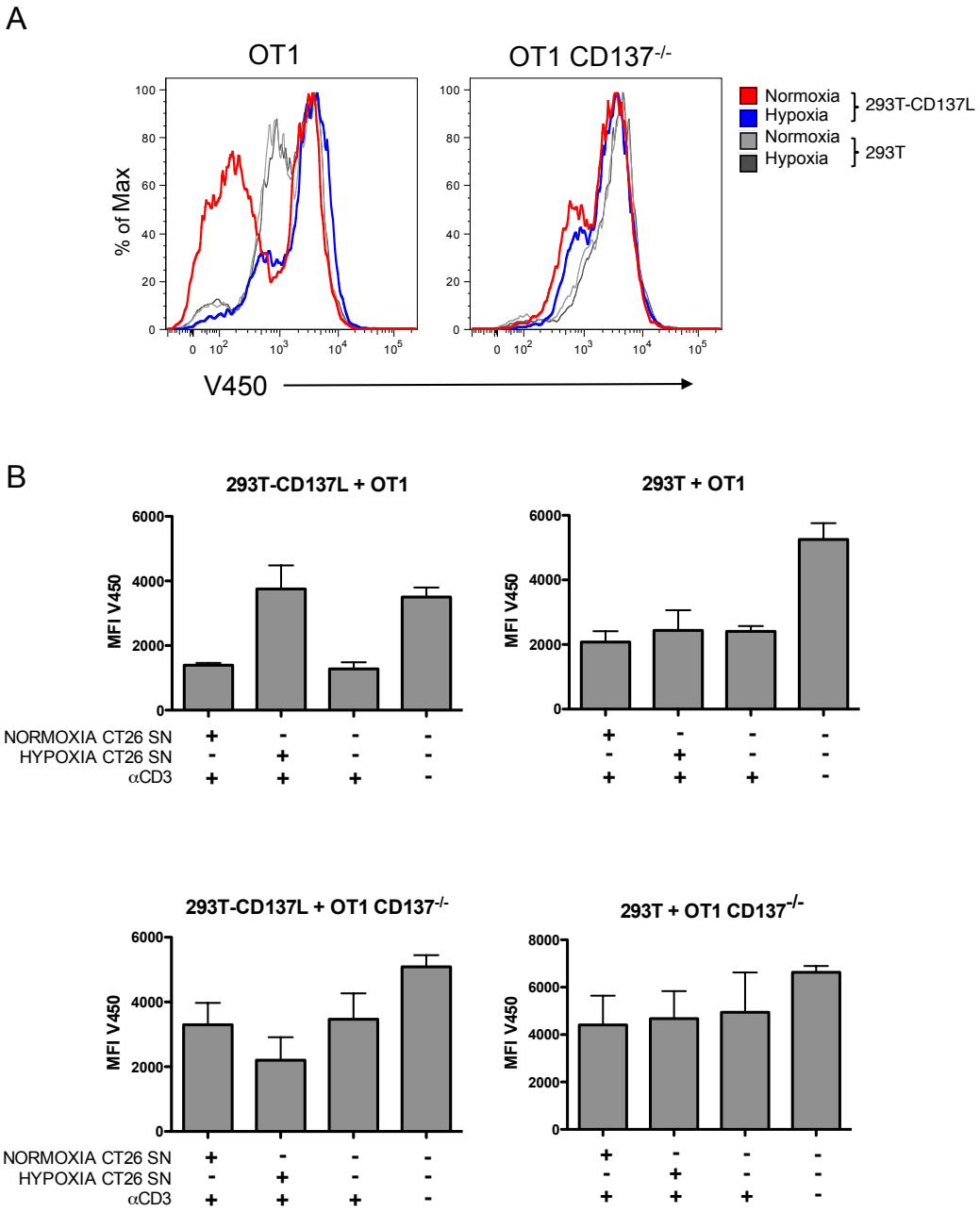


FIGURE 5. Soluble CD137 in the supernatant of hypoxia-treated CT26 cells blocks CD137L-mediated T-cell costimulation. Cultures of CD8-purified OT1 WT or OT1 CD137$^{-/-}$ T cells were

stimulated with anti-CD3 mAb for 48h prior to seeding in co-cultures with 293T transfected or not with CD137L as indicated. T cells were pre-loaded with Violet 450 fluorescent dye and dilution of the fluorescent dye was used as a surrogate marker of T-cell proliferation. Cultures were conditioned with cell culture supernatants (1/16 diluted) of normoxia or hypoxia treated CT26 cells. (A) Representative flow cytometry histograms showing the extent of inhibition of proliferation by the sCD137-containing supernatants at 72 h of coculture. (B) Pooled data of two independent experiments identically performed. MFI: mean fluorescence intensity; SN: supernatant.

Our interpretation of these results is that under hypoxia tumor cells produce a soluble CD137 moiety in order to block CD137L-mediated co-stimulation of primed $CD137^+$ T lymphocytes, some of which are likely to be specific for tumor antigens.

CD137 silencing in hypoxic CT26 tumor cells renders them more immunogenic

Polyclonal stable transfectants of a CD137 shRNA were generated in CT26 tumor cells by lentiviral transfection. Such transfectants were unable to upregulate surface CD137 in response to 1% O_2 (Figure 6A). More importantly, CD137 RNA including the soluble form was almost completely silenced (Figure 6B). To study if CD137 loss results in higher immunogenicity under 1% O_2, hypoxia pre-exposed shControl and shCD137 transfectants were inoculated subcutaneously in BALB/c WT mice and Rag2IL2R$\gamma^{-/-}$ syngenic mice. As can be seen in Figures 6C and D, shCD137 transfectants progressed at slower pace and three out of eight mice underwent complete spontaneous rejections. By contrast, such tumors rapidly grafted and progressed in Rag2IL2R$\gamma^{-/-}$immunodeficient mice (Figure 6E) or in immunocompetent BALB/c mice in which CD8 T cells were selectively depleted with a specific mAb (Figures 6C and D).

In conclusion, sCD137 expressed by CT26 tumor cells in response to hypoxia seems to be an important adaptive immune escape mechanism, at least in this tumor model.

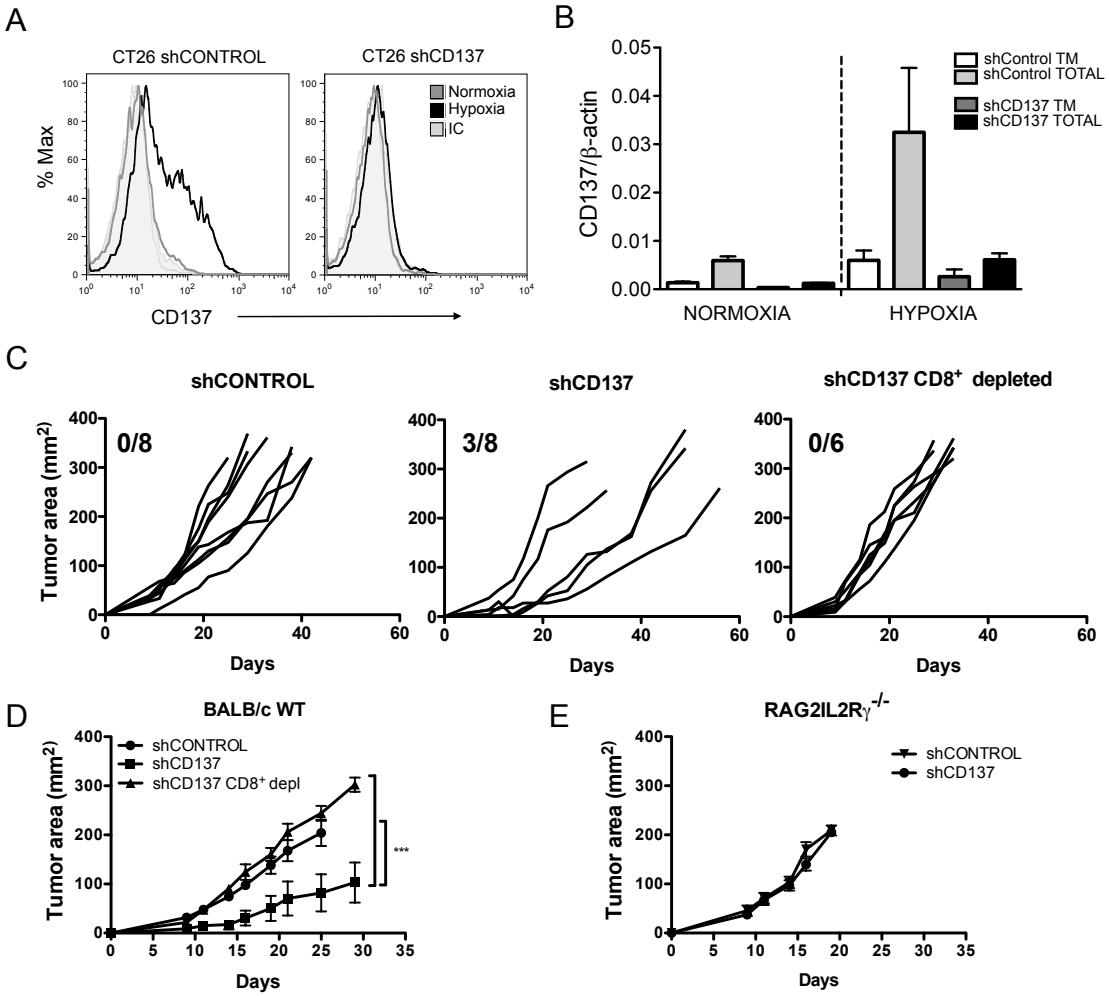


FIGURE 6. CD137 silencing in CT26 tumor cells gives rise to more immunogenic variants. CT26 were stably transfected with lentiviral vectors to express a shRNA targeting CD137 (shCD137) or a scrambled control (shControl). (A) Transfectants were inoculated for 36h in normoxia or 1% O_2 and analyzed by flow cytometry to quantitatively determine the expression of surface CD137. (B) Quantitative RT-PCR analysis of total and transmembrane CD137 mRNA in the indicated transfectants under hypoxic or normoxic conditions as in A. (C) BALB/c wild type immunocompetent mice were subcutaneously inoculated with $5x10^5$ cells of indicated transfectants and tumor sizes were individually followed over time. Tumor cells had been pre-exposed in every case to 1% O_2 for 36h. The fraction of mice spontaneously rejecting their tumors is given in each graph. When indicated, BALB/c mice were mAb-depleted of $CD8^+$T cells. (D) mean±SEM and Statistical comparisons of experiments in A whose results are representative of two independent experiments. (E) Tumor growth of the indicated transfectants in immunodeficient Rag2IL2R$\gamma^{-/-}$ mice. Tm: transmembrane.

