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Study of molecular pathways modulating autophagy in lung tumor cells and their impact on NSCLC tumor progression

Lucas Leonardi

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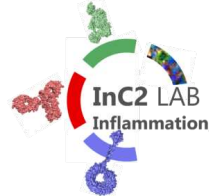
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SORBONNE UNIVERSITE SCIENCES

Ecole doctorale ED394 Physiologie, physiopathologie et thérapeutique

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Centre de Recherche des Cordeliers*

Study of molecular pathways modulating autophagy in lung tumor cells and their impact on NSCLC tumor progression

Par Lucas Leonardi

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Sigles

3D-CRT	Three-dimensional conformal radiation therapy
3-MA	3-methyladenine
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCD	Autophagy-dependent cell death
ADCP	Antibody-dependent cell-mediated phagocytosis
AdenoCA	Adenocarcinoma
ADH	Anti-diuretic hormone
ADP	Adenosine diphosphate
AGO	Argonaute
AIS	Adenocarcinoma in situ
Akt	Protein kinase B
ALFY	Autophagy-linked FYVE
Alk	Anaplastic lymphoma kinase
AMBRA1	Autophagy and beclin-1 regulator 1
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AP	Adaptor protein
ARG1	Arginase
ATG	Autophagy-related genes
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
Baf	Bafilomycin
BAFF	B-cell activating factor
BCG	Bacille Calmette-Guérin

BCL2	B-cell lymphoma 2
BCR	B-cell receptor
BNIP3	BCL2/adenovirus E1B 19kDa protein-interacting protein 3
Breg	Regulatory B cells
CAF	Cancer-associated fibroblast
CAMKKβ	Ca ²⁺ /CaM-dependent protein kinase kinase β
cAMP	Cyclic adenosine monophosphate
Capi	Capivasertib
CAR T	Chimeric antigen receptor T
CCL5	Chemokine ligand 5
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
CfDNA	Circulating free DNA
cGAMP	2',5'/3',5'-cyclic guanosine monophosphate-adenosine monophosphate
cGAS	Cyclic guanosine monophosphate-adenosine monophosphate synthase
CHIKV	Chikungunya virus
circRNA	Circular RNA
Cis	Cisplatin
CK2	Casein kinase 2
CMA	Chaperone-mediated autophagy
c-MET	Tyrosine-protein kinase Met
COPD	Chronic obstructive pulmonary disease
COPII	Coat protein complex II
CQ	Chloroquine
CSC	Cancer stem cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL10	C-X-C motif chemokine 10

CYP7A1	Cholesterol 7-alpha-monooxygenase
DAMP	Damage-associated molecular pattern
DAPK	Death-associated protein kinase
DC	Dendritic cells
DD	Death domain
DDR	DNA-damage response
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DRAM	Damage-regulated autophagy modulator
dsRNA	Double-stranded RNA
E2F	E2 transcription factor
EBRT	External beam radiation therapy
EBSS	Earle's Balanced Salt Solution
EC	Endothelial cell
E-cad	E-cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIF2A	Eukaryotic translation initiation factor 2A
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
ERK	Extracellular signal-regulated kinases
ESCRT	Endosomal sorting complexes required for transport
FasL	Ligand de Fas
FDA	Food and drug administration
FIP200	FAK family kinase-interacting protein of 200kDa
FOXA2	Forkhead box protein A2

FOXO	Forkhead box class O
FYCO1	FYVE and coiled-coil domain-containing protein 1
GF	Growth factor
GITR	Glucocorticoid-induced TNFR-related gene
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte macrophage colony stimulating factor
GOPC	Golgi-associated PDZ and coiled-coil motif-containing protein
Gp	Glycoprotein
Gy	Gray
HCQ	Hydroxychloroquine
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factors
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1
HOPS	Homotypic fusion and protein sorting-tethering complex
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HSV	Herpes simplex virus
HTT	Huntingtin
HUVEC	Human umbilical cord endothelial cell
IASLC	International Association for the study of Lung Cancer
IAV	Influenza virus
ICD	Immunogenic cell death
IFNAR	Interferon- α/β receptor
IFN-I	Type 1 Interferon
IFNγR	Interferon gamma receptor
IKK	I κ B kinase
IL	Interleukin
IMRT	Intensity modulated radiation therapy

IRAK	Interleukin-1 receptor-associated kinase
IRE1	Inositol-requiring enzyme 1
IRF	IFN regulatory factor
IRGM	Immunity-related GTPase family M protein
IRS1	Insulin receptor substrate 1
ISGF3	IFN-stimulated gene factor 3
JNK	Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
KIR	Killer-cell immunoglobulin-like receptor
KO	Knockout
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAG-3	Lymphocyte activation gene 3
LAMP	Lysosomal-associated membrane protein
LAP	LC3-associated phagocytosis
LC3	Microtubule-associated protein 1A/1B-light chain 3
LCAC	Large cell carcinoma
LCI	Lithium chloride
LIR	LC3-interacting region
LKB1	Liver Kinase B1
LncRNA	Long non-coding RNA
Loxo	Loxoribin
LRR	Leucine-rich repeat
LRRC59	Leucine-rich repeat-containing protein 59
MAGE-3	Melanoma-associated antigen 3
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MDSC	Myeloid-derived suppressor cells

MEK	Mitogen-activated protein kinase kinase
MEKK3	Mitogen-activated protein kinase kinase kinase 3
MET	Mesenchymal-epithelial transition
MeV	Measles virus
MHC	Major histocompatibility complex
MIA	Minimally invasive adenocarcinoma
MIF	Macrophage migration inhibitory factor
miRNA	Micro RNA
MMP-3	Matrix metalloproteinase-3
MPL	Monophosphoryl lipid A
mTOR	Mammalian target of rapamycin
MUC-1	Mucin-1
MVB	Multivesicular body
MyD88	Myeloid differentiation primary response 88
MYXV	Myxoma virus
NBR1	Neighbor of BRCA1
ncRNA	Non-coding RNA
NDP52	Nuclear dot protein 52
NEMO	NF- κ B essential modulator
NF-κB	Nuclear factor-kappa B
NIK	NF- κ B- inducing kinase
NK	Natural killer cells
NKT	Natural killer T cells
NLR	Nod-like receptor
NLRP3	NOD-like receptor family pyrin domain containing 3
NOD-SCID	Nonobese diabetic / severe combined immunodeficiency
NOS2	Nitric oxide synthase 2
NOX2	NADPH oxidase enzyme 2

NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung cancer
OPTN	Optineurin
OS	Overall survival
OV	Oncolytic virus
Oxali	Oxaliplatin
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen associated molecular patterns
PARP-1	Poly (ADP-ribose) polymerase 1
PB1	Phox and Bem1
PC	Furin-like proprotein convertase
PD-1	Programmed cell death 1
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PE	Phosphatidylethanolamine
PERK	PRKR-like endoplasmic reticulum kinase
PET-Scan	Positron emission tomography scan
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-Phosphate
Picti	Pictilisib
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type 3
PKA	Protein kinase A
PKC	Protein kinase C
PKCβ	Protein kinase C beta
PKR	Protein kinase R
PLEKHM1	Pleckstrin homology domain-containing family M member 1

PN	Polynuclear neutrophils
PP2A	Protein phosphatase 2A
PRAT4A	Protein-associated with TLR4
PRR	Pattern Recognition Receptors
RAB	RAS-associated binding
RabGAP	Rab GTPase-activating proteins
Rb	Retinoblastoma-associated protein
RHEB	Ras homolog enriched in brain
RIG-I	Retinoic acid-inducible gene I
RLH	RIG-I-like helicase (RLH)
RLR	RIG-1-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SBRT	Stereotactic body radiation therapy
SCF^{b-TRCP}	Skp, cullin, F-box containing complex
SCLC	Small cell lung cancer
SH2	Src-homology 2
SIGIRR	Single immunoglobulin IL-1R-related molecule
SKIL	SKI like proto-oncogene
SMER	Small molecule enhancer of rapamycin
SNAP29	Synaptosomal-associated protein 29
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
SOCS	Suppressor of cytokine signaling
SQCLC	Squamous cell carcinoma
SQSTM1/P62	Sequestosome 1
SRS	Stereotactic radiosurgery

ssRNA	Single stranded RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
STX17	Syntaxin-17
TAA	Tumor-associated antigens
TAB	TAK1 binding protein
TAK	TGF- β -activated kinase
TAK	Transforming growth factor beta-activated kinase
TAM	Tumor-associated macrophages
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TF	Transcription factor
TFEB	Transcription factor EB
TGFβ	Transforming growth factor beta
TIR	Toll/Interleukin-1 receptor
TKI	Tyrosine kinase inhibitors
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alpha
TP53	Tumor protein 53
TRAF	Tumor necrosis factor receptor-associated factors
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
TREML4	Triggering receptor expressed on myeloid cells like 4
TRIF	TIR-domain-containing adapter-inducing interferon- β
TSC	Tuberous sclerosis
UBQLN2	Ubiquilin 2

ULK	Unc-51 like autophagy activating kinase
UNC93B1	Uncoordinated 93 homolog B1
UPR	Unfolded protein response
VAMP8	Vesicle-associated membrane protein 8
v-ATPase	Vacuolar ATPase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VMAT	Volumetric modulated arc therapy
VPS	Vacuolar protein sorting-associated protein
VSV	Vesicular stomatitis virus
WHO	World health organization
Wort	Wortmaninn
WT	Wild type
ZEB1	Zinc finger E-box-binding homeobox 1
α1.3-Gal	Carbohydrate alpha-1,3-galactosyltransferase

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INTRODUCTION

1 Cancer

1.1 Definition and epidemiology

Cancer is a disease characterized by the continuous proliferation of abnormal cells that can be initiated in almost every organ and then spread in others, leading to organ dysfunction and, eventually, patient death. Cancer statistics keep rising despite the tremendous progress in cancer prevention and treatments. It is the second leading death factor worldwide, responsible for nearly ten million in 2020. Among the most prevalent cancer worldwide, breast, lung, and colorectal cancers are the top 3, whereas the deadliest are lung, colorectal, and liver cancers. In 2020, more than 18 million new cancer cases were diagnosed, and incidence has been predicted to keep increasing (**Figure 11.**)¹. Actually, epidemiologists predict a significant increase in cancer incidence from 18 million in 2020 to 29.4 million in 2040².

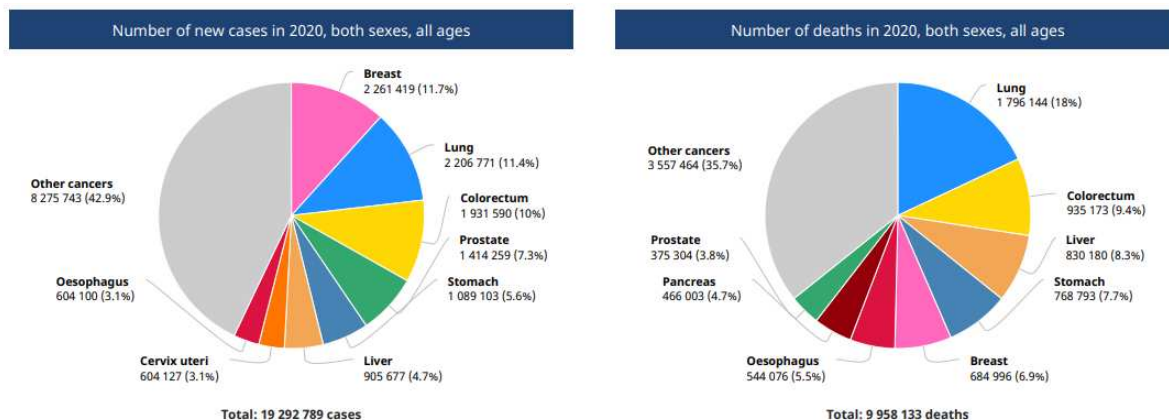


Figure 11. Statistics showing the cancer incidence and the mortality induced in function of cancer type in 2020 (Taken from International Agency for Research on Cancer, World Health Organization) ¹.

1.2 Carcinogenesis

Cancer is not just one disease but a set of more than 100 diseases characterized by the continuous proliferation of abnormal cells in the organism that can occur in almost all organs and then spread in others during a process called metastasis. Although they share similar characteristics and hallmarks, they can differ according to their biology, aggressiveness, and response to treatments.

Cancer is a genetic disease in which the accumulation of successive genomic alterations leads to the formation of a mass of abnormal cells called tumor³. The mechanism by which a normal cell becomes tumoral is called carcinogenesis, and it is a multi-step process, including tumor initiation, promotion, progression, and metastasis. Although genomic alterations, mostly deoxyribonucleic acid (DNA) mutations, permanently occur in normal cells (10^5 DNA mutations per day for each cell), fortunately, no abnormal cells appear due to an efficient DNA repair mechanism⁴. However, in very few cases, this process fails to cope with all those alterations, and some DNA mutations are not removed. Given genome is the core of the cell, coding for proteins that dictate cellular processes necessary for the good functioning of the cell, genomic mutations can disrupt protein function that eventually leads to an abnormal cell (tumor initiation)⁵. The selection and maintenance of this genomic mutation follow Darwinian selection. Actually, if the genomic mutation of a normal cell results in the acquisition of biological advantage, like the inactivation of tumor suppressors or the abnormal activation of oncogenes, this mutation will be selected and transmitted to its progeny⁶.

Consequently, due to clonal selection, these mutated cells will take the lead over the other cells (tumor promotion). In addition, successive driver mutations can occur before the end of the clonal selection, resulting in intratumoral heterogeneity⁷. Over time, the accumulation of advantageous DNA modifications called driver mutations allows the transformation of a normal cell into a malignant cell characterized by the progressive acquisition of the different cancer hallmarks necessary for its well development (tumor progression). Finally, with the acquisition of a more aggressive and invasive phenotype, tumor cells can spread from the primary tumor site to other organs through the blood or lymph vessels (metastasis)^{8,9}.

1.3 Hallmarks of cancer

The Hallmarks of cancer are summarized in the **figure I2** and commented in the following sections

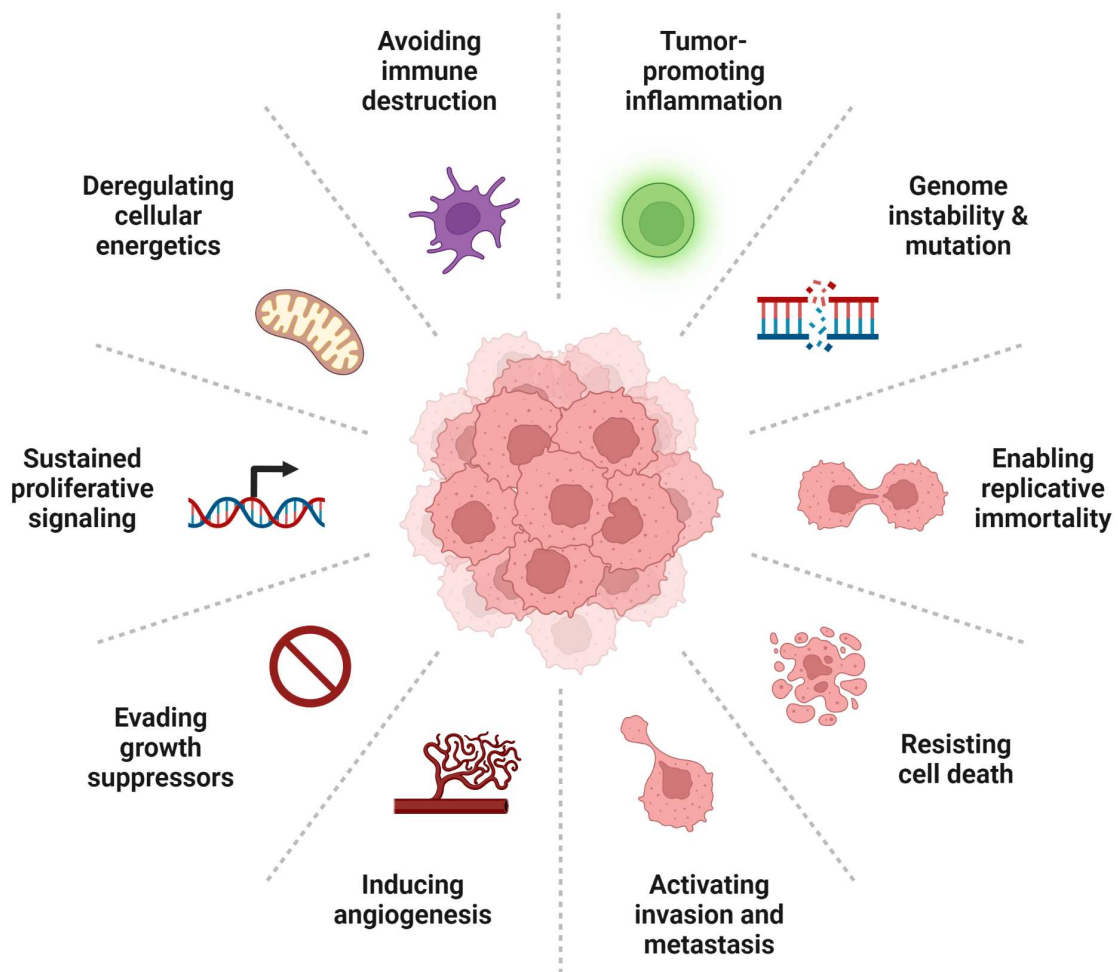


Figure 12. Hallmarks of cancer (Performed on biorender)

1.3.1 Genome instability and mutation

To maintain its integrity and function, cells must keep their genome unmodified. In physiological conditions, cells divide themselves during a process called replication. Although cellular machinery is very efficient in replicating cellular genome, some errors of copy or mutations can occur^{10,11}. In normal cells, DNA mutations detrimental to their homeostasis and functions are efficiently removed, whereas, in malignant cells, these mutations are conserved as they confer a biological advantage. Actually, although genetic modifications can occur in normal cells, efficient DNA repair mechanisms mainly cope with that. When they are insufficient to remove DNA errors, other homeostatic processes, notably apoptosis, are induced¹². However, tumor cells follow Darwin's law of natural selection. As normal cells, malignant cells are constantly subjected to DNA modifications that can confer to the recipient cell a selective biological advantage over others, allowing them to proliferate faster or better survive and eventually take the lead in the local tissue environment. Those beneficial

mutations explain other cancer hallmarks, like the continuous proliferation and the resistance to cell death⁷. Although that selective advantage is beneficial at the cell scale, at the scale of the organism, it is detrimental, and that explains in normal cells, protection mechanisms from that phenomenon have been developed. In cancer cells, those resistance mechanisms are completely altered, amplifying the mutation-generating loop and accelerating the constant selection of advantageous mutated tumor cells. For example, Tumor protein 53 (TP53), a protein that controls genome integrity, is frequently mutated in cancers, leading them to escape from DNA repair, senescence, or apoptosis¹³. That mechanism also explains the important heterogeneity between tumor cells.

1.3.2 Tumor-promoting inflammation

It is now well accepted that immune cells can infiltrate tumors and are a central component of cancers¹⁴. Although their presence inside the tumor was initially only viewed as an attempt of the immune system to eradicate cancer, studies have demonstrated that immune cells could paradoxically promote tumor progression by inducing inflammation. Actually, immune cells can secrete molecules that help tumor cells to develop their other hallmarks. Among them, there are growth factors, survival factors, proangiogenic factors, and ECM modifying enzymes that allow tumor cell invasion and metastasis development¹⁵. In addition, immune cells can release reactive oxygen species, mutagenic molecules, that can impact tumor cells and favor the generation of beneficial genomic mutations¹⁶.

1.3.3 Reprogramming energy metabolism

To have sufficient cellular energy (Adenosine triphosphate or ATP) and metabolic precursors necessary for coping with its continuous proliferation, tumor cells have been demonstrated to modify their metabolism. In normal cells, cellular energy is produced following two metabolic steps. The first one, glycolysis, is a series of biochemical reactions transforming glucose into pyruvate. After that process, this latter usually enters into the Krebs cycle, which consists of a series of high energy producer's biological reactions that are oxygen dependent. However, most pyruvate molecules do not enter the Krebs cycle when oxygen is lacking. They are eventually transformed in a low-energy producer series of reactions, in which pyruvate is converted into lactate and released into the extracellular space. In cancer cells, Otto Warburg first demonstrated that even in aerobic conditions, malignant cells tend to produce energy through that latter mechanism, explaining the use term of aerobic glycolysis¹⁷. That mechanism has dual advantages for tumor cells. It allows them to rapidly produce ATP, and glycolytic intermediates, which can integrate various biosynthetic pathways, especially those that generate nucleosides and amino acids. Those elements will then be used to assemble macromolecules and organelles necessary for developing new cells. Both oncogenes (Ras, Myc), TP53, and external conditions like hypoxia are responsible for this metabolism switch^{18,19}. To compensate for its deficit in cellular energy production, tumor cells have been described to upregulate glucose

transporters, like glucose transporter 1 (GLUT1) and glycolytic enzymes, to facilitate glucose uptake and promote aerobic glycolysis^{20,21}.

Interestingly, it has been observed in various cancers the coexistence of two subpopulations of tumor cells according to their metabolism²². Tumor cells under hypoxia realize glycolysis leading to lactate production and evacuation into the extracellular space. In a symbiotic way, the second population, more oxygenated, takes it and uses it for other biochemical reactions to produce energy.

1.3.4 Sustaining proliferation

Among the main characteristics of cancer, the most intuitive one is its capacity to proliferate continuously. In homeostatic conditions, normal cell growth and proliferation are tightly regulated to maintain the good architecture and functioning of the tissue and organ. That mechanism is mediated by binding molecules, called growth factors, on membrane receptors. The interaction triggers signaling pathways that lead to cell progression into the cell cycle. Often these pathways also influence cell survival or energy metabolism. Cancer cells, by different means, deregulate that proliferation control leading them to proliferate non-stop. Among the various means described, there is the secretion of growth factors in an autocrine way by tumor cells or in paracrine by other cells composing the tumor microenvironment (TME). Also, tumor cells can overexpress growth factor receptors rendering them very sensitive to growth factors. Finally, mutations in the growth factor coding gene or other genes coding downstream signaling components can lead tumor cells to have these growth factors and associated signaling pathways constitutively activated, overcoming the need for growth factor and its receptor interaction²³.

1.3.5 Evading growth suppressors

In addition to induce and sustain continuous proliferation, cancer cells must overcome the actions of cellular protein-coded genes, called tumor suppressor genes, that integrate various internal and external signals and negatively regulate cell growth and proliferation. Among these genes, the two most known are the retinoblastoma tumor suppressor gene (Rb) and tp53²⁴. This latter decides if, according to the external and internal conditions (like DNA damages), the cell can proliferate or, alternatively, enters into senescence or die by apoptosis. Rb is a cell cycle checkpoint that determines, according to external signals, whether the cell should enter or not into the cell cycle. These genes are frequently mutated in cancer cells, rendering them inefficient in ensuring their function and leading cancer cells to proliferate continuously.

1.3.6 Resisting cell death

The induction of cell death by apoptosis is a natural mechanism tightly regulated by various external and internal signals like nutrient availability or DNA damage. Actually, that process allows if the cell experiences DNA damage to be eliminated to maintain homeostasis²⁵. Apoptosis can be induced through two distinct pathways, the extrinsic and the intrinsic pathways. The extrinsic pathway is mediated by the expression of the

death receptor or its ligand (Fas/FasL), induced when the cell is too damaged. The intrinsic pathway involves the mitochondria and the release of a molecule called cytochrome c. Those two pathways both meet and induce a similar effector pathway that ultimately leads to caspase activation and apoptosis²⁶. In the cancer context, the intrinsic pathway is the prominent actor of apoptosis. Its induction can be either positively modulated by BAX and BAK or negatively by the BCL-2 family of regulatory proteins (BCL-2, BCL-xL, BCL-w, MCL-1, A1). Cancer cells have developed several resistance mechanisms to overcome that barrier to grow and progress. Among them, cancer cells overexpress Bcl-2 family members and downregulate Bax and Bak²⁷.

In addition, TP53 can integrate various internal and external signals like DNA damage and respond by apoptosis induction. However, although these inducer stresses exist all along tumor development and treatment, TP53 is frequently mutated in cancer cells, which renders it inefficient in inducing apoptosis¹³.

1.3.7 Enabling replicative immortality

In an organism, each cell has a natural life expectancy. Actually, after a certain number of cell cycles have been performed, the cell stops proliferating and enters into a viable non-proliferative state called senescence or dies by apoptosis. That biological clocking device is mediated by the shortening of the non-coding area, called telomeres, at the extremity of chromosomes that protects the coding region of the genome from being degraded by enzymes. As the cell enters into the cell cycle, its telomeres are shortened. When the cell has too proliferated, the telomeres reach a limited size, leading the cell to either enter into senescence or die by apoptosis²⁸. Conversely, cancer cell proliferation is not limited by that mechanism. Contrary to normal cells, malignant cells constitutively express an enzyme called telomerase that can add telomere repeat segments to the ends of telomeric DNA, rendering them immortalized²⁹.

1.3.8 Inducing angiogenesis

Like normal cells, tumor cells require blood vessels to receive nutrients and oxygen and, in exchange, evacuate metabolic waste and carbon dioxide. The formation of new blood vessels, called angiogenesis, addresses these needs. As the cancer progress, the tumor cells face two difficulties. They need more and more nutrients for their development, and as cancer grows, they are pushed away from the blood circulation. These two factors lead tumor cells to promote the formation of new blood vessels to cope with those difficulties.³⁰ Although natural, angiogenesis is continuously stimulated by the tumor cells, but the vascular architecture resulting is abnormal and leaky. That aberrant vascular endothelium inside the tumor renders both nutrient and oxygen intake and metabolic waste and carbon dioxide evacuation very difficult. That mechanism gradually transforms the extracellular space into an acidic and hypoxic environment, two factors that promote angiogenesis and tumor progression³¹. Among the inducers, oxygen lacking or hypoxia, and oncogenes, promote that phenomenon through the overexpression of the causative ligand of angiogenesis, the vascular

endothelial growth factor A (VEGF-A) that binds its receptor, the vascular endothelial growth factor receptor (VEGFR)³².

1.3.9 Activating invasion and metastasis

In homeostasis conditions, normal cells are strongly anchored into their extracellular matrix (ECM) to maintain the architecture and function of tissues and organs. In contrast, tumor cells, due to their continuous proliferation, have to develop in other parts than the primary tumor site and develop metastasis³³. That mechanism is a multistep process mediated by biological transformation into the tumor cells. Among these transformations, the best characterized is the downregulation by malignant cells of E-cadherin, a protein involved in the cell-to-cell attachment and the anchorage of the cell to the ECM. However, tumor cells upregulate adhesion molecules that are physiologically involved in cell migrations, notably during inflammation. N-cadherin, which allows neurons to migrate during organogenesis, is upregulated by many tumors. The switch performed by tumor cells to migrate from an epithelial to a mesenchymal phenotype is called epithelial-mesenchymal transition (EMT)³⁴. It is a critical process in tumor cell metastasis development. Then, tumor cells undergo different steps to eventually develop metastasis. Among them, there is the local invasion realized by tumor cells secretion of proteases that degrade the ECM and the intravasation of cancer cells into nearby blood and lymphatic vessels^{35,36}. Inside them, malignant cells migrate from the primary tumor site to others, go to the new tumor site (extravasation) and then, during the colonization step, form new tumor masses called micrometastasis that grow to become macrometastasis³⁷. Intravasation and extravasation are two processes that are possible thanks to the modifications and alterations realized by tumor cells on the cells composing the endothelial and lymphatic vasculatures³⁸.

1.4 Tumor microenvironment

When people think about cancer, they picture it as a mass of numerous tumor cells, whereas in reality, it is so far different from that simple view. Actually, inside a tumor, cancer cells coexist with various other components in a complex and continuously evolving environment called the tumor microenvironment (TME). This environment comprises different cell types that communicate with each other and their extracellular matrix through the release of molecules and vesicles to ensure the good functioning of the TME. Although its composition varies depending on the tumor type, some components are common for all cancers. It includes tumor cells, immune cells, stromal cells, blood vessels, and extracellular matrix (ECM)³⁹.

1.4.1 Immune cells and cancer: an evolving interrelation with the time that impacts tumor progression

1.4.1.1 *Inhibition of cancer onset through the implementation of an anti-tumor immune response*

Within the TME, immune cells are a crucial modulator of cancer progression. Actually, it limits cancer onset by developing an anti-tumor immune response. To maintain homeostasis, organisms have developed an organization of different cell types and molecules, called the immune system, that can recognize external and internal danger and remove it. As a central component of the TME, the infiltrated immune cells play an essential role in controlling tumor progression. Considered abnormal, tumor cells can be recognized by the immune system and eliminated⁴⁰. The importance of the immune system in limiting tumor occurrence, progression, and metastasis development has been demonstrated in various tumor models, in which immunosuppression both resulted in an increased likelihood of developing cancers and increased tumor mass and metastasis^{41,42}.

Immune cells are usually categorized into innate and adaptative immune cells. Although involving different cell types and leading to distinct immune responses, the two systems are essential and complementary to protect the organism from external or internal dangers like cancer cells. Innate immunity is a non-specific defense mechanism in which the immune cells composing it recognize common components of abnormal cells, remove them, and induce a second immune response, more robust and extremely specific, called adaptative immunity. Actually, through their receptor, adaptative immune cells can recognize elements very specific to one abnormal cell, called antigens. In addition, at the end of the immune response, adaptative immunity can keep a record of the danger, which allows if the antigen reappears to respond more quickly and stronger. That mechanism is called immune memory⁴³.

Innate immunity, which is the first response put in place after recognition of the danger, is composed of polynuclear neutrophils, macrophages, and dendritic cells. These cells, called phagocytes, can recognize through different surface membrane receptor molecules present in the tumor cells and not in normal cells (eat me signal). After the recognition, the tumor cell is phagocytosed and destroyed, and tumor-associated antigens (TAA) are presented on specific cell membrane molecules that are recognized by the adaptative immune cells, the major histocompatibility complex (MHC) molecules, to induce the adaptative immune response, effective for eliminating tumor cells⁴⁴.

In addition to their role in antigen presentation, phagocytes and epithelial cells have various immune receptors called Pattern Recognition Receptors (PRR) that can recognize a broad spectrum of elements from stressed cells as cancer cells, the damage-associated molecular pattern (DAMP). After recognition, two main signaling pathways, the nuclear factor-kappa B (NF- κ B) and the type I Interferon (IFN-I), can be stimulated, respectively, leading to the secretion of pro-inflammatory cytokines and IFN-I that are crucial for the activation of the adaptative immunity. In addition to the

scavenger receptor and the mannose receptor, PRR includes the nod-like receptor (NLR), RIG-1-like receptor (RLR), and toll-like receptors (TLR). Each receptor has a specific type of ligand⁴⁵.

In addition to the phagocytes, anti-tumor innate immunity also includes natural killer (NK) cells that can be activated upon recognition of cell membrane modifications like the downregulation of MHC-I expression or upregulation of cellular stress molecules at the tumor cell membrane leading to its elimination. That killing activity is mediated by the secretion of two proteins, perforin, and granzyme, in which the first favors the entry into the target cell of the second protein that acts in activating caspase and inducing apoptosis⁴⁶.

A set of 30 proteins called the complement system can also recognize tumor cells directly or indirectly (through antibody recognition). After recognition of the target and activation of one of the three complement pathways, a series of cleavage reactions occur, which lead the abnormal cell to be lysed. Moreover, other intermediate components of the complement cascade produced upon activation of the complement system favor the induction of anti-tumor immunity. Actually, C3a and C5a can be recognized by other immune cells and promote their activation. C3b, another by-product of the complement cascade, called opsonin, facilitates abnormal cell phagocytosis⁴⁷.

After the implementation of innate immunity, an adaptative one is developed thanks to the action of specific cells called antigen-presenting cells (APC), which are the macrophages, the dendritic cells (DC), and the B cells. Actually, these cells have the remarkable capacity to phagocyte abnormal entities like tumor cells, degrade them, and present some specific elements on the MHC molecules that will be then recognized by the major orchestra of the adaptative immune response, the CD4 T cells (signal 1). In addition, APC strongly expresses molecules necessary for the activation of T cells, called co-stimulatory molecules (signal 2). By its great capacity to migrate from the danger site, like the tumor site, to organs where T cells reside (mainly the lymph nodes), DC is the main APC and the top inducer of the adaptative immune response. In addition, to the two signals emitted by the DC to the T cells, DC is also responsible for secreting cytokines that will activate and orientate the T cell phenotype and activity to confer the best T cell immune response against the danger identified by DC (Signal 3)⁴⁸. Those three signals emitted by the DC to T cells will be then recognized by the latter, which will respond according to the type of T cell. Actually, the CD4 T cell will respond by amplifying the adaptative immune response by releasing a cocktail of cytokines necessary for the activation, proliferation, and effector activity of CD8 T cells and B cells⁴⁹.

CD8 T cell is the pivotal actor of the cellular mediated immunity whereas B cell is the crucial orchestra of the humoral mediated immunity. Although the B cell is an APC that recognizes specific antigen on its own, it is also a key player in the adaptative immune response, which requires for its activation, amplification, and its effector functions the help from CD4 T cells⁵⁰. Cellular-mediated immunity is the strongest immune response

against cancerous cells. After interacting with the CD4 T cells and the APC, a CD8 T cell-specific for one antigen gets activated, proliferates, and then migrates to the danger site. Upon recognizing the abnormal cells expressing their specific target antigen through their MHC-I, it induces the death of the target cell. The death caused by the CD8 T cell can be either mediated by the expression of the cell death receptor that interacts with its ligand on the target cell membrane (Fas/Fas Ligand) or as the NK cells by the release of perforin and granzyme molecules⁵¹.

B cells, whose primary purpose is to produce antibodies that have diverse functions, lead them to be at the crossroads of various immune responses. Specific antibodies directed against the antigen can either directly neutralize abnormal cells by favoring NK cell activation and lysis activity through a process called antibody-dependent cell-mediated cytotoxicity (ADCC) or by activating other immune responses. Among those responses, antibodies can activate the complement system and facilitate phagocytosis through a mechanism called antibody-dependent cell-mediated phagocytosis (ADCP) or promote the secretion of pro-inflammatory cytokines⁵⁰. Recently, it has been demonstrated that T cells and B cells can be localized in ectopic structures inside the TME, called tumor lymphoid structures or TLS. Although the implementation of an adaptive immune response usually occurs in secondary lymphoid organs, in the cancer context, it can be initiated in similar structures present inside the tumor site called TLS. Their formation is not specific to cancer, but their induction is caused by chronic inflammation, and they have been described in various pathologies, including autoimmune diseases and infections⁵². TLS are spatially well-organized and comprises T and B cell zones and germinal centers that favor local adaptive anti-tumoral immune response. In the cancer context, TLS has been reported to be present in the tumor stroma and be associated with a better prognosis for patients⁵³.

1.4.1.2 Escape and hijacking of the anti-tumor immune response as a hallmark of cancer

Although the initial function of immune cells consists in eliminating tumor cells, considered abnormal cells, tumor cells can hijack and modify them to escape from the anti-tumor immunity. That mechanism put in place over time is a multistep process called cancer immunoediting (**Figure 13.**).

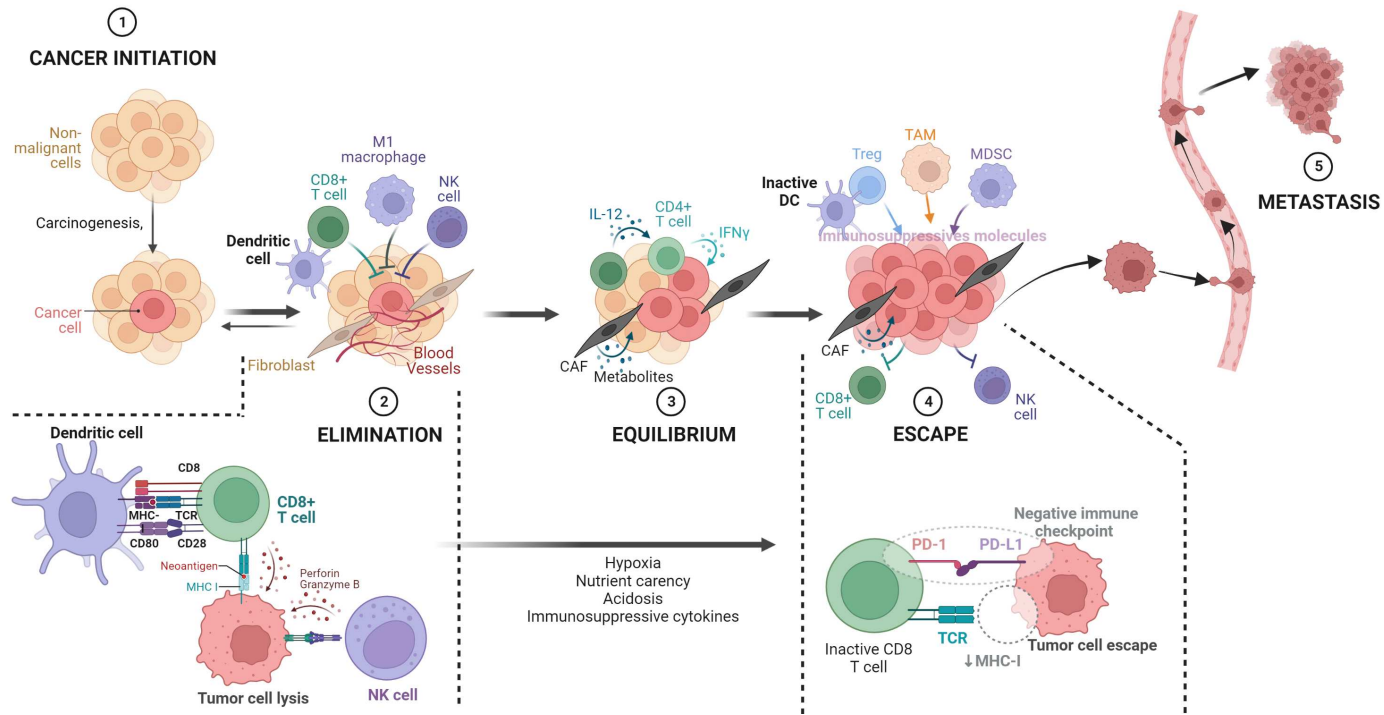


Figure 13. The phenomenon of carcinogenesis and the changing of the interrelation between cancer and its microenvironment over time (Performed on biorender).

Actually, when a normal cell becomes tumoral, in most cases, the immune cells remove it before the cancer is established, explaining why despite the high rate of mutations that can lead cells to become tumoral, no tumor is formed (elimination step). Unfortunately, tumor cells gradually develop resistance mechanisms that render the immune system less efficient in lysing tumor cells, leading to a situation where the battle between tumor and immune reaches an equilibrium, and the tumor extent remains stable (equilibrium step). Eventually, thanks to the development of escape mechanisms from immunity, tumor cells take advantage over the immune system that is no longer capable of shrinking cancer progression (escape step)⁵⁴. The resistance mechanisms are diverse and impact both the infiltration of immune cells within the TME and their function. Actually, to survive and grow, tumor cells must avoid their elimination by the immune system. For this purpose, cancer cells have developed various strategies that impact the infiltration of immune cells within the TME and their function⁵⁵.

The first strategy consists in limiting immune infiltration inside the TME. To perform that, tumor cells boost ECM production, a physical barrier preventing immune infiltration⁵⁶. When the immune cells have succeeded in entering the tumor site, cancer

cells have developed other strategies to prevent their killing action. One of them consists for tumor cells in preventing antigen presentation on MHC-I molecules, which is necessary for the immune system to recognize them as abnormal. To do that, tumor cells either perturb the antigen processing or its association with MHC molecules, notably by downregulating MHC-I molecules⁵⁷.

Another strategy consists in upregulating inhibitory immune checkpoints and their ligands both in tumor and immune cells. Although inhibitory immune checkpoint expression is a natural mechanism induced following danger elimination to stop the immune response, tumors hijack this process to render immune cells ineffective. The most known inhibitory immune checkpoints are the cytotoxic-T lymphocyte associated protein 4 (CTLA-4), the programmed cell death 1 (PD-1) and its ligands, programmed cell death ligand 1/2 (PD-L1/PD-L2)⁵⁸. In addition to that resistance mechanism, tumor cells can secrete multiple immunosuppressive molecules, such as the transforming growth factor beta (TGF β) or lactate, inhibiting the anti-tumor activity of infiltrating immune cells^{59,60}.

Eventually, another means mediated by tumor cells to escape from immune elimination consists in both recruiting immune cells that exert immunosuppressive activity, notably on the cytotoxic lymphocytes, such as the regulatory T cells (Treg) and the myeloid-derived suppressor cells (MDSC), or turning anti-tumor immune cells into immune cells exerting pro-tumoral effects⁶¹. It is worth noting that although the TME has very few infiltrated B cells in contrast with T cells, and the impact of B cells on anti-tumor immunity is still controversial, a recent subset population of B cells, called regulatory B cells or Bregs, which exert immunosuppressive activities and pro-tumoral function has been discovered^{62,63}. Concerning the hijacking of the immune cells by the tumor microenvironment, it has been well described that the tumor-associated macrophages (TAM), which are usually the most abundant immune cell type⁶⁴ within the TME, switch from an anti-tumor actor (M1 phenotype) into a pro-tumoral mediator (M2 phenotype), favoring tumor progression through different means⁶⁵. Therefore its abundance in multiple cancers, notably lung cancer, has been associated with a bad prognosis⁶⁶. Similarly, polynuclear neutrophils (PN), which are usually efficient in limiting the cancer onset or eliminating the disseminated tumor cells, are also changed into a pro-tumoral phenotype within the TME^{67,68}.

Besides, hypoxia and nutrient carency, two characteristics of the tumor site, take part in that process and render immune cells anergic and inefficient in killing tumor cells^{69,70}. This latter mechanism is notably observed for the NK, natural killer T cells (NKT), and CD8 T cells, which are usually three efficient tumor-killing immune cells associated with a good prognosis in many types of cancer. Actually, although they can infiltrate the stroma, they are usually not in direct contact with tumor cells, which limits their cytotoxic activity. In addition, those which have succeeded in infiltrating the TME share an anergic phenotype rendering them inefficient in eliminating tumor cells and blocking tumor progression^{71,72}. Concerning the CD8 T cells, their incapacity to remove cancer cells is notably mediated by the expression and the action of negative immune checkpoints⁷³.

Eventually, given their pivotal role in the induction and amplification of an efficient anti-tumor immune response, the impairment of dendritic cells and CD4 T cells within the TME is responsible for both their incapacity to implement the anti-tumor immune response as well as the transformation of CD4 T cells into an immunosuppressive cell, the Tregs^{74,75,76}.

1.4.2 Other cellular components of the tumor microenvironment

1.4.2.1 Cancer-associated fibroblast (CAF)

Although its origins can differ, CAF is often derived from tissue-resident fibroblast. When a tumor occurs, malignant cells produce TGF β and other molecules that can drive that differentiation⁷⁷. Other cell types composing the TME can differentiate into CAF, such as adipocytes and endothelial cells. The pro-tumoral impact of CAF on tumor cells is visible at every step of cancer development, acting as a promoter of tumor cell proliferation, migration, metastasis, and immune evasion. When the TME becomes hypoxic, CAF metabolism is altered, resulting in the secretion of nutrients, like ketones, necessary for malignant cells to survive and proliferate. Also, CAF can secrete molecules, like chemokine ligand 5 (CCL5) or C-X-C motif chemokine 10 (CXCL10) and a broad range of growth factors, such as the hepatocyte growth factor (HGF), that change the metabolism of tumor cells, favoring their proliferation and the metastasis development. In addition, CAF participates in the development of metastasis through different means. CAF induces EMT in malignant cells through the secretion of TGF β . It also stimulates its migration capacity and angiogenesis through the secretion of matrix metalloproteinase-3 (MMP-3), which degrades E-cadherin (E-cad) and promotes the release of vascular endothelial growth factor (VEGF). Another contribution of CAF in tumor progression is mediated by the secretion of ECM components that organize themselves in fibrovascular cores, limiting anti-tumor immune cell infiltration and drug diffusion, leading to cell survival and immune evasion⁷⁸.

1.4.2.2 Endothelial cells (EC)

As a basal element of the vascular endothelium, EC is a critical element of the TME and participates in promoting tumor growth. Actually, not only vascular endothelium allows each cell of the organism to cope with its nutrient supply and, in exchange, remove waste produced by the same cells, it also permits the immune cells to enter or exit from the tissue. It also takes part in the formation of new blood vessels or angiogenesis. However, when cancer occurs, as cancer grows and reaches a 1-2mm³ in volume, some parts of the tumor cells are not well supplied with oxygen and nutrients, and tumor wastes are not well evacuated, resulting in a progressive hypoxic and acidic TME, advantageous for inactivating anti-tumor immune cells. To overcome that difficulty, tumor cells, through the secretion of pro-angiogenic factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) induced by hypoxia, promote the formation of new vessels³⁰. However, new blood vessel formation in the TME is impaired in their maturation step,

leading to a leaky vasculature⁷⁹. That imperfect architecture has several pro-tumoral consequences. In addition to fail in coping with hypoxia and acidosis in the TME, it prevents drug delivery to tumor cells, participating in tumor cell resistance^{80,81}. Besides, that leaky vasculature results in weaker junctions between the EC, allowing tumor cells to escape from the primary site to reach circulation and establish metastasis in other organs⁸². Moreover, EC is a very plastic cell that can change its phenotype in CAF through the action of cytokines to promote migration and invasion of the tumor cells during the metastatic process⁸³.

1.4.2.3 Lymphatic endothelial cells

Similarly to angiogenesis for EC, tumor cells can secrete pro-angiogenesis factors, like VEGFC and D, that promote the development of new lymphatic vessels during a lymphangiogenesis process. Although tumor cells can invade existing lymphatic vessels, that mechanism facilitates it and acts as a tumor metastasis promoter⁸⁴. Emerging evidence tend to demonstrate that the lymphatic endothelial cell could promote tumor growth in other ways, notably in altering the anti-tumor immune response⁸⁵.

1.4.2.4 Pericytes

Pericytes are a cell type that envelopes the surface of endothelial cells and acts both in vascular endothelial support and in its remodeling during angiogenesis. Besides, pericytes are also involved in attracting immune cells from the blood circulation to the tumor site and their activation⁸⁶.

1.4.2.5 Adipocytes

In certain types of cancer, another cell type is comprised in the TME. Those cells, called adipocytes promote tumor progression through the secretion of metabolites, growth factors, proteins, and hormones. Actually, in breast cancer, where the importance of adipocytes in tumor progression has been the most described, these cells have been demonstrated to modify their lipidic metabolism to fuel lipids in malignant cells. Also, adipocytes secrete a hormone called leptin that favors tumor cell proliferation. In addition to those two mechanisms, adipocytes can also secrete a great number of metalloproteases that modify ECM to promote tumor cell invasion⁸⁷.

1.4.3 Non-cellular components of the tumor microenvironment

1.4.3.1 Extracellular matrix (ECM)

ECM not only acts as a physical scaffold putting together each cell type composing the TME, but it is also a structure comprising a great number of molecular messengers that allow each cell type to communicate with each other in a working way⁸⁸. By using this way, tumor cells hijack the TME functioning for its own benefits, promoting its survival, proliferation, immune evasion, and metastasis development. In addition to the secretion of molecules that impact the phenotype and functioning of each cell type composing the TME for the tumor cell benefit, cancer cells also physically modify the

TME and the ECM for the same purpose⁸⁹. To prevent anti-tumor immune cells infiltration in the TME, tumor cells promote the secretion of fibrous proteins (collagen, elastin), glycosaminoglycans, proteoglycans, and glycoproteins by diverse cellular components of TME, mainly CAF, that constitute a physical barrier⁵⁶. In addition, it prevents the distribution of anti-cancer drugs, limiting the efficacy of anti-tumor treatments⁹⁰. That large ECM deposit, called desmoplasia, can account for up to 60% of the tumor mass and is strongly associated with a bad prognosis in cancer patients.

Conversely, when the tumor cells metastasize, tumor cells to escape from the ECM, but also CAF and TAM produce and secrete MMP. These proteases degrade the ECM, facilitating their migration and metastasis development⁹¹. Moreover, the TME comprises many secreted molecules that impact tumor development. Actually, ECM contains growth factors, angiogenic factors, and chemokines that favor angiogenesis and tumor progression. In addition, inside the TME, tumor cells and non-malignant cells of the TME whose functions have been modified and hijacked to promote tumor progression, secrete immunosuppressive cytokines and molecules that perturb the anti-tumor immune cells, which eventually lead to immune evasion⁸⁹.

Finally, cellular components of the TME can, at a distance, shape the ECM of distant organs to favor metastasis development⁹². Soluble factors, and notably exosomes, mediate that mechanism.

1.4.3.2 Exosomes

Exosomes, microvesicles comprised between 30 and 200nm in size, are secreted by the cell whose content is the reflection of this latter (proteins, ribonucleic acid (RNA), DNA, lipids...)⁹³. They are an emerging means to favor crosstalk between cancer cells and other cells of the TME and promote tumor progression⁹⁴. Actually, exosomes have been described to promote tumorigenesis, tumor proliferation, angiogenesis, metastasis, and resistance to conventional anti-tumor therapies⁹⁵⁻⁹⁹. Actually, in the lung cancer context, tumor cells initially sensitive to icotinib have been described to acquire resistance to that drug through a transfer of the oncogene MET by exosomes. In addition, MET also promotes migration and invasion properties of tumor cells¹⁰⁰. Moreover, some tumor stress conditions like hypoxia have been shown to boost exosome production by cancer cells, facilitating the transition of stromal cells into CAF¹⁰¹. Exosomes have also been demonstrated to favor communication between cells composing the TME and the immune microenvironment¹⁰². Its impact on anti-tumor immunity is controversial and depends on the studies. Actually, in some papers, exosomes have been shown to facilitate NK cells activation, whereas in others, they favor NK cell dysfunction through another way^{103,104}.

1.4.3.3 Circulating free DNA (cfDNA)

cfDNA is a cell-free, very short double-strand DNA fragment (<200 bp) that is released both by dead and living cells¹⁰⁵. In the TME context, those elements are secreted by malignant and non-malignant cells. It participates in cell-to-cell contacts and, as a horizontal gene transfer means, can modify the biology of the recipient cells, favoring

the tumor progression and metastasis¹⁰⁶. Although the precise molecular mechanisms have not been unveiled yet, some studies suggest that an overexpression of pro-metastatic genes could mediate those effects through a Toll-like receptor 9 (TLR9) / Myeloid differentiation primary response 88 (MyD88) independent pathway or by an increase in exosome uptake¹⁰⁷. Moreover, radiation therapy treatment favoring cfDNA release could promote radioresistance and chemoresistance when chemotherapies are combined. In addition, cfDNA from dying tumor cells can integrate genomes of non-malignant cells that promote oncogenesis and metastasis development through a process called genomestasis involving DNA damage and inflammation¹⁰⁶. As a reflection of tumor cell biology, cfDNA has become a cancer biomarker, with a great deal of information concerning mutations that could allow clinicians to adapt the treatment in a more personalized way and without biopsy intervention¹⁰⁸.

2 Lung cancer

2.1 Organisation of the lungs

Like other big animals, humans are complex organisms within all cells are not in direct contact with the environment and its nutrients. Given that each cell composing the organism requires nutrients (mainly glucose) and dioxygen to produce cellular energy essential to their function, the human organism is organized in different systems (formed by various organs) that allow each cell to have nutrients from the environment, produce energy and evacuate wastes outside the organism¹⁰⁹.

Among them, there is the respiratory system, whose lungs are central organs. Given its function of favoring gas exchange between the environment and the cells of the organism (thanks to the bloodstream and the circulatory system), the lungs are vital organs. It allows each cell of the organism to receive oxygen and, in exchange to evacuate from the cell to the environment, the carbon dioxide produced following cellular energy production. The respiratory system is composed of two portions, the conducting and the respiratory portion¹¹⁰. The conducting portion comprises the nose, oropharynx, larynx, and trachea, which brings oxygen from the environment to the respiratory part. That latter portion includes a conduct called the bronchi that divides itself into bronchioles within the lungs and then in more than 300 million units, called alveoli, where gas exchange occurs with the bloodstream and the capillary network. Then the oxygenated blood is transported to tissues and cells through the arterial system. In addition, the poorly oxygenated blood that is also highly concentrated in carbon dioxide drives through the veinal system to the alveoli, where the latter gas is evacuated, and the blood gets reoxygenated. That air exchange between the environment and the bloodstream through the lungs is allowed by an innervated respiratory muscle called the diaphragm. The lungs are further divided into individual lobes that comprise the alveoli: three for the right lung and two for the left one. That organization of the lungs with the alveoli significantly increases the exchange surface

between the respiratory system and the circulatory one, with approximately 80 square meters of surface area dedicated to gaseous exchange¹⁰⁹.

All the respiratory tree is lined by an epithelium whose composition and organization change according to the disposition and function of the organ in the respiratory system. Actually, the conducting portion of the respiratory system is lined by a ciliated pseudostratified columnar epithelium composed of three major cell types: ciliated, non-ciliated secretory cells (goblet and neuroendocrine cells), and basal cells¹¹¹. The proportion of each cell type varies according to the progression in the respiratory tree. Basal cells are a type of cells that supports the attachment of the goblet and ciliated cells to their basal lamina and therefore maintain the epithelium organization. Neuroendocrine cells that are present in a cluster in the bronchial mucosa can secrete various molecules through the release of granules. That includes catecholamine and polypeptide hormones like calcitonin and serotonin. It represents a tiny proportion of the mucosal epithelium, nearly 3%¹¹². The term pseudostratified refers to an epithelium composed of a single layer of cells whose nuclei are not aligned at the same plane and appear as multiple layers. As the lungs are an exchange surface between the harmful environment and the organism, the incoming air must be removed from foreign particles and pathogens to limit infection that could cause damaging inflammation and tissue injuries. A collaboration mediates that role between the goblet cells and the ciliated cells. Actually, the goblet cells sense pathogens coming from the environment and produce mucus to trap the pathogen. Then the mucus is evacuated outside the respiratory tree by ciliated cells' action during a mucociliary elevator mechanism¹¹³. Therefore, the roles of that portion of the respiratory tree are multiple: bringing air from the environment to the lungs, warming it, and cleaning it from pathogens.

As the degree of ramification within the respiratory tree continues, the epithelium progressively changes from pseudostratified to simple cuboidal in the bronchioles, whose most cells are non-ciliated¹¹¹. Finally, alveoli possess a lining of thin squamous epithelium optimal for gas exchange. That epithelial comprises two other cell types: type I and type II pneumocytes. The central role of type I pneumocytes is to realize the gas exchange between the airway and the bloodstream, whereas type II pneumocytes act as a support to allow gas exchange. Actually, the main role of type II pneumocytes is to produce surfactant, which has the double advantages of preventing alveoli from collapsing during exhalation and reducing the surface area between the type I pneumocyte and the capillary, therefore, promoting the gas diffusion between the respiratory and the circulatory systems¹¹⁴.

2.2 Different types of lung tumor

Lung cancer comprises two histological subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), respectively accounting for 15 and 85% of total lung cancer¹¹⁵. They differ from one another depending on the origin of the tumor, the

histology, the genetic alteration profile, the aggressiveness, and the response to anti-tumor treatments.

