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IMPROVING NEUROBLASTOMA THERAPY BY TARGETING INTRA-TUMORAL HYPOXIA AND IMMUNE CHECKPOINT AXIS PD-1/PD-L1

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I hereby confirm that the PhD thesis entitled “Improving neuroblastoma therapy by targeting intra-tumoral hypoxia and immune checkpoint axis PD-1/ PD-L1” has been written independently and without any other sources than cited. All necessary ethical approvals have been obtained in accordance with the EU and National laws (on the use of clinical samples and on the Care and Use of laboratory animals, where applicable).

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Dedications

To my father, Claude Sauvage.

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LIST OF ABBREVIATIONS

5-hmC:	5-hydroxy-methylcytosine
α-SMA:	α-smooth muscle actin
Acetyl-CoA:	acetyl coenzyme A
ADCC:	antibody-dependent cellular cytotoxicity
AIFs:	Arterial Input functions
APCs:	antigen-presenting cells
APM:	Antigen Processing Machinery
ARG1:	Arginase I
ARNT:	aryl hydrocarbon receptor nuclear translocator
ASCT:	autologous stem cell transplant
AVG:	Number of Averages
BET:	bromodomain and extraterminal domain
BETi:	BET protein inhibitors
bFGF:	basic fibroblast growth factor
bHLH-PAS:	basic-helix-loop-helix-PER-ARNT-SIM homology
BM:	basement membrane
CA:	carbonic anhydrase
CAR(s):	chimeric antigen receptor(s)
CCL:	C-C motif chemokine ligand
CCR7:	CC-chemokine receptor 7
CD40L:	CD40 ligand
CDCC:	complement-dependent cellular cytotoxicity
CDCP:	complement-dependent cellular phagocytosis
cDCs:	conventional DCs
cDNA:	complementary DNA
CDP:	common dendritic cell progenitor
CLIP:	class II invariant chain peptide
CLP:	common lymphoïd precursors
CMC:	complement-mediated cytotoxicity
CMP:	common myeloïd precursors
CNS:	central nervous system
CSF1:	colony stimulating factor 1
CSF1R:	colony stimulating factor 1 receptor
CT:	Computer Tomography
CTA:	Contrast Agent
CTL(s):	cytotoxic T lymphocyte(s)
CTLA-4:	cytotoxic T lymphocyte-associated antigen 4
CXCCL:	C-X-C motif-chemokine ligand
CXCL:	C-X-C motif ligand
DAF:	decay-accelerating factor
DAMPs:	damage-associated molecular patterns
DCE-MRI:	Dynamic Contrast-Enhanced Magnetic Resonance Imaging
DCs:	dendritic cells
DLI:	donor lymphocyte infusion
DMSO:	Dimethyl Sulfoxyde
DNAM-1:	DNAX accessory molecule (also called CD226)
ECs:	endothelial cells
EES:	extravascular extracellular space
EMT:	epithelial-to-mesenchymal transition
ER:	endoplasmic reticulum
ET:	Echo Train
FA:	Flip Angle
FDCs:	follicular dendritic cells
FDG:	fluorodeoxyglucose
FGF:	Fibroblast growth factor

Fig:	figure
FLASH:	Fast Low Angle Shot
Flt3L:	FMS-related tyrosine kinase 3 ligand
FSE:	Fast Spin Echo
fTE:	First Echo Time
gal-1:	galectin-1
Gd:	gadolinium
GEMMs:	genetically engineered mouse models
GM-CSF:	Granulocyte Macrophage Colony Stimulating Factor
GWAS:	Genome Wide Association Study
HIFs:	hypoxia-inducible factors
HLA:	human leucocyte antigen
HMGB1:	high-mobility group box 1
HR:	high-risk
HRE:	hypoxia responsive element
HSC:	hematopoietic stem cell
HSCT:	hematopoïetic stem cell transplantation
ICAM-1:	intracellular adhesion molecule-1
ICI:	immune checkpoints inhibitors
IDO:	indoleamine-pyrrole 2,3 dioxygenase
IFN(s):	interferon(s)
IFP:	interstitial fluid pressure
Ig G:	immunoglobulin G
IGF-1:	insulin growth factor-1
IL(s):	interleukin(s)
iNOS:	inducible nitric oxide synthase
IP:	intraperitoneally
ITAMs:	immunoreceptor tyrosine-based activation motifs
ITIMs:	immunoreceptor tyrosine-based inhibitory motifs
iTregs	induced Tregs
JmJ:	jumonji domain
KIR(s):	Killer-cell immunoglobulin-like receptor(s)
LDH:	Lactate Deshydrogenase
LFA-1:	leukocyte function-associated antigen-1
Mab:	monoclonal antibody
Mabs:	monoclonal antibodies
MAC:	Membrane Attack Complex
MAPK:	mitogen-activated protein kinase
MBL:	mannose-binding lectin
MDSCs:	myeloid-derived suppressor cells
MHC:	Major Histocompatibility Complex
MIBG:	metaiodobenzylguanidine
MICA/B:	MHC class I chain-related protein A/B
MIF:	migration inhibitory factor
miRNAs:	microRNAs
MLL5:	mixed lineage leukemia 5
MMP(s):	matrix metalloproteinase(s)
moDCs:	monocyte-derived DCs
MRI:	Magnetic Resonance Imaging
NB:	neuroblastoma
NCA:	numerical chromosomal aberrations
NCCs:	neural crest cells
NCRs:	natural cytotoxicity receptors
NE:	Number of Experiments
NFA:	Number of Flip Angles
NK:	natural killer
NKG2D:	natural-killer group 2, member D
NO:	nitric oxyde

NOE:	Number Of Echoes
Nrp-1:	Neuropilin-1
NSE:	Neuron Specific Enolase
ODD:	oxygen-dependent degradation domains
OMS:	opsomyoclonus syndrome
PAMPs:	pathogen-associated molecular patterns
PCR:	Polymerase Chain Reaction
PD-1:	Programmed cell Death protein 1
PD-L1:	Programmed death-ligand 1
PD-L2:	Programmed death-ligand 2
PD(O)Xs:	Patient-derived (orthotopic) xenografts
pDCs:	plasmatoïd DCs
PDGF(s):	Platelet-derived Growth Factor(s)
PDH:	pyruvate dehydrogenase
PDK1:	Pyruvate dehydrogenase kinase 1
PET:	Positron Emission Tomography
PGE2:	prostaglandin E2
PHD:	proline hydroxylases
PI3K:	phosphoinositide 3-kinase
PTLD:	post-transplant lymphoproliferative disease
pVHL:	von Hippel-Lindau protein
PVR:	polio virus receptor (also called CD155)
RA:	retinoic acid
RG:	Respiratory Gating
ROS:	reactive oxygen species
RT-qPCR:	Quantitative reverse transcription Polymerase Chain Reaction
s:	soluble
SC:	subcutaneous
SCA:	segmental chromosomal aberrations
scFv:	single-chain fragment variable
SD:	Scan Duration
SDF-1α:	stromal-derived factor 1α
Sema3A:	Semaphorin3A
SG:	sympathetic ganglia
sGD2:	soluble form of the oncoantigen GD2
sHLA-G:	soluble form of HLA-G
Sl:	Number of Slices
SR:	Spatial Resolution
T1w:	T1 weighted
T2w:	T2 weighted
TA:	tumor antigens
TAD:	transactivation domains
TAMs:	tumor-associated macrophages
TAP:	transporter associated with antigen processing
TCA:	tricarboxylic acid cycle
Tconv:	CD4+ conventional T lymphocytes
TCR:	T cell receptor
TDMVs:	tumor derived microvesicles
TE:	Echo Time
TGF-β:	transforming growth factor-β
Th:	T helper
TH:	tyrosine hydroxylase
TIGIT:	T cell immunoreceptor with Ig and ITIM domains
TILs:	tumor-infiltrating lymphocytes
TME:	tumor microenvironment
TNBC:	triple-negative breast cancer
TNF:	tumor necrosis factor
TR:	Repetition Time

TRAIL:	TNF-related apoptosis inducing ligand
Treg(s):	T regulatory lymphocyte(s)
TRes:	Time Resolution
TSP-1:	thrombospondin-1
ULBPs:	UL16-binding proteins
VEGF:	vascular endothelial growth factor
VEGFR:	vascular endothelial growth factor receptor
XCL1:	X-C Motif Chemokine Ligand 1

SUMMARY

Neuroblastoma (NB) is a heterogeneous pediatric cancer mostly affecting (very) young children and having still a tremendous prognosis in the stage 4 metastatic form [1, 2]. The most important progress in NB therapy has been achieved with the introduction of anti-GD2 monoclonal antibody (Mab) immunotherapy, yet the prognosis of high-risk (HR) forms remains very bad (survival rates inferior to 50% at 5 years) [3, 4]. However, the benefit achieved with anti-GD2 Mab highlights the importance of the immune system in NB.

On the other side, similarly to other solid tumors, hypoxia negatively impacts NB outcome [5]. Hypoxia is linked to the immunosuppressive tumor microenvironment (TME) and contributes to many of the immune escape mechanisms developed by cancer cells [6-8]. One of these involves the expression of Programmed death-ligand 1 (PD-L1) by cells present in the TME and the resulting binding to Programmed cell Death protein 1 (PD-1) on T cells leading to dampened T cell responses [9, 10].

Immune checkpoints inhibitors (ICI) have been developed and have substantially improved adult cancer therapy, but monotherapy in pediatric settings have been disappointing [11].

Based on these observations and on pre-clinical studies having shown a benefit of combinatorial therapies including ICI, we decided to investigate the effectiveness of a combinatorial therapy associating ICI (anti-PD-1 Mab) to JQ1 (an epigenetic drug able to impair hypoxic responses) in a HR NB mouse model, the TH-*MYCN* model.

We showed that JQ1 prolongs mice survival and induces a drastic tumor volume decrease even in well-developed tumors. It also contributes to vessels normalization and decreases tumor hypoxia. Moreover, it modulates tumor immune landscape, decreasing PD-L1 expression on dendritic cells (DCs) and activating T cells. Combination with anti-PD-1 blocking led to a synergistic effect and prolonged mice survival compared to JQ1 monotherapy.

INTRODUCTION

1. Neuroblastoma: a pediatric heterogeneous tumor

Pediatric malignancies epidemiology differs from adult cancers, with the most frequent neoplasia encountered in children being leukemia (and mostly acute lymphoblastic leukemia), central nervous system tumors and lymphomas [12]. But under the age of one, NB is the most frequent cancer [13].

NB affects mostly (and almost exclusively) young children as 90% of cases are diagnosed before 5 years of age and 98% of cases before the age of 10 [3, 12]. The incidence is about 1 case for 100 000 children in France (corresponding to 120-150 cases per year in France) [3]. It represents about 8% of all pediatric cancers [12]. Despite this, NB is responsible for about 15% of deaths due to cancer in this part of the population [14]. The age at diagnosis, the stage of the disease and the biologic characteristics of the tumor account for the prognosis which can be very good for some spontaneously regressing tumors or disastrous in the case of metastatic *MYCN*-amplified disease [3]. Progress has been made in the treatment notably with the introduction of immunotherapy but new therapeutics, less toxic, are urgently needed for the most aggressive forms of NB [3].

1.1 The etiology and origin of neuroblastoma

NB etiology is still the subject of intensive research. Indeed, based on epidemiological studies, no environmental factors have been formally incriminated as responsible of the development of the disease to date [13, 15] but an association between environmental exposures and NB development has been suggested by some studies, such as drug or hair dyes use during pregnancy [13].

NB appears to be a developmental disease, with a multitude of genetic and epigenetic factors adding to environmental exposures leading to the disease emergence [15].

The cellular origin of this embryonal tumor is also still source of debate. Indeed, NB arises from neural crest cells precursors that will form the sympathetic nervous system, but the precise cell source and oncogenic events that lead to this malignant disease are still being investigated and partially identified. Based on the normal neural crest development and evolution, deep understanding of this phenomenon will probably lead to new therapeutics and even could lead to prevention in some cases [14, 16, 17].

1.2 Normal development of the sympathetic nervous system

Human prenatal development involves three important structures: endoderm, mesoderm and ectoderm [18]. Pluripotent cells from these tissues will progressively proliferate, migrate and differentiate into all tissues necessary for a normal life; this phenomenon is tightly controlled by different precise gene expression patterns.

During the embryonic development, the ectoderm will give rise to the neurectoderm and the process of neurulation will start leading to the formation of the nervous system [14]. During this phenomenon, the neural plate forms the neural tube whereas the cells doing the junction between the extremities of the neural plate and the epidermidis give rise to the neural crest. Neural crest is a transient structure in vertebrates that gives rise to sympathetic and parasympathetic neurons, adrenal medullary cells, enteric neuronal system, sensory neurons, glia, melanocytes and facial cartilage [14, 17, 19]. NB is thought to originate from neural crest cells (NCCs) as it often arises from adrenal medulla or in the paravertebral sympathetic ganglia [17]. Different oncogenic events interfering at different times during the normal course of NCCs migration and differentiation account for disease development [19]. The origin of NB is summarized in Fig.1.

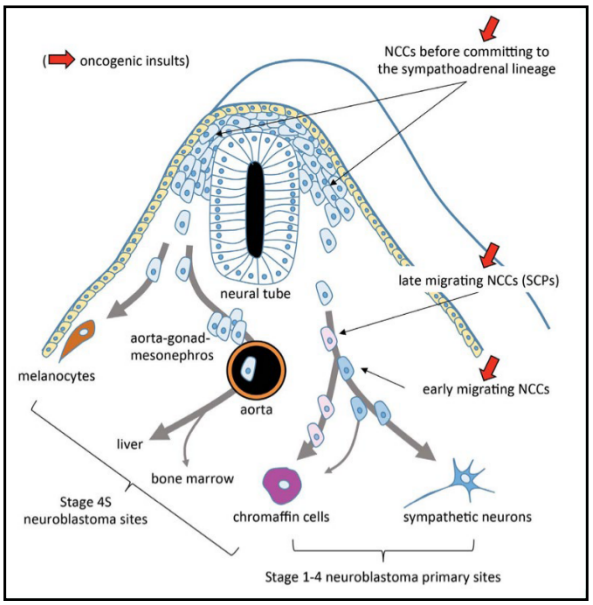

Fig. 1: Origin of NB. From [19].

1.3 Genetic and epigenetic initial events leading to NB induction and progression

Intensive research is still ongoing to uncover the initiation mechanisms leading to the disease [19]. Few recurrent genetic abnormalities have been evidenced in NB, underlying a difference with adult malignancies, in which such mutations are regularly

found (for example, BRCA1 mutations in breast cancer or KRAS mutations in colon cancer) [15]. However, different studies have revealed that somatic mutations in NB occur in *ALK*, *PTPN11*, *MYCN*, *ATRX* and *NRAS* genes [15, 20]. Much more frequently, numerical chromosomal aberrations (NCA) corresponding to ploidy abnormalities and segmental chromosomal aberrations (SCA) including genes amplifications, deletions or gains are observed and correlate with prognosis [15]. The paucity in mutations observed in NB suggests a preponderant role of abnormal gene sets expression and disturbed epigenetic regulation during development [12, 19-22]. Understanding the fundamental events responsible for NB emergence will certainly conduct to more targeted therapies with less toxicities [14]. Genetic changes and different expression patterns account for the differences observed in the disease's evolution, which could be very good with spontaneous regression or maturation in ganglioneuroma, or disastrous with progression to incurable disease [12]. In rare situations, NB can be bilateral, affecting both adrenal glands, and suggesting in this case a familial predisposition [23]. Indeed, germline mutations in *PHOX2B* or in *ALK*, have been identified in these rare situations, and probably contribute to tumorigenesis [13, 23]. Moreover, some single nucleotide polymorphisms (SNPs) have also been shown to contribute to the development of NB [23].

1.3.1 MYCN: a main oncogenic driver in NB

MYCN gene is localized at 2p24.3 [3], and encodes for the MYCN protein which is a trascription factor implicated notably in cellular proliferation. It belongs to the family of *MYC* oncogenes, together with *c-MYC* and *MYCL* [24, 25]. Among these oncogenes, *c-MYC* has been intensively studied at the functional level and is implicated in a variety of blood and solid cancers [25]. On the other side, overexpression of *MYCN* mostly occur in neural-derived solid cancers, such as NB, but also in non-neural tumors (for example, hematological malignancies) [25]. *MYCN* amplification is often present in HR form of the disease, and clearly correlates with poor prognosis [12]. *MYCN* C-terminal domain interacts with the Max protein, leading to the active form of the complex responsible for the interaction with specific DNA sites and the transcriptional activity of *MYCN* [25, 26]. *MYCN* has an important physiologic role during embryonal development, and is normally expressed in neuronal precursors where it contributes to cellular proliferation [26]. In order to control its activity, the expression of *MYCN* is tightly regulated at the transcription level, at the mRNA level but also post- translationally at the protein level [26]. Epigenetic mechanisms play an important role in this regulation [24].

1.3.2 ALK

ALK encodes for a receptor tyrosine kinase present at the cell surface and having a

role in the developing nervous system [12]. This gene is frequently involved in NB, with about 10% of sporadic cases presenting an *ALK* mutation or amplification [15]. *ALK* can be affected in different ways, including translocations impacting the locus of *ALK*, mutations leading to *ALK* constitutive activation (F1174L and F1245V are somatic mutations whereas R1275Q mutation can be somatic but is also found in the familial forms of NB), *ALK* amplification or copy number gain of *ALK* without any mutation or overexpression without any amplification/copy number gain [26].

1.3.3 Other rare mutations

Genes implicated in chromatin remodeling can be mutated in NB: we can cite for example, *ATRX* gene mutation leading to loss-of-function (in older patients), *ARID1A*, *ARID1B*, or rearrangements in the promoter of *TERT* [12, 13, 26].

1.3.4 Segmental chromosomal aberrations (SCA)

In addition to *MYCN* amplification, other frequent SCA are found in poor prognosis NB tumors. Indeed, 17q gain is frequently observed in HR tumors; PPM1D (a p53 induced phosphatase that negatively regulate p53), survivin and some other genes could have a role as they are located in this region [15]. 11q LOH and 1p loss also correlate with poor outcome; these regions contain tumor suppressor genes which could play a role in tumorigenesis [15].

1.3.5 MicroRNAs (miRNAs) and NB

miRNAs are small non-coding RNAs that act post-transcriptionally to regulate gene expression [26]. They bind to complementary sequences on target mRNAs preventing their translation in proteins or leading to their degradation [26]. miRNAs deregulation is a frequent phenomenon in cancer and is present also in NB [26]. Different miRNAs families have been extensively studied in NB, but this is beyond the scope of this study.

1.3.6 Familial predisposition to NB

ALK and *PHOX2B* germline mutations have been well described in familial cases of NB [23]. More recently, *KIF1Bβ* germline mutations have also been identified in some families of NB patients [27]. Many syndromes are also implicated in the risk of developing cancers, including NB; we can cite for example Li-Fraumeni syndrome, Noonan syndrome and congenital central hypoventilation syndrome [26, 27]. Recently, thanks to the Genome Wide Association Study (GWAS), polymorphisms in some genes have been identified with a potential contributing role to disease initiation, but also to

tumor progression in NB sporadic cases [13]. We can cite for example polymorphisms in *LIN28B*, *LMO1* and *BARD1* [13, 21-23].

1.4 Clinical presentation of the disease

Symptoms are correlated to the site of origin, the loco-regional extension of the disease and the presence or absence of metastasis [3, 12]. Most of children affected by NB presented with an abdominal mass arising from adrenal medulla (in 65% of cases) or from a sympathetic ganglia (SG) [1, 3]. In a small part of cases, tumors from SG can also be found in the chest region, or in the neck or pelvic region [1, 3]. In rare situations, NB can be bilateral, affecting both adrenal glands, and suggesting in this case a familial predisposition as mentioned above [1, 3].

Sometimes NB can be discovered by chance during a routine medical exam [3, 12]. Abdominal masses will cause abdominal discomfort or pain and distension and can also induce arterial hypertension [3, 12]. In SG from neck or thoracic region, the child can present a Claude-Bernard- Horner syndrome or some respiratory distress resulting from the compression by the mass [3]. Tumors originating from the paraspinal ganglia can also invade the medullary canal leading to medullar compression syndrome which constitutes an emergency [3, 12].

When NB is diagnosed, children are often already in a metastatic state [3]. Metastatic sites include the liver, the bone marrow, the bones, the skin, the central nervous system, the lungs and the testis [3, 12, 23]. The metastatic disease can cause alteration of the general state with fever and painful lesions, but also bleeding, pallor or infection resulting from bone marrow replacement [3, 12, 13]. One classic but rare sign of metastatic NB is the raccoon eyes syndrome: periorbital bone metastases cause bruising around the eyes, and could be associated with proptosis [3, 12]. In the 4S NB, a particular metastatic form of the disease affecting very young children and having a good prognosis, presentation can include massive hepatomegaly leading to respiratory distress [12].

NB can also manifest with paraneoplasic syndrome. Opso-myoclonus-ataxia syndrome results from auto-immune damage induced by anti-GD2 auto-antibodies, leading to cerebellar ataxia, myoclonus and rapid multidirectional conjugate eyes movements [3, 12, 13]. Although the overall prognosis is good, children often have sequelae in terms of psycho- motor and language delay and behavioral trouble [12]. This particular kind of NB responds to immunosuppressive therapy [12].

Mature tumors such as ganglioneuroblastoma and ganglioneuroma can also lead to symptoms of severe water diarrhea due to Vasoactive Intestinal Peptide secretion; in this case, surgical resection of the tumor leads to cure [3, 12, 13].

1.5 NB diagnosis

NB is suspected based on the symptoms and clinical signs presented by the child. Complementary exams notably include urinary catecholamines excretion measurement, blood Neuron Specific Enolase (NSE) and Lactate Deshydrogenase (LDH) dosage [3], and radiological examination including Magnetic Resonance Imaging (MRI) and/or scan of the primary site [3].

Metastatic state evaluation includes bone marrow involvement assessment (bilateral bone marrow aspirations and biopsies), and ^{123}I- metaiodobenzylguanidine (MIBG)-scintigraphy; if this last one results negative, a Positron Emission Tomography (PET)-Computer Tomography (CT) with ^{18}F-fluorodeoxyglucose (FDG) will be performed [3, 12]. ^{99}mTc- diphosphonate scintigraphy bone scan can help to identify bone metastasis [12].

Diagnosis confirmation results from histological analysis of a tumor sample: this sample is preferentially taken in the primary tumor, but if not accessible, other tissue sample can contribute to the establishment of the diagnosis [12]. However, sufficient material is needed as immunohistological analysis must be done, but also genomic profile of the tumor and *MYCN* status assessment [12]. In case of bone marrow involvement, bone marrow aspirates and biopsies could be a valuable option to confirm the diagnosis [12].

Tumors range from mature and differentiated state, represented by ganglioneuroma and ganglioneuroblastoma to undifferentiated and immature lesions (NB) [3]. The presence and abundance of schwanian stromal cells also contribute to tumor histopathological classification [12].

1.6 Risk assessment and group classification

Histopathological classification and tumor genomic profile contribute to the stratification of the disease [3]. The main prognosis factors include age at diagnosis, genetic and genomic factors and the presence or absence of metastasis [13].

The age at onset of the disease correlates with prognosis and very young children (under 18 months) often have a better outcome [3]. A particular form of NB, called 4S, arises in children under the age of one year and includes a primary tumor with metastases involving skin, liver or bone marrow (with less that 10% involvement); these tumors can spontaneously regress [28]. In adolescents and adults, the disease is very rare but despite the fact that the form is more indolent, the prognosis is very bad and the disease is often fatal [3, 12].

Cytogenetic abnormalities impact also the prognosis: NCA correlate with good prognosis, with an overall survival at 5 years and an event free survival superior to 90% [3]. On the other side, SCA are associated with poor prognosis, with a survival of about

50% at 5 years [3]. HR tumors harbor *MYCN* amplification in about 50% of cases, and despite the progress done in treatments, overall survival remain less than 50% at 5 years [3]. HR patients die from disease progression or relapse and suffer of long-term sequelae potentially leading to death [26].

1.7 Principles of NB therapy

Depending on the patient's classification in the different risk group, the therapeutic options range from patient's monitoring to very heavy treatment, as summarized in the Fig. 2 [3].

Low risk NB	Observation, surgery or chemotherapy
Intermediate risk NB	Chemotherapy, surgery, radiotherapy, +/- maintenance treatment
HR NB	Induction chemotherapy, high dose chemotherapy followed by autologous stem cell transplant (ASCT), surgery, radiotherapy, maintenance treatment including immunotherapy

Fig. 2: NB treatment according to risk group classification. Translated and adapted from [3].

The standard of care of HR children include:

- An induction phase associating different chemotherapeutic molecules (etoposide, carboplatin, cisplatin, cyclophoshamid, vincristine); the response of this phase is particularly important for the prognosis;
- Two additional chemotherapy cycles including topotecan, vincristin and doxorubicin in patients having an insufficient metastatic response at the end of induction;
- Local surgery and radiotherapy on the primary tumor;
- Consolidation therapy (high-dose chemotherapy regimen including busulfan and melphalan) followed by ASCT rescue;
- Maintenance therapy with 13-cis-retinoïc acid (to induce cell differentiation) and immunotherapy (anti-GD2 Mab) [3].

Relapsed patients benefit from different combinatorial conventional therapies but prognosis remain very poor in this case [3].

As all the conventional therapies (including chemo- and radio-therapy) are not specific for the cancer cells, they lead to a lot of side effects involving normal tissues [12]. New, more selective drugs and therefore less toxic are being introduced in clinical trials, particularly for relapsed patients, and results are promising. In this view, epigenetic

drugs and immunotherapy will be discussed more in detail in the next sections. As new therapies are urgently needed, compounds with promising anti-NB activity need to be tested in pre-clinical studies. The next section summarizes the NB mouse models available to conduct such studies.

1.8 Murine models of neuroblastoma

Animal preclinical models are still essential nowadays to test new anti-cancer therapeutic drugs [29]. Different NB murine models are available, and each of them present advantages and drawbacks. Importantly, caution is needed when results are translated in clinics because despite of similarities between murine tumors and human disease, differences exist.

An ideal disease model should present a profile highly similar to human pathology at different levels: tumor location and metastasis, but also on the histopathological and genetic alterations aspect [22]. Animals should develop tumors rapidly and penetrance should be high; TME and immune responses should mimic the human ones [22].

NB murine models include subcutaneous (SC) models, orthotopic models and genetically engineered mouse models (GEMMs).

In SC models, NB murine cell lines are subcutaneously injected in syngeneic mice to form tumors. Another possibility is to inject human NB cell lines in immunodeficient mice. These models are often time- and money-saving, but reflect poorly the tumor microenvironment (TME) of « real » tumors, and cell lines often differ from the initial cancer cells as they have been passaged for long periods [29]. Moreover, in case of a xenograft model, immune responses cannot be studied as mice are immunodeficient [29, 30]. Cells can also be injected in orthotopic localization, in the adrenal medulla, in this case better reflecting the NB TME [31].

Finally, different GEMMs based on the NB oncogenic driver events have been developed including the well-described and mostly used TH-*MYCN* mouse model, created by Weiss *et al* [32]. In this model, the human *MYCN* transgene is placed under the control of the rat tyrosine hydroxylase (TH) promoter which activity is increased by the rabbit β-globin enhancer, leading to *MYCN* overexpression specifically in NCCs (Fig. 3) [22, 32].

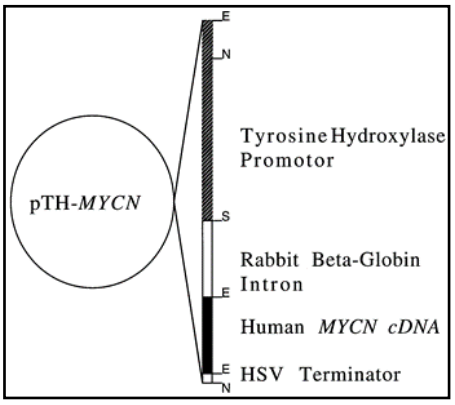

Fig. 3: "Construct used for generating tissue-specific expression of the MYCN transgene. A cDNA for human MYCN was ligated downstream of the rat TH promotor. The rabbit β-globin enhancer was used to enhance expression, and a herpes simplex virus thymidine kinase gene sequence was used as a transcription terminator. The transgene was cleaved from vector sequences using NsiI. E, EcoRI; S, SalI; N, NsiI". Legend and figure from [32].

This model mimics childhood HR *MYCN*-amplified NB: indeed, tumor histology, genetic alterations and expression patterns are similar to those present in HR patients [22, 32-34]. Lesions appear spontaneously in orthotopic locations and are mostly abdominal tumors, but are predominantly developed from the sympathetic ganglia and not in the adrenal medulla, making a difference with children tumors [26]. Few bone marrow metastases are observed accounting for another difference with human disease [32, 34]. A high variability in disease penetrance is noted for hemizygous mice, depending on the mouse background, and the disease development window is large; on the other side, homozygous mice frequently develop NB early after birth; for example, on a 129X1/SvJ background, 50% of hemizygous mice present tumors between 6 to 20 weeks, whereas 100% of homozygous mice develop tumors between 4 and 7 weeks [22, 32, 34]. Therapeutic window is very short for homozygous mice compared to hemizygous ones [34]. These data are summarized in Fig. 4.

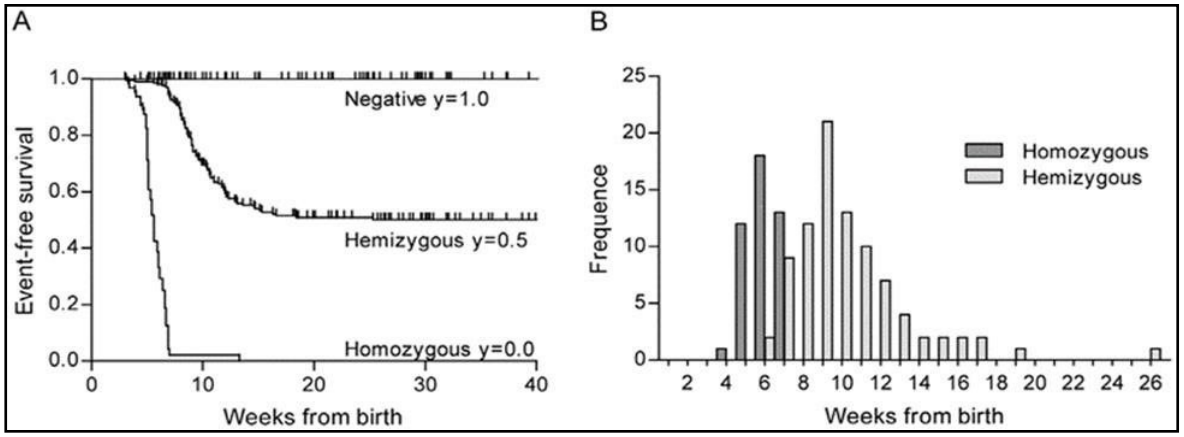

Fig. 4: Tumor development of TH-MYCN mice on the 129X1/SvJ background. From [34].

TH-*MYCN* model allows to study immunotherapy as animals are immunocompetent; however, one major disadvantage is its time- and money-consuming characteristics [22]. Nonetheless, it remains frequently used in pre-clinical studies and many drugs have already been tested with different degrees of efficacy, including epigenetic drugs [22].

Based on other genetic events having a role in NB tumorigenesis, other GEMMs have

been developed by crossing transgenic mice with TH-*MYCN* mice [22]. For example, TH-*MYCN*/ ALK^{F1174L} represents ultra-HR NB cases, and has been generated taking into account that *ALK* gain-of-function mutations are known to contribute to tumorigenesis, and exerts a synergistic effect with *MYCN* amplification [22]. The DBH-iCre/CAG-LSL-Lin28b model is another example; it is a conditional transgenic model of mice developing tumors in sites similar to human NB. The relation between *LIN28B* gene polymorphism as well as *LIN28B* gene amplification or overexpression and NB has been evidenced by different studies, as mentioned above [22].

A more personalized approach for cancer treatment will require the use of more tailored animal models. Patient-derived xenografts (PDXs) mice models have already been developed from NB patients, but as animals are immunodeficient, immunotherapy studies cannot be performed [22, 30]. However, humanized mice could resolve this issue in the future [22].

2. The complexity of tumorigenesis: the hallmarks of cancer

2.1 Introduction to the hallmarks of cancer

The oncogenesis is a highly complex process involving different mechanisms that can be classified in broad categories, common to most of malignant diseases. Insights in this very sophisticated phenomenon allow the development of new therapeutic strategies able to target each event contributing to cancer development and progression. The model of cancer hallmarks developed by Hanahan *et al* is continuously refined taking into account the results of all comprehensive works and represents an excellent tool to deeply investigate the underlying mechanisms from cancer initiation to overt disease [35, 36].

Of note, it is now well demonstrated that tumors are made of an elaborated tissue including not only cancer cells but also normal cells, largely contributing to the aggressiveness of malignant cells and playing an active role in the disease instead of being just passive [35].

2.2 The hallmarks of cancer

First, cells acquire an abnormal capability to proliferate breaking the cell-cycle homeostasis that guarantees an equilibrium between proliferation and period of quiescence [35]. This uncontrolled proliferation occurs through various processes, such as sustained proliferative signals, evasion from growth suppressors or resistance to cell death [35].

As cancer masses rapidly grow, they need increased blood supply, delivering oxygen and nutrients and evacuating cellular metabolic wastes [35]. The formation of tumor neovascularization is generally termed « tumor angiogenesis » and includes different processes [35]. Vasculogenesis refers to the formation of neovasculature from the incorporation into the tumor bed of endothelial progenitors which are produced in the bone marrow and are circulating in the bloodstream [26]. Classical angiogenesis concerns the sprouting of new vessels from already existing ones [26]. Finally, vascular mimicry is the ability of cancer cells to create a new vascular network by mimicking endothelial cells [26]. These three mechanisms co-exist in NB tumor neovasculature formation (Fig. 5) [26].

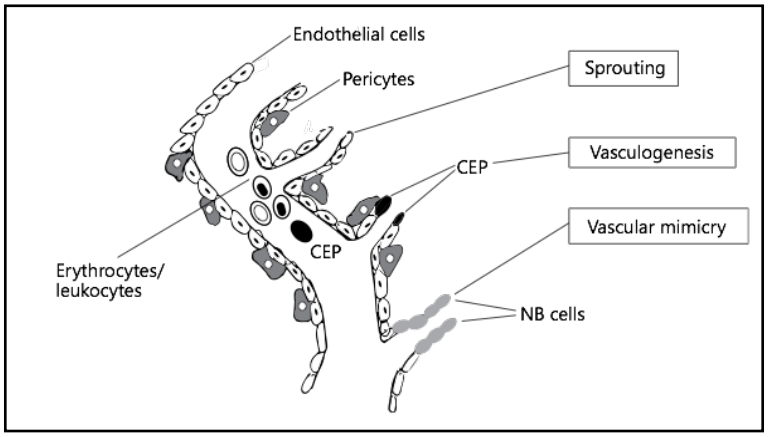

Fig. 5: Mechanism of angiogenesis in NB. From [26].

Angiogenesis results from the balance between pro- and anti-angiogenic factors. As pro- angiogenic factors in cancer are produced in excess, tumor angiogenesis gives rise to abnormal chaotic vessels, presenting structural and functional defects, with a heterogeneous distribution, ranging from enlarged and leaky vessels with poor pericytes coverage to glomeruloid aspect vessels (Fig. 6) [37].

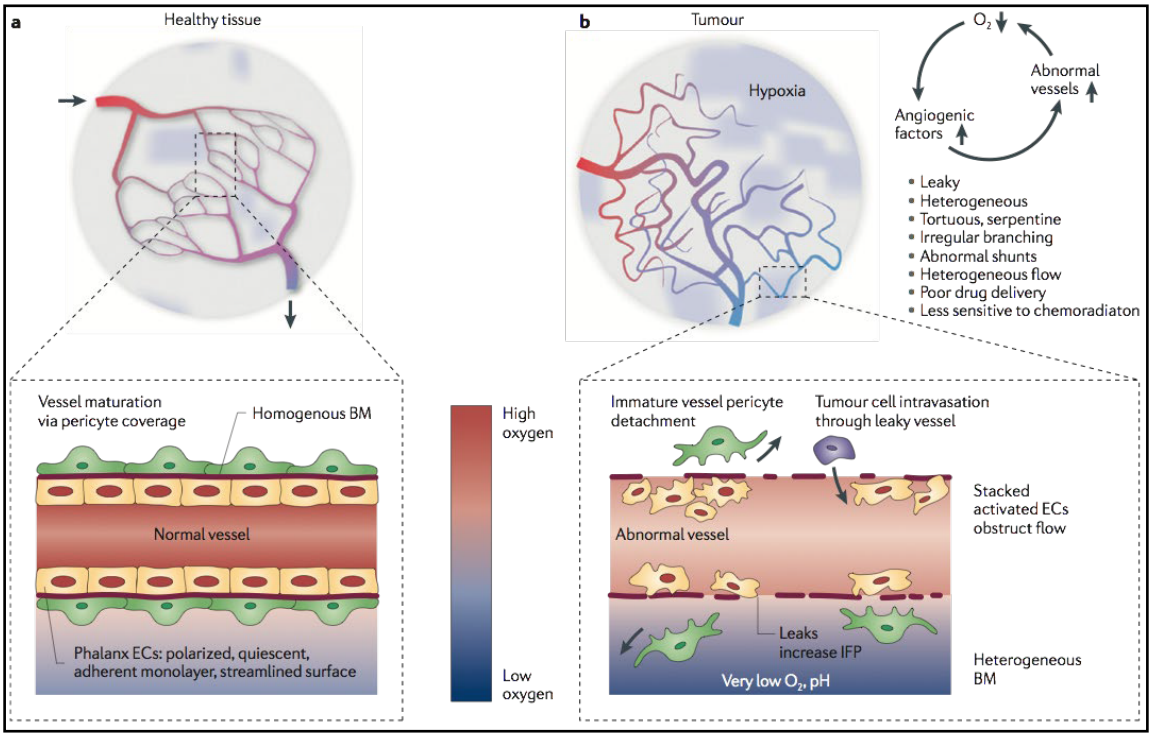

Fig. 6 showing the differences between normal vessels in healthy tissues and tumor vessels in cancer lesions. BM: basement membrane, ECs: endothelial cells, IFP: interstitial fluid pressure. From [37].

Blood flow is impaired in some tumor areas, contributing to poor tumor perfusion and increasing tumor hypoxia, leading to a vicious cycle [37, 38]. Hypoxia contributes also

to chemotherapy and radiotherapy resistance as the reactive oxygen species (ROS) production needed for the therapeutic benefit of these treatments is decreased [39]. As blood flow is low, chemotherapeutic drugs and immune cells can hardly reach the tumor [37, 39]. Moreover, cancer cells can easily enter the blood flow through leaky vessels and metastasize to distant sites [37].

Pro-angiogenic factors include vascular endothelial growth factor (VEGF)-A, which binds to its receptor, VEGFR-1, -2 or -3 leading to angiogenesis [35]. Other pro-angiogenic molecules are represented by basic fibroblast growth factor (bFGF), angiopoietin and platelet-derived growth factors (PDGFs) [35]. Pro-angiogenic factors production is induced by hypoxia and metabolic changes such as acidosis, or by oncogenic and inflammatory signaling pathways [35, 37]. On the other side, angiogenesis inhibitors such as thrombospondin-1 (TSP-1), angiostatin and endostatin exert the opposite effect [35].

In NB, poor prognosis forms are often highly vascularized and pro-angiogenic factors including VEGF family members and PDGF-A, have been correlated to more aggressive forms of the disease [26, 40]. In NB cell lines, hypoxia leads to increased VEGF-A production promoting endothelial cells proliferation [26]. On the other side, activin A dampens endothelial cells proliferation and is associated to good- prognosis forms of NB [26]. To note, *MYCN* impedes activin A expression whereas Schwann cells (which are abundant in good prognosis forms) secrete angiogenesis inhibitors [26].

Cancer cells are able to invade neighboring tissues and metastasize to distant sites notably through the process of epithelial-to-mesenchymal transition (EMT), which is favored by hypoxia and acidosis [35, 37]. Non-cancer cells secrete cytokines but also enzymes (i.e., matrix metalloproteinases, MMPs) that also facilitate dissemination [35]. Finally, the TME in the colonized tissue greatly impacts the development of metastatic cancer cells and their development into macroscopic tumors [35].

Reprograming energy metabolism is a hallmark reflecting the need for cancer cells to adapt their metabolism to their fast chronic growth requirements [35]. Glycolysis is favored for their glucose metabolism, even under aerobic conditions; this is called « aerobic glycolysis » or Warburg effect [35]. To compensate for the lower energy production, they uptake more glucose by upregulating its transporters, such as GLUT-1 [35]. Moreover, under hypoxic conditions, hypoxia-inducible factors (HIFs) are stabilized and promote glycolysis [35]. Even if glycolysis is not so efficient for ATP- production, it might confer the possibility for malignant cells to derive intermediate molecules to the nucleic acids and protein synthesis, which are essential to rapidly dividing cells [35].

The progress of the disease implies that cancer cells evade the immune destruction [35]. Over the past few years, it has been demonstrated that hypoxia and immune escape are highly interconnected phenomenon, suggesting that targeting hypoxia could improve immunotherapies, paving the way for combinatorial therapies [6-8].

The immune system in physiologic conditions and in cancer will be detailed in the next

sections, with a particular attention to NB immune escape mechanisms; then, hypoxia and metabolic reprograming will be the subject of the next sections, and finally, the link between hypoxia and immune evasion will be reviewed.