DISCUSSION

Tumor exploitation of immune system mechanisms to evade immune surveillance is currently considered a hallmark of cancer (41). Importantly, tumors may induce immune escape mechanisms when undergoing immune attack or stress as previously described for instance in the case of adaptive acquisition of PD-L1 (42, 43) and IDO expression (44). Tumors in mice and humans consistently show a degree of hypoxia at least in the core of primary or metastatic lesions. It is likely that hypoxia would be more pronounced under immune attack due to vascular disruption by secreted cytokines such as IFNγ and TNFα (45). Evolutionary adaptation of tumors to cope with immunity ought to target the mechanisms that enhance the immune response (2). In this study we show that tumors can cunningly tamper with the CD137/CD137L co-stimulatory system which has the potential to elicit potent cytotoxic immune responses against cancer (46), as schematized in Figure 7.

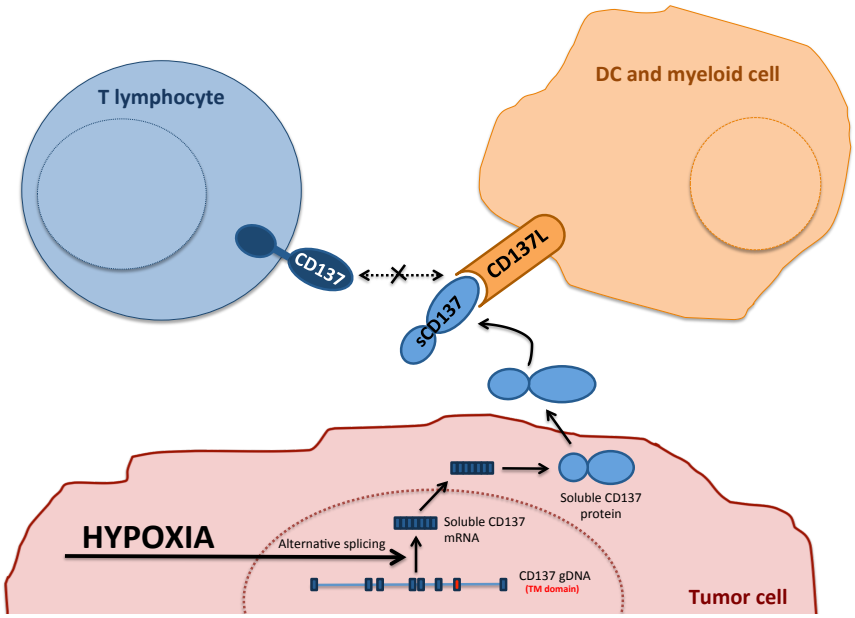


FIGURE 7. Graphical interpretation of the experimental results. Schematic representation of the postulated mechanism underneath the experimental observations. TM: transmembrane; sCD137: soluble CD137

Our results extend prior findings in the sense that CD137 expression is enhanced by hypoxia on T lymphocytes and endothelial cells in a HIF-1α-dependent fashion (33). This feature is not exclusive to CD137 since it has also been observed with OX-40 (CD134), a close co-stimulatory relative of CD137 in the TNFR family, which is also clearly upregulated by hypoxia (47).

Expression of CD137 on grafted tumor cells is as intense as to be capable of inducing anti-CD137 antibodies in CD137-/- mice that do not appear in tolerized wild-type mice. The induction of antibodies in CD137-/- mice is so potent that it is convenient for raising monoclonal antibodies covering different epitopes on the CD137 molecule.

In an attempt to define a role for sCD137 in the tumor setting, we explored the possibility that it would block CD137L mediated co-stimulation. Indeed, our results clearly demonstrate the existence of such a mechanism, since we show that sCD137 produced in hypoxic cultures or by in vivo grafted tumor cells binds to CD137L and disrupts its co-stimulatory functions on primed $CD8^+$ T lymphocytes. CD137-CD137L interactions are postulated to be important in immune synapses between antigen presenting cells and T lymphocytes (48) and sCD137 may compete and disrupt such co-stimulatory events. In addition, some human tumor cell lines have been shown to weakly express CD137L (49) and the local presence of sCD137 would block its pro-immune functions.

Our observations have implications for cancer immunotherapy with anti-CD137 mAb, since the administered antibodies, in addition to signaling via CD137 on T and endothelial cells, should neutralize the sCD137 moieties which otherwise would obstruct CD137L functions. Moreover, such a mechanism helps to explain why single chain Fv anti-CD137 antibodies attached to the plasma membrane of tumors

(11, 29) are more efficacious than the natural CD137 ligand (10, 50, 51) expressed at the same location. The explanation would be that CD137L is amenable to inhibition by sCD137, whereas agonist anti-CD137 antibodies are not. This concept could be relevant to refining reported attempts based on gene therapy with CD137L (50) or on the use of CD137L-Ig fusion proteins (52).

Our experiments with CD137-silenced variants of CT26 clearly demonstrate that these mechanisms are involved in immunoescape. When CT26 tumor cells could not up-regulate CD137 in response to hypoxia, tumors were rejected or slowly progressed in contrast with control variants. This effect critically required CD8 T cells.

Consistent with our conclusions and interpretations, sCD137 is also observed in patients suffering autoimmune conditions such as rheumatoid arthritis (53). In this case, the production of sCD137 probably constitutes a negative feedback mechanism to attenuate damaging self-reactivity through CD137L blockade. Other possible mechanisms of sCD137 include reverse signaling via CD137L, but these would probably require sCD137 multimeric forms (34).

Further studies are warranted to rank the relative importance of this new mechanism among other immunosuppressive and tolerogenic mechanisms deployed by malignances (5). The dependency of this immunosuppressive mechanism on hypoxia is quite intriguing and indicates a common theme (3), according to which immune subversion can be enhanced when the tumor senses that it is under hypoxic stress. How splicing for the soluble form of CD137 is favored in the tumoral transcriptional scenario, or modulated by hypoxia, needs to be defined at the molecular level.

In our view, the aberrant secretion of sCD137 is clearly a simple and effective

trick used by tumors to tackle a critical pathway of T-cell activation and memory differentiation (54). In this sense, production of a blocking molecule for CD137L by hypoxic tumor cells most likely pursues immune escape.

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CD69 is a direct HIF-1α target gene in hypoxia as a mechanism enhancing expression on tumor-infiltrating T lymphocytes

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ABSTRACT

CD69 is an early activation marker on the surface of T lymphocytes undergoing activation by cognate antigen. We observed intense expression of CD69 on tumor infiltrating T-lymphocytes that reside in the hypoxic tumor microenvironment and hypothesized that CD69 could be, at least partially, under the control of the transcriptional hypoxia response. In line with this, human and mouse CD3-stimulated lymphocytes cultured under hypoxia (1% O_2) showed increased expression of CD69 at the protein and mRNA level. Consistent with these findings, mouse T lymphocytes that had recently undergone hypoxia *in vivo*, as denoted by pimonidazole staining, were more frequently $CD69^+$ in the tumor and bone marrow hypoxic tissue compartments. We found evidence for HIF-1α involvement both when using T-lymphocytes from inducible HIF-$1\alpha^{-/-}$ mice and when observing tumor infiltrating T-lymphocytes in mice whose T cells are HIF-$1\alpha^{-/-}$. Direct pro-transcriptional activity of HIF-1α on a newly identified hypoxia response element (HRE) found in the human CD69 locus, was demonstrated by ChIP experiments. These results uncover a connection between the HIF-1α oxygen sensing pathway and CD69 immunobiology.

INTRODUCTION

The C-type lectin CD69 is the first membrane-attached glycoprotein induced upon T and NK cell activation (1, 2). For this reason, its expression is frequently used as a read out for recent T cell activation (3). The transcriptional control of CD69 has been studied in depth and gene expression is mainly driven by NF-κB, EGR, ATF/CREB and AP-1 binding to the promoter of both human and mouse CD69 (4-6). In spite of its rapid induction, the functions of CD69 are difficult to interpret. To begin with, the ligand or the ligands for CD69 have remained elusive for a long time and only recently CD69 has been reported to bind galectin-1 (7) and to S100A8/S100A9 both in a glycosylation-dependent manner (8). The outcome of such ligations is even less clear. When CD69 is perturbed by monoclonal antibodies, ligation can either enhance (9) or inhibit T lymphocyte activation and proliferation (9, 10) but the downstream events have been only poorly clarified. Availability of CD69 knockout mice (11) showed that the phenotype is quite unaltered baseline, although a regulatory role in the differentiation was unraveled by controlling Th17 differentiation in pathogenically relevant disease models (12, 13). Recently, CD69 has been found associated to the amino acid transporter LAT1-CD98, regulating Tryptophan uptake, AhR activation and IL-22 secretion in skin T lymphocytes, contributing to the development of psoriasis (14). In addition, a repressive role on NK function has been reported and exploited for antitumoral effects in RMA-S lymphoma-engrafted mice (15, 16).