2.2.1 Small cell lung cancer (SCLC)

SCLC, also classed as neuroendocrine tumors, start from the neuroendocrine cells present in the lung and are extremely aggressive due to rapid dissemination into submucosal lymphatic vessels and regional lymph nodes without a bronchial invasion. Its immunohistochemical markers are synaptophysin, chromogranin, and CD56/NCAM^{116,117}.

2.2.2 Non-small cell lung cancer (NSCLC)

Although less aggressive, NSCLC is often diagnosed at a later stage. Actually, it is considered that only 25% of NSCLC patients are diagnosed at stage 1 or 2. Based on the 2004 World Health Organisation (WHO) classification, NSCLC comprises three main types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, respectively accounting for 40%, 25 to 30%, and 10 to 15%¹¹⁸.

2.2.2.1 Adenocarcinoma (AdenoCA)

AdenoCA is the most prevalent subtype of NSCLC (accounting for 40% of total lung cancers). It begins in the peripheral lung tissue and, more specifically, in the epithelial cells of segmental bronchi. AdenoCA is either characterized by mucus formation or by distinct growth patterns such as glandular/acinar growth, papillary differentiation, or a single layer spread along the alveolar septum and bronchioles (Bronchioalveolar carcinoma). Contrary to other types of lung cancer, it grows slower. Based on histologic differences, AdenoCA was firstly segregated into different subcategories. Among them, we can note the well-differentiated fetal adenocarcinoma, the mucinous adenocarcinoma, the mucinous cystadenocarcinoma, the signet ring adenocarcinoma, and the clear cell adenocarcinoma. The 2011 International Association for the study of Lung Cancer (IASLC) and the 2015 WHO have shed light on the importance of classifying AdenoCA according to the extent of invasiveness. Actually, we can note adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma^{119,120}. AIS comprises a lepidic pattern with a diameter smaller than 3 cm. If it exceeds 3 cm, a rare phenomenon, it is called lepidic predominant AdenoCA, suspect AIS^{119,120}. MIA is defined by a diameter and an invasion size, respectively, smaller than 3 and 5 cm. If the tumor size is larger than 3 cm, a rare phenomenon, it is called lepidic predominant adenocarcinoma suspect MIA^{119,120}. Invasive AdenoCA is now classified using five main patterns: acinar, lepidic, papillary, micropapillary, and solid adenocarcinoma¹²¹.

When the adenocarcinoma identification at the light microscope is too tricky, it can be carried out by immunohistochemistry through the identification of specific markers, TTF-1 and Napsin A. (CK7)¹²²

2.2.2.2 Squamous cell carcinoma (SQCLC)

SQCLC tends to be located centrally, arising in the main bronchi. Although previously diagnosed by the presence of keratinization or intercellular bridges, the 2015 WHO classification has identified keratinizing, non-keratinizing, and basaloid SQCLC^{119,120,122}. Its immunohistochemistry markers are p40, CDK5/6, and p63.

2.2.2.3 Large cell carcinoma (LCAC)

LCAC tends to begin in the outer regions of the lungs. It behaves similarly to SCLC, with a rapid spread. It takes its name from the large size of the tumor cells composing lung cancer. It is an exclusion diagnosis. Actually, it is an NSCLC in which neither the characteristics of adenocarcinoma nor squamous cell carcinoma is observable. The WHO/IASLC classification has recognized different variants of LCAC. Among them, we can note the large cell neuroendocrine carcinoma, the basaloid carcinoma, the lymphoepithelioma-like carcinoma, the clear cell carcinoma, and the large cell carcinoma with rhabdoid phenotype^{119,120}.

2.3 Etiology and prevention

Like every cancer, lung tumor is a genetic disease that takes its origin from genomic modifications inside a normal cell. Although these modifications are usually removed by an efficient DNA repair mechanism, in very few cases, they are not⁵. If the genomic alteration gives an advantage to the cell over the others, that modification can persist and be transmitted to the progeny. The accumulation of genomic mutations can progressively modify the normal cell and turns it into a malignant one⁷. In addition to the DNA repair mechanism, the immune system can recognize and eliminate these abnormal cells before the onset of cancer¹²³. These two combined processes explain why despite the significant number of daily genomic modifications, the human host mostly does not develop cancer. However, some environmental factors can increase the likelihood of developing genomic mutations and, by extension, cancer. As in various cancers, diet habits influence the likelihood of developing lung cancer, with some dietary elements acting positively whereas others negatively^{124–127}. Although some factors are common for all malignancies, like immunodeficiency (innate or acquired) and aging, which both favor cancer onset, other factors are more specific to cancer, like smoking, air pollution, occupational exposure, and the history of previous lung affections^{128–131}. These factors promote tumorigenesis by directly favoring oxidative stress that leads to DNA damage or indirectly through the induction of chronic inflammation^{132,133}. That latter mechanism explains why pathogens causing chronic inflammation are responsible for almost 15% of cancers¹³⁴.

2.3.1 Genetic susceptibility

Given lung cancer is a genetic disease, the impact of familial genetic inheritance on lung cancer onset has been investigated. Analysis and metanalysis have evaluated

the association between gene mutations and an increased risk of developing lung cancer. To confirm its impact, studies demonstrated that having a near relative suffering from lung cancer is associated with an increased risk of developing cancer^{135,136}. Among the genetically inherited mutations responsible for that increased susceptibility, some affect crucial mechanisms of cell homeostasis, preventing the normal cell from becoming malignant (uncontrolled proliferation and apoptosis). These mutations, called driver mutations, favor lung tumor onset^{137,138}. Moreover, other mutations in genes coding for the nicotine receptor, a cigarette component, affect the human carrier's behavior and influence its smoking susceptibility and habits (smoking addiction)^{139,140}. Since smoking is the leading cause of developing cancer, its increased exposition naturally raises the onset of lung cancer. Eventually, other genes are associated with an increased risk of developing lung tumors, notably genes necessary for the activation of procarcinogens¹⁴¹.

2.3.2 Smoking

Given its position and function in the organism, the lungs are continuously exposed to substances present in the environment. The main risk factor for lung cancer is tobacco. Actually, the likelihood of developing lung cancer depends on various parameters, including the frequency of smoking and the number of cigarettes consumed per day. It has been assessed that persons who smoke are between 13 and 23 times more likely to get lung cancer¹⁴². In addition, death by lung cancer is strongly associated with smoking activity, around 80 to 90%. Although nicotine by itself is not a carcinogen, it is associated with more than 50 substances considered like this that are responsible for DNA modifications¹⁴³. Cigar and pipe smoking have also been observed to be a facilitating factor for lung cancer¹⁴⁴. Moreover, person who do not smoke but are often in contact with a person who did it, are also subjected to the negative impact of smoking. That mechanism called secondhand smoking increases the risk of developing cancer by almost 25% in nonsmokers¹⁴⁵. As a result, to limit lung cancer spread, the world health agency highly recommends people to stop smoking. A study demonstrated that smokers who quit for more than 15 years have a risk of developing lung cancer decreased by 80 to 90% compared with persons who continue to smoke¹⁴⁶.

2.3.3 Air pollution

In big cities or areas where there is traffic congestion and pollution accumulation, the exposition of the population of that pollution, notably emissions of polycyclic aromatic hydrocarbons, is considered as a lung cancer risk factor. American and European studies based on multiple cohorts (ESCAPE study) have demonstrated that particulate matter in ambient air is associated with an increased risk of developing lung cancer, notably adenocarcinoma¹⁴⁷. In a study, in Great Britain, 8% of lung cancer cases were attributed to air pollution¹⁴⁸. Lipsett, Campleman, and Bhatia estimated in a solid study that persons occupational exposed to diesel exhaust (notably persons working in the trucking industry) have a 30 to 50% risk increased of developing lung cancer^{149,150}.

Given the current situation and the planned tendency in terms of air pollution increase, the incidence of lung cancer will naturally increase.

2.3.4 Occupational exposure

In addition to smoking and air pollution, occupational exposure is also a risk factor for developing lung cancer¹⁵¹. Lung cancer is considered the cancer in which occupational exposure has the biggest impact¹⁵². Among agents, occupational exposures to substances like heavy metals, radon, asbestos, arsenic derivative products (present in insecticides, herbicides, and anti-fungal products), and beryllium oxide (used for X-ray or radiation technology) promotes lung cancer incidence^{153–157}. Given that lung cancer is influenced by various factors, determining the percentage of lung cancer attributable to that exposition is difficult. One study has evaluated that occupational exposure accounts for 10% of lung cancer onset and was confirmed in a study performed in Great Britain^{148,158}.

2.3.5 Previous lung affection

Pulmonary diseases associated with chronic lung inflammation are also linked with an increased risk of developing lung cancer. Actually, chronic obstructive pulmonary disease (COPD), which is by far the most common comorbidity in patients who have lung cancer (Prevalence between 30 and 70%), has been associated with an increased risk of developing lung cancer¹⁵⁹. Other affections (caused by infections or not) like pneumonia, tuberculosis, and chronic bronchitis, responsible for chronic inflammation within the lungs, have also been associated with lung cancer emergence¹⁶⁰. Actually, infection by *Chlamydia pneumonia* (the agent responsible for acute respiratory affection) or by *Mycobacterium tuberculosis* in patients has been observed to increase the risk of developing lung cancer^{161,162}. Although those organisms are not oncogenic, the persistence of the microorganism and the chronic inflammation induced, responsible for an increase in reactive oxygen species (ROS) and DNA damage, could be the way by which they favor the development of lung tumors¹⁶³.

2.4 Symptoms

2.4.1 Common symptoms

Most lung tumors do not cause signs or symptoms at the early stages. The symptoms occur when the disease is already advanced. It includes difficulties in breathing (shortness of breath), unstoppable cough (that can be accompanied by blood), hoarseness, fatigue, weight loss accompanied by a loss of appetite, and chest pain¹⁶⁴.

When the tumor spreads in other organs, it can be responsible for other symptoms like the swelling of lymph nodes (a structure that comprises immune cells), jaundice (yellowing of the skin and eyes during metastasis in the liver), or some nervous perturbations like headache, weakness of arm or leg and balance or seizure problems during metastasis in the brain¹⁶⁵.

2.4.2 Rare specific syndromes

Some lung cancers can also be responsible for specific syndromes like the horner syndrome, the superior vena cava syndrome, and the paraneoplastic syndromes. The horner syndrome is caused by lung cancer developing in the upper part of the lungs (called Pancoast tumors, more likely NSCLC than SCLC) and results from the fact that it can affect specific nerves in the eye and the face. The symptoms are a smaller pupil and the drooping or weakness of the upper eyelid¹⁶⁶.

The superior vena cava syndrome takes its name from the fact that lung tumors during their growth can press on the large vein called the superior vena cava present in the upper part of the right lung, which drives blood from the head and arms to the heart. That mechanism is responsible for blood to back up in the veins causing swelling in the face, arms, and upper chest. If it affects the brain, it can lead to headaches and a change in consciousness. This syndrome can sometimes become life-threatening and needs to be treated immediately¹⁶⁷.

Paraneoplastic syndromes are a set of syndromes caused by hormone-like substances secreted by lung tumors that can enter the bloodstream and affect distant organs (more likely associated with SCLC than NSCLC). Sometimes the symptoms observed are the first signs of lung cancer. However, given that it leads to symptoms in other organs, lung cancer is not suspected to be the cause, and therefore the diagnosis of lung cancer is not realized¹⁶⁸. Among the syndromes, there is the syndrome of inappropriate anti-diuretic hormone and the Cushing syndrome caused respectively by the production of ADH, a hormone that leads the kidneys to hold water, destabilizing the salt level in the blood and the release of adrenocorticotrophic hormone, that causes the adrenal glands to produce cortisol. The syndrome of inappropriate ADH can lead to fatigue, muscle weakness or cramps, restlessness, and confusion. It can become life-threatening if untreated, leading to seizures and coma¹⁶⁹. The symptoms of Cushing syndrome include weight gain, weakness, and fluid retention that can lead to high blood pressure and diabetes¹⁷⁰. Lung tumors can also cause hypercalcemia, leading to thirst, frequent urination, vomiting, nausea, and weakness¹⁷¹.

In addition, SCLC can lead the immune system to attack its nervous counterpart causing two well-known syndromes: the Lambert-Eaton syndrome and the paraneoplastic cerebellar degeneration, both resulting in muscle disorders, the latter leading also to trouble speaking^{172,173}.

2.5 Diagnosis

Lung tumors are often asymptomatic during the first cancer stages and become symptomatic when the tumor has already progressed or developed metastasis. That explains why lung cancer patients are often diagnosed at an advanced stage and therefore are more challenging to treat and heal. Only 23% of cancer patients are diagnosed at an early stage when the 5-year survival rate is around 59%, whereas the

great majority are latter stage cancer when the 5-year survival rate is much lower. As a result, lung cancer has a poor 5-year survival rate, around 22.3%¹⁷⁴. Actually, most early-stage cancers are discovered incidentally during a radiology test ordered for other reasons. More rarely, symptoms of lung cancers occur, and a thoracic scan is ordered to determine whether there is a tumor or not. Then, the diagnosis must be confirmed by recuperating a piece of the tumor during a process called biopsy.

Different methods are proposed depending on the localization and the extent of the tumor. Actually, if cancer has reached the bronchi or the lymph node, the piece of the tumor is taken under local anesthesia during a bronchial fibroscopy. If the cancer is not localized in the bronchi or lymph nodes, a puncture of the malignant part is realized under local anesthesia during a thoracic scan. More rarely, if neither fibroscopy nor thoracic puncture is possible, surgery can be needed to take the biopsy. Then, the piece of the tumor is sent to an anatomic pathology laboratory and examined by microscopy to determine the type of lung tumor and the cancer stage (based on the TNM classification). In addition, a positron emission tomography (PET)-Scan, which consists in revealing the tumor metabolism, is ordered to determine the extent of cancer and the possible presence of metastasis within the organism¹⁷⁵.

2.6 TNM classification

To determine the advancement and the severity of cancer, clinicians have elaborated a classification that facilitates them in managing their patients. Actually, that ranking, called TNM classification, is helpful to give some indications of prognosis and to adapt the treatments for patients. Although the classification remains quite similar, it is not used to evaluate all cancers. Actually, in lung tumors, that categorization is used for NSCLC but not SCLC. That classification is based on three parameters:

- The T describes the size of the primary tumor.
- The N assesses the extent of involvement of regional lymph nodes.
- The M evaluates the presence or not of metastasis.

According to the extent of the gravity of each parameter, a number is added. The more the number is high, the more severe the parameter is. According to the combination of the three parameters called TNM subsets, the NSCLC is ranked from stage I to stage IV. TNM subsets with similar prognoses are put in the same stage, and the more it is high, the more the cancer has progressed¹⁷⁶.

However, SCLC does not suit that TNM classification, but it is ranked in two stages: Limited or extensive according to the extent of the primary tumor, the latter characterized by the spread beyond the supraclavicular areas¹⁷⁷.

2.7 Treatments: from hope to the appearance of resistance

The choice of the cancer treatment for a lung cancer patient is decided after a concertation between the patient and his doctor. It is based on different factors: the

type of lung tumor, the cancer stage, the global health condition, and the patient's choice.

2.7.1 Cancer stage and treatments

Depending on the lung cancer stage, the treatment provided to the patient is different¹¹⁸. Actually, the standard treatment for early-stage disease (stage I and II) is surgery and is associated with high 5-year survival rates, almost 70% for stage I and 40% for stage II. For some patients, usually from stage IIA to IIIA, adjuvant therapy is realized after surgical resection to maximize the elimination of the remaining tumor cells, prevent tumor recurrence and prolong patient survival. Adjuvant therapy can be chemotherapy, radiation therapy, or targeted therapy. For IIIA patients, adjuvant chemotherapy is the standard treatment and has been shown to significantly prolong the overall survival of the patient (an increase of 5-6% in 5-year survival rate compared with surgery alone)¹¹⁸.

Radiation therapy is the primary treatment for patients refusing surgery or with unresectable tumors.

Surgery is no longer the standard therapy for patients who suffer from late-stage disease (stage III and IV), accounting for more than 70% of NSCLC patients. Stage III disease is very heterogeneous, from a resectable tumor with microscopic metastasis in the lymph nodes to an unresectable one with multiple nodal invasions. The 5-year survival is very low, between 2 and 15%¹⁷⁸. Concerning the treatments, according to the tumor location and the possibility of surgery, radiation therapy, chemotherapy, and surgical resection can be used to cure the patient. The standard treatment for unresectable stage IIIA patients is either a combination of chemotherapy and radiation therapy or radiation therapy alone. The first option has been shown to decrease patient death by 10% compared with the latter. Concerning stage IIIB patients whose 5-year survival rate is very low, between 3 and 7%, the regimen treatment is the same as unresectable stage IIIA patients¹¹⁸. Treatments for stage IV patients, that account for 40% of the newly diagnosed NSCLC patients, can be diverse according to many factors, including notably the comorbidity and the molecular alterations of cancer. Actually, the various medical options are:

- The combination of different chemotherapies.
- The combination of chemotherapy with targeted therapy.
- Palliative radiation therapy.

In addition, surgery could also be planned to reduce disease-related symptoms.

2.7.2 Surgery

Surgical resection, which consists in removing all the tumor part, remains the standard treatment and the more successful medical option for lung cancer patients, notably early-stage patients (stages I and II). The efficacy of the surgery is based on two conditions: the tumor must be totally resectable and so confined to the lungs, and the patient must be able to tolerate that intervention. The first issue is addressed by determining the cancer stage through biopsy and imaging studies. The evaluation of

patient factors manages the second one. Different types of surgical resection are proposed according to the extent of the tumor and the health condition of the lung cancer patient. Actually, from the smallest to the largest, there is the wedge resection, which consists in removing a small part of the lungs, the segmental resection, the lobectomy consisting in removing a complete lobe of a lung, and eventually, the pneumonectomy, in which an entire lung is removed. Sometimes, the nearby lymph nodes are also removed if a risk of the tumor spreading within it is suspected.

Usually, when the patient can tolerate it, surgeons, to realize a complete resection, perform the ablation of an entire lobe instead of realizing limited resections like wedge resection or segmentectomy. This protocol has been demonstrated to decrease tumor recurrence and favors long-term survival in lung cancer patients. With time, the techniques have evolved and have become less and less invasive. Video-assisted lobectomy is one example, among others. That evolution has allowed expanding the surgical resection option to people whose surgery was not firstly recommended, notably elderly people with meaningful long-term survival¹⁷⁹.

When there are risks that some tumor cells have not been removed, an adjuvant therapy which consists in applying radiation therapy or chemotherapy to eliminate the remaining cancer cells and prevent cancer recurrence, is recommended¹⁸⁰.

After surgery, some complications can occur (estimated to 1 out of 5 patients with lung cancer surgical resection) and include infection or inflammation of the lungs (pneumonia), excessive bleeding, or a blood clot in the leg (thrombosis) that can migrate to the lungs and causes a pulmonary embolism¹⁸¹.

2.7.3 Radiation therapy

Radiation therapy is a technique that consists in using high-energy rays or particles (such as X-rays or protons) to eliminate cancer cells. Depending on the purpose, it can be applied before or after surgery or proposed alone as a standard treatment. Actually, before surgery, radiation therapy can be performed to shrink the tumor and facilitate its resection. It can also be used after surgery to eliminate the remaining tumor cells and limit cancer recurrence. Radiation therapy can also be proposed as the standard treatment for lung cancer patients, especially when the tumor has spread too far to have surgery or when the patient is not able to tolerate a surgical resection. The dose of radiation therapy depends on the type of lung cancer. Actually, for NSCLC, the usual total amount is 66 Gray (Gy), whereas, for SCLC, it is comprised between 54 and 66 Gy dispatched in 5 to 7 weeks. It also depends if the treatment is given once a day or twice. Actually, for SCLC, if the treatment is given twice a day, the total dose is reduced to 45 Gy for 3 to 4 weeks¹⁸². Finally, radiation therapy can also be used as a palliative treatment in later cancer stages to reduce symptoms associated with cancer. In addition, for SCLC patients, radiation therapy to the brain is recommended as prophylaxis to limit the recurrent lung tumor spreading in the brain (Usual total dose: 25 Gy for two weeks). Several techniques are used to administer radiation therapy according to the localization of the emission source of radiation. Actually, radiation therapy can be external or internal, also called brachytherapy¹⁸³. External radiation

therapy comprises different techniques: external beam radiation therapy (EBRT) and its newest versions, and stereotactic body radiation therapy (SBRT). EBRT is the most often technique used in radiation therapy to treat patients and consists in focusing radiation from outside to cancer in a procedure similar to an X-ray in which the radiation dose is stronger. Although the standard regimen varies depending on the patient and the purpose of the radiation therapy, it is usually given 5 days a week for several weeks. Actually, for curative treatment, the radiation therapy course is between 4 and 7 weeks (20 to 32 sessions), whereas, for a palliative one, it is reduced to 1 to 5 sessions. The treatment is painless, and each session lasts 10-15 minutes a day.

Newer EBRT techniques have been developed to help clinicians target the tumor more specifically and limit damage to nearby healthy tissues, therefore limiting the side effects. These comprise the three-dimensional conformal radiation therapy (3D-CRT), the intensity modulated radiation therapy (IMRT), the volumetric modulated arc therapy (VMAT), and the stereotactic radiosurgery (SRS). The 3D-CRT, by the use of advanced computers, can precisely determine the cancer localization which helps to target efficiently the tumor by radiation from several directions. The IMRT is quite similar to the 3D-CRT, with the great additional advantage of having the possibility to adjust the strength of the beams. That technique is often used when the tumor is near an essential structure like the spinal cord. Although VMAT is very similar to the IMRT, it differs in its capacity to rotate around the body and delivers the radiation to the tumor mass very quickly and precisely. The SRS differs from the other techniques because the treatment is given in only one session but can be repeated if needed. It can be used instead of or along surgery when the tumor has spread to the brain. There are two different versions of the SRS. The first one consists in receiving 200 beams of radiation on cancer from different angles over a few minutes to hours. The second one is a type of SRS where a linear accelerator that creates radiation moves around the head of the patient and targets the tumor from different angles. SBRT, used to treat both early-stage lung cancer and cancers that have spread to other organs like the brain, has a different functioning than the EBRT technique and its newest versions. Actually, instead of giving a small dose of radiation each day for several weeks, SBRT considerably increases the amount of radiation in fewer session treatments. In practice, the patient is put in a designated body frame, limiting the movement of the lung tumor during breathing, and the cancer is targeted from different angles.

In addition to external radiation therapy, the internal one, called brachytherapy, also exists. Whereas external radiation therapy is used to cure lung cancer patients, the internal one is mainly used for a palliative purpose, shrinking the tumor from the airway to relieve the symptoms. The process consists of putting a small source of radioactive material directly into cancer or its nearby airway through a bronchoscope or after surgery. Given the distance of action is very short, it does not cause damages to the surrounding healthy tissues. In addition, the material is then removed after a short time. In some instances, small radioactive elements are left in place, and the radiation worsens over several weeks.

After radiation therapy, different side effects can occur, like fatigue, pain in the chest, redness and soreness of the skin, hair loss, persistent cough, and difficulties in swallowing¹⁸⁴.

2.7.4 Chemotherapy

Chemotherapy as radiation therapy is a therapeutic strategy against lung cancer cells that can be used for different purposes. Actually, chemotherapy can be administered before surgical resection to shrink tumor mass and make removing it easier. It can also be used alone or in combination with radiation therapy when surgery is no longer an option. In addition, chemotherapy can also be used after surgery to eliminate the remaining tumor cells and prevent cancer recurrence. Finally, it can also be used as a palliative treatment to reduce the symptoms of the later-stage lung tumor patient. Chemotherapy is a drug that can be administered intravenously or orally for weeks or months with breaks in between to recover from the effects of the drugs. That is called a cycle. The number of chemotherapy cycles depends on the type of lung cancer and tumor stage. Most lung cancer patients treated by chemotherapies are later-stage cancer patients and need 4 to 6 chemotherapy cycles over a period of 3 to 6 months. After that period, for patients that have had a good response to their chemotherapies drugs, some clinicians prone a maintenance therapy to keep cancer in check and prolong the patient survival. Adjuvant and neoadjuvant chemotherapy is usually given for 3 to 4 months.

The chemotherapy regimen given to the lung cancer patient depends on the tumor stage. The standard chemotherapy regimen to treat early-stage lung cancer is a combination of two chemotherapy drugs that often include cisplatin or carboplatin, platinum-salt based chemotherapies, and one other drug. In rare cases, other combinations that do not integrate these drugs, like gemcitabine with vinorelbine or paclitaxel, are used. Conversely, later lung cancer patients who are often in poor overall health or elderly might not tolerate the combination chemotherapy and are treated by a single chemotherapy drug. Depending on the type of lung tumor cancer, the combination of the chemotherapeutic drugs given to the patient is different. Actually, although for both SCLC and NSCLC patients, the regimen standard is a combination of drugs, including either cisplatin or carboplatin plus one other drug, that second drug administrated is different between SCLC and NSCLC. Usually, SCLC patients are treated either by a combination of cisplatin or carboplatin with etoposide or by a combination of carboplatin and gemcitabine and are good responders to chemotherapies. For NSCLC patients, the usually additional chemotherapy drugs administrated either in combination with cisplatin or carboplatin are paclitaxel (also called taxol), vinorelbine, gemcitabine, etoposide, and pemetrexed^{185,186}.

Given its presence intravenously, chemotherapeutic drugs can both act on the tumor mass and healthy cells and therefore be responsible for the occurrence of different side effects after chemotherapy treatment, like fatigue, constipation and diarrhea, nausea, vomiting, hair loss, and increased susceptibility to infections. In addition, some chemotherapeutic drugs can have specific effects. Actually, cisplatin, paclitaxel, or

vinorelbine can be responsible for nerve damage, mainly in hands and feet, that can lead to symptoms like pain, weakness, burning, and sensitivity to cold. These side effects usually go away progressively after the end of the treatment. Sometimes, clinicians, in complement to the chemotherapy, give some vitamins to reduce the side effects¹⁸⁷.

2.7.4.1 Platinum-salt based agents

Cisplatin, the initial platinum-salt based agent, is a neutral platinum complex with two chloride and ammonia groups. Inside the cell, its structure is modified by biochemical reactions, rendering it more reactive to DNA. The platinum complex can react with purine bases of DNA (guanosine and adenosine nucleotides), particularly in the N7 position, which is mostly responsible for cross-linking of adjacent guanine bases leading to a significant distortion in the DNA double helix. Upon this error being detected, proliferation is stopped, and DNA repair mechanisms are implemented, and based on the extent of the damage, the cell is repaired or eliminated by apoptosis. Although this molecule is more prone to enter and act in intense proliferative cells as tumor cells, it also can impact normal cells resulting in various toxicities¹⁸⁸.

Over time, other platinum-salt based agents have been developed to improve efficacy and reduce toxicities, giving birth to second and third-generation products, respectively, carboplatin and oxaliplatin. As efficient as cisplatin, carboplatin is easier to administrate and less toxic¹⁸⁹. In addition, although oxaliplatin is responsible for fewer DNA adducts, it is more efficient than cisplatin and carboplatin in initial responder cancers. Also, it has anti-tumor activity in some cancers, while cisplatin and carboplatin show no efficacy¹⁹⁰.

However, initially responsive to these drugs, tumor cells rapidly acquire resistance to a broad spectrum of platinum-salt based agents. Although the mechanisms underlying this phenomenon are not yet fully understood, some studies suggest a reduced cellular drug accumulation, an increase in the DNA damage repair system and the detoxification process, or a decrease in apoptosis¹⁹¹.

2.7.4.2 Agents targeting the cell architecture

Microtubules, a vital component of cellular architecture, are a protein complex whose importance in cell homeostasis is proportional to the diversity of mechanisms it mediates. As a crucial component of the cytoskeleton, microtubules are essential for cell growth and division, intracellular trafficking, and cell motility. Microtubules are multimeric proteins composed by the polymerization of monomeric subunits called tubulin. Five tubulin families coexist in the cell: α , β , γ , δ , ϵ , in which the three latter are present exclusively in the centrosomes and are not involved in microtubule dynamics. On the contrary, microtubule dynamics, meaning its polymerization and depolymerization, essential for cell homeostasis, is mediated by the heterodimers α and β tubulin previously assembled in the cytosol. Microtubule polymerization is realized by the addition of new $\alpha\beta$ tubulin subunits to the plus ends of the microtubule.

β -tubulin has a GTP molecule whose hydrolysis and energy release is essential for incorporating the heterodimer into the microtubules¹⁹².

To fight against cancer, clinicians have developed agents targeting the microtubules. Although all of them target the β tubulin, soluble or incorporated into the microtubules, according to their site of binding to this subunit, they change the tubulin conformation, but their impact on microtubule dynamics is different. Therefore, two classes of agents can be defined. One class prevents microtubule polymerization (vinca alkaloids, notably vinorelbine), whereas the second prevents their depolymerization (taxanes, including paclitaxel (taxol)). By impairing microtubule dynamics, both classes of agents result in cell division arrest and apoptosis. Given the role of microtubules in other cellular processes, agents targeting them can also disturb cell signaling and vesicular trafficking, which can have potent anti-tumor effects. Actually, they have been demonstrated to perturb the trafficking into the nucleus of several proteins involved in DNA damage repair, explaining why these agents have been shown to work in synergy with radiation therapy or with chemotherapies targeting the DNA. In addition, these agents can be used to prevent metastasis by impacting targeted cells' migration capacity. Although this molecule is more prone to enter and act in intense proliferative cells as tumor cells, it also can impact normal cells resulting in various toxicities like neurological and haematological ones. As against other treatments, cancer cells can acquire resistance to these agents. Although the mechanisms underlying this phenomenon are not yet fully understood, some studies suggest a reduced cellular drug accumulation, mutations on tubulin rendering agents inefficient, an increase in the detoxification process, or a decrease in apoptosis¹⁹³.

2.7.4.3 Gemcitabine

After being transported inside the cell by specific membrane transporters, gemcitabine, a deoxycytidine analog initially inactivated, is successively phosphorylated to become activated and exert its anti-tumor action. Its primary mechanism of action consists in being incorporated into DNA and structurally preventing DNA elongation. Also, that mechanism prevents gemcitabine from being removed to DNA. That process leads to a blocking in proliferation and triggers apoptosis. In addition to that anti-tumor mechanism, gemcitabine also directly inhibits the activity of two proteins, the ribonucleotide reductase (involved in nucleotides synthesis) and the deoxycytidylate deaminase, making gemcitabine more likely to be activated and incorporated into DNA. Given its different means of action, gemcitabine treatment is responsible for a lower toxicity profile than other chemotherapies and has been demonstrated to work in synergy with other chemotherapeutic drugs¹⁹⁴.

2.7.4.4 Other chemotherapies

By targeting topoisomerase II, a necessary enzyme for DNA replication, etoposide or doxorubicin, prevents cell replication and eventually leads to cell death¹⁹⁵.

Pemetrexed is an inhibitor of the folate metabolism, purine, and pyrimidine synthesis required for DNA replication and RNA synthesis. That drug, as the others, leads to cell cycle arrest and cell death¹⁹⁶. By preventing the availability of nucleotides required for DNA repair after platinum-salt based agents, pemetrexed is often given in combination, with a good overall survival in non-squamous NSCLC patients¹⁹⁷.

2.7.4.5 Emergence of resistance

Unfortunately, although efficient in the first instance, the efficacy of these different anti-cancer therapeutics is compromised by the lung tumor cell resistance to these treatments that can be natural or acquired following exposition to the drug. That resistance, whose subjacent mechanisms are not yet fully understood, explains why despite the emergence of new therapeutic drugs, the 5-year survival rate remains low¹⁹⁸. In addition to determining the mechanisms by which the tumor becomes resistant to the therapy, one of the big issues in lung cancer therapeutics is to find biomarkers that could predict the patient's response to the different therapeutics.

Unfortunately, like a wide variety of solid tumors, most lung cancer patients are resistant to chemotherapeutic drugs. Depending on the type of lung cancer, that resistance is naturally inherent or acquired following treatment administration. Usually, SCLC initially responds well to chemotherapies but rapidly develops resistance that eventually causes tumor recurrence and disease progression. NSCLC is intrinsically resistant to certain anti-cancer drugs¹⁹⁸. Actually, in one study of more than 3000 lung tumor samples from NSCLC patients, resistance to carboplatin was documented in 68% of samples, and cisplatin resistance was observed in 63% of the cases. Besides resistance to etoposide, gemcitabine, vinorelbine, and paclitaxel were respectively reported in 63, 72, 42, and 40% of lung tumors¹⁹⁹. Often, the resistance is a multi-resistance against drugs with a similar antineoplastic action mechanism.

That resistance is often responsible for an increase in the drug dosage that leads to an aggravation of the side effects without conferring better anti-tumor efficacy and outcome. That problem is aggravated by the fact that most lung cancer patients are later stage diagnosed, and therefore chemotherapy is the standard treatment. Actually, when the tumor has already spread, surgery and radiotherapy are no longer alternatives to cure cancer. That resistant mechanism explains why despite the development of new chemotherapeutic drugs, the 5-year survival rate remains less than 15% for NSCLC and 5% for SCLC. Although the molecular mechanisms conferring the resistance of lung tumor cells to chemotherapies have not been fully elucidated yet, some mechanisms have been proposed. Among them, there is the overexpression of transporters participating in the drug efflux outside the cell, the genetic and epigenetic heterogeneity of the tumor cells that could explain the difference in tumor cell response to chemotherapies, and the presence of a subpopulation of tumor cells called the cancer stem cells (CSC) that are naturally resistant to the chemotherapy drugs²⁰⁰.

2.7.5 Targeted therapy

In addition to surgery, radiation therapy, and chemotherapies, another type of drugs is used to fight cancer. That type of treatment is based on molecular alterations that occur during carcinogenesis. Actually, when a healthy cell becomes cancerous, some receptors or proteins kinase associated with pathways that favor its division and the cancer progression are mutated and hyperactivated. The principle of that class of drugs is to target that specific mutated proteins to limit their progression. This type of treatment relies on molecular alterations present in tumor cells but absent in normal cells, so it is called targeted therapy²⁰¹. This class of drugs, only used for NSCLC patients, not SCLC, is prescribed when the NSCLC has already spread to the lymph nodes or other organs or when chemotherapy has failed, and cancer has recurred²⁰². During the diagnosis of NSCLC, mutations that can explain the different common alterations are searched, and based on that, a treatment is recommended. It is established that up to 60% of NSCLC adenocarcinoma and 50-80% of NSCLC squamous cell carcinoma have a known oncogenic driver mutation. The different signaling pathways that can be mutated and hyperactivated are the RAS-RAF-MEK-ERK, MAPK, PI3K-AKT-mTOR, or JAK-STAT, which all of them lead to uncontrolled lung tumor cell growth and proliferation. Identification of the oncogenic driver mutation has allowed the successful design of therapeutics blocking the mutated component of the signaling pathway. In NSCLC adenocarcinoma, the main mutated oncogenic drivers are the epidermal growth factor receptor (EGFR), the Kirsten rat sarcoma viral oncogene homolog (KRAS), the anaplastic lymphoma kinase (ALK) and BRAF²⁰².

EGFR is a tyrosine kinase receptor present in the cell membrane whose function is to bind growth factors and trigger signaling pathways, including the RAS-RAF-MEK-ERK, the MAPK, or the PI3K-AKT-mTOR that ultimately induce cell division and proliferation. In up to 40-80% of NSCLC patients, EGFR expression is upregulated. Actually, the two main EGFR gene mutations, exon 19 deletions, and the L858R, are responsible for constitutive activation of the EGFR signaling without ligand binding, which leads to a hyperactivation of the receptor and, therefore, a tremendous proliferation²⁰³. To block its activity, drugs called tyrosine kinase inhibitors (TKI) like erlotinib or blocking antibody-like cetuximab have been developed with great results^{204,205}.

KRAS, a GTPase protein that transduces growth signals from multiple tyrosine kinases like EGFR and MET, is often mutated in NSCLC patients (mainly KRAS G12C mutation), around 30% of adenocarcinoma and 4% of SCLC. KRAS mutations that have been described lead to constitutive activation of the protein, hyperactivation of the signaling pathways, and ultimately increased growth and proliferation²⁰⁶. Blocking therapeutic agents of KRAS like sotorasib or salirasib were developed but unsuccessful. Other drug alternatives have been designed and focused on the downstream components of the different signaling pathways, the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR²⁰⁷.

In addition, ALK is an enzyme mutated in up to 3-7% of NSCLC patients. That mutation causes hyperactivation of the enzyme that leads cancer cells to grow and divide. To

tackle its activity, targeted therapy drugs have been developed like crizotinib²⁰⁸. Also, BRAF mutations (mainly BRAF V600E) leading to its hyperactivation and causing an increase in cell growth and division can be observed in almost 5% of NSCLC patients. To tackle that hyperactivation, blocking drugs have been designed as dabrafenib and trametinib²⁰⁹. Other oncogenic driver mutations, rarer, have also been described, like the mesenchymal-epithelial transition (MET) factor amplification, the ROS1 rearrangements, and the RET fusion, respectively accounting for 5% of lung adenocarcinoma, 2% of NSCLC and 2% of NSCLC adenocarcinoma²⁰².

Another therapeutic strategy consists in starving the tumor cells by preventing the development of new vessels bringing nutrients inside the tumor or angiogenesis. Some success has been observed for anti-angiogenic factors, as in colorectal or ovarian cancers. Although many factors regulate angiogenesis, two key growth factors are crucial: the vascular endothelial growth factor (VEGF) and the platelet-derived growth factor (PDGF)²¹⁰. In NSCLC, targeting angiogenesis seems promising as two pivotal phase III trials have demonstrated that using an anti-VEGF (bevacizumab) combined with a standard chemotherapy regimen is more efficient than chemotherapy alone²¹¹. However, serious side effects have been revealed after bevacizumab treatment, from thromboembolism, and hypertension to fatal pulmonary hemorrhagic events, limiting their clinical application.

It is now well established that lung cancer cells during the treatment can acquire resistance to TKI through the appearance of mutations to the EGFR or the ALK gene, rendering drugs entirely inefficient for fighting cancer. In response, derivatives of erlotinib and crizotinib have been developed²⁰¹. Although more specific in targeting cancer cells than chemotherapy or radiation therapy, targeted therapy can cause some side effects. Fatigue, constipation or diarrhea, nausea, and flu-like symptoms are the more common²¹².

2.7.6 Immunotherapy

Contrary to the other anti-tumor therapies that directly target the tumor cell, a new type of drug called immunotherapy, which consists in boosting the elimination of cancer cells by the immune system, has emerged with promising results. Although the immune system was initially viewed as a protective system against pathogens, it is also a major player in eliminating abnormal cells such as tumor cells. Its importance in controlling tumor progression has been demonstrated for numerous tumors and has led to the integration of the composition of the tumor immune system as a crucial prognosis factor for cancer patients (Immunoscore). Although the immune system is initially efficient in eliminating tumor cells and controlling their spread, as cancer grows and the tumor cells develop immunosuppressive means of action, the immune system reaches an equilibrium with the cancer cells and then ultimately fails to control the tumor growth. The immune system is composed of various cells and molecules interacting with each other and their target to eliminate pathogens or abnormal cells like cancer cells. That mechanism is a multistep process that begins with the release of tumor antigens after tumor cell death, which are captured, and processed on MHC

molecules by the DC that migrates to the tumor-draining lymph node. There, the DC primes the anti-tumor T-cells that migrate to the tumor to induce a robust anti-tumor immune response and remove the tumor cells, therefore reinforcing the virtuous circle. In addition, as evoked previously, lung cancers, like all solid tumors, are not strictly composed of tumor cells, immune system cells are also present inside it. Given its role of identifying and removing the foreign and abnormal (tumor cells) elements considered as danger signals from the healthy normal cells, the immune system is tightly regulated to avoid an hyperactivation that could lead to unwanted targeting and eventually healthy tissue damages. That immune cell activation is notably negatively regulated by molecules present on the cell membrane, called the immune checkpoints, whose the most studied are PD-1, PD-L1, and CTLA-4. Its inhibitory activity is mediated by the binding of the immune checkpoint with its ligands that can be present on other immune cells or tumor cells. Tumor cell, either by directly expressing immune checkpoints or their ligands (PD-L1/L2) or by indirectly forcing their expression on immune cells through the release of immunosuppressive molecules, is the leading actor that prevents the immune system from completely eradicating the tumor. Therefore, many emerging studies have focused on designing antibodies that block the interaction between immune checkpoints and their ligands (immune checkpoints blockade (ICB) antibodies), with promising results in many tumors²¹³.

2.7.6.1 PD-1

PD-1 is an inhibitory molecule present in T-activated cells, B cells, NK cells, and monocytes whose ligands are PD-L1 and PD-L2, expressed by tumor cells and antigen-presenting cells. Interaction between PD-1 and its ligands is responsible for inhibiting the T cells survival, proliferation, and activation. In addition, it also promotes the differentiation of CD4 T cells into an immunosuppressive Treg phenotype²¹⁴. The efficacy of monoclonal antibodies blocking PD-1 has been demonstrated in various tumors, notably lung cancer. Among them, the two most known are nivolumab and pembrolizumab.

Nivolumab, a human IgG4 monoclonal antibody to PD-1, has been demonstrated to be efficient in various clinical trials. In NSCLC patients, nivolumab was shown to be efficient alone in tumor elimination. It was demonstrated to be more efficient than docetaxel in the first instance or after chemotherapies treatment failure, improving both the median overall survival (OS) and the progression-free survival (PFS). However, no clinical benefits were observed when nivolumab was added to a platinum-salt based drug. The efficacy of the monoclonal antibody seems to be independent of PD-L1 expression^{215,216}.

Pembrolizumab is another monoclonal antibody targeting PD-1 used in clinical, and that has been demonstrated to be more efficient than docetaxel (OS and PFS increased). In contrast to nivolumab, the efficacy of pembrolizumab depends on PD-L1 expression. In 2015, the Food and drug administration (FDA) approved the drug to treat advanced-stage patients whose disease has progressed after chemotherapy and that tumor cells express PD-L1. In addition, NSCLC patients receiving pembrolizumab have been

demonstrated to have a better response, increased overall survival, and progression-free survival than patients treated with platinum-salt based chemotherapy. Besides, severe adverse events reported in patients treated with pembrolizumab were at least two times less than in patients treated with chemotherapy²¹⁷.

Although the anti-PD-1/PD-L1 has conferred great clinical benefits in numerous cancers, patients do not all benefit from that. Although some patients respond well to immunotherapies, a great number do not, therefore raising the importance of determining biomarkers in patients that could predict the response to the therapy. Expression of PD-L1 is a known marker of response to anti-PD-1/PD-L1 treatments²¹⁸.

2.7.6.2 PD-L1

Programmed cell death ligand 1 is an inhibitory molecule that is upregulated in cancer and whose function is to bind PD-1 and block T cell activation. To overcome that inactivation, clinicians, like for PD-1, has developed monoclonal antibody targeting that molecule, whose efficacy has already been demonstrated and has led to their approval by FDA. Among them, the most known are durvalumab and atezolizumab, two human IgG1 monoclonal antibodies.

FDA has approved the use of durvalumab for stage III NSCLC patients with unresectable tumors whose patients have responded well to chemotherapies and radiation therapy. The efficacy of atezolizumab has been reported in monotherapy for patients expressing PD-L1. In addition, it has been demonstrated to be more efficient than docetaxel in increasing the overall survival of NSCLC patients. Based on those clinical results, FDA has approved the use of atezolizumab for advanced-stage NSCLC patients whose disease has progressed after platinum-salt based chemotherapy²¹⁹.

2.7.6.3 CTLA-4

Cytotoxic T-lymphocyte-associated protein 4, or CTLA-4, is a molecule expressed on the surface of T cells that negatively regulates its activation. Actually, CTLA-4 acts as a competitive inhibitor, preventing the activation of T cells through the interaction of activating receptors present at their surface (CD28) with their ligands present on DC (CD80 and 86). By preventing this interaction, CTLA-4 downregulates the T helper cell activity and promotes the Treg phenotype, therefore disrupting the implementation of a robust anti-tumor immune response²²⁰. To overcome that inhibition, monoclonal antibodies blocking CTLA-4 have been designed, and some of them have already been approved by the FDA to fight cancer. Among them, ipilimumab, a humanized monoclonal antibody, has demonstrated significant results in numerous cancers, notably melanoma. Although a clinical trial is ongoing to determine its potential in lung cancer treatment, in a phase II clinical trial, in which chemotherapies (carboplatin/paclitaxel) in combination with the monoclonal antibody were compared with chemotherapies alone, the combination regimen was more efficient. Actually, the median progression-free survival was 5.68 months for the combination regimen compared with 4.63 for chemotherapies alone. Compared to placebo, ipilimumab seems to work only on squamous cell carcinoma, not others²²¹. The main adverse

events were immune-related, such as enterocolitis, hyperthyroidism, and hypophysitis. In practice, that drug is never given alone, it is always delivered in advanced-stage lung cancer patients in combination with chemotherapies or with another immune checkpoint blockage like an anti-PD-1/PD-L1 antibody.

2.7.6.4 Anti PD-1/CTLA-4 combination

As the CTLA-4 axis prevents the selection and expansion of different antigen-targeting anti-tumor T cells (repertoire) and the PD-1/PD-L1 axis prevents T cell activation of T cells already selected, the rationale of targeting both, through a combination treatment to synergize clinical benefits have been evaluated. Unfortunately, in a phase I/II study, the combination between anti-PD-1 (pembrolizumab) and an anti-CTLA-4 (ipilimumab) in NSCLC has demonstrated no better clinical response than pembrolizumab alone. However, toxicities results were significantly more critical than those caused by each monoclonal antibody treatment separated²²².

2.7.6.5 Other immunotherapies

In addition to the most known immune checkpoint blockade PD-1/PD-L1 and CTLA-4, other immunotherapies have been tested for their anti-tumor activity in NSCLC. Although clinical trials are ongoing, promising results have already been shown. The most known are the lymphocyte activation gene 3 (LAG-3), the Killer-cell immunoglobulin-like receptor (KIR), OX-40, and the glucocorticoid-induced TNFR-related gene (GITR). LAG-3 is an inhibitory immune checkpoint present in various immune cells, which is upregulated in a broad range of cancers, leading to T cell dysfunction²²³. Actually, in preclinical studies, the combinatory treatment of an anti-LAG-3 with an anti-PD-1 in NSCLC has demonstrated better results than the monotherapy anti-PD-1: increasing the anti-tumor response, tumor regression, and reducing the autoimmune related adverse events²²⁴. These promising results were reinforced by a phase II clinical trial in metastatic patients²²⁵. KIR is an inhibitory molecule at the NK cell membrane that prevents its anti-tumor cell lysis²²⁶. Given its targeting has demonstrated efficacy in preclinical studies, a phase I clinical trial including anti-KIR in NSCLC patients is ongoing^{227,228}. GITR is a costimulatory molecule that promotes the inhibitory activity of Treg cells²²⁹. Although the action of monoclonal antibody targeting GITR appeared to be inefficient in restoring an anti-tumor immunity, use in combination with radiation therapy and anti-PD-1 has demonstrated promising results^{230,231}. In the same way, a phase I clinical trial consisting in evaluating the efficacy and safety of that combination in metastatic lung carcinoma patients is ongoing²³². Finally, targeting other inhibitory molecules like the indoleamine 2.3 dioxygenase (IDO), the T cell immunoglobulin, and ITIM domain (TIGIT) appeared deceptive in NSCLC patients^{233,234}.

2.7.6.6 Limitations of anti-immune checkpoint blockade immunotherapies

Despite having demonstrated very interesting clinical results in various types of cancer, and in particular, in NSCLC patients, only a minority of patients respond in the long term to ICB immunotherapies and have clinical benefits. Although the percentage

differs between studies, it is estimated that about 20% of NSCLC patients respond to ICB²³⁵. That situation is due to the initial or acquired presence of intrinsic and extrinsic resistance mechanisms in the tumor cells. Among the inherent resistance mechanisms, tumor cells have been shown to reduce MHC-I expression, which limits their recognition by cytotoxic T cells²³⁶. In addition to this mechanism, the immune microenvironment, i.e., the density and phenotype of the immune cells present in the TME, greatly influence the effectiveness of ICB immunotherapies.

Thus, a characterization of the immune environment before treatment is necessary to predict the response of ICB and adapt the treatment. Based on the immune landscape within the TME, tumors can be classified into 3 categories: tumors without pre-existing anti-tumor immunity (immune desert), tumors with T cells accumulated at the periphery of the tumor (immune-excluded), and tumors with high infiltration of T cells that display an exhausted phenotype (inflamed). According to the profile of immune infiltration within the TME, another treatment has to be added to the ICB. Actually, for tumors weakly infiltrated by T cells (immune desert and immune-excluded), a therapy boosting both respectively induction of anti-tumor T cells and infiltration of T cells from the margin to the tumor site needs to be added to the ICB for boosting ICB efficiency. This additional treatment consists either in adding a cancer vaccine or using an agent inducing cell death in an immunogenic way, the immunogenic cell death (ICD)^{237,238}. Given the weak percentage of cancer patients and, notably, NSCLC patients responding to ICB, it is crucial to determine potential biomarkers of ICB responsiveness in cancer patients. Among the potential biomarkers, only PD-L1 has been both validated and routinely used to predict patient responsiveness to ICB. However, its accuracy must be improved for predicting patient responsiveness to ICB in all cases. It must be considered with other biomarkers like the mutational burden, specific genetic alterations, or the immune infiltration within the tumor site²³⁹.

Another limitation of the use of immunotherapies is based on its toxicity. Actually, given its action of overcoming the control mechanisms of the immune response, some immune-related adverse events like auto-immunity syndromes can occur following immune checkpoint blockade antibody administration. Although generally very moderate (mainly fatigue, nausea, loss of appetite, and diarrhea or constipation), in certain rare cases, more severe symptoms can appear and be life-threatening. Among autoimmune side effects, the most common are hypophysitis (<1%), hypothyroidism (1-7%), rash (4-13%), pneumonitis (3-5%), colitis (<1%), hepatitis (<1%) and renal insufficiency (<1%). Therefore, in addition to the treatment, clinicians can order corticosteroids that inhibit the immune system activation to limit those adverse events²⁴⁰. In addition, some patients can also have an allergic-like reaction following infusion of the monoclonal antibodies, whose symptoms can include fever, flushing of the face, and chills.

2.7.7 Other therapeutic strategies

2.7.7.1 Adoptive T cell therapy

In this therapy, the own T cells of the cancer patient are removed from their body during a process called plasmapheresis and genetically modified to express a specific receptor, the chimeric antigen receptor T (CAR T). That structure has the great advantage of combining the two signals necessary for the activation and the tumor cell lysis activity by T cells in one single design. Actually, CAR T allows both the recognition of the specific tumor antigen and the induction of the co-stimulation signal. Along with time, that structure has evolved, and new drug generations also include the expression of cytokines or activating ligands that amplifies the immune response induced. After being modified, T cells are amplified and reinjected into the patient to enhance the anti-tumor response. Although that new type of therapy has demonstrated remarkable results that have led to FDA approval for lymphoma and certain forms of leukemia, its impact on lung cancer has not been clearly shown yet. Actually, most clinical trials on CAR T cells in lung cancer are in phase I/II²⁴¹. Actually, the application of CAR T cells for solid tumors is still very challenging for two main reasons. The first one is the challenge for CAR T cells to access and penetrate inside the tumor microenvironment. The second one is the difficulty in coping with the immunosuppressive tumor microenvironment that inactivates the activity of CAR T cells, suggesting that the success of CAR T cells in treating solid tumors will come through a combination of CAR T cell with another treatment, like the immune checkpoint blockade immunotherapies.

2.7.7.2 Cancer vaccines

Another strategy that has been developed is to promote the implementation of a de novo anti-tumor immune response. That strategy is called cancer vaccines and comprises antigen-specific and cell-based vaccines, whose efficacy has already been assessed in lung cancer. Most of them have been developed for NSCLC, and a much smaller proportion for SCLC²⁴². Antigen-specific based vaccines consist in inducing specific anti-tumor immunity against specific TAA injected. Several have been developed, which the most known are the melanoma-associated antigen 3 (MAGE-3) and mucin-1 (MUC-1) associated vaccines. Although the MAGE-3 antigen is expressed in 35-42% of NSCLC, no clinical benefits were observed in the phase III trial for the MAGE-3 vaccine^{243,244}. Although MUC-1 is also overexpressed in lung tumors, its associated cancer vaccine has not demonstrated significant clinical improvement in phase III trial^{245,246}.

Conversely, cell-based vaccines aim to induce an immune response against a broad spectrum of tumor-associated antigens through the injection of irradiated tumor cells. Usually, those cells are also genetically modified to express cytokines, chemokines, or costimulatory molecules that favor the induction of a robust immune response. Although most of them succeed in inducing a robust anti-tumor immune response, they fail to improve clinical parameters for lung cancer patients. The most known are

Belagenpumatucel-L, composed of allogeneic NSCLC cell lines modified to express the TGF β antisense gene, and GVAX, in which the tumor cells secrete granulocyte-macrophage colony-stimulating factor (GM-CSF)^{247,248}.

2.7.7.3 Oncolytic viruses

In the fight against cancer, researchers have made continuous progress in designing therapeutics that are more efficient and targeted to avoid side effects. In this way, they have begun using oncolytic virus (OV) properties²⁴⁹. Actually, many natural viruses can specifically target cancer cells without infecting healthy ones to replicate and kill. Once cell death occurs, virions are released and amplify the phenomenon by targeting the neighboring tumor cells. The reasons for which oncolytic viruses target specifically tumor cells are multiple. Among them, there is notably the natural tropism of certain OV for tumor cells due to the overexpression of the virus surface receptor (coxsackievirus in breast cancer). In addition, other OV hijacks for its development the resistance to cell death and the unstoppable proliferation properties of tumor cells, promoting the preferential infection of tumor cells.. Although the type of cell death induced following OV infection differs according to the virus, most of them are able, in addition to their killing capacity, to induce a robust anti-tumor immunity through the release of immunogenic molecules. That type of cell death is called immunogenic cell death (ICD).

Moreover, some OV can also be genetically engineered to express cytokines or specific tumor antigens to boost the anti-tumor immune response induced. So far, only one OV has been approved by the FDA, the T-vec, which is an herpes simplex virus (HSV) virus genetically modified to express a pro-inflammatory cytokine, the GM-CSF, in addition to its killing activity in melanoma tumor cells. Concerning the use of OV in lung cancer, three preclinical studies have been realized, one on SCLC and the two others on NSCLC. The first one demonstrated that in SCLC, oncolytic myxoma virus (MYXV) selectively targets and kills tumor cells in a way that boosts the anti-tumor immune response²⁵⁰. In NSCLC context, coxsackievirus B3 has been shown to do the same²⁵¹. Also, HSV OV virus, the NV1066 has been shown to efficiently eliminate cancer cells²⁵².

2.7.8 Limitation of anti-tumor therapies

Although there are many innovative treatments available, such as immunotherapies, in addition to conventional treatments used in the clinic, such as radiation, chemotherapy, and targeted therapies, none of them can completely eliminate the tumor at this time. That mechanism can be explained by the fact that some tumor cells seem to adapt to these treatments and resist to death induced by these different treatments. This situation tends to increase the doses in patients without completely eliminating the tumor, which in turn leads to increased toxicity. Given the importance of this public health problem, many studies have attempted to determine the molecular mechanisms involved in tumor cells, which are responsible for their resistance to various anti-cancer treatments²⁵³. Among the explanatory processes, many teams have been interested in the autophagy phenomenon, a mechanism in all cells that acts

as a global response to various stresses affecting the cell to maintain its homeostasis and survival²⁵⁴. Interestingly, it has been shown that all treatments cause cellular stresses that promote the induction of protective autophagy for the tumor cell, allowing its survival. Thus, many clinical trials now combine conventional anti-cancer treatments with inhibitors of the autophagy mechanism to boost their efficacy, with interesting initial results^{255–257}.