Recently, two more hallmarks have been included in the model of Hanahan *et al*: indeed, unlocking phenotypic plasticity is achieved by cancer cells in different ways, including dedifferentiation, stemness state maintenance or trans-differentiation, allowing them to avoid the anti-proliferative state commonly associated to differentiation [36]. The role of senescence is still being investigated but clearly constitute a hallmark of cancer cells: contrary to the common idea that senescent cells exert a tumor suppressing role, recent studies have underlined that depending on the cancer and tissue context, senescence could promote tumor growth, notably through a particular secretory phenotype associated to the senescent state [36].

All these hallmarks of cancer result from enabling characteristics, including genomic instability, non-mutational epigenetic reprogramming, tumor-promoting inflammation, and finally polymorphic microbiomes [35, 36].

Genomic instability and the resulting high mutability of cancer cells confer them advantageous tumor-driving mutations, giving them a selective benefit [35, 36]. However, epigenetic regulation also greatly impacts genes expression contributing to the acquisition of cancer cells hallmarks [36].

Inflammatory cells in the TME provide molecules able to sustain the hallmarks capabilities, such as growth factors, pro-angiogenic factors and enzymes contributing to the remodeling of the extracellular matrix [35]. Moreover, ROS produced by some immune cells exert a mutagenic action increasing genetic alterations in cancer cells [35]. This way, inflammation is considered as an enabling characteristic which can promote tumor evolution and progression [35].

Microbiomes present in the intestine but also in other tissues and even in tumors still attract interest in oncology, and they are now included in enabling factors impacting the acquisition of cancer cells hallmark capabilities [36]. For example, some bacteria exert a mutagenesis- promoting action on intestinal epithelium resulting in disrupted genomic integrity [36]. They also can directly stimulate epithelial cells proliferation, contributing to sustain proliferating signals [36]. They modulate immune responses, impacting cytokines and chemokines secretion profiles [36]. So, genomic instability, tumor-promoting inflammation and polymorphic microbiomes are intertwined enabling characteristics of cancer diseases [36].

3. The immune system in cancer: the dual face, friend or foe

3.1 Introduction to immunology

The knowledge in the field of immunology and in particular in oncoimmunology is still growing leading to new therapeutics development and survival improvement.
The main function of our immune system is to protect us against bacterial, fungal and viral infections, but also against cancer development [12, 41]. It plays also a role in tissue scarring and regeneration [41]. To achieve this, complex interactions and strong cooperation take place between the innate and the adaptive immune system [41].
First, the general way the immune system develops and works will be briefly described and then its dual role in cancer (fighting but also sometimes promoting tumors) will be emphasized with a particular attention to NB immunology, including immune escape mechanisms and immunotherapy [42, 43].

3.2 The innate and adaptive immune system

3.2.1 Immune system components

Leucocytes derive from bone marrow precursors: classically, myeloid cells (including notably granulocytes (neutrophils, monocytes/macrophages) and dendritic cells (DCs)) arise from the common myeloid precursor (CMP) and lymphoid cells (represented notably by T and B lymphocytes and natural killer (NK) cells) come from the common lymphoid precursors (CLP) [41]. The innate immune system involves epithelial and mucosal barriers, rapidly acting immune cells (such as for example phagocytes (neutrophils, monocytes/macrophages), DCs, NK cells) and the complement system [41]. Innate immunity and in particular DCs is essential to the initiation of the adaptive immune response; moreover, it contributes to cleaning of dead cells and tissue repair [41].
Innate immune cells receptors (localized on the cell membrane, in the cytosol or in endosomes) recognize pathogen-associated molecular patterns (PAMPs) (molecules or part of molecules present on pathogens but absent or different on normal mammalian cells) and damage-associated molecular patterns (DAMPs) (molecules coming from host cells which are necrotic or damaged), but not normal components of the host cells [41].
The complement system involves a complex enzymatic reaction cascade initiated directly on the target cell membrane or by binding to antibodies fixed on antigens. This finally results in the formation of C3b ad then the production of pro-inflammatory C3a and C5a fragments, and the formation of the Membrane Attack Complex (MAC) that forms a channel lysing the target cell [41]. Decay-accelerating factor (DAF) among other proteins regulates complement activation in order to avoid damage to normal host cells

[41].

Adaptive immunity involves naïve T and B lymphocytes which mature in the central lymphoid organs (the thymus and the bone marrow respectively), and then circulate via the blood and the lymph to become activated in the secondary lymphoid organs (represented by lymph nodes, spleen and lymphoid tissue associated to skin and mucous) where they encounter their specific antigen in the presence of co-stimulatory signals [41]. Upon activation, lymphocytes become either effector cells with high functional activity or memory cells that will be ready to promote an immune response if they encounter again the antigen for which they are specific [41].

3.2.2 Immune system activation

The activation and start of innate and adaptive immune responses is a tightly controlled phenomenon, and results from a balance between activating signals and inhibitory ones, which can be disrupted in pathologic conditions such as cancer, when inhibitory signals are increased and activating ones decreased [12, 41, 44, 45]. Of note, co-stimulation signals mainly coming from antigen-presenting cells (APCs, represented by DCs, macrophages, B cells and follicular DCs) are required to elicit immune reactions [41].

Importantly, cytokines orchestrate all the immune responses [41]. These soluble proteins are secreted by a large panel of cells including immune, endothelial and stromal cells and act in a paracrine, autocrine or endocrine way [41]. Depending on the context, the resulting effect of a given cytokine can be pro- or anti-inflammatory [41]. The main cytokines families include interferons (IFNs), interleukins (ILs), tumor necrosis factor (TNF) and transforming growth factor-β (TGF-β) [41].

NK and T cells are major immune actors in NB [45, 46], and so the way they become activated will be emphasized. On the other side, NB tumors contain virtually no B cells [4], and so B cells and humoral immunity will not be detailed here. As some mechanisms responsible for immune tolerance can be subverted by cancer cells to evade the immune system attack, the central and peripheral tolerance development in physiologic state will be detailed.

1. *Innate immune system activation: the example of NK cells activation*

NK activation takes place when the balance between activating signals overwhelms inhibitory ones [41]. NK cells activating receptors binds to molecules expressed at the surface of cells infected by intracellular pathogens (virus or bacteria), cells presenting irreparable DNA damage or cancer cells expressing danger signals [41]. This leads to the phosphorylation of their cytoplasmic part containing immunoreceptor tyrosine-based activation motifs (ITAMs) finally resulting in the production of cytokines (mainly interferon gamma, IFNγ) and the exocytosis of cytotoxic granules, killing the target cell

by perforing its membrane [41]. NK cells can also kill their target cell by expressing TNF molecules or ligands able to bind to death receptors present on the target and provoke target apoptosis (for example, NK cells express Fas-Ligand or TNF-related apoptosis inducing ligand (TRAIL)) [47]. Importantly, NK cells don't need any priming to exert their killer function [47].

Among activating receptors on NK cells, natural-killer group 2, member D (NKG2D) receptor binds to UL16-binding proteins (ULBPs), MHC class I chain-related protein A (MICA) and MICB; DNAX accessory molecule (DNAM-1 or CD226) binds to CD155 (polio virus receptor, PVR) and CD112 (Nectin-2); the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 recognize B7-H6, MLL5 (mixed lineage leukemia 5) and an unknown ligand respectively [12, 41, 44, 45]. CD16 (FcγRIII) also belongs to activating receptors and recognizes cells coated by immunoglobulin G (Ig G) leading to antibody-dependent cellular cytotoxicity (ADCC) [12, 41, 45].

As mentioned above, NK cells also harbor inhibitory receptors that counteracts the effect of engaged activating receptors; indeed, these have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on their cytoplasmic part exerting the opposite effect of ITAMs once phosphorylated [41, 45]. Killer cell immunoglobulin-like receptors (KIRs) recognize Major Histocompatibility Complex (MHC) class I molecules expressed on all nucleated cells delivering an inhibitory signal to NK cell [12, 41, 45]. CD94/NKG2A heterodimers also belongs to NK cells inhibitory receptors and recognize non-classical human leucocyte antigen (HLA)-E [12, 41, 45]. CD96 and T cell immunoreceptor with Ig and ITIM domains (TIGIT) share the same ligands as DNAM-1, but exert an inhibitory effect [47].

Depending on the net result of activating and inhibitory signals delivered to NK cells, the target cell will or not be lysed [47]. Indeed, healthy cells express some activating ligands but as they also harbor MHC class I molecules, the inhibitory signal overwhelms the activating one and the cell is not destroyed [45, 47]. On the other side, in the case of decreased inhibitory signals (such as for example downregulation of MHC class I molecules, the « missing-self theory ») or upregulation of stimulatory ligands on an abnormal cell (for example, a cancer cell), the net result of the balance is in favor of NK cells activation [47] (Fig. 7).

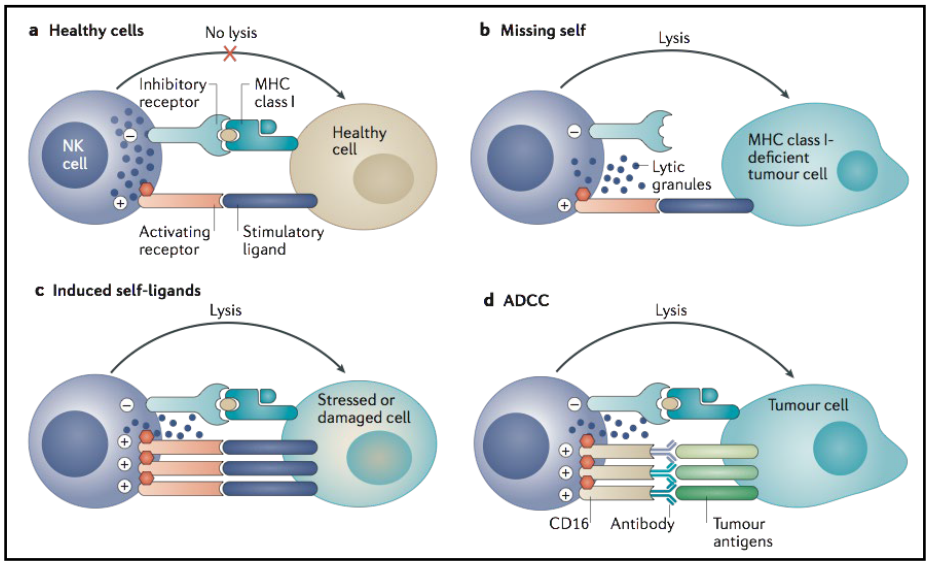

Fig. 7: The functions of NK cells. « (a) A balance of signals delivered by activating and inhibitory receptors regulates the recognition of healthy cells by natural killer (NK) cells. (b) Tumor cells that downregulate major histocompatibility complex (MHC) class I molecules are detected as 'missing self' and are lysed by NK cells. (c)Tumor cells can overexpress induced stress ligands recognized by activating NK cell receptors, which override the inhibitory signals and elicit target cell lysis. (d)Tumor antigen-specific antibodies bind to CD16 and elicit antibody-dependent NK cell-mediated cytotoxicity ». Legend and figure from [47].

Of note, some NK receptors are also present on other immune cells: for example, NKG2D is expressed on CD8+ T lymphocytes, DNAM-1 is also found on myeloid cells and some T cells, and CD16 also contribute to ADCC by activated macrophages [47].

2. *Adaptive immune activation: the example of T cell-mediated immunity*

Microbes or their antigen are captured by DCs in tissues and activate other innate immune cells such as macrophages, leading to pro-inflammatory cytokines secretion, finally activating and modifying DCs phenotype [41]. This results in DCs maturation, with increased expression of MHC as well as co-stimulatory molecules, and migration to the lymph nodes (notably through CC-chemokine receptor 7 (CCR7) signaling) where they can initiate naïve T cell priming [41]. The majority of T cells harbor an $\alpha\beta$ T cell receptor (TCR) able to bind their specific antigen only when peptide fragments are loaded on MHC molecules, a property called « MHC restriction » [12, 41].

MHC molecules are proteins expressed on cell membranes with an extracellular cleft where antigen-derived peptides bind and can be presented to T lymphocytes. Importantly, T cells harboring an $\alpha\beta$ TCR recognize peptides only if they are loaded on the right MHC molecules; in other words, T cells will not recognize peptides presented

by genetically foreign MHC molecules [12].

All nucleated cells express Class I MHC molecules, displaying peptides from cytosolic proteins degraded by the proteasome and containing an invariant region binding to the CD8 co-receptor on T cells [41]. DCs, B-cells and macrophages express Class II MHC molecules, displaying peptides from extracellular proteins and containing a domain that binds to the co- receptor CD4 on T cells [41].

MHC molecules are expressed on cell surface only if they are bound to peptides resulting from the breaking of entire proteins under the Antigen Processing Machinery (APM) activity [41]. Cytosolic proteins coming from pathogens or from abnormal nuclear or cytosolic proteins present in cancer cells are tagged with ubiquitin and degraded by the proteasome [12, 41]. Under inflammatory cytokines action (notably IFN-γ), the subunits of the proteasome are replaced by more efficient ones: LMP2 replaces β1 subunit, LMP7 replaces β5 and LMP10 take the place of β2 [44]. Peptides generated from protein proteolysis are then transported by the transporter associated with antigen processing (TAP), which is formed by an heterodimer of TAP1 and TAP2, to the endoplasmic reticulum (ER) where they can be loaded on MHC class I molecules stabilized by the tapasin protein (links the TAP and the unbound class I MHC molecules) [12, 41, 44]. Class II MHC molecules cannot bind these peptides because the associated invariant chain block their peptide cleft [41]. The complex formed by MHC class I molecule and peptide is then transported to the cell membrane for CD8+ T cell presentation [12, 41].

APCs (DCs, macrophages and B lymphocytes) internalize extracellular proteins into endosomes; these are then fused with lysosomes and proteolysis into peptides occur [41]. On the other side, class II MHC molecules are produces in the ER and are associated with a protein, the invariant chain which contains the class II invariant chain peptide (CLIP) that block the peptide cleft of class II MHC molecules so that they cannot bind peptides in the ER (these are intended to bind class I MHC) [41]. Class II MHC molecules with the invariant chain go through the Golgi and then join the endolysosomes; in this compartment, only the CLIP fragment of the invariant chain remains attached to the MHC molecules and then, under the action of a protein called DM, it is exchanged with the peptides resulting from the proteolysis of the extracellular proteins [41]. Class II MHC molecules loaded with peptides are then transported to the cell membrane and are presented to CD4+ T cells. Fig. 8 [41] summarizes the intracellular processing of protein antigens.

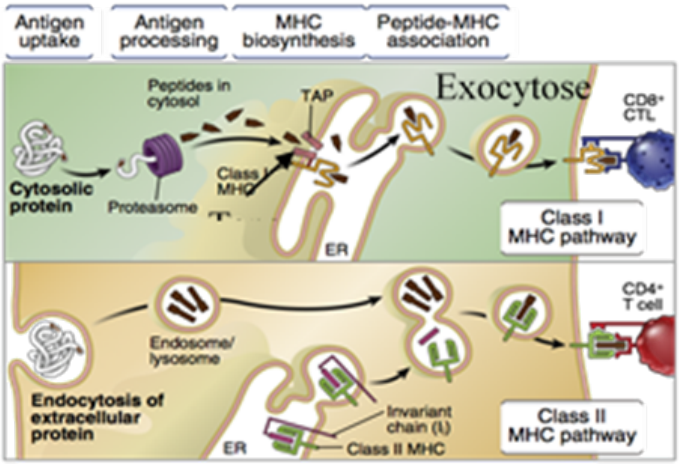

Fig. 8 summarizing the intracellular processing of protein antigens. Adapted from [41].

DCs are also able to present exogenous peptides antigens attached to class I MHC molecules to naïve CD8+ T cells in lymph nodes, a process called cross-presentation [41]. This phenomenon is essential as pathogens infect non DCs cells and naïve CD8+ T cells require activation through interaction with APCs in lymph nodes to become effector cells [41]. Infected cells cannot migrate to lymph nodes, and the process of cross-presentation resolve this issue: DCs ingest pathogen antigen or fragments of infected cells and through cross- presentation process activates naïve T CD8+ cells after migration in the lymph node [41]. The same phenomenon accounts for the activation of naïve CD8+ T cells by DCs after that they have ingested dead cancer cells or tumor antigens [41]. Indeed, cancer arise in each cellular type, and priming of naïve T cell is required to activate them; after activation has taken place, effector cytotoxic T lymphocytes can recognize tumor antigen presented on class I MHC molecules by cancer cells themselves and exert their function of killer. Fig. 9 illustrates the cross-presentation phenomenon [41].

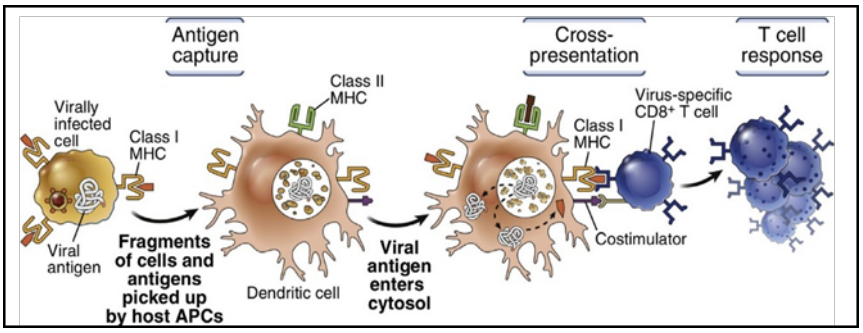

Fig. 9 illustrating the cross-presentation process. From [41].

Furthermore, co-stimulation of naïve T cells by CD28 engagement with its ligand B7-1 (CD80) or B7-2 (CD86) on APCs is essential to activate naïve T cells. B7 expression on APCs increased only in the inflammatory context, avoiding harmful unnecessary T cell activation. Other pathways involving Toll-ike receptors and their ligands and CD40 (on APCs) interaction with CD40 ligand (CD40L) on activated T cells promotes co-

stimulation molecules expression [12, 41, 48].

Once TCR and co-stimulation signals are engaged, T cells transform in effector cells expressing cytokines. Depending on the cytokines pattern expression, CD4+ T cells are sub- divided into helper T cells (Th) Th1, Th2 and Th17 subsets [12, 41]. A subset of CD4+ cells leave the secondary lymphoid organ to join the infection/inflammation site and recruit other immune cells whereas the remaining cells differentiate into T follicular helper cells which stimulate B lymphocytes in the lymphoid follicles. CD8+ T cells express cytotoxic proteins and are poised to kill target cells; they also produce cytokines, such as IFNγ [12, 41]. Perforin and granzymes are essential molecules released by CD8+ cells and acting by perforing the target cell membrane and inducing its apoptosis respectively [12, 41]. But cytotoxic T lymphocytes (CTLs) also induce apoptosis by expressing molecules able to bind apoptosis- inducing receptors on taget cells (for example, FasL which bind to Fas receptor) [12, 41]. Some CD4+ and CD8+ T cells become memory T cells, ready to respond if they are reexposed to the antigen they are specific for [41].

3. Central and peripheral tolerance, homeostasis and the role of immune checkpoints

Self-tolerance mechanisms are essential to avoid the development of auto-immune diseases [49]. Central tolerance which occurs in the thymus involves the death by apoptosis of immature CD4+ and CD8+ lymphocytes recognizing and interacting strongly with self-antigen (negative selection), or the development of some of these CD4+ lymphocytes into T regulatory lymphocytes (Tregs) (CD4+ CD25+ FoxP3+) [41, 49]. However, not all self- reacting lymphocytes are eliminated nor develop into regulatory cells, and peripheral tolerance mechanisms allow to control their activation [41, 49]. Peripheral tolerance mechanisms act at different stages of the T cell cycle [49, 50]. Ignorance refers to the fact that T cells can ignore the antigen they are specific for because of its low abundance (for example, in the case of nascent tumors) of due to its inaccessibility to naïve T cells [49]. At the stage of priming, insufficient co-stimulatory signals delivering fosters T cells anergy, whereas chronic antigen exposure leads to T cell exhaustion [49]. Self-antigens reacting mature T cells may also be deleted by apoptosis, resulting from an imbalance in pro-apoptotic factors in confront of anti-apoptotic ones: indeed, when T cells recognize their antigen, proapoptotic factors are produced but normally counterbalanced by antiapoptic ones resulting of the co-stimulation signals; in case of insufficient co-stimulatory signals, pro- apoptotic factors are preponderant and lead to T cells death. Moreover, binding of self- antigens may induce apoptosis mediated by couple of molecules such as Fas and Fas-ligand. Finally, suppression by Tregs play an important role in maintaining peripheral self-tolerance [41, 49].

Inhibitory receptors are expressed on T cells and contribute to self-tolerance. Moreover, they limit T cell activation and the inflammatory response maintaining in this way the immune homoeostasis [44, 48]. In addition, they take part in immune escape mechanisms in cancer *(see below)* [44, 48].

These inhibitory receptors are called immune checkpoints, and the first to have been evidenced are the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 receptor or CD152), which has for ligands CD80 and CD86, and programmed cell death protein 1 (PD-1 or CD279) that binds PD-L1 and PD-L2 [12, 44, 48].

CTLA-4 constitutive expression on Tregs contributes to increase their immunosuppressive functions [41, 44]. It competes with CD28 for binding with B7 proteins, and has a higher affinity than CD28 [44]. When the expression level of B7 proteins is low as it is the case in non-infectious situation such as presentation of tumor antigen (TA) *(see below)* or self- antigen by APCs, B7 proteins engage preferentially with CTLA-4 leading to downregulation of immune responses [41]. On the other side, B7 expression level is high in infections and thus can bind to CD28 also reversing the balance to an activating signal [41]. Tregs also suppress immune responses by inhibitory cytokines secretion (inhibiting DCs, macrophages and lymphocytes), and by consuming Il-2 via their IL-2 receptor (inhibition of other T cells) [41]. Of note, CTLA-4 expression on conventional CD4+ T helper lymphocytes occur when they are activated limiting their activity and so contributing to maintain homeostasis [44].

PD-1 expression on CD4+ and CD8+ T cells first reflects the activation of effector T cells, as it's expressed in response to activating signals such as engagement of the TCR with its specific antigen [44, 48]. As antigen is cleared, PD-1 expression decreased and the situation returns to the steady state [48]. But under chronic T cell stimulation (in case of tumor or chronic viral infection, or even when self-antigens are presented by APCs), PD-1 expression persists and can lead to a state of exhaustion, characterized by reduced effector function of T cells and decreased cytokine production as well as distinctive markers expression [4, 48]. Then, PD-1 binds to its ligands (PD-L1 or PD-L2) activating an enzymatic cascade finally counteracting the activating signals provoked by TCR and CD28 engagement [41, 48].

3.3 Immune system and cancer

3.3.1 Introduction to the immunology of cancer

The interplay between immune system and cancer tissues has been suggested more than 100 years ago, but it's only in the 1950's that the idea of an active role of immune cells in controlling cancer development has raised more enthusiasm [12, 41-43]. Since then, the understanding on how the immune system works has made a huge step and its role in cancer is still being uncovered leading to substantial progress in therapy, with prolonged survival and less toxicities.

Immunity clearly contributes to protect against malignancies [42, 43]. Indeed, our immune system defends us against viral infections and some cancers they may induce [42, 43]. Moreover, it contributes to control the inflammation induced by infections, and thus to protect us against the cancer-promoting role of inflammation; uncontrolled chronic inflammation would induce a genomic stress and lead to a high cellular proliferation rate, to angiogenesis and to tissue invasion paving the way for cancer development [42, 43].

Several arguments (in humans but also in animal studies) sustain the hypothesis that immunity is also able to eliminate tumor cells [12, 42, 43]. First, paraneoplastic syndromes are a set of symptoms not directly caused by the cancer cells invasion, but resulting from the production of endocrine mediators by cancer cells or from an immune cross-reactivity between normal tissues and cancer tissue witnessing in this last case that an immune reaction has developed [42, 43, 51]. Secondly, immune-compromised patients have more cancers than immune-competent ones, including viro-induced cancers but also other types of malignancies such as skin cancers [12, 42, 43]. Thirdly, patients harboring tumors with a high number of tumor-infiltrating lymphocytes (TILs) have a better prognosis [4, 42, 43]. For example, correlating with this observation, in colorectal cancer, patients harboring high microsatellite instability, and therefore high tumor mutational burden, show high CD8+ tumor infiltration and better prognosis [43]. Another clinical argument sustaining the role of immune system in human cancer comes from the success observed with the introduction of immunotherapy in oncology [12, 42, 43]; moreover, in the case of allogeneic hematopoietic stem cell transplantation (HSCT) for leukemia, donor lymphocytes are able to recognize host cancer cells and the resulting graft-versus-leukemia effect could positively impact the patient outcome [12, 52]. Even more, when relapse occurs after HSCT, donor lymphocyte infusion (DLI) can reverse the course of the disease [12, 52]. Evidence also comes from animal experiments: it has been demonstrated that mice previously immunized against a given tumor do not develop tumor when re-challenged by the same tumor cells [42]. This has led to the discovery of tumor antigens (TA).

Based on these observations, the hypothesis of cancer immunoediting has been suggested. Indeed, it was observed that tumors developed in immune-competent mice present a lower immunogenicity than tumors developed in immune-deficient ones [12, 35, 42, 43]. This difference resulted from the pressure exerted by immune cells on transformed/cancer cells, and led to the idea that the immune system not only watches for cancer cells but also shapes them; doing so, immunity can result in cancer cells eradication (protective role) but it could also lead to cancer cells adaptations finally resulting in a tumor-promoting action [12, 42, 43]. Cancer immunoediting will be detailed in the next section.

3.3.2 Cancer immunoediting: three steps

1. Elimination phase

The elimination phase consists in the eradication of nascent cancer cells by the immune system when intrinsic cellular processes such as DNA repair, senescence and apoptosis have failed to do so [42-44]. Indeed, in this case, nascent abnormal cells develop into transformed cells expressing danger signals that can alert the immune system and lead to immune responses capable of eliminating cancer cells before they form overt tumors (immunosurveillance) [42-44]. These danger signals include cytokine secretion or expression of DAMPs and stress ligands. In this elimination phase, cooperation between innate and adaptive immune systems plays a major role [42-44].

2. Equilibrium phase

When elimination phase failed to eradicate transformed cells, these ones enter a phase of dormancy, in which their growth is tightly controlled by the adaptive immune system, with CD4+ and CD8+ T cells, IL-12 and INFγ playing a major role [44]. Importantly, cancer cells are exposed to an important immune pressure in this phase, giving sense to the « editing » process [44].

3. Escape phase

Cancer cells may evolve under the pressure exerted by the immune system and because of their genomic instability [44]. This leads to the escape from the immune system. Many mechanisms are involved in this process. First, cells may dampen the « danger » signals: by loosing TA expression, decreasing MHC class I expression, altering the cellular peptide processing machinery (TAP,…). These events make the cells « hidden » from immune cells [44]. Cancer cells can also evade immune-induced apoptosis by regulating the expression of pro- or anti-apoptotic factors (for example, increased expression of the anti-apoptotic protein BCL-2). Finally, tumor cells can induce an immunosuppressive TME by several ways. For example, they may secrete immunosuppressive cytokines; they may recruit immunosuppressive cells at the tumor site, such as Tregs (which secrete IL-10 and TGF-β), or myeloid-derived suppressor cells (MDSCs) which contribute to dampen anti-tumoral lymphocyte action [43, 44]. Cancer cells may also express inhibitory immune checkpoints such as PD-1/PD-L1 and CTLA-4; this last point will be discussed in details below. The escape phase finally results in overt disease [43].

Importantly, cancer cells may skip a step and directly enter in the ultimate phase, or remain in the equilibrium phase with no overt disease for years or even throughout life [42]. On the other side, depending on some particular circumstances (for example,

exposition to immunosuppression or on the contrary to immunotherapy), cells can proceed to the next step or turn back in the previous one [42]. Fig. 10 summarizes the cancer immunoediting concept.

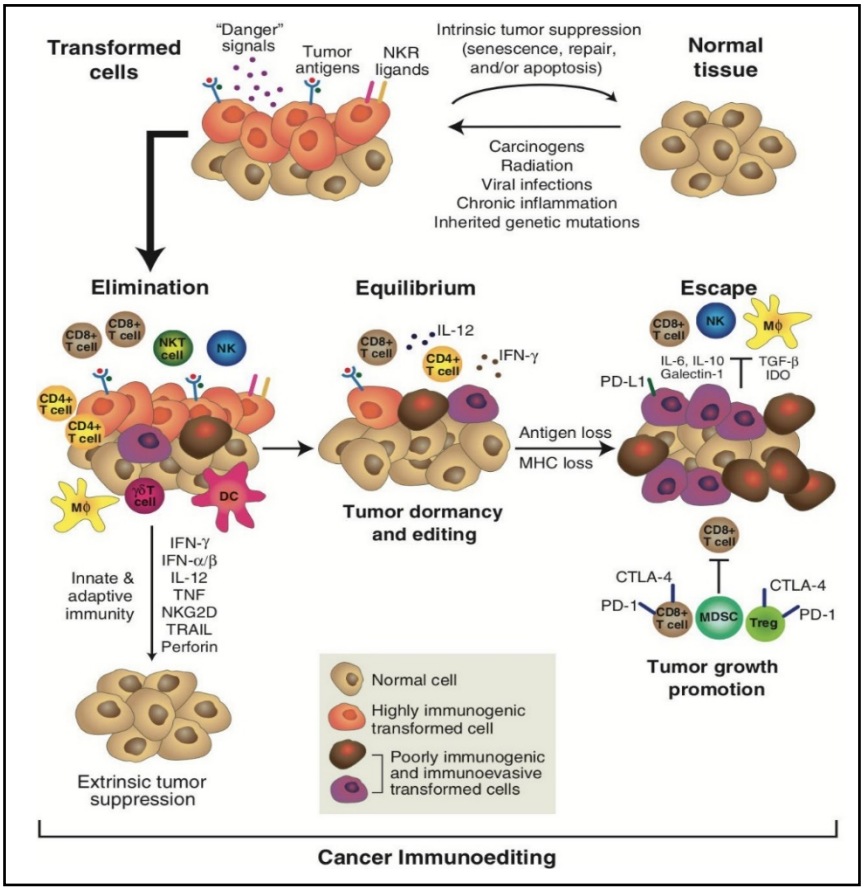

Fig. 10: The cancer immunoediting concept. From [42].

3.3.3 TA and the recognition of cancer cells by the immune system

As detailed in the introduction to immunology chapter, the cancer cells recognition by the immune system starts with the release of tumor (neo)antigens by dying or dead cancer cells [6]. TA led to the recognition of tumor cells as « foreign cells » by the immune system. Antigens that can be processed into peptides and loaded on MHC molecules to stimulate a T cell response (in particular, a CTL immune response) are probably the most relevant ones [41].

TA includes neoantigens, that is to say proteins expressed from mutated genes (passenger or driver mutations) or from viral genes in the case of virus-induced cancers, and self- antigens, represented by proteins resulting from normal genes aberrantly expressed or overexpressed in transformed cells (including oncofetal and differentiation antigens) [12, 41, 42]. In this last case, although the protein is normal (self-antigen), it can evoke an immune reaction because it is considered as foreign by

the immune system due to its aberrant expression (for example, embryonic proteins elicit immune reaction in adults due to their aberrant expression at this age) [12, 41, 42]. This gives also evidence that self-reacting T cells are not completely eliminated by tolerance mechanisms, but the remaining autoreactive T cells are low-avidity lymphocytes and may be not able to product a robust immune reaction [12].

For immunotherapy, the ideal target TA should be expressed at high level in cancer cells to strengthen the interaction with TCR, but should be absent or expressed at very low level on normal tissues to avoid off-tumor undesirable effects. It should give rise to immunogenic peptides that will be loaded and presented by MHC molecules to TCR [41]. Moreover, among products of mutated genes, those from driver mutations raise more interest as they are essential for tumor growth and thus less prone to be downregulated as an immune escape mechanism [12]. In addition, it could be hypothesized that neoantigens, contrary to self- antigens, would be able to elicit T cells reactions that are not subject to self-tolerance mechanisms and so should contribute to more robust immune reactions [49]. But at initial tumor development stages, in the absence of inflammation, the lack of co-stimulatory signals often leads to T cells hyporesponsiveness [49].

The process of tumor recognition by CTLs is the same as pathogen recognition: APCs (DCs) ingest cancer cells or tumor antigens, which after their processing into peptides are loaded on MHC-I and presented to naïve CD8+ T cell in the draining lymph node [41]. The activation of naïve CD8+ T cells to produce effector CTLs requires additional co-stimulation signals or the contribution of MHC-II-restricted CD4+ T cells also activated by DCs [41]. DCs express co-stimulatory molecules following the activation of innate immune responses due to the release of DAMPs by dying tumor cells lacking blood supply or exposed to chemotherapy [41]. When CTLs are produced, they can migrate to any tumor site and attack cancer cells expressing the specific antigen without the need of helper T cell or co-stimulation signals [41]. Even if CTLs clearly play a major role in anti-tumor immunity [53], other immunocompetent cells such as CD4+ T cells, NK cells, macrophages and B cells actively participate to cancer cell eradication.

3.3.4 The dual role of the immune system in cancer: a complex crosstalk between TME cells

Depending on the TME conditions (including metabolic changes) and mainly cytokines they are exposed to, immune cells present with different phenotypes, promoting anti-cancer responses or on the contrary sustaining tumor growth. The main immune cell types present in the TME in cancer in general and their potentially dual role in cancer (depending on their differentiation and polarization phenotype) will be briefly described. We will also describe how the immune landscape and immune escape strategies are

able to divert the immune system and make it an ally to promote cancer growth.

Macrophages found in tumors, named tumor-associated macrophages (TAMs), take their origin in circulating monocytes but also in resident macrophages that have infiltrated the surrounding tissue during embryogenesis [54]. Of note, the contributing role to cancer progression of embryonic-derived TAMs is not yet fully understood [54]. Another source of TAMs is represented by the mononuclear myeloid-derived suppressor cells (M-MDSCs) *(see below)* that convert into TAMs under the action of different chemokines [54].

TAMs present plasticity properties and their phenotype is highly depending on the molecules and namely cytokines and metabolites present in the TME [6, 54]. These molecules are produced by the cancer cells but also by non-cancer cells such as other immune cells present in the TME [54]. TAMs can differentiate into M1-TAM, promoting tumor eradication by several mechanisms, such as releasing ROS and nitric oxyde (NO) or acting by ADCC [6, 54]. On the other hand, M2-differentiated TAMs harbor a tumor and metastasis promoting role. Indeed, they produce MMPs and pro-angiogenic factors (such as for example VEGF-A) favoring the extracellular matrix degradation and a chaotic vasculature [54]. They also sculpt the tumor immune landscape through the expression of different cytokines: for example, C- C motif chemokine ligand (CCL) 22 contributes to attract Tregs in the tumor bed, dampening T cells immune responses [54]. TAMs also negatively impact CD8+ T-lymphocyte proliferation through induced metabolic changes involving notably arginase and nitric oxide synthase (iNOS) [54]. To note, hypoxia promotes a M2-phenotype contributing to tumor immune escape; this point will be detailed later in the section related to the interaction between hypoxia and immunosuppression.

In summary, TAMs are mainly involved in immunosuppression in the TME [55]. On the other side, DCs play a main role in initiating T cell responses against cancer [55].

DCs are classified into different subsets taking into account their origin, their expression markers, their localization and their function [56]; the main subsets include conventional DCs (cDCs), with sub-type 1 and 2 (cDC1s and cDC2s), plasmatoïd DCs (pDCs) and monocyte- derived DCs (moDCs) [55, 56]. Depending on the transcription factors expression profile in the CMP, progenitors cells differentiate into monocytes, next able to give rise to moDCs, or into common dendritic cell progenitor (CDP) able to differentiate in cDCs or in pDCs [55].

The most studied DCs in cancer to date are the cDC1s, which are known to play an important role in stimulating anti-tumor responses; indeed, cDC1s and in particular migratory cDC1s are highly performant for MHC cross-presentation and largely contribute to the priming and activation of CD8+ T-cells [55, 56]. The cytokine secretion by cCD1s greatly impact the infiltration but also the effector function of T lymphocytes, with the CXC-chemokine ligand 9 (CXCCL9) and 10 (CXCCL10) attracting CTLs into the tumor bed, and IL-12 and type I Interferon (IFN) playing a crucial role in enhancing

T cells effector function [55, 56]. The crosstalk between cDC1s and NK cells has also been underlined in recent studies [55]. Indeed, NK cells are major players to attract cDC1s into the TME through FMS-related tyrosine kinase 3 ligand (Flt3L), CCL5 and X-C Motif Chemokine Ligand 1 (XCL1) action. On the other side, NK cells secrete IFNγ in response to the cDC1s IL-12 production contributing to the anti-tumor reaction [55].

cDC2s mostly present endogenous antigen via MHC class II molecules to CD4+ lymphocytes supporting their helping role in CD8+ lymphocyte immune reaction [55]. However, the role of cDC2s as well as the one of pDCs and moDCs in human cancer need further studies to be better characterized [55].

In the particular setting of the immune pressure exerted by the immune system on nascent cancer cells, some variants develop and use strategies to drive DCs to immune tolerance induction [56]. For example, cancer or immune cells in the TME (including DCs) can express immune checkpoints ligands able to dampen T cell activity [9, 10]. Moreover, metabolic changes (such as for example indoleamine 2,3-dioxygenase 1 expression and the resulting L-Tryptophan depletion which can be induced in DCs by CTLA4/CD80 and 86 interaction) also contribute to reduce CTLs and NK cells activity while promoting the differentiation of immunosuppressive Tregs [56]. Cancer cells produce cytokines and metabolites able to impact DCs infiltration, differentiation or function, such as for example VEGF, IL-6 or prostaglandin E2 (PGE2), finally resulting in a compromised anti-tumor phenotype in DCs [56].

Immature myeloid cells leaving the bone marrow infiltrate different organs where they differentiate into granulocytes, DCs or macrophages [28, 44]. Under the action of different attractive molecules produced notably in the TME, immature myeloid cells also colonize tumor tissues where their differentiation is blocked whereas they retain the capacity to proliferate [44]**.** MDSCs refer to this heterogeneous group of immature myeloid cells, which are only observed in pathological situations such as cancer disease [6, 28]. These cells are Gr1+ and present some similarities with neutrophils (PMN-MDSC, polymorphonuclear - MDSC) or monocytes (M-MDSC, mononuclear MDSC) [6, 28]. They also express some surface markers shared by monocytes, TAMs and neutrophils [6]. They acquire immunosuppressive properties resulting from different mechanims: ROS generation preventing T cell recruitment and activation but favoring their apoptosis; depriving T cells from essential amino acids, such as L-arginine leading to T cell proliferation arrest; secretion of immunosuppressive mediators (TGF-β, IL-10, …) impacting NK cells function and inducing Tregs, and finally PD-L1 expression activating Tregs but dampening effector T cells [6, 10, 28, 44]. Moreover, MMPs and pro-angiogenic factors secreted by MDSCs contribute to tumor growth and expansion [6].

As mentioned in the introduction to immunology above, NK cells activation take place depending on the net result of the balance between activating and inhibitory signals [47]. Moreover, cytokines present in the TME contribute to this activation [47]. In

response, NK cells also produce a lot of inflammatory signals, including cytokines and chemokines, impacting other immune cells infiltration, activation and proliferation [47]. One major cytokine produced by activated NK cells is IFNγ, impacting the expression of MHC class II molecules by APCs, activating macrophages and favoring CD4+ Th1 phenotype [47]. Once again, cytokines and molecules exerting an immunomodulatory activity present in the TME can dampen NK cells activity, such as for example TGF-β or PGE2 [47]. Due to the pressure exerted by the immune system, cancer cells often dampen their MHC class I expression to escape the CD8+ T cell attack [47]. This renders themselves vulnerable to NK cell lysis as MHC class I molecules cannot engage with KIRs anymore, reversing the balance to activating signals [47]. However, in this context, the immunosurveillance exerted by NK cells can fail due to the dampening of activating signals by cancer cells [47].

T lymphocytes present in the TME include CD4+ conventional T lymphocytes (Tconv), Tregs and CTLs. Tconv contribute to tumor eradication. First, they play a role in the secondary lymphoid organ where they contribute to enhance the CD8+ priming through stimulating the DCs co-stimulatory functions, cytokines production (notably IL-12) and maturation (through CD40-CD40L interaction) [57]. Due to the CD4+ help, CD8+ priming occur even for low- affinity lymphocytes, allowing a larger CD8+ cohort harboring a TCR specific for TA to be primed [57]. Tconv at the tumor site greatly impact the TME cytokines landscape notably by producing IFNγ contributing to support CTLs and IL-2 stimulating NK cells [57].

As mentioned in the introduction to immunology, Tregs promotes self-tolerance and in the context of cancer, exert a deleterious immunosuppressive activity. In the cancer TME, Tregs take their origin mainly from the thymus, but could also derive from the Tconv conversion (induced Tregs, iTregs) under the action of tumor cells TGF-β production [57]. Tregs dampen the immune system attack against cancer cells, and notably decrease the DCs maturation and alter the CTLs as well as the macrophages activity through immunosuppressive cytokines secretion (for example, IL-10, IL-35 and TGF-β) [57]. Tregs also promote angiogenesis and are trapped in hypoxic masses through CCL28 pathway [57, 58].