A role in T cell trafficking was suggested by experiments showing that CD69 downregulates the chemotactic receptor $S1P_1$ from the cell surface acting in *cis* (17). According to such a model, CD69 upregulation would prevent T cells from exiting

lymph nodes in response to sphingosine-1-phospate (S1P) gradients, to transiently ensure permanence in the lymphoid tissue during antigen-driven activation (18). Interestingly, tissue-resident memory T cells express CD69 and this $S1P_1$-CD69 functional crosstalk can be involved in preventing recirculation of such a memory non-migratory T-cell subset (19, 20). Other authors have reported that CD69 is involved in the generation and persistence of long-lasting memory T cells in the bone marrow microenvironment (21, 22).

Hypoxia is known to profoundly affect the physiology of cells of the immune system through the heterodimeric HIF (α/β) transcription factors (23, 24). The HIF-1α and HIF-2α system mainly senses hypoxia by means of control of their post-translational degradation. Prolyl hydroxylases (PDH 1-3) functionally sensitive to availability of O_2 at physiological levels, hydroxylate HIF-1α and HIF-2α and as a result these proteins are targeted for proteasomal degradation following a K48 poly-ubiquitination reaction catalyzed by von Hippel-Lindau tumour suppressor protein (VHL), an E3 ubiquitin ligase (25-29). Therefore, in hypoxic conditions HIFα cannot be hydroxylated resulting in the stabilization of the HIFα subunits. Furthermore, pharmacological inhibitors of PHDs, such as dimethyloxaloylglycine (DMOG), as well as Vhl or PHDs gene inactivation result in constitutive stabilization of the HIF transcription factors in normoxic conditions (27, 30-33) Moreover, HIF-1α activation can be also triggered by NF-κB-dependent activation of HIF-1α proximal promoter, which has been particularly relevant in both myeloid (34-36) and lymphoid cells (37-40). An additional layer of modulation of HIF-1α levels in immune cells is controlled by metabolic changes in the microenvironment, including adenosine concentration (41) and oxygen free radicals (42). Upregulation by HIF-1α has also been reported for OX40 and CD137 as activation-promoting cell surface markers expressed by T cells

(43, 44). Such an effect of hypoxia explains more intense patterns of expression on the surface of tumor-infiltrating T-lymphocytes since tumors are hypoxic as a result of insufficient blood supply in a high-demanding metabolic tissue environment (44).

In this study we report that CD69 is a direct transcriptional target of HIF-1α under physiologically relevant conditions. Hypoxia upregulates CD69 expression explaining its presence on tumor-infiltrating T lymphocytes, suggesting that the functions of CD69 could be involved in the adaptation of activated T and NK lymphocytes to low O_2 availability.

MATERIAL AND METHODS

Mice and Cell lines

Female C57BL/6 and BALB/c wild type mice (6-7 weeks old) were purchased from Harlan Laboratories. C57BL/6 Tg(TcraTcrb)1100Mjb(OT-1) x B6SJL-Ptprc^{a}Pep3^{b}/boyJ(CD45.1) mice were bred in our laboratory. BALB/c MMTV-*NeuT* transgenic mice were purchased from Jackson Laboratories and bred in our facilities. *Hif-1α*^{floxed}-UBC-*Cre*-ER^{T2} mice and their counterparts *Hif-1α*^{WT}-UBC-*Cre*-ER^{T2} were generated by crossing B6.129-*Hif-1α*^{tm3Rsjo}/*J* mice (Jackson Laboratories, stock no. 007561) with *Tg*(UBC-*Cre*/ER^{T2})*1Ejb*/*J* mice (Jackson Laboratories, stock no. 008085). Mice carrying *Hif-α* loxP-flanked alleles were crossed with *dLck-Cre* mice to obtain T cell specific gene deletion. Mice were backcrossed over ten generations to the C57Bl6 background. All animal procedures were approved and conducted under institutional ethics committee guidelines (study number 003/16).

Mouse CT26 colon carcinoma and RENCA renal carcinoma cell lines were obtained from American Type Culture Collection (ATCC). MC38 colon carcinoma and B16OVA melanoma cell lines were kindly provided by Dr. Karl E. Hellström (University of Washington, Seattle, WA) and Dr. Lieping Chen (Yale University, New Heaven, CT), respectively. Cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Sigma-Aldrich), 100IU/mL penicillin and 100ug/mL streptomycin (Gibco) and 5x10^{-5}M 2-mercaptoethanol (Gibco), with the exception of LLC that was cultured with high glucose DMEM (Gibco) supplemented with 10% FBS. B16OVA cultures were additionally supplemented with Geneticin (400μg/mL, Gibco).

In vitro T-lymphocytes activation studies

Splenocytes obtained from C57B/L6 mice were activated with plate-bound anti-CD3ε (0.5μg/mL, clone 145-2C11, Biolegend) and, when indicated, with soluble anti-CD28 (1μg/ml, clone 37.51) at $2.5x10^6$ cells/mL. In order to silence *HIF-1α* gene, splenocytes from *Hif-1α*^{floxed}-UBC-*Cre*-ER^{T2} mice and their corresponding controls were cultured with 5μM 4-hydroxytamoxifen (Merck) for 48h following activation under either 21% or 1% O_2 atmospheres (altitude 449m over sea level).

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors and isolated from total blood by Ficoll gradients. Afterwards, cells were stimulated in 12-well plates precoated with anti-CD3ε (1μg/mL, clone OKT3) at $2.5x10^6$ cells/mL. Human NKs cells were isolated from peripheral blood by Ficoll gradients, following purification with NK Cell Isolation kit by negative selection in an automacs device (Miltenyi Biotec).

In both mice and human experiments, cells were cultured for indicated times at 37ºC in a 1% O_2 atmosphere in the H35 Hypoxystation (Don Whitley, West Yorkshire, UK) incubator in order to study hypoxia conditions. In hypoxic mimicking experiments, dimethyloxaloylglycine (DMOG, Enzo Life Sciences, NY, USA) was added to lymphocyte cultures at a concentration of 0.2mM for 48h.

In vivo studies

BALB/c, C57BL/6 or conditional HIF-1α$^{-/-}$ mice were inoculated subcutaneously in the flank with $5x10^5$ tumor cells of the indicated origin in 50 μL of PBS. When tumor areas reached 100 mm^2, including the spontaneous breast carcinomas from Her2/NeuT, mice were sacrificed and the spleen and tumor were excised in order to obtain splenocytes and tumor-infiltrating T lymphocytes. Isolated

tumors were incubated with Collagenase-D and DNase-I (Roche) for 15 minutes at 37ºC, followed by mechanical disaggregation and filtration in a 70-μm cell strainer (BD Falcon, BD Bioscience). Tumor-infiltrating lymphocytes were isolated from stromal cells in a 35% Percoll gradient.

For pimonidazole experiments, B16OVA-bearing mice for 12 days were transferred intravenously with splenocytes obtained from OT1CD45.1. Two days following lymphocyte transfer, mice were injected i.p. with the hypoxic marker pimonidazole hydrochloride (60mg/kg, Hydroxyprobe-1 Plus Kit, Hydroxyprobe Inc) and four hours later, mice were euthanized to prepare single-cell suspensions from bone marrow and tumor. To detect lymphocytes that had undergone hypoxia, cells were fixed with Cytofix/Cytoperm (BD Bioscience), washed with PermWash (BD Bioscience) and incubated with a FITC-MAb1 (45) after a surface staining of CD69.

Flow cytometry and antibodies and reagents

After treating with FcR-Block (anti-CD16/32 clone 2.4G2; BD Biosciences Pharmingen), mouse T cell suspensions were extracellularly stained with the following antibodies purchased from Biolegend: CD3-PEC7 or FITC (17A2), CD8-BV510 (53-6.7), CD4-BV421 (RMA-5), CD45.1-PerCPC5.5 (A20), CD45.2-APC (104) CD69 PE (H1.2F3), CD137-biotin (17B5), Syrian Hamster IgG Biotin (SHG-1) and Armenian Hamster IgG PE (HTK888) as isotype-matched negative control.

Cultured PBMCs were pretreated with Beriglobin and surface stained with the following antibodies: CD4-BV421 (RPA-T4), CD8-BV510 (5K1) purchased from BD Bioscience, CD3-FITC (UCHT-1), CD4-PerCPC5.5 (OKT4), CD69-PE (FN50), NKp46-APC (9E2), CD16-PB (3G8), FOXP3-AF647 (150D), CD25-APC (BC96) and mouse IgG1-PE and AF647 (MOPC-21) and purchased from Biolegend.

Either Zombi NIR Fixable viability kit (Biolegend) or 7AAD (Biolegend) were used as a live/dead marker. True-Nuclear™ Transcription Factor Buffer Set (Biolegend) was used for FOXP3 staining experiments. Cell acquisition was carried out with FACSCanto II and Fortessa (BD Biosciences) and FlowJo (Treestar) software was used for data analysis and presentation.

RNA purification, reverse transcription and qRT-PCR assays

Total RNA was extracted from splenocytes or PBMCs using a Maxwell 16LEV simplyRNA tissue kit (Promega). Reverse transcriptions were performed with M-MLV reverse transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was carried out with iQ SYBR green supermix in a CFX real time PCR detection system (Biorad). PCRs included primers for mouse *CD69* cDNA (fw: 5'-AGGCTTGTACGAGAAGTTGGA-3', rev:5'-AGTTCACCAGAATATCGCTTCAG -3'), mouse PGK1 cDNA (fw: 5'-GTTCCTATGAAGAACAACCAG-3', rev: 5'-CATCTTTTCCCTTCCCTTCTTCC-3') and human CD69 cDNA (fw: 5'-AAATCTGTGTCAGTGGATGC-3', rev: 5'-TCATTCTTCTCATTCTTGGG -3'). Expression data were normalized by comparison with levels of RPLO (mouse and human: fw: 5'-AACATCTCCCCCTTCTCCT-3', rev: 5'-GAAGGCCTTGACCT TTTCAG-3'). The expression of each transcript was represented according to this formula $2^{\Delta Ct\ (Ct\ RPLO\ -\ Ct\ CD69\ or\ PGK1)}$, where Ct corresponds to *cycle number*.