3 Autophagy : a pivotal homeostatic mechanism for cancer

3.1 Different types of autophagy

Autophagy, whose name comes from the greek, auto, and phagein and signifies eating itself, is an intracellular catabolic mechanism discovered by Christian de Duve over 60 years ago. Although studies aiming to understand the mechanism of autophagy and its regulation have been initially performed in yeast, this biological process is ubiquitous, suggesting that it could play an essential role in cell physiology. So far, this mechanism has been described to be orchestrated by 32 autophagy-related genes (Atg) conserved among the eucaryotes.

Autophagy is a general term describing the cell's capacity to bind substrates and deliver them to the lysosome for degradation. Then, the resulting molecules are used by the cell to promote its survival and growth. Three types of autophagy can occur in eucaryote cells: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (hereafter called autophagy) (**Figure I4.**).

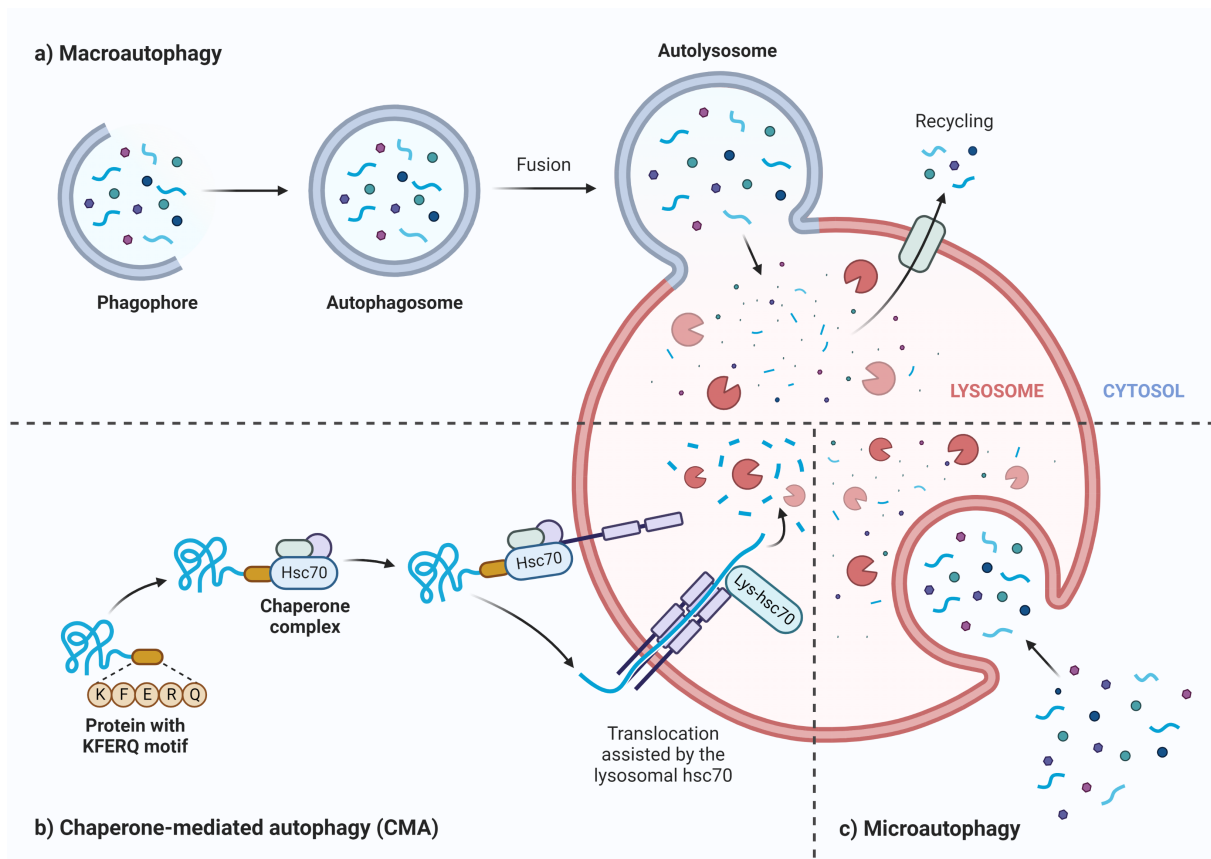


Figure 14. Three different types of autophagy pathways (Performed on biorender).

These different types differ depending on the machinery implemented in the cell, the cargo bound's nature, and the regulatory pathways involved²⁵⁸. The CMA is a process in which proteins containing a KFERQ amino acid sequence are recognized by the heat shock proteins acting as chaperone proteins. Then the resulting complex is translocated to the lysosome thanks to the interaction with the lysosomal-associated membrane protein (LAMP)-2A²⁵⁹. Microautophagy differs from CMA by the fact that the lysosome directly takes up the cytosolic substrates through the invagination of its membrane. Macroautophagy is a multistep process in which the cytosolic substrates are locked up in specific double membrane vacuoles called autophagosomes that eventually fuse with lysosomes²⁶⁰. During my thesis, I focused on macroautophagy, which I will refer in this manuscript as the global term autophagy.

3.2 Autophagy, a multistep process

Autophagy begins with the formation of a piece of membrane called isolation membrane or phagophore, whose origin is still controversial but seems to result from different organelles, notably the endoplasmic reticulum (ER) and/or the Golgi apparatus and/or endosomes. Then, after this initiation phase, the expansion phase occurs, wherein the phagophore expands to engulf intracellular cargo in a double membrane vacuole called the autophagosome. Then, the autophagosome fuse with

the lysosome, wherein proteases degrade substrates, and the by-products of degradation are reused by the cell for homeostasis²⁶¹ (**Figure I5.**).

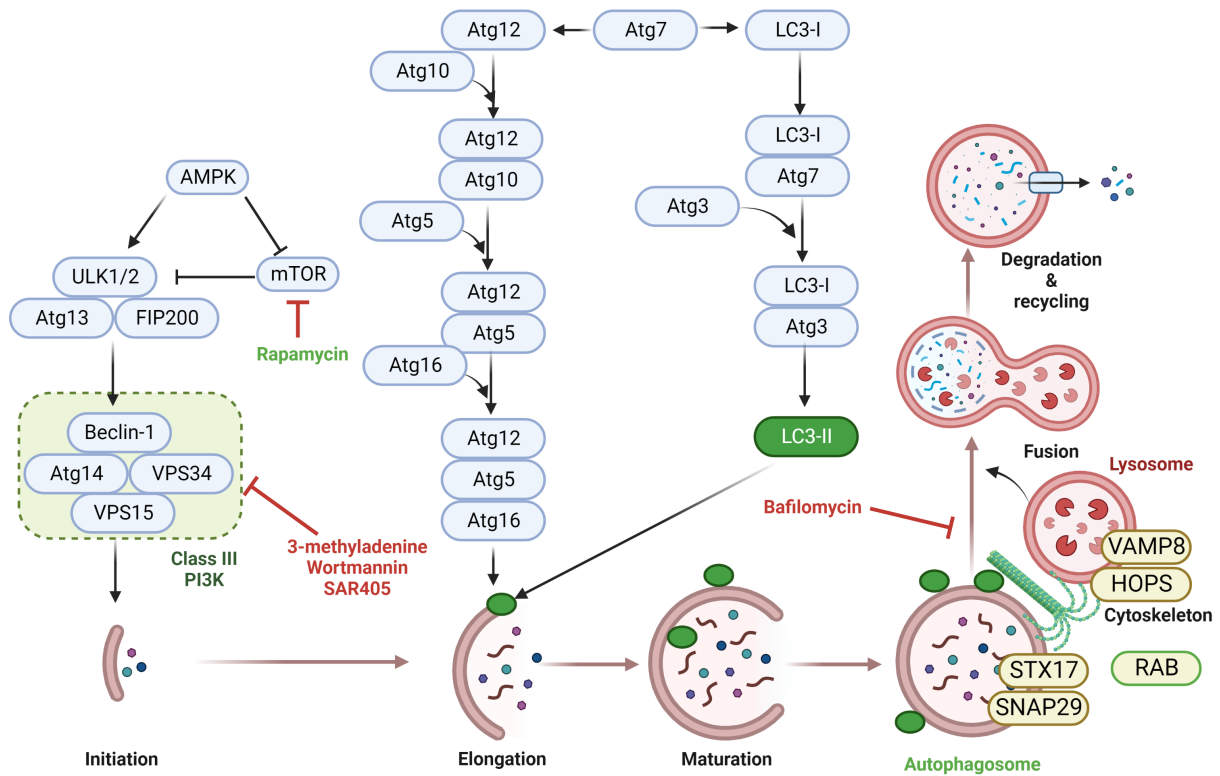


Figure I5. Schematic view of the macroautophagy process, the different genes involved as well as the different autophagy modulators used in that project (Performed on biorender).

In mammals, autophagy is initiated in a specific area of the ER called the omegasome, wherein the phagophore emerge. Then the piece of membrane keeps growing by the membrane supply coming from other organelles like the trans-Golgi, the late endosomes, and the mitochondria or from the plasma membrane, which seems to derive from the specific area of the organelles with the ER (ER-organelle contact site). That emergence is mediated by a protein complex called Unc-51-like kinase 1 (ULK1) and composed of ULK1, the RB1-inducible coiled-coil protein 1 (FIP200), ATG13, and ATG101. Upon activated, that complex stimulates and recruits to the omegasome, another proteic complex, the class III phosphatidylinositol 3-kinase (PIK3C3), composed of the BECLIN-1, the activating molecule in Beclin-1-regulated autophagy protein 1 (AMBRA1), ATG14, the vacuolar protein sorting (VPS)15 and VPS34. Upon activated, that complex leads to the production of a pool of phosphatidylinositol 3-phosphate (PtdIns3P) that recruits the WD repeat domain phosphoinositide-interacting (WIPI) proteins present on ATG9 positive vesicles and the zinc-finger FYVE domain-containing protein 1, as well as the recruiting the ATG5-ATG12-ATG16L1 complex, both favoring the phagophore growth. That complex setup is allowed by the activation of ATG12 by ATG7, its conjugation to ATG5 by ATG10, and the recruitment of ATG16L located at the membrane of the omegasome through its interaction with WIPI2. Upon gathering, that complex activates the processing of a key protein of phagophore

elongation and autophagosome formation, ATG8 or microtubule-associated protein light chain 3 (LC3), its homolog in mammals. That latter protein is present in the cytosol under an inactive form, and to become active, the action of other proteins is needed. LC3 inactive form (LC3-I) is cleaved at their C-termini by ATG4, leading to the exposition of a glycine residue required for LC3 conjugation with phosphatidylethanolamine (PE), a necessary step to become activated (LC3-II). That step is performed by ATG3, whose activation depends on the ATG5-ATG12-ATG16L1 complex. The addition of the PE group allowed LC3 to be anchored to the autophagosome membranes, whose presence is critical for cargo selection and degradation. In addition to its PE group, LC3 has an LC3-interacting region (LIR) domain that allows it to recruit autophagic receptors containing those domains. These latter proteins can then recognize a broad range of cargoes possessing ubiquitin groups, binding and confining them into autophagosomes for degradation and recycling. The nature of the cargo bound can be non-selective or very selective and depends on the stress the cell has to cope with. After the formation of the autophagosome, the ATG proteins bound to the outer membrane are gradually removed by ATG4. The only protein that remains attached to the autophagosome membranes (both outer and inner) is LC3. That property makes it being the main and more reliable autophagy marker (**Figure 15.**).

During the maturation step of autophagy, two types of machinery are recruited to allow the autophagosome fusion with the lysosome. The first one is the kinesin network of the cytoskeleton, which is necessary for bringing together autophagosome and lysosome. The second complex mobilized is the machinery required for their fusion, involving molecules present on autophagosomes, the syntaxin 17 (STX17) and the synaptosomal-associated protein 29 (SNAP29) and on lysosome, the vesicle-associated membrane protein 8 (VAMP8) and the homotypic fusion and protein sorting (HOPS). That step is managed by LC3, which realizes the link between the autophagosome to kinesins through the action of adaptor proteins like FYVE and coiled-coil domain-containing protein 1 (FYCO1). Besides, it also recruits thanks to pleckstrin homology domain-containing family M member 1 (PLEKHM1), the HOPS complex. Furthermore, RAB proteins, notably RAB7, are also involved in autophagosome fusion with the lysosome to give birth to autolysosome. Lysosomal hydrolases then degrade the cargo, and the products derived from that degradation are released in the cytosol by lysosomal permeases and reused by the cell. To become activated, the lysosomal proteases must be in an acidic environment, whereas the autophagosome lumen is not an acidic milieu. It has been suggested that before fusion with the lysosome, autophagosome fuse with early and late endosome to reduce the pH inside it and facilitate lysosomal hydrolases action after autophagosome fusion with the lysosome. Moreover, LAMP-1 and LAMP-2 at the lysosome membrane are critical for autophagy, as a deletion impairs autolysosome maturation²⁶¹.

Initially thought to be essential for autophagy, an ATG5/ATG7 independent autophagy pathway has also been observed in various healthy and malignant cell types. Although it results in autophagosome and autolysosome formation, the mechanism is quite

different from the ATG5/ATG7 dependent pathway. In this alternative pathway, LC3 processing does not occur, and autophagosome is formed by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes in a RAB9-dependent manner. Although the alternative pathway has not been fully elucidated, it seems it can be activated by starvation, and P53 is a central regulator²⁶².

3.3 Autophagy, a mechanism that can be highly selective

Initially viewed as a non-selective degradation mechanism, engulfing varied contents to supply macromolecules to starving cells, autophagy has been described to target specific proteins or organelles depending on the stress whose cell has to cope with. Since the discovery of autophagic receptors linking the LC3-anchored autophagosome to the cargo, autophagy has been shown to target macromolecules like glycogen, lipids, or protein aggregates through glycophagy, lipophagy, and aggrephagy or organelles composing the cell for the degradation and recycling. Each organelle can be degraded through an autophagy-specific mechanism, like the mitochondria (through mitophagy), the ER (through ER-phagy), the nucleus (nucleophagy), the peroxisome (pexophagy), and even the lysosome (lysophagy). Besides, exogenous elements like pathogens can be recognized and degraded through a mechanism called xenophagy. Depending on the stress whose the cell has to cope with, like starvation or infection by pathogens, the signaling induced will be different, as well as the machinery recruited. As a result, different substrates will be recognized by the autophagic receptors and degraded by autophagy. It is now well established that, on the one hand, several autophagic receptors can realize the same type of specific autophagy (like xenophagy))²⁶³.

So far, seven autophagic receptors have been discovered: sequestosome 1 (SQSTM1 or P62), optineurin (OPTN), ubiquilin 2 (UBQLN2), Neighbor of BRCA1 (NBR1), autophagy-linked FYVE (ALFY), nuclear dot protein 52 (NDP52), and Huntingtin (HTT). The most described autophagic receptors, P62, OPTN, NBR1, and NDP52, harbor both the Ubiquilin-binding domain and a LIR domain, allowing them to recognize cargo polyubiquitinated and engulf them into autophagosomes. The addition of polyubiquitin is a critical signal for the destiny of the substrate. Depending on the organization of the polyubiquitin chain, the substrate destiny will be different. Actually, the polyubiquitin chain resulting from the addition of newly ubiquitin residues on lysine 48 or 63 will lead, respectively, to the substrate for degradation by the proteasome or by the lysosome through autophagy. Autophagic receptors are tightly regulated by phosphorylation, ubiquitination, and oligomerization, impacting the type of specific autophagy induced. To sum up, depending on the nature of the stress, the autophagic receptor recruited and the post-translation modification within it can be different, impacting the type of specific autophagy induced by the autophagic receptor^{263,264}.

3.3.1 P62

P62 is the best-characterized autophagy receptor that can mediate different types of specific autophagy, like aggrephagy and xenophagy. It can also regulate autophagy itself. Besides being an autophagy receptor, it is also a hub of diverse signaling pathways such as amino-acid sensing, oxidative stress and the DNA damage response. In addition to have a TIR and Ub-binding domains, which are necessary to realize the link between the autophagosome and the polyubiquitinated substrate, P62 also possesses other domains that are targeted by various kinases (like the casein kinase (CK) 2 and the Tank-binding kinase (TBK) 1), both impacting its function and regulation. For example, phosphorylation of P62 at S403 by CK2 and TANK-binding kinase 1 (TBK1) in the Ub-binding domain increases the affinity of the autophagic receptor to its polyubiquitinated substrate²⁶⁵. In contrast, phosphorylation at S351 in the Kelch-like ECH-associated protein 1 (KEAP1) interacting region motif by mTOR disturbs the KEAP1-Nuclear factor erythroid 2-related factor 2 (NRF2) interaction. As a result, KEAP1 is then degraded by autophagy, and NRF2 is translocated to the nucleus, wherein it induces the expression of many genes involved in the antioxidant response. Furthermore, P62 can also be ubiquitinated at K7 in the Phox and Bem1 (PB1) domain, eliciting the regulatory mechanism of redox homeostasis. In a nutrient-rich environment, P62 can also be phosphorylated at T269 and S272 by p38D-Mitogen-activated protein kinase kinase kinase 3 (MEKK3), facilitating P62-TRAF6-mammalian target of rapamycin complex 1 (mTORC1) complex formation that eventually leads to mTORC1 activation and cell growth regulation. Like the other autophagic receptors, depending on the stress whose cell has to cope with, P62 is differently post-translated modified, and the response induced is also impacted. P62 also has a TRAF6 domain that allows it to interact with the NF- κ B pathway-dependent component TRAF6²⁶⁵.

3.4 Autophagy, a tightly regulated process

3.4.1 Modulation of the autophagy process by cellular stresses

While autophagy is present in every cell at the basal level, various stresses can induce it in cells to maintain homeostasis. Although initially described as a mechanism for the cell to cope with starvation, autophagy can be generated in the cell upon different stresses, notably hypoxia, oxidative stress, ER stress, and pathogen infection²⁶⁶.

3.4.1.1 Nutrient carency

During nutrient starvation, the cell can sense the poorly nutrient environment and, as a result, promotes the increase of catabolic degradation through autophagy induction that eventually allows the cell to survive. That data integration is mediated by two signaling cascades exerting inhibitory activity on autophagy: the mTORC1 and the RAS-cAMP-PKA pathways. mTOR complex comprising mTORC1 is a hub, integrating data about nutrients and growth factor availability and, in response, facilitating cell

growth, cell cycle progression, and protein synthesis²⁶⁷. In a basal state, these signaling pathways are activated and inhibit autophagy. However, when the nutrient quantity decreases in the environment, these pathways are inhibited and therefore, autophagy is induced. In basal state, mTORC1 exerts its inhibitory activity on the initiation complex of autophagy, the ULK1 complex²⁶⁷. However, in a poor nutrient environment, mTORC1 gets inactivated by the action of Ras-related small GTPases that facilitate the interaction of the complex with an activator, the Ras homolog enriched in the brain (RHEB)²⁶⁸.

Another signaling pathway is involved in nutrient sensing, the RAS/cAMP-dependent protein kinase A (PKA)²⁶⁹. In a nutrient-rich environment, small GTPases RAS1 and RAS2 are activated and favor cAMP production by adenylyl cyclase. In return, cAMP binds a regulatory PKA subunit, Bcy1, and releases it from PKA, leading to its activation. Although its impact on autophagy in mammalian cells has not been clearly elucidated, its inhibitory action on autophagy in *S cerevisiae* was demonstrated. In yeast, PKA inhibits autophagy through inhibitory phosphorylation of ATG1 protein, involved in the autophagy initiation²⁶⁹.

3.4.1.2 Growth factor availability

Another determinant factor for autophagy is the presence of growth factors (GF) in the extracellular milieu that initiates signaling pathways leading to autophagy inhibition²⁶¹. Although the pathways involved differ from that induced upon nutrient carency, they both converge on the major repressor of autophagy, the mTORC1 complex. In the presence of insulin and insulin-like growth factors, their binding on their specific receptors at the plasma membrane leads to the autophosphorylation of the receptor on their tyrosine residues. After that biochemical change, the insulin receptor substrate (IRS)1 and IRS2 are recruited, phosphorylated, and activated, favoring the binding of adaptor proteins like elements of the class I PtdIns3K that lead to the generation of PIP3^{270,271}. As a result, PKB/AKT are recruited and activated, and they phosphorylate the tuberous sclerosis complex (TSC) 2 protein. As a consequence, TSC2 is no longer able to interact with TSC1 and form the inhibitory complex that represses RHEB-promoting activity on the mTORC1 complex²⁷². On the contrary, in the absence of growth factors, that signaling pathway is not activated, RHEB activity is inhibited as well as the mTORC1, and autophagy is induced²⁷³.

Another pathway linking the growth factor and autophagy was elucidated and involves RAS that activates the class I PtdIns3K and, as a result, leads to the recruitment of proteins as previously described, that eventually activates mTORC1 and inhibits autophagy²⁷⁴. RAS was also involved in another pathway that links amino acid availability and autophagy. In an amino acid-rich environment, its binding on its receptor inhibits RAS and its downstream effector, RAF-1 activities. Consequently, the latter can no longer activate the MAPK/ERK1/2 (MEK1/2) and ERK1/2 proteins that act as a repressor of autophagy²⁷⁵. Therefore in the absence of amino acids, autophagy is induced.

3.4.1.3 Cellular energy

Cells to grow, synthesize macromolecules, and realize the activities necessary for ensuring their function need cellular energy, which is mainly ensured by a nutrient-rich energy molecule hydrolysis called Adenosine triphosphate (ATP). Cell metabolism can be modified depending on the cell needs and the compounds available in the medium. Actually, in a rich environment context and when ATP level is sufficient, cells will be more likely to realize anabolism, meaning ATP hydrolysis in its hydrolyzed forms, adenosine diphosphate (ADP), and monophosphate (AMP) to produce macromolecules. These biological reactions lead to energy release that the cell will then use to synthesize macromolecules necessary for its function and growth. However, in a poor environment and low ATP level, the cell will tend to perform catabolism and autophagy to produce ATP from macromolecule degradation. The energy sensing and the choice of the type of metabolism performed are realized by the 5'-AMP-activated protein kinase (AMPK) and mTOR complex. A reduced ATP level in the cell leads to the activation of the liver kinase B1 (LKB1), that in turn phosphorylates and activates the TSC1/2 complex that inhibits mTOR and induces autophagy²⁷². Besides its indirect effect on autophagy induction, the AMPK pathway has been shown to directly target autophagy induction through the phosphorylation and activation of ULK1²⁷⁶. In addition to favoring ATP production through autophagy and catabolism induction, the LKB1-AMPK pathway also triggers cell cycle arrest by activating P27, a cyclin-dependent kinase inhibitor²⁷⁷.

3.4.1.4 Hypoxia

Physiologically cells to function normally require dioxygen, whose level can variate depending on the tissue from 3 to 7.4%, with an average of 5%. Below this level, the oxygen condition is called hypoxia and is a characteristic of most tumor cells²⁷⁸. As dioxygen is required for the cell to produce energy efficiently in mitochondria, hypoxia is considered as a danger signal whose cell must cope with²⁷⁹. Cell response to hypoxia through autophagy induction is mediated through two pathways. The first one is as the oxygen level is decreased, the ATP production is reduced, and as a consequence, the LKB1-AMPK pathway is induced²⁸⁰. The second pathway is mediated by the hypoxia-inducible factor-1 (HIF-1) response. HIF-1 is a transcription factor whose expression is induced under hypoxia²⁸¹. HIF-1 increases the expression of hundreds of genes, impacting biological processes like angiogenesis, mitochondria biogenesis, and autophagy. HIF-1 promotes the expression of BCL-2 interacting protein 3 (BNIP3) that disturbs the BECLIN-1-BCL-2 complex that is present in the cytosol at the basal state and inhibits autophagy initiation. BNIP3 competes with BECLIN-1 for binding BCL-2, leading to BECLIN-1 release from BCL-2 inhibitory action and autophagy induction²⁸¹. Another transcription factor, the E2F, has been shown to induce BNIP3 expression during hypoxia. That transcription factor is under the negative control of the Rb tumor suppressor protein²⁸². After hypoxia, autophagy induction, and more specifically mitophagy, is a cell response to prevent ROS formation that could eventually lead to DNA damage and cell death^{283,284}.

3.4.1.5 Oxidative stress

Cells can also have to cope with intracellular stresses like oxidative stress, meaning the ROS formation in the mitochondria that causes DNA damage. That generation of ROS leads to autophagy induction. That mechanism is mainly observed in cancer cells rather than nonmalignant cells and can be explained by the capacity of normal cells to cope with that oxidative stress through the increase of the antioxidant system expression, notably the catalase, the superoxide dismutase, and the redox system²⁸⁵. The ROS capacity to induce autophagy, notably mitophagy, is mediated through the oxidation by ROS of the cys81 residue on ATG4, leading to the inactivation of its protease activity and the promotion of the LC3 conjugation²⁸⁶. Besides, ROS has also been observed to activate poly ADP-ribose polymerase-1 (PARP-1) that promotes autophagy through the activation of the LKB1-AMPK pathway. PARP-1 is also activated upon DNA damage²⁸⁷.

3.4.1.6 Endoplasmic reticulum stress

The main function of ER is to perform the folding of newly synthesized proteins and facilitate their migration to their action site. In certain situations, too many unfolded proteins accumulate within the ER, exceeding its folding capacity. That mechanism is called ER stress and is triggered by different stimuli. Among them, there are glucose deprivation, hypoxia, and oxidative stress²⁸⁸. The glucose deprivation and hypoxia lead to lower energy production by the cell, and a lower quantity of ATP attributed to the chaperone proteins, which are essential for the good folding of proteins²⁸⁹. Oxidative stress through decreasing disulfide bond formation is also responsible for ER stress²⁸⁵. Besides ER being a Ca²⁺ reservoir organelle, an efflux of the calcium from the ER to the cytosol also results in ER stress²⁹⁰. The cell induces autophagy through the unfolded protein response (UPR) to cope with that detrimental stress²⁹¹. Different pathways have been demonstrated to link ER stress and autophagy, and the pathway involved depends on the organism studied and the ER stimuli. Two kinases can sense the accumulation of misfolded cytosolic proteins and trigger distinct signaling pathways that activate specific transcription factors that induce LC3 conjugation and autophagy. Those kinases are the inositol-requiring enzyme 1 (IRE1), which activates the JNK, and the protein kinase RNA-like ER kinase (PERK), which activates the eukaryotic initiation factor 2 a (EIF2a)^{292,293}. Besides, during ER stress, Ca²⁺ efflux from the ER to the cytosol occurs, leading to the activation of the calmodulin-dependent kinase kinase- β (CaMKK β) that stimulates the AMPK pathway and autophagy induction²⁹⁰. The increase of Ca²⁺ cytosolic level induces the phosphorylation and activation of protein kinase C theta (PKC theta) that promotes LC3 conjugation and autophagy²⁹⁴. Another pathway was observed and involved the activity of the calmodulin-regulated serine/threonine kinase death-associated protein kinase (DAPk). After ER stress sensing, DAPk gets activated and, in turn, phosphorylates BECLIN-1, which leads to its dissociation from BCL-2 and autophagy induction²⁹⁵. The autophagy induction following ER stress is a means for the cell to compensate for the accumulation of

proteins through the induction of a destructive catabolism mechanism and, at the end, promote cell survival²⁹⁶.

3.4.1.7 Pathogen infection

Another autophagy-inducing mechanism is pathogen infection. The most described pathogens for autophagy induction are bacteria and viruses²⁹⁷. Autophagy induction following pathogen infection can be realized directly *via* the recognition of the pathogen by specific receptors or indirectly by inducing stresses in the infected cell that lead to autophagy induction (ER stress and oxidative stress).

3.4.1.7.1 Direct induction by immune receptors

After virus entry, delivery of its viral content inside the cell is subject to autophagy induction. That mechanism is mainly mediated by PRR and, more specifically, TLR^{298,299}. Pathways involved in TLR stimulation-induced autophagy seem to depend on the TLR and/or on the cell model. Actually, it was observed in lung cancer cells that TLR3 and TLR4 stimulation induced autophagy through a mechanism involving TRIF and TRAF6³⁰⁰. In murine macrophages, while all TLR family has been shown to induce autophagy, TLR7 is the biggest autophagy inducer and induced autophagy through MyD88 activation²⁹⁹. Another study precised that TLR7 stimulation-induced autophagy resulted from p38 or MEK/ERK ½ pathways activation and ROS production increase. Although the underlying mechanisms by which TLR activation leads to autophagy induction are not well understood, several studies have demonstrated an important impact of this process on anti-microbial response or in tumor progression. For instance, Delgado et al. observed that TLR7 stimulation-induced autophagy promoted the degradation of *Mycobacterium tuberculosis* in macrophages. In lung tumor, activation of autophagy by TLR3 and 4 favors tumor migration and metastasis.

RLR has also been demonstrated to induce autophagy through BECLIN-1 activation, as in the Sendai virus infection context upon dsRNA recognition³⁰¹. Protein kinase R (PKR), which also recognizes dsRNA, induces autophagy through the phosphorylation and activation of the eukaryotic translation initiation factor (eIF2)³⁰². Autophagy is also induced through BECLIN-1 activation by the cytosolic DNA sensor cGAS upon HSV-1 infection³⁰³. During cell infection by the measles virus (MeV), a membrane receptor, CD46, has been shown to promote autophagy after virus binding. Upon fixation of the virus, BECLIN-1 is recruited and activated through a scaffold protein named Golgi-associated coiled-coil motif-containing (GOPC), leading to autophagy induction³⁰⁴. Besides, autophagy induction upon MeV infection can also be recognized through an autophagy-associated protein called immunity-associated GTPase family M (IRGM)³⁰⁵. The fusion of the virus to the plasma membrane has also been shown to induce autophagy, as in HIV infection. Actually, the fusogenic activity of its two envelope glycoproteins, (GP)120 and GP41, has been shown to induce autophagy in uninfected CD4 T cells^{306,307}.

3.4.1.7.2 Autophagy induction as an indirect by-product of cellular stresses caused by infection

Meanwhile autophagy is induced by pathogen recognition, the resulting stresses initiated within the cell by the infection also stimulate autophagy induction. ER stress and oxidative stress, two known factors to induce autophagy, have been shown to be a by-product of viral infection. For example, the chikungunya virus (CHIKV) has been shown to induce both stresses that lead to autophagy initiation in the infected mouse embryonic fibroblasts³⁰⁸.

During pathogen infection, direct and indirect induction occur and work together to elicit a strong autophagy response. Besides, recognition of the virus by the cell can promote the secretion of pro-inflammatory cytokines, which in turn induce autophagy³⁰⁹. In addition, a great number of viruses have been described to impact mTOR pathway, which in turn lead to autophagy modulation in infected cells³¹⁰.

3.4.2 Transcriptional regulation of the autophagy process

In addition to being induced by protein-protein interactions, autophagy can also be stimulated through an increase in gene expression of proteins involved in the autophagic cascade. That mechanism was firstly observed in yeast, where nitrogen starvation resulted in the upregulation of Atg8p expression, the homologous of mammalian lc3³¹¹. Since that discovery, many transcription factors upregulating autophagy genes that effectively lead to autophagy have been discovered.

3.4.2.1 TFEB pathway :

Transcription factor EB (TFEB) is a transcription factor (TF) that belongs to the family of basic helix-loop-helix leucine zipper (bHLH-Zip) TF like MYC. It comprises two domains: a DNA-binding domain and an HLH plus a leucine zipper domain required for its dimerization and activity³¹². Its binding to DNA is performed by recognizing a specific sequence (CACGTG) plus specific motifs that flank this sequence, GTCACGTGAC (CLEAR). Promoters of many autophagy and lysosomal genes have these particular sequences. As a result, TFEB induces the expression of various autophagy genes involved in different steps of the autophagy mechanism: initiation (Beclin-1, wipi, Atg9b), elongation (Map1lc3b and Atg5), cargo capture (Sqstm1 coding for P62) and fusion with the lysosome (Uvrag and Rab7)³¹³. TFEB activity is mainly controlled by its subcellular localization: inactive in the cytosol versus effective in the nucleus. That localization is regulated through phosphorylation and dephosphorylation by kinases and phosphatases whose activities are controlled by the environmental medium³¹². Among the kinases described to target TFEB, mTOR is the main one. Actually, upon nutrient starvation, mTOR is inactivated, TFEB is dephosphorylated and translocated to the nucleus wherein it induces the expression of various autophagy genes³¹⁴. Besides, mTOR can also inhibit TFEB transcriptional activity by activating the zinc finger transcription factor harboring a SCAN domain (ZKSCAN3) that represses the expression of the autophagy genes when nutrients are available³¹⁵. Also, under the same condition, TFEB nuclear localization is inhibited by ERK2, which gets

activated³¹⁶. TFEB can be activated upon ER stress³¹⁷. Efflux of Ca²⁺ from the ER to the cytosol favors the activation of calcium and calmodulin-dependent serine/threonine phosphatase that facilitates TFEB translocation into the nucleus³¹⁸. Moreover, AKT and protein kinase C β (PKC β) also impact TFEB activity by decreasing its stability^{319,320}. TFEB also gets activated upon oxidative stress by activating the protein phosphatase 2A (PP2A)³²¹. To sum up, TFEB activation is a general response of the cell to cope with the stresses it faces by increasing autophagy genes expression and autophagy.

3.4.2.2 FOXO pathway

The class O of forkhead box transcription factors (FOXO) comprises the FOXO1 and 3 that have been demonstrated to modulate the expression of autophagy genes. The activity of these two transcription factors is inhibited by AKT, a kinase that gets activated in response to growth factors and insulin stimulation. Upon nutrient starvation, AKT gets inactivated. As a result, FOXO1 and 3 are not phosphorylated and are no longer blocked into the cytosol. This mechanism leads to the translocation of FOXO to the nucleus, facilitating the expression of various autophagy genes³²². Besides, FOXO1 has also been shown to induce autophagy independently of its transcriptional activity. Upon nutrient starvation or oxidative stress, FOXO1 gets acetylated, promoting its interaction with Atg7 to induce autophagy³²³.

3.4.2.3 P53

P53, the main tumor suppressor, has also been demonstrated to promote autophagy gene expression upon DNA damage. In addition to favoring autophagy by activating the AMPK pathway through the expression of Sestrin 1 and 2, it induces the transcription of the damaged-regulated-modulator protein (DRAM) that also promotes autophagy^{324–326}. Besides, it also influences the transcription of various genes involved in the autophagy machinery (notably Ulk1/2) or its regulation (Lkb1)^{327,328}. P53 has also been demonstrated to control the expression or activity of transcription factors involved in autophagy genes expression, such as FOXO3a or TFEB^{329,330}.

3.4.2.4 E2F1/NF- κ B pathway

E2F1 and NF- κ B are two transcription factors that compete for binding BNIP3 promoter. As evoked previously, BNIP3 is a protein that competes with BECLIN-1 for BCL-2 binding. NF- κ B is a repressor of BNIP3 expression, whereas E2F1 is an activator^{282,331}. In physiological conditions, NF- κ B binds the BNIP3 promoter and represses its expression³³². However, upon hypoxia, E2F1 takes the lead on NF- κ B for binding the promoter. As a result, during hypoxia, BNIP3 expression is increased, the BECLIN-1-BCL-2 complex is disturbed, BECLIN-1 is released, and autophagy is induced²⁸¹. Besides, E2F1 has also been shown to favor the expression of other autophagy genes like Ulk1 or Atg5³³³.

3.4.2.5 Epigenetic regulation of autophagy

In addition to both the protein-protein interaction and the regulation of expression of the autophagy genes by specific transcription factors, epigenetic modifications are another way by which autophagy is regulated within the cell. Epigenetic modifications consist of biochemical modifications (methylation, acetylation, deacetylation) on histones, basal protein components around which the DNA is wound. Depending on the modification occurring on histone, the accessibility for the transcription factors or the regulators to the promoter and enhancer is modified as well as the gene expression. Many studies have demonstrated that upon cellular stresses like nutrient starvation, epigenetic modifications occur and affect the expression of autophagy genes and the autophagy process³³⁴.

3.4.2.6 Long non-coding RNA-induced autophagy modulation

Finally, another emerging mechanism by which autophagy is modulated is through the action of non-coding RNA (ncRNA), which are RNA sequences that are not translated into protein³³⁵. However, these ncRNAs have a crucial role in regulating gene expression. Depending on their structure, they can be classified as linear, like the microRNA (miRNA) or the long non-coding RNA (lncRNA), or as circular RNA (circRNA). miRNA are single-stranded RNA sequences of 18-25 nucleotides. Their main role is to bind specific coding RNA sequences thanks to their complementary sequence, to recruit the argonaute protein (AGO), and eventually lead to the degradation of the RNA targeted or to the inhibition of its translation into protein. A miRNA is not specific to a unique sequence, it can inhibit the expression of hundreds. miRNA are RNA sequences coded in the genome, and it is estimated that 1 to 5% of the human genome is attributed to miRNA expression. Thanks to their function, miRNA actions can affect many biological processes, notably the autophagy mechanism. For example, miR-93 targets ULK1 under hypoxia, leading to autophagy inhibition³³⁶.

As the miRNA, the lncRNA is single-stranded RNA that is not translated into protein and whose function is to regulate the expression of various genes. However, the sequence length and the means of action are different. lncRNA are longer than miRNA, usually above 200 nucleotides. Their means of action also differ from the miRNA. Contrary to miRNA, which only targets coding RNA, lncRNA, through its structure, can bind both DNA, RNA, and protein. As a result, lncRNA can affect the gene expression at each step: preventing the transcription, inhibiting the RNA translation, and modulating the protein activity³³⁷. Both miRNA and lncRNA have been shown to modulate the expression of the autophagy genes, both positively and negatively, and as a result, to impact the autophagy mechanism^{338,339}. For instance, under energy stress, lncRNA NBR2 has been shown to interact with AMPK and, as a result, lead to autophagy induction³⁴⁰.

3.5 Functions of autophagy

3.5.1 Canonical functions of autophagy

As previously mentioned, autophagy is a mechanism induced in cells following various stresses to manage them and maintain cell survival. The main function of autophagy is to degrade non-essential, aged, or damaged proteins and organelles in a more or less selective way. After that degradation step, the resulting molecules produced are reused by the cell, which leads autophagy being a crucial means for cells to meet their metabolic needs, especially during nutrient deficiency, hypoxia, or energy deficiency²⁵⁴. The importance of autophagy in coping with metabolic stress has been demonstrated notably in autophagy-deficient mice, which fail to survive neonatal starvation³⁴¹. The accumulation of misfolded proteins and damaged organelles is detrimental to the homeostasis of the cell, and autophagy, by promoting their degradation, also helps to maintain their survival²⁵⁴. It is also important to note that by limiting oxidative stress, autophagy inhibits inflammasome activity and inflammation, thus promoting the cell's genomic integrity^{342,343}. The autophagic process also protects cells from harmful foreign elements, such as pathogens, by degrading them. Elements from the pathogen, called antigens, are then loaded to MHC-I, which promotes the elimination of infected cells by immune cells and the organism's integrity³⁴⁴. In addition to its role in coping with internal and external dangers and, by this way, preventing cellular stresses inducing cell death, the autophagy machinery can directly target molecular actors of cell death pathways. Autophagy induction could promote cell survival or cell death depending on the intensity or duration of the stress. By degrading specific inhibitors of each cell death pathway, autophagy has been shown to promote these different cell death modalities. Actually, by respectively degrading K-Ras and, by extension, inhibiting MAPK signaling survival pathways, or ferritin, a cellular iron binding, autophagy has been shown to favor apoptosis or ferroptosis in different cancer models^{345,346}. Also, by degrading ROS scavenger catalase, a crucial cell enzyme in the elimination of ROS, autophagy has been shown to promote necrosis in mice fibroblasts³⁴⁷. It is interesting to note that the induction of a complete autophagy influx is not necessary for the autophagic process to induce cell death. Actually, autophagosomes have been shown to serve as a platform for caspases activation and apoptosis induction³⁴⁸. Although autophagy in certain conditions can cause cell death, the primary role of autophagy is to promote survival. In addition to coping with cellular stresses, autophagy can negatively regulate molecular actors involved in the signaling pathways of apoptosis induction. Actually, autophagy has been observed to prevent TRAIL-induced apoptosis by degrading an actor of cell death, the cleaved caspase-8³⁴⁹. As one of the two apoptosis pathways is mediated by mitochondria which releases an effector mediator that eventually leads to apoptosis, autophagy has been shown to prevent that cell death pathway by inducing mitochondria degradation or mitophagy³⁵⁰. Also, it is interesting to note that BECLIN-1, a central protein involved in canonical autophagy, is physically connected with the anti-apoptotic protein BCL-2 and inhibits the action of each other. However, when autophagy is induced, BECLIN-1 is activated and recruited to the autophagy machinery, and as a result, BCL-2 is released and

exerts its anti-apoptotic function³⁵¹. It is also interesting to note that in addition to the canonical autophagy function in preventing cell death, proteins encoding genes of the autophagy cascade can prevent cell death independently of their function in canonical autophagy. For example, ATG5 and ATG12 have been shown to regulate apoptosis independently from autophagy, as well as ATG7 through its interaction with p53^{352–354}.

3.5.2 Non-canonical functions of autophagy machinery and autophagy genes

3.5.2.1 LC3-associated phagocytosis (LAP)

In addition to its primary role of degradation and recycling, autophagy and its molecular components can mediate other functions like LC3-associated phagocytosis (LAP)³⁵⁵. Although LAP is another pathway by which cargo can be degraded through delivery to the lysosome, it differs from the classical autophagy degradation pathway at different points³⁵⁶. First, contrary to canonical autophagy which is present in all cells, LAP is a specific mechanism to phagocytes. Then, the nature of the cargo engulfed and degraded is different, intracellular for autophagy versus extracellular for LAP. Through this mechanism, the cell can degrade extracellular pathogens and live or dead cells. Secondly, to be implemented, LAP requires the stimulation of a membrane receptor. Among the receptors, TLR engagement has been demonstrated to induce LAP. In addition, the nature of the vacuole containing the cargo is also different, a double membrane vacuole for autophagy versus a single membrane for LAP. Finally, the machinery mobilized for autophagy is different from that required for LAP. To be realized, LAP needs NADPH-oxidase (NOX2) to generate ROS, some components of the BECLIN-1 complex, the phosphatidylinositol 3-Phosphate (PI3P) generation, and the LC3-conjugation to the single membrane of the phagosome. Contrary to autophagy, LAP does not require either the ULK1 initiation complex or the ATG14 of the BECLIN-1 complex. However, LAP needs Rubicon, a protein exerting an inhibitory effect on autophagy. LAP, like autophagy, has been demonstrated to have a crucial role in cell homeostasis as a deficiency leads to the development of autoimmune diseases, probably due to the defect in eliminating dead cells^{356,357}.

3.5.2.2 Exocytosis

Autophagy machinery can also target the cargo to the plasma membrane or the extracellular space, acting as a critical component in cell secretion^{355,358}. There are different ways by which autophagy facilitates addressing of the cargo to the plasma membrane and/or to the secretion, gathered in a general term called secretory autophagy: the unconventional secretion, the exocytosis of secretory granules, the exosome release, and the retromer dependent trafficking^{355,358}.

Unconventional secretion takes its name from the secretion of proteins that do not pass through the classical secretion mechanism. Contrary to that latter, unconventional secretion involves neither the presence of a signal peptide in the protein that drives it to the plasma membrane nor their secretion into the extracellular space or the conventional transit through the ER-Golgi apparatus to reach the plasma membrane.

The mechanism involves the protein binding that has to be addressed to the membrane or secreted in autophagosomes. Although neither the precise molecular mechanism nor the proteins required are well understood, autophagosomes seem to have specific Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) and syntaxins that facilitate fusion with the plasma membrane rather than the lysosome³⁵⁹. That secretion was notably observed in mammalian cells after inflammasome activation to permit the secretion of IL-1 β and IL-18^{360,361}. It is interesting to note that in a recent paper, another type of secretion, called LC3-dependent extracellular vesicle loading and secretion, has been described. That unconventional secretion is mediated by autophagy-related proteins involved in LC3 conjugation and was described to promote the secretion of RNA binding proteins (RBP) and small ncRNA through the release of extracellular vesicles³⁶².

Autophagy machinery was also described to be essential for the exocytosis of secretory granules like the lysozyme secretion by the Paneth cell, an intestinal cell type whose secretory activity is critical to limit intestinal microbiota expansion³⁶³. The importance of autophagy in that mechanism was partly discovered by the association between a polymorphism in ATG16L1 and Crohn's disease, characterized by an intestinal microbiota out of control and chronic intestinal inflammation³⁶⁴. The effective role of autophagy machinery in that process was then confirmed by the defect in exocytosis of the lysozyme-containing granules in Atg deficient Paneth cells³⁶⁵. Although the autophagy machinery mobilized is still unclear, lysozyme has been demonstrated to be present in LC3-positive vacuoles before exocytosis in the Paneth cell³⁶³. In another specialized secretory cell, the osteoclast, the secretion of cathepsin K into the extracellular space requires autophagy machinery but differently³⁶⁶. Actually, rather than targeting the vacuole-containing cargo, the autophagy machinery labels the plasma membrane with LC3 to facilitate the binding of the vacuole and the secretion of its content into the extracellular space. In that type of secretion, the vacuole is not an autophagosome but a lysosome.

Another way by which autophagy impacts the secretion of intracellular content is through the release of exosomes. In that type of secretion, the role of autophagy is still undetermined. In certain studies, it acts as a negative regulator, limiting the release of exosomes when autophagy is induced and conversely. That type of secretion seems to act as a compensatory mechanism for the cell when autophagy is inhibited³⁶⁷. The exosome secretion is increased to reduce cell death induced by proteotoxicity. That mechanism was notably observed for the release of pathogenic protein cargoes like the α -synuclein, prions, and the amyloid precursor protein involved in diverse neurodegenerative diseases³⁶⁸⁻³⁷⁰. However, in other studies, autophagy genes seem to promote the release of exosomes. Actually, ATG5 has been observed to disrupt the vacuolar-ATPase (v-ATPase) to limit late endosome acidification and favor exosome production³⁷¹. Besides, the ATG3-ATG12 conjugate has been shown to interact with the endosomal sorting complexes required for transport (ESCRT) protein ALIX and facilitate exosome release³⁷². The exosome secretion involves the fusion of the multivesicular body (MVB) with the plasma membrane. Eventually, autophagy has

been shown to modulate the endosome-to-cell surface recycling pathway. During metabolic stress like starvation or hypoxia, LC3 can interact with the Rab GTPase-activating protein (RabGAP), TBC1D5, and disturb its interaction with the retromer, impairing its inhibitory effect on the latter. As a result, the retromer can associate with the endosomal membrane and allow the translocation of proteins to the plasma membrane. That mechanism was notably described for the translocation of GLUT1, a glucose transporter, to the plasma membrane during starvation³⁷³.

3.5.2.3 Other functions

Through its role in degradation and secretion, autophagy by different means has been shown to limit inflammation and promote cell survival^{374,375}. In addition to its role in degradation and secretion, some proteins of the autophagy cascade can also have distinct roles, interacting with other cellular pathways like the cell-death pathways, cell cycle regulation, and immune signaling³⁷⁶. For example, ATG7, independently of its function in autophagy, can regulate P53-dependent cell cycle arrest and apoptosis³⁵⁴. UVRAG is involved in DNA repair³⁷⁷. Besides, many autophagy proteins have been shown to regulate inflammation independently of the autophagy cascade. Among them, ULK1 is activated after 2',5'/3',5'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthesis by cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) and inhibits the stimulator of Interferon genes (STING)-dependent cytokine production³⁷⁸.

4 Autophagy: a double edged sword in cancer

Given its roles in maintaining cell homeostasis and survival through its different means of action, it is not surprising that a defect in basal autophagy has been shown to favor the development of various diseases like neurodegenerative diseases (due to the accumulation of protein aggregates within the neurons), inflammatory disease as the Crohn's disease, infection susceptibility and also cancer³⁷⁹. Since autophagy has been shown to impact each step of carcinogenesis, it seems to act as a double-edged sword in cancer development, depending on the type of tumor and the stage. Generally, in the early stage, autophagy is considered as an anti-tumor mechanism, limiting the transformation of a normal cell to a malignant cell. However, autophagy acts as a potent pro-tumoral process when the tumor is established, facilitating tumor cell survival, proliferation, metastasis development, resistance to conventional anti-tumor therapies, and also escape from the immune system³⁸⁰.

4.1 Autophagy limits the tumorigenesis

First pieces of evidence linking autophagy defect and cancer onset have been revealed by the high percentage of mutations in gene expression related to autophagy in cancer

patients versus healthy ones. Among the genes, Bif-1, Beclin1 and UVRAG have been shown to be mutated in various cancers^{381–384}. Beclin1 is monoallelically deleted in breast, prostate, and ovarian cancers in 40 to 75%³⁸³. *In vitro* and *in vivo*, cancer cells deficient for Beclin1, UVRAG, and Bif-1 increase cell proliferation and tumor progression, demonstrating that autophagy is a key process to inhibit cancer onset^{383,385,381}. Another evidence is the development of spontaneous liver cancers in mice deficient for Atg5 and Atg7. That emergence of the tumor was then demonstrated to be associated with the presence of damaged mitochondria and oxidative stress in autophagy-deficient hepatocytes³⁸⁶. Autophagy deficiency is responsible for a defect in damaged mitochondria elimination and preventing ROS formation that can damage the DNA^{387,388}. Besides, upon autophagy inhibition, the autophagic receptor P62 is no longer degraded through the autophagy process and is accumulated in the cytosol. As a result, P62 can interact with the NRF2-KEAP1 complex present in the cytosol, bind KEAP1 and release the master regulator of the antioxidant response, the nuclear factor erythroid 2 like 2 (NRF2)³⁸⁹. NRF2 then translocates in the nucleus, promoting expressing genes involved in the antioxidant response, eventually favoring tumor survival and growth³⁹⁰. As another facilitating tumor development mechanism, P62 can trigger the NF- κ B and induce chronic inflammation. In an autophagy-deficient cell, P62 deficiency reduces tumor development, demonstrating this pathway's importance in cancer onset³⁹¹.

Also, autophagy is a known repressor of a protein complex responsible for inflammation and named nod-like receptor family pyrin domain containing 3 (NLRP3 or inflammasome). The inflammasome's activity is inhibited by the elimination of its ligand (damaged mitochondria) and by direct targeting of its structure by autophagy³⁹². Finally, autophagy prevents the cell from dying by necrosis, a type of cell death associated with inflammation, under unfavorable conditions³⁹³. Mechanistically, in a normal cell, basal autophagy promotes damaged mitochondria elimination and inhibits ROS accumulation and inflammation. As a result, basal autophagy protects the cell from becoming mutated and tumoral by maintaining genome integrity³⁹⁴. It is worth noting that key oncogenes like Akt or Bcl-2 inhibit autophagy, whereas tumor suppressor genes like Pten, p53, and Tsc1/2, often mutated in cancer, induce the autophagy process³⁹⁵. In KRAS-driven NSCLC, autophagy inactivation by Atg5 silencing has been shown to increase the number and the surface of hyperplastic regions and adenoma, demonstrating the importance of the autophagy process in preventing the formation of tumor *de novo*³⁹⁶.

4.2 Autophagy promotes tumor progression and metastasis development

In contrast to its role in tumor formation, autophagy promotes cancer progression through different means when the tumor is established, promoting tumor cell survival, proliferation, migration, and resistance to anti-tumor therapies³⁹⁷.

4.2.1 Survival/Proliferation

Among the pro-tumoral effects, autophagy is a critical component for the cancer cell to cope with the various stresses it faces within the TME, promoting its survival and proliferation. Cancer cells have been shown to present highly autophagic activity and are more dependent on that biological process than normal cells. That dependence is likely due to the adverse conditions within the TME (nutrient carency and hypoxia) and to the high energetic and metabolic demands required by the tumor cells for its continuous proliferation^{398,399,400}. Autophagy inhibition by genetic inactivation within the tumor cell leads to a decrease in tumor progression and an increase in cell death upon exposure to environmental stresses⁴⁰¹. In response to those stresses, autophagy is induced within the cell to deal with the high metabolic and energetic demands of cancer cells and to decrease the ROS level, promoting cancer cell survival and proliferation³⁹⁸. For instance, tumor cells deficient for autophagy have been shown to be unable to catabolize lipids upon nutrient carency. Therefore they are more sensitive to cell death upon starvation⁴⁰². In non-starvation conditions, autophagy impairment has been shown to prevent lipids degradation and reduce energy production, limiting the proliferation of hepatocellular carcinoma cells⁴⁰³. Also, the addition of glutamine or glutamate in the medium of starving Atg7-deficient KRAS lung tumor cells has been shown to promote their survival, demonstrating the importance of autophagy in the metabolic adaptation of tumor cells upon starvation⁴⁰⁴.

In addition to being increased by the different stresses, autophagy level is also increased within the tumor cell by molecular pathways often disturbed during carcinogenesis. Among them, the RAS pathway, frequently mutated in cancers, notably in lung cancer, leads to an increase in autophagy⁴⁰⁵. Inhibition of that process through Atg7 silencing within the lung cancer cell is responsible for an accumulation of defective mitochondria and a metabolism impairment that eventually leads to an increase in cell death and a reduction in tumor cell proliferation⁴⁰⁶. Autophagy has also been shown to inhibit P53, a tumor suppressor that leads to cell cycle arrest or apoptosis, facilitating cancer cells' continuous proliferation⁴⁰⁷.

Autophagy induction in KRAS mutated cancer cells also increases glycolysis⁴⁰⁸. That mechanism can notably be explained by the fact that autophagy induction has been demonstrated to facilitate glucose uptake by increasing its transporter expression (GLUT1) to the plasma membrane upon starvation. Actually, blocking the autophagy flux has been related to a decrease of the transporter expression at the plasma membrane through its retention within late endosomes³⁷³. That autophagy facilitating transporter membrane expression was also described in pancreatic cancer for the cystine transporter, favoring cysteine metabolism. Actually, when autophagy is inhibited, the cystine transporter is blocked within the lysosome⁴⁰⁹. As another adapter mechanism to starvation, hexokinase-2 has been shown to change the metabolic pathway from glycolysis to an autophagy-dependent pathway by inhibiting mTORC1, to facilitate cancer cell survival and proliferation⁴¹⁰. Similarly, it has been demonstrated that autophagy can regulate glycolysis in liver cancer cells by selectively degrading hexokinase 2⁴¹¹. Starvation-induced autophagy within the malignant cells in bladder

cancer also facilitates lactate dehydrogenase A expression, which eventually promotes lactate production and cancer cell survival⁴¹². The acid stress induced by lactate production is then coped by tumor cells through autophagy-induced lipogenesis, which supports their survival⁴¹³.