CTLs represent the major actor of immune cancer cells killing [53]. After their priming in the secondary lymphoid organs and their conversion to effector T cells, they reach the tumor bed where they are attracted by several cytokins and chemokines (for example, CXCL9, CXCL10, CCL5) and infiltrate through ICAM-1 (intracellular adhesion molecule-1)/LFA-1 (leukocyte function-associated antigen-1) binding and selectins interactions [59]. They recognize their specific antigen presented on MHC class I molecules on cancer cells which is then killed through granzyme and perforin-containing granules releasing by T cells or by activating death receptors on the target cell (through the interaction of FasL on T cells with Fas receptor for example) [53]. However, each step of CD8+ T cells activity is governed by stimulatory factors (including

cytokines, chemokines, ligands/receptors interactions, growth factors, MHC class I and co-stimulatory signaling,…) but can also be counteracted by inhibitory signals emerging from the particular conditions (metabolic and immune landscape,…) of the TME [53]. One of these dampening signals include the interaction between immune checkpoints ligands and their cognate receptor, including the interaction between PD-1 on T cells and its ligands on cancer cells or immunosuppressive immune cells [53]. The importance of PD-1/PD-L1 axis in cancer will be detailed in the next section.

3.3.5 The PD-1/PD-L1 axis in cancer: anergy and exhaustion state of T cells and tumor immune escape

The importance of the PD-1/PD-L1 axis in self-antigen immune tolerance development and in T cells anergy induction has been described in the introduction. PD-1/PD-L1 axis also plays a main role in the dysfunctional hyporesponsive states of T cells observed in cancer, namely anergy and exhaustion, and the accent will be put on these two phenotypic differentiation of T cells [49].

Indeed, naïve CD4+ and CD8+ T cells priming in the context of nascent cancer mass is often suboptimal, resulting from the insufficient co-stimulatory signals provided by a poor- inflammatory context and the consecutive suboptimal activation of APCs [49]. This leads to an early dysfunctional state similar to T cells anergy [49]. Then tumors progress to bulking mass with a larger TA load (self-antigens or neoantigens resulting from neomutations) more able to elicit immune reactions [49]. However, in this case, TCR continuous cancer antigen exposure promotes an exhausted phenotype of CD8+ T cells similar to that observed in chronic viral infections (late dysfunctional state) (Fig. 11) [49].

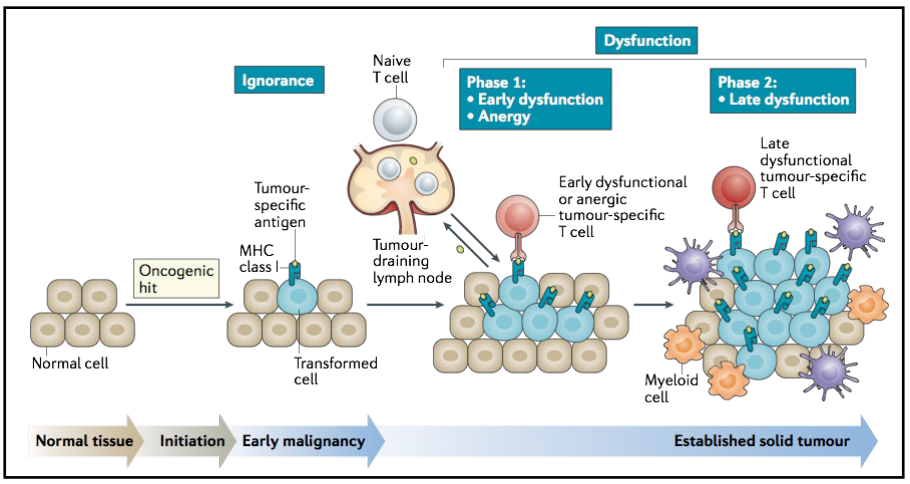

Fig. 11: From [49].

To note, early dysfunctional T lymphocytes represents a plastic state, as they can be

« reinvigorated » and regain their effector functions [49]. On the other side, late dysfunctional T lymphocytes have definitively lost their cytotoxic functions. Both states are characterized by PD-1 expression, but in the late dysfunctional state, more inhibitory receptors are expressed [49]. The phenotypic appearance of T cells, both in normal states (naïve, effector or memory states) and in dysfunctional ones (anergy-like and exhausted) depends on transcriptional program expression but also on epigenetic regulation (Fig.12) [49].

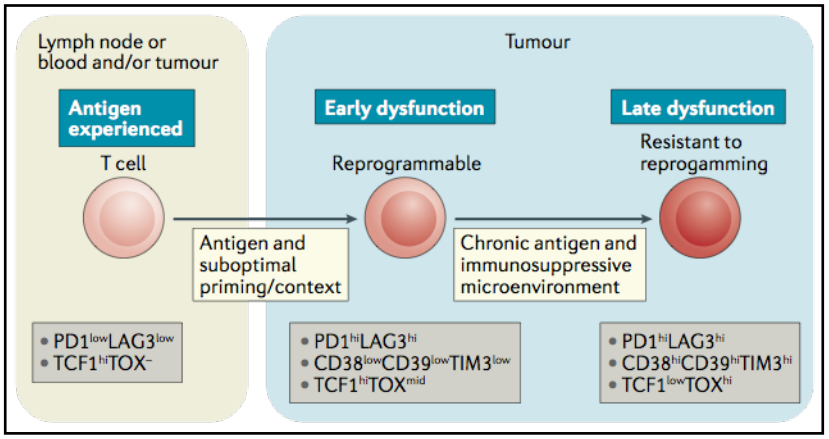

Fig. 12: From [49].

To remember, PD-1+ not only represents an exhaustion marker but also reflects T cells activation as it is present in a transitory way during naïve to effector state activation, in order to limit CD8+ lymphocytes overstimulation [44, 48, 49].

However, when CD8+ T cells fail to eliminate cancer cells, many factors contribute to the CD8+ lymphocytes dysfunction [49]. The immunosuppressive TME including immunosuppressive cytokines (such as IL-10 and TGF-β), metabolic changes (enzymes expression such as indoleamine-2,3-deoxygenase, changes resulting from hypoxia,…), and immunosuppressive cells (such as MDSCs, TAMs and Tregs) dampen T cells responses. Cancer cells also evade CD8+ attack in several ways: by down-regulating their MHC class I molecules expression making them hidden for CTLs, by loosing some TA expression, by impairing the antigen presentation machinery, and finally by expressing inhibitory ligands such as PD-1 ligands (PD-L1 and PD-L2) [49]. Many mechanisms account for the increased expression of PD-L1, such as for example IFNs secretion or HIFs stabilization under hypoxia [6, 10, 48]. PD-L1 (and PD-L2)/PD-1 axis have so appeared to be a suitable target in order to reinvigorate T cells reactions, and monoclonal antibodies (Mab) able to block immune checkpoints have been developed, first tested in the pre-clinical setting and next introduced in clinics with a high success rate in various type of adult cancers [48]. Among these immune checkpoint inhibitors (ICI), nivolumab and pembrolizumab target the PD-1 molecule whereas atezolizumab blocks PD-L1; these ICI have first demonstrated clinical efficacy in

advanced forms of melanoma and some lung cancers and have been next introduced in the treatment of many other cancer types [60]. Nonetheless, responses are observed only in a small part of patients (20-40%) and are not always sustainable over time [60]. Biomarkers able to predict the individual patient benefit from anti-PD1/anti-PD-L1 therapy are needed, and few have been strongly established to date. Among them, PD-L1 expression on tumor cells (including cancer cells and APCs) is reported as a valid biomarker for response but is still imperfect [60]. Indeed, some PD-L1+ cancer do not respond, and other PD-L1 negative tumors respond well to PD-1/PD-L1 axis blockade [48, 61]. Another correlation has been established between a high somatic mutational burden and better ICI response [62]. Once again, despite this, some patients harboring tumors with a low mutation rate may respond to ICI [48]. One hypothesis in this case is that antigen recognized by T cells are self-antigen present on cancer cells, and ICI allow to remove the brakes of self-antigen recognizing T cell tolerance [48]. In conclusion, new biomarkers helping to predict patient response are strongly needed. To reach better responses rates among patients, ICI have been tested in combination to other (immuno)-therapies with promising results [63]. The combinatorial therapy could include strategies aiming to increase the mutational burden (radiation or tumor vaccination), epigenetic drugs, metabolism targeting drug or other immunotherapies (Mab, CAR T cell,,…). Recent studies tend to demonstrate that late-dysfunctional CD8+ lymphocytes (exhausted T cells) in the TME are not reinvigorated after ICI therapy, but instead of this, reprogrammable T lymphocytes (early dysfunctional T cell or other functional tumor-antigen recognizing T cells) replace exhausted CD8+ lymphocytes in the tumor bed accounting for the effect of ICI [49, 64]. However, more studies are needed to fully unravel the T cells origin accounting for ICI success (Fig. 13).

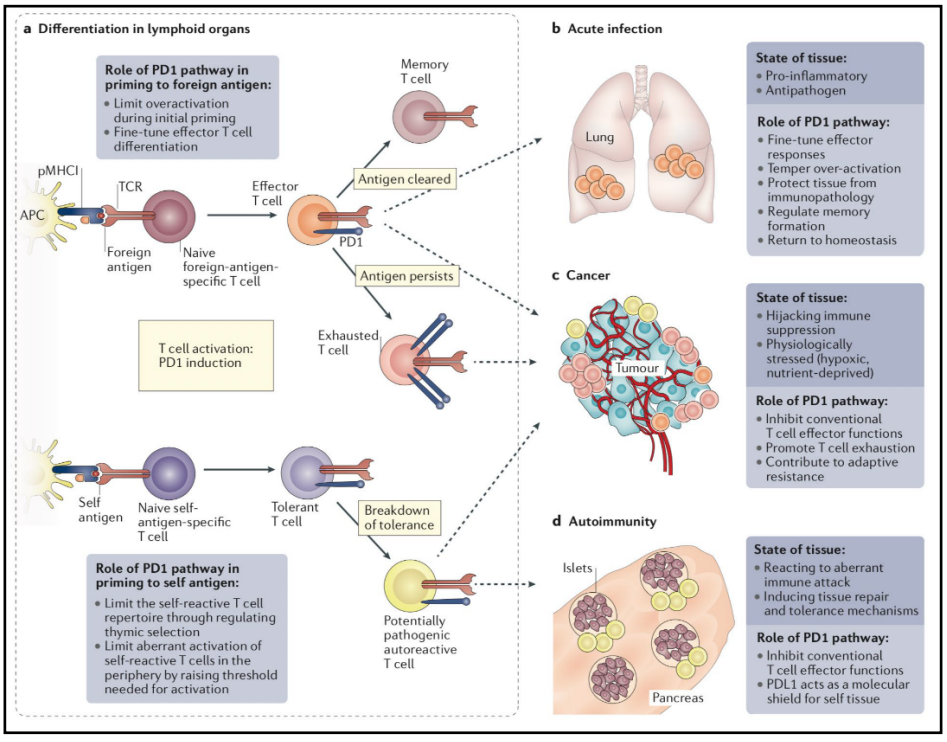

Fig. 13 illustrating the roles of PD-1 in acute infection, tolerance and cancer. From [48].

3.4 Neuroblastoma, immunity and immune escape mechanisms

Clinical observations have highlighted the importance of immune system in NB and evidenced that NB cells can elicit immune responses in host.

First, some patients present with a particular form of NB called opsomyoclonus syndrome (OMS) [12]. This paraneoplastic syndrome is due to anti-neural antibodies that recognize not only tumor cells but also normal neural cells leading to neurologic disabilities [12, 65]. These tumors are immune infiltrated (including B and T lymphocytes infiltration, and the presence of lymphoid follicles containing FDCs and macrophages) demonstrating an immune response against the disease [12, 65, 66]. Patients with OMS harbor an excellent oncologic prognosis but can keep severe sequelae resulting from the anti-neural auto-immunity [12, 13, 21, 67]. Secondly, spontaneous regression is often observed in infants presenting a MS (or 4S) stage NB [21, 67]. It seems that immune system could play an important role in this evolution, as the immune cell landscape of tumors from these young children is different compared to older ones harboring non self-regressing disease but the precise role of immune system in 4S regression is not yet fully understood [4, 21, 67]. Thirdly, immunological studies have demonstrated that NB are infiltrated by immune cells, and some correlations between lymphocyte infiltration, their function, *MYCN* amplification status and the patient prognosis have been underlined [4]. Finally, pre-clinical studies including immunotherapeutic agents have given promising results, and clinical trials including anti-GD2 monoclonal antibodies has led to substantial progress in overall survival of NB patients *(see immunotherapy chapter)* [65].

However, to progress, NB cells need to induce immune tolerance and escape the immune attack [4, 26]. Uncovering their immune escape strategies will certainly contribute to therapeutic progress. Further studies are needed to gain more insights into the immune interplay between NB cells and host immunity; deep understanding of 4S spontaneous regression and OMS pathogenesis will probably help to precise the mechanisms involved. Immunotherapy takes part of the standard treatment of HR forms, but relapses are frequent and the overall survival remains less than 50%. In the next future, combination therapies will probably help to avoid immune resistance, and immunotherapies aiming to develop immune memory would help to prevent relapses [4].

3.4.1 Immunogenicity of NB and TA: the low mutational burden of NB

NB tumors are poorly immunogenic and harbor few neo-antigens [4]. TA having an impact on immunotherapeutic drug development include GD2, B7H3, ALK, survivin and

tyrosine hydroxylase (TH) [26].

3.4.2 The immune landscape of NB tumors

Immunological studies of human NB tumors are not easy to conduct as NB is a rare disease and as primary tumor biopsy is often performed after chemotherapy treatment, skewing the analysis of immune cells landscape. Indeed, immune cells are impacted by the heavy chemotherapeutic treatment and few living cellular material is available.
Moreover, discrepancies are founded in the literature due to the different techniques used to assess tumor immune cell content and/or function and to the small number of samples available.
Nonetheless, some results indicate that TILs are present in NB tumors. Lymphocytes include T CD4+ and CD8+ cells, NK cells, but also NKT, iNKT cells and γδ T cells; B cells are not preponderant [4]. TIL density correlates with the patient outcome and stage of the disease: patients having higher CD3+ TIL infiltration have better prognosis [4]. The correlation between TIL density and disease stage is still poorly understood; tumor differentiation may play a role, as it accounts in part for disease stage and may impact tumor immunogenicity. For example, undifferentiated NCCs do not express Class I MHC molecules and thus are less immunogenic, possibly leading to less TIL infiltration [4].
Subsets of TILs infiltrating NB tumors have also been studied, but conflicting results are reported [4]. Prognosis have been reported to correlate with the CD4+ / CD8+ intratumoral ratio and with the CD4+ T cells subsets (Th1 versus Th2) present in the tumor, but opposite results arise from different studies [4]. Tregs usually induce immunosuppression and thus are associated with poor prognosis in cancer [4]. However, in NB, better outcome has been correlated with higher FOXP3 expression at diagnosis [4]. This seems counterintuitive as Tregs exert immunosuppressive functions, but in this case, FOXP3 expression possibly reflects Tconv activation and not the presence of Tregs; one could also hypothesize that higher FOXP3 expression could reflect higher T cell infiltrates in tumors in general, which often correlates to good prognosis [4].
MYCN amplification seems to correlate with reduced tumors immune infiltration but also with immune cells decreased cytotoxic activity [4, 68]. As *MYCN* amplified tumors often have a low mutational burden, their immunogenicity is reduced and could account at least partly for their low immune infiltration. In addition, *MYCN* amplified NB tumors produce less cytokines possibly giving another explanation for the low TILs presence in these tumors [4]. More studies are needed to precise the correlation and the mechanisms implicated in low immune infiltration in these cases [4].
If the presence of TILs in NB tumors is well established, much less is known about their

function and their specificity for NB cancer cells [4]. Some arguments advocate for a reactivity of TILs against NB cells [4]. First, a small number of untreated patients present differences in the TCR clonality between circulating lymphocytes and TILs, with reduced clonality in TILs TCR suggesting their expansion in response to specific antigens [4]. Secondly, higher activation and proliferation markers expression on TILs have been reported in some studies but these data are not confirmed by other ones; moreover, TILs presented less frequently a naïve phenotype and were predominantly effector memory cells in the TME compared to blood circulating cells [4]. However, as a subset of T cells reside in different human tissues, it's not clear as effector memory T cells in NB are specific for tumor antigens [4]. Thirdly, the importance of NK and CD8+ cells cytotoxic activity have been evidenced in clinical and pre- clinical studies; especially, TILs cytotoxicity may be enhanced with appropriate cytokine stimulation, which may have an interest for NB immunotherapy [4]. Lastly, in *MYCN* non- amplified NB tumors harboring a high *MYCN*-signature at diagnosis, higher expression of exhaustion markers and notably higher expression of CTLA4 and PD-1 were associated to enhanced cytolytic immune signatures [4, 68]. The higher immune checkpoint expression in this case contributes to an immunosuppressive TME limiting the benefit of the TILs infiltration and their cytotoxic activity [68]. These patients may probably benefit of ICI therapy, as TILs are present, have a higher cytolytic activity, but are exhausted [68].

3.4.3 Immune escape strategies in NB

Similar to other cancer cells, NB cells have developed a set of immune escape strategies allowing tumor growth and metastatic lesions development often leading to patients' death in the HR forms. These escape strategies include the decreased or on the contrary the increased expression of surface molecules activating or inhibiting respectively receptors on immune cells, metabolic and enzymes expression changes in NB cells impacting the metabolites present in the TME and more broadly in blood, and the release of soluble factors (including proteins such as cytokines and chemokines but also gangliosides) impacting the immune cells TME landscape and functions.

A first strategy developed by NB cells to avoid immune attack is to render themselves hidden for T and NK cells.

TA peptides elicit T cells responses only if they are loaded on MHC molecules and presented in this way to effector T lymphocytes. Therefore, NB cells often dampen their expression of MHC class I molecules, rendering them invisible by circulating T cells and leading to poor immune infiltration [21, 26, 44, 45, 65, 68]. Moreover, the expression of antigen processing machinery molecules such as TAP-1 or LMP-7 and -2 is also downregulated, contributing to a decreased immunogenicity [21, 26, 44, 45]. NB cells

are also able to express immune checkpoints such as CD200 and PD-L1, dampening the T lymphocytes responses [4, 69].

Decreased MHC class I molecules should elicit NK cell response against NB cells, but cancer cells have developed strategies to reverse the balance between activating and inhibitory signals in favor of dampening the NK cell attack: inhibitory signals (such as B7H3) on cancer cells are expressed whereas they down-regulate their activating ligands expression (such as MICA-A, MICA-B, ULBP 1 and 3 and DNAM-1 ligands) [26, 44, 45, 65].

NB cells and immune cells infiltrating the tumor also secrete soluble factors including cytokines and chemokines but also gangliosides dampening the immune cancer responses and impacting the TME immune landscape [26, 44].

For example, among immunosuppressive cytokines, we can cite macrophage migration inhibitory factor (MIF) that has been reported to be overexpressed and secreted by NB cells and to correlate with poor prognosis from of the disease [70]. In NB, MIF contributes to dampen T cell immune responses [70]. TGF-β1 derived from NB cells and from monocytes and IL-6 derived from monocytes impairs NK cells IL-2 activation decreasing their cytotoxic activity, their ability to perform ADCC and the IFNγ secretion [71].

Glycan-binding protein galectin-1 (gal-1) is expressed by NB cells and exerts immunosuppressive function on effector T cells, induces T cell apoptosis and impairs DCs maturation and function so contributing to downregulate immune responses against NB cancer cells [26, 44, 72].

The soluble form of the oncoantigen GD2 (sGD2) is also produced by NB cancer cells and impacts DCs function dampening MHC class II and co-stimulatory molecules expression on their surface, reducing immunostimulatory cytokines production and increasing immunomodulatory cytokines production [4].

HLA-G molecules belongs to HLA class Ib family and are known to be expressed by a vast majority of cancer cells [26, 44]. In NB, cancer cells instruct blood monocytes to produce the soluble form of HLA-G (sHLA-G), resulting in higher plasmatic concentrations of sHLA-G in NB children, levels that were correlated with the occurence of relapse [73, 74]. sHLA-G dampens the NK cells and CTLs activity notably by leading to cytotoxic effectors apoptosis [73, 74].

MHC class I chain-related gene A protein (MICA) is released by NB cells as a soluble (s) form, s-MICA, and has been found in NB patients serum [26, 44, 45]. s-MICA has immunosuppressive properties, as it decreases NGK2D expression on circulating T cells surface and dampens the NK cells action against MICA positive NB cancer cells [45].

Neuroblasts express B7-H6, the ligand of the NKp30 receptor on NK cells, and the shed B7- H6 contributes to the downregulation of NKp30 receptor expression on NK cell surface and so dampens NK cells responses, representing an additional immune escape

mechanism [75]. Vanichapol *et al* have reported that high-mobility group box 1 (HMGB1) expression is up- regulated *in vitro* in the SK-N-SH cell line and this results in Treg differentiation induction. This overexpression was also found in 11% of patients listed in the NCBI GEO database and correlates to poor overcome [76]. Among other tumor-promoting roles, HMBG1 could be a major player in the NB immunosuppressive TME by its Tregs differentiation promoting activity [76].

Through arginase II overexpression, and the resulting local but also systemic arginine depletion, NB cells are able to suppress T lymphocytes proliferation and activation as well as myeloid cells activity [77]. This mechanism could at least accounts in part for the lymphopenia often observed in NB children harboring bulking masses at diagnosis (prior to any treatment) and obviously contributes to the impaired anti-NB immune responses, even those resulting from adoptive Chimeric Antigen Receptor (CAR)-T cell therapies (*see below*) as they also require arginine for their activity [77].

In addition to exert direct effects on immune cells functions, cytokines and chemokines in the TME also contribute to attract regulatory and immunosuppressive cells in the TME, mainly represented by TAMs, MDSCs and Tregs [28]. These cellular populations presence in NB tumors correlate to poor prognosis forms of the disease [28].

Circulating monocytes, after being attracted in tumors by different chemokines such as colony stimulating factor 1 (CSF1), CCL2 and CCL20, differentiate into TAMs and polarize into M1 or M2 phenotype [26, 28]. To note, hypoxia favor the production of these attractant cytokines [28]. Dismal prognosis metastatic NB patients often harbor tumors infiltrated by M2-polarized TAMs, which exert immunosuppressive function, in contrast to good prognosis forms, which often show immunostimulatory M1 TAMs infiltrated tumors. The tumor- promoting roles of M2 TAMs results from their ability to sustain angiogenesis, to favor metastases by enhancing cancer cell invasion and by preparing metastatic sites, and by blocking T cells and NK cells function and favor Tregs and MDSCs attraction and activation [26, 65]. To do so, they produce immunosuppressive cytokines (IL-6, IL-10 and TGF-β), VEGF, MMPs, enzymes such as arginase, indoleamine dioxygenase and iNOS and express immune checkpoint ligands such as PD-L1 [28]. To note, hypoxia but also non-oxygen dependent HIF-1 and HIF-2a stabilization favor M2-polarization of TAMs [28]. Fig. 14 summarizes the immunosuppressive activity of TAMs.

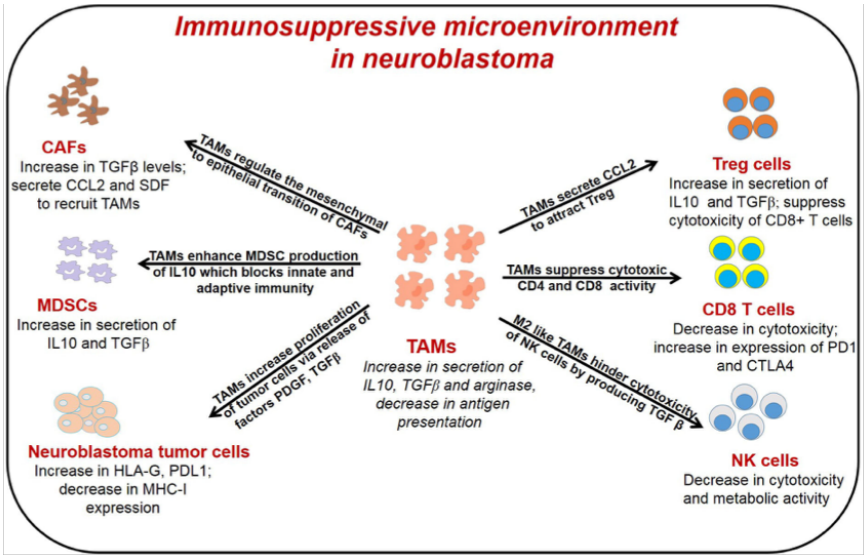

Fig. 14 illustrating the immunosuppressive activity of TAMs. From [28].

MDSCs are attracted in the NB TME by different chemokines such as CXCL1, CCL7 and CCL2. They exert their immunosuppressive properties by several mechanisms, resulting in impaired CD4+ and CD8+ actvity, as well as NK cells and DCs activity [28, 44]. T cell proliferation and function are impacted by the depletion in essential amino acid surch as L- arginine or by ROS and NO. IL-10 and TGF-β produced by MDSCs favor Tregs but dampen NK cells activation and function [28, 44]. MDSCs also impact the differentiation as well as the migration of DCs and doing so, impaired CD8+ lymphocytes activation [28, 44]. Tregs in NB express CTLA-4 and contribute to APCs maturation inhibition; moreover, they produce immunosuppressive cytokines (notably IL-10 and TGF-β) and are able to kill effector T lymphocytes by mechanisms involving granzymes and perforins (Fig.15) [28, 44].

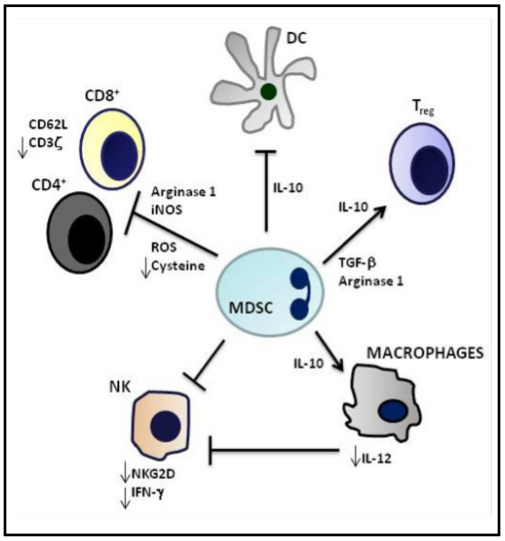

Fig. 15 illustrating the immunosuppressive actions of MDSCs. From [44].

Immune escape strategies in NB are summarized in Fig. 16.

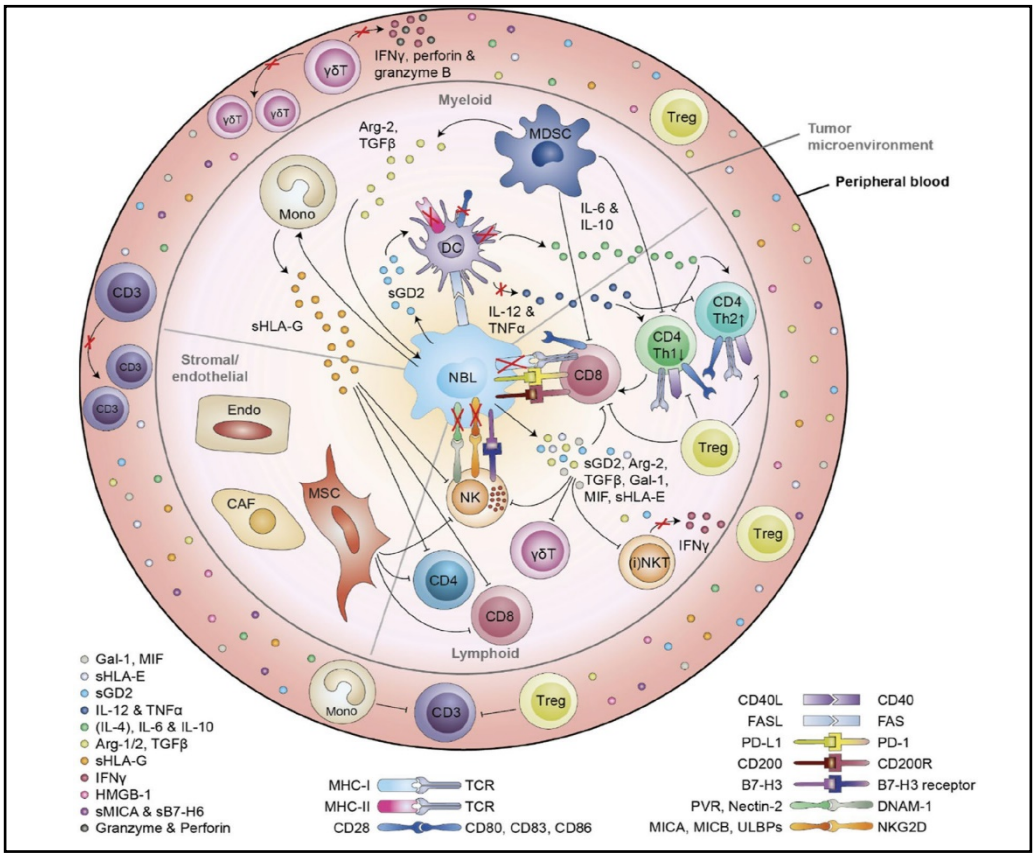

Fig. 16 summarizes the complex immune escape mechanisms encountered in NB. From [4].

3.4.4 PD-1/PD-L1 axis in NB

The importance of the PD-L1/PD-1 axis in pediatric solid tumors and in particular in NB has been the subject of several studies over the past recent years. However, these studies have led to conflicting conclusions [78].

Aoki *et al* have reported low PD-L1 expression in pediatric malignancies, including neuroblastoma [79]. On the other side, recent publications demonstrate that PD-L1 is expressed in NB [69, 78, 80-82]. The discrepancy in PD-L1 expression in NB studies could results from several issues. First, technical reasons could be an explanation as different techniques have been used to detect PD-L1, and even when assessed by the same technique, the reagents used could be different (for example, different antibodies are used for Fluorescence activated cell sorting (FACS)) [83]. Secondly, PD-L1 expression is not a static process and evolves over time, responding to stimuli produced in the TME [84], such as for example IFN$\gamma\gamma$ which up-regulates PD-L1 expression [69].

In addition, PD-L1 expression represents a valuable NB prognostic biomarker. Indeed, Melaiu *et al* have reported the important prognostic value of PD-L1 combined to HLA-1 expression assessment [78] and Majzner *et al* reported that PD-L1 is expressed in a consistent proportion of NB (14%) and correlates with poor survival for HR patients [82]. Zuo *et al* have also reported a tendency to worse prognosis in PD-L1 positive NB patients; moreover, they were the first to compare the PD-L1 expression status before and after chemotherapy in a small number of patients [83]. Patients with a positive status before and after treatment clearly had a worse outcome that PD-L1 negative remaining ones [83]. Of note, no significant correlation has been observed between *MYCN* amplification status and PD-L1 expression [69], but Melaiu *et al* reported that *MYC* and *MYCN* are involved in PD-L1 expression regulation, suggesting that targeting the two main oncodrivers *MYC* and *MYCN* could target one of the NB immune evasion mechanism [78]. Remarkably, PD-L2 is rarely expressed by NB cells [69].

Immune effectors cells in NB also participate to the immune evasion through the PD-L1/PD- 1 axis. Indeed, HR patients tumors present a high percentage of PD-1 positive CD8+ TILs [81]. In addition, PD-L1 and PD-L2 positive macrophages and DCs have been observed, contributing to dampening the immune reaction against cancer cells [69]. So, even if NB cancer cells are PD-L1 negative, patients could benefit from ICI targeting the PD-L1/PD-1 axis [69].

Pre-clinical evidence for a benefit of blocking PD-L1/PD-1 axis in NB has been recently underlined in some studies [85, 86]. Mao *et al* have demonstrated a synergistic activity and a drastic tumor reduction in TH-*MYCN* mice receiving a combinatorial therapy associating BLZ945 (a blocker of the macrophage colony-stimulating factor receptor CSF-1R on myeloid cells) and PD-1/PD-L1 targeting antibodies [85]. To note, ICI alone was not sufficient to delay tumor progression [85]. Siebert *et al* showed an up-regulation of PD-L1 on NB cells and of PD-1 on effector cells after anti-GD2 Mab treatment, and combining anti-GD2 and ICI led to synergistic effect [86]. Based on these results, two relapsed refractory NB patients benefited from anti-GD2 (dinutuximab beta) and anti-PD-1 Mab (nivolumab) combinatorial therapy leading to complete remission in one patient and a good partial response in the other one [87].

In conclusion, new pre-clinical studies to assess the efficacy of combining PD-1/PD-L1 axis blockade with other immunotherapeutic drugs or small inhibitory compounds need to be conducted. In this way, new therapeutic schedules will emerge and enter clinical trials in order to improve the overall disastrous prognosis of HR NB patients.

3.5 Immunotherapy in cancer

Immunotherapy for adult cancers has led to substantial progress in survival and continue to raise great interest, notably with combinatorial treatments [63]. The major aim of immunotherapy is to reactivate or increase existing immune responses against

cancer cells, or to induce them [52]. Both innate and adaptive immune responses can be enhanced leading to anti-tumor effect: indeed, innate immunity stimulate adaptive immunity leading to specific responses and potentially to memory immunity against the tumor disease. Immunotherapy for pediatric patients is challenging but arouse still more interest as great progress has been achieved notably for patients harboring NB and leukemia or lymphoma [52].

First, pediatric tumors are less immunogenic than adult cancers, as they have a low mutational burden [52]. Tumor neoantigens are scarce, but progress in technologies and particularly in sequencing will extend the field of targetable molecules in the next future, with a more personalized medicine [52]. Nonetheless, under the immune pressure, cancer cells can develop new immune escape strategies, and among these, antigen loss could represent a limitation to target immunotherapy long-term efficacy [52]. Secondly, toxicities resulting from immunotherapy such as auto-immunity and cytokine release syndrome and their management need to be carefully assessed in children [52]. Thirdly, immunotherapies and notably adoptive cellular therapies are not available in all care centers and extend technology facilities could be very expensive [52]. Finally, how to include immunotherapy in the standard of care of pediatric tumors has still to be determined: the right timing to administrate this new class of drugs is still the source of debate [52]. Tumor volume but also immune-depression resulting from conventional cancer treatments can influence immunotherapy responses and have to be taken into account [12].

The mechanism of action of each class of immunotherapeutic compounds will be next briefly described, as well as their potential role in NB therapy.

3.5.1 Immunotherapeutic agents and immunotherapy in NB

In the field of NB, the disialoganglioside GD2 represents a fundamental target for immunotherapy and has led to strong therapeutic progress [65]. It is an oncofoetal differentiation antigen that belongs to the glycolipids family [65]. Its expression takes place during fetal life and after is restricted to central neurons, peripheral nerves and melanocytes (accounting for some of the side effects of anti-GD2 mABs therapy such as pain, *see below*) [65]. GD2 is also present at the NB cell surface in primary and metastatic lesions and leads to cellular proliferation and migration via MEK/ERK and PI3K/AKT signaling pathways [65]. It is often expressed at high density on NB cells and almost no antigen loss has been observed, making it suitable for immunotherapy [65]. As GD2 is not a protein molecule, it can not be processed to be loaded on classic MHC molecules to stimulate T cell immunity, but as a carbohydrate antigen it can be targeted by antibodies [41, 65]. GD2 represents also an attractive molecule for CAR-T cell therapies *(see below)* [65].

A broad spectrum of immunotherapeutic agents have been developed and can be classified into 3 large groups: vaccines, monoclonal antibodies (Mabs) and adoptive cellular therapies [12, 52].

1. Vaccines

« Vaccines » include different approaches, ranging from the administration of tumor peptides simultaneously with adjuvants, to DCs vaccines [12]. DCs vaccines are obtained when immature DCs are primed ex-vivo with tumor antigen (tumor cell lysates or dying tumor cells or tumor RNA), leading to their maturation rendering them able to present tumor peptides loaded on class I or II MHC molecules [12]. Another technique use mature DCs incubated with antigen peptides which bind to MHC molecules directly [12].

Vaccines can dampen tumor growth or have a role in preventing relapses, but are often unable to lead to bulky masses regression [52]. Vaccination therapy has been tested in clinical trials for NB patients, and some studies are still on-going; nevertheless, no long-term benefit has been demonstrated until now [26].

2. Mabs

Mabs exert their anti-cancer activity by several mechanisms [12]:

- They can induce apoptosis by binding a cell signaling receptor;
- They can compete for binding to a growth factor receptor depriving the cancer cell from its survival signal or bind the growth factor itself;
- By coating the cancer cell, they can induce ADCC as their Fc fragment can activate Fc receptors on innate immune cells;
- They can activate the complement system by binding to cancer cell surface leading to cell death due to the MAC assembly (complement-mediated cytotoxicity, CMC); as the opsonins produced are then deposited on cancer cells and recognized by complement receptors on NK cells, neutrophils and macrophages, complement-dependent cellular
  phagocytosis (CDCP) and complement-dependent cellular cytotoxicity (CDCC) can also occur;
- They can be coupled to a (pro-) drug, a radioisotope or a toxin, delivering this active agent at the tumor site and thus lowering systemic side effects;
- They can block immune checkpoints, releasing the brakes for immune cell activation;
- They can bridge cancer cells to immune cells, on onse side by binding to a specific antigen on the cancer cells and on the other side by binding and recruiting immune cells (bi- or - tri- specific antibodies engineering).

In NB, immunotherapy with anti-GD2 mABs has revolutionized the standard treatment and led to a substantial progress in overall survival [65]. Several mABs targeting GD2 have been developed [65]:

- The mouse IgG3 3F8 and 14.18;
- Dinutuximab (ch14.18), which results from the switching of 14.18 to IgG2a class and the chimerization with human IgG1-Fc;
- Dinutuximab-β (ch14.18/CHO) resulting from the production of ch14.18 in Chinese hamster ovary cells.

Mouse IgG3 mABs act mainly by CMC mechanism, whereas human IgG1 mABs exert their activity mostly by ADCC [65]. The two families have impacted positively the survival in clinical studies, and now Dinutuximab is integrated in the standard of care of HR NB patients [65]. It has been tested in combination with GM-CSF and IL-2, in order to stimulate innate effector cells and increase its efficacy, but IL-2 has not demonstrated additional effects, suggesting that maybe ADCC from NK cells is not the leading mechanism of action [65]. Now, Dinutiximab is given in combination with 13-cis-retinoic acid and in some care centers with Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) in the post-consolidation phase of NB treatment [65]. To note, Mabs anti-GD2 therapy has to be given in the minimal residual disease setting as it is not efficacious on bulky masses [65]. Nonetheless, combination with chemotherapy induction is tested in clinical trials and could contribute to initial responses and impact survival [65].

Side effects include pain, fever and possible anaphylaxis [65]. Moreover, antibodies directed against the murine or chimerized Mabs can develop and abolish their activity [65]. In order to circumvent these issues and reduce side effects, new humanized anti-GD2 mABs have been produced and are being tested in clinical trials [65].

Conjugated anti-GD2 Mabs also arouse great interest [65]. Pre-clinical studies are on-going with anti-GD2 Mabs coupled to liposomes in order to selectively deliver anti-cancer drugs to NB cells; in this perspective, pegylated anti-GD2 immunoliposomes loaded with YM155 (a survivinn inhibitor) or with etoposide have given promising results [65]. Clinical trials with anti- GD2 Mabs labeled with ^{131}I have revealed poor pharmacokinetics profiles, but intrathecal administration for central nervous system (CNS) metastasis leads to substantial antitumor responses [65]. Finally, cytokines fused to the heavy chains of anti-GD2 Mabs aim to avoid systemic side effects by delivering these mediators at the tumor site [65]. Responses observed in preclinical animal models were better when these conjugated compound were intratumoral administrated compared to systemic injection [65].

Bispecific antibodies anti-GD2 x anti-CD3 thus targeting GD2 and recruiting T cells bypassing the MHC restriction issue are now tested in phase I/II clinical trials [65].

In addition, combinatorial immunotherapies including anti-GD2 Mabs with molecules

able to fight the NB immunosuppressive TME are now tested in pre-clinical and clinical trials with promising results [11]. Indeed, immune checkpoints engagement on innate immune effectors dampen ADCC activity [65]. CTLA-4 and PD-1 are part of these inhibitory signaling pathways [65]. ICI monotherapy has given disappointing results, with no observed tumor regression and even progression under treatment [11]. However, preclinical studies combining immunotherapies or immunotherapy with drugs targeting cancer induced immunosuppression mechanisms appear to act synergistically [85, 86, 88]. This has led to clinical trials testing ICI combinations or anti-GD2 Mabs and ICI combinatorial therapies. Results from these studies are currently awaited.

Other NB targets raise great interest. Among them, B7-H3, also overexpressed in primary and metastatic NB tumors, has led to the development of Omburtamab [65]. As liver toxicity resulted from its systemic administration, radionucleide conjugated-Omburtamab is now injected by intrathecal via Omaya reservoir and shows efficacy against CNS metastases [65]. Enoblituzumab, another B7H3 targeting Mab, has no liver toxicity [65]. Bispecific antibodies B7-H3 x CD3 are also being investigated [65].

3. Adoptive cellular therapies

a) T-cell therapy

T cell therapy aims to deliver tumor-specific T cells to the patient, with increased function and number [52]. T cell therapy have demonstrated its efficacy even in presence of extended tumors, and long-term effect has been observed [26, 52].
T cell- therapy include [52]:

- TILs previously harvested and expanded ex-vivo, that can be reinfused to the patient, increasing the preexisting immune response;
- Tumor antigen specific CTLs could also be selected and expanded ex-vivo and then given back to the patient; this approach has led to progress in the treatment of EBV- associated lymphomas and post-transplant lymphoproliferative disease (PTLD);
- Autologous T cells can also be harvested and genetically modified, to express a TCR specific for a tumor antigen. Then, lymphocytes are given back to the patient after lympho depleting treatment in order to favor engraftment. However, this technique
  implies a MHC restricted recognition limiting its use as many cancer cells dampen their MHC-I molecules expression to escape immune attack. Alternatively, CARs have been genetically engineered: these molecules contain an extracellular domain similar to the single-chain fragment variable (scFv) of Ig and able to bind to specific cancer antigen, and an intracellular signaling element leading to the T

cell activation, bypassing the need for MHC presentation and co-stimulation signals. CARs are classified into first, second and third generation as they have CD3ζ, CD3ζ and CD28 or 4-1BB, or CD3ζ and two co-stimulatory signals as intracellular domain respectively (illustrated in Fig. 17).