Chromatin Immunoprecipitation Assay (ChIP)

For ChIP assays, human T lymphocytes were activated by anti-CD3ε mAb and cultured under normoxic or hypoxic conditions for 12 hours. Subsequently, cells were fixed with formaldehyde that was stopped by the addition of glycine. Cell pellets were

resuspended in a membrane lysis buffer. Nuclei pellets from these lysates were resuspended in a SDS sonication buffer and were sonicated to shear the DNA under conditions established. Next samples were diluted in a Triton dilution buffer and pre-cleared with protein G sepharose. An "input sample" was removed and stored from each sample, while the rest was immunoprecipitated with a rabbit polyclonal anti-HIF-1α antibody (Abcam, ab2185) or a rabbit normal IgG control antibody (Cell Signaling Technology, 2729). Immunocomplexes were recovered by addition of protein G sepharose to the samples that were then sequentially washed with several buffers and eluted with an elution buffer. DNA-protein cross-linking was reversed in the input and eluted samples and DNA was purified and resuspended in water. Immunoprecipitated DNA was quantified by PCR using the following primers: *CD69* proximal promoter: fw 5'-CAAGCTTTCTGTTTCCTGCATTC-3'; rev 5'-TCGCTTCTTCCCTGGTGACT-3'; *PDK1* positive control: fw 5'-CGCGTTTGGATTCCGTG-3'; rev 5'-CCAGTTATAATCTGCCTTCCCTATTATC-3'. *PDK1* negative control: fw 5'-GTGGGATGGTATCGTGATGGT-3'; rev 5´-TTTGGCCAACCTCCTTCCT-3´.

Statistics

Prism software (GraphPad Software) was used to analyze statistical differences of CD69 mRNA and protein expression by applying the Mann Whitney U test or the Wilcoxon paired test. P values <0.05 were considered significant.

RESULTS

Tumor-infiltrating T lymphocytes express CD69

The presence and function of tumor-infiltrating T lymphocytes has been found to be critical for the outcome of human neoplasia as originally reported in ovarian and colorectal cancer (46-48). At least a fraction of tumor-infiltrating T lymphocytes is known to be recognizing tumor antigens (49) and is expected to undergo TCR-CD3-mediated activation.

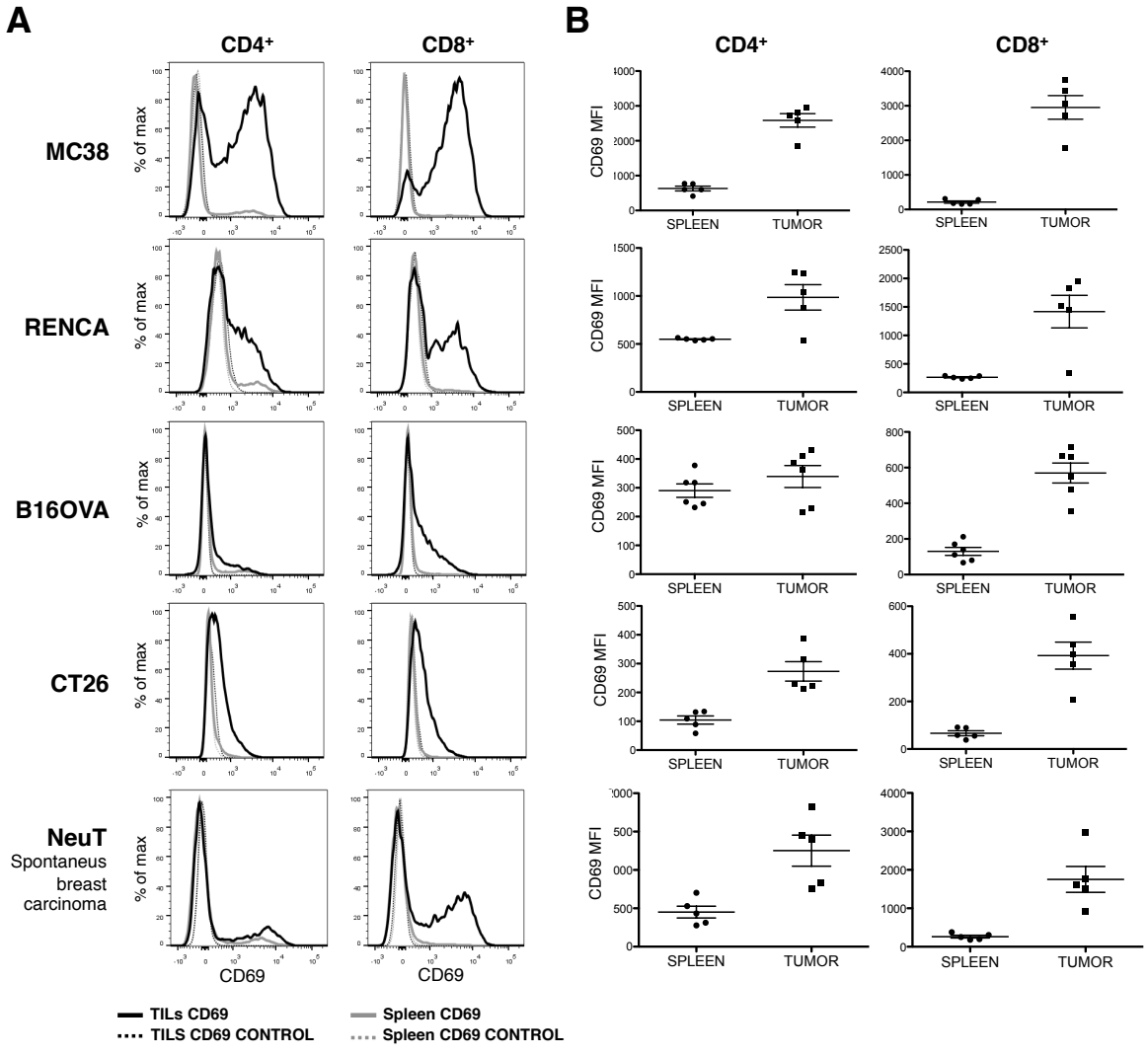
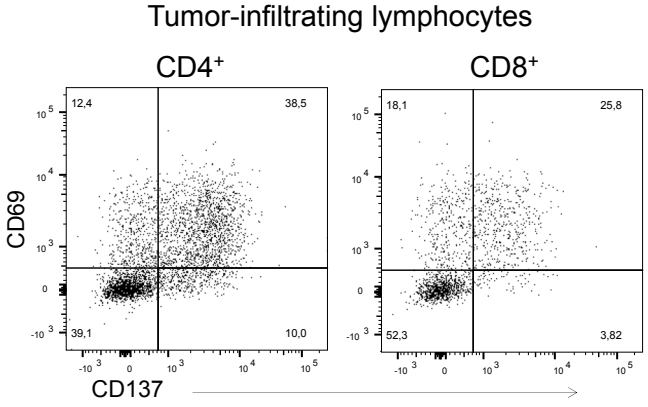


FIGURE 1. Tumor-infiltrating T lymphocytes in hypoxic tumors express surface CD69. Tumors from mice engrafted with the indicated syngeneic tumors or developing spontaneous breast cancer in Her2/NeuT transgenic mice, were surgically excised when reaching a diameter above 10 mm, conditions that we previously reported to result in hypoxia (44). CD69 expression was assessed by flow cytometry on single cell suspensions derived from the tumors upon gating of CD4 and CD8 T

lymphocytes. MFIs (Mean of fluorescence intensity) are shown for individual mice (B) and representative FACS histograms are shown (A). n=5 tumor bearing mice per experiment (Mean±SEM)

Flow cytometry analyses of $CD4^+$ and $CD8^+$ T lymphocytes infiltrating mouse tumors showed CD69 on their plasma membrane at higher levels than those obtained from spleen lymphocytes (Figure 1). CD69 expression was maximal in MC38 colon carcinoma, although with variable intensity, in every case the presence of CD69 was detectable (Figure 1).

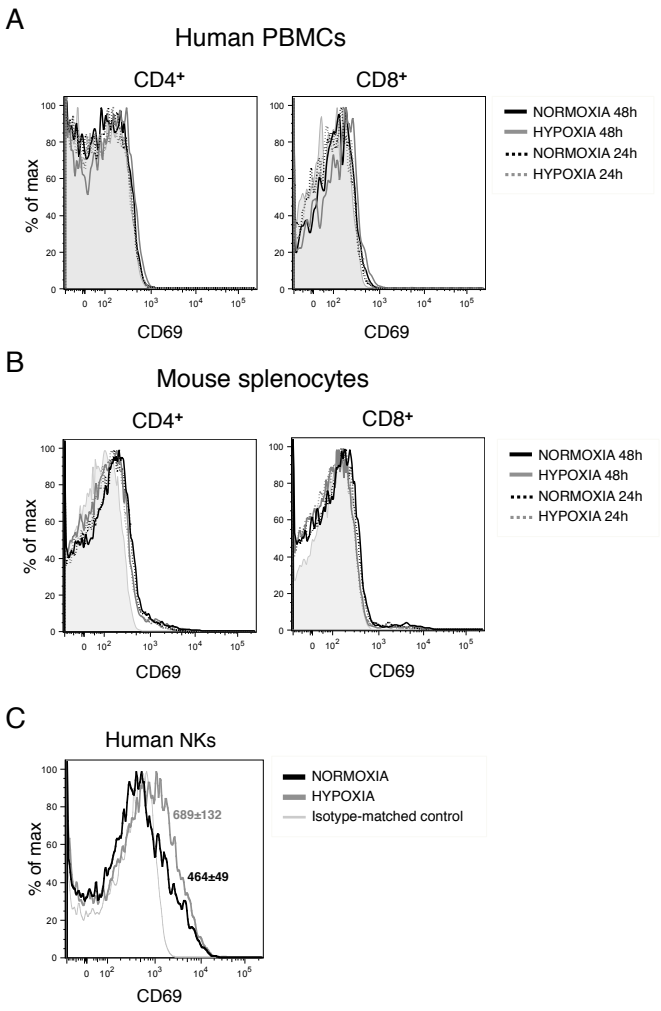
We have previously reported that because of hypoxia, CD137 is upregulated on T lymphocytes (44). The fact that CD137 and CD69 are frequently co-stained on tumor-infiltrating T lymphocytes (Supplementary Figure 1) prompted us to explore whether CD69 expression was also somehow connected to hypoxia.