In addition to the impact of autophagy in supplying nutrients within cancer cells, cancer-associated fibroblast (CAF) is also a critical actor in that process. Actually, within the TME, cancer cells and CAF collaborate to maximize nutrient supply for the malignant cells. Actually, tumor cells have been shown to induce autophagy in CAF through ROS transfer. As a result, metabolism within CAF is changed from oxidative phosphorylation (OXPHOS) to glycolysis, therefore promoting the synthesis of high-energy metabolites like the glutamine and lactic acid to carry out the tricarboxylic acid (TCA) cycle within cancer cells, eventually facilitating tumor growth under adverse conditions. This mechanism is called the reverse Warburg effect⁴¹⁴. Autophagy in CAF is also crucial for their activation and the production of proline and collagen that promotes tumor growth⁴¹⁵. In pancreatic cancer, autophagy in CAF has also been described to facilitate nucleoside secretion, eventually leading to cancer cell proliferation⁴¹⁶. It is worth noting that cancer cells, through their metabolic change from OXPHOS to aerobic glycolysis (Warburg effect), lead to ROS production and increased lactate secretion⁴¹⁷. By contrast with non-malignant cells, the presence of lactate within the TME is beneficial for tumor cells, facilitating their survival, notably under glucose-deprivation conditions⁴¹⁸. Moreover, as evoked previously, ROS can induce autophagy through different pathways (FOXO3, NRF2, HIF-1, and TIGAR). As a result, autophagy promotes its elimination, and removes oxidized molecules and damaged mitochondria, eventually favoring cell survival⁴¹⁹.

Regardless of its importance in maintaining metabolism and energy production in cancer cells upon nutrient carency, autophagy has also been shown to inhibit the apoptosis pathway, notably through the degradation of mitochondria by mitophagy⁴²⁰. Therefore unsurprisingly, autophagy inhibition is responsible for tumor cell death increase in various cancer models⁴²¹.

Autophagy induction by TFEB has also been shown to promote cancer cell proliferation through different mechanisms. Although the mechanistic underlying this phenomenon is not fully understood, a study has demonstrated in hepatocellular carcinoma that autophagy induction is responsible for corticotropin-releasing hormone-binding protein degradation, overcoming its negative regulation on the cyclin B2-CDK1 complex⁴²². Another one has unveiled that TFEB-induced autophagy is essential to the pro-tumoral activity of the non-enzymatic heparanase in gastric cancer cells⁴²³. As a result, proliferation is increased through complex activation. Conversely, it is worth noting that two recent papers have observed that autophagy could restrain cancer cell proliferation^{424,425}.

4.2.2 Angiogenesis

During cancer development, the more the cell proliferate, the more they are moved away from the vasculature system and the source of nutrients, and the more they suffer

from starvation and hypoxia. As a response, both factors facilitate the development of new vessels from the pre-existing ones during a phenomenon called angiogenesis through the secretion of pro-angiogenic factors. Then these molecules, like VEGF, bind specific membrane receptor of endothelial cell and promotes angiogenesis. That process is a key step for cancer to keep growing and to develop metastasis. Although hypoxia and oxidative stress, which are the main inducer of angiogenesis, also stimulate the autophagy process to cope with those stresses, the impact of autophagy in angiogenesis is not clear⁴²⁶. In certain papers, autophagy in endothelial cells (EC) seems to promote angiogenesis, whereas, in others, it inhibits it. In a non-tumoral model, *in vitro* and *in vivo* autophagy induction by rapamycin has been shown to promote the EC proliferation, migration, and formation of new vessels, whereas autophagy inhibition by 3-methyladenine (3-MA) prevents it⁴²⁷. Autophagy also seems important for EC homeostasis and the migration of EC and angiogenesis induced after VEGF stimulation, as autophagy inhibition has been shown to impair angiogenesis⁴²⁸. Interestingly, in bladder cancer, tumor cells have been shown to promote angiogenesis through the autophagy-dependent secretion of extracellular vesicles targeting EC⁴²⁹. However, autophagy in EC has also been shown to degrade critical actors of the angiogenesis process. Actually, one paper has described that in EC, autophagy can degrade the main pro-angiogenic enzyme, the hyaluronan synthase 2, responsible for the production of hyaluronan, eventually impairing angiogenesis⁴³⁰. Besides VEGFR2, a receptor of the pro-angiogenic factors VEGF present at the plasma membrane of EC can also be degraded by autophagy, damaging new vessel formation⁴³¹.

4.2.3 Epithelial-mesenchymal transition and metastasis development

4.2.3.1 Epithelial-mesenchymal transition (EMT)

During cancer development, its expansion is limited physically by the tissue length. To continue to develop, cancer cells can detach from the extracellular matrix (ECM), migrate from the primary site to another one through the vasculature system, and create a second tumor site wherein they could grow during a process called metastasis⁴³². The impact of autophagy in this multistep process is not fully elucidated. As the first step, cancer cells must realize their EMT, during which they lose their adherence capacity to gain mobility and invasive capabilities, a necessary switch for metastasis development. Depending on the cancer, autophagy seems to positively or negatively impact the EMT process⁴³³.

As the first sign of EMT, the degradation of E-cadherin (E-cad), an anchoring molecule of the tumor cell to the tumor site, is mediated by autophagy in various types of cancer, notably lung cancer^{434–436}. Furthermore, TGF β signaling, which is essential for the EMT process, seems to depend on the autophagy mechanism. Actually, in lung tumor cells deficient for autophagy, both EMT transcription factors activation and cell markers expression are impaired, leading eventually to a defect in cancer cell migration. Autophagy inhibition in cancer cells is responsible for decreasing TGF β receptor internalization to endosomes and decreasing TGF β signaling⁴³⁷.

However, in certain studies, a negative impact of autophagy on EMT has been described. For example, in colon cancer cell lines, autophagy inhibition has been shown to decrease E-cad expression while increasing the expression of vimentin and twist1, meaning that EMT has occurred. In parallel, the migration and invasion capacities of the autophagy-deficient malignant cells are increased⁴³⁸. As another example, *in vitro* and *in vivo* in the glioblastoma model, autophagy has been shown to inhibit EMT through the degradation of c-Met⁴³⁹.

4.2.3.2 Tumor migration and metastasis

To migrate from the primary cancer site to another, cancer cells must detach themselves from the ECM. While that detachment usually results in apoptosis in a process called anoikis, autophagy in cancer cells makes them resistant to that cell death induction signal^{440–442}. In addition to promote tumor cell survival during anoikis, many papers have demonstrated that autophagy acts as a promoter of migration and invasion of cancer cells, notably in hepatocellular carcinoma, breast, and gastric cancer, involving distinct pathways^{443–445}. One paper has observed that mitogen-activated protein kinase kinase 3, an important modulator of p38, JNK and ERK pathways, degradation by autophagy in gastric tumor promoted metastasis development⁴⁴⁶. In another study, the role of mesothelial cells in metastasis development in gastric cancer has been demonstrated. Upon hypoxia-induced autophagy, mesothelial cells have been observed to secrete VEGFA that binds the VEGFR receptor on gastric cells. As a result, ERK and JNK pathways are activated, integrin α 5 and fibronectin are expressed, and metastasis development is promoted⁴⁴⁷.

Interestingly, CAF has also been described to promote cancer cell motility and invasion capacities and metastasis development through different mechanisms. In breast cancer, it has been observed that this mechanism is mediated by autophagy through a cardiotrophin1/STAT3/AMPK pathway⁴⁴⁸. Another metastasis-facilitating pathway dependent on autophagy in breast CAF has been described, involving NOX4/NRF2⁴⁴⁹. CAF can also mediate metastasis development by facilitating the invasion capacity of cancer cells through the secretion of extracellular vesicle autophagy-dependent. That mechanism has been shown to involve both the NF- κ B induction and the secretion of pro-inflammatory cytokines like IL-8 and metalloproteinase MMP9⁴⁵⁰.

However, it is worth noting that in contrast to the pro-metastasis impact, autophagy has also been described to act as an anti-metastasis mechanism in some studies. Actually, autophagy is known to impair, through the tyrosine-protein kinase Met (MET) degradation, the associated receptor tyrosine kinase signaling that promotes motility and invasion capacities of tumor cells⁴⁵¹. In breast cancer, autophagy has been shown to degrade neighbor to BRCA1 (NBR1), and as a result, metastasis development is impaired⁴⁵². In colorectal cancer cells, autophagy inhibition by a decrease of STAT3 nuclear expression has been demonstrated to promote cancer progression and metastasis development⁴⁵³. It is interesting to note that an autocrine regulation of the autophagy process is responsible for inhibiting metastasis development in melanoma and colon adenocarcinoma⁴⁵⁴.

In lung cancer cells, autophagy seems acting as a pro-tumoral mechanism, positively regulating each step of the metastasis development involving different signaling pathways. Actually, in addition to prevent anoikis, autophagy induced in lung cancer cells also facilitates EMT^{455,436}. During this process, lung cancer cells acquire migration and invasion capacities through different mechanisms, either involving apelin molecule or through the inhibitory acetylation of Snail^{456,457}. Interestingly it has been demonstrated that *in vitro* TLR3/4-induced autophagy is another means facilitating migration and invasion of lung cancer cell lines and involving TRAF6 ubiquitination³⁰⁰. It is worthy noting that in a very recent paper, a study has unveiled that particulate matter leads to ROS-induced autophagy, TRIM37 degradation, TRAF6 protection and inflammation-induced neutrophils infiltration, which favors metastasis development⁴⁵⁸.

4.2.4 Cancer stem cells maintenance

Furthermore, autophagy is also a crucial actor in the homeostasis and functions of a small cancer cell subpopulation called cancer stem cells (CSC), characterized by its capacity for self-renewal and differentiation. CSC are involved both in tumor initiation, metastasis development, and resistance to anti-tumor therapies⁴⁵⁹. In ovarian CSC, autophagy level increase has been shown to promote self-renewal and chemoresistance capacities through FOXA2 upregulation⁴⁶⁰. In gastric CSC, autophagy increase promotes chemoresistance through the induction of the Notch signaling pathway⁴⁶¹. As a mechanism of stemness maintenance, lung CSC has been shown to degrade P53 by autophagy, therefore facilitating Zinc finger E-box-binding homeobox 1 (Zeb1) expression and stemness of CSC⁴⁶².

Autophagy inhibition in those cells also impairs the self-renewal ability of CSC. Actually, in breast cancer, inhibition of autophagy by Beclin-1 or Atg12 silencing has been shown to reduce the stemness of CSC^{463,464}. As a result, autophagy inhibition has been shown to reduce the CSC population and sensitize tumor cells to anti-cancer therapies, as observed in lung cancer⁴⁶⁵.

4.3 Autophagy and resistance to anti-tumor treatments

As a crucial mechanism of cell survival, autophagy has been described to be induced in cancer cells following most anti-cancer treatment, and as a result, reduce treatment efficiency, eventually leading to anti-cancer treatment resistance. That resistance has been observed for radiation therapy, chemotherapy, and targeted therapies in various types of cancer and involved different pathways⁴⁶⁶.

4.3.1 Radiation therapy

Although the underlying mechanisms are not really understood, autophagy has been described as taking part in resistance to radiation therapy in various types of cancer²⁵⁵. In a recent paper, P53-induced autophagy following radiation therapy was demonstrated to be involved in the resistance of radiation therapy in ovarian and

leukemia cancer cell lines⁴⁶⁷. In NSCLC cancer cell lines, radiation therapy-induced ROS production has been shown to stimulate the autophagy process and activate the NRF2 pathway, eventually leading to radiation resistance⁴⁶⁸.

4.3.2 Chemoresistance

Although chemotherapies can differ in their means of action, most of them can induce protective autophagy in various cancer types that limit chemotherapy effectiveness. The mechanism of induction can also vary depending on the chemotherapy²⁵⁶. In multiple cancer types and notably in NSCLC, autophagy induction by the ERK pathway is involved in chemoresistance to various chemotherapies, notably cisplatin and temozolomide^{469–471}. In lung cancer cells, hypoxia-induced autophagy has also been shown to lead to cisplatin resistance⁴⁷². Paclitaxel (taxol) has also been shown to induce protective autophagy in NSCLC cancer cells by activating BECLIN-1⁴⁷³. Although its impact in lung cancer cells is yet unknown, in colorectal cancer cells, oxaliplatin has been shown to induce autophagy by activating the inhibitory activity of c-Myc on the expression of miR-27b-3p that negatively regulates the expression of ATG10⁴⁷⁴. Gemcitabine-induced autophagy has been shown to restrain treatment efficiency in lung cancer cells by inhibiting the expression of pro-apoptotic proteins and increasing the expression of the anti-apoptotic protein, BCL-2⁴⁷⁵. As another example, vinorelbine has been shown to induce protective autophagy that keeps in check the ROS level in NSCLC cancer cells and, as a result, limits chemotherapy-induced apoptosis⁴⁷⁶. Depending on the cancer type, the pathway involved in protective autophagy induction following the chemotherapy treatment can also differ. For example, chemoresistance to 5-FU has been shown to involve the AMPK pathway-induced autophagy in various cancer types, such as gastric cancer and glioblastoma^{477,478}. By contrast, protective autophagy induction following 5-Fluorouracil (5-FU) treatment is mediated by the p53 pathway in colorectal cancer cells⁴⁷⁹. As a result, inhibition of autophagy has been shown to increase the efficacy of chemotherapies⁴⁸⁰.

4.3.3 Targeted therapies

The main targeted therapy consists in blocking the EGFR signaling, which is responsible for cancer cell growth and whose pathway is often overactivated in various types of cancer⁴⁸¹. Both erlotinib, gefitinib, and icotinib, three tyrosine kinase inhibitors of the EGFR receptor, have been shown to inhibit the EGFR signaling pathway by inhibiting the transduction signal of growth factor binding on the EGFR receptor of the cancer cell. Both have also been described to induce autophagy in the targeted cells, and as a result, the efficacy of the therapy is decreased⁴⁸². In NSCLC cancer cells, erlotinib has been described as concomitantly inducing apoptosis and autophagy. Erlotinib-induced autophagy has greatly decreased cell death caused by the targeted therapy in NSCLC cells with EGFR-activating mutations⁴⁸³. Given the importance of autophagy in resistance to targeted therapy, a combination regimen of erlotinib and autophagy inhibitor has demonstrated much better efficacy than targeted therapy alone⁴⁸⁴. In NSCLC, autophagy inhibition has been shown to allow the complete

elimination of cancer cells by erlotinib⁴⁸⁵. Although the mechanism underlying this phenomenon is not understood, ER stress modulation seems involved. A recent paper has described that in NSCLC cancer cells, autophagy-induced icotinib resistance has been shown to involve a STAT3/FOXM1/ATG7 signaling and is non-sensitive to autophagy initiation blocking by 3-MA but sensitive to autophagy maturation blocking by chloroquine (CQ)⁴⁸⁶. Moreover, gefitinib efficacy is mainly decreased by autophagy in NSCLC. Therefore its inhibition facilitates the efficiency of the targeted therapy⁴⁸⁷. The targeting of another growth factor receptor, the fibroblast growth factor receptor, in NSCLC has also been shown to induce protective autophagy and, as a result, limit targeted therapy efficacy⁴⁸⁸. Similarly, autophagy inhibition has been shown to facilitate cell death caused by the treatment⁴⁸⁹. It is worth noting that targeted therapy against RAS has been shown to induce protective autophagy through RAF-MEK-ERK inhibition in various cancer cells, which limits therapy effectiveness⁴⁹⁰. Moreover, in NSCLC cancer cells, ALK targeting has been shown to induce protective autophagy⁴⁹¹.

Another pathway mainly disturbed in cancer to promote tumor growth is the PI3K/AKT/mTOR pathway⁴⁹². In various types of cancer, autophagy inhibition combined with the inhibitor of the PI3K/AKT/mTOR pathway has demonstrated increased efficacy than the targeted therapy alone. Autophagy influx seems essential for resistance to the targeted therapy as both the inhibition of the elongation or maturation autophagy steps have increased cancer cell sensitivity to the treatment⁴⁹³. It is worth noting that an inhibitor of the maturation step, hydroxychloroquine (HCQ), has been described to increase cancer cell death when combined with the targeted therapy in an autophagy-independent way⁴⁹⁴.

4.4 A contrasted impact of autophagy in the anti-tumor immune response

4.4.1 Autophagy prevents inflammation and anti-tumor immune response

4.4.1.1 Autophagy limits inflammatory-inducing pathways

Although the impact of autophagy has not been demonstrated as a modulator mechanism of responsiveness to immunotherapy, the autophagy process seems to act as a negative regulator of inflammation within the cancer cells, limiting the implementation of a robust anti-tumoral immune response. In a recent paper, a study demonstrated the importance of Beclin-2 in limiting inflammation within cells and carcinogenesis. By targeting MEKK3 and TAK1 for degradation, it inhibits ERK and NF- κ B pathways, limiting proinflammatory cytokines secretion. Loss of Beclin-2 is responsible for elevated proinflammatory cytokine production and spontaneous lymphoma development in mice^{495,496}. Also, in a recent study on lung cancer cells, autophagy has been shown to prevent cGAS-STING stimulation by mitochondrial DNA after irradiation. As a result, type I interferon as well as CCL5 and CXCL10 production

and secretion in those cells are decreased, inhibiting the CD8 T cell efficiency and the anti-tumor immune response⁴⁹⁷.

It is also now well established that autophagy can prevent a proteic complex present within the cell, which promotes inflammation, the inflammasome. By degrading PAMP, DAMP, and cytokines that are the activating signals of inflammasome or by directly degrading components of the inflammasome, autophagy limits inflammasome activation⁴⁹⁸. For example, in melanoma and colorectal cancer, autophagy inhibition through the targeting of PI3KC3/Vps34 has been shown to promote CCL5 and CXCL10 secretion that favors NK and T cell recruitment within the tumor bed. As a result, combining the autophagy inhibitor with the immune checkpoint anti-PD-1/PD-L1 has demonstrated better clinical benefits than the immune checkpoint blockade alone⁴⁹⁹. Additionally, it was shown that the proto-oncogene SKIL in NSCLC cells promotes their escape from the immune system and the cancer progression by upregulating autophagy⁵⁰⁰. However, it is worth noting that in another study, autophagy seems to prevent the recruitment or differentiation of the immunosuppressive Treg cells within the tumor site⁵⁰¹.

Another example demonstrating the negative role of autophagy in developing a robust anti-tumor immune response is the secretion of macrophage migration inhibitory factor (MIF) and the polarization of macrophages into an anti-tumor phenotype (M1 phenotype) when autophagy is inhibited⁵⁰². Cancer cells in hepatocellular carcinoma and glioma have also been shown under hypoxia to secrete molecules that induce autophagy in macrophages and lead them to become pro-tumoral (M2 phenotype)^{503,504}. It is worth noting that in macrophages, autophagy deficiency causes LAP impairment, meaning that dying cancer cells are not eliminated. As a result, STING pathway stimulation induced IFN-I production, and the anti-tumor immune response are elicited⁵⁰⁵.

4.4.1.2 Autophagy modulates MHC-I and immune checkpoints expressions in cancer cells

Autophagy has also been demonstrated to modulate the membrane expression of molecules that can impact the recognition of cancer cells by immune cells and their elimination, therefore impacting the anti-tumor immune response. Among the molecules whose membrane expression seems to depend on autophagy in cancer cells, the MHC and the immune checkpoints molecules are the most described. While autophagy is involved in the antigenic presentation into the MHC class I (MHC-I) and MHC class II (MHC-II) as well as cross-presentation in a non-tumoral model, cancer cells negatively regulate MHC-I molecules membrane expression in cancer cells of different tumor types^{506–508}. In pancreatic and endometrial cancer, autophagy has been shown to respectively degrade MHC-I and its transactivator, impairing MHC-I membrane expression on cancer cells that eventually lead to cancer cells' evasion from the anti-tumor immune response^{509,510}. However, a recent paper demonstrated that in bladder cancer cells, the blocking of the autophagy at the maturation step has been shown to decrease MHC-I expression, suggesting that autophagy could promote

MHC-I expression in this model⁵¹¹. Besides, in NSCLC cancer cells, radiotherapy has been shown to promote MHC-I expression through autophagy induction⁵¹².

Furthermore, autophagy in cancer cells has also been demonstrated to impact immune checkpoint expression. Very little information is available concerning the involvement of autophagy in the expression of PD-L1, an inhibiting immune checkpoint and the conclusions vary according to the tumor model studied. Actually, studies have shown that autophagy negatively impacts PD-L1 expression in bladder and cholangiocarcinoma cancers^{513,514}. In contrast, a recent study demonstrated that in lung tumor cell models, autophagy, by promoting the activation of STAT3 signaling, could induce the expression of PD-L1⁵¹⁵. In addition, autophagy has also been shown to degrade a costimulatory molecule, B7H3, in breast cancer cells and be involved in the degradation of an inhibitory immune checkpoint present on CD8 T cells, the CTLA-4⁵¹⁶.

4.4.1.3 Autophagy prevents tumor cell from being eliminated by the immune cells

Autophagy in cancer cells has also been shown to protect malignant cells from being eliminated by NK and CD8 T cells. Hypoxia-induced autophagy has been demonstrated to safeguard tumor cells from T cells through a mechanism involving STAT3⁵¹⁷. Besides, autophagy has also been described to protect tumor cells from NK cytotoxicity by two distinct means, the elimination of granzyme B and the degradation of connexin 43, which are necessary for NK cells to exert their anti-tumor activity^{518,519}. Autophagy can also control cytokine expression and secretion, and, conversely, cytokines can modulate the autophagy process. Some of them activate the autophagy process like IL-2, whereas others inhibit it like IL-10, with consequences on cancer cell progression⁵²⁰. In a recent study, CD8 T cell-secreted IFN ψ has been shown to repress autophagy in lung cancer cells and, as a result, decrease tumor cell survival⁵²¹.

To sum up, although the impact of autophagy has not been demonstrated as a modulator mechanism of responsiveness to immunotherapy, autophagy takes part in both intrinsic and extrinsic mechanisms of resistance to immune checkpoint blockade immunotherapies (ICB). Actually, although the very few data concerning NSCLC in the literature seem to be the perfect opposite, autophagy was shown to degrade MHC-I expression in pancreatic cancer cells, therefore preventing their recognition by immune cells⁵⁰⁹. Also, some preliminary studies seem to demonstrate that PD-L1, the only biomarker so far used to predict the responsiveness of patients to PD-1/PD-L1 ICB, can be negatively regulated by autophagy^{522,523}. It is also important to note that autophagy limits inflammation as another means of protecting the cell. This mechanism constitutes an additional brake for developing an anti-tumor immune response, the infiltration of immune cells into the TME, and the efficacy of ICB³⁹². A study has already demonstrated the interest of combining an ICB with an autophagy inhibitor with promising preclinical results, linked to an increased anti-tumor immune cell infiltration and activity within the tumor site⁵²⁴.

4.4.2 Autophagy-induced cell death promotes anti-tumor immune response

Although the central function of autophagy is to promote cell survival with multiple interconnections with cell death pathways already described, a prolonged stress condition or a stress too intense can lead to cell death. That mechanism called autophagy-dependent cell death or ADCD, is a process in which a large portion of the cytosol is engulfed in autophagic vacuoles⁵²⁵. Although the explaining mechanism of ADCD has not been clearly described, the main hypothesis is that cells, to cope with intense or prolonged stress, perform more and more autophagy and, at the end, lead the cell to die because of severe metabolic or energetic deficiencies. ADCD is a type of cell death whose autophagy inhibition prevents cell death⁵²⁵. Autophagy has also been shown to facilitate cell death by specifically targeting substrates that lead to specific cell death or indirectly by using autophagosomes to assemble death-inducing complexes^{526,527}. Actually, by impairing the P62-NRF2 pathway and degrading ferritin that is a cellular iron protein, and the ROS scavenger catalase, an enzyme participating in the elimination of ROS, autophagy has been shown to respectively elicit apoptosis, ferroptosis, and necrosis^{528–530}. Autophagy is also required for inducing cell death in an immunogenic way, meaning that an anti-tumor immune response is elicited upon cancer cell death. That type of cell death is called immunogenic cell death (ICD) and is characterized by releasing tumor-associated antigens (TAA) and immunogenic molecules like high mobility group box 1 (HMGB1) and ATP and by calreticulin membrane exposure. As a result, DC gets activated, and TAA is processed on MHC molecules by autophagy in those APCs, eventually eliciting a potent anti-tumor immune response⁵³¹. In glioblastoma cancer cells, autophagy is required for the release of HMGB1 following chemotherapy treatment⁵³². Also, autophagy is essential for releasing ATP following cancer cell death⁵³³. Even though autophagy is not required for treatment-induced cell death, it appears necessary for the immunogenicity of the dying cells. ATP release seems critical as autophagy-competent dying cancer cells but not autophagy-deficient ones, succeeded in attracting DC and T lymphocytes within the tumor bed following chemotherapeutic treatment⁵³⁴. A recent paper highlighted the importance of the autophagy mechanism in releasing proteoglycan decorin by dying tumor cells through ferroptosis. As a result, this ligand binds the advanced glycosylation end-product-specific receptor on macrophages and triggers a pro-inflammatory cytokine secretion that promotes the implementation of an anti-tumor immune response⁵³⁵.

4.5 Targeting autophagy in cancers: therapeutic strategies and clinical studies

4.5.1 Autophagy inhibition as a strategy to overcome resistance to treatments

4.5.1.1 *Autophagy inhibitors used in clinics and research*

Both for biological and clinical purposes, autophagy inhibitors have also been developed. It is worth noting that autophagy can be inhibited at any step of the autophagy influx. However, most of the inhibitors target proteins involved in other biological functions and are not strictly specific to the autophagy process, forcing the experimentators that use those molecules to be cautious in interpreting the results obtained. The autophagy inhibitors' specificity also seems to depend on the concentration and the incubation time⁵³⁶. Among them, the most used autophagy repressors are the PI3K inhibitors. In mammalian cells, three classes of PI3K exist. While class I PI3K is a repressor of autophagy, PIK3C3 is essential for initiating the autophagy process. The class II PI3K has shown no impact on the autophagy process⁵³⁷. PI3K inhibitors such as 3-methyladenine (3-MA), wortmannin (wort), and LY294002 have been shown to inhibit the autophagy process through the repression of the class III PI3K^{538–540}.

As the class III PI3K necessary for the induction of autophagy acts downstream of the class I PI3K inhibitory activity on the autophagy process, the expected overall effect of these inhibitors is to block the autophagy mechanism. However, it is worth noting that depending on the condition and the time of incubation, 3-MA can both positively or negatively regulate autophagy. Actually, under nutrient-rich conditions and a prolonged time of incubation, 3-MA can promote autophagy⁵³⁸. Wortmannin differs from 3-MA by the fact that it has been shown to have a persistent inhibitory effect on class III PI3K and a transient inhibitory effect on class I PI3K⁵³⁹. LY294002 has also been shown to induce autophagy through class I PI3K inhibition⁵⁴¹. To overcome misinterpretations of autophagy involvement due to the use of PI3K inhibitors, Pasquier et al. designed the only inhibitory drug specific to the autophagy process, the SAR405. By inhibiting the VPS34, the latter is also an inhibitor of the class III PI3K but is more specific than the other agents previously described⁵⁴². It is now widely accepted that under 10 μ M, SAR405 represses only the class III PI3K and autophagy without affecting the class I and II PI3K.

Other autophagy inhibitors targeting the initiation or the elongation steps have been developed, such as SBI-0206965, which targets ULK1 and NSC185058, repressing ATG4 activity^{543,544}. Cycloheximide, an inhibitor of protein synthesis, has also been shown to prevent starvation-induced autophagy through an mTOR-dependent pathway⁵⁴⁵. Another class of agents that are mainly used to evaluate the importance of a complete autophagy flux in the cell is the vacuolar-type H⁺ ATPase (V-ATPases) inhibitors. V-ATPase is found within membranes of many organelles, notably lysosomes, endosomes, and secretory vesicles, playing a crucial role in organelle

function, notably by promoting their acidification⁵⁴⁶. As the most used V-ATPase inhibitor, bafilomycin A1 (baf), by preventing the acidification of lysosomes and endosomes, has been shown to impair autophagosome fusion with lysosome and, therefore, autophagosome degradation. As a result, autophagosomes accumulate within the cell after baf treatment⁵⁴⁷.

Furthermore, other agents have been described as inhibiting autophagy at the process's last step. Those agents, called lysosomal lumen alkalizers, inhibit autophagosome degradation by impairing lysosome acidification and, therefore, proteases activation. Among them, the most known and used are chloroquine (CQ) and its derivative, hydroxychloroquine (HCQ), which are already used as anti-malarial therapeutic agents⁵⁴⁸. Lys01 and Lys05, new derivatives of CQ, have been shown to exert 10-fold more potent autophagy inhibitory activity than HCQ⁵⁴⁹. Finally, upon fusion of the autophagosome with the lysosome, autophagosome degradation can be prevented by a class of agents called the acid protease inhibitors that leads to the accumulation of autolysosomes within the cell. Among the lysosomal proteases and hydrolases that take part in the degradation of autophagosome cargo, cathepsins are one of the leading actors⁵⁵⁰. Among the acid protease inhibitors, both E64d and pepstatin A have been shown to inhibit autophagy at the last step by repressing cathepsins activities. While E64d has been demonstrated to inhibit cathepsins B, H, and L, pepstatin A represses cathepsins D and E⁵⁵¹. It is worth noting that using those agents to evaluate the autophagy process seems complicated by confocal microscopy and through the visualization of autophagosomes represented by GFP-LC3 puncta. It was demonstrated that despite the inhibitory action of those agents on autophagosome cargo destruction, the GFP-LC3 is degraded, resulting in an unexpected decrease in the visualization of autophagosome number after treatment⁵⁵².

4.5.1.2 Preclinical and clinical studies aiming at inhibiting autophagy to boost therapeutics efficiency

Given the critical role of autophagy in promoting cancer progression and resistance to most of the conventional anti-tumor therapies in various types of cancer, many studies have focused on combining an anti-tumor treatment with an autophagy inhibitor. Although different autophagy inhibitors have demonstrated efficiency in boosting therapeutics treatments, only the already approved FDA autophagy inhibitor, chloroquine, and its less toxic derivative, hydroxychloroquine, which both inhibit autophagy at a later stage, are used in ongoing clinical trials. Among the autophagy inhibitors having demonstrated efficiency in boosting anti-cancer therapeutics, 3-MA has been shown to increase sorafenib efficiency in hepatocellular carcinoma, while SAR405 has been demonstrated to promote PD-1/PD-L1 ICB efficiency in colorectal cancer and melanoma through autophagy inhibition^{499,553}. Spautin-1, which inhibits autophagy through Vps34 degradation, has been shown to increase imatinib efficacy in chronic myeloid leukemia⁵⁵⁴. Lys05 also increases sunitinib efficiency in clear-cell ovarian carcinoma⁵⁵⁵. Another autophagy inhibitor, chemically designed from HCQ to increase autophagy inhibition efficacy, anti-tumor efficiency, and reduce toxicity, the ROC-325 has been shown to increase the anti-tumor activity of azacitidine and mice

survival without leading to increased toxicity in acute myeloid leukemia⁵⁵⁶. In addition to those autophagy inhibitors, more specific ones like ATG4 or ULK1 inhibitors have been designed. First preclinical studies involving those agents have demonstrated encouraging results, and further experiments should be performed to evaluate their efficacy^{557,558}. Although autophagy inhibition has already been shown to be efficient in partially overcoming the resistance and boosting therapies efficiency in preclinical studies, clinical studies aiming at evaluating its safety and efficacy are ongoing (exclusively phase I and II studies)⁵⁵⁹. The effectiveness of combining treatment-induced autophagy (most chemotherapies, radiation therapy, targeted therapies...) and an autophagy inhibitor has demonstrated both safety and better efficacy in various types of cancer than the anti-tumor therapy alone⁵⁶⁰. The first clinical evidence of the therapeutic efficacy of targeting autophagy in cancer therapy comes from a trial where glioblastoma patients receiving CQ plus the combination of radiotherapy and temozolomide alkylation exhibited higher median survival than patients treated with the combination alone⁵⁶¹. A study comprising 73 NSCLC or breast cancer patients with brain metastasis exhibited higher control of brain metastasis when the radiation therapy treatment was combined with CQ, with no increase in toxicity⁵⁶². Also, in a phase Ib/II clinical trial, combining chemotherapy with HCQ has been demonstrated to reverse chemotherapy resistance in advanced NSCLC⁵⁶³. Combining targeted therapy and autophagy inhibitors has also been demonstrated to be clinically relevant. As in pancreatic cancer, targeting the RAS pathway has been shown to induce autophagy, combining an inhibitor of RAS and HCQ has demonstrated *in vivo* better efficiency in eliminating tumor cells⁵⁶⁴. Impressive results were obtained in BRAF-mutated cancer cells by combining a BRAF inhibitor with CQ in glioma. The combination was demonstrated to decrease metastasis development and improve patient survival⁵⁶⁵.

Although CQ and HCQ, in combination with anti-tumor therapies, are usually well tolerated, in most cases, it leads to modest clinical benefits. Various reasons can explain that mechanism: firstly, the lack of selectivity of CQ and HCQ that also impacts other biological processes like secretion. Also, like all treatments, its administration leads to the distribution of the autophagy inhibitor to unwanted non-malignant cells. It can be easily believed that autophagy inhibitors can also impact immune cells, impairing their function and the implementation of a robust anti-tumor immune response. Finally, in response to autophagy inhibition, cells have developed compensatory mechanisms like the DNA damage response (DDR) to promote survival, limiting autophagy inhibition-induced cell death⁵⁶⁶.

4.5.2 Boosting autophagy as a strategy to promote immunogenic cell death (ICD)

However, it is worth noting that contrary to the widely used strategy consisting in inhibiting autophagy to improve the effectiveness of anti-tumor treatments, an emerging field of research consists in boosting autophagy induction to induce cell death in an immunogenic way (ICD)⁵⁶⁷. Both chemotherapies like cisplatin, oxaliplatin, and taxanes, radiation therapy, and targeted therapy like crizotinib have been shown

to induce ICD, the involvement of the autophagy following those treatments has not been precised⁵⁶⁸⁻⁵⁷¹. Even though cancer cells can dye independently of autophagy, studies have demonstrated that autophagy is essential for releasing ATP and HMGB1, two key immunogenic molecules released after ICD^{532,533}. Moreover, Michaud et al. clearly showed that upon methotrexate treatment, autophagy-competent cancer cells were more likely to be completely eliminated than autophagy-deficient ones. That difference was explained by the lower capacity of dying autophagy-deficient cancer cells to release immunogenic molecules and to attract DC and T lymphocytes than the autophagy-competent cancer cells⁵³⁴. The main way currently used to induce autophagy and boost ICD consists in inhibiting the central repressor of autophagy, mTOR (rapamycin and its analogs, temsirolimus and everolimus), or activating its antagonist, AMPK (metformin)⁵⁷². After having demonstrated anti-cancer efficiency in preclinical studies, those non-specific autophagy inducers are currently used in ongoing clinical trials against various cancer types alone or in combination with other treatments. For example, everolimus combined with bevacizumab or gefitinib in advanced papillary renal cell carcinoma or triple-negative breast cancer has demonstrated better efficacy than each treatment alone^{573,574}.

However, given that most clinical trials performed on NSCLC patients consisting in combining an inhibitor of mTOR with another anti-cancer treatment are phase I, very few data concerning the efficacy of this combination regimen are available. Although NSCLC patients well tolerate those treatments, the few data concerning their effectiveness are not really encouraging. Actually, the combination of everolimus or metformin with chemotherapies has respectively demonstrated modest clinical improvement or a worse treatment efficacy and greater toxicity^{575,576}. Similarly, the combination of everolimus with erlotinib in a phase II failed to show better efficiency and increased toxicity for NSCLC patients⁵⁷⁷. Also, many clinical trials consisting in testing the efficacy of combining one of those autophagy inducers with radiation therapy, chemotherapy, and immunotherapy are currently ongoing on NSCLC patients. For example, one clinical trial on NSCLC patients assesses the efficiency of combining rapamycin with an immunotherapy drug, the durvalumab⁵⁷⁸. The failure of mTOR inhibitors to improve the effectiveness of anti-cancer treatment can notably be due to the non-specificity of those agents, therefore, immune cells which are dependent on mTOR are negatively impacted by those drugs.

Another emerging strategy to boost ICD consists in starving cancer cells through different regimens: starvation, fasting-mimicking diet (FMD), caloric restriction, and caloric restriction mimetics (CRM)⁵⁷⁹. FMD involves either a reduced caloric intake with a change in diet (usually a decrease in proteins and increase in fat proportion called ketogenic diet) or intermittent fasting⁵⁸⁰. By contrast with its other analogs, CRM reproduce the same biological and biochemical changes in cancer cells and the same anti-cancer effectiveness but without impacting the global nutritional status of cancer patients. Therefore this new type of treatment could be easier physiologically and psychologically tolerated by cancer patients⁵⁸¹. Although the molecular targets and the resulting biochemical changes can differ depending on the treatment, all those

treatments have been shown to promote cancer cell death by ICD through autophagy induction^{582,583}. Therefore the efficacy of combining those treatments with other ICD inducers or ICB has been tested. In lung cancer, Pietrocola et al. demonstrated *in vivo* that in lung cancer, CRM, like the hydroxycitrate, an inhibitor of ATP citrate lyase, was sufficient to reduce tumor mass through an increase of anti-tumor activity within the tumor site. In addition, he showed that combining various CRM with chemotherapies promoted the latter's effectiveness through ICD induction. That mechanism was only observed in autophagy-competent tumor cells and not in autophagy-deficient, therefore demonstrating the requirement of autophagy in that process⁵⁸⁴. Another study showed that combining crizotinib in an ALK-positive lung tumor with an FMD boosted targeted therapy efficiency⁵⁸⁵.

Given that numerous preclinical studies have been performed on various cancer with very promising results, clinical trials involving in most cases, FMD, in combination with chemotherapies-induced ICD or with ICB or both have been carried out with encouraging results. Concerning lung cancer management, two clinical trials are ongoing. The first one consists in combining chemotherapies (cisplatin/carboplatin + pemetrexed) plus metformin with FMD⁵⁸⁶. In the second clinical trial, NSCLC patients are treated with FMD, in combination with carboplatin and pemetrexed plus an ICB, the pembrolizumab⁵⁸⁷. Additional clinical trials are ongoing in other types of cancer and should precise the rationale of using those starvation-induced autophagy agents in clinics.

Autophagy-induced ICD has also been demonstrated for oncolytic viruses (OV). For example, the Newcastle disease virus (NDV) has been shown to induce the release of ICD molecules like HMGB1, Heat shock proteins 70/90, and calreticulin exposure in autophagy-competent lung cancer cells but not in autophagy-deficient ones, suggesting that autophagy is a critical process for ICD induction following lung cancer infection by NDV. Besides, treatment of lung cancer-bearing mice with supernatants from NDV-infected autophagy-competent lung cancer cells is sufficient to significantly reduce tumor progression, suggesting that the agents released are enough to promote the implementation of an anti-tumor immune response⁵⁸⁸. Also, it should be noted that even if other OV, naturally or genetically modified to induce a most significant autophagy response, have been shown to die in an autophagy-dependent way, the impact on the immune response has not been evaluated therefore limiting the application of the ICD term^{589–591}.

4.5.3 Clinical study of autophagy in lung tumor

As evoked previously, autophagy in NSCLC has been described as a double-edged sword depending on the cancer stage. Although initially acting as a mechanism limiting cancer onset, autophagy favors tumor progression when the cancer is established. Despite numerous preclinical studies showing a detrimental effect of autophagy in tumor progression and mice survival, relatively few studies have demonstrated its impact on cancer patients. This situation comes from the difficulty of having specific

biomarkers that specifically reflect a change in the level of autophagy in tumor cells, which is necessary to evaluate this mechanism's impact on cancer patients' survival.

Among the techniques mainly used to study the correlation between the autophagy level and cancer patients' clinical data, the immunohistochemical staining of autophagic proteins is the most used. Among them, most studies have focused on the analysis of LC3 and p62 proteins. In most of the studies on different types of cancer, the expression of the LC3 protein is associated with a poor prognosis in patients and reduced survival, which can be explained by an increased proliferation and development of metastasis^{592,593}. In lung cancer, only two studies have been performed with contrasting results. While a study demonstrated that LC3 expression was not associated with NSCLC patients' survival, another one showed that LC3 expression in puncta, named Stone-like structures (SLS), was associated with a bad prognosis for patients^{594,595}. As the p62 protein is an autophagy receptor that is degraded during the autophagy process, its accumulation is often considered as an impaired autophagy process, and its expression is often associated with a worse overall survival for patients in various cancer types⁵⁹⁶. Concerning NSCLC, different studies have been carried out to determine the potential correlation between the expression level of this protein with NSCLC patients' survival. All studies demonstrated that p62 expression was associated with higher aggressiveness and bad patient prognosis^{597,598}. However, it is interesting to note that contrary to the two other autophagy proteins, Beclin-1 expression has been associated with a better prognosis for NSCLC patients⁵⁹⁹.

As an emerging means of evaluating autophagy impact in cancer patients and in contrast to studying the expression of autophagy genes, more and more studies are focused on studying the expression of molecular actors that can regulate the autophagy process, like the long non-coding RNA. Actually, in NSCLC patient, Jiang et al. identified 16 autophagy-related lncRNA whose expressions have a prognostic value for NSCLC adenocarcinoma patients⁶⁰⁰.

To sum up, the impact of the autophagy level in NSCLC patients is not well established and depends on the protein expression measured. Besides, it has yet to be discovered, apart from classical living conditions reigning in the TME, how autophagy can be modulated in an NSCLC context. Finally, given that autophagy is an essential mechanism for the function of all cells composing the TME, the variation of the autophagy activity can have various and contrary impacts on tumor progression depending on the cell types studied. To the best of my knowledge, only a few studies have focused on studying the impact of the autophagy level only on malignant cells to study its effect on NSCLC patient survival. During my Ph.D., I tried to answer those three issues to determine how in an NSCLC context, autophagy can be modulated on malignant cells and what the impact is on tumor progression and patient survival. Thanks to bioinformatic studies on a public cohort of NSCLC patients in which transcriptomic analysis was performed, correlation studies between autophagy genes and other genes were carried out. Among the genes that strongly correlate with autophagy genes both in bulk and in lung tumor cells, we have observed that TLR7

was one of the top, suggesting a potential molecular link between autophagy and TLR7. That observation was particularly interesting for three reasons. The first one is that in previous work, the lab team has demonstrated that TLR7 expression in NSCLC patients was associated with a bad prognosis^{601,602}. The second reason is that TLR has already been shown to induce autophagy in a non-tumoral model, with TLR7 responsible for the biggest induction²⁹⁹. The last one is that various TLRs, not TLR7, have been shown to promote multiple pro-tumoral effects through autophagy induction^{300,603}.

5 Toll like receptor 7: An immune receptor to modulate autophagy in lung cancer?

5.1 Nature and distribution

5.1.1 The TLR family

Although their discovery is ancient and was discovered in drosophila, their role in immunity was found later. Initially, researchers thought that TLR was a receptor involved in embryonic development, as it appeared necessary to implement the dorso-ventral polarization. Latter, its importance in immunity was revealed as a deficiency in that receptor or molecules involved in its signaling has led to an increase of mortality in drosophila, associated with an impairment of anti-microbicidal peptides synthesis. Latter, an analog of that receptor was discovered in humans, the TLR4, and then others were gradually revealed. TLR analogs were also found in mice. Nowadays, respectively 10 and 12 TLR are described in humans and mice. Among them, the TLR1 to 9, except TLR8, are conserved in humans and mice, each recognizing specific ligands identical in the two species. In mice, because of a viral insertion into its coding gene, TLR10 is non-functional, whereas, in humans, TLR11 to 13 have been removed from the genome⁶⁰⁴.

As the first line of defense, both immune cells (dendritic cells, macrophages, B cells) and non-immune cells (epithelial cells and fibroblasts) express receptors that recognize a broad range of dangers (external and internal) and, in response, induce the expression and secretion of molecules that will directly or indirectly (through the implementation of an immune response) remove the danger. These receptors are called PRR and comprise the TLR. While TLRs are mainly present at the cell membrane, some are exclusively present in endosomes (**Figure 16**).

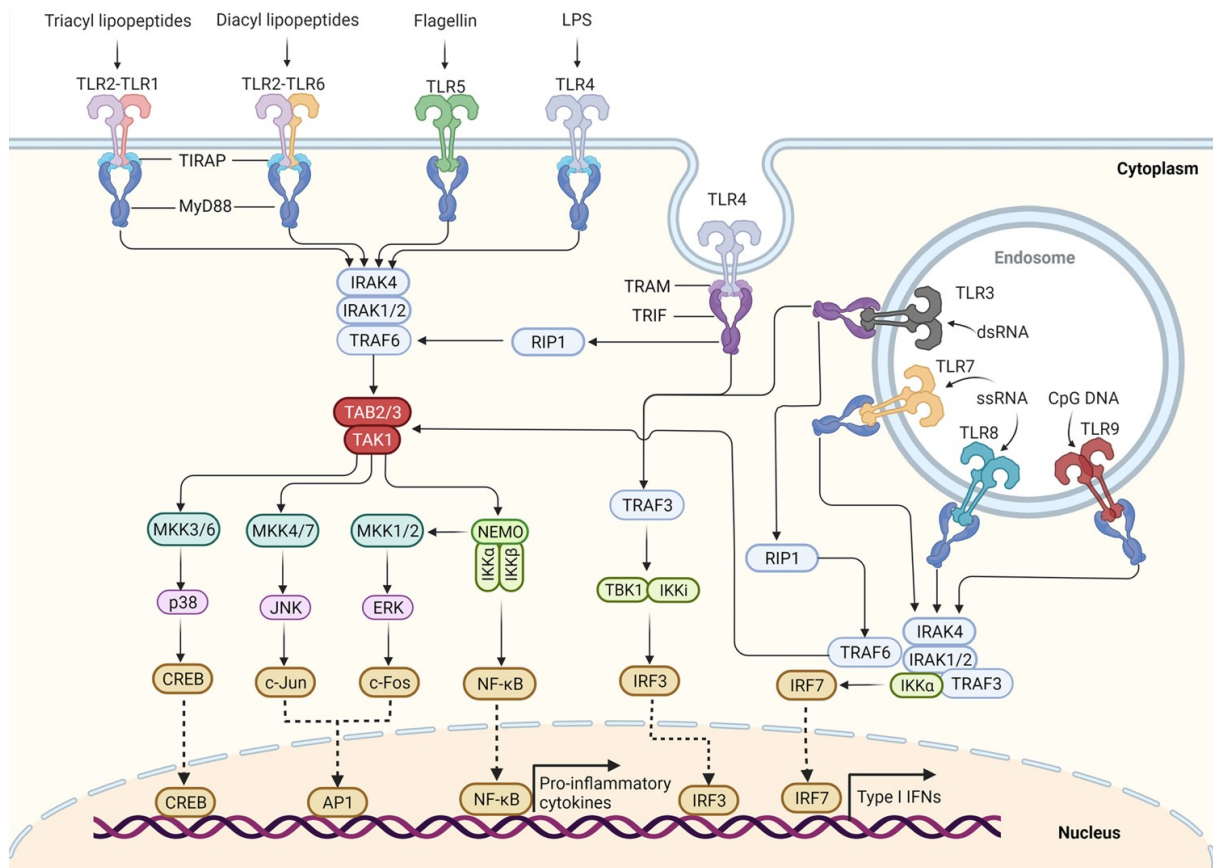


Figure 16. Distribution of TLRs within cells and description of TLRs ligands and signaling pathways induced (Performed on biorender).

Those latter TLRs are TLR3, 7, 8, and 9. Although initially described as a receptor recognizing pathogens elements (PAMP) exclusively, an increasing number of studies have demonstrated that, in the absence of pathogens, both TLR present at the cell membrane and in the endosomes can recognize elements released by live cells or upon they die. These elements, called DAMP, are considered as a danger by the cell since, physiologically, these elements are not released by the cell and do not trigger TLR activation. Therefore, their abnormal presence is the reflection of a pathological situation. Both membranous and endosomal TLR recognize PAMP, while the recognition of DAMP is mainly mediated by TLR present in endosomes through the sensing of self-nucleic acid. TLR can identify a broad range of microbes that can enter the organism (bacteria, viruses, fungi, and parasites). Given that most pathogens must enter cells to survive and proliferate, endosomal TLR can also discriminate pathogens by recognizing differences in the nucleic acid between the cell host and the pathogen invader⁶⁰⁴.

Although their localization and their ligand can differ, TLRs share a similar structure. Their extracellular or endosomal domain contains between 19 to 25 copies of the leucine-rich repeat (LRR) motif. Each LRR is a 24 to 29 amino acid sequence, including the leucine-rich sequence. Each has a β -strand and an α -helix connected by loops that organize the domain similar to a horseshoe, necessary for recognizing the ligand. Although the structure of the domain is conserved among the TLR, they can recognize

a broad range of structurally different ligands. That ligand recognition site is then connected to the intracellular domain necessary for signal transduction by a transmembrane domain. That intracellular domain comprises a conserved region of nearly 200 amino acids, named the toll/interleukin-1 receptor (TIR) domain. Within this domain, three conserved boxes are present and are crucial for implementing transduction signaling. These different boxes have a 20 to 30% of sequence homology with each other. By crystallography studies, the TIR structure was revealed and comprised five stranded parallel β -sheet at the center, surrounded by six α -helices, where loops realize the interconnection between helices and strands. For example, the BB loop that links the strand β - β and the helix α - β is an essential component with boxes 1 and 2 for the TIR interaction with the first component of the signaling pathway, the adaptor protein. After ligand binding, the TLR operates a conformational change that renders accessible the TIR domain to the molecular actors of the signaling pathway and, first of all, the adaptor proteins⁶⁰⁵.

5.1.2 TLR7, a receptor for DAMP and PAMP tightly regulated

Like the other TLR, TLR7 is expressed both in immune cells and in non-immune cells like fibroblasts, endothelial, and epithelial cells^{606–609}. Actually, TLR7 is an immune receptor expressed constitutively in specific immune cells like plasmacytoid dendritic cells (pDC) or B cells. In contrast, its expression is very low but increased following danger sensing in macrophages, hepatocytes, epithelial cells, and keratinocytes in an IFN-I dependent pathway⁶¹⁰. TLR7 is an endosomal receptor that recognizes naturally single-stranded RNA (ssRNA) but also small synthetic molecules, the imidazoquinoline derivatives (including resiquimod and imiquimod) and guanine analogs (notably loxoribine) through two different sites. These two sites work synergically, leading to a bigger stimulation when the two sites are bound⁶¹¹. As it recognizes ssRNA, it is a pivotal actor in both DAMP and PAMP sensing. TLR7 has been demonstrated to identify both ssRNA viruses (Vesicular stomatitis virus (VSV), influenza A virus (IAV), respiratory syncytial virus (RSV), and human immunodeficiency virus (HIV)) and RNA bacteria as group B streptococcus^{612,613}. In addition, TLR7 recognizes cellular ligands as ssRNA released following cell death or various non-coding RNA (ncRNA) secreted by live cells^{614,615}.

Given the capacity to recognize self elements, perturbations in TLR and notably in TLR7 signaling have been linked with the development of autoimmune diseases⁶¹⁶. Evolution has developed several mechanisms of protection to reduce this dysregulation, notably leading the TLR responsible for endogenous ligands recognition to be localized in endosomes and not in the membrane. That process renders endosomal TLR less likely to become activated by endogenous ligands and to induce chronic inflammation favorable for autoimmune diseases and tumor development. As an amplifying mechanism, free ssRNA or ssDNA are rapidly degraded by nucleases in the extracellular space⁶¹⁷. In addition to the mechanisms that mediate TLR7 signaling regulation, the delivery of TLR7 ligands is also a tightly regulated process. Actually, following viral infection or after agonist release by living cells or upon their dye, the

TLR7 ligands have to be transported to the endosome for signaling. As for some viruses infecting cells, TLR7 agonists can enter the cells through specific membrane receptor-mediated endocytosis^{618,619}. The importance of that regulatory mechanism has been demonstrated in autoimmune diseases where self-DNA is transported inside the cell to the endosomes thanks to the associated binding immunoglobulin and its recognition by a membrane receptor, CD32, in B cells. Besides, the activity of immunoglobulin also protects self-DNA from being degraded by nucleases in the extracellular space⁶¹⁹.

In addition to its localization that renders it less likely to become activated by endogenous ligands, TLR7 signaling activity is tightly regulated through two processes. First, except in specific immune cells like plasmacytoid dendritic cells (pDC) or B cells whose TLR7 expression is constitutive, its expression in other expressing cells like macrophages, hepatocytes, epithelial cells, and keratinocytes is very low but increased following danger sensing in an IFN-I dependent pathway⁶¹⁰. The second reason is to become activated in the endosome, it has to migrate from the endoplasmic reticulum (ER (site of production)) to the endosome compartment (site of action), and that migration is well orchestrated⁶²⁰.

In cells expressing TLR7, its expression is mediated following NF- κ B signaling. Then, after its transcription in the nucleus, TLR7 is translated into the ER, where ER-resident chaperone proteins, named gp96 and protein-associated with TLR4 (PRAT4A), are present. These two proteins are required to realize the fine conformation for TLR7, necessary for ensuring its function, before exiting the ER^{621,622}. At this stage, TLR7 is maintained in the ER. TLR7 is then managed in the ER by two proteins, the leucine-rich repeat-containing protein 59 (LRRC59) and the uncoordinated 93 homolog B1 (UNC93B1). These two proteins allow the packaging of TLR7 into coat protein complex II (COPII)-coated vesicles to exit the ER and translocate to the Golgi⁶²³. In addition to TLR7, other proteins are packaged in that vesicles, including UNC93B1 and the adaptor proteins (AP). It is interesting to note that the own stimulation of the TLR7 by imiquimod or other receptors of the same family, the TLR4 and 9, has been shown to promote ER exit and the endosomal localization of TLR7 in murine myeloid cells⁶²⁴.

In this later organelle, UNC93B1 ubiquitinates TLR7 and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) / endosomal sorting complexes required for transport (ESCRT) machinery to permit TLR7 transport from the Golgi to the endolysosome⁶²⁵. Other proteins intervene to transfer the TLR7 in specific sorted endosomes according to the molecular pathway induced following TLR7 stimulation, either NF- κ B or IFN-I. These proteins are the adaptor protein (AP)-3 and 4. AP-3 translocates TLR7 into IFN-I production-specific endosomes, whereas AP-4 is necessary for the translocation of TLR7 into endosomes wherein the NF- κ B response is elicited^{626,627}. Although TLR7 has reached the endosome wherein it realizes its inflammation-promoting actions, the TLR7 receptor is under an inactive form and has to become activated through a well-organized mechanism. TLR7, as for all endosomal TLR, has to be cleaved by specific proteases that are activated under endosomal

acidification to become functional. After translocation of the TLR7 to the endosomes, endosomal acidification occurs, which allows the activity of proteases like the furin-like proprotein convertase (PC) in humans, whereas, in mice, this mechanism is mediated by the cathepsins and the asparagine endopeptidases. These proteases cleave TLR7 inside its N-terminal ectodomain, separating the N-terminal ectodomain from the rest of the TLR7 structure, eventually converting TLR7 from an inactive form into an active one. The N-terminal ectodomain is already linked with the C-terminal ectodomain through disulfide bonds^{628,629}.