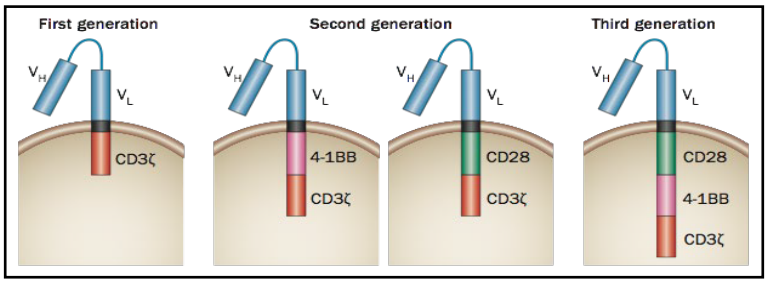

Fig. 17: CARs. From [52].

b) NK-cell therapy

NK cell adoptive therapy uses autologous NK cells previously expanded ex-vivo or allogenic NK cells in the setting of a haploidentical stem cell transplant; in this case, selection of mismatch for KIR-KIR ligands could increase tumor immune attack [26]. CARs expressing NK cells have also been genetically engineered, by-passing the effect of inhibitory or activating molecules expressed on cancer cells [26]. Studies are still on-going [26].

4. Hypoxia in cancer

Hypoxia represents a common hallmark of most solid tumors and correlates with unfavorable prognosis [89]. Fast growing masses need higher oxygen and nutrients supply but often harbor a chaotic vasculature not able to fully encounter their requirements, leading to a low oxygen pressure in some tumor areas [35, 37, 38]. All hypoxic cells in the TME face these issues by reprogramming their metabolism and changing their gene pattern expression, notably through Hypoxia-Inducible-Factors (HIFs) activity which influences many process involved in tumor progression [38].

4.1 HIFs family members and their regulation

HIFs transcription factor family is the major signaling mediator of hypoxia [38]. It is represented by three isoforms: HIF-1, HIF-2, and HIF-3 [38]. Each isoform is composed by a constitutively expressed HIF-1β subunit (also named ARNT for aryl hydrocarbon receptor nuclear translocator) and an α subunit (respectively HIF-1α, HIF-2α or HIF-3α), which is regulated in a O_2-dependant manner [38].

HIF-1 is the main mediating factor to adapt to tissue oxygen level [90]. Its level mainly depends on post-translational regulation and is notably affected by oxygen availability as illustrated in Fig. 18 [38].

Indeed, under normoxic conditions, proline (P) and asparagine (N) residues on the oxygen- dependent degradation domains (ODD) and transactivation domains (TAD) respectively are hydroxylated by the proline hydroxylases (PHD) activity [38]. Of note, PHD need oxygen as substrate, whereas they use 2-oxoglutarate and ferrous ions (Fe^{2+}) as co-factors [38]. The main PHD isoform playing a role in HIF-1α hydroxylation is PHD2 [91]. After asparagine residues hydroxylation, the co-activator P300 cannot bind to the TAD anymore whereas the von Hippel-Lindau protein (pVHL) binds to the hydroxylated proline residues, recruiting an E3 ubiquitin-ligase finally resulting in the ubiquitination and proteosomal degradation of the HIF-1α protein [38]. Moreover, in normoxia, factor inhibiting HIF (FIH) also mediates HIF-1α TAD hydroxylation, impairing the interaction with transcriptional co-activators and so preventing HIF target genes expression [91].

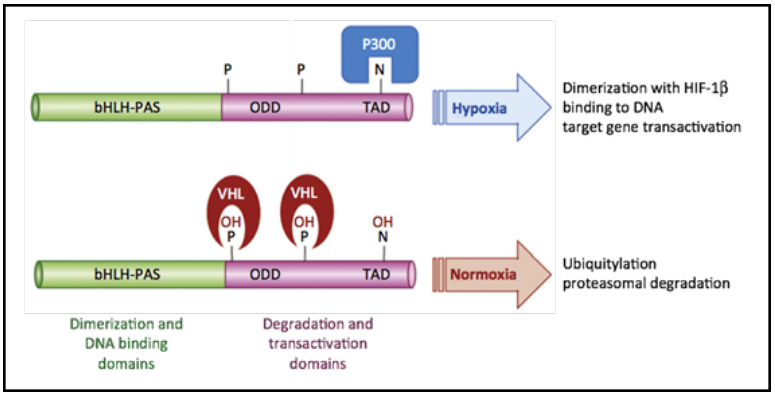

Fig. 18: HIF-1α oxygen-dependent hydroxylation. From [38].

On the contrary, under hypoxia, hydroxylation does not take place and the co-activator P300 can be recruited; the stabilized HIF-1α subunit can form dimer with HIF-1β through the basic- helix-loop-helix-PER-ARNT-SIM homology (bHLH-PAS) domain activity, allowing the binding to the hypoxia responsive element (HRE) in the promoter of HIF target genes and finally leading to their expression [38]. Under prolonged hypoxic conditions, however, a natural antisense HIF controls its mRNA level [38]. Of note, other mechanisms account for HIF-1 expression and activity. Indeed, oxygen-independent mechanisms also contribute to HIF-1 expression: for example, growth factors (such as insulin growth factor-1 (IGF-1), hormones (such as insulin), inflammation molecules (such as TNFα (tumor necrosis factor α) or oncogenes induce HIF-1α expression and subsequently its target genes transactivation [38, 91]. The majority of these oxygen-independent regulatory mechanisms finally converge to MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase)/Akt pathways [91]. Therefore, HIF-1 expression and stabilization reflects tumor hypoxia but can also been observed under normoxic conditions, a state commonly called « pseudo-hypoxia » [6].

Recently, epigenetic events interfering with HIFs stability and transcriptional activity have been identified as a third regulation mechanism. For example, notably in different NB cell lines, HIF-1 up-regulates the expression of ten-eleven-translocation 5-methylcytosine dioxygenase (TET) 1 enzyme, which is responsible for the increase of 5-hydroxy- methylcytosine (5-hmC) in regulatory regions of HIF specific genes, facilitating their expression under hypoxic conditions [92]. Other similar epigenetic mechanisms contributing to the increase of HIF-induced transcriptional program occur in a HIF-dependent manner: jumonji domain (JmJ) histone demethylases induces histone modifications and chromatin remodeling in HIF-regulated genes such as Carbonic Anhydrase (CA) IX or Pyruvate dehydrogenase kinase 1 (PDK1) leading to their increased expression under hypoxia; interestingly, JmJ histone demethylases belongs to HIFs targets [92]. Therefore, HIF can transcriptionally induce enzymes involved in epigenetic regulation of HIF target genes transcription [92]. Uncovering all

the epigenetic pathways implicated in hypoxia and HIFs responses could positively impact cancer therapies. For example, retinoic acid (RA) takes part in the standard maintenance therapy of HR NB but fails to have a great anti-tumor activity. However, when RA is combined to the DNA-demethylating compound 5-aza- deoxycytidine, HR NB tumors growth is impaired [93, 94]. In this case, HIF-2α transcriptional program seems to be increased, linking HIF-2α to a tumor suppressor role in the context of NB [93, 94]. HIF-2α regulation occurs in an oxygen-dependent manner similar to HIF-1α. On the other side, HIF-3α is regulated at the transcriptional level; it has been demonstrated that due to alternative splicing, different HIF-3α variants are generated and could exert opposite function in hypoxic condition [95].

4.2 HIFs target genes and regulation of cancer progression mechanisms

Hundreds of HIF direct target genes have been identified using genome wide chromatin immunoprecipitation combined with DNA microarray (ChIP-chip) or DNA sequencing (ChIP- seq) [96, 97]. HIF-1 binds to the HRE in the enhancer or promoter regions of its target genes [98-100]. HIF-1α and HIF-2α share structurally similarities in their bHLH-PAS domains but present differences in their TAD [101]. So, they have common target genes but also regulate a unique set of genes [102]. HIFs are involved in many process linked to cancer progression, such as angiogenesis, metabolic shift, extracellular matrix degradation, epithelial-to-mesenchymal transition, invasion and metastasis, cellular stemness maintenance and tumor immune escape (Fig. 19) [38].

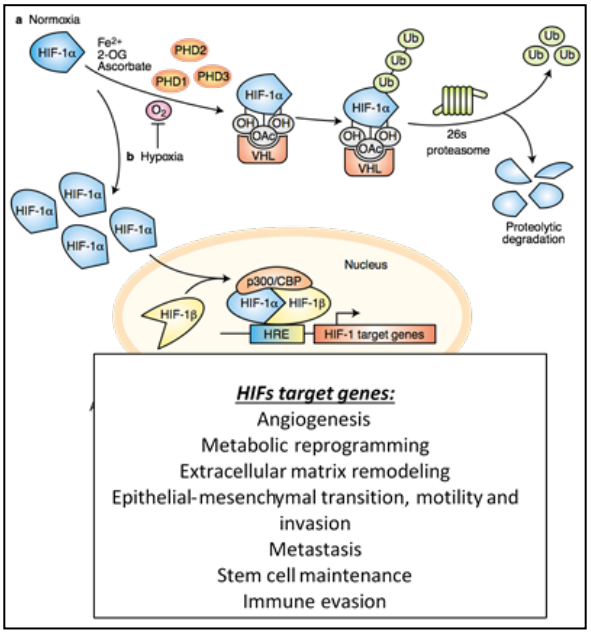

Fig. 19: HIF-1α proline oxygen-dependent hydroxylation. Adapted from [38] and [91].

4.3 Hypoxia, HIFs and angiogenesis

The main angiogenesis genes regulated by HIFs include VEGF-A and CXCL12 [38]. As angiogenic factors in cancer are produced in excess, this results in poor quality vessels, presenting structural and functional defects, and so contributing to poor tumor perfusion [38]. Strategies targeting VEGF-A or its receptor have been clinically tested, but present some limitations; indeed, impairing angiogenesis could lead to more hypoxia resulting in increased HIFs activation and generating more pro-angiogenic factors [38]. Combinatorial therapies aiming to block VEGF-A and its receptor and HIFs could in this context be more promising [38]. In NB, bevacizumab alone has not provided substantial results, but combinatorial therapies with immunotherapies and/or chemotherapy could impact NB outcome; however, (pre)-clinical studies are still ongoing [65, 103].

4.4 Hypoxia, HIFs and metabolic reprogramming

One important aspect of the metabolic shift observed in cancer cells concerns the decreased oxidative metabolism and increased glycolytic pathways, mainly orchestrated by HIFs [35]. HIF-1 targets include glucose transporters such as GLUT-1, allowing increased cellular glucose uptake, which is needed for glycolysis [104]. Glycolytic enzymes are also up-regulated, such as lactate dehydrogenase A (LDH-A) which converts pyruvate to lactate, avoiding the transformation of pyruvate into acetyl coenzyme A (acetyl-CoA) [89, 104]. PDK1 is also up-regulated in response to HIF-1 and it phosphorylates pyruvate dehydrogenase (PDH)-E1α, leading to its inactivation and also accounting for the decreased acetyl-CoA generation [89, 104]. The reduced level of acetyl-CoA impairs mitochondrial function and in particular the tricarboxylic acid cycle (TCA) as Acetyl-CoA is the first metabolite involved in this cycle [89, 104]. HIF-1 also decreases other essential mitochondrial enzymes activity through diverse mechanisms, such as induction of miRNAs expression or by promoting mitophagy (mitochondrial autophagy) [104]. The lactate and protons overproduction induced by this metabolic shift and the potential pH dysregulation is also controlled by HIF-1 as it promotes the expression of ion exchange transporter or pH regulatory enzymes, such as CAIX [39]. CAIX contributes to the acidification of the extracellular milieu while maintaining an increased intracellular pH [39]. Extracellular acidosis leads to the death of surrounding cells, giving an advantage to cancer cells, and promotes tumor progression in several ways such as for example extracellular matrix remodeling or inhibition of immune cells infiltration or activation [39, 105]. As CAIX expression occurs almost exclusively in tumor tissues and not in normal ones, its inhibition has aroused interest, with promising results obtained in (pre)- clinical studies using blocking monoclonal antibodies or sulfonamide derivatives inhibitors in different adult cancer

types [39]. Combinatorial therapies including immunotherapies and molecules targeting CAIX also raise interest [39]. Of note, not only cancer cells but also immune cells in the TME are greatly impacted in their metabolism by hypoxia [6].

4.5 Hypoxia, HIFs, immunosuppressive TME and cancer immune escape

The role of hypoxia in the cancer immune response has been the subject of many studies. It has been shown that hypoxia and nutrient-deprivation in the TME mainly dampens immune reactions against cancer, but the phenomenon is very complex, as hypoxia can also contributes to immunogenic cell death having so an immunostimulatory function [7]. Moreover, hypoxia and HIFs could also favor innate and adaptive immune responses depending on the context: for example, in the bacterial infectious setting, infected tissues are often hypoxic and HIF-1α plays an important role to sustain the phagocytes bactericidal immune function [106]. At infected hypoxic sites, CTLs also show increased effector molecules expression as well as co-stimulatory and inhibitory receptors (such as for example CTLA-4), and ablation of HIF-1α in T lymphocytes decreases effector cytokines levels as well as cytolytic effector molecules (such as for example IFN gamma or TNFα and granzyme B respectively) [107, 108]. Nonetheless, in the malignant diseases setting, the net result of hypoxia tips the balance to immunosuppression [8, 109].

Indeed, if the hypoxic-induced metabolic changes in cancer cells help them to cope with oxygen deprivation, allowing their outgrowth even in hard conditions, on the other side hypoxia, HIFs and the resulting metabolic changes (such as lactate accumulation and pH decrease for example) act by several mechanisms to negatively impact immune cells :

- they lead to the production and expression of different immunomodulatory compounds (including immunosuppressive cytokines and immune checkpoints);
- they impact immune cells infiltration (on one side, immunosuppressive immune cells are attracted, but on the other side the chaotic vasculature accounts for the poor circulation of some immune effectors);
- they influence the polarization of immune cells (favoring a pro-tumor phenotype);
- they impact immune cells proliferation and function;

all of these mechanisms finally result in the dampening of the immune response against cancer cells [6-8, 109] . Of note, as HIF-1α stabilization also results from oncogenic pathways activation, it can be difficult to distinguish effects resulting from hypoxia or from pseudo- hypoxia [6, 8].

4.6 Hypoxia, HIFs and myeloid cells in cancer

Myeloid cells and in particular monocytes/macrophages are attracted in hypoxic areas by several mechanisms.

HIF-1α leads to the secretion of different chemokines and cytokines by cancer cells, such as for example CXCL12 (also called stromal-derived factor 1α, SDF-1α), CCL5, VEGF-A and endothelins [110, 111]. It promotes also the expression of their cognate receptors on macrophages (for example, CXCR4 for CXCL12 and CCL5) [111]. Moreover, Semaphorin3A (Sema3A) is expressed in cancer cells under hypoxia, and attracts TAMs by binding to the Neuropilin-1 (Nrp-1) receptor finally leading to VEGFR-1 transactivation [112]; once macrophages reach hypoxic zones, hypoxic repression of TAMs Nrp-1 expression occurs leading to their entrapment in hypoxic areas [112]. Hypoxic TAMs then acquire an immunosuppressive M2-phenotype, expressing lower levels of MHC-II molecules and producing immunosuppressive factors [111, 112]. Moreover, HIF-1α stabilization in TAMs promotes the angiogenic switch and chaotic vasculature development supporting tumor progression and metastasis through the action of VEGF-A [6]. HIF-1α also regulates the MMP9 expression by hypoxic tumor macrophages, resulting in the degradation of extracellular matrix, releasing the sequestered VEGF-A which becomes then available to interact with its receptor VEGFR2 [110].

HIF-1α induces CSF1 secretion by cancer cells attracting CSF1R+ macrophages but also MDSCs in tumors, increasing metastasis and immunosuppression [113].

Myeloid cells and in particular macrophages largely contribute to suppress T cells function notably through arginine depletion and NO generation [114]. Indeed, Doedens *et al* have demonstrated that HIF-1α in myeloid cells leads to Arginase I (ARG1) and in a minor extent to inducible iNOS finally resulting in T cell suppression [114]. To note, under hypoxic conditions, indoleamine-pyrrole 2,3 dioxygenase (IDO) and PGE2 are also upregulated in macrophages, also taking part in their immunosuppressive activity [114]. MDSCs in the TME are able to differentiate into TAMs; HIF-1α plays a major role in this process [115]. HIF-1α accounts also for the increased expression of inhibitory ligands such as PD-L1 on MDSCs [10]. Indeed, hypoxia through HIF-1α-dependent mechanism has been reported to increase the expression of PD-L1 in several cancer types, including melanoma, breast cancer and prostate cancer [9], but Noman *et al* have demonstrated that this effect not only occurs on cancer cells but also on MDSCs, DCs and macrophages, resulting in T cells immune function suppression [10]. HIF-1α

acts by binding to the HRE located in the PD-L1 promoter [10]. Interestingly, after blocking PD-L1 with targeted Mab under hypoxic conditions, the IL-6 and IL-10 MDSCs secretion which was up-regulated by hypoxia decreases, contributing partly to restore T cell functions [10]. This study highlights the potential benefit of targeting HIF-1α and PD-L1 for cancer treatment.

Samanta *et al* have reported the damaging effect of HIF-1$\alpha\alpha$ in triple-negative breast cancer (TNBC) cell lines and its link with PD-L1 expression: indeed, hypoxia but also chemotherapy act through HIF-1α to promote the expression of immunosuppressive genes including PDL1, CD73 and CD47 on TNBC cells [116]. In this way, adaptive immune reactions are dampened as PD-L1 binds to PD-1 on effector cells, and as CD73 favors the conversion of AMP to adenosine which binds to A2A receptor on T cells also leading to anergy or death [116]. Moreover, innate immunity is also impacted as adenosine favors DCs immunosuppressive cytokines production and reduces their ability to elicit adaptive immune reactions [116]. Adenosine also contribute to attract MDSCs [116]. On the other side, CD47 binds to SIRPα on macrophages blocking phagocytosis [116]. These studies suggests a potential benefit to combine HIFs inhibitors to ICI, as HIFs acts by several mechanisms and blocking only one pathway could be not sufficient to stop cancer growth [116].

4.7 Hypoxia, HIFs and DCs in cancer

The effect of hypoxia on DCs is controversial in the literature, but this may reflect the fact that studies are mainly conducted in inflammatory disease settings, and inflammation in tumors differs from inflammation in other pathologic conditions [6, 117]. However, Mancino *et al* have reported that hypoxia uncouple the DCs role in eliciting adaptive immune responses in confront to their role in tissue repairing and inflammatory functions [118]. Indeed, hypoxia, notably through impairing CD80, CD86 and MHC Class II molecules expression on DCs, resulted in reduced DCs maturation and ability to elicit T cells activation [118]. CCR7 was not up-regulated under hypoxic conditions, also contributing to reduced migration to the draining lymph node so impairing priming of naïve T cells [118]. To note, VEGF hypoxia-induced secretion also contributes to the dampening of DCs maturation [118]. On the other side, hypoxia promoted the expression of pro-inflammatory cytokines (such as IL-1β and TNF-α) as well as CCR5 expression resulting in the homing of DCs into the peripheral tissues [118]. Nonetheless, other studies have reported opposite results; for example, in the study of Köhler *et al* CD86 and MHC Class II molecules expression was not impaired under hypoxic conditions and the cytokines secretion profile revealed notably a decreased

IL-10 production [119]. This contrasts with another study showing that HIF-1α supports the production of immunosuppressive cytokines by DCs, such as IL-10 and TGF-β, resulting in Tregs induction in a inflammatory bowel disease mouse model [120]. The IL-12 downregulation by hypoxia on the other hand has been underlined by several studies, and leads to impaired CD4+ Th1 immune responses and CD8+ T cell expansion in a Leishmania infection mouse model [121, 122]. Nonetheless, other studies are strongly needed to fully unravel the interlink between hypoxia, HIF-1α and DCs maturation, migration and cytokine secretion as divergent results are reported far to date.

4.8 Hypoxia, HIFs and TILs in cancer

Hypoxia greatly inhibits the anti-tumor activity of NK cells in several ways. *In vitro* experiments have shown that NK cells adapt to hypoxia by accumulating HIF-1α [123].

In hypoxic conditions, NK cells exposed to activating cytokines such as IL-2 are not able anymore to up- regulate their activating receptors expression including NGK2D or the NCRs (namely NKp46, NKp44 or NKp30) [123]. Despite this, NK cells retain their ability to attack the target cells through ADCC as CD16 expression is slightly changed under hypoxia [123]. Of note, cancer cells are also able to secrete tumor derived microvesicles (TDMVs), and Berchem *et al* have shown that TDMVs derived from hypoxic cancer cells are taken-up by NK cells and lead to the dampening of their activation and cytotoxic function; this results in part from the hypoxia- induced TGF-β production by cancer cells, which is then transported to NK cells through TDMVs finally leading to decreased NGK2D expression [124]. Moreover, miR23a is also up- regulated in hypoxic microvesicles, contributing to the decrease observed in CD107a expression, which is a well-described NK cells activity marker [124].

In the TME, hypoxic cancer cells undergo autophagy, an adaptive phenomenon leading to the degradation of cytoplasmic proteins into autophagosomes [125]. Baginska *et al* have demonstrated that under hypoxia, cancer cells autophagy results in degradation of granzyme B released by NK cells finally decreasing their cytolytic activity [125]. This process occur also *in vivo* in melanoma and breast adenocarcinoma mouse models [125].

T cells functions are greatly negatively impacted by the unfavorable conditions in the TME. Indeed, hypoxia results in metabolic changes such as lactate accumulation and carbonic anhydrases activity resulting in an acidic pH, abnormal metabolites accumulation (for example, increased extracellular adenosine production or reactive nitrogen species), important nutrients deprivation (for example, glucose and arginine depletion, tryptophan catabolism induced by IDO and finally hypoxia also promotes the expression of inhibitory ligands (for example, PD-L1 on myeloid and cancer cells)

impacting T cells function) [10, 109, 116, 126].

Studies have demonstrated an enhanced effector function in hypoxic T cells [107, 108], which is in concordance with the fact that glycolysis plays an important role in CD8+ effector functions [127], but in the cancer TME, this is largely counterbalanced by the negative impact of hypoxia, HIFs and the resulting metabolic changes in which T cells are immerged [8, 109]. For example, a strong acidic extracellular milieu blocks the MCT-1, the transporter of lactate in T cells, resulting in altered cytolytic functions [127]. As glucose is highly consumed by cancer cells and as its up-take is favored by the up-regulation of GLUT-1 under the control of HIF, T cells in the TME result deprived from this essential nutrient [128]. Moreover, as mentioned above, iNOS and ARG1 induction under hypoxia in myeloid cells contribute also to dampen T cells functions [114]. Hypoxic or apoptotic cells in the TME release ATP which is then converted by CD73 (expressed by cancer cells and Tregs) or CD39 (expressed by Tregs) into adenosine, a potent inhibitor of T cells immune responses through the binding of A2A receptors [116, 126]. Tryptophan is catabolized into kynurenine by IDO, expressed in immunosuppressive TME cells such as TAMs and MDSCs or by cancer cells in response to hypoxia. Tryptophan depletion reduces T cells activation, whereas kynurenine contributes to Tregs differentiation and downregulation of the CD3 zeta chain of the TCR impacting negatively effector T cells function [129]. HIF-1α through the increased PD-L1 expression on cancer but also MDSCs dampens T cells immune reactions against cancer cells [9, 10].

Of note, T cells and in particular CTLs concentrate in normoxic tumoral areas while avoiding hypoxic zones, as demonstrated by the study of Manaster *et al*, underlying once again that vascular normalization (eventually combined to immunotherapy) could contribute to immune tumor eradication [130]. On the other side, Tregs are attracted in hypoxic tumors through the interaction of their CC-chemokine receptor 10 (CCR10) with the hypoxia-induced CC-chemokine ligand 28 (CCL28) [58]. The VEGF secretion by stromal cells also contributes to trap Tregs by binding to their Nrp-1 receptor [111]. Of note, Tregs secrete VEGFA thereby contributing to tumor angiogenesis and promoting cancer tolerance [58]. Hypoxia induces TGF-β secretion by malignant cells and TGF-β also attracts Tregs into the tumor bed [111]. Clambey *et al* have also demonstrated that hypoxia and HIF-1α favor the induction of Tregs through up-regulating FoxP3 mRNA and protein expression, a mechanism that requires TGF-β; this effect was observed *in vitro* and *in vivo* as well [131]. Of note, the role of HIF-1α in promoting Tregs differentiation instead of inducing Th17 phenotype could be context-dependent and particularly impacted by the surrounding cytokine environment [131, 132]. Indeed, Dang *et al* have suggested that induced Tregs and Th17 derive from the same CD4+ precursor, but that depending on the cytokine landscape of their surrounding

environment, CD4+ cells differentiate into Th17 or Tregs [132]. However, in contrast to the study of Clambey *et al*, Dang *et al* showed that HIF-1α promotes the degradation of FoxP3 as it binds to FoxP3 leading to its proteosomal degradation, a data that was not confirmed in the study of Clambey *et al* [131, 132], underlying that further studies are needed to fully unravel the interconnection between HIF-1α, Tregs and FoxP3.

In summary, hypoxia largely contributes to immune escape mechanisms in cancer cells allowing to evade NK and adaptive immune cells attack, and promotes angiogenesis. These hypoxia-induced mechanisms contribute to the hypoxia-driven acquired resistance to immunotherapy, underlying the potential synergistic effect that could result from combinatorial therapies including immunotherapeutic and hypoxia-targeted drugs [7, 8].

5. HIFs and hypoxia in NB

The role of hypoxia in NB progression and aggressiveness has been the subject of many studies. Hypoxia promotes cell stemness and HIF-2α is associated with a pseudohypoxic stem cell phenotype [133-138]. However, the respective role of HIFs factors in NB prognosis and their respective association to the disease stage is still investigated. Recent studies highlight a more preponderant role of HIF-1α in poor-prognosis forms [139, 140] *(see discussion)*.

5.1 HIFs inhibitors and combinatorial therapies

Inhibitors of HIF-1α and HIF-2α have been designed to target hypoxia. PX-478 (S-2-amino- 3-4- N,N ,-bis(chloroethyl)amino]phenyl propionic acid N -oxide dihydrochloride) for example acts by decreasing HIF-1α mRNA levels, and by inhibiting HIF-1α protein translation and deubiquitination [141, 142]. PT2385 disrupts the formation of the heterodimer HIF-2α/ARNT; this compound has already given good results in preclinical model of clear cell renal carcinoma, particularly when combined with PD-L1 blockade [143-145]. However, PT2385 has led to disappointing results in NB [146, 147].

Recently, Bromodomain and extraterminal domain (BET) protein inhibitors (BETi) have demonstrated an activity against hypoxic responses [148]. JQ1 belongs to this class of epigenetic-acting drugs [149].

BET proteins, including BRD4, contains bromodomains that bind to acetylated lysine residues on histone tails and play the role of « chromatin readers » [149]. They associate to other proteins and transcription factors and form a complex able to induce the transcription or repression of genes [149]. BETi, by binding to the bromodomain,

prevent the interaction with the acetylated lysine histone residues (Fig. 20) [149]. When tested in pre-clinical neuroblastoma mouse models, JQ1 showed a drastic effect on survival [150].

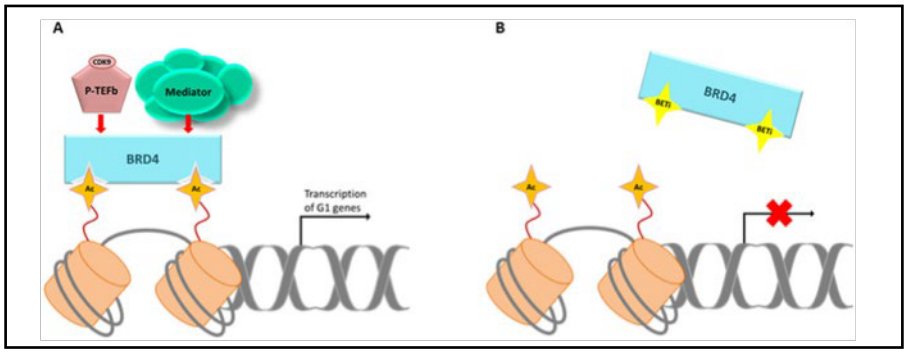

Fig. 20 showing the mechanism of action of BET inhibitors. BETi, BET inhibitor; Ac, acetylated lysine residues; and P-TEFb, positive transcription elongation factor b. From [149].

SCOPE AND AIMS OF THE THESIS

The main aim of this research project is to assess the effect of targeting intra-tumoral hypoxia in association to PD-1/PD-L1 immune checkpoint blockade immunotherapy in a HR NB transgenic mouse model.

HR NB patients still harbor a very bad prognosis, but the introduction of anti-GD2 therapy has allowed improving their outcome, highlighting the importance of immunity in NB [3, 4]. On the other side, major progress has been achieved in adult cancers with the introduction of ICI [48]. However, ICI in monotherapy for pediatric malignancies has given disappointing results, with no effect in NB when used alone [11].

Hypoxia contributes largely to the immunosuppressive TME and correlates to poor-prognosis in solid tumors in general and in NB in particular [5-8]. On the other side, many studies highlighted the pejorative role of PD-L1 in NB, favoring cancer cell immune escape [78, 82, 83]. The recent literature has evidenced the role of HIF-1α in inducing PD-L1 expression on cancer cells and on MDSCs under hypoxic conditions [9, 10, 151]. Moreover, in clear cell renal carcinoma cell lines with defective function of pVHL resulting in constitutive expression of HIF-2α, Ruf *et al* have demonstrated that PD-L1 is also a HIF-2α target, suggesting that associating PD-L1 blockade therapy to HIF inhibition could represent an attractive option [152].

Based on these observations, our project aims to assess the impact of targeting HIFs on the improvement of PD-L1/PD-1 axis blockade therapy in a HR NB transgenic mouse model, the TH-*MYCN* model. The ultimate goal is to provide strong evidence for conducting clinical trials in the pediatric setting, as data obtained in adults are not directly transferable to children due to differences between adult and pediatric malignancies [11].

To target hypoxia, JQ1 BETi has been selected for its multifaceted activity described in the literature [148, 153] and based on our *in vitro* results (*see below*). Moreover, JQ1 has already proven efficacy in NB mouse models, notably in the TH-*MYCN* model [150].

The PD-L1/PD- 1 axis blockade will be achieved using anti-PD-1 Mab.

In order to treat mice developing similar tumor volume and to assess the response to treatment, Magnetic Resonance Imaging (MRI) has been performed. The T2 weighted images show a hyperintense tumor and allow a precise measure of the tumor volume as previously described in other studies [29, 154-156]. Tumor volume have been assessed before treatment and at different times during drug therapy leading to more objective response rate evaluation.

The second aim of the project is to confirm the effect of JQ1 on the impairment of hypoxic responses in the particular setting of NB.

JQ1 has led to hypoxic responses impairment in triple negative breast cancer [148] and ovarian carcinoma and in melanoma cell lines [157]. To our knowledge, the impact of JQ1 on hypoxia has not yet been addressed in the NB setting. The evaluation of this effect will be performed by using real-time qPCR and western blotting (HIFs and HIFs target expression), and by developing techniques such as Magnetic Resonance Imaging (MRI) relaxometry. Relaxometry relies on the variation of the transverse relaxation rate R2* (R2*= 1/T2) whose changes depend on paramagnetic species concentration (such as deoxyhemoglobin concentration) [155]. Studies suggest that changes in T2 relaxation could reflect changes in tumor hypoxia [154].

The third aim of the project is to study the effect of JQ1 on vessel normalization in NB tumors.

The study of the normalization of chaotic vessels and of the re-oxygenation of the tumor bed will be performed by usual techniques such as CD31 and α-SMA staining on tumor sections. Dynamic contrast-enhanced-MRI (DCE-MRI) will be set up, with the collaboration of the in vivo imaging facility of the LIH, in order to assess vascularization *in vivo*.

Briefly, for DCE-MRI, a first session of scans without contrast agent is performed, followed by scans acquired after Gadolinium (Gd) intravenous injection. Gd concentration modifies the T1 relaxation; dynamic images are registered over time. Gd exits the vessels toward extravascular extracellular space (EES) in a way more or less dependent on vessel leakage degree. Then Gd turns back into the vessels. A mathematical analysis is then performed and pharmacokinetic parameters such as K^{trans} and K_{ep}, representing respectively the volume transfer constant between plasma and EES and the efflux rate constant between EES and plasma are assessed, reflecting vessel permeability [158]. As MRI is already implemented for tumor assessment in paediatric malignancies including NB, DCE-MRI could in the future become a useful tool to evaluate tumor vascularization [158].

Finally, we will analyze the impact of JQ1 on immune landscape of NB tumors.

Tumors will be analyzed using flow cytometry and large-scale cytokine array. Emphasize will be put on the impact of JQ1 on PD-L1/PD-1 expression on immune cells infiltrating NB tumors.

MATERIALS AND METHODS

6. In vitro experiments

6.1 Cell culture

NHO2A cell line is a murine cell line isolated from homozygous TH-MYCN neuroblastoma mice, kindly provided by Dr Claudia Flemming, Children's Cancer Institute Australia for Medical Research, Sydney, Australia, and Prof. Ursula Kees, Telethon Institute for Child Health Research, Perth, Australia. These cells grow as adherent colonies and are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin
/streptomycin in humid atmosphere at 37°C and 5% CO2. Sima and CHP-134 are human neuroblastoma cell lines derived respectively from a *MYCN*-amplified stage III and stage IV human neuroblastoma and purchased from DSMZ, Germany. These cells are maintained in culture in the same conditions as NHO2A cells, with the only difference being the concentration of fetal bovine serum (20% for Sima and CHP-134).
For experiments, all cells were seeded in 6 well plates and as they reached 50% confluence, they were treated with with JQ1 drug at 0.5 or 1 µM either under normoxic conditions or in hypoxic conditions (humid atmosphere with 0.1% pO2 and 5% CO2), for 24 or 48h. JQ1 was purchased from Selleckchem and dissolved in DMSO at a concentration of 500 mM for the stock solution, and then working solutions were prepared by diluting the stock solution into RPMI 1640 medium.

6.2 Protein extraction for western blotting

Cells were washed with DPBS with CA/Mg (Lonza) and lysed with 30 µl RIPA/well (Ripa Lysis Buffer, 10x, Millipore). The RIPA buffer was diluted 10x and supplemented with protease inhibitor (cOmplete Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich). 40 µg of proteins were separated on a SDS-PAGE gel and transferred onto a nitrocellulose membrane. Protein detection was done by the following primary antibodies: HIF-1α (D2U3T) Rabbit mAb (Cell Signaling), CAIX (Novus), Monoclonal Anti-β-Actin antibody produced in mouse (Sigma). Secondary antibodies include anti-rabbit antibody (Jackson).

6.3 RNA extraction and reverse transcription

Medium was removed and cells were washed with DPBS containing calcium and

magnesium (Lonza), lysis solution was added and total RNA extraction was performed according to the manufacturer's protocol (NucleoSpin RNA Plus, Macherey-Nagel). Reverse transcription was performed on 200 ng of total RNA for NHO2A cells and 500 ng of total RNA for Sima and CHP-134 using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol.

6.4 RT-qPCR

Quantitative PCR was performed on 10 times diluted cDNA using SYBR Master Mix according to the manufacturer's protocol (Takyon Low ROX SYBR Master Mix, Eurogentec); the qPCR was done on a ViiA 7 Real-time PCR system (Applied Biosystems) and data was calculated with the Quant Studio Real Time PCR software. Fold change were calculated using the $2^{-\Delta\Delta CT}$ method. The following primers were used: mCAIX and hCAIX from Qiagen; sequences used for mouse 18s are F: 5'-GAA TCG AAC CCT GAT TCC CCG TC-3' and R: 5'-CGG CGA CGA CCC ATT CGA AC-3'; and for human B actin: F: 5'-GGT GGC TTT TAG GAT GGC AAG-3' and R: 5'-ACT GGA ACG GTG AAG GTG ACA G-3'.

7. In vivo experiments

7.1 Transgenic TH-MYCN mouse model

All *in vivo* experimental protocols were approved by the ethical committee of the LIH under the agreement number LECR-2018-01. After ordered Cryopreserved sperm from hemizygous TH-*MYCN* mouse (129X1SvJ) from NCI mouse repository (strain number: 01XD2), resuscitation was performed by Janvier Labs into 129S2/SvPasOrlRj females. Pups obtained from the next breedings were weaned at 3 weeks of age. Genomic DNA extraction from distal mouse tail biopsy was done with NucleoSpin Tissue kit from Macherey-Nagel according to the manufacturer's protocol. TaqMan PCR was performed using the TaqMan™ Universal Master Mix II, with UNG Kit with mouse *TERT* as the reference gene (VIC; Quencher TAMRA) and a TaqMan human *MYCN* Probe (FAM; Quencher NFQ). CopyCaller Software was used to analyze the results and discriminate the wild type, hemizygous or homozygous status of the animal.

7.2 Magnetic Resonance Imaging (MRI)

Images acquisition

MRI was performed on a 3T preclinical horizontal bore scanner (MR Solutions, Guilford,

UK), equipped with a quadrature volume coil designed for mouse body imaging. Animals were placed prone in the cradle and maintained asleep during the duration of the scans, using 2- 3% isoflurane mixed with oxygen. The body temperature was kept constant at 37°C and breathing was monitored throughout the scan sessions. The MRI protocols and parameters used are described **in Table 1**.

Sequence	Purpose	Parameters	Number of mice treated	Number of mice control
FSET2w	Tumour detection, delineation and volume measurement	SR: 156μm×161μm×1500μm, SI: 16, TE: 68ms, TR: 5000ms, ET: 8, AVG: 3, SD: 7min45sec, RG ON	9	6
FSET1w	Tumour detection, delineation and volume measurement	SR: 156μm×159μm×1500μm, SI: 16, TE: 11ms, TR: 1000ms, ET: 4, AVG: 4, SD: 4min12sec, RG ON		
MEMS	T2 relaxometry map	SR: 156μm×208μm×1500μm, SI: 16, fTE: 15ms, NOE: 10, TR: 3000ms, AVG: 1, SD: 9min36sec, RG ON		
MGE	T2* relaxometry map	FLASH, SR: 313μm×313μm×1500μm, SI: 16, fTE: 4ms, TR: 600ms, NOE: 10, AVG: 1, SD: 1min16sec, RG ON		
MFA	T1 relaxometry map	FLASH3D, SR: 313μm×313μm×1500μm, SI: 16, TE: 3ms, TR: 40ms, NFA: 6, AVG: 1, FA: 5/10/15/20/25/30, SD: 8min11sec, RG ON.		
DCE FLASH 3D	Perfusion and vessels permeability parameters	FLASH3D, SR: 313μm×625μm×1500μm, SI: 16, TE: 3ms, TR: 4ms, NE: 250, TRes: 0.25s, AVG: 1, FA: 10, SD: 17min04sec, CTA: 0.5mmol/kg Dotarem injected i.v after 60″, RG OFF.	7	4

Table 1: MRI Protocols. SR: Spatial Resolution, SI: Number of Slices, TE: Echo Time, TR: Repetition Time, ET: Echo Train, AVG: Number of Averages, SD: Scan Duration, RG: Respiratory Gating, fTE: First Echo Time, NOE: Number Of Echoes, FA: Flip Angle, NFA: Number of Flip Angles, FLASH: Fast Low Angle Shot, NE: Number of Experiments, TRes: Time Resolution, CTA: Contrast Agent.

For relaxometry and DCE experiments, homozygous mice were regularly clinically assessed by abdominal palpation and when abdominal mass was detectable, mice were randomly assigned to JQ1 treatment group, receiving 50 mg/kg/day intraperitoneally (IP) for 2 days (diluted in 10% dimethyl sulfoxyde (DMSO), in 10% (2-Hydroxypropyl)-β-cyclodextrin, Sigma- Aldrich) or to control group receiving vehicle IP (10% DMSO in 10% (2-Hydroxypropyl)-β- cyclodextrin, Sigma-Aldrich) for 2 days. Anatomical series (FSE T1w and FSE T2w) were used to screen the animals and calculate tumor volumes. Relaxometry and perfusion series were acquired to assess physiological parameters in the tumors before and after treatment; of note, for perfusion sequences (DCE), mice received Dotarem 0,05 mmol/kg into the tail vein (5 $\mu\mu$l/g of a 0,01 mmol/ml solution of Dotarem diluted in physiologic saline added with heparin). Anatominal and relaxometry data were acquired for nine mice in the treatment group and six mice in the control group, whereas perfusion data was acquired for seven mice in the treatment group and four mice in the control group Acquisitions were done just before starting treatment (day 0) and repeated just before sacrifice (day 2). Only the

abdominal part of tumors was taken into account.

7.3 Data analysis and Statistics

Tumor volume was defined as the part of the abdominal tumor visible on T2-weighted MRI. Tumor volumes are expressed in mm^3. Relaxometry data were analyzed in nordicICE (NordicNeuroLab, Bergen, Norway). Maps of the relaxivity parameters T_1, T_2 and T_2* and corresponding relaxivity rates R_1 (1/ T_1), R_2 (1/ T_2) and R_2* (1/ T_2*) were derived from the respective relaxometry series. R_2 and R_2* are considered as surrogate markers of hypoxia [154, 155]. Pharmacokinetic (PK) analysis of the DCE-MRI data was also performed using nordicICE. The extended Toft's model was used to assess perfusion and vessels permeability parameters in animals for both groups. Arterial Input functions (AIFs) were detected from the heart of the mice. Quantification of the perfusion parameters maps generated was performed in Matlab 2019a (MathWorks, MA, USA), and perfusion parameters in the tumours were normalized to values in the liver. Median values are reported for the treatment and controls groups. A Student's t-test was used to assess the statistical significance between groups, calculated in GraphPad Prism 8.0.1; p values < 0.05 were considered statistically significant.