SUPPLEMENTARY FIGURE 1. Double staining for surface CD69 and CD137 on gated CD4 and CD8 tumor-infiltrating T lymphocytes retrieved from MC38 bearing mice.

Hypoxia upregulates CD69 in human and mouse T lymphocytes

In order to explore if hypoxia regulates CD69 expression, human peripheral blood mononuclear cells (PBMCs) and mouse splenocytes were cultured for 24h and 48h under normoxia conditions and in a 1% O_2 atmosphere. No significant upregulation of surface CD69 was observed (Supplementary Figure 2), a finding similar to that we also reported for CD137 (44).



SUPPLEMENTARY FIGURE 2. Hypoxia without TCR-CD3 stimulation does not induce CD69 expression on T lymphocytes while it does so on human NKs. Human PBMCs (A) and mouse splenocytes (B) were cultured under normoxia or hypoxia (1% O_2) as in Figure 2 but without CD3 stimulation. Results represent CD69 surface staining in gated CD4 and CD8 T cells. (C) Surface expression on immunomagnetically sorted human $CD16^+CD56^+$ NK cells retrieved from peripheral blood of healthy donors. Histograms include the intensity for CD69 (Mean±SEM) in two analyzed individuals following a 48h culture under normoxia or 1% O_2 hypoxia.

Next, we assessed if hypoxia can cooperate with a CD3-driven T-cell activation to promote CD69 expression. As shown in Figure 2, CD69 was upregulated at the surface protein (Fig 2 A and B) and at the mRNA levels (Fig. 2 C and D) both in human T cells and mouse splenic T-cells when exposed to hypoxia during 48h. Likewise, activated human $CD4^+FOXP3^+$ also increased CD69 protein expression when PBMCs were cultured under hypoxic conditions (Supplementary Figure 3). Interestingly, hypoxia upregulated CD69 surface expression as a single stimulus in human purified NK cells (Supplementary Figure 2C).

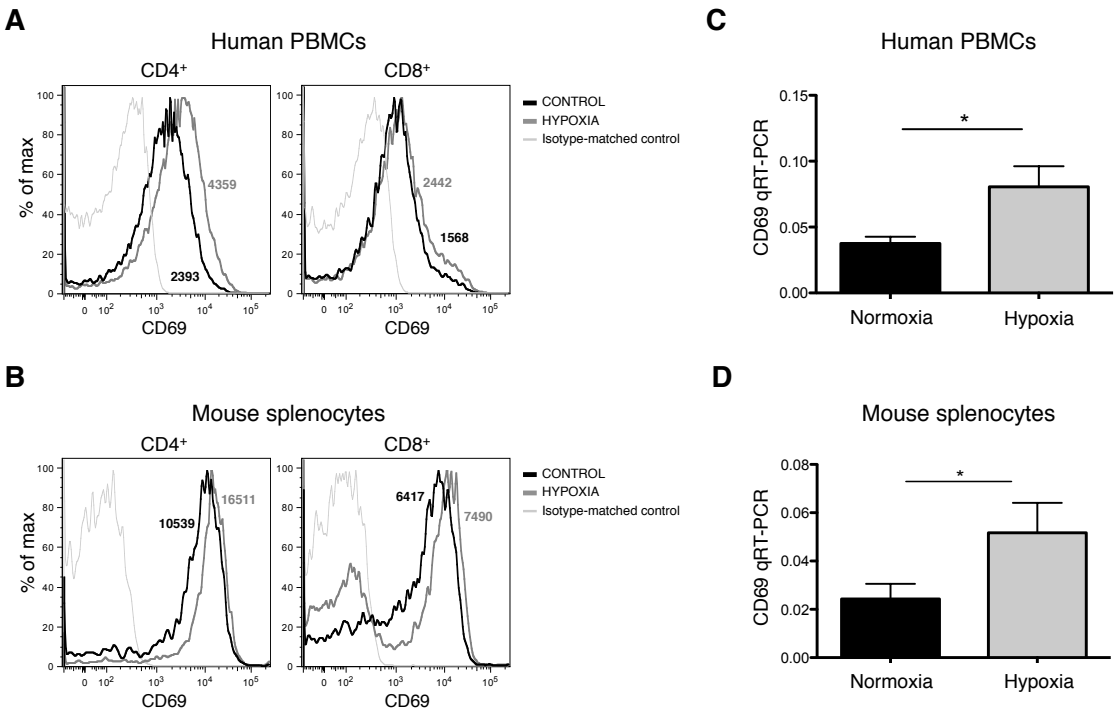
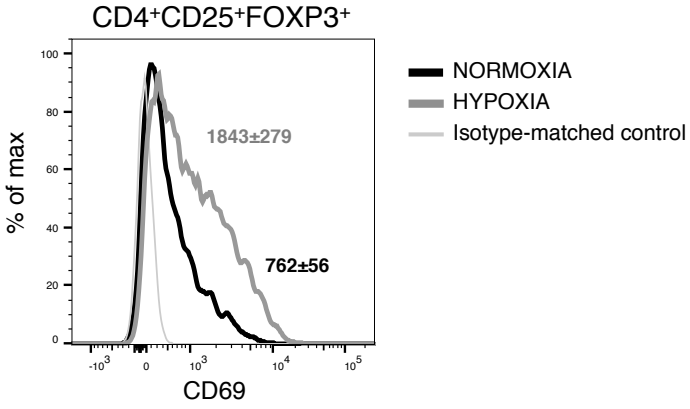


FIGURE 2. Hypoxia upregulates CD69 expression on T lymphocytes undergoing stimulation via TCR-CD3. Human PBMCs (A) or mouse splenocytes (B) were cultured for 48h under normoxia or hypoxia while being activated with a plate-bound anti-CD3ε mAb. Histograms show a representative experiment out of six performed indicating the mean of intensity of CD69 immunofluorescence on gated CD4 and CD8 T cells. C and D represent a quantitative RT-PCR analysis of CD69 mRNA expressed in the corresponding human and mouse samples. Bar diagrams represent six cases each (Mean±SEM). *P<0.05 (Mann Whitney test)



SUPPLEMENTARY FIGURE 3. Surface expression of CD69 on gated FOXP3$^+$CD25$^+$CD4$^+$ T lymphocytes from CD3-activated PBMCs cultured under normoxic or hypoxic conditions (n=3).

Taken together, these results highlighted a link between hypoxia and CD69 expression. Hypoxia can potentially exert such a function through a variety of

mechanisms. To gain further insight into the underlying mechanism, we substituted hypoxia by DMOG chemical inhibition of the prolyl hydroxylases (PHD 1-3) (26, 50), that are critical in the control of HIF stability and function (51), thereby mimicking the effect of hypoxia. We observed that DMOG treatment replicated the findings observed under low O_2 conditions in human and mouse $CD4^+$ and $CD8^+$ T lymphocytes (Figure 3) pointing to the involvement of the HIF transcriptional machinery in CD69 upregulation.

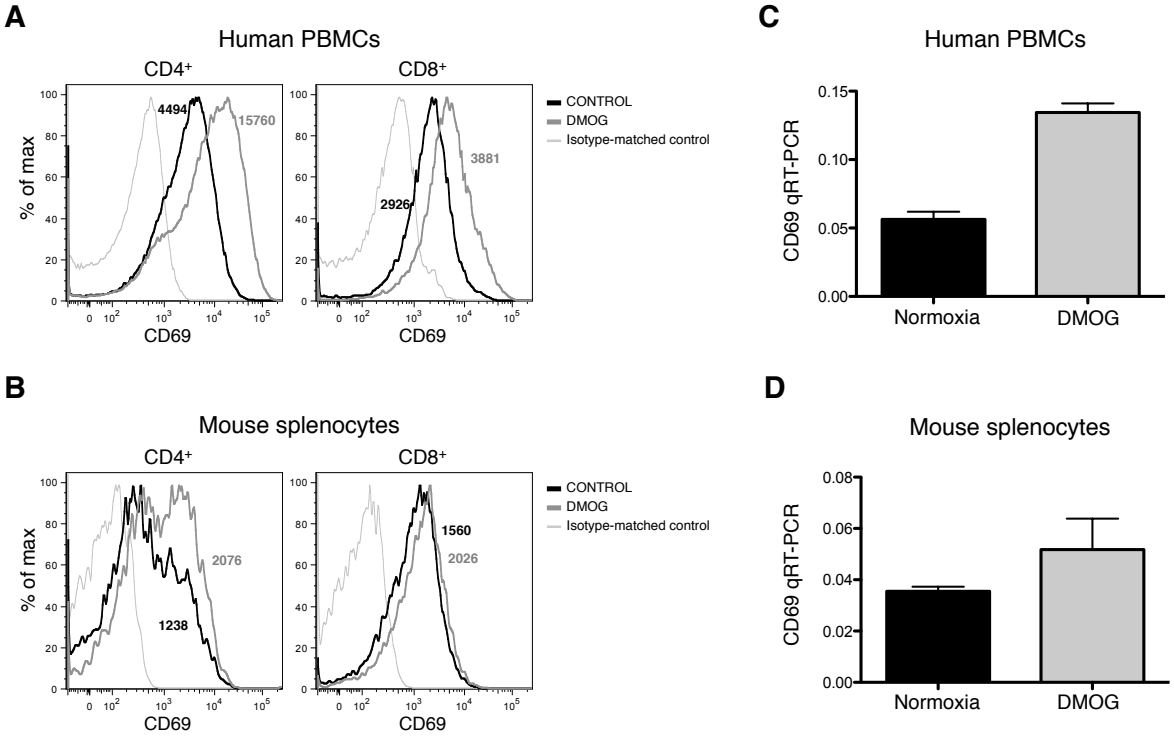


FIGURE 3. DMOG upregulates CD69 mimicking hypoxia by inhibition of prolyl hydroxylases. Experiments were carried out as in Figure 2 but culturing the indicated human PBMCs (A) and mouse splenocytes (B) in the presence of DMOG to inhibit PHDs 1-3. Histograms show an experiment representative of three performed. (C, D) Quantitative RT-PCR assessment of the CD69 mRNA levels in the three cases (Mean±SEM).