5.2 A pivotal actor of TLR signaling

Although the molecular actors recruited can differ according to the TLR, they all induce the same intracellular pathway leading to the mobilization of three molecules producer signaling pathways: the nuclear factor-kappa B (NF- κ B), the type 1 interferon (IFN-I) and the MAPK pathways (**Figure 16.**). The induction of these pathways results in the secretion of various molecules that are, by their direct or indirect (through the induction of an immune response), essential for removing the external or internal danger. When a TLR recognizes its ligand, its binding leads to a conformational change in the TLR structure that allows a homo or heterotypic interaction of TLR and the recruitment of proteins necessary for signal transduction. The first molecules recruited are the adaptor molecules, MyD88 and the TRIF, whose recruitment differs according to the TLR activated. Except for TLR3, all the TLRs (including the TLR7) perform their signaling through myeloid differentiation primary response 88 (MyD88), whereas TLR3 recruits TIR-domain-containing adapter-inducing interferon- β (TRIF) to ensure its signaling. TLR4 can recruit both and realize its signaling through a MyD88-dependent or independent pathway⁶⁰⁵.

5.2.1 Nuclear Factor-kappa B (NF- κ B) pathway

After its ligand binding and the resulting conformational change, in most cases, TLR recruits at its site, MyD88 (MyD88-dependent pathway), which has the particularity to comprise structurally two key domains, a TIR domain that allows its recruitment to the intracytoplasmic part of the TLR and a death domain (DD) that permits its association with downstream signaling molecules of the cascade, that also have a DD domain. Then, after being recruited, MyD88 facilitates interleukin-1 receptor-associated kinase (IRAK)4 binding and activation, leading to IRAK1 phosphorylation. That phosphorylation activates IRAK4, that in turn phosphorylates itself, favoring tumor necrosis factor receptor-associated factors (TRAF)6 recruitment. The latter protein is characterized by two key domains necessary to ensure its function. TRAF6 comprises a structure called TRAF-C that allows interaction with the upstream actors of the cascade. Moreover, at his N-terminal extremity, it contains a coiled-coil domain (called TRAF-N) comprising a structure named RING, which is essential for activating downstream molecular actors of the cascade, like TGF- β -activated kinase 1 (TAK1),

TAK1 binding protein (TAB)1, and TAB2. In addition to being a key actor of the NF- κ B signaling, TRAF6 has also been demonstrated to act as a signaling mediator for the TNF-receptor superfamily and directly interact with members of that family, like cluster of differentiation (CD)40 and TRANCE^{605,630}.

Then IRAK4 phosphorylates IRAK1, which leads to IRAK1 and TRAF6 dissociation from the TLR receptor and forms together a new complex with TAK1, TAB1, and TAB2, within TAK1 and TAB2 are phosphorylated, and IRAK1 degraded. The resulting complex is then translocated to the cytosol, which associates with two proteins, UEV1A, and UBC13, leading to the ubiquitylation of TRAF6 and the TAK1 activation. As a result, mitogen-activated protein kinase (MAPK), including Jun N-terminal kinase (JNK) and I κ B kinase (IKK) complex, are phosphorylated and become activated. The IKK complex comprises two catalytic subunits called IKK α and β and one regulatory subunit named IKK γ or NF- κ B essential modulator (NEMO). After activation, this complex acts on the main inhibitor of the NF- κ B complex, known as I κ B. NF- κ B includes P50, P52, C-REL, relA (p65), and relB proteins. NF- κ B complex comprises transcription factors acting in homodimers or heterodimers, mostly through P50/P65 combination. Usually, NF- κ B is maintained in an inactive form inside the cytosol by the negative action of I κ B. Upon IKK activation, I κ B is phosphorylated, ubiquitinated, and degraded. As a result, the NF- κ B complex is translocated from the cytosol to the nucleus, wherein it induces the expression of various genes whose functions are numerous and diverse. Molecules produced following the NF- κ B pathway can both modulate cell behavior like its survival and proliferation, and facilitate the implementation of an immune response by releasing pro-inflammatory cytokines and chemokines. Additionally, TLR3 and TLR4 experienced an NF- κ B signaling MyD88-independent pathway in which TRIF replaces MyD88. In contrast to a MyD88-dependent pathway, the cytokine production is delayed as the MAPK activation. This classical pathway for NF- κ B activation that involves either MyD88 or TRIF has been described following tumor necrosis factor alpha (TNF α) or IL-1 stimulation or after TLR, T-cell receptor (TCR), and B-cell receptor (BCR) engagement.

Recently, some studies suggest an alternative pathway for NF- κ B activation involving the NF- κ B-inducing kinase (NIK) protein that phosphorylates and activates IKK α . As a result, the precursor protein p100 is phosphorylated, ubiquitinated, and degraded by the Skp, cullin, F-box containing complex (SCF $^{\beta}$ -TRCP complex), leading to the release of the NF- κ B complex, its activation and translocation to the nucleus, and the transcription of the target genes. This alternative pathway is less frequent than the first and occurs after stimulation by particular stimuli such as lymphotoxin beta, B-cell activating factor (BAFF), and cluster of differentiation 40 ligand (CD40L)^{605,630}.

5.2.2 Type I Interferon (IFN-I) pathway

Although similar in their signaling, each TLR leads to the secretion of a specific profile of cytokines that permit the immune system to develop the most appropriate immune response against the pathogen-ligand recognized by the receptor. Although all the

TLRs have been described to induce NF- κ B signaling and the secretion of pro-inflammatory cytokines, some have also been shown to trigger a IFN-I response. In addition to the retinoic acid-inducible gene I (RIG-I)-like helicase (RLH) family, the TLR4 and all the endosomal TLR have been described to implement an IFN-I response. That response is the first anti-viral response implemented by the cells after infection by a virus. Following viral infection in immune or non-immune cells, the virus is recognized and leads to intracellular signaling and the production and secretion of IFN-I⁶³¹. Then after being released in the extracellular medium, this cytokine can either act in an autocrine or paracrine way and lead to the expression of hundreds of molecules exerting anti-viral actions. Mechanistically IFN-I binds membrane receptor composed of a heterodimer of interferon- α/β receptor (IFNAR)1 and 2. After binding, IFN-stimulated gene factor 3 (ISGF3) is activated, that in return activates the signal transducer and activator of transcription 1 and 2 (STAT1/2) and the IFN regulatory factor (IRF) 9. Eventually, this later translocates to the nucleus and induces the transcription of many genes exerting anti-viral actions. In addition to the expression of proteins with a direct anti-viral impact, other proteins induced affect the global immune response, favoring the maturation of DC, the cellular and humoral mediated immune response, and the recruitment of other immune cells to the danger site through the release of chemokines⁶³².

Depending on the TLR, the induction of IFN-I expression does not imply the same intracellular machinery, including different adaptor proteins and transcription factors. TLR4 and TLR3 have been described to stimulate IFN-I production through TRIF and IRF3, whereas TLR7 and 9 use the MyD88 protein and IRF7. IRF7 has been described to be interconnected with the NF- κ B signaling TLR7 induced. Actually, in the basal state, IRF7 forms a complex with TLR7, MyD88, and TRAF6, whereas upon stimulation by a TLR7 ligand, IRF7 is phosphorylated by IRAK1 and becomes activated. Then, it translocates into the nucleus to induce IFN-I expression. Contrary to IRF3, whose expression in various cells is constitutive, IRF7 is weakly expressed, but its expression can be induced by the IFN-I produced thanks to IRF3 action. In contrast, to ensure its function, pDC constitutively expresses high level of IRF7⁶³¹.

5.2.3 MAPK pathway

Given that the MAPK pathway is an intermediate between the extracellular signals present and the cell behavior, this signaling can be induced by diverse stimuli, notably after TLR stimulation. This pathway involves 3 families of kinase, the mitogen-activated protein kinase kinase kinase (MAPKKK) that phosphorylate and activate the mitogen-activated protein kinase kinase (MAPKK) that in turn phosphorylate and activate the MAPK. Then, the MAPK also phosphorylate many diverse proteins that exert different functions in the cell. The MAPK is a family of kinases comprising 6 members in mammals sorted into 3 groups: the JNK 1/2/3, the p38 $\alpha/\beta/\gamma/\delta$, and the ERK1/2, ERK3/4, ERK5, and ERK7/8⁶³³.

Although the activation of ERK has often been associated with Ras and MEK proteins, it can also be independent of Ras, notably in an inflammatory condition and after TLR

stimulation^{634,635}. After TLR engagement, ERK is phosphorylated and, in turn, phosphorylates and activates various differentially expressed transcription factors such as JUN, FOS, EGR-1, and MYC, which lead to the expression of numerous genes⁶³⁶. As a result, depending on the cell type, it mediates various biological processes like survival, proliferation, migration, and angiogenesis⁶³⁷.

In addition, after TLR stimulation, the JNK can be activated, and as a result, the transcription factors, like c-Jun. JNK and c-Jun play a key role in Ras-mediated tumorigenesis⁶³⁸. C-Jun has notably been demonstrated to inhibit p53 expression⁶³⁹. Among the many functions regulated by it, JNK seems to have a crucial role in DNA repair as JNK inhibitors action in cells treated by chemotherapies results in cell incapacity to repair DNA damages⁶⁴⁰. In contrast with NF- κ B, JNK seems to promote apoptosis, but the NF- κ B signaling gene products often repress JNK activity⁶⁴¹.

p38 is another JNK stimulated after TLR stimulation. Among its targets, it has been demonstrated to activate MK2, which switches on the small heat shock protein Hsp27⁶⁴². Among its roles, the p38 pathway is a crucial regulator of cell cycle progression, cell survival, and growth. p38 has been shown to facilitate p53-mediated apoptosis, whereas its inhibition during cells treated by chemotherapies enhances apoptosis, suggesting that p38 in this condition acts as a pro-survival inducing pathway^{643,644}.

5.2.4 Interconnection with other pathways

Besides inducing NF- κ B and IFN-I signaling, TLRs stimulation modify the activity of interconnected signaling pathways, notably, the phosphatidylinositol 3-kinase (PI3K) pathway. As many of its components interact with TLR molecular actors, the PI3K pathway is intrinsically linked with TLR signaling. Through its Src-homology 2 (SH2) domain, the PI3K p85 regulatory subunit interacts with a specific motif on TLR composed of Tyrosine-Xaa-Xaa-Methionine, Xaa meaning any amino acids⁶⁴⁵. That particular sequence is present only in TLR1, 2, and 6. Besides binding directly to the TLR, PI3K pathway components can bind downstream effectors of the TLR signaling. PI3K can specifically recognize a sequence present in the C terminal domain of MyD88, composed of Tyrosine257-Lysine258-Alanine259-Methionine260. TLR4 stimulation has been shown to favor this interaction through the phosphorylation of tyrosine residue⁶⁴⁶. Also, MyD88 has been demonstrated to interact with a downstream effector of the PI3K pathway, protein kinase B (AKT). Although the physical link has been well established between components of the TLR and the PI3K pathways, the impact of one pathway on the other must be elucidated. In one study, TLR4 stimulation has been demonstrated to promote AKT activity in a MyD88-dependent pathway. Also, in TLR4 and IL-1R stimulation conditions, p85 was essential for implementing the NF- κ B signaling, whereas it was useless for the induction of the same pathway following TNF stimulation⁶⁴⁷. This study suggests that these two pathways work together in TLR4 and IL-1R conditions: the stimulation of one pathway activates the other. However, another study opposes that hypothesis, as p85 inactivation in DC has led to

an increased expression of IL-12, suggesting that PI3K could act as a negative regulator of TLR signaling in DC⁶⁴⁸.

5.3 Regulation of TLR signaling

5.3.1 Regulation mechanism of TLR signaling shared by all members of the TLR family

Although not directly involved in intracellular signaling, some proteins are essential for TLRs stimulation. Among them, CD14, initially described as a protein containing specific motifs necessary for TLR4 endocytosis and TRIF signaling, is critical for TLR to induce the secretion of pro-inflammatory cytokines⁶⁴⁹. However, given the physiological importance of keeping inflammation under control, TLRs are tightly regulated in the cell to avoid excessive inflammation. Several mechanisms and proteins have been demonstrated to negatively control TLRs signaling (**figure 17**).

Among them, there are IRAK-M, the suppressor of cytokine signaling 1 (SOCS1), the MyD88 short (MyD88s), the single immunoglobulin IL-1R-related molecule (SIGIRR), and ST2⁶⁵⁰⁻⁶⁵⁴. Actually, as a mechanism of limiting inflammation, TLRs can enter into a tolerance phase after continuous stimulation by their ligands, leading them to no longer respond to a ligand. That mechanism, described in various cell types, is mediated by an upregulation of MyD88 pathways inhibitors, such as IRAK-M and Src homology 2 domain-containing inositol-5-phosphatase-1⁶⁵⁵. Similarly, the inhibitory process on TLR can also occur after continuous stimulation to another TLR and prevent the induction of an exaggerated inflammation that could have detrimental consequences for the organism⁶⁵⁶. Actually, immune cells previously treated with TLR4 inducer were no longer responsive to TLR7 ligand⁶⁵⁶. Contrary to the other members of its family, whose expression is constitutive and ubiquitous, IRAK-M expression is induced following TLRs stimulation and is restricted to monocytes and macrophages so far. Functionally, it inhibits the formation of the IRAK1-TRAF6 complex necessary for the TLR signaling by preventing the dissociation of the IRAK1-IRAK4 complex⁶⁵⁰. SOCS1 is another inhibitory molecule of TLR signaling whose expression is increased by cytokines. As its impact on inhibiting TLR signaling has been clearly demonstrated, the underlying mechanism still needs to be elucidated. Yet SOCS1 has been shown to interact with IRAK1 and IRF7⁶⁵¹. As a means of limiting TLRs activation, following TLR4 stimulation, monocytes have been described to upregulate a non-functional variant of MyD88, named MyD88s, that acts as a competitive inhibitor for the functional MyD88⁶⁵². Finally, other molecules like SIGIRR and ST2 have also been demonstrated to negatively affect TLRs signaling. Although physical interaction between SIGIRR and ST2 and the effectors of the signaling pathway have been observed, respectively, with

TLR4, IRAK1, TRAF6, and MyD88, the precise mechanisms underlying their inhibitory activity still need to be discovered^{653,654}.

5.3.2 Specific regulation mechanism of TLR7 signaling

After being transformed into a functional form, TLR7 signaling intensity is also positively or negatively modulated through the interaction with proteins. Also, TLR7 signaling is dependent on its interaction with the triggering receptor expressed on myeloid cells like 4 (TREM4). Genetic deficiency of this protein in DC is responsible for a decrease in TLR7 endosomal localization, TLR7-MyD88 interaction, and as a result, for a reduction of TLR7-mediated signaling and cytokine production, suggesting that TREM4 could favor TLR7 stability⁶⁵⁷. In addition, although TLR7 expression is facilitated through the IFN-I induction produced following the stimulation of other TLR, these PRRs can, by direct interaction, negatively regulate the TLR7 activity. TLR9 as TLR8 was demonstrated to act as a competitive inhibitor of TLR7 for its fixation to the Unc93B1 protein, necessary for TLR7 trafficking to the endosomes⁶⁵⁸.

Besides, although TLR8 recognizes the same ligands as TLR7, its importance in inducing inflammation is weak, as, in mice, its inhibition has demonstrated no negative effect on inflammation in response to its ligands⁶⁵⁹. On the contrary, TLR8 seems more likely to act as an inhibitory regulatory mechanism of TLR7 signaling. Its genetic inactivation in mice was responsible specifically in DC for an increased inflammation due to a hyperactivation of the NF- κ B signaling and overexpression of TLR7. This phenomenon could explain why in mice, the genetic deficiency of TLR8 leads them to develop autoimmune diseases. Mice double deficient for TLR7 and TLR8 do not develop autoimmune disorders⁶⁶⁰. Although the underlying mechanism that explains the inhibitory action of TLR8 on TLR7 has not been fully elucidated, TLR8 has been shown to be physically associated with TLR7 to inactivate it.

Molecules and pathways involved in the regulation of TLR7 expression are summarize in **figure I7**.

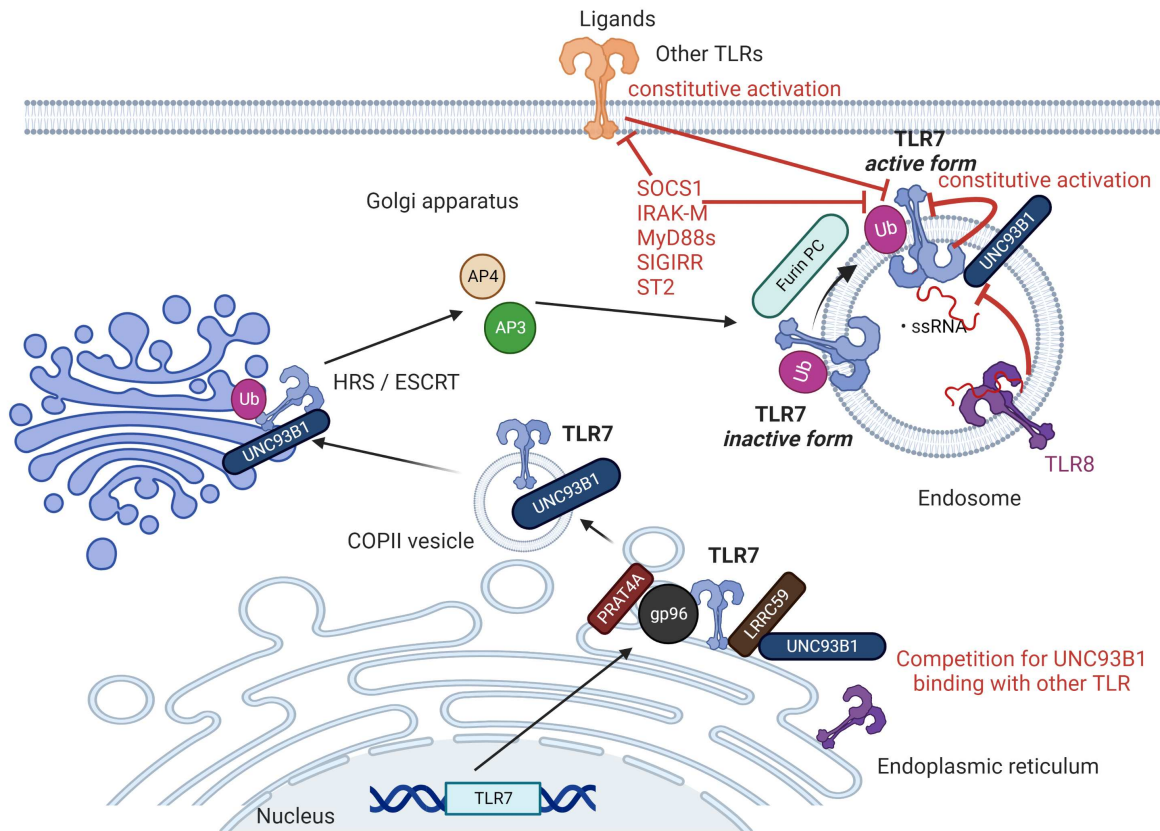


Figure 17. TLR7 trafficking and regulation of its activity (Performed on biorender).

5.4 TLR : a double edged sword in cancer therapy

5.4.1 TLR ligands : a deceptive therapeutic option in cancer treatment

TLR is a critical component of immune response induction in various immune cells. As TLR stimulation favor DC activation and NK, macrophages, B cells, and T cells activity, they are necessary for the induction of a robust anti-tumor immune response. Many studies have tried to use TLR ligands as adjuvants in vaccines or to implement a bigger anti-tumor immune response.

Despite the diversity of TLR and the possibility of combination regimen, so far, only three TLR agonists have been approved by FDA: the bacille Calmette-Guérin (BCG (Ligand of TLR2, 4, and 9)), the monophosphoryl lipid A ((MPL), TLR4 agonist) and the imiquimod (TLR7 ligand) for clinical use in human. BCG and imiquimod are respectively used for immunotherapy in situ bladder carcinoma and superficial basal cell carcinoma. In contrast to BCG and imiquimod, which act in curative, MPL works in preventive and is used as an adjuvant in the Cervarix formulation, a vaccine against human papillomavirus 16 and 18 that are oncogenic⁶⁶¹. Although various TLR agonists have been described to elicit an anti-tumor immune response in preclinical studies, in clinical trials, they fail to improve the progression-free survival or the overall survival of cancer patients⁶⁶¹.

5.4.2 TLR have a dual role in cancer development

The failure of TLR-based therapies can be explained by the fact that the TLR are not exclusively expressed by immune cells. They are also expressed in epithelial, endothelial cells, fibroblast, and even in tumor cells^{662,663}. Although the immune cells express the whole spectrum of TLR, whose stimulation usually elicits anti-tumoral effects, the TLR expression by tumor cells is dependent on the tumor, and generally, their stimulation lead to a pro-tumoral response. Therefore, depending on cancer and the TLR targeted, the TLR stimulation can be either pro-tumoral or anti-tumoral⁶⁶⁴.

Actually, endothelial cells (EC) have been described to express a broad range of TLR, and that expression depends on the tissue in which the endothelial cells are comprised. Human umbilical cord EC (HUVEC) express a high level of TLR1 to 4 and a low level of TLR5 to 10, whereas human EC composing the aorta highly express all the TLR except the TLR3 and 9. In non-tumoral models, their stimulation has been demonstrated to favor EC's invasion and migration capacity, therefore leading to angiogenesis. This process is mediated by the secretion of MMP proteins, pro-angiogenic factors like VEGF or their receptor VEGFR, or by the release of pro-inflammatory cytokines. In addition, the activation of the PI3K-Akt pathway in EC (notably following TLR5 stimulation) has also been described to favor angiogenesis⁶⁶⁵.

In addition, although epithelial cells from whom the tumor takes its origin naturally express TLR, their expression is significantly increased during tumorigenesis. That mechanism could be explained by the fact that chronic inflammation is a key driver of tumorigenesis and the leading cause of TLR expression. Therefore TLRs and chronic inflammation in tumorigenesis act as a positive loop, the stimulation of one naturally promotes the increase of the other. Generally, no matter the TLR activated in tumor cells, its stimulation leads to pro-tumoral effects. TLR stimulation favors tumor progression through different means, implying different intracellular pathways in tumor cells. Among them, there are the inhibition of apoptosis, the increase in their invasion, migratory and metastasis capacity, the induction of angiogenesis, and the evasion from the immune system. Interestingly, TLR stimulation-induced autophagy has been shown to mediate some of those pro-tumoral effects in diverse types of cancer^{300,666}. Therefore TLRs stimulation in tumor cells both lead to intrinsic and extrinsic changes that eventually lead to tumor progression^{667,663}.

5.4.3 TLR and lung tumor progression

To date, TLR1/2/3 and 5 stimulation have demonstrated anti-tumoral effects, whereas TLR4 and TLR7 stimulation have pro-tumoral effects⁶⁶⁴. Anti-tumoral effects in lung cancer following TLR stimulation could have different biological explanations. TLR1 and TLR2 stimulation lead to a decrease in Treg activity and an increase in the lysis activity of cytotoxic lymphocytes, whereas TLR3 stimulation favors the transition of macrophages from an immunosuppressive phenotype to an anti-tumoral phenotype^{668,669}. Besides, TLR5 stimulation has been described to favor the infiltration of anti-tumoral polynuclear neutrophils into the TME⁶⁷⁰.

In lung cancers, transforming epithelial cell has a higher expression of TLR4, 5, 7, and 9 than the normal epithelial cell^{601,671,672}. The impact of TLR9 activation is controversial. In one study, it has been shown to decrease immunosuppressive immune cells and boost the anti-tumor immune cells⁶⁷³. In contrast, in another study, TLR9 stimulation leads to the secretion of a broad range of molecules that could promote tumor progression⁶⁷⁴. TLR4 and TLR7 stimulation have been described to promote lung tumor progression by various means⁶⁷⁵. TLR4 stimulation has been shown to promote their survival and proliferation⁶⁷⁶. Also, TLR4 stimulation promotes their escape from the immune response through the secretion of immunosuppressive and pro-angiogenic cytokines (TGF β , VEGF, and IL-8) that implies p38 MAPK activation and by the resistance of TNF- α or TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis that is NF- κ B dependent⁶⁷⁷. TLR4 stimulation has also been shown to favor tumor cell motility and metastasis development in a p38 MAPK and extracellular signal-regulated kinase (ERK) dependent way⁶⁷⁸. In addition, TLR stimulation promotes the secretion of cytokines and chemokines that will recruit and activate pro-tumoral cellular components like the CAF or the endothelial cell to boost angiogenesis. Finally, TLR stimulation in tumor cells will also favor the recruitment and activation of immunosuppressive cells while inactivating anti-tumor immune cells^{667,663}.

5.5 TLR7: a pro-tumoral actor in NSCLC

5.5.1 TLR7 and cancers progression

TLR7 can be expressed in immune cells (monocytes, neutrophils, pDC, and B cells) and malignant cells (colorectal, esophageal squamous, lung, myeloma, and pancreatic ductal tumor cells). Studies have demonstrated an anti-tumoral impact of stimulating TLR7 in various cancers like hepatocellular carcinoma, breast cancer, melanoma, and leukemia. TLR7 activation has been shown to increase the lysis activity of T lymphocytes and NK cells or the sensibility of tumor cells to them⁶⁷⁹⁻⁶⁸¹. Also, TLR7 stimulation favors the activation of pDC, exerting anti-tumoral effects^{682,683}. However, the impact of TLR7 on tumor progression is still controversial in some tumors, such as pancreatic ductal carcinoma. Indeed, while studies described that TLR7 stimulation increased the lysis activity of $\gamma\delta$ T lymphocytes against tumor cells⁶⁷⁹, it has also been shown that its activation promoted tumor progression by favoring survival, proliferation, and expression of pro-tumoral molecules like TGF β ^{684,685}. In lung tumor cells, our team and others demonstrated that TLR7 has several pro-tumoral effects, impacting tumor growth in several aspects.

5.5.2 TLR7 expression is associated with a bad prognosis in NSCLC patients

In lung cancer, TLR7 is expressed in immune cells, including pDC, CD, macrophages, B cells, NK cells, and tumor cells. Systemic injection of TLR7 ligand has been demonstrated to induce a robust anti-tumor immune response observable by the

activation and maturation of DC, the increase of NK, NKT, and T cytotoxic lymphocytes, and by a decrease of immunosuppressive Treg⁶⁸⁶. Although TLR7 is a good inducer of anti-tumor immune response, its use in clinics has not proved anti-tumor efficacy⁶⁶¹. That failure can be partly explained by the fact that TLR7 is also expressed by the malignant cells, and its stimulation on tumor cells has various pro-tumoral effects^{667,663}.

Thanks to immunohistochemistry staining performed on slides from NSCLC patients untreated or treated with different chemotherapies (Combination of cisplatin with gemcitabine or cisplatin with vinorelbine), it has been demonstrated that the lung cancer patient's survival was correlated with the expression of TLR7 on lung tumor cells. TLR7 expression in lung tumor cells was associated with a bad prognosis for lung cancer patients at early or later stages and with a worse response to chemotherapeutic treatments. The more the NSCLC patients express TLR7 in their lung tumor cells, the less they respond to chemotherapies and survive⁶⁰¹.

5.5.3 TLR7 promotes survival and resistance to chemotherapies

From that clinical observation, Cherfils et al. have investigated *in vitro* the underlying mechanisms. After having demonstrated that both adenocarcinoma and squamous NSCLC cells express TLR7, whose expression is variable according to the patient, Cherfils et al. showed, using different TLR7 agonists (CL264 and loxoribine), that the lung tumor cells stimulated for TLR7 were more resistant against a broad range of chemotherapies (cisplatin and carboplatin alone or in combination with doxorubicin and navelbin) used in clinic than the non-stimulated tumor cells⁶⁰². These results were then confirmed using different mice models, like the wild type (WT), the nonobese diabetic / severe combined immunodeficiency (NOD-SCID), and the TLR7 knockout (KO) mice, and by grafting lung tumor cells. Both in untreated and chemotherapies treated mice, the tumor progression was increased after TLR7 stimulation compared with the non-stimulated condition, meaning that TLR7 stimulation in lung tumor cells promotes their survival during adverse conditions or following chemotherapeutic treatments. The same results were observed in WT versus NOD-SCID or TLR7 KO mice, suggesting that the immune system would have minimal impact on the survival and chemoresistance observed following TLR7 stimulation. Intrinsic changes in the malignant cells could mainly mediate these effects⁶⁰¹.

Mechanistically, they demonstrated that MyD88 is essential for TLR7-induced lung tumor cell chemoresistance. So far, the mechanisms underlying this phenomenon have not yet been understood. An explanation proposed is the increase of the anti-apoptotic protein BCL-2 expression observed following TLR7 expression, which could explain the resistance to cell death induced by chemotherapies drugs or adverse conditions. Similarly, Cherfils et al. observed the same results after TLR8 stimulation⁶⁰². However, the impact of BCL-2 in this chemoresistance has been questioned because TLR3 stimulation in these same cells has been shown to induce BCL-2 but not chemoresistance. Chemoresistance could result from the large secretion of molecules and pro-inflammatory cytokines released after TLR7 stimulation

in an NF- κ B dependent way. That hypothesis has been reinforced by the fact that TLR7 and TLR8 stimulated lung tumor cells have a cytokine secretion profile similar and different from that observed following TLR3 stimulation⁶⁰². Among the genes upregulated in lung tumor cells following TLR7 and TLR8 stimulation, there are notably: the cholesterol 7-alpha-monooxygenase (Cyp7A1), coding an enzyme involved in drug metabolism and detoxifying system that could promote chemoresistance against a large number of chemotherapies used. Also, a chemokine receptor, CCR4, is increased, which has already been demonstrated to be essential for lung tumor cells to develop metastasis in the bone marrow, suggesting that TLR7 could promote by this way metastasis development. In addition, both TLR7 and TLR8 stimulation lead to pro-inflammatory cytokines secretion, notably IL-6, IL-8, GM-CSF, and nitric oxide synthase 2 (NOS2), which has been demonstrated to be involved in the survival and proliferation of tumor cells⁶⁰².

5.5.4 TLR7 promotes metastasis development through notably MDSC recruitment

In another study, Dajon et al. demonstrated that in addition to induce malignant intrinsic changes required for lung tumor cells to survive under adverse conditions and chemotherapies, TLR7 stimulation in these cells has also been shown to increase the number of MDSC that promote tumor progression *in vivo*⁶⁸⁷. By using WT, NOD-SCID, and TLR7 KO mice, Dajon et al. demonstrated that this rise in MDSC number was the consequence of their recruitment by malignant cells following TLR7 stimulation mediated by the release of CCL2 and GM-CSF in the TME. Then, the importance of this immunosuppressive cell recruitment was evaluated using a depleting antibody to remove the MDSC from the NSCLC TME. As a consequence of the MDSC depletion, the tumor progression was decreased as well as the development of metastasis. In addition to favor the recruitment of MDSC, TLR7 stimulation in malignant cells also lead to changes in gene expression, notably with the increased expression of genes involved in the EMT and eventually to metastasis like E-cadherin, vimentin, SNAIL-1/2, and TWIST. That change in gene expression profile is also observable in adenocarcinoma NSCLC patients strongly expressing TLR7 that have an EMT and metastatic gene signature compared with those that weakly express TLR7⁶⁸⁷.

5.5.5 TLR7 is continuously exposed physiologically to its ligands

Interestingly Cherfils et al., by comparing the gene expression profile of lung tumor cells from NSCLC patients with those obtained *in vitro* in lung tumor cell lines after TLR7 or TLR8 stimulation, realized that the gene expression profiles were identical⁶⁰². These results strongly suggest that lung tumor cells from NSCLC patients have been stimulated by TLR7 and TLR8 ligands present in the TME, notably RNA viruses or RNA released by living cells or after their death. In addition, the correlation between TLR7 expression in malignant cells and survival prognosis both in untreated NSCLC patients and in patients treated by chemotherapies suggests that in the TME of lung cancer, malignant cells are continuously exposed to ligands that stimulate TLR7 and

mediate its pro-tumoral effects. So far, neither the TLR7 ligands involved nor the intracellular machinery induced by TLR7 to realize its pro-tumoral effects are completely understood yet.

One of my Ph.D. projects is based on the hypothesis that autophagy is the missing link between TLR7 stimulation and the induced pro-tumoral effects. That hypothesis is based on three main observations: i) TLR stimulation has already been demonstrated to induce autophagy in the murine macrophage model, whose TLR7 is among the other components of the TLR family, the one that leads the biggest induction²⁹⁹; ii) autophagy has been described to take part in every step of cancer development, mainly acting as a pro-tumoral mechanism when the tumor is established⁶⁸⁸; iii) in other types of cancer, TLR stimulation has already been shown to promote tumor progression through various means, notably by inducing autophagy^{300,689}.

RESULTS

1 Ph.D. objectives

The research team in which I carried out my thesis seeks to understand the molecular pathways that can impact the biology of lung cancer cells and the tumor microenvironment and, ultimately, the progression of non-small cell lung cancer (NSCLC). In previous work, the team demonstrated that during the process of tumorigenesis, TLR7 expression is induced in lung tumor cells and is associated with a poor prognosis and a lower response of NSCLC patients to chemotherapies. Although they demonstrated that TLR7 stimulation in lung tumor cells mechanistically promotes survival and chemoresistance to various anti-tumor chemotherapies, metastasis development, and recruitment of immunosuppressive cells within the tumor microenvironment, the underlying mechanism is yet unknown. During my Ph.D., I have hypothesized that autophagy, an intracellular catabolic mechanism essential for cell homeostasis, could be the missing link between TLR7 and the observed pro-tumoral effects. That hypothesis is based on three main observations: i) TLR stimulation has already been demonstrated to induce autophagy in the murine macrophage model, whose TLR7 is among the other components of the TLR family, the one that leads to the biggest induction²⁹⁹; ii) autophagy has been described to take part in every step of cancer development, mainly acting as a pro-tumoral mechanism when the tumor is established⁶⁸⁸; iii) in other types of cancer, TLR stimulation has already been shown to promote tumor progression through various means, notably by inducing autophagy^{300,689}. Although autophagy is present in all cells, tumor cells appear to depend more on autophagy to meet their metabolic needs and survive the adverse conditions in the tumor microenvironment. Autophagy in tumor cells has also been shown in numerous tumor models to promote resistance to various anti-cancer treatments commonly used in the clinic, the development of metastases, and escape from the immune system.

Therefore, it is not surprising that more and more studies are demonstrating that autophagy level is associated with a worse survival for cancer patients in various cancer types, and in the meantime, an increasing number of preclinical and clinical studies aim to target autophagy are ongoing. Despite these observations, very few data on the importance of autophagy in lung tumor cells and tumor progression are available. Therefore, after having previously set up in the laboratory the different tools allowing the study of autophagy, in particular, the stable transfection of lung tumor cells with the GFP-LC3 construct, as well as the different protocols, the objectives of my thesis consisted in (i) studying the molecular pathways by which autophagy could be regulated in NSCLC lung tumor cells and (ii) evaluating the impact of autophagy on NSCLC tumor progression and in patients

2 Study 1: Autophagy-related gene signature reflects proliferative and immune phenotype status of adenocarcinoma lung tumor cells

2.1 Summary and novelty

Autophagy, by its role in maintaining cell homeostasis, has been described as a key actor in cancer development. While its effect depends on the type and the stage of the tumor, autophagy has been demonstrated to promote cancer progression when the cancer is established by favoring survival, proliferation, and metastasis development but also resistance to conventional anti-tumor treatments and escape from the immune system. As evoked previously, little is known about the impact of autophagy on NSCLC progression.

Furthermore, it is important to note that the relevance of the autophagy genes expression in tumor patients has been very poorly understood, especially in NSCLC. In this study, we decided to investigate the expression of the autophagy genes in NSCLC and determine the impact it might have on the behavior of tumor cells, the immune microenvironment, tumor progression, and patient survival using transcriptomic analysis datasets.

In that article, we used a public cohort of NSCLC patients for which the whole components of the TME have been analyzed for their transcriptome (TCGA) and performed a differential expression analysis of the human autophagy database (HAD), which gathers all the genes related to autophagy, comparing the non-tumoral adjacent tissues versus tumoral tissues. Given that differences in autophagy gene expression profiles were too high between the different histological subtypes of NSCLC, we focused our analysis only on adenocarcinoma, which is the most represented subtype in NSCLC patients. Based on the profile of autophagy genes expression, we found that 35 genes were differentially expressed between adjacent tissues and tumor samples, with 23 genes that were decreased and 12 genes that were increased in the tumor. Interestingly, two clusters of tumor patients appeared (cluster 1 and cluster 2) based on this gene expression. Cluster 1, which in comparison with cluster 2 expresses more the signature B (genes increased) and less the signature A (genes decreased), is also associated with a worse overall and disease-specific survival of patients (**Figure 2 of paper**). By analyzing more precisely the whole gene expression profile between the two clusters of patients, we observed that cluster 1 exhibited more genes related to catabolism and proliferation than cluster 2 (**Figure 3 of paper**). Besides, we also demonstrated that cluster 1 expressed fewer genes related to immune response and, based on the MCP counter, are less infiltrated by T cells, B cells, dendritic cells, and neutrophils. We also noticed that the T cells exhibited a more exhausted phenotype through the overexpression of negative immune checkpoint in cluster 1 as compared to cluster 2 (**Figure 4 of paper**). Then, using another transcriptomic dataset from a public cohort of NSCLC patients for which the major components of the TME have been sorted (malignant cells, immune cells, fibroblasts, and epithelial cells), we

observed that the signature B was predominantly expressed by the malignant cells, while signature A was expressed in all cell types (**Figure 5 of paper**). By focusing our study on malignant cells and comparing their signature B gene expression profile, we noticed that patients were categorized into cluster 1 (higher signature B) and cluster 2 (lower signature B). Besides, as previously, we observed that cluster 1 exhibits more genes related to catabolism, proliferation, and also immune exhaustion by the increased expression of negative immune checkpoints like CD274 (PD-L1 encoding gene) or LGALS9 (Galectin9-encoding gene). In addition, tumors composed by malignant cells from cluster 1 are less infiltrated by T cells and B cells (**Figure 6 of paper**). Interestingly, by analyzing the global gene expression in these clusters, we showed that cluster 1 (for which malignant cells exhibited higher proliferative and immunosuppressive status) expressed much more autophagy genes than cluster 2, suggesting a link between autophagy of tumor cells and their proliferative/immunogenic status. The bioinformatics link between autophagy and proliferation in adenocarcinoma lung cancer cells was then confirmed by *in silico* analysis where adenocarcinoma lung tumor cells genetically deficient for autophagy expressed less genes related to proliferation than the autophagy competent adenocarcinoma lung tumor cells. Besides, *in vitro*, autophagy inhibition by drugs targeting both at the initiation step or the autophagy maturation step was confirmed to decrease the proliferation of adenocarcinoma lung tumor cells (**Figure 7 of paper**).

To sum up, through this project, we have determined an autophagy gene signature that can reflect the autophagy level within adenocarcinoma lung tumor cells, give us information about the proliferative and immune infiltrating capacities of adenocarcinoma NSCLC tumors as well as predictive indications about adenocarcinoma NSCLC patient survival.

Article

Autophagy-Related Gene Signature Highlights Metabolic and Immunogenic Status of Malignant Cells in Non-Small Cell Lung Cancer Adenocarcinoma

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Simple Summary: The role of autophagy in lung cancers is still controversial, mainly because the visualization of autophagy levels in patients remains challenging. One interesting approach consists of studying autophagy at the transcriptomic level. In this line, many transcriptomics analyses performed on autophagy genes focused on the discovery of new biomarkers to predict the efficiency of antitumor therapies. However, the majority of these studies were based on global transcriptomic analysis of the whole tumor microenvironment, and few investigations have been performed on malignant cells themselves. The goal of this study was not to determine another new predictive signature based on autophagy-related genes. Instead, we investigated the expression of autophagy genes to understand the involvement of this process in lung cancer homeostasis. Specifically, we discovered a new autophagy signature that correlates with the metabolic and immunogenic status of malignant cells, supporting the relationship between autophagy and tumor growth in lung cancer patients.

Abstract: Autophagy is a self-degradative mechanism involved in many biological processes, including cell death, survival, proliferation or migration. In tumors, autophagy plays an important role in tumorigenesis as well as cancer progression and resistance to therapies. Usually, a high level of autophagy in malignant cells has been associated with tumor progression and poor prognosis for patients. However, the investigation of autophagy levels in patients remains difficult, especially because quantification of autophagy proteins is challenging in the tumor microenvironment. In this study, we analyzed the expression of autophagy genes in non-small cell lung (NSCLC) cancer patients using public datasets and revealed an autophagy gene signature for proliferative and immune-checkpoint-expressed malignant cells in lung adenocarcinoma (LUAD). Analysis of autophagy-related gene expression profiles in tumor and adjacent tissues revealed differential signatures, namely signature A (23 genes) and signature B (12 genes). Signature B correlated with a bad prognosis and poor overall and disease-specific survival. Univariate and multivariate analyses revealed that this signature was an independent factor for prognosis. Moreover, patients with high expression of signature B exhibited more genes related to proliferation and fewer genes related to immune cells or immune response. The analysis of datasets from sorted fresh tumor cells or single cells revealed that signature B is predominantly represented in malignant cells, with poor expression in pan-immune population or in fibroblast or endothelial cells. Interestingly, autophagy was increased in malignant cells exhibiting high levels of signature B, which correlated with an elevated expression of genes involved in cell proliferation and immune checkpoint signaling. Taken together, our analysis reveals a novel autophagy-based signature to define the metabolic and immunogenic status of malignant cells in LUAD.

Keywords: autophagy; autophagy-gene signature; NSCLC; adenocarcinoma



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1. Introduction

Lung tumors are among the most common in the world and are one of the leading causes of cancer deaths worldwide [1]. Among them, NSCLC accounts for 85% of all lung tumor cases and it is mainly composed of lung squamous cell carcinoma (LUSC) and adenocarcinoma (LUAD) [1]. Although the available therapeutic arsenal, including surgical procedures, chemotherapy, targeted molecules and immunotherapy, have allowed undeniable progress in lung cancer treatment, the 5-year survival rate of NSCLC patients remains unsatisfactory. This relative inefficiency is mainly due to the lack of information about the tumor microenvironment during treatment decision. For this reason, many investigations have focused on the discovery of a new prognostic assessment method to help individualized treatment of NSCLC patients [2].

Autophagy is a cellular process associated with the prevalence and progression of lung cancer [3,4]. Autophagy is a conserved catabolism pathway that plays a key role in the maintenance of cellular homeostasis. It is a multistep mechanism, consisting of the formation of the phagophore that elongates and engulfs targeted proteins or organelles in a double-membrane vesicle called the autophagosome, and finally fuses with late endosomes and/or lysosomes [5]. This process is orchestrated by a large variety of proteins, including the autophagic proteins (Atg), organized in complexes. Autophagy induction is modulated by two protein complexes, the ULK1/2 (unc51-like autophagy activating kinase) and the Beclin-1/PI3KC3 (class III phosphatidylinositol 3-kinase) complexes. Once activated, these complexes recruit other proteins involved in the elongation and formation of autophagosomes, including the two conjugated systems Atg12-Atg5-Atg16L and LC3. After completion, the mature autophagosome fuses with lysosomes to form autolysosomes, wherein the sequestered materials and organelles are degraded by lysosomal enzymes [6]. Then, the degradation products are recycled for cell synthesis biological processes. Autophagy is one of the most important survival mechanisms under stress conditions and is involved in cellular homeostasis and proliferation [5]. Several studies have demonstrated links between autophagy and carcinogenesis, highlighting a dual role for autophagy in cancer. Depending on the tumor model and/or tumor state, autophagy may have pro- or anti-tumor effects. In the initial stage of cancer, autophagy protects normal cells from tumorigenesis by preventing DNA damages and mutations [3]. In established solid tumors, autophagy has been shown to favor tumor development by enhancing tumor growth, cell survival, resistance to platinum-based chemotherapy and metastasis formation [7]. Autophagy may also interfere with immunotherapy, since some studies showed a link between autophagy and immune checkpoint activity and/or expression, including CTLA-4, IDO and PD1/PD-L1 [8,9]. Autophagy also has a critical function in tumor immune cells and tumor immune response, promoting the immunogenic cell death of tumor cells and favoring immune cell activation and proliferation [10]. Meanwhile, autophagy in cancer-associated fibroblasts (CAFs) promotes tumorigenesis by providing nutrients to the cancerous cells and by favoring epithelial to mesenchymal transition, angiogenesis and stemness [11].

Many transcriptomic analyses performed on autophagy genes have focused on the discovery of new biomarkers to predict the efficiency of anti-tumor therapies and to guide individualized treatment in NSCLC patients [12–16]. However, the majority of these studies are based on global transcriptomic analysis of the whole tumor microenvironment, and few investigations have been carried out on malignant cells themselves. Regarding the global effect of autophagy on cells infiltrating the tumor microenvironment, it is important to determine a signature to identify the functional status of each cell type. In this study, we explore the relationship between 232 autophagy-related genes and biological pathways related to tumor progression in multiple LUAD datasets. Comparing tumors with adjacent tissue, we identified two signatures composed of twenty-three (signature A) and twelve (signature B) genes, and these signatures were correlated with survival, tumor metabolic status and immunology factors in LUAD patients. RNA sequence profiling of flow-sorted malignant cells, endothelial cells, immune cells and fibroblasts from freshly resected pri-

primary human NSCLC reveals that signature B was mainly expressed by malignant cells. The predominant expression of signature B in malignant cells was validated in the single cell sequencing data analysis. Deeper investigations supported the correlation between autophagy with tumor cell proliferation and immune checkpoint expression in malignant cells, highlighting the impact of autophagy in tumor cell progression and its potential role in immunotherapy. Therefore, our study provides a new autophagy-related signature that predicts the biological status of malignant cells in LUAD patients.

2. Materials and Methods

2.1. Dataset Source, Pre-Processing and Workflow

The workflow of our bioinformatic analysis is summarized in Figure 1. LUNG and LUAD gene expression datasets and associated clinical information were obtained from The Cancer Genome Atlas (TCGA) and Gene-Expression Omnibus (GEO) databases. The download gene expression profiles from TCGA met the following conditions: (1) the primary site was “bronchus and lung”; (2) the program was “TCGA”; (3) the disease type was “adenomas and adenocarcinomas” and/or “squamous cell neoplasms”; (4) the data category was “transcriptome profiling”; (5) the data type was “Gene Expression Quantification”; and (6) the workflow type was “HTSeq-FPKM”. TCGA-LUNG samples used for this analysis included 110 normal samples and 1019 tumor samples and TCGA-LUAD included 59 normal samples and 517 tumor samples. In addition, we downloaded series matrix files and platform files of four datasets, including one for global TME (GSE31210), one for RNA sequence profiling of flow-sorted malignant cells, endothelial cells, immune cells and fibroblasts from resected primary human NSCLC (GSE111907), one for single-cell analysis (GSE123904) and one for A549 cells invalidated by siRNA for atg5 and ulk1 genes involved in the autophagy process (GSE73158). The basic information regarding all databases is provided in Table 1.

The fragments per kilobase million (FPKM) values were converted into the transcripts per million (TPM) data using the R package “limma”. R (version 4.0.3, R core team, <https://www.r-project.org>, accessed on 5 June 2022) was used to process data. Processing for GSE datasets will be explained in the appropriate section.

2.2. Autophagy Signature and Clustering Analysis

We used the human autophagy database (HADd) to analyze the differential expression of autophagy-related genes ($n = 232$) between normal and tumor tissue. Differentially expressed genes were based on $\log_{2}FC > 1$ or < -1 and adjusted p -value < 0.05 using “limma” package in R. LUNG or LUAD samples were grouped into clusters according to their expression of autophagy signature genes (genes differentially expressed between adjacent and tumor samples). Kaplan–Meier survival curves were applied to each cluster and log-rank tests were performed to compare the overall survival (OS) and disease-specific survival (DSS) between clusters. Univariate and multivariate Cox analysis was performed to analyze the hazard ratio of clusters. Similar analysis was performed for individual genes of autophagy signature.

2.3. Functional Annotation Enrichment

To determine the variation of biological pathways between clusters, the differential expression analysis of whole genome between two clusters was performed. Differentially expressed genes were based on $\log_{2}FC > 1$ or < -1 and adjusted p -value < 0.05 using “limma” package in R. Based on DEG analyses, gene set enrichment analysis (GSEA) and Gene Ontology (GO) enrichment analysis were performed using the “Enrichr” website (<https://maayanlab.cloud/Enrichr/>, accessed on 5 June 2022) and the results were plotted using the “GOplot” R package. We selected the function and pathways with a strict p -value < 0.05 . For “circle plot”, we selected important pathways or functions involved in anabolism or catabolism.

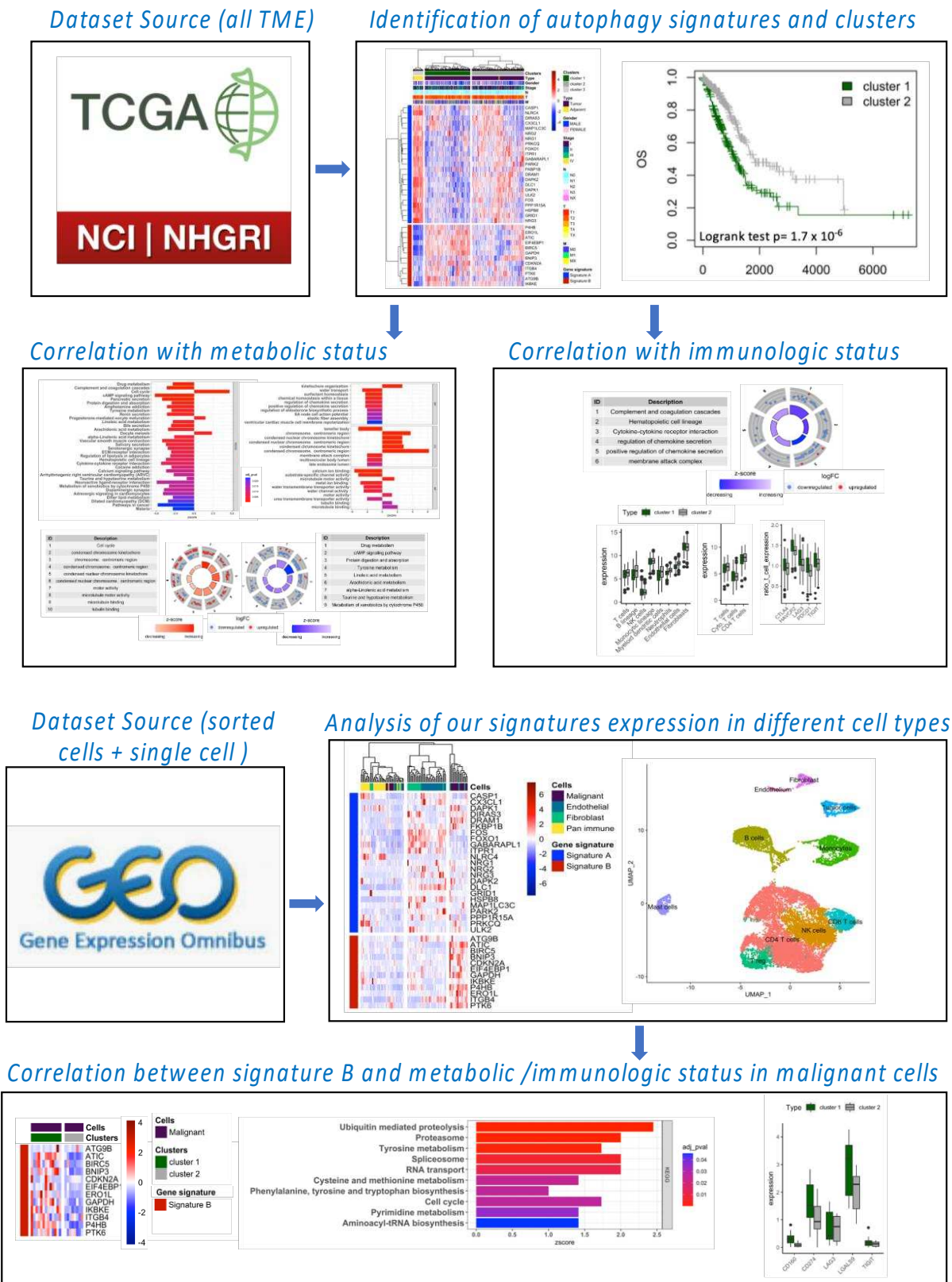


Figure 1. Workflow of bioinformatics analysis. Datasets were obtained from TCGA and GSO databases. Differential expression analysis was performed for 232 autophagy genes (human autophagy database) between tumor and adjacent tissue, and clusters of patients were examined in terms of survival and other clinical features. Correlations between autophagy clustering and metabolism or immunologic pathways were studied. The autophagy signature was studied in flow-

sorted malignant, endothelial, immune and fibroblast cells from freshly resected primary human NSCLC and in single-cell sequencing data, showing a predominant expression of signature B in the malignant cell population. The correlation between autophagy clustering and metabolic or immunological status was confirmed for malignant cells.

Table 1. Main information regarding our four datasets. NA means that we do not have the data. TME signifies tumor microenvironment.

Datasets Source	Platform	Samples Types	Subtype	Stage	Number of Samples
TCGA: LUNG	Illumina RNAseq	All TME	LUAD + LUSC	I/II: 879 III/IV: 250	1129
TCGA: LUAD	Illumina RNAseq	All TME	LUAD	I/II: 879 III/IV: 438	576
GEO: GSE31210	GPL17553 Illumina Hiseq 2000	All TME	LUAD	I/II: 138	226
GEO: GSE111907	GPL17553 Illumina Hiseq 2000	Sorted Cells	LUAD	NA	21–23
GEO: GSE123904	GPL16791 Illumina HiSeq 2500	Single Cells	LUAD	NA	8 patients (18,124 cells)
GEO: GSE73158	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	A549	LUAD cells line	NA	12

2.4. Immune Cell Infiltration, Stromal Cell Population and Exhaustion Marker Expression Analysis

For immune cell infiltration and the stromal cell population, we applied the microenvironment cell population-counter (MCP-count) method [17]. A total of 10 cell signatures were calculated to determine T cells, cytotoxic T cells, CD4+ T cells, B cell lineage, NK cells, monocyte lineage, myeloid dendritic cells, neutrophils, endothelial cells and fibroblasts. To determine the expression of exhaustion markers in T cells, we calculated the expression of *CTLA-4*, *HAVCR2*, *LAG3*, *PDCD1* and *TIGIT* genes according to the median of T-cell expression in TCGA.

2.5. Autophagy Signature Expression in Sorted-Cell Fresh Tumor Samples Datasets and Single Cell Analysis

RNA sequence profiling of flow-sorted malignant cells (EPCAM⁺ CD45⁻ CD31⁻), endothelial cells (CD31⁺ CD45⁻ EPCAM⁻), immune cells (CD45⁺ EPCAM⁻) and fibroblasts (CD10⁺ CD45⁻ EPCAM⁻ CD31⁻ CD10⁺) from freshly resected primary human NSCLC (GSE111907 dataset) were used to calculate expression levels of autophagy signature A and B on different cell populations. For these analyses, we only selected adenocarcinoma subtypes. We analyzed 21 malignant samples, 22 pan-immune samples, 23 endothelial samples and 22 fibroblasts samples. Samples were clustered according to their expression of autophagy signature gene and data were visualized by “pheatmap” R package. For single-cell analysis, we utilized GSE123904 datasets. We used only primary tumor samples for this analysis (LX653, LX661, LX675, LX676, LX678, LX680, LX682 and LX684). A total of 18,124 cells were analyzed. Single cell clustering and dimension reduction were performed by R package “Seurat”. The principal component analysis (PCA), “FindNeighbors” and “FindClusters” packages were employed to construct the cell clustering. The “UMAP” package was used to visualize data, and we utilized the “FeaturePlot” function from “Seurat” to visualize the expression of the autophagy signatures. Cell clusters were annotated

according to the gene expression in each cluster revealed by the “FindAllMarkers” function of “Seurat” package.