7.4 Western Blot on protein extracts from tumors

Tumors from the Relaxometry/DCE-MRI experiment were dissected from euthanized mice and frozen straight away in isopentan. Tumors were reduced to small pieces without previous thawing. RIPA was added and samples were sonicated and then centrifuged. The supernatant was used for western blotting. 80 µg of proteins were separated on a SDS-PAGE gel and transferred onto a nitrocellulose membrane. Protein detection was done by the following primary antibodies: HIF-1a (D2U3T) Rabbit mAb (Cell Signaling), CAIX (Novus), N- Myc Antibody (Cell Signaling)Monoclonal Anti-β-Actin antibody produced in mouse (Sigma). Secondary antibodies include anti-rabbit antibody (Jackson).

7.5 Immunohistochemistry on tumors

Homozygous mice developing tumors were treated either by JQ1 50 mg/kg IP or vehicle for 2 days, and then abdominal tumors were dissected and immediately put in paraformaldehyde 4% and then paraffin-embedded. Hematoxylin, CD31 and α-smooth muscle actin (a-SMA) staining was performed by Histowiz.

7.6 Cytokine/Chemokine profiling

Two homozygous mice with palpable tumors were treated with JQ1 50 mg/kg or vehicle IP for two days and were sacrificed 24h after treatment stop. Tumors were collected and immediately frozen in isopentan. They were then processed using the Proteome Profiler Array, Mouse XL Cytokine Array Kit (R&D Systems, Catalog Number ARY028) according to the manufacturer's instructions.

7.7 Fluorescence Activated Cell sorting (FACS) in vivo

Fluorescence Activated Cell sorting (FACS, ARIA) was performed to assess the immune landscape of tumors. Homozygous mice underwent regularly abdominal palpation and when abdominal mass was detectable, mice were assigned to JQ1 treatment group or control group. Tumors were collected after 3 days of JQ1 treatment at 25 mg/kg/day (diluted in 10% DMSO, in 10% (2-Hydroxypropyl)-β-cyclodextrin, Sigma-Aldrich) or vehicle (10% DMSO in 10% (2-Hydroxypropyl)-β-cyclodextrin, Sigma-Aldrich). Only the abdominal part of the tumor was taken into account as thoracic part was not resectable when present.
After tumor dissociation using a 100 µm cell strainer and a syringe piston to crush the mass in DMEM complete medium, cells were centrifuged for 10 min at 4°C. ACK lysis buffer (Lonza) was used to lyse red blood cells. Cell count was performed. After blocking FC- receptors for 5 minutes with CD16/CD32 appropriate antibodies were used to stain cells. The following list includes antibodies used: Live/Dead near IR (life technology), BuV395 Rat anti- mouse CD45 (BD Horizon), PE-CF594 Hamster anti-mouse CD279 PD1 (BD Horizon), APC anti-mouse CD274 (Biolegend), APC-R700 Rat anti-mouse CD11b (BD Horizon), BV510 Hamster anti-mouse CD3e (BD Horizon), PerCP Cy5.5 anti-mouse CD4 (eBiosciences Lif Tech), BV570 anti-mouse CD8A (Biolegend), BV421 anti-mouse NK1.1 (Biolegend), BV786 Rat anti-mouse CD25 (BD Horizon), BV605 Hamster anti-mouse CD69 (BD Horizon), BV605 anti-mouse F4/80 (Biolegend), BV785 anti-mouse Ly-6G (Biolegend), BV421 anti-mouse Ly- 6C (Biolegend), PE-Cy7 anti-mouse CD11c (eBiosciences Lif Tech), PerCP Cy5.5 anti-mouse CD206 (Biolegend), Alexa Fluor 488 anti-mouse/rat FOXP3 (eBiosciences Lif Tech). Data from lymphoid and myeloid populations were collected using multicolor flow cytometry and FlowJo software served for analysis of these data.

7.8 Survival curve

Homozygous TH-*MYCN* mice developing tumors were detected by abdominal palpation and then T2-weighted MR images were performed in order to confirm the

presence of NB and to determine the total tumor volume (abdominal part and thoracic part when present). When tumor reached a volume between 400 and 800 mm3, mice were randomly assigned to one of the following 4 groups: vehicle + isotype, vehicle + anti-PD-1, JQ1 + isotype, JQ1
+ anti-PD-1. JQ1 treatment was given at 25 mg/kg/day IP (diluted in 10% DMSO, in 10% (2-Hydroxypropyl)-β-cyclodextrin, Sigma-Aldrich) for seven consecutive days, meanwhile control mice received the vehicle (10% DMSO, in 10% (2-Hydroxypropyl)-β-cyclodextrin, Sigma-Aldrich). Isotype (InVivoMAb rat IgG2a isotype control, anti-trinitrophenol, BE0089, BioXCell) or anti-PD-1 monoclonal antibody were administered IP at 10 mg/kg/day each three days until endpoint was achieved, ie mice spontaneously died or mice reached human endpoint (signs of discomfort or tumor volume of 2000 mm3). Tumor volume was precisely measured using T2-weighted MR images. MRI was performed just before starting treatment, and then at day 4, 8 and 15. Then depending on the evolution of tumors, images were repeated at regular intervals until reaching endpoint volume.

7.9 Statistics

Statistical analysis was performed using GraphPad 5.0.

RESULTS

8. Article 1:

The BET protein inhibitor JQ1 decreases hypoxia and improves the therapeutic benefit of anti-PD-1 in a high-risk neuroblastoma mouse model

Delphine Sauvage[1], Manon Bosseler[1], Elodie Viry[1*], Georgia Kanli[2*], Anais Oudin[3], Guy Berchem[1,4] and Olivier Keunen[2], Bassam Janji[1#]

Cells 2022, 11, 2783.

NB is still a poor-prognosis childhood malignancy in the HR forms [13]. Several recent studies have highlighted the importance of PD-L1 expression in NB [69, 78, 81-83]. In adult oncologic patients, combinatorial therapies including ICI [63] pave the way for introduce such combination in pediatric cancer treatment.

On the other side, as in other tumors, hypoxia correlates to poor prognosis in NB [139, 140]. Moreover, recent studies have demonstrated that hypoxia greatly impacts immune reactions against tumors and largely contributes to create an immunosuppressive TME, notably through increased PD-L1 expression [6-8, 10].

In our study, we assess the effect of a combinatorial therapy associating JQ1 to anti-PD1 Mab in a well described transgenic NB mouse model, the TH-*MYCN* model [32]. We first demonstrated that JQ1 leads to impaired hypoxic responses in NB *in vitro*, and these results correlate with the *in vivo* experiments. Indeed, CAIX expression was decreased in tumors from JQ1 treated animals and MRI relaxometry data suggest decreased tumor hypoxia after JQ1 therapy. Moreover, as demonstrated by immunohistochemistry staining on tumors, JQ1 contributes to vessels normalization. DCE-MRI data reveal the same tendency. JQ1 also modulates the immune landscape of NB by lowering PD-L1 expression on immune cells and activating T cells as shown by the increase expression of PD-1 on CD8+ cells.

Finally, combinatorial therapy associating JQ1 with anti-PD-1 monoclonal antibody led to better tumor response and significant prolonged survival in TH-*MYCN* mice. This study could pave the way to clinical trials associating epigenetic drugs with ICI for treating children NB.

Author contributions:

Author 1: Delphine Sauvage: conception and design of the study, collection of the in vitro and in vivo data, data analysis and statistics, paper (text and figures) writing.

Author 2: Manon Bosseler: collection of the in vitro and in vivo data, paper revision.

Author 3: Elodie Viry: collection of the in vitro and in vivo data, data analysis and statistics, paper revision.

Author 4: Georgia Kanli: setting of the MRI technique, collection of the in vivo data, MRI

(relaxometry and DCE MRI) data analysis and statistics, paper writing.

Author 5: Guy Berchem: conception and design of the study.

Author 6: Olivier Keunen: setting of the MRI technique, collection of the in vivo data, MRI (relaxometry and DCE MRI) data analysis and statistics, paper revision.

Author 7: Bassam Janji: conception and design of the study, data analysis and statistics, paper (text and figures) writing.

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The BET Protein Inhibitor JQ1 Decreases Hypoxia and Improves the Therapeutic Benefit of Anti-PD-1 in a High-Risk Neuroblastoma Mouse Model

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Abstract: Anti-programmed death 1 (PD-1) is a revolutionary treatment for many cancers. The response to anti-PD-1 relies on several properties of tumor and immune cells, including the expression of PD-L1 and PD-1. Despite the impressive clinical benefit achieved with anti-PD-1 in several cancers in adults, the use of this therapy for high-risk neuroblastoma remains modest. Here, we evaluated the therapeutic benefit of anti-PD-1 in combination with JQ1 in a highly relevant TH-MYCN neuroblastoma transgenic mouse model. JQ1 is a small molecule inhibitor of the extra-terminal domain (BET) family of bromodomain proteins, competitively binding to bromodomains. Using several neuroblastoma cell lines in vitro, we showed that JQ1 inhibited hypoxia-dependent induction of HIF-1α and decreased the expression of the well-known HIF-1α downstream target gene CA9. Using MRI relaxometry performed on TH-MYCN tumor-bearing mice, we showed that JQ1 decreases R2* in tumors, a parameter associated with intra-tumor hypoxia in pre-clinical settings. Decreasing hypoxia by JQ1 was associated with improved blood vessel quality and integrity, as revealed by CD31 and αSMA staining on tumor sections. By analyzing the immune landscape of TH-MYCN tumors in mice, we found that JQ1 had no major impact on infiltrating immune cells into the tumor microenvironment but significantly increased the percentage of $CD8^+$ $PD\text{-}1^+$, conventional $CD4^+$ $PD\text{-}1^+$, and Treg $PD\text{-}1^+$ cells. While anti-PD-1 monotherapy did not affect TH-MYCN tumor growth, we showed that combinatorial therapy associating JQ1 significantly decreased the tumor volume and improved the therapeutic benefit of anti-PD-1. This study provided the pre-clinical proof of concept needed to establish a new combination immunotherapy approach that may create tremendous enthusiasm for treating high-risk childhood neuroblastoma.

Keywords: neuroblastoma; immune checkpoint inhibitors; PD-1/PD-L1; immunotherapy; hypoxia; epigenetic drugs; JQ1; combinatorial therapy; tumor vasculature

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1. Introduction

Neuroblastoma (NB) is the most frequent cancer type among childhood extra-cranial pediatric solid tumors and is responsible for 13% of cancer deaths in children younger than 15 years [1–3]. NB leads to abdominal, pelvic, thoracic, or cervical masses [1,2,4–6], and metastatic NB can infiltrate bones, bone marrow, lymph nodes, liver, lungs, skin, and rarely some other organs [2,4,6].

MYCN amplification is the major genetic alteration found in 20–30% of NB [7,8] and correlates with an unfavorable aggressive phenotype and treatment failure. Other genetic

alterations have also been described, including (i) germline gain-of-function mutation of ALK (anaplastic lymphoma receptor tyrosine kinase) gene, reported as a predisposition factor for familial neuroblastoma [9,10]; (ii) somatic mutations of ALK, observed in 14% of high-risk neuroblastoma [11]; (iii) loss-of-function genetic alterations of ATRX (alpha-thalassemia and mental retardation, X-linked) and TERT (telomerase reverse transcriptase) genes, respectively described in 10% and 25% of patients, [12,13]; and (iv) familial mutations of PHOX2B (paired-like homeobox 2b) gene [2,5,6,14].

The International Staging System (INSS) is currently used to classify NB in six stages (Stage 1, 2A, 2B, 3, 4 and 4S) (reviewed in [15]). NB stage 4 is the most aggressive and characterized by metastasis formation. However, NB stage 4S is a localized tumor with limited metastasis. Typically, 4S neuroblastoma spontaneously regresses in young infants (<1 year of age) by a mechanism that is not fully understood. In addition to the INSS, NB patients can be stratified into low, intermediate, and high-risk disease groups depending on the age at diagnosis, stage, histology, MYCN status, and tumor cell ploidy (reviewed in [16]). MYCN amplification remains one of the major predictors of high-risk disease [8] and poor clinical outcomes [1,4–6]. About 60% of NB patients develop a high-risk form despite heavy and intensive treatment. The prognosis of high-risk neuroblastoma is below 50% survival at 5 years [17].

Several therapeutic options are currently used to treat high-risk NB, including a high dose of chemotherapy followed by autologous stem cell transplant, surgery, radiotherapy, and immunotherapy [18]. However, these conventional therapies are frequently associated with severe side effects. Therefore, developing a new therapy to improve prognosis and reduce side effects for high-risk neuroblastoma is urgently needed.

Accumulating evidence recognizes that targeting hypoxia represents a therapeutic strategy for high-risk NB. HIF-1α is the major transcription factor, which is stabilized in cells in response to hypoxia. Under normoxia, HIF-1α is degraded by an O_2-dependent mechanism involving a prolyl hydroxylase (PHD)–von Hippel-Lindau (VHL) proteasome pathway [19]. In addition, HIF-1α can be degraded by O_2/PHD/VHL-independent mechanisms through interactions with the receptor of activated protein kinase C (RACK1) following the inhibition of heat-shock protein 90 (HSP90) [20]. The high expression of hypoxia-inducible factor (HIF)-1α was correlated with poor prognosis of NB and found to be upregulated in MYCN amplified tumors and cell lines [21]. Moreover, the expression of HIF-1α downstream target gene CA9 was reported to be associated with poor survival in high-risk NB [22]. Furthermore, it has been described that RACK1 is among the top 10 genes associated with an unfavorable prognosis in NB. RACK1 depletion negatively affects the proliferation, invasion, and migration of NB cells, indicating that, similar to HIF-1α, targeting RACK1 represents a new therapeutic option for NB [23].

Bromodomain and extra-terminal domain (BET) protein inhibitor JQ1 has recently been reported to improve the survival in pre-clinical neuroblastoma mouse models, impact several cellular pathways, including MYCN expression [22], and impair hypoxic responses in triple-negative breast cancer [24].

In this study, we investigated in vitro the impact of JQ1 on the hypoxic status of several NB cell lines by assessing the expression of HIF-1α and its downstream target gene CAIX. By using the relevant TH-MYCN pre-clinical NB model, we evaluated in vivo the hypoxic status and growth of TH-MYCN tumors treated with JQ1 alone or in combination with anti-PD-1. Our results showed that JQ1 treatment impaired the expression of HIF-1α and CAIX in NB cells and improved the therapeutic benefit of anti-PD-1 in TH-MYCN.

2. Material and Methods

2.1. Cell Culture

The murine NHO2A cell line was established from homozygous TH-MYCN neuroblastoma mice. NHO2A cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a humid atmosphere at 37 °C and 5% CO_2. SIMA and CHP-134 cell lines are human neuroblastoma cells derived from

MYCN-amplified human stage III and stage IV neuroblastoma, respectively. These cell lines were grown in the same conditions as NHO2A cells but with 20% fetal bovine serum. SIMA and CHP-134 cells were purchased from DSMZ Germany. NHO2A cells were kindly provided by Claudia Flemming (Children's Cancer Institute Australia for Medical Research, Sydney, Australia) and Ursula Kees (Telethon Institute for Child Health Research, Perth, Australia). For all experiments, cells were seeded in 6-well plates and treated when they reached 50% confluence. NHO2A and CHP134 cells were incubated for 24 h in hypoxic conditions (0.1% pO_2), and SIMA cells were incubated for 48 h under the same conditions. Cells were treated with a JQ1 drug at 0.5 or 1 μM (Selleckchem, Planegg, Germany). A stock solution of JQ1 (500 mM) was prepared by dissolving JQ1 in DMSO and diluted in RPMI 1640 medium for working solutions.

2.2. Protein Extraction for Western Blotting

Cells were washed with DPBS (Lonza, Bornem, Belgium) and lysed with 30 μL/well of RIPA Lysis Buffer (Millipore, Darmstadt, Germany). They were supplemented with protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, Hoeilaart, Belgium). Proteins (40 μg) were separated on an SDS-PAGE gel and transferred onto a nitrocellulose membrane. The following primary antibodies were used for protein detection: anti-HIF-1α mAb (D2U3T, Cell signaling, Leiden, The Netherlands), anti-CAIX (Novus, Abingdom, UK), and anti-β-actin monoclonal antibody (Sigma, Overijse, Belgium). Secondary antibodies included anti-rabbit antibodies (Jackson, Ely, UK). Tumors were harvested from euthanized mice and immediately frozen in isopentane. Tumors were reduced to small pieces without previous thawing. RIPA was added, and samples were sonicated and centrifuged. The supernatant was used for Western blotting. Proteins (80 μg) were separated on an SDS-PAGE gel and transferred onto a nitrocellulose membrane. Protein detection was performed as described above.

2.3. RNA Extraction, Reverse Transcription, and RT-qPCR

The culture medium was removed, cells were washed with DPBS containing calcium and magnesium (Lonza, Bornem, Belgium), lysis solution was added, and total RNA extraction was performed using NucleoSpin RNA Plus (Macherey-Nagel, Hoerdt, France) according to the manufacturer's protocol. Reverse transcription was performed on 200 ng of total RNA for NHO2A cells and 500 ng of total RNA for Sima and CHP-134 using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, St. Leon, Germany) according to the manufacturer's protocol. qPCR was performed on 10× diluted cDNA using SYBR Master Mix according to the manufacturer's protocol (Takyon Low ROX SYBR Master Mix, Eurogentec, Seraing, Belgium); the qPCR was done on a ViiA 7 Real-time PCR system (Applied Biosystems, Ulm, Germany) and data were calculated with the Quant Studio Real-Time PCR software. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. The following primers were used: mCAIX and hCAIX from Qiagen; mouse 18s forward (5′-GAA TCG AAC CCT GAT TCC CCG TC-3′), mouse 18s reverse (5′-CGG CGA CGA CCC ATT CGA AC-3′), human beta-actin forward (5′-GGT GGC TTT TAG GAT GGC AAG-3′), and human beta-actin reverse (5′-ACT GGA ACG GTG AAG GTG ACA G-3′).

2.4. In Vivo Experiments and Transgenic TH-MYCN Mouse Model

All in vivo experimental protocols were approved by the internal ethical committee of LIH and the national authority of the country under the agreement number LECR-2018-01/LUPA2019/72. We used a transgenic TH-MYCN (tyrosine hydroxylase-MYCN) mouse model, genetically engineered to overexpress MYCN under the control of a rat TH-promoter. The overexpression of MYCN occurred in neural crest cells, which resulted in tumors closely resembling human neuroblastoma in terms of tumor localization and histology, genomic aberrations, and gene expression [25].

After ordering cryopreserved sperm from hemizygous TH-MYCN mouse (129X1SvJ) from the NCI mouse repository (strain number: 01XD2), resuscitation was performed by Janvier Labs into 129S2/SvPasOrlRj females. Pups obtained from the subsequent breeding were weaned at 3 weeks of age. Genomic DNA extraction from tissue biopsy was done using the NucleoSpin Tissue kit from Macherey-Nagel according to the manufacturer's protocol. TaqMan PCR was performed by TaqMan™ Universal Master Mix II, using UNG Kit for mouse TERT reference gene (VIC; Quencher TAMRA, Ulm, Germany) and TaqMan human MYCN Probe (FAM; Quencher NFQ, Ulm, Germany). CopyCaller Software was used to analyze the results and define the animal's wild type, hemizygous, or homozygous status.

2.5. Immunohistochemistry on Tumors

Tumors were fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin. Formalin-fixed, paraffin-embedded tumor sections (5 µm thick) from untreated or treated tumors were stained using H&E, anti-CD31, and anti-aSMA antibodies. HistoWiz Company (Brooklin, NY, USA) performed tumor sections and staining.

2.6. Magnetic Resonance Imaging (MRI) and Images Acquisition

An MRI was performed on a 3T pre-clinical horizontal bore scanner (MR Solutions, Guilford, UK), equipped with a quadrature volume coil designed for mouse body imaging. Animals were placed prone in the cradle and maintained sleep during the duration of the scans, using 2–3% isoflurane mixed with oxygen. The body temperature was maintained at 37 °C, and breathing was monitored throughout the scan sessions. The MRI protocols and parameters used are described in Table 1.

Table 1. MRI protocols used to monitor TH-MYCN tumor volumes.

Sequence	Purpose	Parameters	Number of Mice in Group Treated with	
			JQ1	Vehicle
FSE T2w	Tumor detection, delineation and volume measurement	SR: 156 µm × 161 µm × 1500 µm, Sl: 16, TE: 68 ms, TR: 5000 ms, ET: 8, AVG: 3, SD: 7 min 45 s, RG ON	9	6
FSE T1w	Tumor detection, delineation and volume measurement	SR: 156 µm × 159 µm × 1500 µm, Sl: 16, TE: 11 ms, TR: 1000 ms, ET: 4, AVG: 4, SD: 4 min 12 s, RG ON		
MEMS	T2 relaxometry map	SR: 156 µm × 208 µm × 1500 µm, Sl: 16, fTE: 15 ms, NOE: 10, TR: 3000 ms, AVG: 1, SD: 9 min 36 s, RG ON		
MGE	T2* relaxometry map	FLASH, SR: 313 µm × 313 µm × 1500 µm, Sl: 16, fTE: 4 ms, TR: 600 ms, NOE: 10, AVG: 1, SD: 1 min 16 s, RG ON		

SR: spatial resolution, Sl: number of slices, TE: echo time, TR: repetition time, ET: echo train, AVG: number of averages, SD: scan duration, RG: respiratory gating, fTE: first echo time, NOE: number of echoes, FLASH: fast low angle shot, NE: number of experiments, TRes: time resolution.

Homozygous mice underwent regular abdominal palpation. When abdominal mass appeared, the mice were randomly assigned to the JQ1 treatment group, receiving JQ1 diluted in 10% DMSO and 10% (2-Hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) or a control group receiving the vehicle by an intraperitoneal route. The dosing and treatment schedules are reported in the figures. Anatomical series (FSE T1w and FSE T2w) were used to screen the animals and calculate tumor volumes. For relaxometry assessment, only the abdominal part of the tumor, which was visible on a T2-weighted MRI, was considered, and the tumor volume was reported in mm^3. Relaxometry series was acquired to assess tumor physiological parameters before and after treatment. Anatomical and relaxometry data were acquired for mice just before starting treatment (day 1) and repeated as described in the figures just before sacrifice.

2.7. Data Analysis and Statistics

Relaxometry data were analyzed in nordicICE (NordicNeuroLab, Bergen, Norway). Maps of the relaxivity parameters T2 and T2*; corresponding relaxivity rates R2 (1/T2) and R2* (1/T2*) were derived from the respective relaxometry series. R2 and R2* are surrogate hypoxia markers [26,27]. Median values were reported for the JQ1 treatment and control groups. A Student's *t*-test was used to assess the statistical significance between the groups, calculated in GraphPad Prism 9.4.0 (San Diego, CA, USA); *p*-values < 0.05 were considered statistically significant.

2.8. Fluorescence-Activated Cell Sorting (FACS) In Vivo

Immune phenotyping of tumors was assessed by Fluorescence-Activated Cell sorting (FACS, ARIA, Becton Dickinson, Dorp, Belgium). Homozygous mice were subjected to regular abdominal palpations, and when abdominal masses appeared, mice were randomly assigned to the control and JQ1 treatment groups. Tumors were collected after 3 days of treatment with the vehicle or JQ1. Only the abdominal part of the tumor was considered.

After tumor dissociation, using a 100 µm cell strainer and a syringe piston to crush the mass into a DMEM complete medium, cells were centrifuged for 10 min at 4 °C. ACK lysis buffer (Lonza, Basel, Switzerland) was used to lyse red blood cells, and a cell count was performed. After blocking FC-receptors for 5 min with CD16/CD32, appropriate antibodies were used to stain the cells. The following antibodies were used: live or dead near IR (life technology, Carlsbad, CA, USA), BuV395 rat anti-mouse CD45 (BD Horizon, Cambridge, UK), PE-CF594 hamster anti-mouse CD279 PD-1 (BD Horizon, Cambridge, UK), BV510 hamster anti-mouse CD3e (BD Horizon, Cambridge, UK), PerCP Cy5.5 anti-mouse CD4 (eBiosciences Lif Tech, Merelbeke, Belgium), BV570 anti-mouse CD8A (Biolegend, Amsterdam, The Netherlands), BV605 hamster anti-mouse CD69 (BD Horizon, Cambridge, UK), Alexa Fluor 488 anti-mouse and rat FOXP3 (eBiosciences Lif Tech, Merelbeke, Belgium), APC-R700 rat anti-mouse CD11b (BD Horizon, Cambridge, UK), PE-Cy7 anti-mouse CD11c (eBiosciences Lif Tech, Merelbeke, Belgium), BV605 anti-mouse F4/80 (Biolegend, Amsterdam, The Netherlands), BV785 anti-mouse Ly-6G (Biolegend, Amsterdam, The Netherlands), BV421 anti-mouse Ly-6C (Biolegend, Amsterdam, The Netherlands), PerCP Cy5.5 anti-mouse CD206 (Biolegend, Amsterdam, The Netherlands), Alexa Fluor 488 anti-mouse and rat FOXP3 (eBiosciences Lif Tech, Merelbeke, Belgium), and BV605 hamster anti-mouse CD69 (BD Horizon, Cambridge, UK). Data were collected using multicolor flow cytometry, and FlowJo software was used for data analysis.

2.9. Survival Curves

Tumor-bearing homozygous TH-MYCN mice were detected by abdominal palpation, and T2-weighted MR images were performed to confirm the presence of NB and determine the total tumor volume (the abdominal and thoracic parts when present). When the tumor reached a volume between 400 and 800 mm^3, the mice were randomly assigned to one of four groups untreated, treated with anti-PD-1, treated with JQ, and treated with a combination of JQ1 and anti-PD-1. Treatment dosing and schedule are reported in the corresponding figure. Isotype (InVivoMAb rat IgG2a isotype control, anti-trinitrophenol, BE0089, BioXCell, Huissen, The Netherlands) or anti-PD-1 monoclonal antibodies were administered IP at 10 mg/kg/day every 3 days. Mice were scored every day for signs of discomfort or tumor volume of >2000 mm^3. When such parameters were observed or reached, the animal was euthanized. Tumor volume was measured using T2-weighted MR images. An MRI was performed before starting treatment and then on days 4, 8 and 15. Depending on the tumor development, images were repeated at regular intervals until reaching the endpoint volume. Mouse survival probability was defined using GraphPad Prism, and *p*-values were calculated using the log-rank (Mantel-Cox) test.

3. Results

3.1. JQ1 Impairs Hypoxic Responses in Neuroblastoma Cells In Vitro

We assessed the impact of JQ-1 on the mRNA and protein expression of HIF-1α and CAIX in three neuroblastoma cell lines. NHO2A mouse cells were derived from neuroblastoma tumors of homozygous TH-MYCN transgenic mice. CHP-134 and SIMA cells were derived from patients with neuroblastoma tumors. NHO2A cell lines overexpressed *MYCN*, whereas CHP-134 and SIMA cell lines displayed amplified *MYCN*. Together, these cell lines recapitulated the pathogenesis and features of high-risk neuroblastoma in children. As expected, our results (Figure 1A) showed an accumulation of HIF-1α protein in NHO2A, CHIP-134, and SIMA cells under hypoxia. Such accumulation was associated with an increased expression of HIF-1α downstream target gene CAIX in the three cell lines tested.

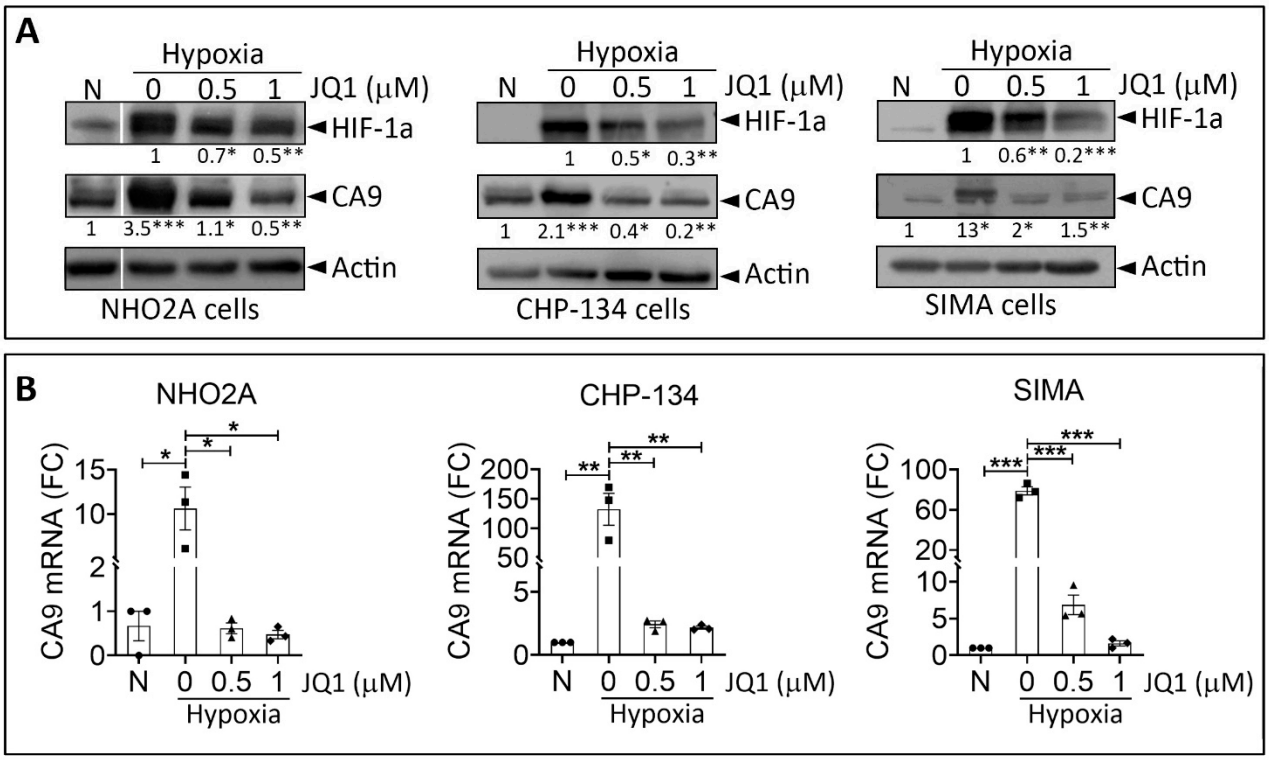

Figure 1. Effect of JQ1 on the hypoxia status of NB cells in vitro. (**A**) Western blot test showing the protein expression of HIF-1α and CA9 in NHO2A, CHP-134, and SIMA cells cultured under normoxia (N) or hypoxia and treated with JQ1 at the indicated concentration. Actin was used as a loading control. The quantification of band intensity corresponding to HIF-1α and CA9 in treated hypoxic cells is reported compared to untreated hypoxic cells and normoxic cells, respectively. (**B**) RT-qPCR measurement of CA9 mRNA in cells described in (**A**). Bars represent means from three independent experiments ± SEM. Statistically significant difference was calculated by unpaired two-tailed Student's *t*-test (* $p < 0.05$, ** $p < 0.01$; and *** $p < 0.001$).

We revealed that treatment of NHO2A, CHIP-134, and SIMA cells cultured under hypoxia with JQ-1 significantly decreased HIF-1α and CAIX protein levels in a dose-dependent manner. Moreover, the mRNA levels of CAIX were significantly decreased in hypoxic cells following treatment with JQ-1 (Figure 1B). Based on these data, we believe that JQ1 impairs hypoxia in neuroblastoma cells in vitro.

3.2. JQ1 Treatment Reduces Hypoxia in TH-MYCN Tumors

To evaluate the impact of JQ1 on the hypoxic status of TH-MYCN tumors, we conducted magnetic resonance imaging (MRI) on homozygous TH-MYCN mice to assess the relaxation rate (R2*) value, previously reported as a surrogate marker of hypoxia [28]. The

treatment schedule with the vehicle (control) and JQ1 is summarized in Figure 2A. The R2* values were determined by MRI before and after the treatment, and the tumor volume was also determined by MRI after the treatment. Figure 2B showed that on day 3, there was a decrease in the tumor volume following two treatments with JQ1 compared with the control, although the difference was not statistically significant. On day 3, following two treatments, we found that the R2* values were significantly increased in the vehicle-treated mice but significantly decreased in the JQ1-treated mice (Figure 2C–E). The R2* ratio of after to before treatment was significantly decreased in JQ1-treated mice relative to vehicle-treated mice (Figure 2F). Our data suggested that JQ1 decreases the hypoxia status of TH-MYCN tumors. These results were supported by our data in Figure 1G showing a significant decrease in the HIF-1α downstream target CAIX in JQ1 treated tumors.

It is now well established that tumor microvascular networks in the hypoxic area display several unique pathological features that can be differentiated from healthy blood vessels. Such characteristics include a high density of leaky, tortuous, and primitive microvessels with poor pericytes' coverage and basement membrane [29]. Based on our data showing that JQ-1 decreased hypoxia in TH-MYCN neuroblastoma tumors, we assessed the quality and integrity of blood vessels in tumors treated with JQ-1. To address this issue, immunohistochemistry of endothelial cell marker CD31 and pericyte marker SMA was performed on the vehicle- and JQ1-treated TH-MYCN neuroblastoma tumors. Figure 2H shows the vehicle-treated tumors displaying a high density of chaotic blood vessels poorly covered by pericytes. Remarkably, JQ1-treated tumors exhibited a lower number and well-structured blood vessels, which were better structured and well-covered by pericytes. Collectively, our data provided evidence that JQ1 reduced hypoxia in neuroblastoma tumors associated with blood vessel normalization.

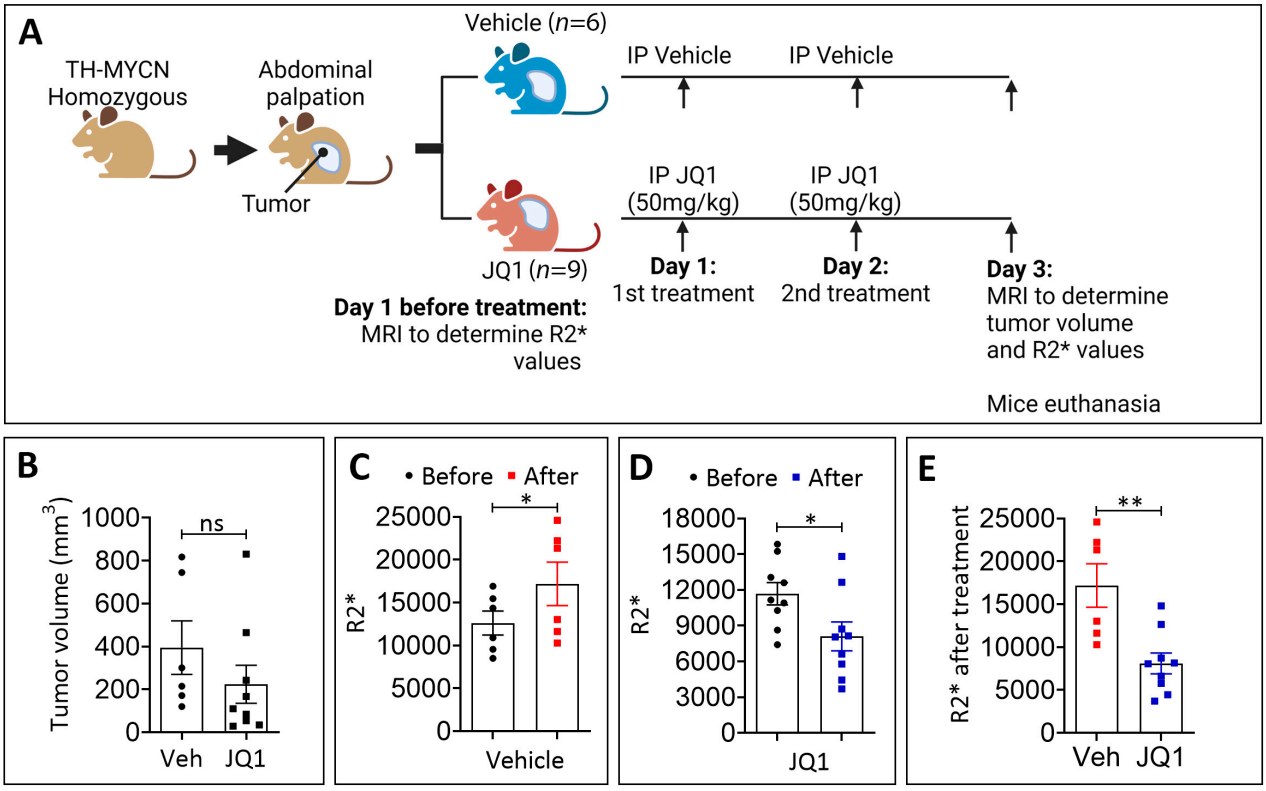

Figure 2. *Cont.*

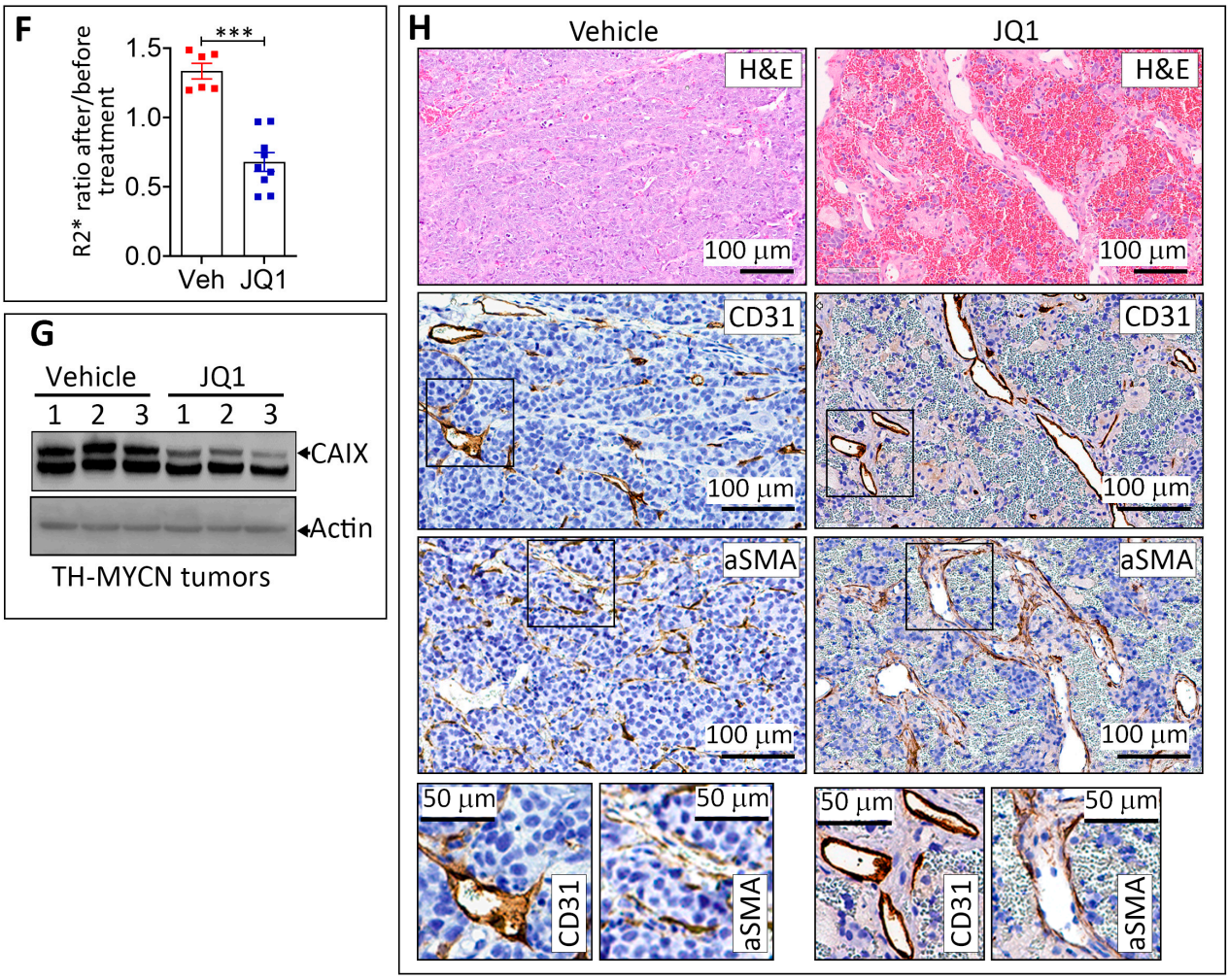

Figure 2. Effect of JQ1 on the hypoxia status and blood vessels in TH-MYCN tumor-bearing mice. (**A**) Experimental design of JQ1 treatment of TH-MYCN tumor-bearing mice showing the schedule and dosing. Following the development of homozygote TH-MYCN tumors, the tumor volume and R2* values were assessed by an MRI in all mice. Mice were randomly assigned to a vehicle-treated group (n = 6) or JQ1-treated group (n = 9). Mice were treated twice with either the vehicle or JQ1 (50 mg/kg) by IP on days 1 and 2. On day 3 post-treatment, an MRI was performed to determine the tumor volume and R2* values. Tumors were harvested for subsequent experiments. (**B**) The volume of TH-MYCN tumors treated with either the vehicle (Veh) or JQ1 as described in (**A**) on day 3. Each dot represents one tumor. Results are shown as mean ± SEM (error bars). Statistically significant differences were calculated compared to the Veh-treated tumors using an unpaired two-tailed Student's t-test (ns: not significant). R2* values in TH-MYCN tumors before and after treatment on day 3 with the vehicle (**C**) and JQ1 (**D**) according to the experimental design in (**A**). The R2* values in TH-MYCN tumors after the treatment with either the vehicle (Veh) or JQ1 are reported in (**E**). The R2* ratio of after to before treatment with the vehicle (Veh) or JQ1 is reported in (**F**). Each dot represents one tumor. Results are shown as mean ± SEM (error bars). Statistically significant differences are calculated using a Mann–Whitney test for (**C**) and (**D**) or an unpaired two-tailed Student's t-test for (**E**) and (**F**) (* $p < 0.05$, ** $p < 0.01$; and *** $p < 0.001$; ns: non-significant). (**G**) Western-blot showing the protein expression of CAIX in three different (1, 2 and 3) TH-MYCN tumors treated with either the vehicle or JQ1 according to the experimental schedule described in (**A**). Actin was used as a loading control. (**H**) Staining of vehicle- or JQ1-treated TH-MYCN tumors described in (**A**) with H&E (upper panels), CD31 (middle panels), or aSMA lower panels. Enlarged images of the zones delineated with black boxes (in CD31 and aSMA stained tumors) are shown (Scale bars 100 or 50 µm).