HIF-1α controls CD69 expression on T lymphocytes undergoing TCR-CD3 activation

To ascertain the involvement of HIF-1α in the regulation of CD69 expression, (43), we compared *HIF-1α* wild type versus *HIF-1α*-deleted splenocytes T-cells from HIF-1α inducible knock-out mice subjected to 1% O_2. *Hif1a* deletion prevented the surface upregulation of CD69 (Figure 4A) as well as CD69 mRNA levels (Figure 4B). The classical HIF-1α target gene *PGK1* also lost its induction under hypoxia as a control (Figure 4C).

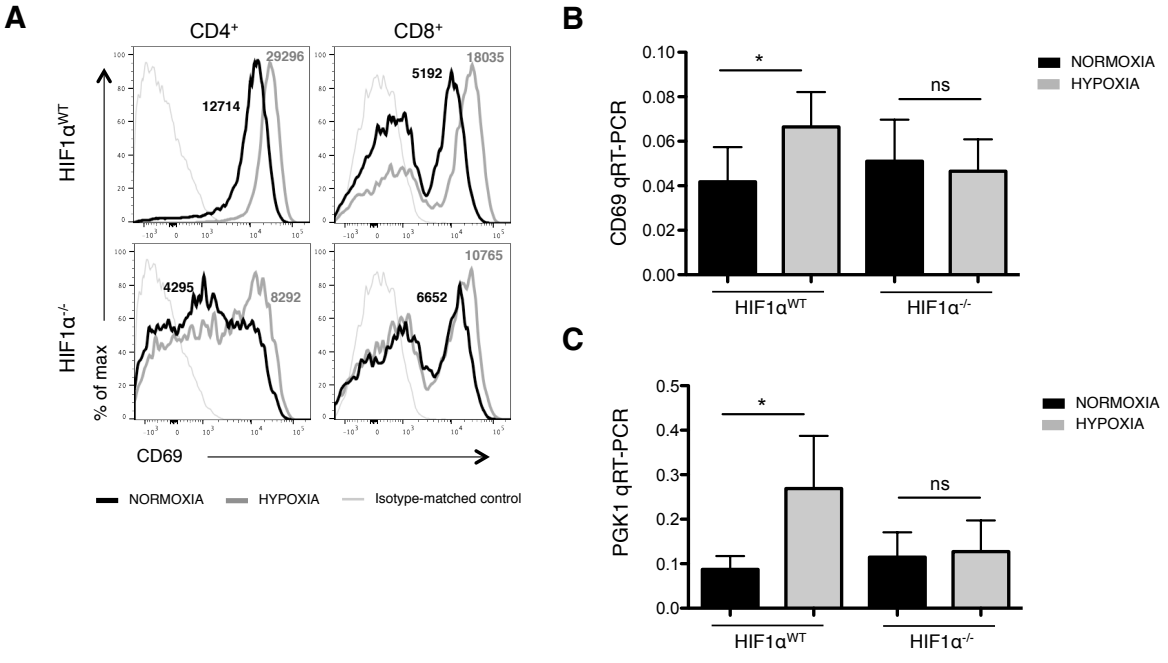


FIGURE 4. CD69 induction by hypoxia is curtailed in T lymphocytes from inducible HIF-1α$^{-/-}$ T cells. Splenocytes from HIF-1α^{floxed}-UBC-Cre-ER or HIF-1α^{WT}-UBC-Cre-ER mice were cultured in the presence of 4-hydrotamoxifen for 48h, subsequently washed and activated for 48h with plate-bound CD3 mAb and soluble CD28 mAb under normoxia or hypoxia as indicated. (A) CD69 staining on gated CD4 and CD8 T lymphocytes in the resulting cultures is shown as FACS histograms representative of seven independent experiments. Mean intensity of fluorescence is shown in each condition. (B) Analyses of CD69 mRNA expression by quantitative RT-PCR and (C) assessment of the classical HIF-1α target gene PGK1 in the same experiments as in B as a positive control. Mean±SEM *P<0.05 (n=7, Wilcoxon signed rank test)

One of the possible explanations of CD69 upregulation by HIF-1α would be direct binding of HIF-1α/HIF-1β to regulatory elements in the *CD69* locus. Sequence analysis revealed that the human *CD69* proximal promoter contains a potential HIF binding site at position -593 bp upstream of the point of transcription initiation (Figure 5A). ChIP assays were performed by quantitative PCR in sequences flanking the candidate HRE (hypoxia response element) in the nuclei of human T lymphocytes activated by anti-CD3ε mAb while cultured under normoxic or hypoxic conditions. Chip analyses to assess HIF-1α binding to the well-recognized HIF-1α responsive gene *PDK1* was used as a positive control (Figure 5B). Our results indicate that HIF-1α binds to the CD69 proximal promoter when T lymphocytes undergo hypoxia (Figure 5B).

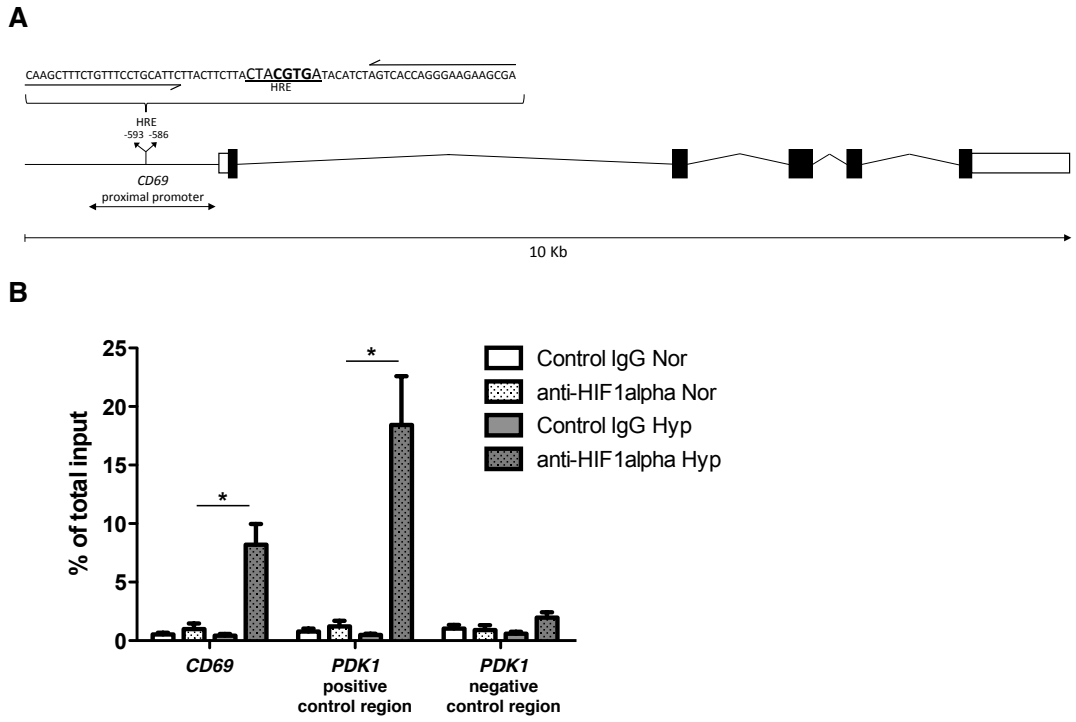


FIGURE 5. HIF-1α binding to the human *CD69* promoter is contingent on hypoxia. (A) Schematic representation of the human *CD69* locus with a newly identified HRE candidate indicating the primers used for DNA amplification in the ChIP assays, its sequence and position at the proximal promoter. Amplicon generated by PCR is shown and the arrows indicate where the primers are localized. (B) ChIP assays using anti-HIF-1α mAb on genomic DNA from PBMCs activated with

plate-bound CD3 for 12 hours under hypoxia or normoxia. Results represent percentage with respect to total unprecipitated DNA input using primers flanking an HRE in the *PDK1* promoter as a positive control and a region without HRE in the same gene as a negative control. The graph shows data from four independent experiments (Mean±SEM). *P<0.05 (Mann Whitney test)

Hypoxia correlates with CD69 expression in vivo

Tissues undergo hypoxia as a result of pathological and physiological conditions. We have previously shown by pimonidazole-based F-MISO positron emission tomography (PET) that isograft murine tumor models are hypoxic (44). In order to track hypoxic lymphocytes isolated from different tissue compartments in mice, we used pimonidazole staining followed by flow cytometry. B16-OVA melanoma-bearing mice were adoptively transferred with TCR-transgenic OT1 CD45.1$^+$ T cells that recognize OVA as a surrogate tumor antigen, and pimonidazole staining was used to detect lymphocytes with a recent history of hypoxia.

Figure 6A shows that transferred T lymphocytes that become CD69$^+$ *in vivo* when reaching either the bone marrow or tumor tissue were more intensely stained by pimonidazole. Endogenous CD69$^+$ CD8$^+$ T lymphocytes showed brighter pimonidazole signals in the bone marrow but not always in the tumor microenvironment.