2.6. Autophagy Clustering Analysis in Malignant Tumor Cells

We selected 22 malignant samples (adenocarcinoma) from the GSE111907 dataset and samples were clustered according to their expression of the autophagy signature B. Gene Set Enrichment Analysis (GSEA) was performed as previously described.

2.7. Gene Set Enrichment Analysis in A549 Deficient for Autophagy

To examine the impact of autophagy genes in global biological processes, we used GSE73158 datasets. We selected three lung adenocarcinoma cell lines, A549 treated with siRNA against *ATG5*, A549 treated with siRNA against *ULK1*, and their respective siRNA control. We performed differential expression analysis for siRNA-treated cells according to their respective control and selected upregulated ($\log_{2}FC > 1$) or down-regulated ($\log_{2}FC < -1$) genes. To perform functional annotation enrichment analyses, we chose genes which were significantly modified in both si*ATG5*- and si*ULK1*-treated cells.

2.8. Cell Culture, Proliferation Analysis and Confocal Microscopy

The human lung adenocarcinoma A549 cell line and the murine adenocarcinoma LLC and carcinoma KP cell lines were cultured in DMEM F-12 medium (Gibco, Waltham, MA, USA) supplemented with 10% FBS (Eurobio Scientific, Ulis, France), 1% non-essential amino acid (Gibco), 1% herpes (Gibco), 1% glutamate (Gibco) and 1% Na^+ /pyruvate (Gibco) in a standard 5% CO_2 incubation atmosphere at 37 °C. The human lung squamous cell carcinoma SK-MES cell line was cultured in EMEM F-12 medium (Gibco) supplemented with 10% FBS (Eurobio Scientific), 1% non-essential amino acid (Gibco), 1% 1% herpes (Gibco), 1% glutamate (Gibco) and 1% Na^+ /pyruvate (Gibco) in a standard 5% CO_2 incubation atmosphere at 37 °C.

For in vitro proliferation assays, 150,000 cells were stained with CFSE (1/500, ThermoFischer, Waltham, MA, USA) for 30 min at 37 °C in PBS and plated in the 6-well plate for 24 h. Cells were cultured for 24, 48, 72 and 96 h in the presence or not of 10 mM of 3-methyladenin (Sigma, Saint-Louis, MO, USA), 100 nM of wortmannin (Sigma), 10 μM of SAR405 (MedChemExpress, Monmouth Junction, NJ, USA), or 100 nM of bafilomycin (Sigma), and stained with live/dead kit (1/100, near-IR, ThermoFisher). Analysis of CFSE staining was performed using the BD LSR Fortessa Cell analyzer. Flow cytometry data were analyzed by FlowJo software.

For in vitro analysis of the autophagy level, 35,000 A549 cells expressing the GFP-LC3 protein were plated in a 24-well plate containing coverslips for 24 h. Cells were then cultured for 24 h in the presence or not of 10 mM of 3-methyladenin (Sigma), 100 nM of wortmannin (Sigma), 10 μM of SAR405 (MedChemExpress), or 100 nM of bafilomycin (Sigma), and were mounted on the slides using glycergel (Dako, Santa Clara, CA, USA). The autophagosomes were observed by confocal microscopy (LSM 710) and enumerated by a personal R script.

2.9. Statistical Analysis

R software (v4.0.3) was used for all bioinformatic statistical analyses, and PRISM software was employed for in vitro experiments. The Wilcoxon test was used to compare the differences between the two groups. The Kruskal–Wallis test was utilized to compare the differences between three groups and above. The survival time of the patient was evaluated by Kaplan–Meier survival analysis, and the different groups were compared by utilizing a log-rank test. Univariate and multivariate Cox regression analysis was used to investigate the independent prognostic factor, employing the “survival” R package. The Benjamin–Hochberg method was used to calculate p -value for FRDs conversation and DEG analyses. Single-cell analysis was performed using R package “Seurat”. Survival curves were performed utilizing R package “survminer”. All heatmaps were generated

by R package “pheatmap”. We employed “GOplot” R package to visualize the functional annotation enrichment analyses. Data visualization was performed using R package “ggplot2”. The R packages utilized in this study could be obtained from “bioconductor”.

3. Results

3.1. Autophagy Gene Expression Was Distinct, According to the Subtype of Lung Tumors

To investigate the impact of autophagy genes in lung tumors, we analyzed the differential expression of autophagy genes (from the human autophagy database) in tumors ($n = 1019$) versus adjacent ($n = 110$) tissues using TCGA-LUNG public cohorts. Among the autophagy-related genes tested ($n = 232$), 23 genes were down-regulated and 16 were up-regulated in tumors as compared to the adjacent tissue (Figure S1A). Further analysis revealed a clear separation of patients into two groups, with 86% of adenocarcinoma in one group and 96% of squamous-cell carcinoma in the other group, suggesting that patients with different histology subtypes expressed very distinct autophagy genes (Figure S1B,C). No differences were observed according to gender, stage of cancer or TNM classification in the two groups of patients (Figure S1B). Given that the autophagy signature is very different according to the tumor subtype, it is important to separately analyze adenocarcinoma and squamous cell carcinoma in this context. In this study, we focused our research on adenocarcinoma subtypes.

3.2. Autophagy-Related Gene Signature in Lung Adenocarcinoma Correlates with an Increase in Anabolic and a Decrease in Catabolic Pathways

Using the TCGA-LUAD cohort, we performed a similar experiment to that previously described by comparing the autophagy gene expressions between tumor ($n = 517$) and adjacent tissue ($n = 59$). Twenty-three genes were down-regulated and 12 were up-regulated in tumor samples as compared with the adjacent tissues (Figure 2A). Two clusters of patients differentially expressed autophagy genes (Figure 2B). Patients in cluster 1 comprised the most distinct cluster for autophagy gene expression as compared with the adjacent tissue (Figure 2C). Moreover, cluster 1 expressed significantly more up-regulated genes (called signature B) and fewer down-regulated genes (called signature A) as compared to cluster 2 (Figure 2D). Univariable analysis for the hazard ratio revealed that some genes involved in signature A (*NLRC4*, *CX3CL1*, *MAP1LC3C*, *DRAM1*, *DAPK2*, *DLC1*, *DAPK1* and *HSPB8*) were associated with a good prognosis (Figure 2E). In contrast, some genes in signature B (*ERO1L*, *ATIC*, *EIF4EBP1*, *BIRC5* and *GAPDH*) were associated with a bad prognosis. Interestingly, cluster-related autophagy genes were an independent factor significantly associated with a bad prognosis (Figure 2G). These observations were supported by the analysis of the overall survival (OS) and disease-specific-survival (DSS), showing that patients in cluster 1 have a lower survival rate as compared to those in cluster 2 (Figure 2G). Of note, while no distinction has been found according to gender, stage or TNM classification, patients in advanced stages (stage III/IV or T3/4 or N2/3) were more represented in cluster 1 than cluster 2 (Figure 2H).

To further investigate the implication of autophagy gene signatures in lung adenocarcinoma, we performed an analysis of the differential expression of all the genes between cluster 1 and cluster 2 and observed that 743 genes were down-regulated and 223 were up-regulated in cluster 1 as compared to cluster 2 (Figure 3A). KEGG and GO analyses showed that many biological pathways were significantly impacted by the differential gene expression profiles between the two clusters (Figure 3B). Among these pathways, several genes implicated in anabolism pathways were increased in cluster 1 as compared to cluster 2, including genes involved in cell proliferation (e.g., “Cell cycle” or pathways related to chromosome or microtubule activity) (Figure 3B–D). On the contrary, genes implicated in catabolic processes were globally decreased in cluster 1, including genes involved in “drug metabolism”, “cAMP signaling pathways” or “Protein digestion and absorption” (Figure 3D). These results suggest that in cluster 1, cellular metabolic processes are more activated than in cluster 2, with potentially a higher rate of cellular proliferation.

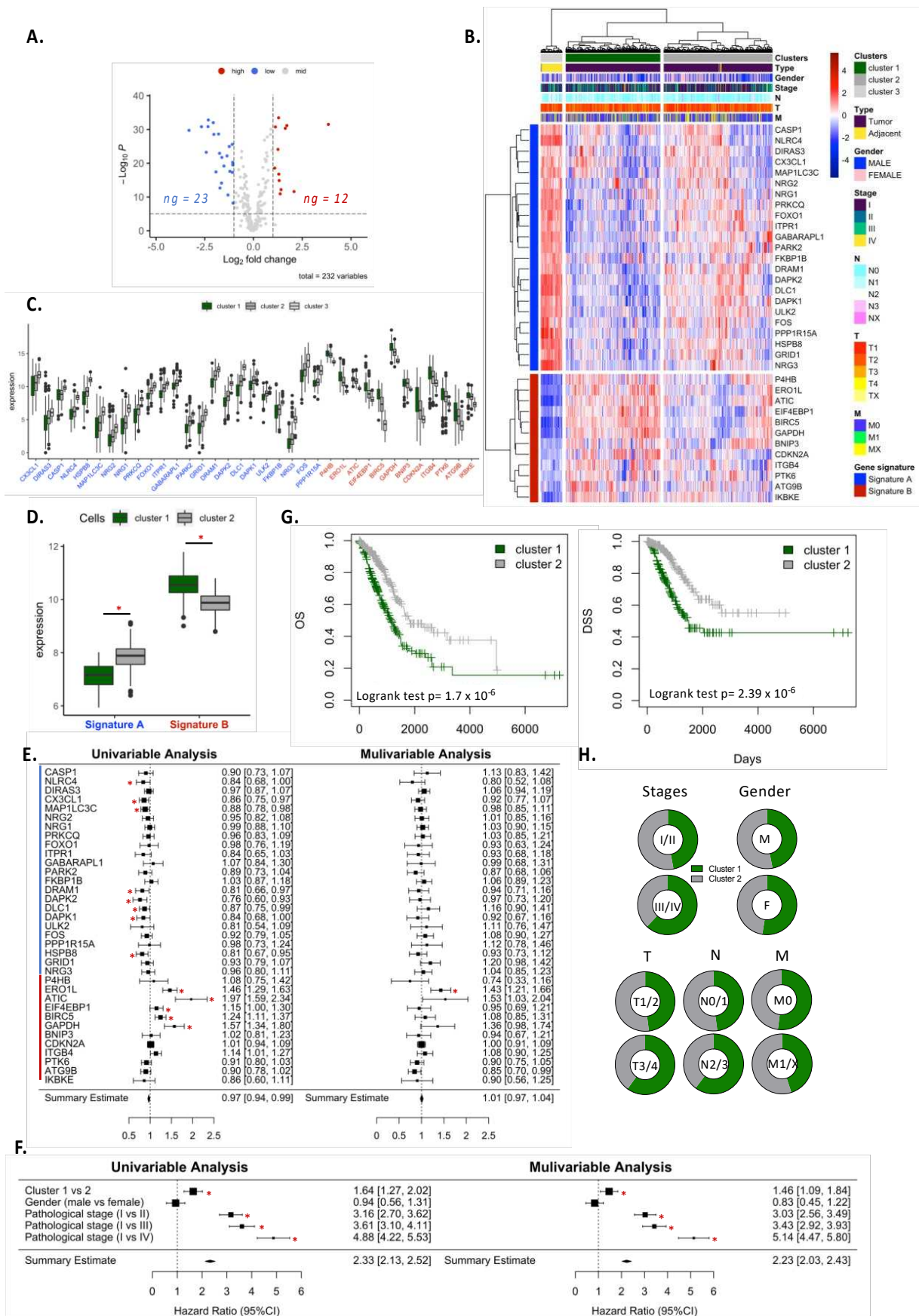


Figure 2. Autophagy genes screening and cluster analysis in lung adenocarcinoma. (A) Differential expression analysis for the human autophagy database ($n = 232$ genes) of TCGA-LUAD patients

between tumor ($n = 517$) and adjacent tissue ($n = 59$). (B,C) Thirty-five differentially expressed genes of autophagy in TCGA-LUAD datasets. (D) Expression of signature A and B in clusters 1 and 2 in TCGA-LUAD datasets. (E,F) Autophagy signature gene expression, clustering of patients, clinicopathological and OS of univariate and multivariate Cox regression analysis in the TCGA-LUAD datasets. (G) Kaplan–Meier OS and DSS curve in the TCGA-LUAD dataset. (H) Distribution analysis of clusters according to stages, gender and TNM classification in the TCGA-LUAD datasets. *, p -value < 0.05.

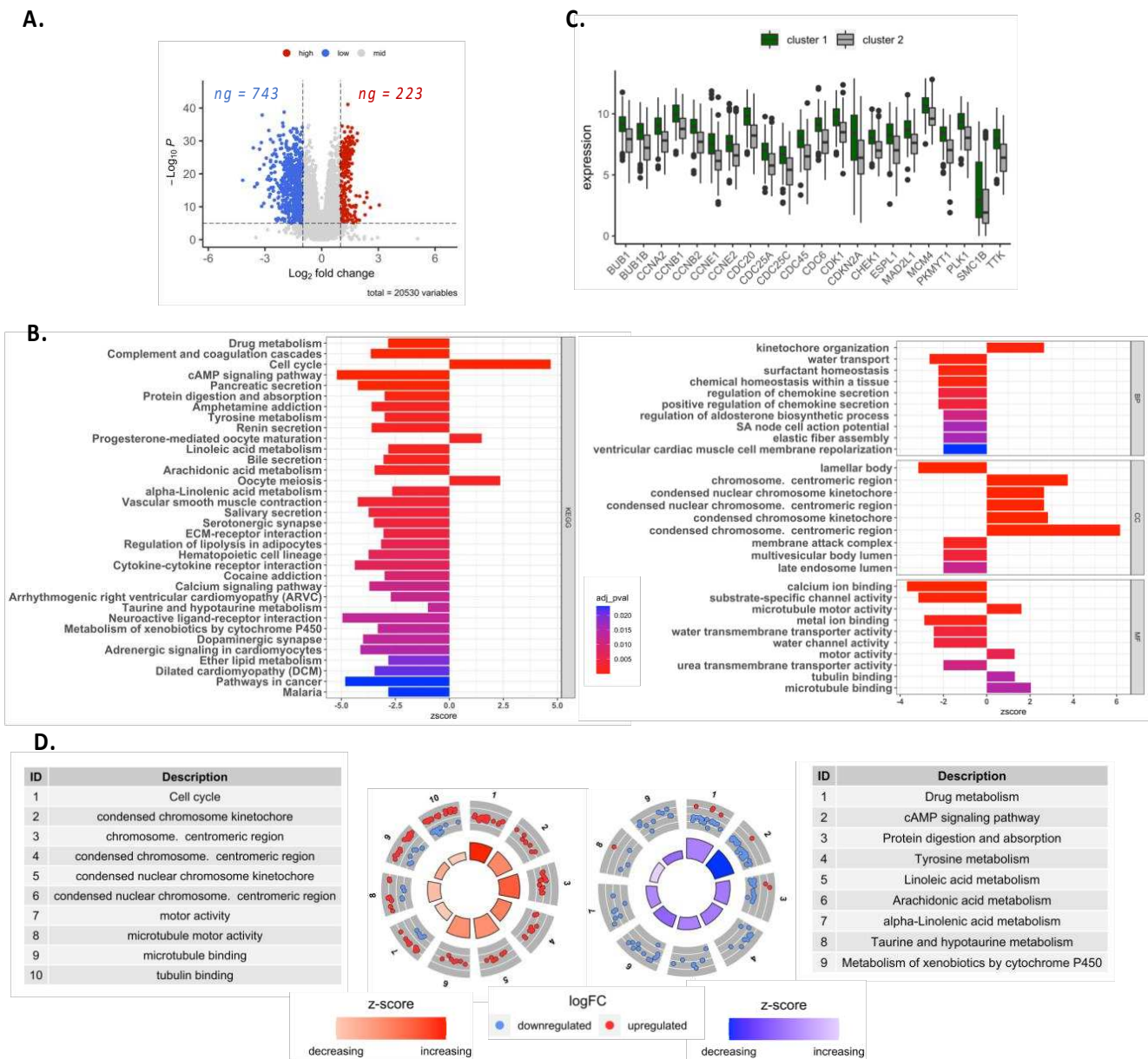


Figure 3. Differential gene expression and enrichment analysis for clusters. (A) Volcano plot for differential expression analysis in all genomes (cluster 1 versus cluster 2) in the TCGA-LUAD datasets. (B) Differential gene GO and KEGG enrichment analysis, BP stands for biological processes, CC stands for cellular components, and MF stands for molecular function. (C) Significant expression of genes involved in cell cycle pathways. (D) Representation for gene expression in GO and KEGG enrichment analysis for anabolism (left) and catabolism (right) pathways.

3.3. Autophagy-Related Gene Signature Highlighted a Decrease in Immunity-Related Pathways and an Increase in Exhaustion Genes

Besides metabolic pathways, some genes involved in immunity were also differentially expressed between cluster 1 and cluster 2 (Figure 3B). Indeed, a decreased expression of genes involved in the complement cascade, hematopoietic cell lineage and cytokine/chemokine pathways was observed in cluster 1 as compared to cluster 2 (Figure 4A–C), suggesting that patients in cluster 1 exhibited a poorly infiltrated tumor microenvironment. To support this result, we evaluated immune cell infiltration in each cluster using the MCP counter method [17]. We observed a significant decrease in genes related to T and B lymphocytes and myeloid dendritic cells, neutrophils and endothelial cells in cluster 1 as compared to cluster 2 (Figure 4D). Further analysis revealed that patients in cluster 1 expressed more genes involved in T-cell exhaustion (*CTLA-4*, *HAVCR2*, *LAG3*, *PDCD1* and *TIGIT*) (Figure 4D). Taken together, our results show that patients with high signature B and low signature A expression (cluster 1) exhibited active cell proliferation but a reduction in immune response, which is consistent with the poor survival rate observed for patients in clusters 1.

To investigate whether the correlation between autophagy signature and metabolic status or immune cell infiltration could be influenced by the stage of the cancer, we performed similar analyses in LUAD-TCGA datasets stratified by tumor stages (stages I/II or stages III/IV). We observed a very similar clustering for patients in both early and advanced stages (with around 90% of similarity for differential expressed genes in the early stages and 85% in the advanced stages) (Figures S2A and S3A). Patients in cluster 1 comprised the most distinct cluster for autophagy gene expression as compared to adjacent tissue. The overall survival (OS) and disease-specific survival (DSS) analyses revealed that patients in cluster 1 have a lower survival rate as compared to those in cluster 2 in the early stages of the tumor (Figure S2B). In more advanced cancers, patients in cluster 1 have a lower DSS, but no significant difference was observed for OS (Figure S3B). For both groups of tumor stages, the KEGG and GO analyses showed that patients in cluster 1 expressed more genes involved in cell proliferation and fewer genes implicated in catabolic processes (Figures S2C and S3C). Patients in cluster 1 were also less infiltrated by immune cells and expressed more genes involved in T-cell exhaustion as compared to patients in cluster 2 (Figures S2D and S3D). These data showed that our autophagy gene signatures correlate with metabolic status and immune infiltration independently of the tumor stages.

Interestingly, we confirmed a correlation between autophagy-related gene signatures and metabolic and immunologic status using another cohort of adenocarcinoma. Applying autophagy signatures in 226 LUAD patients (GSE31210 datasets), we defined two clusters of patients. According to our previous observation, the cluster with a low expression of signature A and high expression of signature B exhibited the worst prognostic value, the most active metabolic status and lowest immune cell infiltration (Figure S4A–D).

3.4. The Autophagy Signature B Was Enriched in Malignant Cells and Revealed Metabolic and Immunogenic Status of Tumor Cells

Using a cohort of adenocarcinoma from GSE (GSE111907), we compared the expression of autophagy gene signatures between tumor-infiltrating cell subsets. In this cohort, RNA-seq profiling of flow-sorted malignant cells, endothelial cells, immune cells and fibroblasts from resected primary human NSCLC was performed. While the global autophagy gene expression was not different between cell subtypes (Figure S5A, Table S1), analyses of our autophagy-related signature revealed that signature B was significantly more expressed in malignant cells as compared to immune, fibroblast or endothelial cells (Figure 5A,B). Signature A was preferentially expressed in fibroblasts and endothelial cells, with a low expression in malignant cells (Figure 5B and Table S2). One gene in signature A (*DRAM1*) and five genes in signature B (*ERO1L*, *ATIC*, *BIRC5*, *BNIP3* and *PTK6*) were significantly more expressed in malignant cells as compared to other cell subtypes (Figure 5C and Table S3). These results were supported using the single-cell transcriptional landscape

of primary lung adenocarcinoma (GSE123904) (Figure 5D and Figure S5B and Table S4). Cells were clustered using weighted nearest neighbor analysis (Seurat) and clusters were annotated according to gene expression (as explained in the Materials and Methods). We then analyzed the expression of autophagy signatures in each cell and observed that tumor cells poorly express genes in signature A and highly express genes in signature B as compared to other cell types (Figure 5E and Table S5).

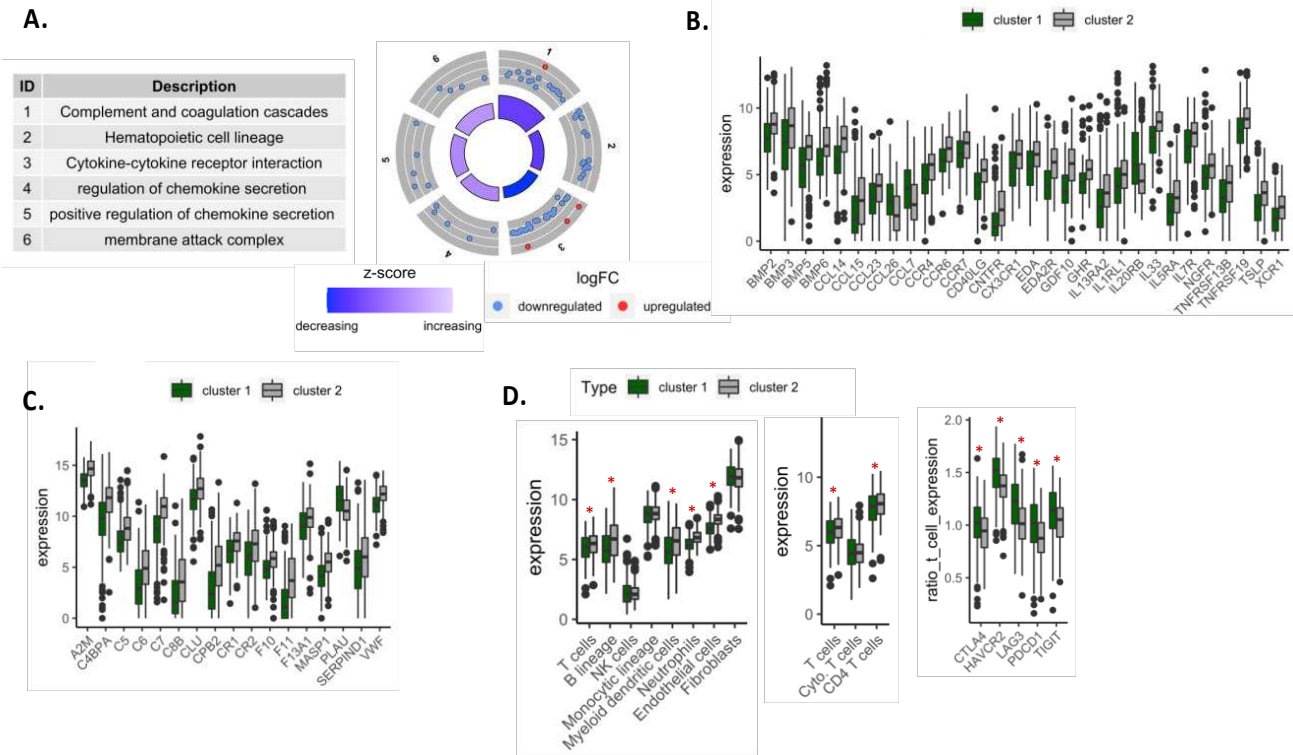


Figure 4. Enrichment analysis related to immune pathways. (A) Representation for gene expression in GO and KEGG enrichment analysis related to immunity. (B,C) Significant expression of genes involved in cytokine-cytokine receptor interaction and complement cascade, respectively. (D) The expression of genes related to immune cells according to MCP counter database in the TCGA-LUAD datasets. *, p -value < 0.05.

Clustering patients (from GSE11907 dataset) for the expression of autophagy signature B in malignant cells also revealed two clusters, with patients in cluster 1 exhibiting more genes in signature B than patients in cluster 2 (Figure 6A). As previously observed for the analysis of the global tumor microenvironment (TCGA dataset), differential gene expression analysis focusing on malignant cells revealed that patients in cluster 1 exhibited more genes involved in cell proliferation and/or anabolism as compared to patients in cluster 2 (Figure 6B). Comparing immune cell infiltration between clusters revealed that patients in cluster 1 were less infiltrated by T and B cells and expressed fewer chemokine-related genes in malignant cells as compared to cluster 2 (Figure 6C,D). Moreover, malignant cells of cluster 1 expressed more *CD274* (PD-L1) and *LGALS9* (galectin 9) genes (Figure 6E). Together, these data suggest that malignant cells expressing high levels of signature B were more prone to proliferating and inducing immune suppression. Interestingly, expression analysis of autophagy genes (from the human autophagy database) showed that cluster 1 exhibited a higher autophagy level as compared to cluster 2 (Figure 6F), underlining a close relationship between the metabolic and immunogenic status of malignant cells and autophagy.

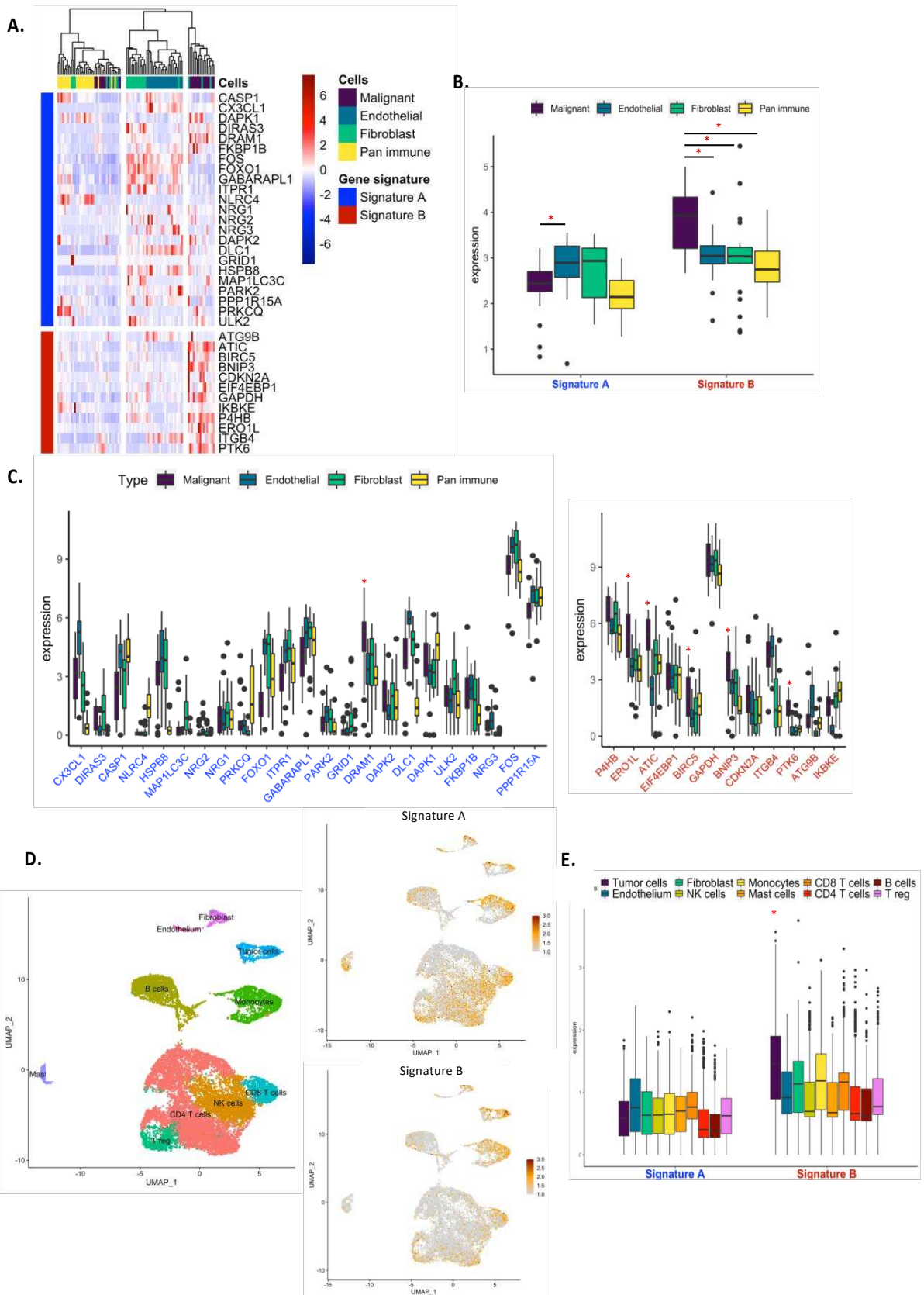


Figure 5. Expression of autophagy-related signatures in different subtypes of TME. (A) Unsupervised clustering of cell types using autophagy gene signatures in the GSE111907 datasets. (B,C) Expression of autophagy gene signatures according to cell types in the GSE111907 datasets. (D,E) Single-cell analysis of autophagy gene signature expression in the GSE123904 datasets. *, p -value < 0.05.

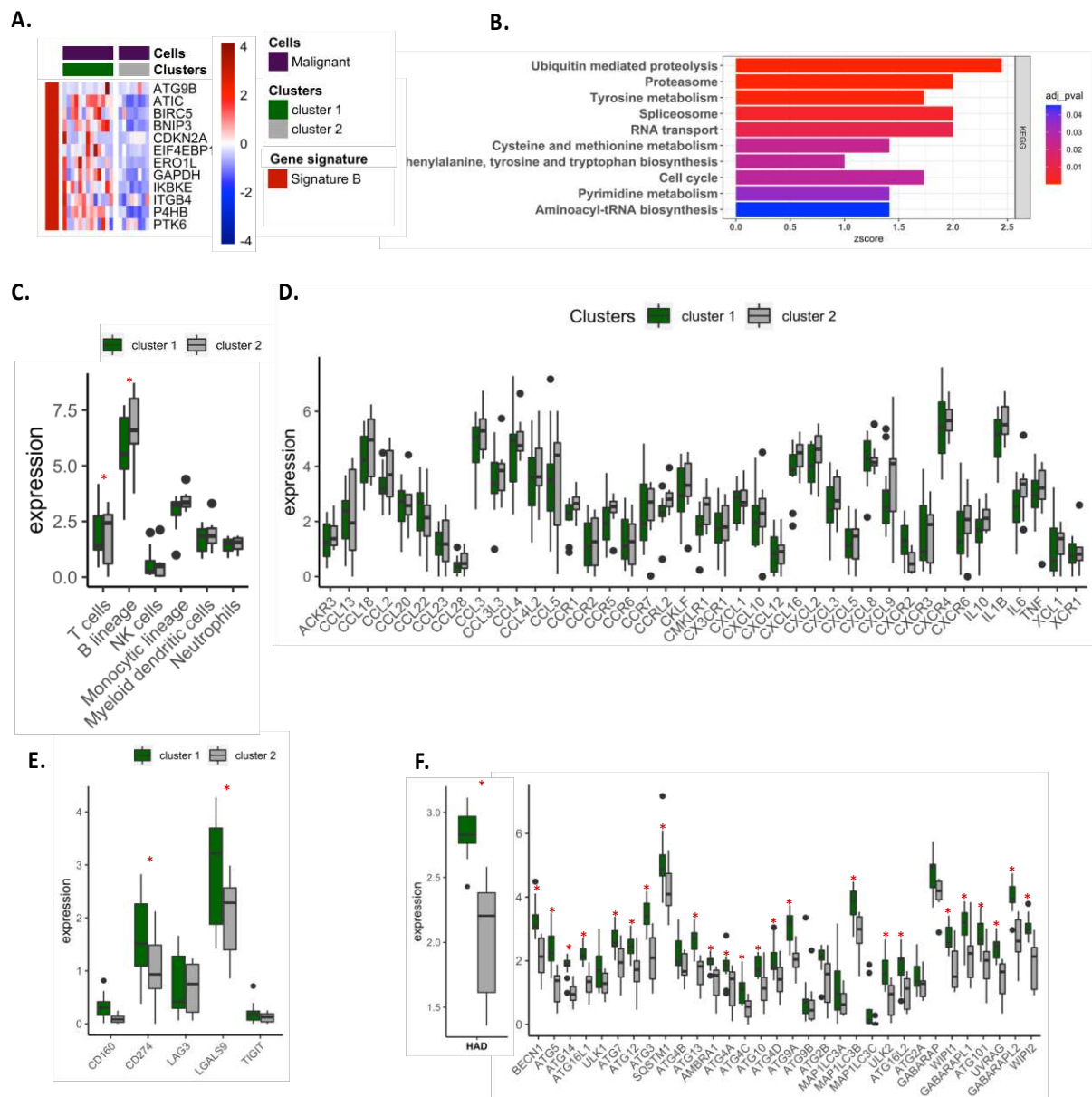


Figure 6. Metabolic and immunologic status of malignant cells according to signature B. **(A)** Unsupervised clustering of patients using autophagy signature B in malignant cells samples using the GSE111907 datasets. **(B)** KEGG enrichment analysis between cluster 1 and cluster 2. **(C–E)** Analyses of immune cell infiltration **(C)**, expression of chemokine-related genes **(D)** and expression of genes involved in immune checkpoint **(E)** in clusters. **(F)** Expression of global (human autophagy database) and autophagy genes in the GSE111907 datasets. *, p -value < 0.05.

3.5. Autophagy Is Required for the Proliferation of Tumor Cells

To demonstrate the impact of autophagy in lung tumor cell expansion, we first compared gene expression in A549 adenocarcinoma cell lines in which autophagy genes (*ATG5* or *ULK1*) had been deleted or not (GSE73158 dataset) (Figure 7A). The deletion of *ATG5* or *ULK1* induced a decreased expression of genes involved in the cell cycle and/or replication, supporting the suggestion that autophagy is required for tumor cell expansion. To confirm this result, we performed in vitro analysis assay for tumor cells proliferation. The inhibition of the autophagy machinery significantly impaired the autophagy level as expected, decreasing the autophagosome number when the initiation of autophagy was inhibited (cells treated with 3-Methyladenin or SAR405) and increasing the accumulation of the autophagy vacuoles when the maturation step was blocked (cells treated with bafilomycin) (Figure 7B).

The cell proliferation of several lung tumor cell lines was drastically decreased when autophagy was inhibited for both the initiation and maturation steps (Figure 7C,D). Taken together, these results demonstrate a strong involvement of autophagy in malignant cell proliferation and metabolism, and support the relevance of using transcriptomic analysis for autophagy genes to analyze the metabolic status of malignant cells.

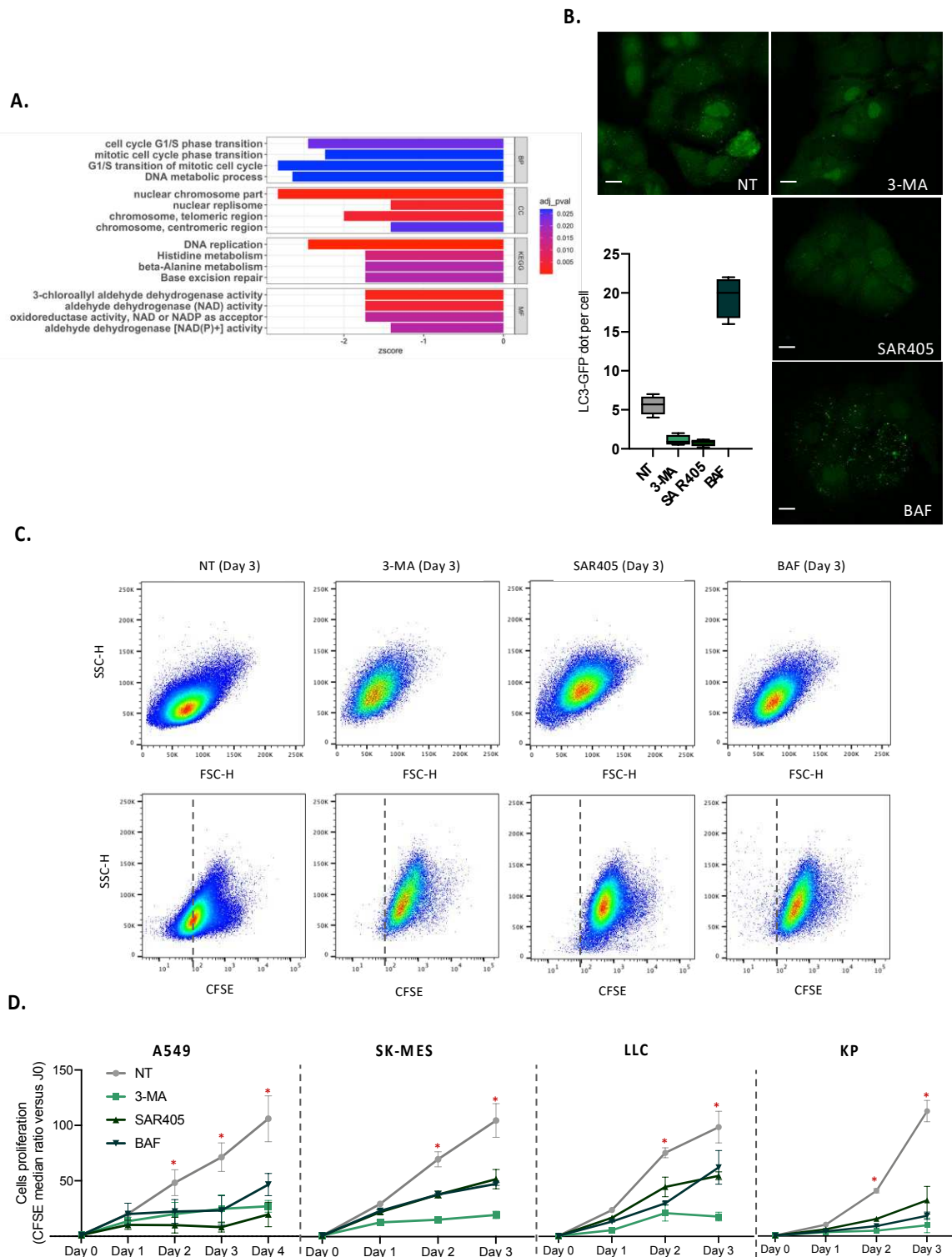


Figure 7. Impact of autophagy in tumor cell proliferation. (A) Differential gene GO and KEGG enrichment analysis in GSE73158 datasets (A549 control versus autophagy deficient), BP stands for

biological processes, CC stands for cellular components, and MF stands for molecular functions. (B) A549-GFP-LC3 cell lines were treated or not with 3-methyladenine (3-MA), SAR405 or bafilomycin (BAF) and the number of autophagosome was evaluated by confocal microscopy. Scale bars represent 10 μ m. (C) A549 cell lines were cultured in media containing CFSE and were treated or not with 3-methyladenine (3-MA), SAR405 or bafilomycin (BAF) for 3 days. Morphological modification and CFSE staining were analyzed by flow cytometry. (D) A549, SK-MES, LLC and KP cell lines were treated as previously described and the proliferation of cells was studied following the CFSE staining. *, p -value < 0.05.

4. Discussion

As a central process of self-digestion and stress adaptation, autophagy has a remarkable impact on tumor development [4,7,18]. It can provide nutrients for cancer cell survival, proliferation and migration, promotes drug resistance and helps tumor cells to evade immune surveillance [4]. In lung cancers, several studies showed that autophagy promotes tumor cell growth and resistance to radiation or chemotherapy [19,20]. However, due to the difficulty of visualizing and quantifying autophagy in tumor patients, the role of autophagy in NSCLC patients is still unclear [20,21].

Gene expression analysis appears to be a relevant approach to analyze autophagy in NSCLC patients. We first conducted our analysis in LUAD and LUSC, which accounted for the majority of NSCLC. Based on the TCGA database, our preliminary exploration revealed that modification for the expression of autophagy genes can be observed in tumor samples, demonstrating that autophagy is particularly active in cancers. Autophagy expression was very dependent on cancer subtypes, and clear clustering of patients has been found between LUAD and LUSC samples. To further investigate autophagy in lung cancers, we focused our analysis on LUAD, which is holding the predominant position among all the pathological types of lung cancer. Performing differential expression analysis of 232 autophagy-related genes between tumor and adjacent tissue samples, we observed two clusters of patients according to the expression of autophagy signature A (23 genes) and signature B (12 genes). Patients in cluster 1, characterized by lower expression of signature A and higher expression of signature B than cluster 2, had the worst overall survival (OS) and disease-specific survival (DSS). Univariate and multivariate COX analyses suggested that autophagy signatures could be an independent feature associated with bad prognosis in patients. We showed that cluster 1 was more metabolically active, expressing anabolism-related genes involved in cell proliferation and migration. On the contrary, cluster 2 exhibited an antiproliferative phenotype, with active catabolism pathways. In addition, cluster 1 samples were less infiltrated by immune cells than cluster 2 and exhibited decreased immune response features. Analyses of autophagy signatures expression in single cells or sorted-cell datasets revealed that signature B was largely expressed by malignant cells, while signature A was preferentially expressed in endothelial and in less extend in fibroblast cells. Signature B expression in malignant cells correlated with an active metabolic feature, a decrease in immune cell infiltration and an increase in the immune checkpoint expression on tumor cells (e.g., *PD-L1* and *Galectin-9*).

Interestingly, signature B correlated with an active autophagy process, supporting the central role for autophagy in tumor proliferation and migration and suggesting an important impact of this process on immune escape.

Previous studies analyzed the expression of autophagy genes in lung tumors and constructed the autophagy-related signature to anticipate the prognosis of LUAD or LUSC patients using the TCGA datasets [15,16,22]. Two studies also determined predictive signatures based on autophagy-associated long non-coding RNAs [14,23]. The goal of this study was not to determine a new predictive signature based on autophagy-related genes. Instead, we investigated the expression of autophagy genes to understand the involvement of this process in lung tumor homeostasis. While previous studies established autophagy signatures in the global TME and correlated the expression of autophagy genes with the survival probability, we focused our analysis on malignant cells, highlighting a

new autophagy signature that could help to understand the metabolic and immunologic status of these cells.

We take advantage of sorted cells and single cell datasets to analyze the expression of our signature and showed that signature B correlated with active metabolic status of tumor cells. Interestingly, the patients with high expression of signature B expressed much higher expression of autophagy genes involved in core machinery, including genes involved in the initiation complex (e.g., *BECN1*, *ULK1*, *AMBA1*) or elongation system (e.g., the majority of ATG genes, *MAP1LC3A*, *MAP1LC3B*, *MAP1LC3C*). These data supported previous studies that described a pro-tumor function for autophagy in lung cancers, favoring the proliferation and migration of tumor cells [3,20,23,24].

Our data also revealed an important impact of autophagy on immune escape, describing that autophagy gene expression can reflect the immune cell infiltration and/or the immunogenic status of malignant cells. Interestingly, the correlation between autophagy genes expression and immune infiltration has also been described in other types of tumors [23,25]. While some studies observed a similar correlation between autophagy and immune checkpoint expression [24,26], future research needs to be developed to carefully understand the impact of autophagy in this context.

Among the genes involved in signature B, some of them reveal a significant prognostic value in TCGA cohorts. We showed that *ERO1L* gene was preferentially expressed by malignant cells, suggesting an important role of this protein in the growth of cancer cells. *ERO1L* has already been demonstrated to play a critical role in NSCLC, promoting cancer development by modulating cell cycle-related molecules [27]. Moreover, recent reports also mentioned that *ERO1L* was implicated in anti-tumor immune response, by preventing T cell-mediated immunity and favoring myeloid suppressor cell activation [28,29]. The expression of *ERO1L* gene in malignant cells could explain, at least in part, the reason for which signature B is associated with a bad prognosis, low infiltration of immune cells and high proliferative rates. Similarly, *ATIC* was much more highly expressed in malignant cells and was associated with a significant prognostic value in our univariate analysis. A recent report demonstrated that *ATIC* facilitates tumor growth and migration by upregulating Myc expression in LUAD [30]. *BIRC5*, an ATG12-ATG5 conjugate interactor has also been found to be expressed predominantly by tumor cells. *BIRC5* was associated with a bad prognosis in lung cancers by favoring mitotic cell cycle-related pathways [31]. In some tumors, *BIRC5* was also correlated with high immune cells infiltration [31]. In LUAD, *BIRC5* gene was inversely correlated with dendritic cells and CD4+ T cell infiltration, observations that we confirmed in our analysis. In our signature A, only *DRAM1* was preferentially expressed by tumor cells. *DRAM1* was associated with p53 and played a critical role in autophagy and apoptosis [32]. However, the biological function of *DRAM1* in lung cancer remains controversial. The study by He Q et al. revealed that *DRAM1* could be a target of FTSJ1 and promotes cancer progression [33]. More recently, another study showed that *DRAM1* inhibits the development of lung tumors by promoting the lysosomal degradation of EGFR [34]. In our analysis, we showed that *DRAM1* was associated with a good prognostic value, suggesting that the expression of *DRAM1* in malignant cells could inhibit tumor growth. Our data highlighted the vital role of these genes expressed by malignant cells in tumor development. While our study strongly suggests a correlation between the expression of these genes and the autophagy level in cancer cells, future investigations should be initiated to understand their role in autophagy modulation in the context of tumor growth.

5. Conclusions

Taken together, our analysis reveals a novel autophagy-based signature to determine the metabolic and immunologic status of malignant cells in LUAD. Our study helps to understand the processes involved in LUAD progression and could be useful for therapeutic intervention in NSCLC patients.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers14143462/s1>, Figure S1: Autophagy gene screening in lung tumors; Figure S2: Autophagy gene screening and cluster analysis in early stages of lung adenocarcinoma. Figure S3: Autophagy gene screening and cluster analysis in advanced stages of lung adenocarcinoma. Figure S4: Analysis of autophagy genes expression in tumor cells subtypes. Figure S5: Analysis of autophagy genes expression in tumor cells subtypes. Table S1: Statistical report for Figure S3A. Table S2: Statistical report for Figure 5B. Table S3: Statistical report for Figure 5C. Table S4: Statistical report for Figure S3B. Table S5: Statistical report for Figure 5E.

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3 Study 2: Physiological TLR7-induced autophagy in NSCLC tumor cells favors tumor progression, resistance to chemotherapies, and nivolumab responsiveness in patients

Given that so far this paper is not published, I will present to you in that section our data as well as the materials and methods.

3.1 Summary

As I previously described, an increasing number of studies have demonstrated that cancer cells are very dependent on autophagy, which exerts pro-tumoral effects by promoting survival, proliferation, resistance to therapies, metastasis development, and immune evasion. Therefore, it is not surprising that more and more studies are demonstrating that autophagy level is associated with a worse survival for cancer patients in various cancer types, and in the meantime, an increasing number of pre-clinical and clinical studies aim to target autophagy are ongoing. In spite of these observations, very few data concerning the importance of autophagy in lung tumor cells and lung tumor progression are available. Therefore this study had two objectives, the first one consisted in determining the molecular pathways by which, in a physiological context, the autophagy level in lung tumor cells is modulated. The second one was to assess the importance of the autophagy level in lung tumor cells on tumor progression by evaluating its possible involvement in various pro-tumoral mechanisms.

3.2 Materials & Methods

3.2.1 WGCNA cluster analysis and genes expression correlation analyses in public cohorts of adenocarcinoma lung tumor patients

The co-expression network was constructed using The Cancer Genome Atlas (TCGA) and Gene-Expression Omnibus (GEO) database. LUAD gene expression datasets and associated clinical information were obtained from The Cancer Genome Atlas (TCGA) and Gene-Expression Omnibus (GEO) databases. The download gene expression profiles from TCGA met the following conditions: (1) the primary site was “bronchus and lung”; (2) the program was “TCGA”; (3) the disease type was “adenomas and adenocarcinomas” and/or “squamous cell neoplasms”; (4) the data category was “transcriptome profiling”; (5) the data type was “Gene Expression Quantification”; and (6) the workflow type was “HTSeq-FPKM”. TCGA-LUAD included 59 normal samples and 517 tumor samples. In addition, we downloaded series matrix files and platform files of GSE111907 datasets, which include the RNA sequence profiling of flow-sorted malignant cells, endothelial cells, immune cells, and fibroblasts from resected primary human NSCLC.

The “WGCNA” package in R was employed for each cohort to establish a gene co-expression network of the 20,000 genes. “GoodSamplesGenes” function was

implemented to examine the quality of raw data. To intensively analyze the functional modules, the adjacency matrix was converted into a topological overlap matrix (TOM), and the dissimilarity matrix between genes was calculated ($\text{dissTOM} = 1 - \text{TOM}$). Hierarchical clustering of dissTOM resulted in genes with similar expression clustered in the same gene module. The minimum number of module genes was set to 40. DynamicTreeCut algorithm was applied to obtain the gene modules, and the modules with high similarity were further merged (**Figure 1B.**).

Module eigengenes (MEs) and gene significance (GS) were employed to identify modules correlated with the expression of the genes of the human autophagy database (HAD, 232 genes) or gene cards (GC, 58 genes; score > 10 with the term “autophagy”). The MEs were considered as the major element of each gene module, and ME expression was recognized on behalf of all genes in a specific module. The module significance (MS) was then interpreted as the average GS of all genes in a given module. The module (i.e., bleu module) with the most absolute MS was defined as the significant module (**Figure 1C.**).

For the bleu module, the gene module membership (MM) was calculated in the hub module, which was used to measure the importance of each gene in the module. Genes with $|\text{GS}| > 0.2$ and $|\text{MM}| > 0.6$ were considered as candidate hub genes. Thereafter, the candidate hub genes were uploaded to the STRING database (<https://string-db.org/>) to establish a protein-protein interaction (PPI) network (**supplemental Figure 1, annex 1**). The top 10 genes with the most ME and PPI are depicted in **Figure 1F**.

3.2.2 Culture of human cell lines and GFP-LC3 transfection of A549

Human lung adenocarcinoma cell line A549 cells were cultured in DMEM /F-12 medium (1X, gibco, 21331-020) implemented with fetal serum (FBS) (biowest, S1810-500) (10%), sodium/pyruvate (100X, gibco, 11360-039) (1%), L-glutamine (100X, gibco, 25030-024) (1%), HEPES (1M, gibco, 15630-080) (1%) and non-essential amino acids (100X, gibco, 11140-035) (1%) and grown in an incubator at 37°C under 5% CO₂.

Human embryonic kidney HEK-blue null and HEK-blue TLR7 cell lines (Invitrogen), cell lines designed for studying the stimulation of TLR7 by monitoring the activation of NF- κ B, were cultured in DMEM+GlutaMAX medium (Gibco, 61965-026), in the presence of FBS (10%), penicillin/streptavidin (1%) (Gibco, 15140-122) and zeocin (100mg/mL) (Invivogen, ant-zn-1). HEK-blueTLR7 cell lines were also grown in the presence of blasticidin (10mg/mL) (Invivogen, ant-bl-1). This antibiotic is necessary for inducing a selection pressure and selecting only cells expressing the TLR7 receptor. These two cell lines were grown in an incubator at 37°C under 5% CO₂.

For GFP-LC3 transfection, A549 cells were infected with a lentivirus (Multiplicity of infection (MOI) of 1 meaning one particle of virus for one cell) containing the GFP-LC3 construct (collaboration with Prof. Guido Kroemer's team) in a medium containing FBS for 48 hours. Cell cloning was then performed using a cell sorter (BD influx), followed by a clonal expansion phase in a culture medium for 6 to 9 weeks. Among the clones

pre-selected based on their GFP expression and viability, we selected one clone more precisely based on its low level of autophagic vesicles in an untreated condition. This clone allowed us to analyze the level of autophagy in tumor cells under different cell conditions.

3.2.3 Stimulation of TLR7 by synthetic agonists and TLR7 blocking by an antagonist

To stimulate TLR7, A549 or A549 GFP-LC3 cells were cultured in cell culture plates of varying size for 24 hours and then grown with 10% FBS medium containing the TLR7 agonist ligands, CL264 (Invivogen, tlr1-c264e-5) (1mM) or loxoribin (Invivogen, tlr1-lox) (Loxo, 1mM) at the times indicated in the experiments. Both agonists act similarly on TLR7. Conversely, to prevent its stimulation, before incubation of A549 GFP-LC3 cells with tumor supernatants, the cells were pre-incubated overnight with a TLR7-specific antagonist (ODN 20958, 2 μ M) (Miltenyi Biotec) and then re-incubated upon addition of the supernatant.

3.2.4 Modulation of autophagy using drugs in lung tumor cell lines

To study the impact of autophagy on different parameters, rapamycin (Sigma, R0395) was always used as a positive control to induce autophagy. Thus A549 or A549 GFP-LC3 cells were cultured in cell culture plates of variable size for 24 hours, then cultured in DMEM F/12 medium with 10% FBS containing rapamycin (1 μ M) for 6, 24, or 48 hours. To study the involvement of autophagy in different processes induced after TLR7 stimulation, different autophagy inhibitors targeting different phases of the autophagy mechanism were used: (i) 3-methyladenine (3-MA., 10mM) (Sigma, M9281), wortmannin (wort., 100nM) (Sigma, W1628) and SAR405 (10 μ M) (MedChemExpress, HY-12481) which are inhibitors of the autophagy initiation complex, targeting the class III phosphatidylinositol 3-kinase (PI3K III); (ii) bafilomycin (Baf., 100nM) (Sigma, B1793), which is a non-specific inhibitor of the maturation phase of autophagy by preventing fusion between autophagosomes and lysosomes. A549 or A549 GFP-LC3 cells were cultured in cell culture plates of varying sizes for 24 hours and then grown in DMEM F/12 medium with 10% FBS containing these different inhibitors for 6, 24, and 48 hours.

Given 3-MA and wort can target both the types I and III PI3K, and to confirm the actual involvement of type III PI3K and autophagy in the effects observed, we used specific inhibitors of the type I PI3K, the capivasertib (Capi., 8nM) (MedChemExpress, HY-15431) and the pictilisib (picti., 3nM) (MedChemExpress, HY-50094).

3.2.5 Quantification of autophagic vesicles by confocal microscopy

Round coverslips previously sterilized with 70% ethanol were placed at the bottom of the wells of a 24-well plate. A549-LC3-GFP cells were then cultured on these coverslips for 24 hours and incubated for 6, 24, or 48 hours with different conditions. After fixation of the cells with fixation solution (BD Cytofix, BD Biosciences, 554655),

for 30 minutes at 4°C, the cells were labeled with DAPI (1/500) (Thermo Scientific, 62248) for 7 minutes at 4°C in the dark. After mounting the coverslips on slides by glycerol (Dako, C0563), visualization of autophagosomes was then observed by confocal microscopy (LSM 710) using ZEN software (ZEISS). For each condition and randomly, about ten images were captured (representing between 100 and 150 cells), and then the number of autophagosomes in the cells was measured using a personal script in the R software.