3.3. JQ1-Treated Tumors Displayed a Higher Infiltration Level of CD8⁺ PD-1⁺, Conventional CD4⁺ PD-1⁺, and Treg PD-1⁺ Cells Compared to the Control

It is now well established that hypoxia impacts the tumor immune landscape [30–32]. Blood vessel abnormalities associated with hypoxia limit or prevent the extravasation of cytotoxic immune cells [33]. Based on our data showing that JQ1 decreased the hypoxia status of TH-MYCN tumors and induced blood vessel normalization, we evaluated the immune infiltration in the control and JQ1-treated tumors. TH-MYCN mice bearing tumors were treated as described in Figure 3A. Tumors were collected on day 4 after three treatments with either vehicle or JQ1 and processed for FACS analysis. To avoid a potential bias in the interpretation of immune infiltration, which could be associated with intra- and inter-tumor heterogeneity and volume, we performed T2-weighted MR images at the time of euthanasia and selected tumors that had a similar average volume and mass to be included in the two groups (untreated and JQ1-treated) (Figure S1).

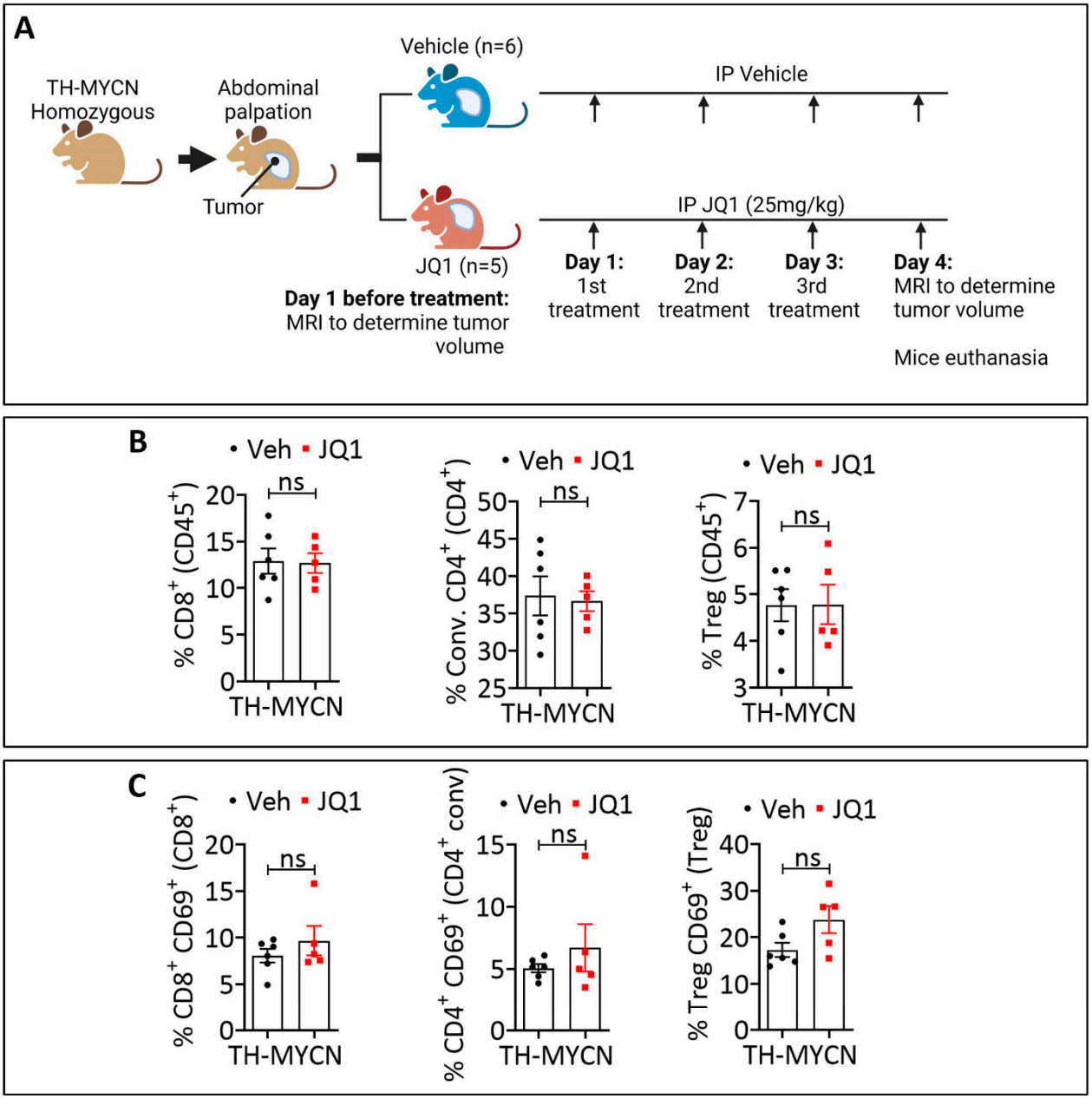

Figure 3. *Cont.*

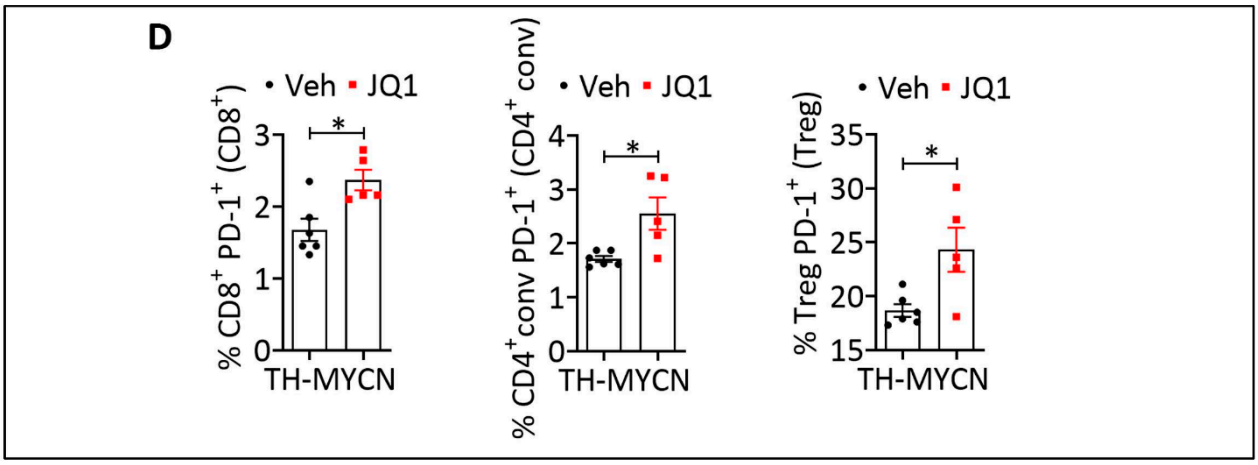

Figure 3. Immune phenotyping of JQ1-treated TH-MYCN tumors. (**A**) Experimental design of JQ1 treatment of TH-MYCN tumor-bearing mice showing the schedule and dosing. Following the development of homozygote TH-MYCN tumors, the tumor volume was assessed by MRI in all mice. Mice were randomly assigned to a vehicle-treated group (n = 6) or a JQ1-treated group (n = 5). Mice were treated three times with vehicle or JQ1 (25 mg/kg) by IP on days 1, 2, and 3. On day 4 post-treatment, MRI was performed to determine the tumor volume, and tumors were harvested for immune phenotyping experiments. (**B**–**D**) Flow cytometry quantification of total (**B**), $CD69^+$ (**C**), or $PD\text{-}1^+$ (**D**) $CD8^+$ T cells, conventional (Conv.) $CD4^+$ T cells and Tregs infiltrating vehicle-treated or JQ-1-treated TH-MYCN tumors at day 4. The defined subpopulations were gated and quantified in live $CD45^+$ cells. Each dot represents one tumor. The data are reported as the average of six or five mice per group. Results are shown as mean ± SEM (error bars). Statistically significant differences (indicated by asterisks) are calculated compared to vehicle-treated tumors using an unpaired two-tailed Student's t-test (ns: not significant *: $p < 0.05$).

We assessed by FACS the infiltration of lymphoid cells (CD4, CD8, and Treg) and myeloid cells (DC and macrophages). In addition, we analyzed the expression of activation and exhaustion markers CD69 and PD-1 on lymphoid cells. Our results revealed no significant difference in the infiltration of total $CD8^+$, conventional $CD4^+$, and neither Treg cells (Figure 3B) nor the infiltration of DC and total macrophages (Figure S2) in the either control or JQ1-treated tumors. Moreover, $CD8^+$, $CD4^+$, and Treg cells infiltrating the control and JQ1-treated tumors expressed a similar level of the activation marker CD69 (Figure 3C). We showed that JQ1-treated tumors exhibited a significantly higher level of $CD8^+$ $PD\text{-}1^+$, $CD4^+$ $PD1^+$, and Treg $PD\text{-}1^+$ cells compared to the control (Figure 3D).

3.4. Combining JQ1 Improves the Therapeutic Benefit of PD-1 in TH-MYCN Tumor-Bearing Mice

Recently, the infiltration of $CD8^+$ T cells expressing high PD-1 has been established as an effective biomarker for the response to immune checkpoint inhibitor therapy across multiple cancers [34]. Consistent with this, we evaluated the impact of combining JQ1 on TH-MYCN tumor response to anti-PD-1. To address this issue, homozygous TH-MYCN tumor-bearing mice were randomly assigned to several groups for treatments with mono (JQ1 or PD-1 alone) or combination (JQ1+PD-1). Mice included in the experiments were those having developed tumor volumes ranging from 400–800 mm^3 based on T2-weighted MR images performed before starting the treatments (Day 1). MRI images also assessed tumor volumes after treatment on days 4, 8 and 15. The different mouse groups and treatment schedules are summarized in Figure 4A. Representative images of one mouse from each group on days 1, 4, 8 and 15 are reported in Figure 4B. As shown on day 1, the tumor mass (delineated by a red line) was comparable in all groups (Figure 4B). However, the tumor volume was significantly reduced on days 8 and 15 in mice treated with a combination of JQ-1 and anti-PD-1 relative to mice treated with JQ-1 alone (Figure 4C). This reduction in the tumor volume was translated into a significant improvement in mice

survival in mice treated with a combination of JQ1 and anti-PD-1 relative to those treated with JQ-1 alone (Figure 4D).

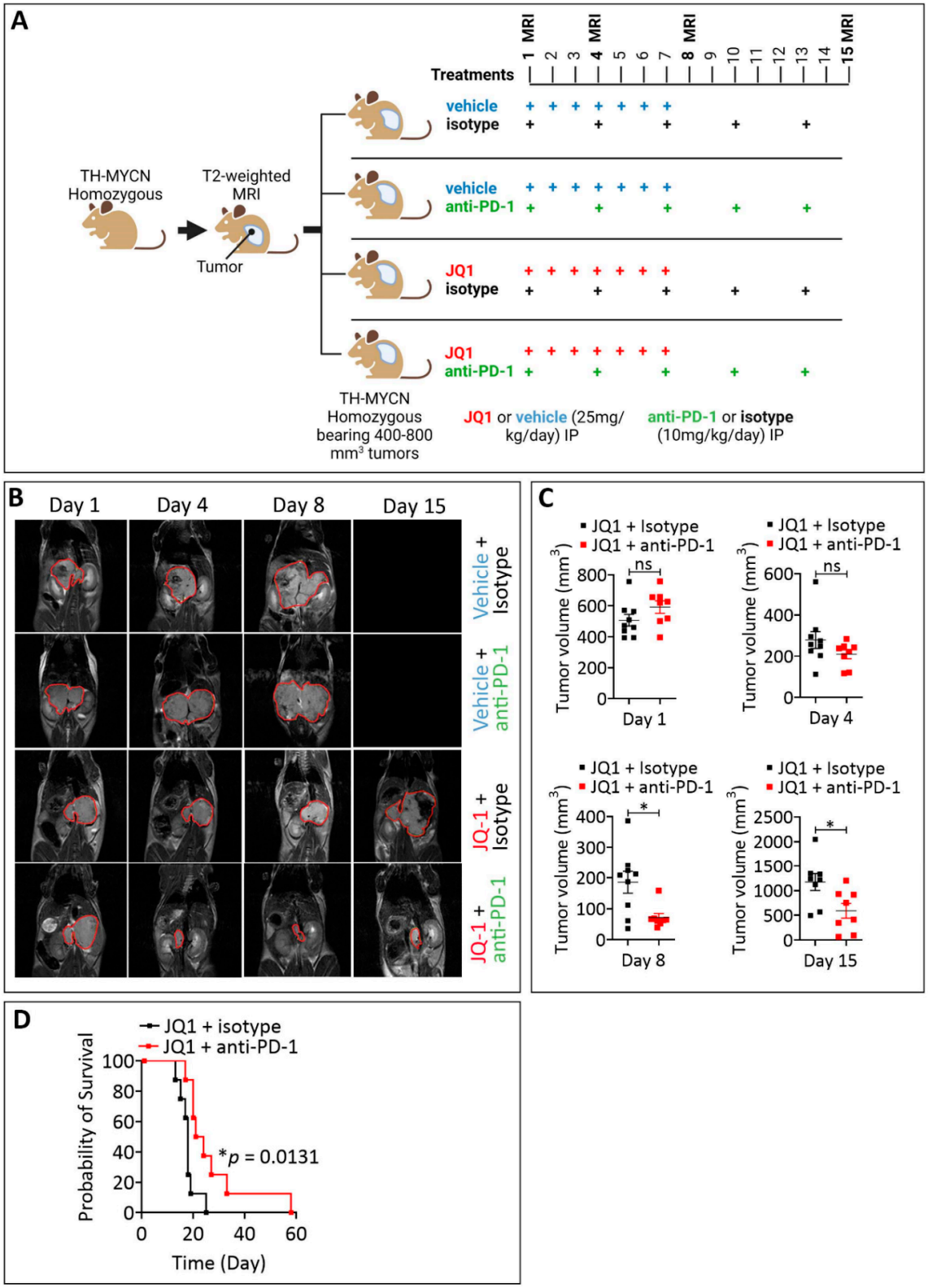

Figure 4. Impact of combining JQ1 and PD-1 on TH-MYCN tumor volume and mice survival. (**A**) Experimental design of JQ1 and anti-PD-1 treatment of TH-MYCN tumor-bearing mice showing

the schedule and dosing. Following the development of homozygote TH-MYCN tumors, the tumor volume was assessed by MRI in all mice. Mice were randomly assigned into four groups: vehicle- and isotope-treated group (n = 9), vehicle and anti-PD-1-treated group (n = 8), JQ1 and isotype-treated group (n = 8), and JQ1 and anti-PD-1-treated group (n = 8). Mice were treated daily from days 1 to 7 with either vehicle (group 1 and 2) or JQ1 (group 3 and 4) and on days 1, 4, 7, 10 and 13 with either isotype (group 1 and 3) or anti-PD-1 (group 2 and 4). After day 15, only the treatment with isotype or anti-PD-1 was continued using the same schedule until the end of experiment (mice euthanasia). JQ1 (25 mg/kg/day) and anti-PD-1 (10 mg/kg/day) were administered by IP. MRI was performed on days 1, 4, 8 and 15 post-treatment and on a regular basis to determine the tumor volume. (**B**) Representative images of TH-MYCN tumor-bearing mice on days 1, 4, 8 and 15 for the groups described in (**A**). On day 15, no images for groups 1 and 2 are provided as animals died. Abdominal tumor masses are delineated in red. (**C**) Volumes of TH-MYCN tumors on days 1, 4 and 8, and in mice treated with JQ1 and isotype or JQ1 and anti-PD-1. Each dot represents one tumor. Results are shown as mean ± SEM (error bars). Statistically significant differences are calculated compared to the control group (JQ1 and isotype) using an unpaired two-tailed Student's t-test (ns: not significant *: $p < 0.05$). (**D**) Mice survival curves were generated from tumor-bearing mice treated with JQ1 and isotype or JQ1 and anti-PD-1. Lack of survival was defined as death or tumor size > 2000 mm^3. The probability of survival was defined using GraphPad Prism, and p-values were calculated using the log-rank (Mantel-Cox) test (* $p \leq 0.05$).

4. Discussion

In this study, we revealed that combining JQ1 improves the benefit of anti-PD-1 in the TH-MYCN NB mouse model. Such improvement could be related to the effect of JQ1 in decreasing hypoxia in TH-MYCN NB tumors. Indeed, JQ1 and derivatives are currently attracting major interest in treating hematological and solid cancers, including pediatric malignancies [35]. We strongly believe that the ability of JQ1 to decrease hypoxia in TH-MYCN NB tumors relies on the impairment of the transcriptional activity of HIF-1α. This concept was supported by: (i) our data showing that treatment with JQ1 decreased the mRNA and protein levels of CA9 in several NB cell lines, including those derived from TH-MYCN tumors; and (ii) previous studies showing that JQ1 impairs hypoxia in triple-negative breast cancer through its ability to interact with HIF-1α and inhibit its transcription activity [24,36].

Remarkably, in all hypoxic NB cell lines, we showed that the protein level of HIF-1α decreased following treatment with JQ1 in a dose-dependent manner. Although the exact mechanism responsible for the decrease of HIF-α protein levels remains unknown, it is tempting to speculate that this could be related to the impact of JQ1 in reactivating the ubiquitin proteasomal system (UPS) responsible for HIF-1α degradation. Additional experiments need to be performed to assess the reactivation of UPS in JQ1-treated cells and tumors.

Since CA9 overexpression is associated with poor survival in NB patients, we believe that JQ1, through inhibiting HIF-1α/CAIX axis, may inhibit the growth of TH-MYCN tumors. However, our results, depicted in Figure 1B, showed that, although there is a clear trend toward a decrease, the average tumor volume between vehicle- and JQ1-treated tumors was not significantly different. This could be related to the narrow therapeutic windows used in the experimental design or to the limited group size. Indeed, the graph depicted in Figure 2B suggests that JQ1 treatment appeared to be efficient in decreasing the volume in many tumors but not in all tumors, which may be caused by biological or experimental reasons. Furthermore, considering the role of RACK-1 in NB invasion and migration, it would be interesting to evaluate the impact of JQ1 on the regulation of RACK-1 in NHO2A mouse cells in vitro and in TH-MYCN tumors in vivo.

Nevertheless, the role of JQ1 in decreasing hypoxia in TH-MYCN tumors has also been supported in vivo by assessing the R2* values using MRI technology. Our results further supported that implementing the R2* assay in the clinic may be beneficial as this is

a non-invasive method to indicate the hypoxic status in pediatric tumors where invasive procedures are always difficult to implement [37].

JQ1 displayed an anti-angiogenic effect by reducing the expression of the angiogenic pathway, the key angiogenic inducer VEGF-A, and the blood vessel count. In keeping with this, we showed that treatment of TH-MYCN tumor-bearing mice with JQ1 seemed to reduce the number of blood vessels while improving their quality and integrity, as revealed by CD31/αSMA staining. Therefore, our data supported the concept that JQ1 showed typical behavior of anti-angiogenic agents in NB tumors.

By assessing the infiltration of major cytotoxic immune cells, our data revealed no impact on the infiltration of CD8, CD4 and Tregs into the tumor microenvironment of TH-MYCN tumors following treatment with JQ1. However, JQ1 treatment significantly increased the expression of PD-1 on $CD8^+$, $CD4^+$ and Tregs. The role of PD-1 expression on $CD8^+$ cells has been extensively evaluated. Initially described as an exhaustion marker, the expression of PD-1 on $CD8^+$ T cells is now reported as a strong predictor of the response to ICB in NSCLC and correlated with increased overall survival [38]. The predictive value of PD-1^{high} $CD8^+$ T cells was also reported across five cancer types in several clinical samples and mouse models (reviewed in [34]). Recently, the combined expression of PD-1 on circulating $CD4^+$ and $CD8^+$ T cells before ICB treatment has been considered to guide therapy for patients with NSCLC [39]. Our data showed a significant increase in $CD4^+$ PD-1+ and CD8 PD-1^+ in the microenvironment of JQ1-treated TH-MYCN tumors highlighting the value of combining JQ1 and anti-PD-1. Indeed, we showed that JQ1 synergizes with anti-PD-1 to elicit a remarkable anti-tumor effect compared with JQ1 or anti-PD-1 alone. We believe that such a synergistic result relied on the effect of JQ1 to activate $CD8^+$ T cells through the upregulation of PD-1. Knowing that NFATc1 is reported to regulate the expression of PD-1 expression in activated T cells [40,41], it would be interesting to determine whether JQ1 regulates NFATc1 in CD8 T cells infiltrating TH-MYCN. Nevertheless, PD-1-overexpressing CD8 T cells engaged PD-L1 on tumor cells, which can subsequently be released by anti-PD-1. Although the underlying mechanism of PD-1 upregulation on CD4, CD8, and Tregs following JQ1 treatment has not yet been investigated, we speculate that such a mechanism relies on epigenetic regulation or an increase in the protein synthesis of PD-1 by JQ1.

Although much remains to be learned mechanistically, our in vivo data are supported by a previous report showing that combining JQ1 and anti-PD-L1 led to a synergistic effect in pancreatic cancer [42]. Moreover, cooperative effects between JQ1 and anti-PD-1 have been reported in Kras+/LSL-G12D; Trp53L/L (KP) mouse models of NSCLC. In this model, combining JQ1 with anti-PD-1 impaired the immunosuppressive activity of Tregs and favored the activation of T cells in the tumor microenvironment. The JQ1–anti-PD-1 combination induced robust and long-lasting anti-tumor responses associated with an improvement in the overall survival compared to each treatment alone [43].

Taken together, our study provided convincing data supporting the concept that combining BET bromodomain inhibition JQ1 with immune checkpoint blockade based on PD-1 offers a promising therapeutic approach for high-risk neuroblastoma displaying MYCN amplification. However, given that not all high-risk NB is MYCN-amplified, it would be interesting to evaluate whether our data described here can be translated to MYCN-non-amplified NB.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11182783/s1, Figure S1: Volume and mass of vehicle- or JQ1-treated tumors; Figure S2: Flow cytometry quantification of total DC and macrophages infiltrating vehicle- or JQ-1.

Author Contributions: Conceptualization, D.S., M.B. and E.V.; methodology, G.K. and O.K.; validation, D.S., M.B., E.V., G.K. and A.O.; investigation, D.S., M.B., E.V., G.K., A.O. and G.B.; resources, D.S., M.B., E.V., G.K., A.O., G.B. and B.J.; data curation, D.S., M.B., E.V., G.K. and O.K.; writing—original draft preparation, D.S., M.B. and B.J.; writing—review and editing, M.B. and B.J.; visualization, B.J.; supervision, B.J.; project administration, B.J.; funding acquisition, B.J. and G.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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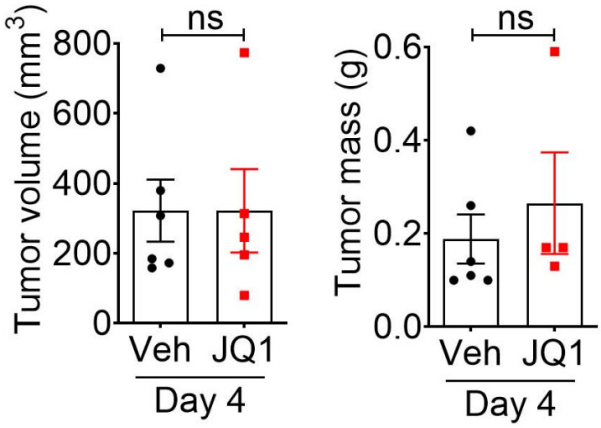
cells

MDPI

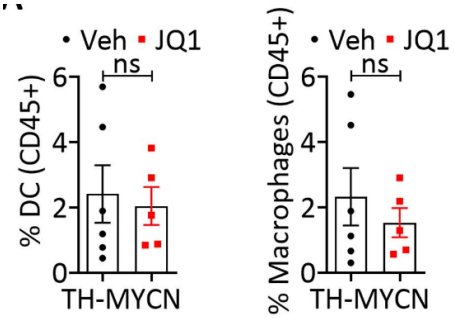
Supplementary Data

The BET Protein Inhibitor JQ1 Decreases Hypoxia and Improves the Therapeutic Benefit of Anti-PD-1 in a High-Risk Neuroblastoma Mouse Model



	Volume	mass (g)
Mean of Vehicle-treated tumors	321.9	0.1883
Mean of JQ1-treated tumors	321.6	0.265
Difference between means (Veh - JQ1) ± SEM	(0.2583 ± 145.7)	(-0.07667 ± 0.1082)

Supplementary Figure S1. Volumes (left) and masses (right) of TH-MYCN tumors at day 4 in mice treated with vehicle (Veh) or JQ1. Each dot represents one tumor. Results are shown as mean ± SEM (error bars). Statistically significant differences were calculated compared to the control group (veh) using an unpaired two-tailed Student's t-test (ns: not significant). The table at the bottom shows the means of tumor volume and mass in vehicle- and JQ1-treated tumors and the difference between means.



Supplementary Figure S2. Flow cytometry quantifies total DC and macrophages infiltrating vehicle-treated or JQ-1-treated TH-MYCN tumors on day 4. The defined subpopulations were gated and quantified in live CD45+ cells. Each dot represents one tumor. The data are reported as the average of six or five mice per group. Results are shown as mean ± SEM (error bars). Statistically significant differences (indicated by asterisks) are compared to vehicle-treated tumors using an unpaired two-tailed Student's t-test (ns: not significant).

9. Additional Results

9.1 DCE-MRI

Homozygous mice with palpable tumors were assessed by MRI to measure the initial tumor volume and perfusion acquisitions were performed after Gadolinium injection. Then, mice were treated either with JQ1 50 mg/kg IP or vehicle for 2 days and then acquisitions were performed again to assess the JQ1 effect on vessel permeability. In total, JQ1 group include 6 mice whereas 3 mice were in the control group.

JQ1 leads to tumor shrinkage, however tumor volumes were similar between the two groups at euthanasia. In addition, tumor perfusion data were normalized using liver values. In this way, the differences observed between JQ1-treated animals and controls could be attributable to JQ1 action and effect possibly resulting from tumor volume changes was minimalized.

We considered the K^{trans} to assess the vessel permeability, as this pharmacokinetic parameter depends on the Gd outside the vessels to the tissue (EES) and so correlates to the leakage of the vessels. The K^{trans} values were decreased in the JQ1 treated mice although the results were not significant (p = 0.08); we observed the opposite effect in the control group, with a significant increase in K^{trans} values after vehicle treatment (p = 0.04) (figure 21 (a)). Moreover, K^{trans} values significantly differ between JQ1 and vehicle mice at the time of euthanasia. Mice treated by JQ1 showed significantly lower K^{trans} values than control ones (p = 0.04, figure 21 (b)). Our results suggest that tumor vessels become less leaky after JQ1 treatment.

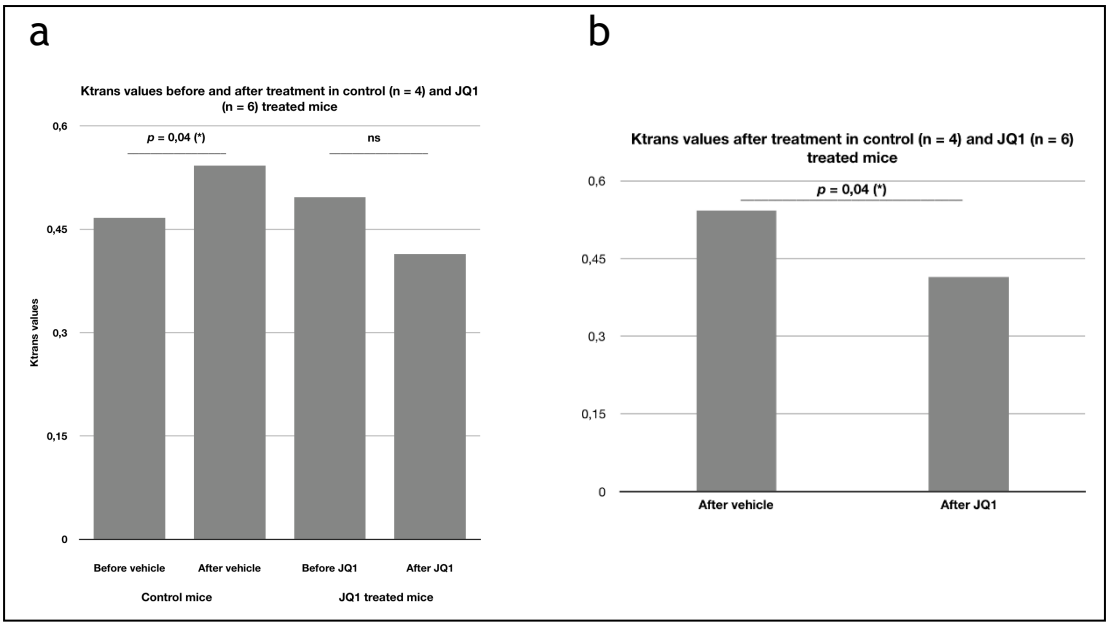


Figure 21: (a) : K^{trans} values before and after vehicle treatment: p = 0.04 (paired student t-

test); K^{trans} values before and after JQ1 treatment: $p = 0.08$ (paired student t-test); (b): K^{trans} values after treatment: JQ1 versus control: $p = 0.04$ (unpaired student t-test).

9.2 Expression of Cytokines/chemokines

To assess the major cytokines/chemokines impacted by JQ1 treatment, we performed large scale cytokine/chemokine study using a Cytokine Array membrane kits. Two homozygous mice with developing tumors were treated for 2 days either with JQ1 50 mg/kg or vehicle. Tumor volume was precisely assessed by T2w MRI sequences just before starting treatment and at the time of euthanasia: JQ1 mouse harbor an initial volume of 507 mm3 which decreased to 258 mm3 after 2 doses of JQ1, whereas vehicle mouse had an initial volume of 286 mm3 and an end-volume of 681 mm3. Of note, vehicle mouse had also a chest mass but for the study only the abdominal tumor was taken into account and harvested for cytokine array.

Cytokine array was performed according to the manufacturer's protocol. After substracting the mean value of the negative control from the mean value of the wells, we normalized each mean value for cytokine with the mean value of the reference spots. We considered the control values as 1, and we reported the values for JQ1 treated mouse to the vehicle treated mouse values and results were expressed as fold-change. We considered fold-change values smaller than 0.5 or greater than 2 for spots exhibiting sufficient intensity. Taking into account these criteria, JQ1 had an impact on Chemokine (C-C motif) ligand 6 (CCL6), Fibroblast growth factor (FGF) acidic and endostatin levels (Figure 22).

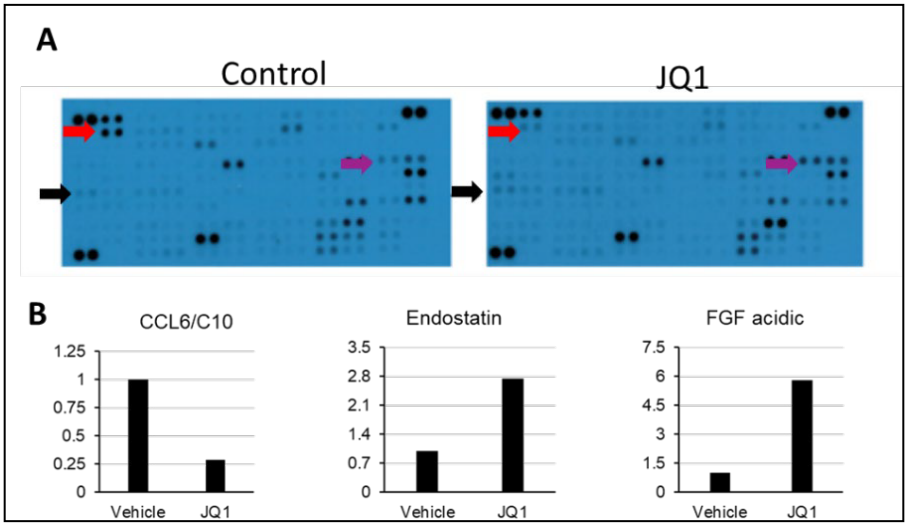


Figure 22: **A:** Cytokine/Chemokine Array of control- and JQ1-treated tumors. Positions of the spots on the array (B3-B4: CCL6 (red arrows); D21-D22: Endostatin (purple arrows); E1- E2: FGF acidic (black arrows). **B:** Quantification of CCL6, FGF acidic and endostatin levels expressed in fold-change considering the control mouse values as 1.

Our cytokine assay results have to be interpreted with caution as the test was done on only two tumor samples. Clearly, additional investigations are needed to confirm these preliminary observations. However, in our experiment, JQ1 downregulate CCL6. The

overexpression of the mouse cytokine CCL6 has been reported to favor the aggressiveness of different cancer cell lines increasing their invasiveness and metastasis dissemination in cancer pre-clinical models [159]. In addition, depending on the cancer tissue, Myc oncoproteins regulate CCL6 [159]. In our cytokine profile, we suggest that the mechanism leading to the decreased CCL6 could be at least in part explained by the downregulation of *MYCN* by JQ1, but this has to be confirmed by further studies.

Endostatin is an endogenous angiogenesis inhibitor, enzymatically cleaved from collagen XIII [160]. It plays a role in different cancer types, and through repressing angiogenesis and contributes to inhibit tumor growth in several cancer pre-clinical models [160]. However, the clinical use of Endostatin is limited as its administration is challenging; indeed, recombinant human Endostatin (Endostar), even if modified and rendered more stable, needs long perfusion time and has to be administered every day [160]. Our cytokine assay suggest that JQ1 could up-regulate Endostatin, and by this way could represent an attractive method to inhibit tumor angiogenesis.

JQ1 seems to up-regulate FGF acidic, also known as FGF1. As other FGFs, FGF acidic is involved in tumor progression as it notably contributes to tumor angiogenesis [161]. However, FGFs activity is context and cancer-dependent, as many studies have also reported the tumor suppressive functions of FGFs [161, 162]. These benefits could result from the differentiation of cancer cells promoted by the FGF receptor binding in particular cellular settings [161]. As JQ1 drastically reduced tumor volume of NB bearing TH-*MYCN* mice, the potential benefit of FGF acidic up-regulation could result from the induction of cancer cells differentiation. Nonetheless, additional studies are strongly needed to unravel the mechanism of action of FGF acidic in the context of NB.

9.3 MYCN expression

We performed a Western Blot to confirm the previous observations reported in the literature in our mouse model. Tumors from homozygous mice treated with JQ1 50 mg/ kg IP or vehicle IP for 2 days were collected and immediately frozen in isopentan. Proteins were extracted according to the method described in the Material and Methods section. JQ1 clearly decreases *MYCN* expression in treated mice as demonstrated on Westen Blot (Figure 23).

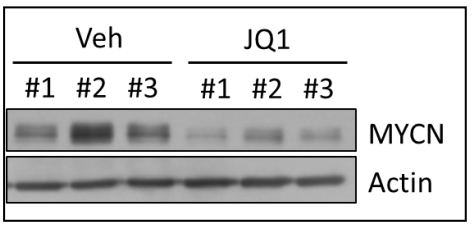


Figure 23: The expression of MYCN protein in vehicle- and JQ1-treated tumors by Western Blot. Actin was used as loading control. Three tumors (#1, #2 and #3) from each group were assessed.

10. Article 2:

Impact of hypoxic tumor microenvironment and tumor cell plasticity on the expression of immune checkpoints

Lequeux, M. Z. Noman, M. Xiao, D. Sauvage, K. Van Moer, E. Viry, I. Bocci, M. Hasmim, M. Bosseler, G. Berchem, B. Janji.

Cancer Letters 458 (2019) 13-20.

This review aims to summarize the current knowledge on the interplay between hypoxia, cancer cell EMT and immune checkpoints expression. Indeed, a strong link between these three actors of cancer cells immune evasion have been established in several studies and the deep understanding of the pathways involved will certainly leads to more selected combined immunotherapies allowing sustainable clinical responses.

Many reports have underlined the importance of the hypoxic TME in promoting cancer immune escape, and recent studies have demonstrated the role of HIFs in immune checkpoints expression: for example, Noman *et al* have shown that HIF-1α binds to the HRE in the PD-L1 promoter leading its increased expression in tumor-infiltrating MDSCs, which results in increased immunosuppressive cytokines production and finally dampen CD8+ T cells proliferation [10]. Hypoxia also promotes PD-L1 expression on cancer cells as reported by Barsoum *et al* [9]. Other studies have revealed that the role of HIF-1α is not limited to the PD-L1 immune checkpoint, but instead accounts also for example for the up-regulation of CD47, CD73 and soluble CD137 in cancer cells [116, 163].

Hypoxia also favors EMT in cancer cells, a state characterized by down-regulation of E-cadherin and a morphological switch rendering cancer cells more mobile and invasive. EMT also directly impacts immune checkpoints expression. For example, Noman *et al* have showed that up-regulation of CD47 occurs through the binding of SNAI1 and ZEB1 to the CD47 promoter in EMT-activated mesenchymal breast cancer cells [164]. Moreover, the link between EMT-activation and ZEB1/miR-200 pathway and the increased of PD-L1 expression in EMT-activated breast cancer cells has also been reported [165].

So, hypoxia, EMT and immune checkpoints are interconnected in cancer to promote an immunosuppressive TME and combinatorial therapies targeting hypoxia and/or EMT in combination to ICI (such as for example PD-1/PD-L1 axis blockade) should give promising clinical results.

Author contribution: I contributed in the writing process of this review, notably the section related to hypoxia.

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Impact of hypoxic tumor microenvironment and tumor cell plasticity on the expression of immune checkpoints

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ABSTRACT

Compared to traditional therapies, such as surgery, radio-chemotherapy, or targeted approaches, immunotherapies based on immune checkpoint blockers (ICBs) have revolutionized the treatment of cancer. Although ICBs have yielded long-lasting results and have improved patient survival, this success has been seriously challenged by clinical observations showing that only a small fraction of patients benefit from this revolutionary therapy and no benefit has been found in patients with highly aggressive tumors. Efforts are currently ongoing to identify factors that predict the response to ICB. Among the different predictive markers established so far, the expression levels of immune checkpoint genes have proven to be important biomarkers for informing treatment choices. Therefore, understanding the mechanisms involved in the regulation of immune checkpoints is a key element that will facilitate novel combination approaches and optimize patient outcome. In this review, we discuss the impact of hypoxia and tumor cell plasticity on immune checkpoint gene expression and provide insight into the therapeutic value of the EMT signature and the rationale for novel combination approaches to improve ICB therapy and maximize the benefits for patients with cancer.

1. Immune checkpoint blockade in cancer therapy

For over a century, scientists have attempted to eradicate cancer cells by activating and harness the patient's immune response. The majority of the cancer immunotherapies developed over the past two decades have been dedicated to the potentiation of anti-tumor adaptive immune response mediated mostly by T lymphocytes. Neither systemic administration of high-doses of interleukin (IL)-2 or autologous T lymphocyte transplantation has yielded very little to no therapeutic benefit and very high toxicity.

After decades of bench research and the resulting deeper understanding of the mechanisms underlying anti-tumor immunity, cancer immunotherapy has now finally moved into the clinic. The immune checkpoint blockade-based cancer immunotherapy revolution has just started and yet it has transformed the field of onco-immunology and the way we treat cancer. Immune checkpoint blockers (ICBs) such as anti-CTLA-4 and anti-PD-1/PD-L1 antibodies have reshaped the clinical approaches to multiple different types of cancers. ICBs have emerged as a revolutionary treatment for cancers including, but not limited to, breast, lung, kidney, bladder, prostate, lymphoma, and melanoma [1,2]. Ipilimumab (anti-CTLA-4) was approved in 2011 and pembrolizumab and nivolumab (anti-PD-1) were approved in 2014 by the U.S. Food and Drug Administration (FDA) for the treatment of advanced melanoma.