To study the involvement of HIF-1α in the control of CD69 expression in the tumor microenvironment, we performed experiments using Lewis lung carcinoma-bearing mice whose loxP-flanked *Hif1a* locus is conditionally deleted by a cre recombinase expressed under the control of the distal lck promoter (*dLck-Cre)* thereby targeting all T-cell lineages. When retrieved from tumors, HIF-1α$^{-/-}$ T cells showed weaker and less frequent CD69 staining than WT counterparts (Figure 6B and 6C). These results show that T lymphocytes which have experienced and sensed hypoxia in their recent past upregulate CD69 *in vivo* in an HIF-1α-dependent fashion.

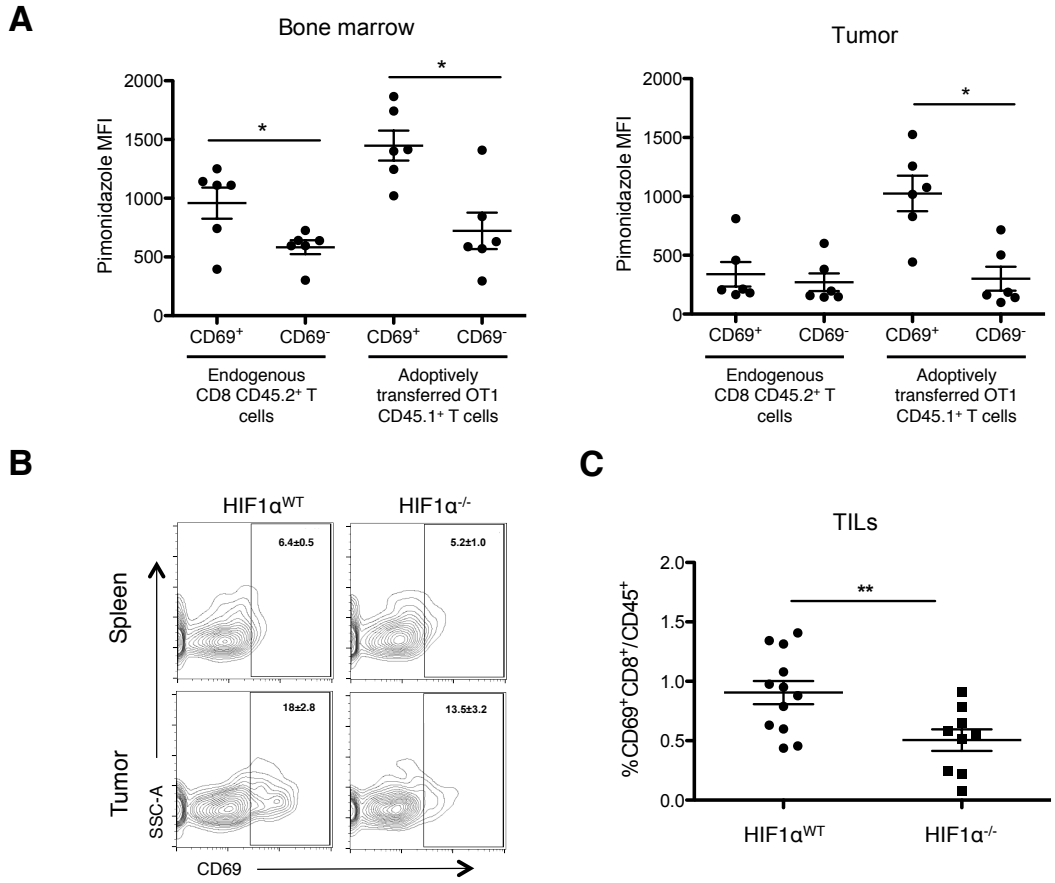


FIGURE 6. Tissular hypoxia determines CD69 expression *in vivo*. (A) Mice bearing B16-OVA subcutaneous tumors were adoptively transferred with total splenocytes from TCR-transgenic OT1 mice congenic for the CD45.1 allele. Forty-eight hours following adoptive transfer, mice were sacrificed and cell suspension from the tumor, and bone marrow were rapidly stained with anti-CD69 and pimonidazole. CD45.1$^+$ and CD45.2$^+$ CD8$^+$-gated T cells were analyzed for CD69 surface expression. Dots represent individual mice (Mean±SEM). *P<0.05 (mann Whitney test). (B) Five HIF-1α^{floxed}-*dLck-Cre* and five HIF-1α^{WT}-*dLck-Cre* mice were subcutaneously engrafted with Lewis lung carcinoma syngeneic tumors and sacrificed when these reached over 10 mm in diameter. Cell suspensions from the tumors were retrieved and CD8$^+$ T lymphocytes were assessed for CD69 specific immunofluorescence. B shows representative SSC/CD69 contour plots indicating percentage of positive CD69$^+$ events and mean±SEM of the five analyzed individual cases per condition. (C) CD69 assessment as in B representing CD69$^+$ CD8$^+$ T lymphocytes referred to total CD45$^+$ leukocytes infiltrating the tumor. Results come from two pooled experiments and are represented as mean±SEM, **P<0.01 (Unpaired test)

DISCUSSION

The functions of T lymphocytes are executed in tissue locations with variable and usually low availability of O_2 and the immune response is known to be strongly influenced by hypoxia, not only acting on T lymphocytes but also on myeloid leukocytes (52, 53) and B cells (54, 55). In this complex scenario, our study highlights that CD69, an early C-lectin receptor, considered a hallmark of T-cell activation upon antigen recognition, is co-regulated by HIF-1α sensing hypoxia as a direct transcriptional target.

Our observations in mouse and human T lymphocytes argue in favor of hypoxia-mediated upregulation of CD69 being a conserved function likely to have an important role in physiology and pathology. Indeed, tissues such as the bone marrow are hypoxic under physiological conditions (56, 57). In infectious diseases, autoimmunity and cancer the inflammatory infiltrate is very often under severe O_2 deprivation (58, 59). Our *in vivo* results clearly demonstrate that lymphocytes residing in hypoxic tissue or with a recent history of hypoxia, as denoted by pimonidazole staining, expressed higher molecular surface densities of CD69.

Our finding of CD69 upregulation by hypoxia was prompted by observations in tumor-infiltrating T lymphocytes which are very often $CD69^+$ and become hypoxic to some extent when entering into the hypoxic tumor microenvironment. However, hypoxia by itself does not upregulate CD69, since in our hands concomitant activation via CD3-TCR is absolutely required. By contrast, NK cells apparently upregulate surface CD69 to some extent under hypoxia without requiring further exogenous. The need for CD3-TCR stimulation probably reflects the interplay of the various

transcription factors jointly involved in the transcriptional regulation of the CD69 promoter (4-6).

The function/s of CD69 besides denoting recent T-cell activation is/are difficult to address. Reportedly, CD69 expression inhibits lymphocyte migration through a crosstalk with the $S1P_1$ receptor. According to this model, $CD69^+$ lymphocytes fail to chemotactically respond to sphingosine-1-phosphate (S1P) gradients and thereby do not depart from lymphoid or inflamed tissues (18, 60-62). These findings are consistent with the expression of CD69 on $CD103^+$ resident memory CD8 T cells that remain in tissue without recirculation via afferent lymphatic vessels or the blood stream (63). In our hands, recently activated T lymphocytes through CD3 stimulation are very poorly attracted by S1P in any circumstance, thus precluding comparative chemotaxis experiments of lymphocytes cultured under hypoxia or normoxia (data not shown).

Another limitation to understanding the function of CD69 is that ligands for CD69 have not been widely studied. Recent evidence suggests specific glycosylation-dependent binding of CD69 to galectin-1 (7) but the functional outcome of such interaction has only been established for Th17 cells. Previous evidence in CD69KO mice suggests an involvement of CD69 in the differentiation of Th17 cells (13) with implications in animal models of autoimmunity (12). Very recently, a role for CD69 in skin gamma/delta T cells and the pathogenesis of psoriasis in an IL-23-induced mouse model has been described (14). How hypoxia shapes these disease models will be an area to explore in the future. Interestingly, many tissues under self-inflicted damage by autoimmunity become hypoxic (64). In the context of cancer and autoimmunity the overall picture is that CD69 behaves as a checkpoint inhibitor that contributes to decreasing the intensity of local inflammation and tissue damage.

Hence our results suggest that tissue O_2 could play a role in such functions as a result of a mechanism of CD69 upregulation conserved between humans and rodents. This mechanism conceivably would mitigate tissue damage in areas of ischemia and hypoxia to favor T cell residence in stressed hypoxic tissues that are likely to be challenged by pathogens.

The control of T-cell gene expression (43) and functions (65, 66) by hypoxia is an entangled but very active area of research. It is becoming very clear that energy metabolism is a key feature regulated in T cells that dictates, among others, effector functions, apoptosis susceptibility and T-cell memory differentiation (65, 67). The hypoxic HIF-mediated regulation of CD69 brings another piece to the puzzle of the adaptive response of T lymphocytes to hypoxia as observed in tumor-infiltrating T lymphocytes. Implications and consequences of CD69 control by the HIF system are therefore potentially far-reaching for T-cell immunology and immunotherapy.

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GENERAL DISCUSSION

The influence of tissue hypoxia in cancer immunobiology and immunotherapy is a largely neglected field. This contrasts with the fact that malignant tumors are often very hypoxic beyond a few microns away from capillary vessels.

The interplay of immune cells in the tumor microenvironment is entangled and the response to hypoxia is functionally different in each cell subset. Hence, the overall balance of the effects of hypoxia is difficult to estimate, since some of the hypoxia driven mechanisms will impair tumor immunology, while others could be in favour of the immune response fighting cancer. The experimental models to explore these effects are artificial and mainly rely on tissue culture in hypoxia chambers, conditional knockout mice for proteins in the HIF response pathway and correlative science in human samples. HIF-independent effects are hard to approach and evidence in human subjects and samples, very difficult to attain.