For co-localization experiments, GFP-LC3 cells were grown under the same conditions as already evoked before. To label lysosomes, cells were treated for 2 hours with lysotracker deep red (50nM) (Invitrogen, L12492) just before the cell fixation step. Co-localization and correlation analysis (Pearson's R coefficient) between green (GFP-LC3, autophagosomes) and red (Lysotracker, lysosomes) pixels was performed using the "coloc2" script in ImageJ software.

3.2.6 Study of autophagy by western blot

A549 cells were seeded in 6 wells plates (400,000 cells in 2 mL of culture medium). After 24 hours, cells were treated, or not, with rapamycin (1µM) or synthetic TLR7 agonists, CL264 (1mM), and loxoribine (1mM) for 6 hours. Two hours before the end of kinetics, bafilomycin (Baf, 100nM) was added to each incubating media condition to block autophagosomes degradation. Cells were then recovered and lysed with RIPA buffer (RadioImmunoprecipitation Assay, ThermoFisher Scientific, 89900) supplemented with PMSF (Phenylmethylsulfonyl fluoride, ThermoFisher Scientific, 78441B) (10µL/mL RIPA) and protease inhibitors (Roche, 04906845001) (1 pellet/mL RIPA) for 30 minutes. After centrifugation (20 000g for 2 minutes at 4°C), cell lysates were recovered, and protein concentrations were assessed by Bradford assay. Migration buffer (NuPAGE LDS Sample Buffer, ThermoFisher Scientific, NP0007) containing DTT (Dithiothreitol, ThermoFisher Scientific, P2325) was added to our samples (1/3 dilution). Then we deposited our different samples (20 µg per well) on 12% density polyacrylamide gels (Invitrogen, NW00120BOX) to migrate our lysates for 40 minutes at 200V. After transfer to nitrocellulose membrane (Invitrogen, IB23002) using iBlot2 (Invitrogen), we incubated our membranes with TBS (Tris Buffered Saline, Interchim, UP74004A) solution containing Tween 20 (Novagen, S7514484 809) (0.1%) (TBS-T) and BSA (Bovine Serum Albumin, Sigma Aldrich, A7906) (5%) for 1 hour to block aspecific sites on membranes. Then we added the primary antibodies anti-LC3 (rabbit, Cell Signaling, 4108) (1/1000) or anti-actin (mouse, Invitrogen, MA1-140) (1/6000) overnight at 4°C with stirring. After washing with TBS-T, the membranes were incubated with TBS-T containing goat anti-rabbit (Covalab, lab0363) (1/1000) or anti-mouse (Covalab, lab0365) (1/1000) secondary antibodies, both coupled to HRP enzyme for 1 hour. Finally, the membranes were put in contact with a revelation solution containing the HRP substrate (ThermoFisher Scientific, 34075), and the result of the enzymatic reaction was observed using iBright technology (ThermoFisher Scientific, CL1000). The ratio of LC3-II to actin bands was measured using ImageJ software.

3.2.7 Infection of A549 cells with single-stranded RNA viruses, IAV, and RSV

A549 and A549 GFP-LC3 cells were cultured for 24h, then infected with single-stranded RNA viruses with respiratory tropism, influenza virus (IAV) (H₃N₂ strain), or respiratory syncytial virus (RSV) (A2 strain) with an MOI variable depending on the experiments and the virus (IAV: MOI of 0.2 or 1 vs. RSV: MOI of 1 or 2), in a medium without FBS for 1h30. Then, the cells were incubated in a medium with FBS for 24 or 48 h. Measurement of the infection rate of A549 cells was performed by flow cytometry (BD, LSR Fortessa) *via* the detection of distinct viral proteins, respectively NP protein for IAV (1/100, Abcam, ab20921) and F protein for RSV (1/100, Novus Biologicals, NB100-63020AF488) The infection rate of A549 GFP-LC3 cells was determined by confocal microscopy (LSM 710) using ZEN software (ZEISS) by visualizing the different viruses, both coupled to mCherry in the cytosol of the cells. Actually, thanks to that construction, when the viruses replicate, mCherry is expressed and gets visible in the infected cell.

3.2.8 Treatment of cells with chemotherapies or specific cell death inducers and measurement of cell death induction by flow cytometry

After having evaluated different chemotherapies and specific cell death inducers doses at 6, 24, 48, and 72 h, an IC₅₀ dose at 24 h, corresponding to the concentration where 50% of cells are dead, was determined for each drug and was used for our different experiments. To study the impact of chemotherapies-induced cell death, cisplatin (Cis., 50μM) (from Cochin Hospital), taxol (from Cochin Hospital, 10μM), or oxaliplatin (Oxali., 200μM, Sigma, 09512) were used for 24 or 48h depending on the experiments. Besides to determine the impact of specific cell death, such as ferroptosis, pyroptosis, and necroptosis, specific inducers were used, such as respectively erastin (Era., 15μM) (Sigma, E7781), polyphillin VI (Pol VI., 8μM) (Selleckchem, S9302) and FTY720 (10μM) (Sigma, SML0700) for 24 or 48h depending on the experiments.

After culturing A549 cells for 24 hours, different treatments were applied to the cells depending on the experiments: treatment with anti-cancer chemotherapies (cisplatin and oxaliplatin) or with specific inducers of other types of cell death (erastin, polyphillin VI, and FTY720) for 6, 12, 24, or 48 hours. After recovery and washing with PBS, cells were labeled with Live dead (1/100) (Invitrogen, L10119), annexin V (1/200), and propidium iodide (PI) (1/100) (Invitrogen, V1345) for 30 minutes at 4°C in the dark. After washing with PBS, the cells were fixed with a fixation solution (Invitrogen, 00-5223-56) for 30 minutes at 4°C in the dark. Finally, the cells were analyzed for their different markers by flow cytometry (BD, LSR Fortessa). Data acquisition and analysis were respectively performed with Diva (BD, Biosciences) and Kaluza (Beckman Coulter) software. According to their labeling, cells were divided into live cells (Live dead-) versus dead cells (Live dead+). Then, the type of death induced was also determined for each of the treatments applied to the lung tumor cells: death by apoptosis (Annexin V+/PI-) or death by other types of death than apoptosis (Annexin V-/PI+).

3.2.9 Measurement of TLR7 ligands concentration by spectrophotometry

In HEK-blue TLR7 cells, which are genetically depleted for all the receptors involved in the induction of the NF- κ B pathway except TLR7, the stimulation of TLR7 promotes the production of the SEAP protein, dependently of NF- κ B. The presence of the SEAP protein can then be quantified by a colorimetric reaction thanks to a molecule that will bind to the SEAP protein and react by inducing a colorimetric response. The importance of the induced colorimetric reaction can then be quantified by measuring the absorbance by spectrophotometry. HEK-blue null cells served as control as they do not express receptors able to activate the NF- κ B pathway.

Then, HEK-blue null and TLR7 cells were incubated with different supernatants from A549 subjected to various treatments (untreated condition, infection by IAV or RSV, or induction of cell death by chemotherapies and different cell death inducers), and the measurement of TLR7 ligands concentration in supernatants was carried out. The same experiment was performed for tumor supernatants from fresh tumors of NSCLC patients. Actually, after surgery, fresh tumors from NSCLC patients were incubated in an RPMI medium with 10% FBS. After 24 hours, the media containing each tumor (hereafter called tumor supernatants) were recovered and stored at -80°C . The presence of TLR7 ligand in this supernatant was analyzed using HEK-blue cell lines. Thus, after 24h incubation of HEK-blue cells with tumor supernatants, 20 μL of cell culture supernatant was mixed with 180 μL of QUANTI-Blue solution (Invivogen, hb-det2) containing the alkaline phosphatase substrate for 1h30 at 37°C and the OD value, reflecting the concentration of TLR7 ligands, was measured at 620nm by the spectrophotometer (infinite 200Pro).

3.2.10 Measurement of immunogenic molecules expression by flow cytometry

A549 cells were seeded in wells of 12-well plates (100,000 cells in 1mL of culture medium). After 24 hours, different treatments were applied or not (NT) to the cells depending on the experiments: TLR7 agonists (synthetic or natural) or chemotherapies alone or combined with TLR7 agonists. After 24 or 48 hours of treatment, cells were treated with live/dead kit (1 μL /million cells) (Invitrogen, L34968) and an Fc receptor blocker to promote the proper binding of the different antibodies to their target antigens, Fc block (1/100) (BD Biosciences, 564219) for 30 minutes at 4°C . Then, the membrane antigens of interest were revealed using antibodies (1/100) incubated with the cells for 45 minutes. The references of the different antibodies and associated isotypes are summarized in a table (**Table 1**). After washing with PBS, cells were fixed and permeabilized thanks to a fixation/permeabilization solution (BD Cytotfix, BD Biosciences, 554655) for 30 minutes at 4°C . Cells were then incubated with different intracellular targeting antibodies diluted in WashBuffer solution (1/100) (Invitrogen, 00-8333-56) for 45 minutes. For each experiment and each kinetic, 2 wells of untreated cells were used to incubate all isotypes of each antibody used. After washing, the cells were passed through the flow cytometer (BD, LSR Fortessa), and for each cell, the intensity of the different fluorochromes coupled to the different antibodies was measured. Thus, the more the cell expresses the specific target antigen of the

antibody, the more the antibody will bind to it and accumulate in the cell, and the higher the intensity of the fluorochrome coupled to the antibody will be. Data acquisition and analysis are then respectively performed with Diva (BD, Biosciences) and Kaluza (Beckman Coulter) software.

TLR7	Rabbit IgG PE-conjugated polyclonal antibody	Novusc Biologicals, NBP2-24761
PD-L1	Mouse IgG2b,k BV421-conjugated monoclonal antibody	BioLegend, 329713
MHC-I	Mouse IgG1,k FITC-conjugated monoclonal antibody	BD Biosciences, 555552
Fas	Mouse IgG1,k APC-conjugated monoclonal antibody	BD Biosciences, 558814
IFN ψ R	Mouse IgG1 FITC-conjugated monoclonal antibody	R&D systems, FAB673F
CD47	Mouse IgG1,k PE-conjugated monoclonal antibody	BD Biosciences, 556046
CD133	Mouse IgG1,k PE-conjugated monoclonal antibody	BD Biosciences, 566593
B7-H3	Mouse IgG1 FITC-conjugated monoclonal antibody	R&D systems, FAB1027F

Rabbit IgG PE-conjugated isotype control	Novus Biologicals, NBP2-24983
Mouse IgG2b,k BV421-conjugated isotype control	BioLegend, 400342
Mouse IgG1,k FITC-conjugated isotype control	BD Pharmingen, 555748
Mouse IgG1,k APC-conjugated isotype control	BioLegend, 400122
Mouse IgG1,k PE-conjugated isotype control	BD Pharmingen, 555749

Table 1. Antibodies and associated-isotypes used for the project.

3.2.11 Cohorts of NSCLC patients

Three retrospective cohorts of NSCLC patients from the thoracic surgery department of Cochin Hospital were used in this study. The first one is composed of NSCLC patients non-treated with chemotherapy (**Table 2.**), while the second one is composed of NSCLC patients treated with various combinations of neoadjuvant chemotherapies (**Table 3.**). The last retrospective cohort of NSCLC patients comprises NSCLC patients before treatment with PD-1 anti-immune checkpoint immunotherapy (**Table 4.**). Clinical data of NSCLC patients composing the three cohorts were collected by clinicians. Clinical parameters of the three cohorts of NSCLC patients used in this study are summarized in tables.

Clinical data cohort 1	Number of patients
N	216
Age	
Median	62 years
Range	19 - 83 years
Gender	
Male	156 (72%)
Female	60 (28%)
Tumor size (mm)	
Median	35
Range	13 - 95
Stade	
IA	34 (16%)
IB	51 (23%)
IIA	34 (16%)
IIB	28 (13%)
IIIA	63 (30%)
IIIB	6 (2%)
Histology type	
ADK	155 (72%)
CE	51 (28%)
CGC	7 (0,05 %)
Smoking status	
< 15PA	44 (20%)
>15 PA	168 (77%)
NA	4 (3%)
BPCO status	
Yes	76 (35%)
No	107 (50%)
NA	33 (15%)

Table 2. Clinical data of NSCLC patients non-treated (cohort 1).

ADK: Adenocarcinoma, CE: squamous carcinoma, CGC: Large cell lung adenocarcinoma

Clinical data cohort 2	Number of patients
N	102
Age	
Median	60 years
Range	39 - 78 years
Gender	
Male	79 (78%)
Female	23 (22%)
Stade	
IIIA	90 (88%)
IIIB	12 (12%)
Histology type	
ADK	49 (48%)
CE	38 (37%)
CGC	15 (15%)
Smoking status	
Yes	84 (83%)
No	18 (17%)
BPCO status	
Yes	62 (60%)
No	40 (40%)
Chimiotherapies	
CDDP + GC	47 (46%)
CDDP + NAV	35 (35%)
CDDP + TAX	14 (13%)
others	6 (6%)
Responders	
Yes	25 (25%)
No	77 (75%)

Table 3. Clinical data of NSCLC patients treated by neoadjuvant chemotherapies (cohort 2).

ADK: Adenocarcinoma, CE: squamous carcinoma, CGC: Large cell lung adenocarcinoma. CDDP: cisplatin, GC: gemcitabine, NAV: navelbine, Tax: taxanes

Clinical data cohort 3	Number of patients
N	20
Age	
Median	64 years
Range	50 - 80 years
Gender	
Male	14 (70%)
Female	6 (30%)
Histology type	
ADK	11 (55%)
CE	4 (20%)
other	1 (5%)
NA	4 (20%)
Smoking status	
Yes	19 (95%)
No	1 (5%)
Responders to IT	
Yes	8 (40%)
No	12 (60%)

Table 4. Clinical data of NSCLC patients treated by immunotherapy anti PD-1, nivolumab (cohort 3).

ADK: Adenocarcinoma, CE: squamous carcinoma, CGC: Large cell lung adenocarcinoma

3.2.12 Staining and analysis of LC3 autophagy protein by immunohistochemistry (IHC) and Halo software

After cutting the slides for each of the NSCLC patient blocks included in the cohorts, the slides were incubated at 37°C to facilitate their deparaffinization in successive clearene baths (Leica microsystems) followed by ethanol baths of decreasing concentration (100%, 90%, 70 and 50%) (VWR). The slides were then unmasked with PT-link (Dako) and incubated in a heating alkaline solution bath (PT-link high). After this step, the slides whose tissue was previously delimited by the Dako Pen (Dako) were put in the automat (Autostainer plus, Dako) to facilitate the same staining conditions for each slide. Thanks to the latter, the slides were first incubated with H₂O₂ (Gilbert) to inactivate endogenous peroxidases, and then the aspecific sites were blocked with a protein block solution (Dako, X0909). The slides were then removed from the automated system and labeled for LC3 protein by incubating the anti-LC3 antibody (Sigma, HPA053767) diluted in a suitable solution (IHC diluent, Enzo) at 4°C overnight. After 3 washes with TTBS, the slides were put back into the automat. In this

second step of the automat, the slides were first incubated with an HRP-coupled anti-rabbit secondary polymer antibody (Polyview plus HRP, ENZ-ACC 103-0000) which emits labeling in the presence of its substrate, for 30 minutes. After washing with TTBS, LC3 labeling was revealed by incubation with a DAB solution (Dako, K3468) for 10 minutes. Nuclei were also labeled with hematoxylin solution (Dako, S3301). After washing with TTBS, the slides were mounted on coverslips with glycergel (Dako, C0563) and stored at 4°C before slides scanning by the nanozoomer at x40 magnification (Hamahatsu). The specificity of our staining was validated by the absence of labeling after incubation with the isotype control (Negative control, Rabbit, Agilent, IR600). After slide scanning, the LC3 staining for each NSCLC patient was analyzed by the Halo software. With the help of anapathologists, this software allowed us to determine the tumor cells within the tumor piece and to eliminate from the analysis the stroma, the necrotic areas, and the void. Then, the analysis of the LC3 labeling for each lung tumor cell was performed, and based on the intensity of their staining, tumor cells were divided into LC3 negative (weak or no staining) versus LC3 positive (strong staining). Thus for each patient composing the cohorts, the proportion of LC3 negative versus LC3 positive tumor cells is determined, and therefore based on the median of the proportion of LC3 negative versus LC3 positive tumor cells, each patient was sorted into LC3 low or LC3 high NSCLC patients.

3.2.13 Study of the correlation between LC3 and TLR7 or PD-L1 expression

For each patient composing the first two cohorts, those of untreated or neoadjuvant chemotherapy-treated patients, the level of TLR7 expression in the patients' tumors was previously measured by TLR7 immunohistochemical labeling. After scanning the slides with the nanozoomer (Hamahatsu) at x40 magnification, and analyzing the TLR7 labeling only on tumor cells with the Halo software, the patients were divided into TLR7 low versus high based on the optimal cut-off, as previously published⁶⁰¹. TLR7 low NSCLC patients include all patients with less than 30% of all tumor cells positive for TLR7. Conversely, TLR7 high patients include patients with more than 80% of all lung tumor cells expressing TLR7.

Similarly, concerning the cohort of NSCLC patients treated by anti-immune checkpoint immunotherapy (anti PD-1, nivolumab), the percentage of PD-L1 expression in the lung tumor cells of each patient was also previously measured by clinicians. Depending on the proportion of lung tumor cells expressing PD-L1, NSCLC patients were sorted into PD-L1 low (proportion of lung tumor cells expressing PD-L1<40%) versus PD-L1 high (proportion of lung tumor cells expressing PD-L1>40%). Then, correlation studies were performed between PD-L1 and TLR7 expression levels of each NSCLC patient of the cohort.

3.2.14 Statistical tests

Data values following a normal distribution are expressed as mean +/- SD. For each experiment, a comparison between the means of the samples in each group was

performed using the parametric Student or Mann-Whitney test. Values $p < 0.05$ were considered statistically significant (*). All data were analyzed with GraphPad company's Prism software or R studio, depending on the experiment.

3.3 Results

3.3.1 The immune receptor TLR7 expression correlates with the autophagy genes expression

To determine the molecular pathways that may modulate the level of autophagy in the tumor cell, we performed a WGCNA analysis that permitted us to calculate a global correlation of genes with the autophagy genes expressions. We used a public cohort of 516 NSCLC patients (TCGA-LUAD) for whom transcriptomic analysis was performed. Correlation studies of the whole transcript with either the human autophagy database (HAD, 232 genes) or the autophagy genes card (GC, 99 genes) were performed, as explained (**Figure 1A.**). WGCNA analysis permitted to cluster genes together based on their expression in patients, facilitating the analysis of a global correlation with our genes of interest. This analysis allowed us to cluster genes in 40 different modules, named by different colors (**Figure 1B.**). Some modules are strongly correlated with the autophagy genes of HAD or GC, like the blue module. Conversely, others are inversely correlated with the autophagy genes, such as the darkgreen modules (**Figure 1C.** and **1D.**).

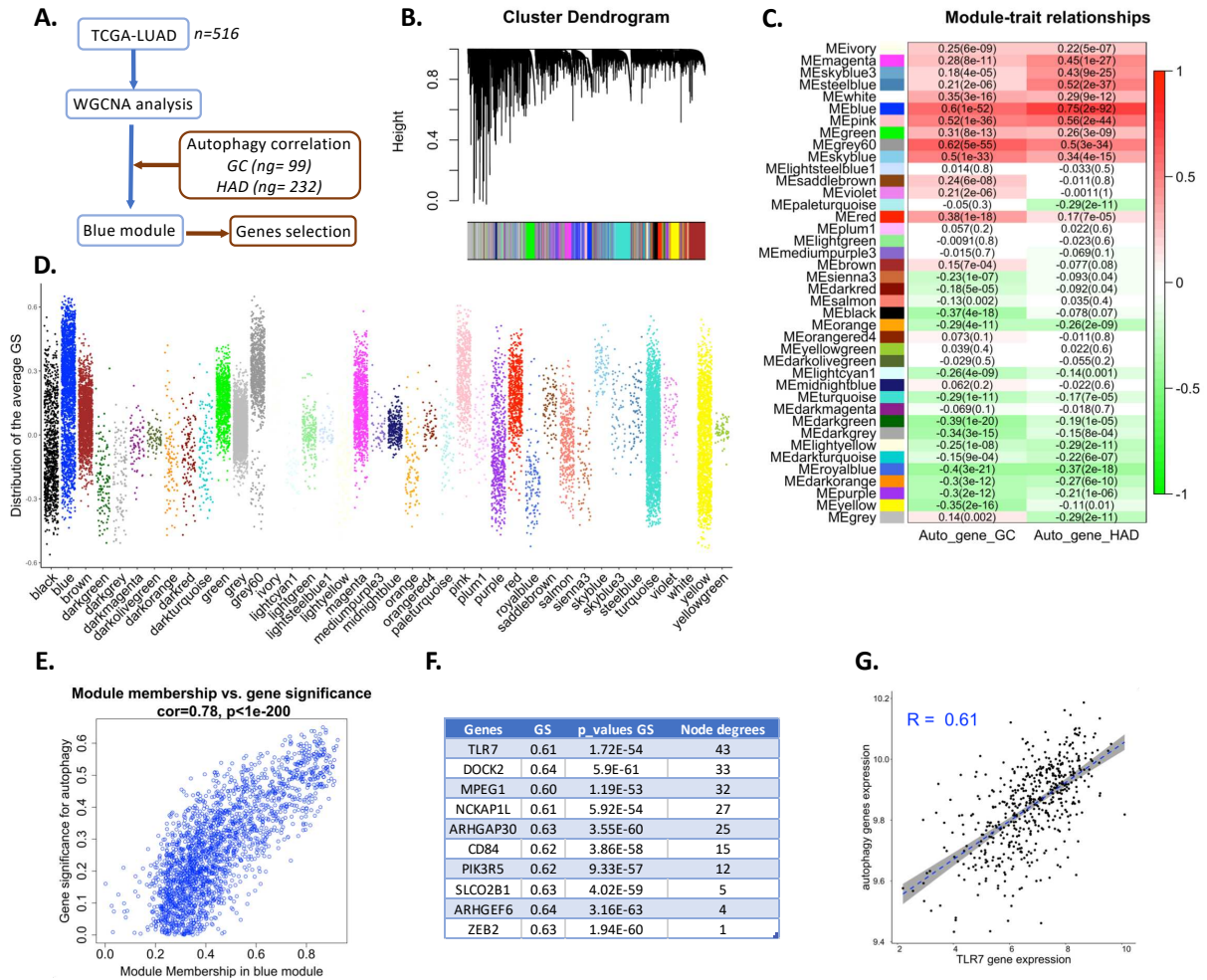


Figure 1. Module identification and genes correlation from WGCNA analysis

A. Strategy of our analysis. A public data cohort of 516 adenocarcinoma NSCLC patients for whom a whole transcriptomic analysis has been performed on the whole cell composing the tumor (TCGA-LUAD) was used, and a WGCNA analysis consisting in clustering genes based on their expression profile, was applied to the cohort. Correlation studies were realized between the different clusterized genes and the autophagy genes from the human autophagy database (HAD, 232 genes) and the autophagy genes card (GC, 99 genes). **B.** Clustering dendrogram of genes to create modules. **C.** Evaluation of the level of correlation of the modules from the HAD and GC. From the weak correlated in green to the strong correlated in red. **D.** Distribution of the average gene significance (GS) for autophagy in function of the module studied. GS represents a correlation score. Each point represents one gene. **E.** Representation of the gene significance for autophagy in function of its module membership in the blue module. **F.** Selection of the top 10 genes comprising the blue module, which are high for GS and high for their number of node degrees. **G.** Study of correlation between the autophagy genes expression (GC) and the TLR7 gene expression and determination of the coefficient of correlation.

Therefore, we selected the blue module as it was the most correlated with both HAD and GC. We then plotted the genes in the blue module according to the correlation score and the higher module membership score, representing the gene's importance in this correlation (based on the correlation with autophagy and the expression in the sample) (**Figure 1E.**). Then, we performed an analysis of the functional protein association networks (STRING) in order to select the gene with a functional protein and calculate the most central (node) protein in this correlated module (**Supplementary Figure S1, annex 1**). A top 10 genes whose expression is

the most correlated with the autophagy genes were selected (**Figure 1F.**). Among these genes, TLR7 strongly correlates with the autophagy genes with a coefficient of correlation of 0.61 and exhibits the most node degrees, suggesting an important role for this protein in the module correlation (**Figure 1F.** and **1G.**).

3.3.2 TLR7 is the member of the TLR family which correlates the most with the autophagy genes in NSCLC malignant cells

Although some TLRs also correlate strongly in transcript with autophagy genes, TLR7 has the highest correlation in expression with autophagy genes ($R=0.61$). Among the other TLRs, TLR4 and TLR8 correlate most strongly with autophagy genes expression ($R=0.58$ and $R=0.56$, respectively). The other TLRs investigated in this study, TLR1/2/3/5/6 and 9, correlated weakly with autophagy genes expression ($R<0.45$) (**Figure 2A.**).

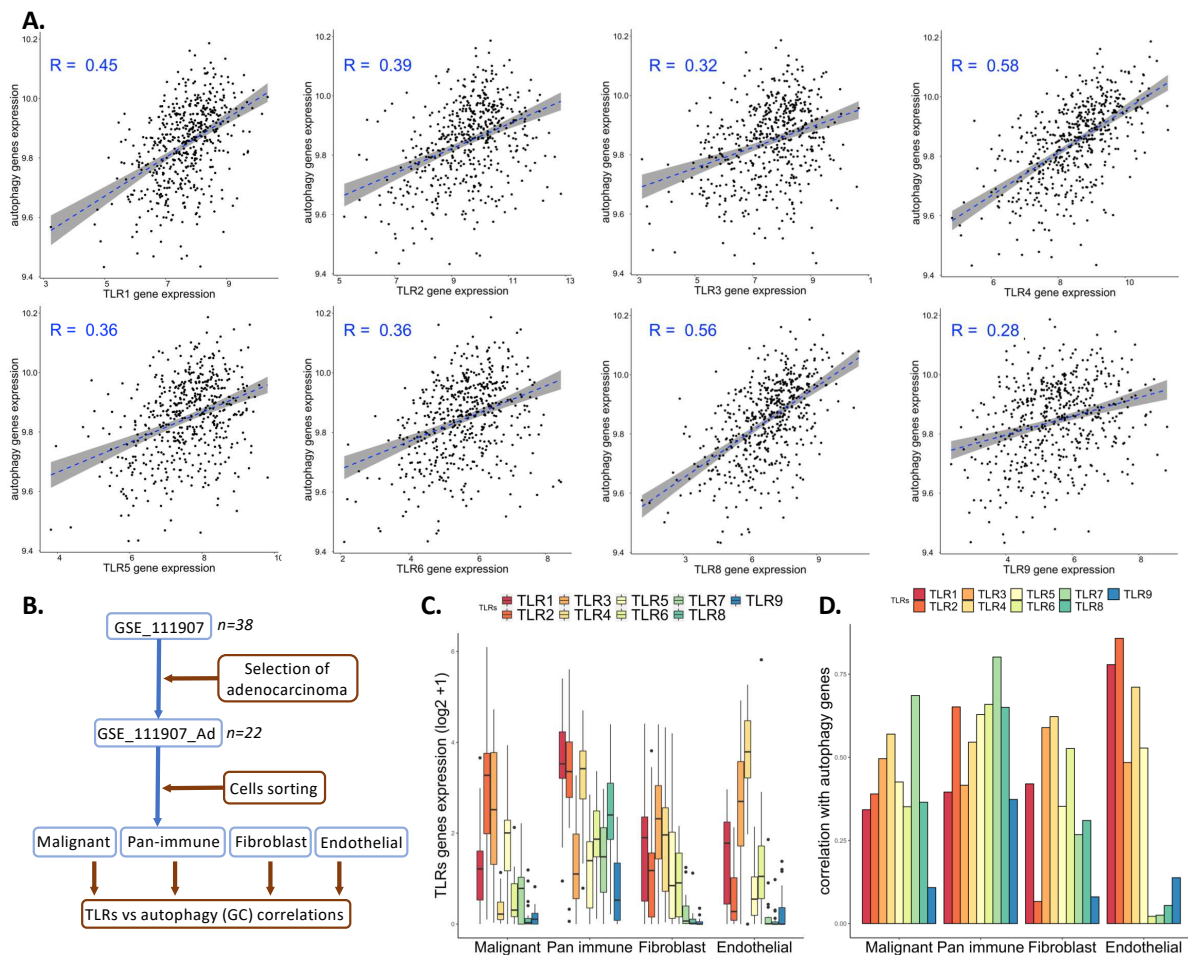


Figure 2. Correlation between TLRs and autophagy genes expression in different cell populations.

A. Clustering of genes from the TCGA-LUAD after WGCNA analysis, the study of the correlation between autophagy genes expression and TLRs genes expression and determination of the coefficient of correlation
B. Strategy on which is based our analysis. A public data cohort of 38 lung tumor patients for whom each cell type composing the tumor microenvironment (TME) has been sorted and for which a whole transcriptomic analysis has been performed on all the sorted cells (GSE) was used, and only the adenocarcinoma NSCLC patients were selected for our analysis ($n=22$). For all the cells composing the tumor microenvironment (malignant, pan-

immune, fibroblast and endothelial cells), correlation studies between autophagy genes, and TLRs genes expression were performed. C. Graph representing the level of TLRs expression in function of the cell type composing the TME. D. Graph representing the level of correlation of autophagy genes with each TLRs genes expression in function of the cell type composing the TME.

This same analysis of the correlation of autophagy genes expression with TLR coding genes was performed on another public cohort of adenocarcinoma NSCLC patients (GSE-111907 dataset, 22 patients). In this cohort, cells composing the TME were sorted as malignant cells (EPCAM⁺ CD45⁻ CD31⁻), pan-immune cells (EPCAM⁻ CD45⁺), endothelial cells (CD31⁺ CD45⁻ EPCAM⁻), and fibroblasts (CD10⁺ CD45⁻ EPCAM⁻ CD31⁻ CD10⁺). Thus, a correlation study of the expression of autophagy genes with the expression of genes coding for TLRs was performed on these different cells, as explained in **Figure 2B**. First, we observed that the expression of the genes encoding TLRs varies strongly according to the cell type (**Figure 2C**). For example, the gene coding for TLR2 is highly expressed in malignant and immune cells but is more weakly expressed in fibroblasts and endothelial cells. TLR7 also has a variable expression depending on the cell type studied. While it is very weakly expressed in fibroblasts and endothelial cells, it is more highly expressed in tumor and pan-immune cells. In immune cells, genes coding for TLR1, 2, and 3 are the most expressed, whereas, in malignant cells, genes coding for TLR1, 2, 3, 5, and 7 are the most highly expressed. Among the whole family of TLR, fibroblasts express mainly genes coding for TLR1, 3, and 4. Endothelial cells express genes coding for TLR3 and 4 more than other TLRs (**Figure 2C**). Similarly, the level of correlation between the autophagy genes expression and TLR-encoding genes differs according to the TLR and cell type studied. Although TLR7 was previously shown not to be the most highly expressed in any of the cell types comprising the TME, it is the one of the TLR family whose expression correlates the most with autophagy genes in both pan-immune and malignant cells (**Figure 2D**).

3.3.3 TLR7 stimulation within lung tumor cells leads to the induction of a complete autophagy process

These bioinformatic approaches suggest a critical role for TLR7 in modulating autophagy level in lung tumor cells. To investigate this potential impact of TLR7 on autophagy in this context, we used a human adenocarcinoma lung tumor line, A549. Autophagy induction was monitored by microscopy by the increase in the number of autophagic vacuoles, characterized by the expression of the LC3 protein. This autophagosome formation is easily visible thanks to a GFP-LC3 construct, stably transfected into cells by a lentivirus (A549 GFP-LC3). Indeed, among all proteins orchestrating the autophagy mechanism, only the LC3 protein will anchor in the membrane of the forming autophagosome and remain there. Thus, in the basal state, the labeling of GFP coupled to the LC3 protein is widely distributed in the cytosol. On the other hand, when autophagy is induced, puncta will be formed in the cytosol of the cells, which are the autophagosomes, as explained in **Figure I5**.

To better understand the link between TLR7 and autophagy in lung tumor cells, we treated A549 GFP-LC3 cells with two synthetic TLR7 agonists, CL264 (1mM) and loxoribin (loxo, 1mM), and the number of autophagic vacuoles was measured at different time points (6, 24 and 48h). At 6h post-stimulation, it was observed that A549 GFP-LC3 cells had a much higher number of autophagosomes per cell than the untreated condition and of a comparable value to those treated with an autophagy inducer, rapamycin (Rapa, 1 μ M) (**Figure 3A.**). Furthermore, it was observed that TLR7 stimulation induced the same pattern of increase in autophagy level over time as the positive control: a peak in autophagy level at 6h post-treatment that then progressively decreases over time with a level that remains higher than the untreated condition at 24 (**Figure 3B.**). It is important to note that the increase in the number of autophagic vacuoles does not necessarily mean induction of the autophagy mechanism but may result from an accumulation of autophagosomes in the basal state following inhibition of the final phase of the autophagy mechanism. To decide between these two hypotheses, we studied the colocalization of autophagosomes with lysosomes after the treatment of lung tumor cells with synthetic agonists of TLR7. For this purpose, thanks to the GFP-LC3 construct, autophagosomes are marked in green, and by using the lysotracker, which permits to stain lysosomes in red, we can see the formation or not of yellow puncta reflecting the colocalization of autophagosomes with lysosomes (autolysosomes). To perform this experiment, a positive control for *de novo* autophagy induction (Rapa, 1 μ M) and a positive control for inhibition of the autophagy maturation, bafilomycin (Baf, 100nM), were used. After obtaining the images, the level of colocalization of autophagosomes (green pixels) with lysosomes (red pixels) was analyzed with the colocal2 script of the ImageJ software, and a Pearson coefficient reflecting the level of correlation was established for each condition. It was thus shown that the correlation profile of autophagosomes with lysosomes of cells treated with synthetic TLR7 agonists is substantially similar to that of the profile obtained for the positive control of autophagy induction (rapamycin) and does not correspond to the profile observed during inhibition of autophagy maturation (bafilomycin). Thus, TLR7 stimulation induces a complete autophagy flux in lung tumor cells (**Figure 3C.**). To confirm the impact of synthetic TLR7 agonists on the autophagy flux, we treated the cells as before with the addition of bafilomycin, blocking autophagosome degradation, and measured the autophagy level in the tumor cells by western blot technique. Indeed, as indicated in the introduction, following the induction of the autophagy mechanism, the autophagy protein LC3 is converted from an inactive form, diffused in the cytosol (LC3-I, 16kDa) to an active form, coupled to the autophagosome membrane (LC3-II, 14kDa), both distinguishable and differentiable by western blot. Here, we observed that both synthetic TLR7 agonists induced an increase in the LC3-II/actin ratio and, thus, an increase in the autophagy level in treated cells, confirming that TLR7 stimulation by synthetic agonists induces a complete autophagy flux in lung tumor cells (**Figure 3D.**).

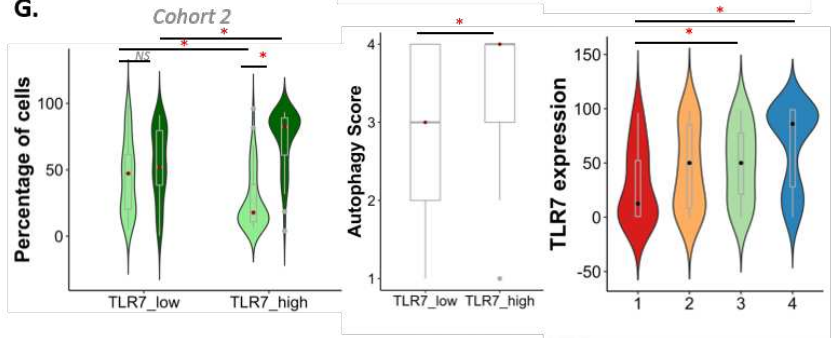
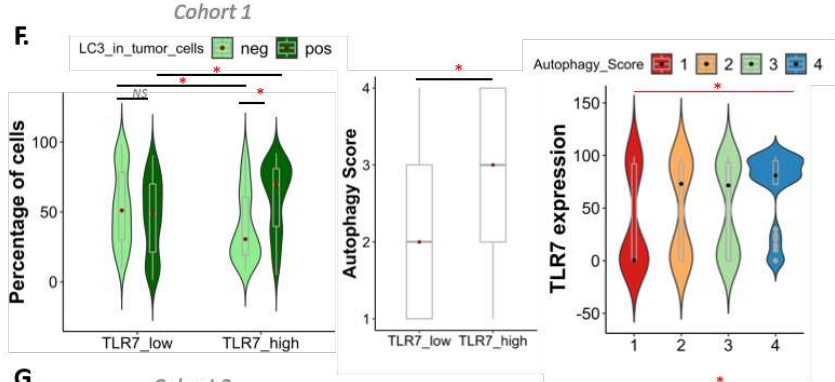
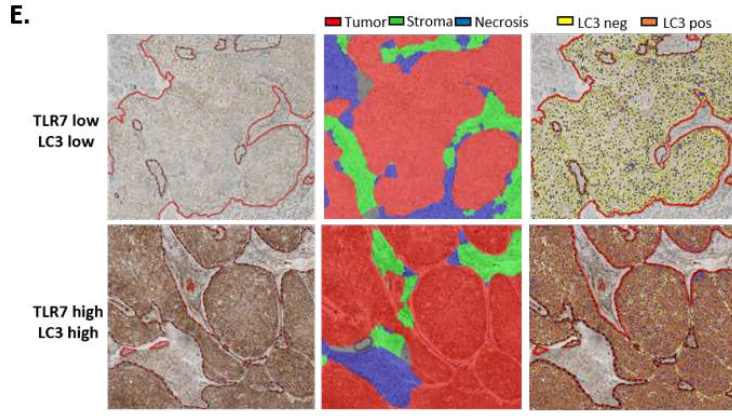
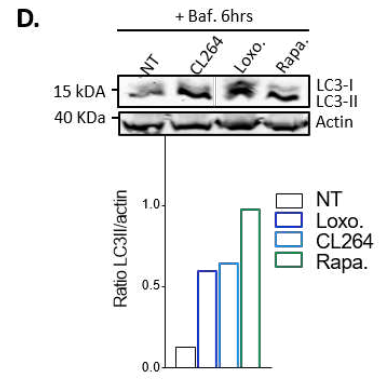
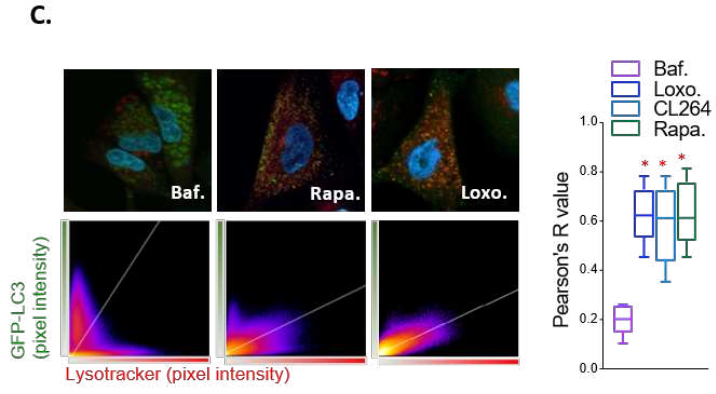
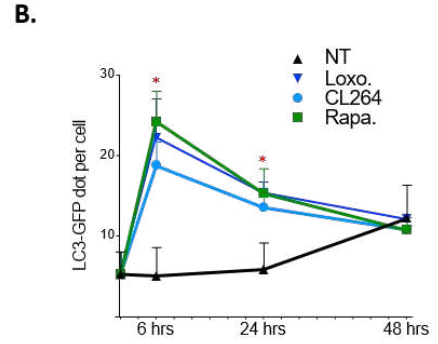
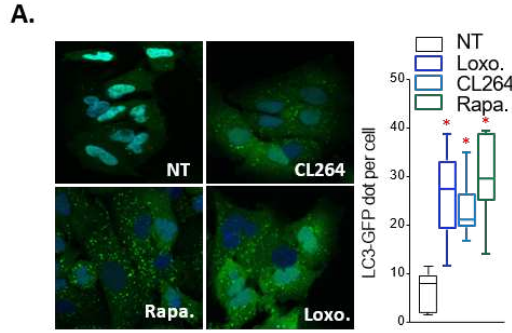


Figure 3. Study of the impact of TLR7 on the autophagy level in lung tumor cells.

A. Example of images of A549 stably transfected with the GFP-LC3 construct (A549 GFP-LC3) treated or not (NT) with an autophagy inducer, the rapamycin (rapa, 1 μ M) or by synthetic TLR7 agonists, CL264 (1mM) and loxoribin (Loxo, 1mM) for 6h. Green puncta represent autophagosomes. The graph represents the quantification of the GFP-LC3 dots per cell in each condition at 6h post-treatment. The analysis of the images was carried out on approximately 100 cells using the ZEN software, and the quantification was performed by R software. The experiment was performed six times. **B.** The same experiment as in A. was performed with different kinetics: 6, 24, and 48h post-treatments. The graph represents the evolution of the GFP-LC3 dots per cell in each condition over time. **C.** Example of images of A549 GFP-LC3 treated or not (NT) with either an inhibitor of the autophagy maturation, the bafilomycin (Baf, 100nM), an autophagy inducer (rapa., 1 μ M) or synthetic TLR7 agonists (CL264 and Loxo., 1mM) for 24h. Lysosomes were marked in red by the addition of lysotracker 90 minutes before the end of the experiment. The graph represents the study of the colocalization of autophagosomes (green puncta) with lysosomes (red puncta) to form autolysosomes (yellow puncta) thanks to the coloc2 script of the ImageJ software and obtention of a Pearson coefficient reflecting the level of colocalization. The experiment was performed three times. **D.** Analysis of autophagy induction by western blot by studying the conversion of LC3-I (inactive form, 16kDa) to LC3-II (active form, 14kDa). Actin is also revealed and used here as a housekeeping protein (42kDa). A549 cells are either treated or not (NT) with an autophagy inducer (rapa., 1 μ M) or with synthetic TLR7 agonists, CL264 and loxoribin (1mM) for 6h. Two hours before cell lysate and western blot, cells were treated with an inhibitor of autophagy maturation, bafilomycin (Baf., 100nM). The LC3-II/actin ratio corresponding to autophagy induction is measured for each condition and plotted. **E.** Analysis of the autophagy level of two NSCLC patients thanks to the LC3 staining within lung tumor cells. After LC3 labelling by immunohistochemistry, the NSCLC slide was scanned by the nanozoomer and analyzed by the self-learning image analysis software Halo. That software allows us, with the help of two anaphysiopathologists, to teach it to recognize the different areas composing the tumor (the tumor cells, the stroma, and the necrosis) thanks to structures and morphologies differences. Then after having differentiated the different parts of the tumor, it measures for each lung tumor cell, the autophagy level. Lung tumor cells were sorted either in LC3 negative (LC3 neg, in yellow) versus LC3 positive (LC3 pos, in orange) lung tumor cells. **F.** At the left : Measurement of the proportion of lung tumor cells LC3 positive versus negative for both TLR7 low (proportion of lung tumor cells expressing TLR7 <78%) versus TLR7 high (proportion of lung tumor cells expressing TLR7 >78%) NSCLC patients of the cohort 1. In the medium : Implementation of an autophagy score ; 1, proportion of lung tumor cells LC3 pos <25% ; 2, 25<x<50% ; 3, 50<x<75% and 4, x>75%. Measurement of the autophagy score in TLR7 low versus TLR7 high NSCLC patients. On the right : Evaluation of the TLR7 expression level in function of the autophagy score. **G.** The same analysis as in F. was carried out on the NSCLC patients from cohort 2, those treated by neoadjuvant chemotherapies. Student's t-test, * p<0.05

Then, we wanted to determine in NSCLC patients if the correlation between TLR7 expression on lung tumor cells and autophagy level was still accurate. For this purpose, two retrospective cohorts of NSCLC patients were used, a cohort of NSCLC patients not treated with chemotherapies (Cohort 1) and a second one within NSCLC patients were treated with neoadjuvant chemotherapy combinations, cisplatin plus vinorelbine or cisplatin plus gemcitabine (Cohort 2). Clinical parameters concerning the cohorts used for our analyses are summarized into tables (**Tables 2 and 3.**). For each patient in the cohorts, biological data, such as the level of TLR7 expression in their lung tumor, and clinical data, such as patient survival, are already known. Depending on the proportion of lung tumor cells expressing TLR7, NSCLC patients were divided into TLR7 high (>78% expression) versus TLR7 low (<78% expression), as previously published⁶⁰¹. The autophagy level of NSCLC patients was measured by analyzing the LC3 staining only in the lung tumor cells using the Halo software, which allowed us, with the help of two anapathologists, to teach the artificial intelligence of the software to recognize the different areas included in the tumor, such as the stroma, the necrosis, and the tumor cells. (**Figure 3E**). Based on the intensity of the labeling and the threshold calculated by the software, the lung tumor cells were divided into LC3 negative (LC3 neg, in yellow) versus LC3 positive (LC3 pos, in orange) (**Figure**

3E.) In the retrospective cohort of NSCLC patients non-treated by neoadjuvant chemotherapies (cohort 1), it was observed that TLR7 high patients had a higher proportion of LC3 pos tumor cells and, conversely, a lower ratio of LC3 neg tumor cells than TLR7 low patients (**Figure 3F**). Based on the proportion of lung tumor cells within the tumor part which are LC3 pos, an autophagy score from 1 to 4 for each NSCLC patient of the cohorts was given (1: <25% of lung tumor cells that are LC3 pos, 2: 25%<x<50%, 3: 50%<x<75% and 4: >75%). We also demonstrated that TLR7 high NSCLC patients have a higher autophagy score than TLR7 low NSCLC patients. Finally, we observed that TLR7 expression in NSCLC patients correlates with the autophagy score (**Figure 3F**). No correlation between the autophagy level and the patient's age, size, and tumor stage was observed (**Supplementary Figure S2A-E, annex 1**). Importantly, the presence of immune cells in the tumor zone did not impact the level of autophagy, meaning that our LC3 staining was not affected by the infiltration of immune cells in the tumor body (**Supplementary Figure S2F, annex 1**).

A similar analysis was performed for the second retrospective cohort of NSCLC patients, those treated by neoadjuvant chemotherapies. Similarly, we observed that TLR7 high patients had a higher proportion of LC3 pos tumor cells and, conversely, a lower ratio of LC3 neg tumor cells than TLR7 low patients. Besides, as previously, TLR7 high NSCLC patients have a higher autophagy score than the TLR7 low NSCLC patients, and the expression of TLR7 was correlated with the autophagy score (**Figure 3G**). These results are consistent with our previous *in vitro* results and suggest that physiologically, TLR7 is stimulated and induces autophagy in NSCLC tumor cells.

3.3.4 The presence of physiological TLR7 ligands in the TME of NSCLC patients induces autophagy in tumor cells

To support this hypothesis, fresh tumors or adjacent tissue from NSCLC patients were incubated for 24 hours in a culture medium to allow the diffusion of molecules present in the tumor microenvironment into the culture medium (called tumor supernatant and control supernatant, respectively). A549 GFP-LC3 cells were then incubated or not with these different supernatants for 6, 24, or 48h, and the average number of autophagosomes per cell, reflecting the autophagy level, was then measured for each culture condition. A positive control of TLR7 stimulation was added to the culture medium (Loxo., 1 mM). Compared with the control condition (RPMI or control supernatant), we observed that lung tumor cells incubated with the tumor supernatant had a higher number of autophagosomes at 6h post-incubation, suggesting the presence of diffuse molecules in the tumor microenvironment favoring the induction of autophagy (**Figure 4A**). Besides, we observed that the autophagy level profile in lung tumor cells treated with the tumor supernatant, although lower compared with the synthetic agonist of TLR7, is similar. This suggests that in a pathophysiological context of lung tumors, diffuse molecules are present within the tumor microenvironment and increase the autophagy level in lung tumor cells, which may have important implications for tumor progression. Interestingly, we observed that the increase of autophagy level in lung tumor cells after incubation with the tumor supernatant was

greatly reduced in tumor cells which has been previously blocked for TLR7 using a specific TLR7 antagonist (ODN 20958, 2 μ M) compared to cells not blocked for TLR7 (Figure 4B.). This observation suggests that the increase in autophagy levels observed following incubation with tumor supernatant is indeed due in part to the TLR7 ligand and its recognition by its receptor.

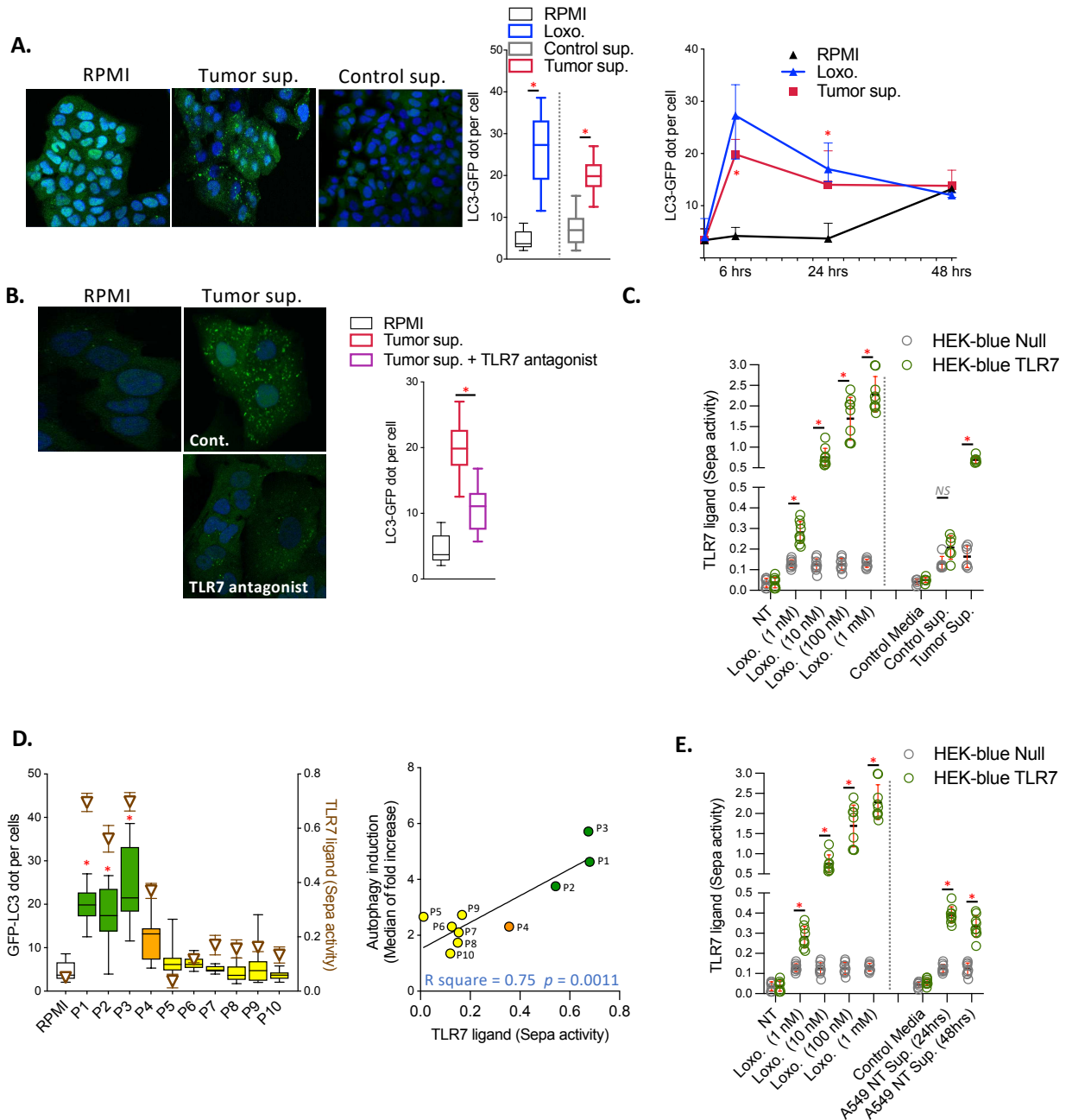


Figure 4. Measurement of the presence of TLR7 ligands in the TME and its impact on autophagy activation in lung tumor cells

A. At the left : Examples of images of A549 GFP-LC3 cells incubated for 6h with either an RPMI medium alone, an adjacent tissue supernatant (Control sup.), or a tumor supernatant (Tumor sup.) that is a medium in which adjacent tissue or fresh tumor from NSCLC patients has been put for 24 hours). Medium : Measurement of the LC3-GFP dot per cell in A549 GFP-LC3 in function of the medium in which the cells are incubated for 6h. On the right : kinetics of LC3 GFP-LC3 dots per cell at 6, 24, and 48h post-incubation in function of the medium, the

cells are incubated. The images were analyzed on approximately 100 cells using the ZEN software, and quantification was performed by R software. The experiment was performed three times. **B.** Examples of images of A549 GFP-LC3 cells treated with either an RPMI medium or a tumor supernatant under two different conditions: an untreated condition (control) or a condition where cells were preincubated with a specific TLR7 antagonist (ODN 20958, 2 μ M) (Miltenyi Biotec) overnight before and at the moment of incubation of the cells with the supernatant, for 6h. Plot representing the average number of autophagosomes in cells pre-incubated or not overnight with the TLR7 antagonist. The experiment was performed three times. **C.** Measurement of the presence of TLR7 ligands through the measurement of the Sepa activity of HEK-blue cells, following the treatment or not (NT) with different doses of TLR7 agonists (Loxo., 1nM, 10nM, 100nM, and 1mM), or after the incubation with a culture media alone (Control media), with an adjacent tissue supernatant (Control Sup.) or with a tumor supernatant (Tumor Sup.). The experiment was performed two times in triplicates. **D.** At the left : Graph describing the GFP dot per cell in A549 GFP-LC3 in function of the tumor supernatant in which the cells are incubated for 6h as well as their concentration in TLR7 ligands, determined through the measurement of the Sepa activity of HEK-blue cells. Tumor supernatants were classified based on their richness in TLR7 ligands: highly concentrated in green, medium in orange, and less concentrated in yellow. On the right : Graph representing the autophagy induction (median of fold increase) observed for each supernatant of NSCLC patient tumors compared with the control condition (RPMI) in function of the concentration in TLR7 ligands within the tumor supernatant (proportional to the Sepa activity induced in HEK-blue cells). The experiment was performed three times in triplicates. **E.** Measurement of the abundance of TLR7 ligands through the analysis of the Sepa activity of HEK-blue cells following the treatment or not (NT) with different doses of TLR7 agonists (Loxo., 1nM, 10nM, 100nM, and 1mM), or after the incubation with a culture medium alone (Control media), or with supernatant from A549 in culture for 24h (A549 NT Sup. (24hrs)) or 48h (A549 NT Sup. (48hrs)). The experiment was performed three times in triplicates. Student's t-test, * : $p < 0.05$.