Despite the exciting and encouraging clinical responses, the majority of patients treated with ICB-based monotherapies (e.g., anti-CTLA-4, anti-PD-1, or PD-L1) only have partial responses and fail to achieve higher objective responses. Only 20–30% of patients with non-small-cell lung carcinoma (NSCLC), renal cell carcinoma (RCC), and melanoma benefited from cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death 1 (PD-1) blockade. ICB-unresponsive patients can be classified into two types: (1) primary resistance patients who did not respond at all and (2) acquired resistance patients who initially responded but later relapsed. It is now well established that enduring therapeutic benefit and prolonged survival can be achieved by combining several ICBs such as anti-CTLA-4 and anti-

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Abbreviations

ANGPTL4	Angiopoietin like 4
BRAFi	B-raf inhibitor
CAFs	cancer associated fibroblasts
ccRCC	Clear cell renal cell carcinoma
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cells
DNMT1	DNA-methyltransferase 1
DNMT3A	DNA methyltransferase 3A
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
EMT-TFs	EMT-Transcription factors
FDA	Food and drug administration
GITR	glucocorticoid-induced TNFR family related gene
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HLA-G	Human leukocyte antigen G
HRE	Hypoxia response element
ICBs	Immune checkpoint blockers
IDO	Indoleamine 2, 3-dioxygenase
IL-6	Interleukin 6
IL-10	Interleukin 10
LAG3	Lymphocyte-activation protein 3
MDSC	Myeloid derived suppressor cells
MET	Mesenchymal-to-epithelial transition
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
NK cells	Natural killer cells
NSCLC	Non-small-cell lung carcinoma
pADC	Pulmonary adenocarcinoma
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
RCC	Renal cell carcinoma
SIRPα	Signal regulatory protein alpha
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TILS	Tumor-infiltrating lymphocytes
TKIs	Tyrosine kinase inhibitors
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
TSP-1	Thrombospondin
VEGFA	Vascular endothelial growth factor A and
VHL	Von Hippel-Lindau

PD-1 [3,4].

2. Hypoxia in the tumor microenvironment

Hypoxia is a hallmark of all solid tumors' microenvironment and it is strongly associated with malignant progression, therapeutic resistance, and poor clinical outcome [5,6]. All solid tumors contain areas of variable oxygen concentration [7]. Within solid tumors, there are areas that are well oxygenated, poorly oxygenated, and, finally, necrotic in which tumor cells have died due to inadequate oxygenation [5].

Tumor cells adapt to the hypoxic microenvironment through the hypoxia-inducible factor (HIF) family of transcription factors. HIFs are heterodimeric proteins composed of an oxygen-sensitive alpha subunit (HIF-1α, HIF-2α, HIF-3α) and a beta subunit (HIF-β/ARNT). Both HIF-1α and HIF-2α are regulated by oxygen-dependent von Hippel-Lindau (VHL)-mediated degradation [6]. HIF-1α and HIF-2α share overlapping target genes and each one also regulates a set of unique targets. These hypoxia-dependent HIF-1α- and HIF-2α-induced genes play important roles in regulating different aspects of tumor biology such as angiogenesis, cell survival, chemo- and radio-resistance, proliferation, tumor cell plasticity, invasion and metastasis, pH regulation and metabolism, resistance to the immune system, and maintenance of cancer stem cells [6,8].

It is well established that cancer progression is not only regulated by the cancer cells but also influenced by the surrounding stroma. The tumor microenvironment (TME), in addition to cancer cells, includes a diversity of cells and factors such as cancer-associated fibroblasts (CAFs), endothelial cells, immune cells, growth factors, cytokines, and extracellular matrix proteins [9]. Loss of HIF-1α and its target gene VEGF-A but not HIF-2α enhanced tumor growth in MMTV-PyMT transgenic mice by reducing vascular density with less leaky vessels and decreased tumor-associated macrophage infiltration [10].

Hypoxia-mediated HIF activation has opposing effects in tumor and stromal cells. HIF1 activation in different cell types within the TME can either promote or repress tumorigenesis; HIF1 activation was shown to be the former in CAF by promoting autophagy and aerobic glycolysis, which in turn provides nutrients to the surrounding cancer cells and, thereby, promotes their growth. In contrast, HIF1 activation exhibited tumor suppressor activity in breast cancer cells [11].

3. Tumor cell plasticity and the epithelial mesenchymal transition

"Epithelial-to-mesenchymal transition" (EMT) was first described as a mechanism driving critical morphogenetic steps in the development of most metazoans and in wound-healing and carcinoma progression [12].

"Epithelial-to-mesenchymal transition" (EMT) is a reversible, dynamic cellular program during which either healthy or neoplastic epithelial cells transform into a more motile, invasive, and aggressive mesenchymal cell type. The resulting mesenchymal-like cells can revert back to the epithelial state, which is known as the mesenchymal-to-epithelial transition (MET). Upon EMT initiation, the expression of epithelial markers (e.g., E−cadherin) is repressed, which leads to the loss of the polygonal, round, cobblestone morphology of epithelial cells. The cells then acquire a spindle-shaped, elongated mesenchymal morphology and express mesenchymal markers (e.g., neural cadherin, vimentin, and fibronectin). EMT is orchestrated by a series of master EMT-inducing transcription factors (EMT-TFs) including ZEB, SNAIL, and TWIST, which inhibit or represses the expression of genes associated with the epithelial state [13]. To quantitatively measure the interplay between EMT and cancer progression, universal computed EMT scoring has been defined from cancer-specific transcriptomic EMT signatures of ovarian, breast, bladder, lung, colorectal and gastric cancers. The EMT score was defined based on the expression of multiple EMT markers including the major EMT-drivers, SNAI1 and ZEB1. Thus, Samples with a positive (high) EMT score were more mesenchymal, whereas those with a negative (low) score were more epithelial [12].

Several cell-intrinsic signaling pathways (TGF-β, WNT, STAT, and NOTCH) also induce EMT-TFs leading to a phenotypic transition to a mesenchymal or partially mesenchymal cell state [14]. Importantly, several stromal cells (e.g., CAFs) and immune cells (e.g., $CD4^+$ helper and $CD8^+$ cytotoxic T cells, Treg, MDSCs, TAMs) in the TME are known to secrete various cytokines (e.g., TGF-β, IL6, TNF-α), chemokines (e.g., CCL18), and growth factors (e.g., VEGF, HGF, and EGF). These secreted factors act in a paracrine fashion to induce EMT in the surrounding

cancer cells either by activating EMT-TFs or by inducing effector molecules that inhibit the epithelial state [13].

Once activated in cancer cells, EMT promotes resistance to cell death inducers including chemotherapy. EMT was shown to contribute to cyclophosphamide resistance in a spontaneous breast-to-lung metastasis model [15]. Similarly, EMT suppression through the deletion of Snail or Twist in a murine model of pancreatic cancer led to increased sensitivity to gemcitabine treatment [16].

EMT supports stemness, immune evasion, immune suppression, and resistance to immunotherapy [13,17–19]. Both human and mouse melanoma cells transfected with snail1 underwent EMT and became more metastatic than their parental cells. Snail1-expressing melanoma cells secreted TGFβ and thrombospondin, which favored the emergence of highly immunosuppressive regulatory T cells. Snail small interfering RNA (siRNA) injected *in vivo* reduced the immunosuppressive and metastatic potential of these melanoma cells [20]. Likewise, the CTL-mediated lysis of MCF-7 snail cells decreased considerably due to the induction of autophagy [21]. Interestingly, tumors that best respond to CTLA-4, PD-L1, and PD1 (e.g., melanomas, renal, bladder and lung cancers [2]) have higher EMT scores [12].

4. Hypoxia-dependent regulation of immune checkpoints in cancer

Hypoxia contributes to the immunosuppressive tumor microenvironment in many cancers by activating intrinsic mechanisms that allow tumor cells to escape from the innate and adaptive immune defenses [8]. Moreover, several lines of evidence suggest that the establishment of this immunosuppressive microenvironment could be attributed to the ability of hypoxia to regulate immune checkpoints. We have previously shown that the mRNA and protein expression of programmed cell death ligand 1 (PD-L1), but not programmed cell death ligand 2 (PD-L2), in myeloid-derived suppressor cells (MDSCs) infiltrating several tumor types, is higher than splenic MDSCs indicating that factor(s) in the tumor microenvironment is (are) involved in the upregulation of PD-L1. We have further identified hypoxia as a major factor involved in the upregulated expression of PD-L1 in tumor-infiltrating MDSCs since the exposure of MDSCs isolated from the spleen to hypoxic conditions dramatically increased the expression of PD-L1 but not PD-L2. Further investigation revealed that, upon its stabilization in hypoxic cells, HIF-1α binds the hypoxia-response element (HRE) located in the proximal PD-L1 promoter. The functional consequences of the overexpression of PD-L1 on the surface of MDSCs include the increased production of IL-6 and -10 under hypoxic conditions and significantly decreased proliferation of $CD8^+$ T cells; taken together, these phenotypic changes are indicative of enhanced immunosuppressive function when MDSCs are exposed to hypoxia. Treatment with an anti-PD-L1 antibody significantly decreased both the expression of IL-6 and -10 and the $CD8^+$ T cells anti-proliferative property of MDSC [22]. Moreover, a combination therapy targeting tumor hypoxia with an antibody against PD-L1 may be beneficial for stimulating the anti-tumor immune response in patients with cancer. Our data were supported by Barsoum et al., who showed that hypoxia upregulates the expression of PD-L1 on the surface of human breast and prostate cancer cells, as well as in mouse melanoma and mammary carcinoma cells. Increased expression of PD-L1 leads to tumor cell resistance to CTL-mediated lysis [23] (Fig. 1A).

In addition to PD-L1, HIF-1α also regulates the expression of macrophage immune checkpoint marker CD47 under hypoxic conditions (Fig. 1A). CD47 is a “don't eat me” signal found on the surface of several cancer cell types. Following its interaction with signal regulatory protein alpha (SIRPα) and thrombospondin-1 (TSP-1), which are expressed by tumor phagocytic cells, the phagocytosis of tumor cells is inhibited by the coordinated blockade of the “eat me” signal and the activation of the “don't eat me” signal [24]. As anti-CD47 blockade-based therapy is now moving into the clinic [25], understanding the molecular mechanism involved in the regulation of CD47 is a highly prioritized area of research toward enhancing cancer immunotherapeutic approaches using CD47 blockade. Thus, it has been shown that HIF-1α regulates the transcription of the CD47 gene by directly binding to its promoter in breast cancer cells. Targeting CD47 increases the phagocytic ability of macrophages against breast cancer cells. According to The Cancer Genome Atlas (TCGA), in an analysis of thousands of patients with breast cancer, CD47 expression is correlated with HIF target gene expression such as angiopoietin-like 4 (ANGPTL4) and vascular endothelial growth factor A (VEGFA). Increased CD47 mRNA levels are associated with decreased patient survival [26]. Furthermore, the induction of HIF-1α by chemotherapy directly activates PD-L1, CD47, and CD73 gene transcription in triple-negative breast cancer (TNBC) making their TME more immunosuppressive by impairing the adaptive anti-tumor immune response (Fig. 1A) [27].

Hypoxia increases the expression of CD137 (4-1BB) in tumor-infiltrating T lymphocytes (TILs) in colon carcinomas, melanoma, and spontaneous breast adenocarcinomas (Fig. 1A). CD137 is expressed on both innate (NK cells) and adaptive immune cells (activated T cells). The CD137 ligand (CD137L) is expressed exclusively on the surface of activated antigen-presenting cells (macrophages, dendritic cells [DCs] and B cells). CD137L binding to CD137 induces a strong antitumor immune response mediated by activated T and NK cells. In HIF-1α knockout T cells, hypoxia fails to induce CD137 expression on the surface of TILs and they remain CD137 negative even when becoming TILs. Combining anti-CD137 agonists results in a synergistic effect on the PD-L1 blockade [28]. The same group also showed that a soluble form of CD137 (sCD137) was induced under hypoxia in multiple murine and human cancer cell lines. This secreted sCD137 blocks CD137L mediated co-stimulation of activated T cells [29].

Human leukocyte antigen G (HLA-G) is another immune checkpoint marker that contributes to tumor immune evasion. A correlation between the expression of HLA-G and poor clinical patient outcome has

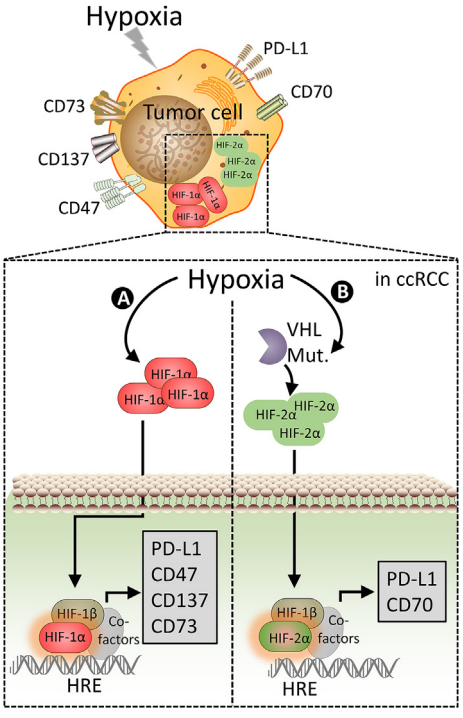

Fig. 1. The impact of hypoxia on the regulation of immune checkpoints in tumor cells. Under hypoxic conditions, stabilized HIF-1α **(A)** or mutated VHL-dependent stabilized HIF-2α in ccRCC cells **(B)** translocates to the nucleus and forms a complex with HIF-1β and its co-factors. This complex binds to the hypoxia-response element (HRE) motif and induces the expression of several immune checkpoint genes such as PD-L1, CD47, CD137, CD73, and CD70.

been described [30]. In melanoma, the expression of HLA-G positively correlated with the hypoxic status of these tumors [31]. In glioma cells, HLA-G gene expression is mediated by HIF-1α through binding to the HRE motif located in exon 2 [32].

In patients with clear cell renal cell carcinoma (ccRCC) and VHL bi-allelic inactivation, the expression of PD-L1 was increased compared to the ccRCC tumors with wild-type VHL. Using 786-O cells expressing different VHL mutants with stabilized HIF-2α, we demonstrated that HIF-2α and PD-L1 expression are positively correlated. Indeed, targeting HIF-2α in ccRCC cells significantly decreased PD-L1 mRNA and protein expression levels. Using chromatin immunoprecipitation and luciferase assays, we found that, similar to HIF-1α, HIF-2α regulates the expression of PD-L1 by binding directly to the HRE motif in the PD-L1 proximal promoter (Fig. 1B). In VHL-mutated RCC4 renal cells that express both HIF-1α and HIF-2α, the knock-down of HIF-1α or HIF-2α or both decreased the expression of PD-L1 suggesting that, in 786-O cells, PD-L1 is a direct target of HIF-2α; however, in RCC4 cells, PD-L1 expression is regulated by both HIF-1α and HIF-2α. These data highlight the rationale behind treating patients with RCC using anti-PD-L1/PD-1 immunotherapies and suggest that the VHL mutation status could potentially be used as a biomarker predictive of RCC response to anti-PD-L1/PD-1 immunotherapy [33]. Another study supported these data by showing a positive correlation between PD-L1 and HIF-2α target genes in ccRCC [34]. CD70 is an immune checkpoint factor from the tumor necrosis factor (TNF) family. The CD70 ligand activates T cells by binding to the CD27 receptor expressed on the surface of these lymphocytes [35]. The overexpression of CD70 has been well documented in RCC and seems to be driven by HIF in ccRCC tumors with defects in pVHL (Fig. 1B). In ccRCC tumors, the overexpression of CD70 seems to correlate with the release of soluble CD27 from the tumor-infiltrating lymphocytes, which thereby prevent T cell activation [36].

Cytotoxic T lymphocytes (CTLs) are the main weapons of destruction against various pathogens and tumor cells. The CTL-mediated anti-tumor immune response is regulated by hypoxia and controlled in part by HIFs and VHL. The deletion of VHL alters the differentiation of effector and memory $CD8^+$ T cells. VHL-deletion also resulted in elevated HIF expression, which sustained CTL effector function. Moreover, hypoxia modulated the expression of critical transcription factors, effector molecules, co-stimulatory receptors (4-1BB, GITR, and OX40) and activation-induced inhibitory receptors (LAG-3 and CTLA-4) in a HIF-1α- and HIF-2α-dependent manner (Fig. 2C) [37]. The hypoxia-mediated regulation of different immune checkpoints in various cancer types is summarized in Table 1.

5. Involvement of EMT in the regulation of immune checkpoints

In addition to its ability to regulate ICBs, several lines of evidence suggest that hypoxia could act as an EMT inducer and that the induction of EMT subsequently regulates the expression of immune checkpoint-associated factors. Indeed, the hypoxia-dependent induction of EMT causes morphological changes and loss of E-cadherin expression in hepatocellular carcinoma cell lines. Moreover, HIF-1α induces the expression of CCL20 in these cancer cells allowing for metabolism changes in macrophages characterized by the expression of indoleamine 2, 3-dioxygenase (IDO) and increased numbers of $Foxp3^+$ regulatory T cells (Treg cells) and subsequent decreased T-cell proliferation [38] (Fig. 3A). It seems that a complex interplay between hypoxia, EMT, and immune checkpoint gene expression exist. Nevertheless, little is known about how tumor cell plasticity regulates the expression of immune checkpoints. Our group has discovered that EMT-TF plays a crucial role in the regulation of PD-L1 [39] and CD47 [40] in human breast cancer cells undergoing EMT.

In keeping with this, we reported that driving EMT by overexpressing EMT-TF in epithelial MCF7 breast cancer cells induced the upregulation of PD-L1. PD-L1 upregulation in mesenchymal breast cancer cells resulted in tumor cell escape from CTL-mediated killing. Surprisingly, treatment of MCF7 with EMT-inducing factor TGF-β, or inhibiting TGF-β signaling in mesenchymal-like MDA-MB-231 cancer cells, had no impact on either mRNA or protein levels of PD-L1. Further investigation of EMT-activated breast cancer cells revealed that the EMT-dependent overexpression of PD-L1 requires the ZEB1/miR-200 axis and SNAI1 but not SLUG (Fig. 3B). Importantly, targeting PD-L1 by siRNA or treating mesenchymal cells with an anti-PD-L1 blocking antibody increased the susceptibility of tumor cells to CTL-mediated killing [39]. These findings were supported by Chen and colleagues who showed that ZEB1 removed the repression of miR-200 on PD-L1 protein levels. Therefore, ZEB1 expression during EMT leads to PD-L1 expression in tumor cells and the subsequent suppression of $CD8^+$ T-cell activity and metastasis. This observation was further experimentally supported in tumors generated from 393P cells overexpressing ZEB1. These tumors exhibited an increase of overall tumor burden, metastasis lung nodules, a significantly reduction in total $CD8^+$ TILs and an increased in exhausted $CD8^+$ T cells (PD-1 + TIM-3 +). Pharmacological treatment of tumors generated from 393P cells overexpressing ZEB1 with ICB (anti-PD-L1) resulted in a reduction in tumor size, metastasis and exhausted $CD8^+$ T cells [41]. In addition to $CD8^+$ T cells, Dongre and colleagues have observed that EMT impacted other immune cell populations. Thus, an increased percentage of suppressive Tregs, pro-tumor M2 markers arginase1 and reduced level of the anti-tumor M1 marker iNOS was observed in mice transplanted with mesenchymal cell line compared to epithelial cell line derived from MMTV-PyMT mouse model. It has been reported that the efficiency of anti-CTLA-4 was only observed in epithelial tumors but not in mesenchymal tumors [42].

More recently, we reported that CD47 is upregulated in EMT-activated breast cancer cells compared with epithelial-like phenotypes. Indeed, CD47 levels were found to correlate with the expression of EMT marker genes in TCGA and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) databases. The overexpression of both SNAI1 and ZEB1 in epithelial MCF7 cells or their inhibition in mesenchymal MDA-MB-231 regulates CD47 expression at both the mRNA and protein levels. Therefore, we concluded that SNAI1 and ZEB1 can directly bind to two E-box motifs in the human CD47 proximal promoter region (Fig. 3B). Finally, we reported that targeting SNAI1, ZEB1, or CD47 rescues macrophage-mediated phagocytosis in mesenchymal MDA-MB-231. Our *in vitro* data highlight CD47 as a direct target of SNAI1 and ZEB1 and its blockade induces the phagocytosis of breast cancer cells undergoing EMT [40]. Overall, based on our data showing that EMT can regulate both CD47 and PD-L1, it is tempting to speculate that combining anti-CD47 and anti-PD-L1 agents will simultaneously reactivate both innate (macrophage checkpoint CD47) and adaptive immunity (T-lymphocyte checkpoint PD-1) and ultimately

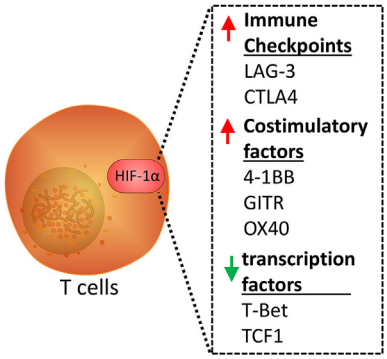

Fig. 2. The impact of hypoxia on the regulation of immune checkpoints in T cells. Through HIF-1a stabilization, hypoxia upregulates the expression of inhibitory immune checkpoints (LAG-3 and CTLA4) and the co-stimulatory factors (4-1BB, GITR, and OX40) but downregulates the transcription factors T-Bet and TCF1. Together, these events promote the effector function of $CD8^+$ T cells under hypoxic conditions.

Table 1
Summary of immune checkpoints that are regulated by either HIF-1α or HIF-2α in different cancer types.

Immune checkpoint	HIF-1 or HIF2	Cancer type	References
PD-L1	HIF-1α	Human breast	[23,27]
		Human prostate	[23]
		Mouse melanoma	[22,23]
		Human kidney	[23]
CD47	HIF-1α	Human breast	[26]
CD73	HIF-1α	Human breast	[27]
CD137 (4-1BB)	HIF-1α	Mouse colon	[28]
		Mouse melanoma	
		Mouse breast	
HLA-G	HIF-1α	Human melanoma	[31]
		Human glioblastoma	
CD70	HIF-1α	Human kidney	[35]
PD-L1	HIF-2α	Human kidney	[33]
CD70	HIF-2α	Human kidney	[36]

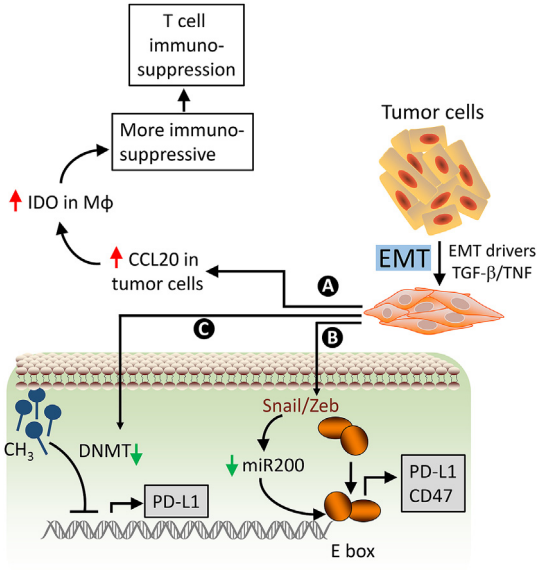

Fig. 3. The impact of EMT on the regulation of immune checkpoints. **A.** Driving EMT leads to an increase of CCL20 in tumor cells and a subsequent increase in indoleamine 2, 3-dioxygenase (IDO). IDO leads to metabolism changes in macrophages and an increase in Foxp3+ regulatory T cells (Treg cells) and a subsequent decrease in T-cell proliferation. **B.** EMT induces associated transcription factors Snail/Zeb. These transcription factors decrease miR-200, thus enhancing the expression of PD-L1. Snail/Zeb can also directly bind to the E box motif in the promoters of PD-L1 or CD47 and induce their expression. **C.** The overexpression of PD-L1 in cancer cells undergoing EMT by TGF-β/TNF-α treatment relies on the downregulation of DNA methyltransferase (DNMT), which prevents the methylation of the PD-L1 promoter.

provide more frequent, durable, and longer lasting responses in patients with breast cancer marked by highly aggressive, mesenchymal, and metastatic tumors. Consistent with our findings, it has been demonstrated that metastatic tumors have high CD47 expression levels compared to primary tumors from patients with melanoma and the *in vitro* blockade of CD47 in metastatic melanoma cells re-activated macrophage-mediated phagocytosis [43].

While the data described above clearly support the involvement of EMT in the transcriptional regulation of PD-L1, new evidence indicates that PD-L1 could also be regulated by epigenetic modification during the EMT process. This regulation is due to DNA demethylation and the NF-kβ pathway through a mechanism involving TGF-β and TNF-α respectively in NSCLC. Indeed, it has been shown that the overexpression of PD-L1 during EMT required both TGF-β and TNF-α treatment. TGF-β treatment decreased levels of DNA-methyltransferase 1 (DNMT1), resulting in PD-L1 promoter demethylation (Fig. 3C). However, TNF-α treatment induced the NF-kB pathway involved in promoting the expression of demethylated PD-L1 promoter in NSCLC [44]. Similarly, in prostate cancer cells, reduced expression of DNMT1 plays a role in the induction of EMT and a cancer stem cell phenotype [45]. In gastric cancer, the overexpression of DNA methyltransferase 3A (DNMT3A) represses E-cadherin expression and promotes cell migration and invasion. Indeed, DNMT3A expression levels are positively correlated with lymph node metastasis and poor prognosis in gastric cancer. Thus, multiple lines of evidence have illustrated the role of the DNMT family in promoting the regulation of immune checkpoints and EMT in a cancer-dependent manner [46].

Recently, Ricciardi and colleagues co-cultured T, B, and NK cells with multiple cancer cell lines undergoing EMT and found decreased lymphocyte proliferation and increased NK and T-cell apoptosis through a mechanism involving IDO but not the Fas ligand pathway [47].

More recently, it was reported that a subtype of breast cancer cells with low expression of claudin showed bidirectional crosstalk between PD-L1 expression and EMT. Indeed, PD-L1 downregulation in claudin-low breast cancer cells leads to CD44 and vimentin downregulation and CD24 upregulation [48].

According to a patient-derived pan-cancer analysis, EMT signatures strongly correlate with drug sensitivity and global molecular alterations at the DNA, RNA, and protein levels. In addition, a high mesenchymal EMT score was associated with high expression of immune checkpoints such as PD1, PD-L1, CTLA4, OX40L, and PD-L2 in 11 cancer types [49]. Additionally, a highly positive correlation exists between EMT and immune checkpoint-related genes including PD-L1, PD-L2, PD-1, TIM-3, B7–H3, BTLA, and CTLA-4 in colorectal cancer [50]. Furthermore, PD-L1 was upregulated in metastatic colorectal cancer compared to primary tumors [51] and this upregulation was closely related to EMT markers in patients with pulmonary adenocarcinoma (pADC) [52]. Indeed, in the high-risk group of patients with hepatocellular carcinoma (HCC), the expression of PD-L1 was correlated with an EMT phenotype and poor survival [53]. In addition, PD-L1 overexpression seems to be associated with developing resistance to a B-RAF inhibitor (BRAFi) in patients with metastatic melanoma [54]. In prostate cancer, N-cadherin upregulation was correlated to an immune regulatory signature comprised of IDO overexpression and increasing numbers of Treg cells [55]. However, therapy-induced E-cadherin downregulation leads to PD-L1 downregulation in lung cancer cell lines suggesting a common pathway for E-cadherin and PD-L1 [56]. Furthermore, the expression of newly emerging immune checkpoints CD276, OX40, and TGFB1 is correlated with the expression of EMT genes in kidney cancer [57]. The EMT-mediated regulation of PD-L1 and CD47 in various cancer types is summarized in Table 2.

6. Conclusions

While the role of EMT in promoting an aggressive and metastatic phenotype in tumor cells is well established and extensively documented, new evidence is accumulating that points to the critical role of

Table 2
Summary of immune checkpoints that are regulated by EMT transcription factors in different cancer types.

Immune checkpoint	EMT	Cancer types	References
PD-L1	SNAI1/ZEB1/miR-200	Human breast	[39]
	ZEB1/miR-200	Human lung	[41]
	SNAI1	Mouse breast	[42]
	TGF-β reduces DNMT1	Mouse lung	[44]
	TNF-α induces NF-κB	Mouse lung	[44]
CD47	ZEB1/SNAI1	Human breast	[40]

this process in the regulation of the expression of immune checkpoint genes. The classification of primary tumors according to their EMT score revealed that mesenchymal-like tumors were highly enriched in immune checkpoint markers compared to epithelial-like tumors. Similarly, the tumor EMT score could be a valuable biomarker for stratifying patients who will benefit from ICB immunotherapies. Moreover, and consistent with the fact that the EMT process could regulate many immune checkpoints, we strongly believe that considering the EMT score of tumors could help tailor immunotherapy-based treatments and, thereby, optimize patient outcome.

In addition to serving as a biomarker for immunotherapy, EMT is a remarkable therapeutic target for cancer treatment [58]. It has been proposed that therapies able to revert the mesenchymal state of a tumor back into an epithelial one can prevent metastasis [59]. However, the complex interplay between hypoxia and EMT should be considered when using inhibitors targeting either mechanism. It will be interesting to know whether combining drugs that target hypoxia or HIF with EMT inhibitors helps to overcome therapy resistance or/and to block tumor cell dissemination and metastasis. We strongly believe that such a combination will have an additive or possibly even synergistic effect in combating those elements, which are critical for cancer's virulence.

Many protocols for tumor immunotherapy in the clinic have not taken into consideration the role of hypoxia and EMT. So far, to enhance the effectiveness of immune checkpoint blockade-based cancer immunotherapies, the combination of various existing immuno-therapeutic strategies have been used. These strategies include the administration of multiple immunotherapies that together confer a more efficacious and longer-lasting response. However, given the wide spectrum of changes that occur during EMT and hypoxia, new combination therapies, including EMT and hypoxia inhibitors, are also being investigated. Indeed, accumulating evidence indicates that improving the therapeutic response to PD-1/PD-L1 blockade in urothelial cancer could be achieved by simultaneously targeting the EMT-inducing TGF-β pathway [60]. The therapeutic value of this combination has also been demonstrated in esophageal squamous cell carcinoma [61]. Additional studies are warranted to determine if other cancer types share these features. Moreover, it has been proposed that targeting the EMT driver AXL would have the dual benefit of being an anticancer therapeutic as well as synergistically activating the antitumor immune response [62,63]. In addition to EMT, recent data showed the ability of reduced hypoxia, achieved using a hypoxia-activated prodrug, to sensitize multiple preclinical models of prostate cancer to the T cell checkpoint blockade. In keeping with these data, a clinical trial is currently ongoing to evaluate the therapeutic benefit of combining hypoxia prodrug TH-302 and anti-CTLA4 against several cancers including prostate, melanoma, and pancreatic tumors (NCT03098160).

As the response to cancer immunotherapy varies considerably from one patient to another, it remains critical to identify biomarkers predicting how patients with cancer will respond to different treatments, notably to the combination therapy given its increased toxicity. While several biomarkers have been identified, including microsatellite instability, the expression of PD-L1 in the tumor as valuable biomarker predicting the response to PD-1/PD-L1-based cancer immunotherapy is still currently debating [64]. Indeed, in certain tumor types, a benefit with immunotherapy was observed irrespective of PD-L1 expression level [65,66].

The number of immunotherapy combination trials initiated each year has been increasing dramatically. However, three major questions arise when designing a successful combination approach to cancer immunotherapy: i) what to combine? ii) how to combine? and iii) when to combine? Nevertheless, all of these clinical trials will soon provide an avalanche of data that should provide insight into how best to tailor cancer immunotherapy treatments for each patient with cancer.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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DISCUSSION

NB is a rare but one of the most deadly cancer in its stage 4 metastatic form in young children [12, 13, 166]. Immunotherapy led to substantial progress over the last decades underlying the important role of immune system in NB [13, 65]. Nonetheless, prognosis remains very poor in HR and very HR forms despite anti-GD2 mAb therapy [13, 166]. Moreover, disabilities resulting from currently administered chemotherapy, radiotherapy and surgery are a subject of concern for long-term survivors [166]. Therefore, new, less toxic, and more targeted treatments still represent today an unmet need.

On the other side, hypoxia is a well-described hallmark of solid tumors, including NB, and largely contributing to the aggressiveness of malignancies and favoring tumor immune escape [5-8]. Moreover, hypoxia through HIF-1α has been involved in the expression of PD- L1 by cancer and stromal cells, leading to the dampening of T cells immune responses against tumor cells [9, 10].

Based on this, in our study, we aimed to assess the effect of a combinatorial therapy associating intra-tumoral hypoxia inhibition to PD-1/PD-L1 axis blockade, in a relevant NB mouse model mimicking the poor-prognosis forms of this disastrous childhood disease. We selected an anti-PD1 MAb to target this immune checkpoint. For hypoxia inhibition, we selected an epigenetic acting drug, JQ1, based on previous reports underlying its benefit in NB mouse models and on its ability to impair hypoxic responses in breast and ovarian cancers [148, 150, 157]. We first demonstrated *in vitro* that JQ1 impairs hypoxic reactions in the particular setting of NB and confirmed this result *in vivo.* We then assessed its activity in vascular normalization and we wanted to describe its impact on NB tumors immune landscape. Finally, we tested the combinatorial therapy associating PD-1 blockade to JQ1, and demonstrated a significant tumor reduction and prolonged survival in the TH-*MYCN* mouse model.

For pre-clinical *in vivo* experiments, we selected the HR NB transgenic mouse model TH- *MYCN*. Of note, because NB is rare, large-scale clinical studies resulting in robust evidence- based conclusions is not easily achievable. Therefore, pre-clinical studies are essential to identify promising combinatorial therapies for clinical testing. Relevant and representative NB models are strongly needed, but to date few animal models recapitulate the human HR disease. TH-*MYCN* is still the best studied and the most representative of childhood HR NB malignancy, but it suffers from some limitations. Indeed, even if *MYCN* is a well-recognized oncogenic driver of the disease, the first events leading to overt NB are still poorly understood, impairing the development of « perfectly mimicking » animal models. Nonetheless, in our study, we choose the TH-*MYCN* mouse model due to its numerous advantages: (1) it mimics anatomy-pathological features but also known genetic events and miR expression of human disease [22, 32]; (2) homozygous mice on a 129SvJ background develop the disease

with a high penetrance (100%) and without treatment all animals die by 6-8 weeks of life [22, 34], data that were confirmed in our study cohort; (3) the tumor develops in orthotopic localization (even if different from the most frequent localization in children) and has a representative TME [22, 32]; (4) TH-*MYCN* mice being immunocompetent, the immune landscape of TH-*MYCN* tumors is similar to human tumors, allowing immunological studies [167]; (5) a precise tumor volume assessment is possible using medical imaging technology such as MRI [22]; (6) moreover, integrating specific MRI parameters in the tumor evaluation allows to assess mass vascularization and hypoxia *in vivo* [154, 155]; (7) TH-*MYCN* model has already been used in several pre-clinical studies with robust and promising results [85, 150]. Obviously, the use of another animal model to confirm our *in vivo* results obtained in TH-*MYCN* mice would be valuable. However, in the case of our study, this was not achievable. Indeed, few models of HR NB exist. Subcutaneous (SC) syngeneic models are not relevant for immunotherapy and hypoxia studies of HR forms, as their TME have been demonstrated to greatly differ from that of orthotopic tumors [31]. Thus, orthotopic syngeneic models could be an alternative; however, using such models requires great ability in microsurgery and skilled staff, which was not possible to develop during the period of our study. SC models using human NB cell lines suffer from the same issue (orthotopic implantation is very challenging), and they have to be implanted in immunocompromised animals, impairing immunotherapy studies. PDXs and patient-derived orthotopic xenografts (PDOXs) would also be relevant but need to be humanized to conduct immunological studies in animals. However, this technology is very expensive and time-consuming. Moreover, stromal cells are still mouse-derived, which could impact the results as interactions between stromal and cancer cells play an important role in tumor outcome [30, 168]. Nonetheless, in the future, humanized PDXs/PDOXs models of pediatric cancer disease will probably allow to develop more personalized therapies and impact the individual patient prognosis [30]. In our case, it was not possible to confirm our results in PD(O)Xs humanized animals as their price and duration of obtaining were limiting/restrictive issues. Finally, new technologies such as 3D structures mimicking NB TME will be more commonly used for *in vitro* testing in the future and will contribute to select the right molecules to test *in vivo* [169]. In summary, we considered TH-*MYCN* is currently the most suitable model for HR pre-clinical studies including immunotherapeutic drugs, justifying this choice in our study.

Hypoxia and HIFs in solid tumors play a major role in several hallmarks of cancer; among others, it promotes immune escape and favors angiogenesis and the emergence of a chaotic vasculature playing a role in the immune escape and in conventional chemo- and radio- therapy resistance [37-39]. In NB, the contributing role of hypoxia to the disease aggressiveness has been well documented, and the respective role of HIF-1α and HIF-2α in tumor progression has been the subject of several studies with

discording results. The role of hypoxia in NB in promoting stem cell immature phenotype has been suggested by Jogi et *al* [133]. In more recent studies, HIF-2α has been suggested to correlate with NB adverse prognosis and with a pseudohypoxic stem cell phenotype [134, 136-138, 170]. However, the preponderant role of HIF-2α relative to HIF-1α in NB poor-prognosis forms has been questioned as some studies underline a strong correlation between HIF-1α and its target gene signature and outcome and differentiation state. Indeed, Qing and *al*. compared the hypoxia response between *MYCN*-amplified and *MYCN* single copy NB cell lines and demonstrated that HIF-1α is the main sub-unit expressed in *MYCN*-amplified cell lines; moreover, HIF-1α expression was high in *MYCN*-amplified tumors compared to non- amplified ones, whereas HIF-2α expression was decreased in *MYCN*-amplified tumors [139]. These data were also confirmed by immunohistochemistry (IHC) in human tumors, underlying the preponderant role of HIF-1α but not of its sibling sub-unit HIF-2α in *MYCN*- amplified HR forms [139]. N-Myc was also found to cooperate with HIF-1α to the Warburg effect in normoxia and allows to maintain a high proliferation rate despite hypoxic conditions [139]. In line with these results, Chen *et al.* have reported that HIF-1α activates the SHH pathway contributing to NB aggressiveness as it plays a role in proliferation, invasiveness and metastases [5]. High expression levels of HIF-1α, SHH and GLI1 assessed by IHC on patients tumor specimens correlate with advanced stages of the disease whereas HIF-1α and GLI1 expression also associate to poorly differentiated NB [5]. The correlation between high expression levels of HIF-1α and tumor growth was further confirmed in xenografts models, as well as an increased tumor vascularization [5]. Westerlund *et al.* even demonstrated a strong correlation between HIF-2α expression and a more pronounced differentiation state of the developing adrenal chromaffin cells, and high HIF-2α expression was also associated to low-risk human tumors and so inversely correlated with HR markers such as *MYCN*-amplification [146]. These results are supported by the fact that blocking HIF- 2α with small inhibitory molecules (such as for example PT2385) did not decrease NB cells growth *in vitro* nor *in vivo* [146, 147]; moreover, retinoic acid (which promotes differentiation) and 5-aza-deoxycytidine (which is a DNA-demethylating agent) lead to reduced NB tumor growth and a more differentiated state, and this effect is dampened when the two drugs are combined with PT2385, suggesting that HIF-2α acts more as a neuronal differentiation

promoter and an onco-suppressor gene [93]. However, results of HIF-2α target gene expression in PT2385 treated NB cell lines differ in the two studies, as Persson *et al.* did not show any effect and Westerlund *et al.* showed a significant decrease of HIF-2α target gene. Of note, in Persson *et al.* study, when PT2385 was combined with siRNAs targeting HIF-1α or ARNT, HIF-2α target genes (such as *VEGFA*) were efficiently downregulated, suggesting that HIF-1α may compensate for HIF-2α inhibition [147]. All these data suggest a preponderant role of HIF-1α in HR NB patients, underlying the potential benefit of HIF-1α targeting therapy. In addition, some authors have even established NB hypoxic signatures which correlate to poor-prognosis forms [171-173].

In our study, we first showed that, under hypoxic conditions, JQ1 decreased HIF-1α protein expression *in vitro*, in three different NB cell lines (the murine NHO2A cell line and human Sima and CHP-134 cell lines, all of them recapitulating HR NB). This result is in line with the observations of Yin *et al*, that demonstrated that under hypoxic conditions, NHWD-870 (a BET family bromodomain inhibitor) decreased HIF-1α mRNA and protein levels in ovarian carcinoma A2780 and melanoma A375 cell lines; in A375 cells, NHWD-870 inhibited BRD4 decreasing its binding to the HIF-1α promoter [157].

We also demonstrated that, under hypoxic conditions, JQ1 decreased CAIX expression *in vitro*, at the mRNA and protein levels in the same three cell lines. CAIX is a target gene of HIF-1α and is almost exclusively expressed in tumor cells, and therefore represents a good hypoxia marker as well as an interesting diagnosis tool and therapeutic target [39]. It correlates with dismal prognosis in different tumor types including NB, and plays a role in metastasis and in cancer stem cells maintenance [39, 105, 174-177]. Interestingly, inhibiting CAIX has demonstrated efficacy on tumor growth and invasiveness in different adult cancer, making CAIX an attractive therapeutic target [39]. In our cell lines, as JQ1 impacts HIF-1α protein level, we can therefore suggest that this accounts for the observed decrease CAIX expression. However, the effect of JQ1 on HIF-1α appears to be cell line dependent, as da Motta *et al* have showed that JQ1 treatment in triple negative breast cancer downregulates CAIX expression without affecting HIF-1α expression neither at the mRNA level nor at the protein level, suggesting that BETi could directly regulate HIF-1α target genes [148].