The oxygen gas pressure experienced by cells in the organism is different from the one that is set in most of the *in vitro* cell culture systems. Not taking this into account is probably a frequent error in many research studies. The atmospheric oxygen pressure that we usually refer to as 'normoxia' corresponds to 21% oxygen. The physiological oxygen pressure in tissues, which frequently differs a lot from normoxia, is called 'physioxia'. In the body, this means that different organs are subjected to several oxygen concentrations, for instance, 0.5% in the epidermis, 5.6% O_2 in the lung or up to 9.5% O_2 in the kidney. Even in the same tissue, cells can experience different oxygen pressures depending on their localization. The term 'hypoxia' should be used to indicate that there is a lower oxygen pressure than in normal conditions or 'physioxia' (Carreau et al. 2011).

In our opinion, mimicking to the closest extent the oxygenation levels that are present in tissues, is relevant to correctly evaluate how cells function under normal and pathological circumstances.

The tumor microenvironment that includes malignant cells, stroma and immune infiltrates is known to be profoundly affected by different physic and chemical conditions such as pH, nutrient availability and oxygen supply. In this complex biological scenario, to understand the interplay between all these elements is crucial since they are likely to be underlying tumor immunity and tumor escape.

In the projects carried out during this thesis, we have tried to dissect how hypoxia influences the biology of immune cells and have focused on two important lymphocyte receptors.

The fact that tumor cells are able to survive in hostile environments is not a new finding. Their ability to adapt their metabolism in order to obtain the highest possible energy was described in 1931 by Dr. Otto Warburg (Otto 2016). But survival is not only a matter of energy; and among these features, malignant cells have developed a series of mechanisms to defend from the immune system (Hanahan and Weinberg 2011). Importantly, tumors may set in motion immune escape mechanisms when undergoing immune attack or hypoxic stress. For instance this is the case of adaptive acquisition of PD-L1 expression (Barsoum et al. 2014), IDO expression (Munn and Mellor 2007) and the secretion of soluble immunosuppressive molecules such as TGFβ (Facciabene et al. 2011) and Gal-1 (Croci et al. 2014).

Here, we have shown that hypoxia induces the expression of the soluble CD137 isoform in tumor cells. These surprising results follow our previously published work in T lymphocytes (Palazon et al. 2012) and endothelial cells (Palazon et al. 2011). The soluble nature of CD137 expressed by tumors was conducive to

interpret its role as a decoy receptor. Soluble CD137 expression in tumor cells is driven by HIF-1α, as experiments carried out with DMOG suggest (data not shown). How alternative splicing for the soluble form of CD137 is modulated by hypoxia needs to be defined at the molecular level.

There are two main implications for immunotherapy in our results:

1. That sCD137 produced by the tumor under hypoxia binds CD137L, an event which leads to blockade CD137L-mediated T cell co-stimulation. Moreover, it could prevent reverse signaling on APC since it requires sCD137 multimeric forms (Lippert et al. 2008), while tumor-derived sCD137 is probably monomeric. We must considerate that our *in vit*ro co-stimulation model is based on culturing activated CD8 T cells with a transfectant expressing the natural ligand for CD137. Hence, further studies are needed to elucidate the impact of the sCD137 produced by tumor cells on the outcome of interactions between $CD137^+$ T cells and $CD137L^+$ APCs.

2. The binding of sCD137 to anti-CD137 mAb successfully used against preclinical mouse models indicates that these mAb would also neutralize the sCD137 moieties which otherwise would obstruct CD137L-mediated co-stimulation. This neutralization produced by mAb could also be a mode of action of anti-CD137 mAbs in the clinic, considering that sCD137 has been detected in the sera of cancer patients (Dimberg et al. 2006). However, in these cases the cellular source of this soluble protein is difficult to be defined due to the fact that both tumor and immune cells are able to produce sCD137.

In our view, the experiments with CD137-silenced variants of CT26 demonstrate that this mechanism is involved in immunoescape at least in mouse models. This effect was observed to be critically dependent on CD8 T cells and on the immunocompetence of the animal. Further studies are needed to elucidate the relative

importance of this new mechanism, which joins to others that are upregulated when the tumor senses that it is under hypoxic stress.

However, in the tumor microenvironment, malignant cells are not the only population capable of adaptation to hypoxia. Hypoxic tumor infiltrating T-lymphocytes (TILs) have been reported to enhance their antitumoral effects by increasing the release of cytotoxic molecules and up-regulating costimulatory receptors (Doedens et al. 2013). In this regard, our results show that the expression of CD69, an early activation marker on T cells, is modulated by hypoxia as a result of being a direct transcriptional target of HIF-1α.

The first hint we observed was that human activated T cells undergoing hypoxia expressed more intensely surface CD69 than if they were cultured under normoxia. Likewise, the results obtained in mouse T lymphocytes indicate that hypoxia-mediated upregulation of CD69 is conserved interspecies and likely to have an important role both in physiology and pathology.

In physiological circumstances, T lymphocytes which circulate around the organism, are exposed to different 'physioxic' conditions that go from 0.5% O_2 present in the skin to 13% O_2 present in arterial blood (Carreau et al. 2011). Indeed, tissues such as the bone marrow have a low oxygen pressure being hypoxic (Nombela-Arrieta et al. 2013). In pathological conditions, such as infectious diseases, autoimmunity and cancer, the inflammatory infiltrate is very often under severe O_2 deprivation. Our *in vivo* experiments show that T lymphocytes which have experienced hypoxia (denoted by pimonidazole staining) either in bone marrow or tumor, express also higher levels of CD69 on their plasma membrane.

However, in T lymphocytes CD69 is not up-regulated under hypoxia in the absence of stimulation via CD3-TCR in a detectable fashion. This fact suggests that

HIF-1α would need the previous action of other transcription factors acting on the CD69 promoter to enhance its expression. Another evidence that supports this hypothesis is that the highest CD69 expression differences between hypoxic and normoxic cultures occur at 48 hours and even at later timepoints. Actually, we (data not shown) and others have observed no differences in its expression during the first 12 or 24 hours following stimulation (Xu et al. 2016). In our view, prolonged expression of CD69 due to hypoxia must have a role in T-cell biology to be discovered as we make progress on CD69 functions.

Upon its induction on the plasma membrane CD69 interacts with $S1P_1$ resulting in the internalization of this chemotactic receptor, thereby preventing its response to sphingosine-1-phosphate (S1P) gradients (Shiow et al. 2006). This mechanism curtails migration and ensures that lymphocytes stay in the secondary lymphoid tissue or inflamed organs. It is noteworthy that both tissues are known to be under low oxygen pressure. Unfortunately, we were not able to demonstrate that the increase of CD69 expression promoted by hypoxia inhibits the migration towards S1P. Our failure is probably due to the fact that activated lymphocytes are very poorly attracted by S1P *in vitro* (data not shown).

CD69 is known to regulate the permanence/egress not only in the lymph node but also in peripheral tissues (Lamana et al. 2011; Mackay et al. 2015). Therefore, it is tempting to speculate that CD69 augmented transcription is behind establishing tissue resident T cell memory. Indeed, CD69 has been also reported as a chief residency marker. It is permanently expressed on $CD103^+$ resident memory CD8 T cells that remain in tissue without recirculation via afferent lymphatic vessels or the blood stream (Fan and Rudensky 2016). Interestingly, the niches where these cells are located have usually very low oxygen levels such as epidermis, bone marrow, thymus

or gut (Hale et al. 2002; Carreau et al. 2011). The generation of this T cell subset commonly occurs under inflammatory conditions where oxygen concentration can be even lower. They have also been described in some tumors (Djenidi et al. 2015). Our results suggest that the hypoxic environment should have a role in augmenting and maintaining CD69 expression on these memory subsets.

Other functions have been described for CD69. Under inflammatory conditions or autoimmunity CD69 has been reported to have inhibitory properties that contributes to decreasing the intensity of local inflammation and tissue damage (Gonzalez-Amaro et al. 2013). Such effects are dependent on the role of CD69 in the regulation of Th17 differentiation. Further research is needed to evaluate the contribution of hypoxia to these functions performed by CD69.

To sum up, the results obtained in these two projects contribute to the current knowledge regarding the hypoxia-mediated regulation of the interplay between tumor and immune cells. Future and ongoing research will integrate the many effects of hypoxia on tumor immunology and immunotherapy. This thesis has added two new pieces to an increasingly complicated puzzle only amenable to systems biology tools.

CONCLUSIONS

1. CD137$^{-/-}$ mice engrafted with the CT26 colon carcinoma cell line showed a retarded tumor growth compared to that in their wild type counterparts.

2. Transplantable mouse and human tumor cell lines express CD137 under hypoxia.

3. Hypoxia induces a soluble CD137 spliced form in tumor cells that is detectable in the supernatant of tumor cell cultures and in the sera of tumor-bearing mice.

4. The soluble CD137 protein produced by tumor cells binds to the natural ligand of CD137 (CD137L) and blocks its co-stimulatory function on lymphocytes.

5. CD137 silencing in hypoxic CT26 colon carcinoma tumor cells renders them more immunogenic.

6. Tumor-infiltrating T lymphocytes express the surface activation marker CD69.

7. Hypoxia upregulates CD69 in human and mouse T lymphocytes undergoing TCR-CD3 activation.

8. The hypoxia-inducible factor HIF-1α partially controls CD69 expression on T lymphocytes undergoing TCR-CD3 activation.

9. The binding of HIF-1α to a newly described hypoxia response element (HRE) in the human CD69 promoter is contingent on hypoxia.

10. The degree of hypoxia, measured by pimonidazole staining *in vivo*, correlates with CD69 expression on T lymphocytes in the tumor and bone marrow microenvironments.

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APPENDIX