As JQ1 impaired hypoxic responses in NB *in vitro*, we wanted to correlate these results in the *in vivo* setting, using the TH-*MYCN* transgenic mouse model. First, we demonstrated by western blot that CAIX protein expression was also decreased *in vivo*

in tumors from TH- *MYCN* mice treated by JQ1. To assess hypoxia in tumors in living animals, we decided to use magnetic resonance imaging relaxometry. Indeed, several studies have suggested the measure of R2* parameter as a surrogate marker of hypoxia [155]. Mice treated with JQ1 showed a significant decrease in median tumoral R2* and a before-to-after treatment R2* ratio pleading for a decrease in hypoxia *in vivo* while we observed the opposite effect in controls. This effect could not be explained only by the decreased tumor volume in the JQ1 treated group as tumor volumes after treatment (JQ1 or vehicle) was not significantly different between the two groups.

Taken together, our data strongly suggest that JQ1 impairs hypoxic responses *in vitro* and *in vivo*, and contributes to decrease hypoxia in NB tumors in the TH-*MYCN* model. Relaxometry MRI with the measure of R2* as a surrogate marker of hypoxia is an attractive procedure but warrants further validation. On the other side, other imaging techniques have proven their efficacy in identifying hypoxic tumors, but usually use radioactive tracers and ionizing radiations (for example, ^{18}F-fluoroerythronitroimidazole positron emission tomography/computed tomography), which is less suitable for pediatrics use. Immunohistological studies could help to validate MRI findings, but only in the pre-clinical setting as biopsies in children are often difficult to obtain, and results have to be interpreted with caution because hypoxia is a dynamic and non a static process; depending on the timing and localization of biopsy during disease course, results can be very divergent.

JQ1 has also been implicated in impairing tumor angiogenesis notably in childhood sarcomas animal models [153, 178]. Although previous studies suggested that JQ1 acts on angiogenesis via c-Myc pathways, Bid *et al* reported that JQ1 impaired angiogenesis through regulation of tumor-derived pro-angiogenic factors and inhibition of endothelial cells invasion in pre-clinical sarcomas models [153, 178].

In our study, we performed a DCE-MRI study on TH-*MYCN* mice, a technique that allows to assess vessels permeability notably through the measurement of $K^{trans,}$ a pharmacokinetic parameter reflecting the transfer of Gd outside the vessels to the EES. Of note, DCE-MRI is a technique already used in clinics and particularly of interest in the pediatrics setting as it represents a non-invasive procedure even if sedation in young children and a venous access for contrast injection are needed [158]. As drugs acting on angiogenesis do not always lead to tumor shrinkage, it becomes important to assess their efficacy measuring other parameters than lesion size only justifying the growing interest in DCE-MRI technique [158].

The results we have obtained in TH-*MYCN* mice showed a tendency to vascular normalization although not significant. This is reflected by a decreased K^{trans} in the JQ1 treated group versus control animals, indicating that vessels are less leaky. The importance of normalization of tumor vessels have been highlighted in different studies, and exceed pure anti-angiogenic benefit [37]. Indeed, studies with anti-VEGFA

monotherapy often lead to vascular regression and hypoxia, favoring cancer aggressiveness and vascular resistance leading *in fine* in chaotic angiogenesis [171]. In NB, combinatorial therapy associating anti- VEGFA Mab and HIF-1α suppressor allows to overcome the anti-angiogenic therapy resistance due to HIF-1α up-regulation [171]. Although one may suggest that normal vessels could contribute to tumor growth by providing more oxygen and nutrients supply, we should keep in mind that drugs (including conventional chemotherapy but also immunotherapeutic drugs) and immune cells can more easily reach cancer cells if vasculature is not chaotic; moreover, well-oxygenated tumor cells divide faster and are more sensitive to chemo- and radiotherapy effects [37]. Furthermore, structurally abnormal vessels favor hypoxia and its downstream effects on tumor aggressiveness [37]. Finally, cancer cells can enter the circulation and disseminate more easily when vessels are leaky [37].
We compared the MRI data to immuno-histo-chemistry analysis on tumors, using CD31 marker for vessels and α-SMA for pericytes coverage identification. JQ1 treated mice harbor well-vascularized tumors, with normal caliber vessels and better pericytes coverage compared to control animals.

Our data are in line with the literature, indicating that JQ1 contributes to normalization of the chaotic tumor vasculature. This is also concordant with the reduction of hypoxia observed in our model. However, the precise mechanism by which JQ1 has led to vasculature normalization remains to be investigated. We analyzed the expression of well-known angiogenic factor VEGFA in our three cell lines (NHO2A, Sima and CHP-134) under hypoxic conditions, and as expected hypoxia tended to increase VEGFA mRNA levels *(data not shown)*, but the effect of JQ1 was variable and not fully reproducible. Of note, VEGF-A protein expression *in vitro* has already been reported to vary differently depending on the NB cancer cell line analyzed and the hypoxia exposition duration so poorly correlating to the *in vivo* setting [173]. However, for *in vivo* evaluation, we decided to perform a cytokine array for angiogenic factors on one treated and one control mice, and again we didn't observe any change in VEGF-A expression *(data not shown)*. Nonetheless, this cytokine array revealed that endostatin was up-regulated by JQ1 treatment; as it acts as an endogenous angiogenesis inhibitor [160], this could reverse the pro- to-anti-angiogenic balance contributing to a better vessels quality. Recombinant endostatin has been suggested to be an efficacious anti-angiogenic therapy, but its administration remains highly challenging due to the need of long perfusion time and to product stability issues [160]. As JQ1 could up- regulate endostatin expression, it could represent an easier therapeutic tool. However, these data must be confirmed *in vitro* and *in vivo* on several mouse tumor samples. Of note, acidic FGF was also up-regulated by JQ1; its role in tumor progression is not yet fully understood, as it has been suggested to promote angiogenesis but also to induce

cancer cell differentiation through FGF-receptor binding resulting in tumor suppressive functions [161, 162]. Once again, further studies to assess the impact of JQ1 on pro- and anti-angiogenic factors balance and so on the tumor vasculature architecture are warranted to confirm our preliminary observation obtained by our cytokine array.

The immunosuppressive role of tumor metabolic factors such as hypoxia has been strongly established by several studies in the past decade, and one of the most studied immune escape mechanism also promoted by hypoxia involves the expression of PD-L1 which binds to PD-1 on T cells resulting in dampened immune reactions [10, 48, 179, 180] .

NB has a low mutational burden, suggesting that TA susceptible to raise an immune response are scarce [4]. However, different studies and clinical evidence have demonstrated that immune reactions against NB cancer cells exist and can be exploited to treat affected children [4, 12, 13, 21, 65, 67]. Importantly, as in other malignancies, immune escape mechanisms in NB play a main role in disease progression [4].

Studies in NB over the presence and the role of PD-L1 have shown contradictory results, but the more recent ones strongly suggest that PD-L1/PD-1 axis plays an important role in HR NB patients [78, 82, 83]. This discrepancy is mostly due to the different techniques used, and clearly underline the absolute necessity to harmonize pre-clinical and clinical practices for PD-L1/PD-1 expression assessment in NB. Of note, another limitation for NB is the small number of patients and the scarcity of tumor material. Moreover, biopsies from the primary tumor site before treatment are not always available as diagnosis can be done on metastatic sites biopsy and immunological studies including PD-1/PD-L1 expression assessment is sometimes performed after patient has started therapy.

Nonetheless, some pre-clinical studies sustain a correlation between PD-L1 expression (combined to other parameters) and NB prognosis, suggesting that targeting PD-L1/PD-1 could impact NB outcome. Combinatorial therapies tested in the pre-clinical setting on NB mice model support this hypothesis [85, 86, 88] and a recent clinical study has reported the efficacy of a combinatorial therapy including an anti-PD-1 Mab in two advanced NB patients [87]. However, biomarkers to identify NB patients more susceptible to respond to PD-L1/PD- 1 axis blockade have yet to be determined, and as PD-L1 expression is not a static but rather a dynamic process impacted by several TME factors, the right timing for PD-1/PD-L1 axis blockade is also still unknown [60] .

Yet, recent studies have highlighted the role of JQ1 on the immune landscape of tumors and on PD-1/PD-L1 axis [78, 181]. To assess the immune landscape changes under JQ1 treatment in the TH-*MYCN* model, we performed FACS analysis on tumor samples from mice treated either with vehicle or with JQ1 for 3 days. Tumor volume at euthanasia were similar in the 2 groups, as we selected mice with larger initial volume for the JQ1 treated group and mice with smaller volumes for control group, to rule out the impact of the tumor volume on the immune landscape analysis. We observed that

JQ1 significantly decreased PD-L1 expression on DCs. This is in concordance with recent literature showing that PD-L1 expression is impacted by BET inhibitors [181]. PD-L1 expression is regulated in several ways, and is a direct target of BRD4 [181], explaining at least in part the mechanism by which BET inhibitors decreased its expression. Yet, HIF-1α downregulation could also have impacted PD-L1 expression in our model as well as the decreased MYCN expression. Further studies are needed to unravel the possibly multiple underlying mechanisms leading to the dampening of PD-L1 expression by JQ1 in NB. We also observed that JQ1 increased PD-1 expression on T cells. As PD-1 is expressed during acute activation of T-cells, this could reflects a more activated T cell state; however, PD-1 also represents an exhaustion marker as its engagement with PD-L1 leads to the dampening of immune reactions [48].

Taken into account the role of hypoxia in NB aggressiveness, the correlation between HIF- 1α and NB poor prognosis, and the role of PD-L1 in NB immune escape, and based on our preliminary results on hypoxia and vascular changes and immune impact of JQ1 in NB, we hypothesized that combinatorial therapy associating JQ1 to anti-PD1 Mab therapy could synergize to impact NB progression in TH-*MYCN* model. We randomly assigned homozygous TH-*MYCN* mice developing tumors with similar initial volumes in one of the following groups: vehicle + isotype Mab, vehicle + anti-PD1 Mab, JQ1 + isotype Mab, or combinatorial therapy associating JQ1 to anti-PD1 Mab. Mice were regularly assessed by MRI for tumor volume. In each group, vehicle or JQ1 were stopped at day 8 whereas isotype or anti-PD1 Mab were continued until death (spontaneous death or euthanasia because of endpoint was reached). Our combinatorial therapy led to a better response (tumor volume shrinkage) at day 8 in the group receiving JQ1 combined to anti-PD-1 Mab in confront to the group receiving JQ1 with isotype Mab. This difference persists even one week after JQ1 interruption but still under anti-PD-1 Mab treatment. Moreover, there was a significant prolonged survival in the combination group compared to the mice receiving only JQ1. In line with the literature, anti-PD-1 alone has no effect neither on mice survival nor on tumor shrinkage.

To explain the benefit of adding anti-PD1 Mab to JQ1 in our model, and based on the preliminary FACS analysis showing an increased PD-1 expression on T cells, we can hypothesize that JQ1 leads to a T cell activation, and even if PD-L1 expression is decreased, this activation could be insufficient to overcome the inactivating effect of the binding to the remaining PD-L1. Adding the anti-PD-1 Mab releases the immune brakes leading to the synergistic response observed in the combination group. In addition, the vasculature normalization induced by JQ1 could have impacted the immune cells function in the TME by allieving hypoxia and nutrient deprivation which induce deleterious immunosuppressive effects. However, additional studies are strongly needed to assess all the mechanisms by which the combinatorial therapy led to a

synergistic effect. Moreover, we did not investigate the mechanism by which PD-1 expression on T cells is increased by JQ1. Based on the mechanism of action of JQ1, we can expect that PD-1 expression in T cells in NB is epigenetically regulated, but this has to be confirmed by new experiments.

To our knowledge, our study is the first to assess the effect of combining JQ1 to anti-PD-1 Mab in a relevant HR NB mouse model, leading to a synergistic effect on tumor volume shrinkage and prolonged survival.

In agreement with the clinical studies reported in the literature, anti-PD1 therapy alone has no effect on tumor progression. ICI used in monotherapy in the pediatric setting has led to high response rate only in a very limited range of cancers, including hyper-mutated tumors developing in patients harboring Constitutional Mismatch Repair Deficiency, and Hodgkin lymphoma [11]. No effect of ICI in monotherapy was observed in NB [11]. As children cancer is different from adult cancers, combinatorial therapies including ICI should be tested only based on strong preclinical or scientific evidence that they could exert a positive activity [11]. Our study demonstrating a synergistic activity of JQ1 combined to anti-PD1 Mab provides rationale for such combination clinical testing in NB HR patients.

Other studies have highlighted a synergistic effect of combinatorial therapies associating ICI to other immunotherapies in NB, such as association to anti-CTLA4 Mab, anti-GD2 Mab, CSF-1R inhibitor or vaccines [85, 86, 88, 182]. In other solid tumors, combination of JQ1 to PD-1/PD-L1 blockade has led to synergistic effect. Indeed, Adeegbe *et al* demonstrated a significant survival progress in a NSCLC GEMM mouse model treated with a combinatorial therapy including JQ1 and anti-PD1 blockade [183]. The synergistic effect was explained on a part by the decreased Tregs infiltration into the tumor bed, alleviating the immunosuppressive effect on effector T cells, and on the other part by the decreased PD-1 expression induced by JQ1 on T cells. Moreover, the combination therapy resulted in a Th1 cytokine pattern correlating with the enhanced T cells effector function and activation. JQ1 in this study had a small impact on the PD-L1 expression on TAMs [183]. Although the authors demonstrated a reduced PD-1 expression on T cells under JQ1 treatment, we observed an opposite PD-1 variation in our study suggesting that JQ1 effect on PD-1 is cancer type dependent. However, we did not analyzed the impact of the combinatorial therapy on the immune landscape in our model, and we did not show any effect of JQ1 on the percentage of the sub-population of lymphocytes in our tumors. Pan *et al* have also reported the superiority of JQ1/anti-PD-L1 blockade therapy in a pancreatic cancer mouse model [184]. Of note, they demonstrated that PD-L1 expression correlates with c-Myc expression, and as JQ1 negatively impacts c-Myc, they attributed the synergistic effect to c-Myc downregulation by JQ1 [184].

In summary, our results of the combinatorial therapy in the NB pre-clinical setting paves the way for clinical testing of such a combination. Unfortunately, as childhood

malignancies are rare diseases, most anti-cancer drugs are primarily tested in adults in clinical trials before entering the pediatric therapeutic strategy [166]. It is the case for BETi which are already included in adult clinical trials since 2012, whereas the first use in children has started in 2019 [185]. Testing BETi in combination to immunotherapies such as ICI in clinical setting is another challenge. In this way, collecting pre-clinical data and identifying response-predicting biomarkers could clearly sustain their use.

CONCLUSION AND PERSPECTIVES

HR NB patients still suffer from a disastrous prognosis. Progress has been achieved over the past decades with the introduction of anti-GD2 Mab therapy reinforcing the evidence of the important role played by the immune system in disease progression. New combinatorial (immuno) therapies with less toxicities but more efficacious are urgently needed, but data collected from adult patients cannot be directly translated into children clinical trials as cancer disease features in adults differ notably on the immunogenicity aspects.

Pre-clinical studies with strong evidence must be conducted, and to date TH-*MYCN* remains the most suitable mouse model for HR forms. However, results obtained in our study still need to be confirmed in another model, and PD(O)Xs using humanized mice could constitute a good alternative. Indeed, NB tumorigenesis is not necessarily driven by *MYCN* overexpression or amplification, and in PD(O)Xs the TME is preserved although stromal cells are not of human origin.

Hypoxia in solid tumors and in particular in NB correlates to poor-prognosis and its role in promoting an immunosuppressive TME is not yet to demonstrate. Hypoxia contributes to cancer immune escape in several ways, such as for example by favoring PD-L1 expression. Therefore, combinatorial therapies aiming to target hypoxia and immune checkpoints stimulate great interest and have already demonstrated synergistic effects in pre-clinical models.

JQ1 is an epigenetic drug having multifaceted activity. Its mechanisms of action involve the binding of BRD4, disrupting the interaction with acetylated lysine histone residues and interfering with gene transcription. JQ1 decreases hypoxic responses, as shown by the HIF- 1α and CAIX reduced expression in our three NB cell lines and the correlation of these results to the observed *in vivo* decreased hypoxia. However, the precise mechanism underlying this effect should be deeply investigated, as dependent on the cell context, JQ1 can act by repressing HIF-1α mRNA, or by repressing HIF-1α target genes expression, or by both. JQ1 also contributes to vasculature normalization, potentially also accounting for the decreased hypoxia observed. JQ1 seems to affect the pro-to-anti-angiogenic factors balance, however our preliminary data should be confirmed conducting a study on a larger number of animals. Finally, JQ1 shapes the immune landscape of tumors by decreasing DCs PD-L1 expression. Of note, in our model, PD-1 expression on T cells was increased by JQ1, paving the way to test a combination of this BETi with anti-PD-1 Mab. Synergistic effect of the combination led to reduced tumor volume and prolonged survival, suggesting a novel NB treatment approach that could be integrated in clinical trials in the next future. However, the next challenge will be to uncover response predicting biomarkers, to define selective criterions for patients that could benefit from this therapy. In NB, tumor material before

any treatment is scarce and the analysis of immune landscape evolution over time (after treatment has started) is challenging.

ANNEX

11. Book chapter:

The Critical Role of Hypoxia in Tumor-Mediated Immunosuppression

Chapter 18

The Critical Role of Hypoxia in Tumor-Mediated Immunosuppression

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Additional information is available at the end of the chapter

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Abstract

Underestimated for a long time, the involvement of the microenvironment has been proven essential for a better understanding of the cancer development. In keeping with this, the tumor is not considered anymore as a mass of malignant cells, but rather as an organ composed of various malignant and nonmalignant cell populations interacting with each other to create the tumor microenvironment. The tumor immune contexture plays a critical role in shaping the tumor immune response, and it is now well supported that such an immune response is impacted by the hypoxic stress within the tumor microenvironment. Tumor hypoxia is closely linked to tumor progression, metastasis, treatment failure, and escape from immune surveillance. Thus, hypoxia seems to be a key factor involved in creating an immune-suppressive tumor by multiple overlapping mechanisms, including the impairment of the function of cytotoxic immune cells, increasing the immunosuppressive properties of immunosuppressive cells, and activating resistance mechanism in the tumor cells. In this chapter, we review some recent findings describing how hypoxic stress in the tumor microenvironment hijacks the antitumor immune response.

Keywords: cancer, hypoxia, immune response, tumor microenvironment, autophagy, tumor plasticity, tumor heterogeneity

1. Introduction

Malignant cells are part of cellular and microenvironmental complexes which both define the initiation, progression, and maintenance of the malignant phenotype. In turn, malignant cells participate in creating a hostile microenvironment characterized by hypoxic areas within the

tumors. Indeed, the oxygen level in the hypoxic tumor is usually lower than that of corresponding normal tissue. The oxygenation level of tumor is likely depending on (i) the initial oxygenation of the tissue; (ii) the degree of the tumor heterogeneity; (iii) the tumor size and stage. **Table 1** summarizes the percentage of oxygen level reported as a median in some healthy organs and their corresponding tumors, as defined by several studies.

Healthy tissue/corresponding cancer	% of oxygen (Median)
Brain/brain tumor	4.6/1.7
Breast/breast cancer	8.5/1.5
Cervix/cervical cancer	9.5/1.2
Kidney cortex/renal cancer	7.0/1.3
Liver/liver cancer	4.0–7.3/0.8
Lung/nonsmall cell lung carcinoma	5.6/2.2
Pancreas/pancreatic tumor	7.5/0.3
Rectal mucosa/rectal carcinoma	3.9/1.8

Table 1. Comparison of the percentage (%) of oxygen level in different healthy tissues and in their corresponding cancers.

It is now widely appreciated that hypoxia is one of the most relevant factor involved in the impairment of the antitumor immune response by damping the cytotoxic function of immune cells. There are numerous studies supporting that hypoxic stress leads to the establishment of immune tolerance of tumor cells by preventing the migration and the homing of immune effector cells into established tumors. Furthermore, hypoxia can also drive tumor cell plasticity and functional heterogeneity and, thus, favors the emergence of more aggressive tumors. Many strategies are emerging for targeting intratumor hypoxia in order to change the immunosuppressive properties of the tumor to a microenvironment able to support antitumor immunity.

2. Hypoxia is the major factor of the tumor microenvironment

The long-lasting tumor immunology research has validated the concept of tumor immunosurveillance. The tumor immunosurveillance consists in the fact that cytotoxic immune cells recognize nascent transformed cells and destroy them before they become clinically apparent. Several types of immune cells are involved in the control of tumors such as immune effector and immune suppressor cells. Thus, cytotoxic T lymphocytes (CTL) belong to the adaptive immune system and they are able to recognize tumor antigens through the T-cell receptor (TCR) [1]. The antigens expressed exclusively by tumor cells are called tumor-specific antigens [2]. In addition to CTL, the tumor immune surveillance involves natural killer (NK) cells that belong to the innate immune system [1]. NK cells recognize tumor cells by mechanisms

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called "missing-self" and "induced-self" [3]. Briefly, NK cells are regulated by a balance of inhibitory and activating signals of surface receptors. Thus, NK cells can kill their target cell depending on the recognized ligand(s). The identification of activating or inhibitory ligands allows NK cells to distinguish between "self" versus "nonself" and "self" versus "altered self" by "missing-self" and "induced-self" recognitions. Indeed, the protection of normal cells from NK cell killing is achieved by balancing the stimulatory signals delivered by stimulatory ligands with inhibitory signals delivered by self MHC class I molecules. When the expression of self MHC class I molecules is lost following cell transformation or infection, the stimulatory signals delivered by the target cell remain unbalanced, leading to the activation of NK cells and lysis of target cells (known as missing-self recognition). Under some circumstances, transformed or infected cells overexpress stimulatory ligands that overcome the inhibitory signals leading to target cell lysis (known as induced-self recognition). It has been reported that both missing-self and induced-self recognition could operate simultaneously. In this case, NK cells display a high ability to discriminate between normal and transformed target cells [4].

In addition to cytotoxic immune cells, the tumor immune contexture contains immune suppressive cells such as myeloid-derived suppressor cells (MDSC) able to inhibit the function of immune effectors. Macrophages and neutrophil granulocytes are also involved in antitumor immunity [5]. These cells display tumor antigens and can stimulate other immune cells such as CTL, NK cells, or antigen-presenting cells (APC) [6]. Although both CTL and NK cells kill their target following the establishment of immunological synapse (IS) [7], the molecular mechanism by which they recognize their target tumor cells is fundamentally different. Two major pathways are used by CTL and NK cells to recognize and destroy tumor cells: (i) through the release by immune cells of cytotoxic granules containing perforin and granzymes and these cytotoxic granules are captured by tumor cells to induce cell death by apoptosis [8], and (ii) through tumor necrosis factor (TNF) superfamily-dependent mechanism [9].

It has been proposed that despite the powerful ability of the immune system to attack cancer cells, tumors can outmaneuver the immune effectors cells and escape the immune surveillance. It is now well documented that the ability of tumor cells to escape immune cell control is most likely resulted from the activation of several resistance mechanisms to evade effective and functional host immune response. Therefore, it stands to reason that established tumors, displaying multiple resistance mechanism, are likely not fully controlled by the immune system. In keeping with this, it is strongly believed that clinically detected cancers have most likely evaded effective antitumor immune responses. Recently, it has been reported that in addition to its role in protecting host against tumor development, the immune system can under certain circumstances sculpt the immunogenic phenotype of well-developed tumors. Such a mechanism favors the emergence of resistant tumor cell clones [10]. Accumulating experimental and clinical evidence suggest that the resistance mechanisms activated in tumor cells are multifactorial and that such resistance mechanisms are primarily evolved and activated in the tumor microenvironment [11]. It appears that hypoxia is the major tumor microenvironmental factor involved in the alteration of the transcriptome and the metabolome of tumor cells as well as their proliferation, survival, and invasion [12].

In this chapter, we summarize some recent findings describing how hypoxic stress in the tumor microenvironment regulates the antitumor immune response and leads to tumor escape from immunosurveillance. We focus on how hypoxia confers resistance to immune attack and impairs tumor cell killing mediated by CTL and NK cells.

2.1. Hypoxia and hypoxia-inducible factors (HIF) regulation

Tumor cells are able to adapt to hypoxic stress through the regulation of the hypoxia inducible factor family of transcription factors (HIFs) [13]. It has been reported for a large number of human cancers that HIFs were overexpressed and such overexpression is associated with poor response to treatment [14]. Moreover, evidence showed a clear positive correlation between enhanced hypoxic expression of HIFs and mortality [13]. Therefore, inhibition of HIFs could represent a novel approach to improve cancer therapies. Currently, efforts are being actively pursued to identify inhibitors of HIFs and to test their efficacy as anticancer therapeutics.

Three isoforms of HIF have been identified: HIF-1, HIF-2, and HIF-3. The hypoxia-inducible factor-1 (HIF-1) is the major factor mediating adaptive responses to changes in tissue oxygen level [15]. Indeed, HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and an O_2-dependent regulated HIF-1α subunit. HIF-1α is a DNA-binding basic helix-loop helix of the PAS family [Per (period circadian protein); Arnt (aryl hydrocarbon receptor nuclear translocator protein); Sim (single-minded protein)] [16]. HIF-1α contains two oxygen-dependent degradation domains (ODDD), one in the N-terminal (N-ODDD) moiety and one in the C-terminal moiety (C-ODDD) [17, 18]. It also contains two transactivation domains (TADs), one N-terminal, which overlaps with the C-ODDD, and one C-terminal [19].

2.2. Regulation of HIF-1 level

The expression level of HIF-1α is determined by the rates of protein synthesis and protein degradation. While the synthesis of HIF-1α is regulated in an O_2-independent manner, its degradation is primarily regulated via an O_2-dependent mechanism. Thus, normoxic cells constantly synthesize HIF-1α protein and degrade it rapidly [17]. It has been shown that under normoxic conditions HIF-1α has a short half-life of less than 5 min [20]. However, under hypoxia or low oxygen level, the degradation of HIF-1α is blocked or dramatically decreased [21]. Under normoxia, HIF-1α is hydroxylated on proline residue 402 and/or 564 in the ODDD by prolyl hydroxylase domain protein 2 (PHD2) [17, 22]. Such oxygen-dependent hydroxylation of HIF-1α results in its binding to the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is the recognition component of an E3 ubiquitin-protein ligase complex that targets HIF-1α for proteolysis by the ubiquitin-proteasome pathway [23].

Enzymes regulating HIF-1α proteasomal degradation were first identified to be related to egl-9 in caenorhabditis elegans and to termed prolyl hydroxylase domain (PHD) enzymes (PHD1, PHD-2, and PHD3) [24, 25]. PHD2 uses oxygen as a substrate, and thus, its activity is inhibited under hypoxic conditions [25]. The inhibition of PHD2 leads to the inhibition of prolyl hydroxylation of HIF-1α and subsequently to the inhibition of HIF-1α-dependent proteasomal degradation. Consequently, HIF-1α rapidly accumulates in the cytoplasm, translocates to the

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nucleus and dimerizes with HIF-1β. The HIF-1α/HIF-1β heteromeric dimer binds to the hypoxia responsive element (HRE) in target genes, recruits coactivators and activates transcription [14] (**Figure 1A**).

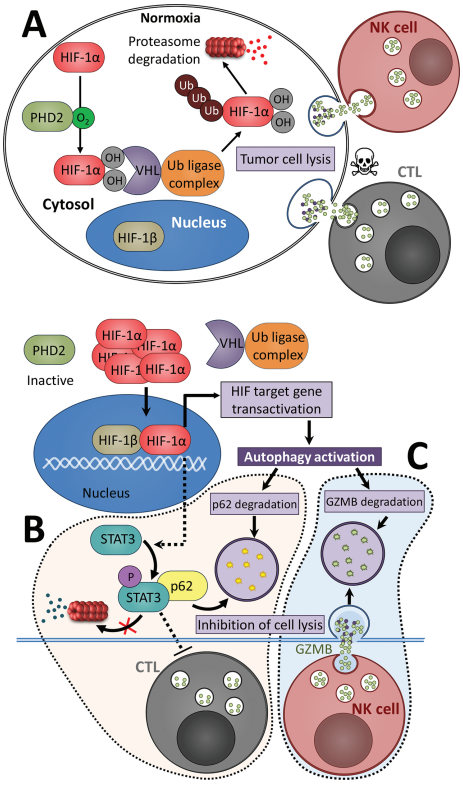

Figure 1. The role of hypoxic stress in the impairment CTL and NK-cell mediated lysis. (A) Under normoxia, the oxygen-sensitive prolyl hydroxylase domain protein 2 (PHD2) hydroxylates HIF-1α subunit. Hydroxylated HIF-1 interacted with Von Hippel-Lindau protein (VHL), subjected to ubiquitination and subsequently degraded by the ubiquitin-proteasome system. Under hypoxic stress, the function of PHD2 protein is blocked, HIF-1α is therefore stabilized and translocated to the nucleus to form heterodimeric complex with HIF-1β to transcriptionally induce the expression of HIF-target genes involved in several pathways such as autophagy. (B) Under hypoxia, STAT3 is phosphorylated at Ser-705 residue in a HIF-dependent manner by a mechanism which is not fully understood. (C) The hypoxia-dependent induction of autophagy leads to the degradation of the adaptor protein p62/SQSTM1, involved in targeting phospho-STAT3 to the ubiquitin proteasome system for degradation. Thus, targeting autophagy accumulated p62/SQSTM1 and therefore accelerated the degradation of phospho-STAT3. The degradation of phospho-STAT3 restores CTL-mediated lysis of tumor cells. In addition, the induction of autophagy in hypoxic tumor cells leads to the selective degradation of granzyme B (GZMB), a serine protease released by natural killer (NK) cells and contained in the cytotoxic granules. Such degradation inhibits NK-mediated lysis of tumor cells.

Using genomewide chromatin immunoprecipitation combined with DNA microarray (ChIP-chip) or DNA sequencing (ChIP-seq) analysis, it has been shown that more than 800 genes involved in several cell functions are direct targets of HIF [26, 27]. HIF-1 activates the

expression of these genes by binding to a 50 base pair cis-acting HRE located in their enhancer and promoter regions [28]. The HREs of all these genes contain the core sequence 5′-[A/G]CGT-3′, which in most cases is ACGTG [29]. It has been reported that HIF transcription factors preferentially bind to specific bases in the 5′ and 3′ proximity of the core that has led to define the following HRE consensus sequence [T/G/C][A/G]CGTG[CGA][GTC][GTC][CTG] [29].

Similar to HIF-1α, the stabilization of HIF-2α is also regulated by oxygen-dependent hydroxylation [30]. This could be related to the fact that HIF-1α and HIF-2α displayed a similar structure of their DNA binding and dimerization domains. However, the major difference between the structure of HIF-1α and HIF-2α is in their transactivation domains [31]. In terms of genes expression, both HIF-1α and HIF-2α share overlapping target genes, and each one also regulates a set of unique targets [32].

In sharp contrast with HIF-1α and HIF-2α, HIF-3α lacks the transactivation domain and could function as an inhibitor of HIF-1α and HIF-2α. It has been reported that the expression of HIF-3α is regulated by HIF-1 [33]. In addition to the regulation of the expression of a large number of genes, HIF family members regulate hypoxia-related microRNAs (HRM) [34] and some chromatin modifying enzymes [35].

3. Intra-tumor hypoxia: a key feature that triggers several resistance mechanisms of tumor evasion from immune surveillance

It has been clearly established that the immune effector activity and the antitumor immune response are significantly regulated by hypoxia. Indeed, hypoxia, via HIF-1α, decreases the susceptibility of lung cancer cells to CTL-mediated killing. It appears that the resistance to CTL is related to the effect of HIF-1α to induce the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in tumor cells by a mechanism involving the vascular endothelial growth factor (VEGF) secretion. These data suggest that following its translocation to the nucleus, HIF-1α cooperates with pSTAT3 to impair lung carcinoma cell susceptibility to CTL-mediated killing [36] (**Figure 1B**). More recently, it has been shown that the expression of the phosphorylated form of STAT3 at Ser-705 residue is tightly controlled by the induction of autophagy in hypoxic tumor cells as the accumulation of pSTAT3 was no longer observed when autophagy was targeted genetically in tumor cells [37]. Autophagy is a catabolic cell degradation process. Autophagy plays an essential role in preventing accumulation of altered cell components [38] and as an adaptive metabolic response to provide nutrients. Recently, an unexpected role of autophagy in shaping the antitumor immune response [39] and the acquisition of resistance to TNFα has been shown [40]. Autophagy is activated under stress conditions such as hypoxia, nutrient starvation, growth factor withdrawal, and endoplasmic reticulum stress. It has been reported that the molecular mechanism by which autophagy regulates the pSTAT3 level involves the protein p62/SQSTM1 the ubiquitin proteasome system [37, 41].

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Another study showed that in addition to the mechanism described earlier, it has been shown that the stem cell self-renewal transcription factor NANOG is also involved in the regulation of CTL-mediated tumor cell lysis [42, 43]. Hypoxia regulates NANOG at both transcriptional and translational levels and targeting NANOG in hypoxic cells restored CTL-mediated tumor cell killing. Furthermore, NANOG depletion results in the inhibition of STAT3 phosphorylation and its nuclear translocation. The hypoxia-induced microRNA (miR)-210 is also involved in the regulation of CTL-mediated tumor cells lysis. In fact, HIF-1 induces the expression of miR-210 which subsequently targets nonreceptor protein tyrosine phosphatase type 1 (PTPN1), homeobox A1 (HOXA1), and tumor protein p53-inducible protein 11 (TP53I11), and thereby decreases tumor cell susceptibility to CTL [44]. In the context of NK-mediated tumor cell lysis, it has been described that hypoxia increases the shedding of the major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA), a ligand for the activating receptor natural killer group 2 member D (NKG2D), on the surface of prostate cancer cells leading to an impairment of NO signaling [45] and subsequent escape of tumor cells from NK- and CTL-mediated killing. MICA expression is also downregulated by HIF-1 in osteosarcoma cells resulting in tumor resistance to NK-mediated lysis [46]. Through the activation of autophagy, it has been recently reported that melanoma and breast tumor cells escape NK-mediated lysis and that targeting autophagy in hypoxic tumor cells was sufficient to restore NK-mediated lysis. In this study, it has been shown that the activation of autophagy under hypoxia was responsible for the degradation of NK-derived granzyme B making hypoxic tumor cells less sensitive to NK-mediated killing [39, 47, 48] (**Figure 1C**). In line with the studies described earlier, it is now well admitted that hypoxic stress in the tumor microenvironment is a key factor involved in the control of antitumor immune response. Beside its role in impairing the function of cytotoxic immune cells, the immunosuppressive effect of hypoxia contributes to the emergence of resistant tumor cells that compromise the effectiveness of the anti-tumor immune response [49].

4. Hypoxia and tumor cell heterogeneity and plasticity

Solid tumors frequently reveal pronounced tumor cell heterogeneity with regards to cell organization, cell morphology, cell size, and nuclei morphology [50]. The molecular mechanisms underlying the phenotypic heterogeneity involve genetic, epigenetic, and environmental factors. It is now well established that hypoxia is an important contributor to intra- and intertumor cell heterogeneity [15, 51] by altering the expression of specific genes involved in cellular phenotype. In this respect, it has been reported that neuroblastoma cells and breast cancer cells lose their differentiated gene expression patterns and develop stem cell-like phenotypes under hypoxic stress [52, 53]. As a low stage of differentiation in neuroblastoma and breast cancer is associated with poor prognosis, it is strongly believed that, in addition to its contribution to tumor heterogeneity, hypoxia-dependent induction of tumor cell dedifferentiation contributes to tumor cell plasticity and aggressiveness.

Several lines of evidence suggest that tumor microenvironment drives stem cell renewal and differentiation. Indeed, poorly vascularized tumors contain hypoxic regions with undifferen-

tiated 'stem-like' tumor cells that survive under control of HIFs [54]. It has been reported that hypoxic stress in colon cancer inhibits the differentiation of tumor cells and maintains their stem-like phenotype [55]. In addition, myofibroblasts stromal cells secrete factors involved in maintaining cancer stem cells (CSC) population in colon cancer [56]. Furthermore, stromal cells drive a CSC phenotype on differentiated cancer cells, allowing a transient morphological heterogeneity observed in several cancers. In this regard, transient phenotypic changes from epithelial to mesenchymal (epithelial-mesenchymal transition (EMT)) or mesenchymal to epithelial (mesenchymal to epithelial transitions (MET)) phenotype, are initially considered as conversions facilitating cell plasticity but have recently gained appreciation as events involved in tumor heterogeneity [57]. In the context of tumor immunity, recent evidence revealed that tumor cell plasticity has serious implications in terms of immunological recognition and killing of the tumor, since such tumor cell plasticity may lead to the emergence of immunoresistant variants [58].

Although the role of the immune system in inhibiting early stages of tumor growth is well established, it is now strongly suggested that the immune system can also facilitate the advanced stages of tumor progression by sculpting the immunogenic phenotype of a developing tumor to favor the emergence of immune-resistant tumor cell variants. This has led to the concept of "immunoediting" which encompasses three phases: elimination, equilibrium, and escape. Thus, immunoediting allows tumors to evade immune destruction by becoming less immunogenic or more immunosuppressive [59]. Such adaptability, achieved through cell reprogramming, reflects an important property of tumors called immune-induced plasticity. While the molecular basis of immune-dependent induction of tumor cell plasticity and its effective contribution to the selection of tumor aggressive variants is still elusive, recent findings have revealed that activated CD8+ T cells can stimulate mammary epithelial tumor cells to undergo EMT and acquire the increased tumorigenic capability and therapy resistance of breast CSCs [60]. In this regard, it has been shown that reciprocal interactions between melanoma and immune cells enhances tumor cell plasticity and drives therapy resistance [61]. Based on these data, it is now well defined that targeting phenotypic plasticity should be considered for the development of novel therapeutic strategies with the ultimate goal to prevent the establishment of a more aggressive phenotype of cancer cells.

5. The clinical significance of targeting hypoxia

For many years, the major issue in the field of cancer immunity was to understand how cancer cells manage to evade immune surveillance despite the presence of a competent immune system. To address this issue, the major focus was on the mechanisms by which tumor cells escape cytotoxic immune cell recognition without considering the impact of the tumor microenvironment. This could partially explain why despite intense investigation, the gains provided by immunotherapy until recently are relatively modest. In addition, accumulating evidence suggests that tumor cell resistance mechanisms are likely evolved in the hypoxic tumor microenvironment. In keeping with this, it is therefore more accurate to consider cancer as a disease of the microenvironment rather than a disease of cells. Although remarkable

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progresses have been achieved over the past two decades regarding the impact of the tumor microenvironment in cancer biology and treatment, its contribution in the development of tumor resistance to immune cell killing remains fragmented.

Emerging data indicates that hypoxia stress within the tumor microenvironment is a key factor involved in the impairment of the antitumor immune response. [62] Therefore, a deep understanding of the molecular mechanism by which hypoxia induces tumor resistance may contribute to the development of more effective tumor immunotherapies.

Consistent with the fact that hypoxia-dependent overexpression of HIF-1α is associated with an increased patient mortality in several cancer types, it stands to reason that inhibition of HIF-1 activity in preclinical studies would have marked effects on tumor growth and survival. In keeping with this, efforts are underway to identify selective inhibitors of HIF-1 and to assess their efficacy as anticancer therapeutics. Currently, two main approaches are used to target hypoxia in tumors, namely bioreductive prodrugs, and inhibitors of molecular targets upon which hypoxic cell survival depends [63, 64]. However, several lines of evidence indicate that the HIF pathway is technically extremely challenging to target. Indeed, the first evidence is that transcription factors in general, including HIF, have long been considered "undruggable," and therefore, no specific inhibitor of HIF has been brought to the market so far. The second evidence is that multiple levels of regulation and signaling pathways converge on and emerge from HIF [65]. Nevertheless, based on the molecular mechanism of HIF-1 protein, it has been suggested that small molecules could be used to inhibit HIF-1 activity through a variety of mechanisms including inhibition of (i) HIF-1α protein synthesis; (ii) HIF-1α protein stabilization; (iii) HIF-1α/β dimerization, and (iv) HIF-1/DNA binding. Two comprehensive recent reviews summarize these mechanisms in detail and give fairly exhaustive lists of the small-molecule inhibitors for each level [15, 66].

Using a cell-based assay, several small-molecule inhibitors of HIF-1 activity have been identified. Briefly, topoisomerase I inhibitors block the expression of HIF-1α via an undefined mechanism [67]. The small molecule YC-1 (3-(5′-hydroxy-methyl-2′-furyl)-1-benzylindazole) was also shown to reduce the level of HIF-1α by a mechanism that has not been established but at least is known to work independently from its function as a stimulator of soluble guanylate-cyclase activity [68]. YC-1 is not in clinical use. The HSP90 inhibitor 17-allyl-aminogeldanamycin (17-AAG) has been reported to induce the degradation of HIF-1α in a VHL-independent manner [69–71]. PX-12 (thioredoxin-1 redox inhibitor) and PX-478 are both inhibitors of HIF-1α protein expression and HIF-1-mediated transactivation [72, 73]. Finally, the disruptor of microtubule polymerization 2-methoxyoestradiol (2ME2) is able to decrease the expression of HIF-1α. Currently, only topoisomerase I inhibitors, camptothecin and topotecan, are clinically approved agents, PX-478, 2ME2, and 17-AAG are under evaluation in clinical trials, whereas YC-1 and thioredoxin-1 inhibitors are not in clinical use.

Despite of the anticancer effects of these agents could be related, in part, to their inhibition of HIF-1, it seems that none of these drugs specifically targets HIF-1. Although such lack of selectivity does not disqualify these drugs as anticancer agents, it enhances the difficulty to correlate molecular and clinical responses in patients. Therefore, the identification of more selective HIF-1 inhibitors in the near future is required and more investigation needs to be

done to identify novel potent and more specific inhibitors targeting clearly defined points in the HIF pathway.

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