To confirm that, we have investigated the presence of TLR7 ligands within the tumor supernatants. This evaluation was performed by measuring the activity of reporter cells for TLR7 (HEK-blue TLR7) after incubation with the different supernatants, whose Sepa activity level is proportional to the concentration of TLR7 ligands. Actually, these cells are genetically depleted for all the receptors involved in the induction of the NF- κ B pathway, except TLR7. Therefore, in the presence of TLR7 ligand, the NF- κ B pathway is activated as well as the reporter gene coding for the SEAP protein, which can react with a substrate and induce a colorimetric reaction. The intensity of that latter can then be quantified and give us an indication of the concentration of the TLR7 ligand present in the medium. A negative control was also used, which expresses the same reporter gene for NF- κ B as the HEK-blue TLR7 but is completely depleted for receptors triggering the induction of this pathway, including TLR7 (HEK-blue Null cell line). First, we used different concentrations of TLR7 synthetic ligands (Loxo. : 1nM, 10nM, 100nM, and 1mM) for 6h to confirm our model, and we observed that the Sepa activity of HEK-blue TLR7 was proportional with the concentration of the TLR7 ligand within the medium (**Figure 4C.**). Conversely, both HEK-blue Null incubated with the different concentrations of TLR7 synthetic agonist and HEK-blue TLR7 incubated with a culture medium (NT) do not exhibit Sepa activity. Therefore, after having demonstrated that our model worked, we incubated these same cells for 6h with either a culture medium (Control Media), a supernatant from adjacent tissue (Control Sup.), or with a tumor supernatant (Tumor Sup.). Contrary to the two other conditions, HEK-blue TLR7 incubated with a tumor supernatant from an NSCLC patient for 6h exhibited a significant Sepa activity compared with the HEK-Null which is quite similar to the Sepa activity induced following incubation of the cells with the TLR7 synthetic agonist at a concentration of 10nM. These results showed that, in the tumor supernatant from NSCLC tumor, TLR7 ligands can be detected (**Figure 4C.**). Besides, using different

tumor supernatants from different NSCLC patients, we have observed that the ability of tumor supernatants to induce autophagy in A549 GFP-LC3 cells was variable depending on the supernatants: low (yellow), medium (orange), and high (green) (**Figure 4D.**). In the meantime, using HEK-blue cells, we demonstrated that the capacity of tumor supernatants to increase the autophagy level in lung tumor cells was proportional to the concentration of TLR7 ligands present within it (**Figure 4D.**).

Interestingly, from our experiments, we observed that culture media incubated with lung tumor cell line, A549 (A549 NT Sup.), for 24 and 48h, are responsible for an increase of Sepa activity in HEK-blue TLR7 and not in HEK-blue Null cells. Therefore, these results demonstrate that lung tumor cells can physiologically secrete TLR7 ligands and could lead to the induction of autophagy in lung tumor cells (**Figure 4E.**).

We have performed different experiments to go deeper into the mechanisms underlying this release of TLR7 ligand. Our main hypothesis was that the dying cell could release, during its death, cellular single-stranded RNA that the TLR7 could recognize. To do that we have used a chemotherapy, the oxaliplatin (Oxali., 200 μ M) for 24 and 48h to induce lung tumor cell death. Its capacity to induce cell death and apoptosis was verified by the double Annexin V / Propidium iodide (PI) staining (**Supplementary Figure S3A, annex 1**). We have measured the percentage of apoptotic cells through the Annexin V+/ PI- cell staining, and cell death was measured by the Live/dead staining. We showed that oxaliplatin-induced cell death according to the time of incubation (with around 70% of cell death at 48 hours), and this cell death was mainly mediated by apoptosis (**Supplementary Figure S3A, annex 1**). Then, by incubating HEK-blue TLR7 and HEK-blue Null cells with the supernatants of A549 treated by oxaliplatin, we demonstrated that, although cell death rate is important both at 24 and 48h in A549, no significant Sepa activity is observable in HEK-blue TLR7 compared with HEK-blue Null. In contrast, tumor supernatants from A549 non-treated (A549 NT Sup.) induce an increase of Sepa activity in HEK-TLR7, as previously described (**Supplementary Figure S3B, annex 1**).

Although apoptosis of lung tumor cells does not seem involved in the release of the TLR7 ligand, we investigated the impact of the other types of cell death that can occur in the tumor microenvironment, such as necroptosis, ferroptosis, and pyroptosis. These different cell deaths are all known to induce immunogenic cell death, characterized by the cell's release of immunogenic molecules. Our hypothesis is that induction of immunogenic cell death favors the release of RNA by the tumor cells that the TLR7 can recognize. For this purpose, lung tumor cells were incubated with specific inducers of the different types of cell death, respectively FTY720 (10 μ M), erastin (Era., 15 μ M), and polyphyllin VI (Pol VI., 8 μ M) for 24 and 48h. For each condition, cell death was measured by Live/dead staining, which positively labels dead cells. Verification of the induction of apoptotic versus non-apoptotic death was then performed by double Annexin V/PI (propidium iodide) labeling: apoptotic death (Annexin V+/PI-) versus non-apoptotic death (Annexin V-/PI+). We observed that both at 24 and 48h, erastin and FTY720 induced a good rate of cell death. Polyphyllin VI is less efficient in killing lung tumor cells at the dose we used. Then, thanks to the double Annexin V / Propidium iodide (PI) staining, we confirmed that all of these inducers induce a non-apoptotic cell death (**Supplementary**

Figure S3C, annex 1). By incubating HEK-blue cells with the supernatants of A549 treated by the different cell death inducers for 24 and 48h, we demonstrated that no significant Sepa activity is observable in HEK-TLR7 compared with HEK-Null (**Supplementary Figure S3D, annex 1**).

Taken together, these results demonstrated that, in the tumor microenvironment, there are physiological TLR7 ligands that can be secreted by alive tumor cells and that ligands modulate autophagy in the malignant cells *via* a TLR7 stimulation.

3.3.5 Autophagy level within lung tumor cells is associated with a bad prognosis for NSCLC patients

Given the importance of autophagy in cancer progression, we investigated the potential impact of this new way of autophagy induction on NSCLC tumor progression. To do that, we analyzed the correlation of our LC3 staining with the clinical data in our retrospective cohort of untreated NSCLC patients (cohort 1).

The proportion of LC3 positive (LC3 pos) versus LC3 negative (LC3 neg) lung tumor cells for each patient in this cohort was measured and determined as previously described. Depending on the ratio of LC3 positive versus negative lung tumor cells, each NSCLC patient was divided into LC3 high and LC3 low according to the median of the percentage of LC3 positive cells (**Figure 5A.**). Subsequently, a correlation analysis of the autophagy level of NSCLC patients with survival was performed for each patient. By Kaplan Meier analysis, we determined that LC3 high patients had a worse survival and an increased hazard ratio than LC3 low patients. Besides, depending on their autophagy score, the NSCLC patient survival and the hazard ratio are impacted. Actually, NSCLC patients with an autophagy score of 3-4 have a worse overall survival and an increased hazard ratio than those with an autophagy score of 1-2 (**Figure 5B.**). These results suggest that autophagy has a poor prognosis in NSCLC patients.

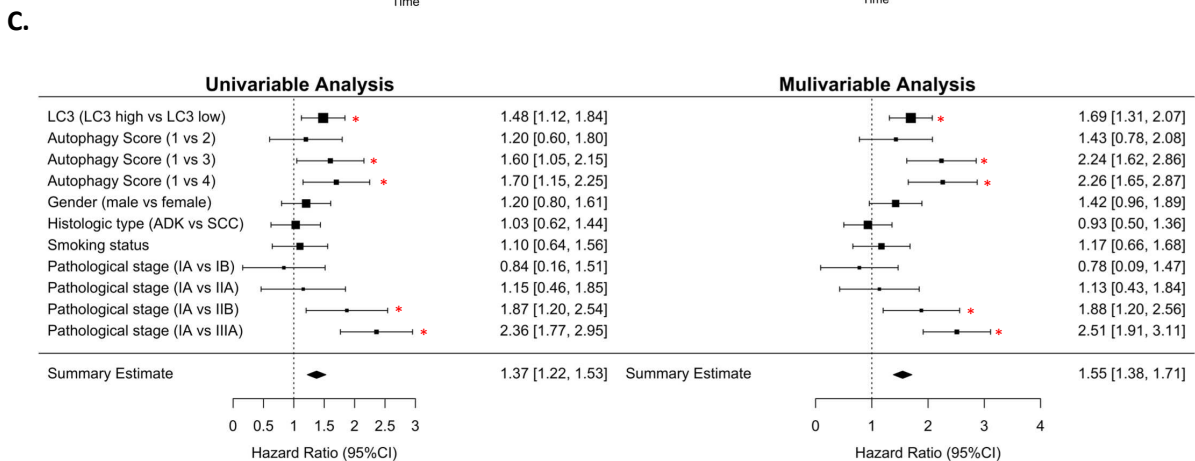
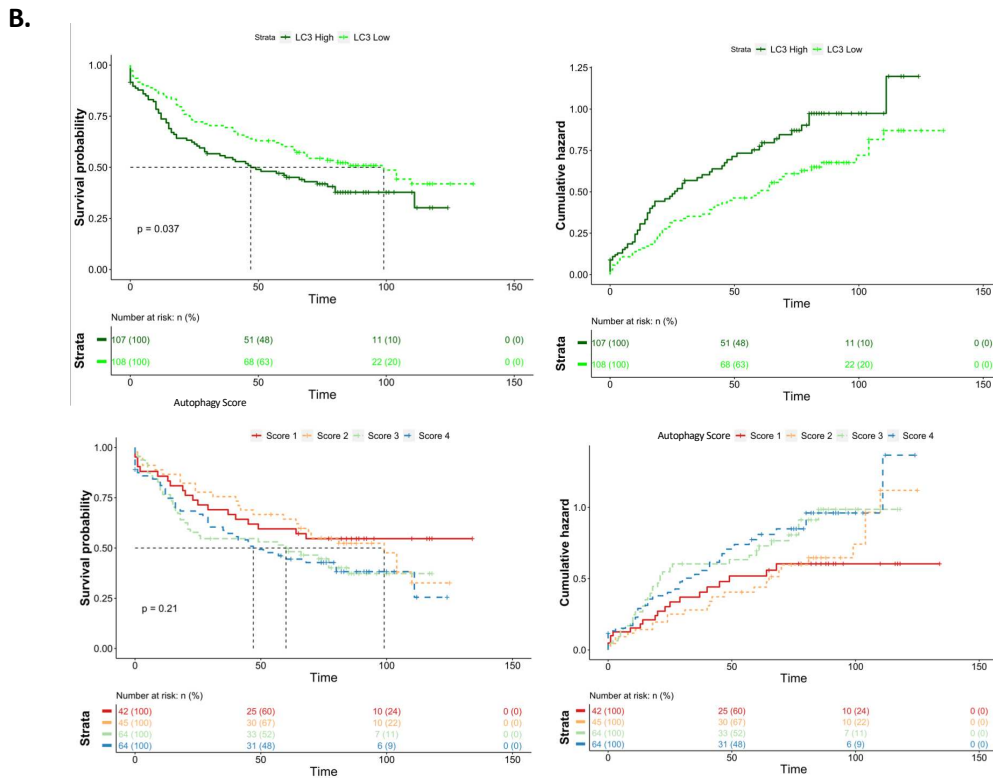
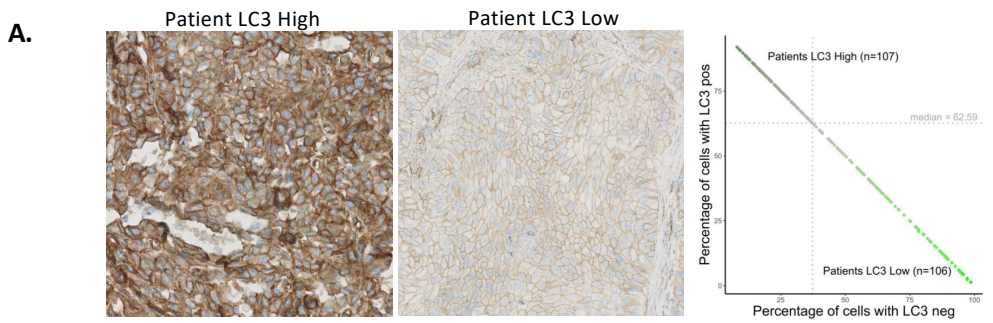


Figure 5. Impact of autophagy level in lung tumor cells on the survival of NSCLC patients.

A. Example of two typical LC3 autophagy protein staining patterns in lung tumor cells obtained from an LC3 high NSCLC patient (left) and an LC3 low NSCLC patient (right). The LC3 high patient is predominantly composed of LC3 positive lung tumor cells (LC3 pos., orange), while the LC3 low patient is mainly composed of LC3 negative tumor cells (LC3 neg., yellow). After immunohistochemical staining of LC3 autophagic protein on slides of NSCLC patients from retrospective cohorts, the slides were scanned by the nanozoomer and analyzed by the Halo software.

After distinguishing the lung tumor cells, the LC3 staining of each lung tumor cell was then divided into LC3 negative versus LC3 positive. Based on the median percentage of LC3 positive versus LC3 negative lung tumor cells in their tumor, each NSCLC patient was distributed either into LC3 high or LC3 low NSCLC patients. **B.** Top left : Kaplan Meier representing the survival probability of patients over time based on whether they are LC3 low or LC3 high. Top right : Kaplan Meier describing the cumulative hazard over time based on the autophagy level of NSCLC patients. Bottom left : Kaplan Meier representing the survival probability of patients over time based on their autophagy score (from 1, the less autophagic, to 4, the more autophagic NSCLC patients). Bottom right : Kaplan Meier describing the cumulative hazard over time based on the autophagy score of NSCLC patients. **C.** Univariable and multivariable analyses of different biological (including whether the NSCLC patients are LC3 high versus LC3 low patients, and the autophagy score) and clinical parameters on survival of NSCLC patients. Student's t-test, *: $p < 0.05$.

These findings were confirmed by both univariate and multivariate analyses, which showed that the autophagy level (through comparing both LC3 neg versus LC3 pos NSCLC patient and autophagy score of 1 versus 3/4) is a significant parameter that impacts the survival of NSCLC patients, as does the cancer stage (**Figure 5C.**) Besides, by measuring both TLR7 and LC3 expression levels in their lung tumor cells, we demonstrated that NSCLC patients highly expressing TLR7 and highly autophagic (TLR7 high, TLR7_h / LC3 high, LC3_h) have the worst overall survival and the biggest cumulative hazard as compared with the NSCLC patients weakly expressing TLR7 and weakly autophagic (TLR7 low, TLR7_low / LC3 low, LC3_l) whose, in contrast, have the better overall survival and the lowest cumulative hazard (**Supplementary Figure S4, annex 1**).

3.3.6 Autophagy level within lung tumor cells is associated with chemoresistance and a worse response for NSCLC patients to chemotherapies

Autophagy level analysis was also performed for the second cohort of NSCLC patients, those treated with neoadjuvant chemotherapy. We observed that non-responders had a much higher proportion of LC3-positive tumor cells than responders (**Figure 6A.**). Conversely, non-responders also had a lower proportion of LC3 negative tumor cells than responders. Besides, by measuring for each NSCLC patient, the autophagy score, we demonstrated that the more the autophagy score is, the more the proportion of non-responders is, with respectively 50%, 60%, 75%, and 95% of non-responders in autophagy score 1, 2, 3 and 4. Moreover, although the autophagy scores seem distributed in the responder patients categories, the non-responders are more likely to have the higher autophagy score (around 56% as compared to 24% in responders). All these results suggest that autophagy in tumor cells is associated with a poor prognosis in NSCLC patients treated with neoadjuvant chemotherapy and promotes chemoresistance (**Figure 6A.**). The same results were obtained when we separately analyzed the two chemotherapies combinations used in that cohort, cisplatin plus gemcitabine and cisplatin plus vinorelbine (**Supplementary Figure S5A.** and **Figure S5B, annex 1**). As before, these findings were confirmed by univariate analysis, which showed that the autophagy level (through comparing both LC3 pos versus LC3 neg patients and autophagy score of 1 versus 4) is a significant parameter affecting survival in NSCLC patients treated with neoadjuvant chemotherapy, as does the number of

cycles of chemotherapy, supporting that autophagy may promote resistance of lung tumor cells to chemotherapy (Figure 6B.).

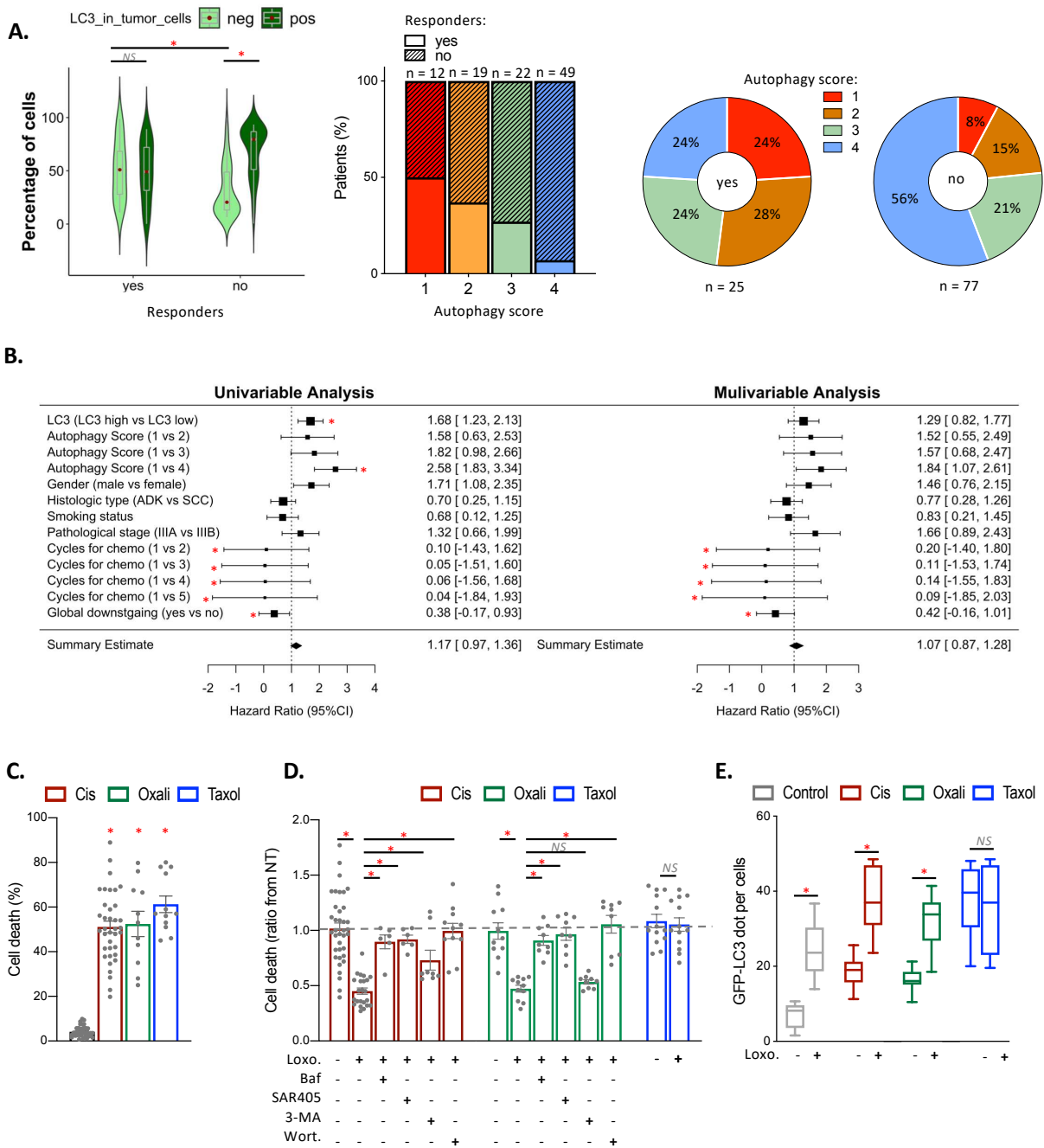


Figure 6. Impact of autophagy level in lung tumor cells on the resistance to chemotherapies in NSCLC patients.

A. At the left : Graph representing the percentage of LC3 positive versus LC3 negative lung tumor cells in NSCLC patients treated with various neoadjuvant chemotherapies in function of their responsiveness to these chemotherapies. In the medium : Distribution of the proportion of NSCLC patients who respond (yes) versus do not respond to chemotherapies (no) in function of their autophagy score. On the right : Proportion of the different autophagy scores of NSCLC patients between responders (yes) versus non-responders to chemotherapies (no). **B.** Univariable and multivariable analyses of different biological (including whether the NSCLC patients are LC3 high versus LC3 low patients, and the autophagy score) and clinical parameters on the survival of NSCLC patients treated with neoadjuvant chemotherapy. **C.** Graph representing the mortality rate of A549 cells treated or not (NT) with different chemotherapies: cisplatin (Cis., 50 μ M), oxaliplatin (Oxali., 200 μ M), and taxol (10 μ M) for 48

hours. The experiment was performed at least four times in triplicates. **D.** Graph showing the mortality ratio of the different combination treatments from that of chemotherapy alone for each chemotherapy at 48 hours post-treatment. In addition to treatment with one of the three chemotherapies, cells were treated or not with a synthetic TLR7 agonist (Loxo., 1mM) alone or with an inhibitor of autophagy initiation, 3-methyladenine (3-MA., 10mM), wortmannin (wort., 100nM), SAR405 (10 μ M) or a terminal phase inhibitor, bafilomycin (baf., 100nM). The experiment was performed at least three times in triplicates. **E.** Graph representing the average number of autophagosomes per A549 GFP-LC3 cell in function of whether or not the cells were treated (NT) with the different chemotherapies (Cis, oxali, or taxol) combined or not with a synthetic TLR7 agonist, loxoribin (Loxo., 1mM) for 6 hours. The analysis of the images was carried out on approximately 100 cells using the ZEN software, and the counting was performed by the R software. The experiment was performed three times. Student's t-test, * $p < 0.05$.

To confirm our results, the A549 lung tumor cell line was incubated with various chemotherapies, two targeting DNA, cisplatin and oxaliplatin, and one, the taxol, preventing microtubule depolymerization. After testing different concentrations at different kinetics, an IC50 dose, corresponding to the dose at which 50% of the cells are killed, at 24 hours post-treatment was determined for each of the chemotherapies: cisplatin (Cis., 50 μ M), oxaliplatin (Oxali., 200 μ M), and Taxol (10 μ M) (**Figure 6C.**).

To determine the impact of TLR7-mediated autophagy in lung tumor cells on resistance to these various chemotherapies, cells were treated with different chemotherapies and simultaneously stimulated for TLR7 with the synthetic agonist loxoribin (loxo., 1mM). After measurement of the number of dead cells by live/dead, it was observed that both for tumor cells treated with cisplatin or oxaliplatin, TLR7 stimulation led to a decrease in the number of dead cells compared to chemotherapies alone and promoted chemoresistance (**Figure 6D.**). This TLR7-induced chemotherapy resistance was not observed in taxol-treated tumor cells. Various autophagy inhibitors were used and added to chemotherapy plus loxoribin combinations to determine the involvement of autophagy in this mechanism. Among these inhibitors, three inhibit the initiation of autophagy flux, 3-Methyladenine (3-MA., 10mM), wortmannin (wort., 100nM), and SAR405 (10 μ M), and one inhibits autophagy in a delayed manner, by preventing the fusion of autophagosomes with lysosomes without inhibiting autophagy initiation, bafilomycin (baf., 100nM). Interestingly, we observed for both cisplatin- and oxaliplatin-treated lung tumor cells that the decrease in mortality induced by TLR7 stimulation was very strongly reduced by the addition of both autophagy initiation inhibitors and bafilomycin. Importantly, as 3-MA and wort were not really specific to autophagy and can also inhibit the kinase PI3K-I (in addition to PI3K-III that was induced in the autophagy process), we tested the effect of several PI3K-I inhibitors (Capiwasertib or capi., a specific inhibitor of AKT and therefore to class I PI3K, at 8nM and pictilisib or picti., a pan-class I PI3K inhibitor, at 3nM) in this TLR7-mediated chemoresistance. We did not observe an effect of these drugs on the chemoresistance induced by TLR7 stimulation (**Supplementary Figure S5C.**). Taken together, these results suggest that the chemoresistance induced following TLR7 stimulation depends on the induction of a complete autophagy flux.

To understand why TLR7 stimulation in lung tumor cells promotes chemoresistance to cisplatin and oxaliplatin but not to Taxol, we analyzed the autophagy level of cells treated with these different chemotherapies, alone or in combination with the TLR7

agonist. First, we observed that for tumor cells, the simple treatment of the cells with the various chemotherapies caused an increase in the autophagy level in these cells, supporting the literature that described an induction of autophagy in several chemotherapies-treated tumors (**Figure 6E.**)⁶⁹⁰. We also confirmed the impact of TLR7 stimulation on the rise in autophagy level in lung tumor cells. Interestingly, we observed that, for lung tumor cells treated with cisplatin or oxaliplatin, the addition of the TLR7 agonist resulted in an even more significant increase in autophagy level as compared to the autophagy of cells treated with chemotherapies alone or TLR7 agonist alone, demonstrating a synergy for the induction of autophagy by this combination. This increase in autophagy level following TLR7 stimulation is not observed for taxol-treated lung tumor cells, where the induction of autophagy in taxol alone was very high. This additional effect on the autophagy level by the TLR7 stimulation in cisplatin- or oxaliplatin-treated cells could explain the chemoresistance effect observed for tumor cells treated with these drugs, which is not observed for cells treated with taxol (**Figure 6E.**). Interestingly, a similar chemoresistance induced by a synergy of autophagy stimulation was previously observed in cisplatin-treated tumor cells exposed to hypoxic conditions⁴⁷².

3.3.7 Autophagy level within tumor cells did not impact immune infiltration

After having demonstrated that autophagy level within lung tumor cells is associated with a worse overall survival in NSCLC patients, we investigated if a critical actor in tumor progression, the immune infiltrate within the tumor microenvironment, could be impacted. By using the autophagy genes signature that we have demonstrated, we shed light that NSCLC patients, mainly composed of lung tumor cells highly expressing the signature, have no statistical differences in terms of gene expression of genes coding for cytokines, chemokines, and their receptors, which are all markers of a potential immune response activity, than NSCLC patients which the lung tumor cells weakly express the signature. (**Supplementary Figure S6A, annex 1**).

Then by using our cohort 1 of NSCLC patients (non-treated), for which a staining for marker of several immune cells has been performed (NK cells, B cells, T lymphocytes (CD8), DC and macrophages (CD68)), we studied the correlation between our LC3 staining and the immune infiltration. No evident correlation between the LC3-positive tumor cells and the different immune cell types tested was observed (**Supplementary Figure S6B, annex 1**). Similarly, no significant differences were observed in the density of immune cells tested between LC3 high versus LC3 low patients (segregated as previously described) (**Supplementary Figure S6C, annex 1**). The same analysis was performed using the autophagy score for NSCLC patients. As observed previously, whatever the autophagy score, no significant change in the immune infiltrate has been described (**Supplementary Figure S6D, annex 1**).

3.3.8 Autophagy level within lung tumor cells promotes PD-L1 expression and favors responsiveness to anti PD-1 immunotherapy

As the immune infiltrate seems unchanged whatever the autophagy level within the lung tumor cells, we investigated what could be the consequences of this autophagy on the immune phenotype of tumor cells themselves, meaning their capacity to be recognized by the immune system and be killed. To do that, thanks to the public cohort of NSCLC patients for which a whole transcriptomic analysis has been performed on the sorted malignant cells (GSE-111907 dataset), we evaluated the expression of various proteins-encoding genes which impact the immunogenicity of the tumor cells (**Figure 7A.**). Among the proteins encoding genes whose expression depends on the gene, some exert pro-tumoral effects (in red), whereas others are anti-tumoral (in green). We found that among the highly expressed proteins encoding genes exerting pro-tumoral effects, the main part were genes encoding for negative immune checkpoints like those coding for galectin-9 (LGAS9), CD47, B7-H3 (CD276) and PD-L1 (CD274). On the contrary, among the highly expressed proteins encoding genes which act negatively on tumor progression, we found those encoding for IFN ψ receptor and the pro-death molecule, Fas (**Figure 7A.**). Then, in order to determine the possible impact of autophagy on their expression, we studied the correlation of the expression level of these different protein-encoding genes with the autophagy genes (GC database). We observed that autophagy genes correlate strongly with proteins encoding genes for IFN ψ receptor, galectin-9, B7-H3, PD-L1, and Fas, suggesting that potentially autophagy level could modulate their expression (**Figure 7B.**). A weak correlation was found with the protein-encoding genes for CD47 ($r = 0.43$), and no correlation was observed with the protein-encoding genes for IDO-1 ($r = 0.12$, data not shown).

We then focused our study on the potential impact of autophagy on PD-L1 expression within lung tumor cells as this protein is largely targeted in immunotherapies. Interestingly, we demonstrated that the TLR7 stimulation in lung tumor cell lines increased PD-L1 expression at 24 and 48h post-stimulation (**Figure 7C.**). Importantly, treatment with the TLR7 agonist also increases the expression of CD47 and Fas without impacting the expression of MHC-I, IFN ψ receptor, B7-H3, or the TLR7 itself (**Supplementary Figure S7A., annex 1**). Intriguingly, we also observed that the combination of the TLR7 agonist with the cisplatin or the oxaliplatin treatments was responsible for a higher increased expression of PD-L1 as compared with TLR7 agonist or chemotherapies treatment alone (**Figure 7D.**). In comparison with cisplatin and oxaliplatin, taxol alone is responsible for a huge increase in PD-L1 expression in lung tumor cells, and the additional stimulation of TLR7 does not improve that expression.

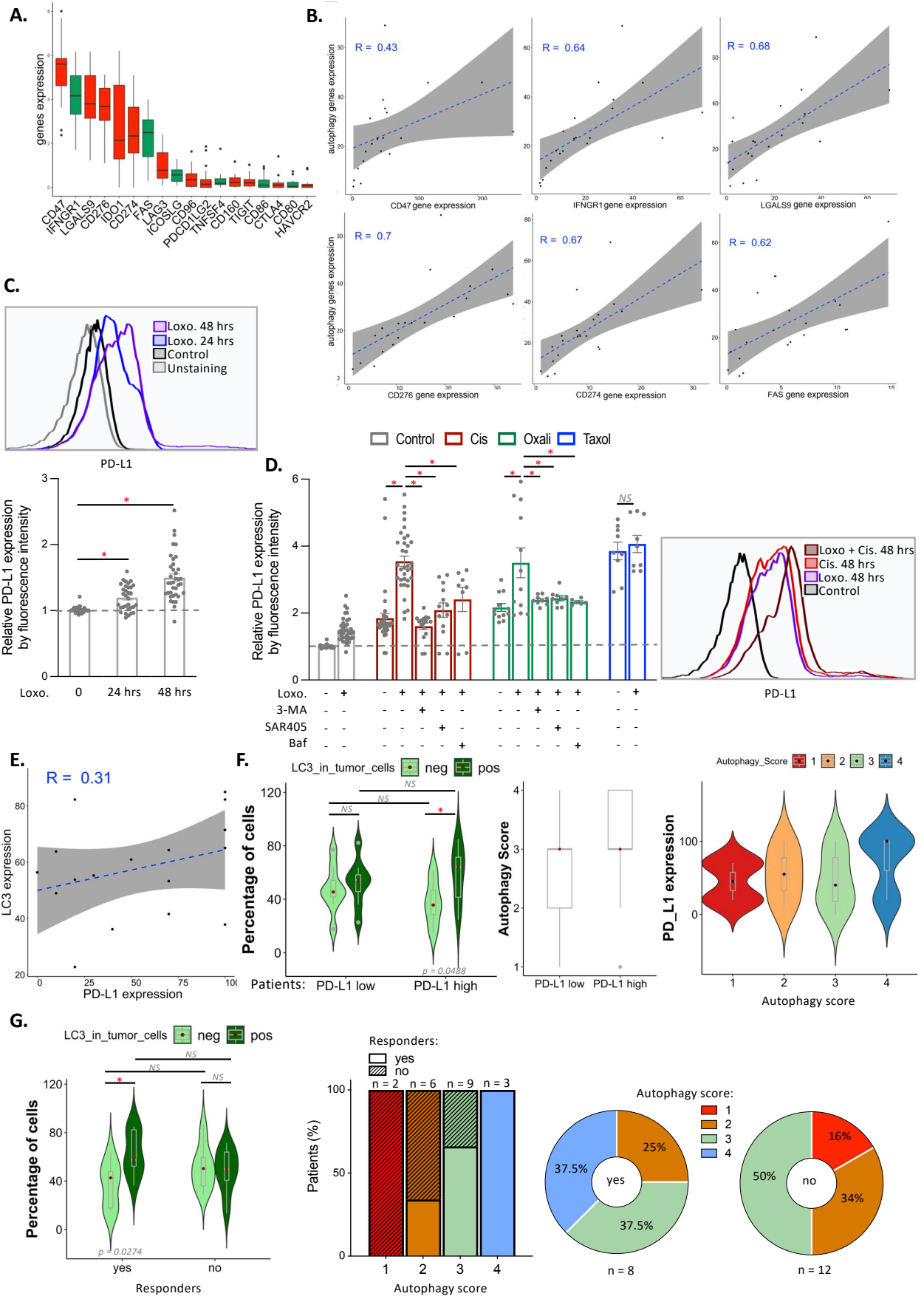


Figure 7. Impact of autophagy level in lung tumor cells immunogenicity and on the response to anti-PD-1 immunotherapy in NSCLC patients.

A. Graph representing the gene expression of different genes in NSCLC tumor cells that can impact their recognition or elimination by the immune cells. The gene expression was evaluated using sorted NSCLC malignant cells (GSE-111907 dataset). In red, genes coding for proteins which exert pro-tumoral effects, and in green, genes coding for proteins which exert anti-tumoral effects. **B.** Study of correlations between the autophagy genes expression (GC dataset) and genes expression. **C.** Top: histogram of PD-L1 fluorescence intensity by flow cytometry in TLR7 synthetic agonist-treated A549 cells (Loxo., 1mM) for 24h and 48h. Bottom: Graph describing the ratio of the relative PD-L1 expression by fluorescence intensity measured by flow cytometry in TLR7 synthetic agonist-treated A549 cells (Loxo., 1mM) from the value observed in non-treated cells, for 24h and 48h. The experiment was performed three times in triplicates. **D.** Left: Graph representing the ratio of the relative PD-L1 expression by fluorescence intensity measured by flow cytometry in TLR7 synthetic agonist-treated A549 cells alone (Loxo. 1mM), or in combination with different chemotherapies from the value observed in non-treated cells, at 48h post-treatment. The chemotherapies used are cisplatin (Cis., 50 μ M), oxaliplatin (Oxali., 200 μ M), and taxol (10 μ M). In addition, different autophagy inhibitors targeting either the initiation step (3-MA., 10mM, and SAR405, 10 μ M) or the autophagy maturation step (Baf., 100nM) were added to these combinatory treatments. The experiment was performed at least three times in triplicates. Right: histogram of PD-L1 fluorescence intensity by flow cytometry in TLR7 synthetic agonist-treated A549 cells alone (Loxo. 1mM), or in combination with cisplatin (Cis., 50 μ M) at 48h post-treatment. **E.** Correlation of LC3 expression in lung tumor cells with PD-L1 expression in these same cells, using a retrospective cohort of NSCLC patients before treatment with immunotherapy anti PD-L1 (nivolumab) (cohort 3). **F.** Left : Graph representing the percentage of LC3 positive versus LC3 negative lung tumor cells in PD-L1 low (proportion of NSCLC tumor cells expressing PD-L1<40%) versus PD-L1 high (proportion of NSCLC tumor cells expressing PD-L1>40%) NSCLC patients. Middle: Graph showing the average autophagy score in PD-L1 low versus PD-L1 high NSCLC patients in the same cohorts. Right : Distribution of the PD-L1 expression level in NSCLC patients in function of their autophagy score. **G.** At the left : graph representing the percentage of LC3 positive versus LC3 negative lung tumor cells in NSCLC patients treated with nivolumab in function of their responsiveness to the immunotherapy. In the middle: distribution of the proportion of NSCLC patients who respond (yes) versus do not respond to nivolumab (no) in function of their autophagy score. On the right : Proportion of the different autophagy scores of NSCLC patients between responders (yes) versus non-responders to the immunotherapy, nivolumab (no). Student's t-test, * : $p < 0.05$.

Given the bioinformatic analysis and the previous results demonstrating the impact of these different treatments on the autophagy level, our hypothesis is that the autophagy level within the lung tumor cell is sufficient to control the expression of PD-L1. Actually, the autophagy level of cisplatin- and oxaliplatin-treated lung tumor cells is increased by the additional stimulation of TLR7, which is responsible for the increased expression of PD-L1 (**Figure 6E** and **7D.**). Again, expression of PD-L1 under the taxol treatment correlated with the level of autophagy observed in this condition, with a high autophagy induction in lung tumor cells treated with taxol alone without synergistic effect after the TLR7 stimulation. Similar observations were made on the expression of some other proteins studied (**Supplementary Figure S7B, annex 1**). With the combinatory treatment, the expression of FAS, MHC-I, CD47, IFN γ receptor, and B7-H3 is induced, while no significant modification was observed for the expression of TLR7. To investigate the impact of autophagy in the increased expression of PD-L1 on tumor cells, we have treated cells with our different autophagy inhibitors (3-MA, SAR405, and baf). We observed that the increased expression of PD-L1 mediated by the combinatory treatment with cisplatin or oxaliplatin plus TLR7 agonist is significantly reduced by the different autophagy inhibitors (**Figure 7D.**). Importantly, inhibitors for PI3K-I did not affect the expression of PD-L1 observed after the combinatory treatment (**Supplementary Figure S7C, annex 1**). Those results demonstrate that the increase

in the autophagy level in lung tumor cells is responsible for an increase in PD-L1 expression.

We then wanted to confirm this observation on NSCLC patients. Therefore, we have used another retrospective cohort of 20 NSCLC patients treated with an anti-PD1, the nivolumab (cohort 3 – biopsies collected post-treatment with nivolumab). As we have done for the two previous cohorts, the autophagy level within lung tumor cells was measured by LC3 staining. We first evaluated the correlation between the LC3 expression and the PD-L1 expression within lung tumor cells, and we observed no significant correlation ($r = 0.31$) (**Figure 7E**). However, depending on the proportion of lung tumor cells expressing PD-L1 according to the median (PD-L1 low for the proportion of lung tumor cells expressing PD-L1<40% and PD-L1 high for the proportion of lung tumor cells expressing PD-L1>40%), we observed that, while the proportion of lung tumor cells positive for LC3 versus negative for LC3 is quite similar in PD-L1 low patients, PD-L1 high patients have significantly more lung tumor cells highly expressing LC3 (**Figure 7F.**, left.). Besides, the autophagy score in PD-L1 high patients seemed to increase as compared with PD-L1 low patients (**Figure 7F.**, middle.). Finally, patients with an autophagy score of 4 tend to highly express PD-L1 compared with those with an autophagy score of 1 (**Figure 7F.**, right). These data strongly suggest that the level of autophagy in lung tumor cells is associated with the expression of PD-L1.

Finally, we wanted to determine if the autophagy level within lung tumor cells could impact the responsiveness of NSCLC patients to nivolumab, an anti-PD1 therapy. For that purpose, we compared the proportion of lung tumor cells positive for LC3 versus negative in responders versus non-responders. Interestingly, we observed that the responders have significantly more lung tumor cells positive for LC3 than negative and have clearly a higher autophagy score than the non-responders (**Figure 7G.**). Taken together, these results demonstrate that the autophagy level within lung tumor cells impacts responsiveness to nivolumab, possibly through an increase of PD-L1 expression in cancer cells.

DISCUSSION

1 Global summary

The research team in which I carried out my thesis seeks to understand the molecular pathways that can impact the biology of lung cancer cells and the tumor microenvironment and, ultimately, the progression of non-small cell lung cancer (NSCLC). During this thesis project, I focused on a homeostatic mechanism that allows the cell to manage a very large part of the stresses it is confronted with, autophagy, and on the impact that the latter could have on lung tumor cells and NSCLC. Although autophagy is present in all cells, tumor cells appear to depend more on autophagy to meet their metabolic needs and survive the adverse conditions in the tumor microenvironment. Autophagy in tumor cells has also been shown in numerous tumor models to promote resistance to various anti-cancer treatments commonly used in the clinic, the development of metastases, and escape from the immune system.

Therefore, it is not surprising that more and more studies are demonstrating that autophagy level is associated with a worse survival for cancer patients in various cancer types, and in the meantime, an increasing number of preclinical and clinical studies aim to target autophagy are ongoing. Despite these observations, very few data on the importance of autophagy in lung tumor cells and tumor progression are available. Therefore, our study had two objectives, the first consisted in determining the molecular pathways by which, in a physiological context, the autophagy level in lung tumor cells is modulated. The second was to assess the importance of the autophagy level in lung tumor cells on tumor progression by evaluating its possible involvement in various pro-tumoral mechanisms.

To determine the molecular pathways that may physiologically modulate autophagy in lung tumor cells from NSCLC patients, we studied the correlation of autophagy gene expression (HAD and GC) with other genes in the genome. By a global analysis of all the cells composing the tumor microenvironment, we could notice that among all the genes, TLR7 is one of those strongly correlating with the expression of autophagy genes. This link was then clarified using a cohort of NSCLC patients for whom tumor cells were sorted and analyzed for their transcriptome, and where we were able to observe in malignant cells that the gene encoding TLR7 correlates very strongly with autophagy genes. This genetic link was then clarified molecularly, *in vitro*, and *ex vivo*, where we demonstrated that TLR7 stimulation in lung tumor cells induces a complete autophagy flux in the latter. We were also able to demonstrate that physiologically, in the tumor microenvironment, natural TLR7 agonists (probably cellular single-stranded RNAs), which could potentially induce autophagy in an autocrine or paracrine manner, were secreted by living tumor cells.

That observation was fascinating, given that in previous work, the team demonstrated that during the process of tumorigenesis, TLR7 expression is induced in lung tumor cells and is associated with a poor prognosis and a lower response of NSCLC patients

to chemotherapies. Although they demonstrated that TLR7 stimulation in lung tumor cells mechanistically promotes survival and chemoresistance to various anti-tumor chemotherapies, metastasis development, and recruitment of immunosuppressive cells within the tumor microenvironment, the underlying mechanism is yet unknown. During this Ph.D., we then focused on determining the possible impact of this new way of autophagy induction in NSCLC tumor cells, mediated by TLR7, on tumor progression and patients.

Using two different approaches, *in silico via* autophagy gene expression analysis and *ex vivo/in vitro*, by measuring the autophagy level in lung tumor cells, we were able to determine that the autophagy level in lung tumor cells had a substantial impact on tumor progression and a poor prognosis in NSCLC patients. Mechanistically, we observed, both *in silico* and *in vitro*, that autophagy in lung tumor cells favored their proliferation and survival, especially following treatment with platinum-salt based chemotherapies. We also showed that autophagy in lung tumor cells seems to negatively impact the anti-cancer immune response. Indeed, by correlating autophagy gene expression in tumor cells with the immune infiltrate of each immune cell type (determined by the MCP counter technique), we observed that the autophagy level of lung tumor cells appeared to negatively impact immune cell infiltration. However, this mechanism was not observed for the patients in a cohort we used in this project. Although the impact of autophagy levels in lung tumor cells on the immune infiltrate has yet to be clarified, we were able to observe, through our two experimental approaches, that autophagy appeared to promote the expression of molecules that can negatively impact the anti-tumor immune response such as the expression of the immune checkpoint, PD-L1. Using different retrospective cohorts of NSCLC patients, untreated, treated with neoadjuvant chemotherapy, and treated with anti PD-1 immunotherapy, and by measuring the autophagy level in lung tumor cells, we were able to observe respectively that autophagy was of poor prognosis for patients, that it was associated with a poorer response of the patients to chemotherapy and inversely with a better response of the latter to anti PD-1 immunotherapy (nivolumab).

To sum up, the work performed during my Ph.D. allowed to understand more precisely the modulation of autophagy in lung tumor cells, as well as its consequences on tumor progression, anti-tumor therapeutics responsiveness, and patient survival. Unlike the targeting of this new way of autophagy induction within lung tumor cells seems quite hazardous, this work encourages using autophagy level measurement in NSCLC patients as a predictive biomarker of tumor progression, resistance to chemotherapies, and responsiveness to nivolumab.

2 How does autophagy is modulated in lung tumor cells in pathophysiological conditions?

2.1 Summary

Both *in silico* by autophagy gene expression analysis, or *ex vivo*, by LC3 autophagy protein expression analysis, on NSCLC patient tissues, we have observed a very high heterogeneity of autophagy levels both between NSCLC patients and within the same patient, between different tumor cells and/or between tumor cells and other cells composing the tumor microenvironment. This huge disparity raises the question of its regulation in the tumor microenvironment, with possible important repercussions on NSCLC tumor progression and in patients. Using *in silico* analyses of public transcriptomic data from NSCLC patients on all cells composing the tumor microenvironment (TCGA) or on individual cells of the microenvironment, sorted out (GSE), we determined that in lung tumor cells, the expression of autophagy genes (HAD and GC) correlates strongly with that of an immune receptor, TLR7. This genetic link was also observed in NSCLC patients, where we found that patients expressing high TLR7 in their lung tumor cells had more highly autophagic cells than those expressing low TLR7, raising a potential physiological regulation of autophagy by this receptor. This link was then clarified by *in vitro* experiments, where we determined that TLR7 stimulation in lung tumor cells induces a complete autophagy flux in the latter. We were also able to determine that in the tumor microenvironment, TLR7 ligands are present physiologically and can generate a complete autophagy flux in malignant cells.

We also demonstrated by further studies that no release of TLR7 ligands is observed regardless of the type of cell death induced. In contrast, tumor cells appear to naturally secrete TLR7 ligands, probably single-stranded non-coding RNAs, under basal conditions. Interestingly, the autophagy level in the latter seems to impact the release of these ligands by the latter, which could constitute a negative feedback loop inhibiting an exaggerated release of TLR7 ligands, continuous stimulation of this receptor, and an overly strong induction of autophagy, which could be detrimental to the cells.

2.2 Intracellular mechanisms underlying TLR7-induced autophagy

Although we have demonstrated in this research that TLR7 stimulation in lung tumor cells induces a complete autophagy flux and that this induction is rapid (less than 6h), the underlying molecular mechanisms remain undetermined (**Figure 3A-D.**). Given the state of art, two hypotheses are possible.

The first one is a direct induction of the autophagy mechanism *via* protein-protein interactions between TLR7-induced signaling and proteins involved in the autophagy process. In their study, Delgado et al., demonstrated that autophagy induction in a murine macrophage model following TLR7 induction was rapid (about 4h) and involved both MyD88, Beclin-1, and Atg5⁶⁹¹. In this same model, autophagy induced following

TLR7 recognition was shown to involve a physical interaction between MyD88 and Beclin-1, and through this process, autophagy induction coincides with the release of the anti-apoptotic protein BCL-2, initially complexed with Beclin-1⁶⁹². This pathway would explain how TLR7 stimulation in our lung tumor cell model promotes autophagy induction and tumor cell survival under chemotherapy treatment. Among other molecular pathways that may be involved in the induction of autophagy following TLR7 stimulation, the NF- κ B pathway and the MAPK pathway involving p38, ERK and JNK appear to be the most likely candidates. Although the impact of these candidates on the autophagy mechanism seems to depend on the cell type studied, all of them have been shown to induce an autophagy mechanism in various tumor and non-tumor models^{693–695}. Furthermore, within the same cell type, here, the murine macrophage, the autophagy induction pathway stimulated by TLR activation can also be different depending on the context. Thus, during a *Listeria monocytogenes* infection, TLR2 has been shown to induce autophagy *via* the activation of the ERK pathway. In contrast, during a *Staphylococcus aureus* infection, autophagy induced following TLR2 stimulation is mediated *via* the activation of JNK^{696,697}.

The second hypothesis consists of an indirect induction of the autophagy mechanism by TLR7 *via* the secretion of cytokines such as IFN-I, which can, in response, induce autophagy^{309,698}. Given the kinetics of the autophagy induction after TLR7 stimulation, we favor the first hypothesis. Further co-immunoprecipitation and western blot studies on control and deficient cells for various proteins of the TLR7 signaling cascade will be required to determine the precise molecular actors involved in autophagy induction following TLR7 stimulation.

2.3 Nature of TLR7 ligands present within the TME and conditions of release: single-stranded RNA released upon non-lethal stress?

In this manuscript, we demonstrated that natural ligands of TLR7 are present in the tumor microenvironment of NSCLC patients, and they induce an increase of autophagy flux in lung tumor cells, mainly *via* recognition by TLR7 (**Figure 4A-D.**). Given the role of TLR7 in recognizing single-stranded RNA and the fact that we also demonstrated that in a basal state, lung tumor cells could secrete physiological TLR7 ligands, we first hypothesized that these ligands are more likely to be single-stranded RNA released physiologically into the TME and whose secretion could be induced either by active secretory pathways in alive cells or be induced following lung tumor cell death (**Figure 4E.**). Indeed, a similar release of cellular RNA and its recognition by TLR7 has been demonstrated in the development of an autoimmune disease, systemic lupus erythematosus (SLE), where following cell death, cellular RNAs are complexed with antibodies, endocytosed in B lymphocytes and plasmacytoid dendritic cells and then recognized by TLR7, which in turn induces inflammation⁶⁹⁹. TLR3 has also been shown to be stimulated in DCs upon recognition of cellular RNA from necrotic cells⁷⁰⁰. Thus,

this proposed mechanism could explain the transfer of TLR7 ligands from the dead to living tumor cells in the context of chemotherapeutic treatment of NSCLC, as well as the chemoresistance induced following TLR7 stimulation *via* the activation of the autophagy mechanism. Although following chemotherapy treatment in various tumor models and radiotherapy treatment in a non-tumor model (keratinocytes), the release of double-stranded RNA that stimulates TLR3 and induces inflammation has been demonstrated, the stimulation of TLR7 following the release of single-stranded RNA has not been yet demonstrated^{701,702}. A similar observation has been observed in non-tumor cells since the apoptosis of endothelial cells promoted the release of extracellular vesicles containing single-stranded RNAs into the extracellular space⁷⁰³. In our model, oxaliplatin-induced apoptosis does not appear to promote the release of TLR7 ligands from the dead cell (**Supplementary Figures S3A. and S3B., annex 1**). In contrast, induction of tumor cell death by this drug seemed to prevent the release of TLR7 ligand in the supernatant.

Since apoptosis is a cell death described as non-immunogenic because it does not promote the expression at the plasma membrane and the release of molecules that can stimulate immune cells and the immune response, such as ATP or HMGB1, and given that other cell deaths can occur in malignant cells in a tumoral context, we wanted to determine if the different immunogenic cell deaths such as ferroptosis, pyroptosis and necroptosis could, in addition to promoting the release of immunogenic proteins, induce the release of single-stranded RNA⁷⁰⁴. As observed for oxaliplatin-induced apoptosis, the different specific cell death pathways inducers in our model do not appear to promote the release of TLR7 ligands from the dead cell (**Supplementary Figures S3C. and S3D., annex 1**). Therefore, regardless of the type of cell death occurring in lung tumor cells, cell death does not appear to promote the release of TLR7 ligands within the TME. The chemotherapeutic treatments used in this research work have all been shown to promote oxidative stress, reticulum stress, and autophagy in various models⁷⁰⁵⁻⁷¹². Although these different stresses and autophagy have been shown in multiple tumor models to stimulate the secretion of extracellular vesicles containing natural TLR7 agonists, the effective recognition of these cellular ligands by TLR7 following anti-cancer treatment has not yet been demonstrated⁷¹³. It is easily thinkable that the absence of TLR7 ligands observed within the TME could be attributable to the dose we used for each chemotherapy. Actually, we can easily imagine that chemotherapies-induced stresses and not cell death are required to promote TLR7 ligands released by the malignant cells. My hypothesis is that lung tumor cells can naturally secrete TLR7 ligands, as we observed, and that release can be modified by cellular stresses induced by non-lethal chemotherapeutic doses. At high doses, chemotherapies could lead to cell death and therefore prevent the secretion of TLR7 ligands, as we observed in that study.

Interestingly, it is important to note that the autophagy level of tumor cells seems to influence the release of TLR7 ligands from living cells. Indeed, incubation of the supernatant from lung tumor cells treated with an autophagy inducer, rapamycin, leads

to a decrease in the activity level of the reporter cells for TLR7 which is proportional to the concentration of the autophagy inducer (**Figure D1.**)

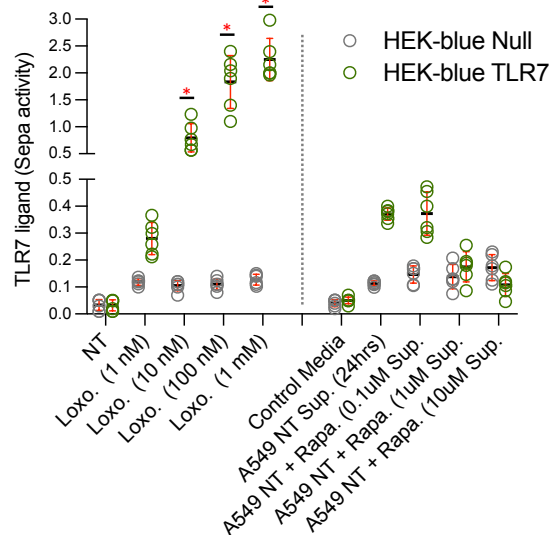


Figure D1. Measurement of TLR7 ligands released by lung tumor cells after treatment by an autophagy inducer

Measurement of the presence of TLR7 ligands through the measurement of the Sepa activity of HEK-blue cells, following the treatment or not (NT) with different doses of TLR7 agonists (Loxo., 1nM, 10nM, 100nM, and 1mM), or after the incubation with a culture media alone (Control media), with a tumor supernatant from A549 cells non-treated (A549 NT Sup.) or with the same supernatant, treated with different concentrations of autophagy inducer (Rapa., 0.1μM, 1μM, 10μM) (A549 NT + Rapa) for 24h. The experiment was performed three times in triplicates.

This mechanism could be a kind of regulatory loop of the autophagy level in lung tumor cells: when cells have a too high autophagy level, the release of TLR7 ligands in the TME, which can subsequently act in autocrine or paracrine to induce autophagy, is inhibited to prevent the induction of too high autophagy level with dramatic consequences for the cell, such as the induction of autophagy cell death.

2.4 Involvement of extracellular vesicles in TLR7 ligands release by NSCLC living tumor cells?

Since the natural agonists of TLR7 present in the TME seem to be single-stranded RNAs of cellular origin, the question of their exocytosis into the extracellular medium arises. Indeed, these RNAs could either be found in the extracellular medium, trapped in extracellular vesicles (e.g; exosomes), or they could be found in the form of free RNA.

The latter hypothesis was studied, in particular, by treating the different supernatants containing TLR7 ligands with RNase, an enzyme degrading free RNA. The impact on the autophagy level in tumor cells was then determined by measuring the number of autophagosomes under each condition by confocal microscopy on A549 GFP-LC3 cells. Although autophagy induction was no longer observed after incubation of cells with RNase treatment of supernatants, RNase treatment of control culture medium (DMEM) and subsequent incubation on A549 GFP-LC3 cells also increased autophagy levels in these cells, making interpretation of the results difficult (data not shown). The RNase present in the supernatants seems to be still active and causes stress in the lung tumor cells which favors the increase of the autophagy level in these cells. Prolonged treatment of the supernatants with RNase could thus lead to damage at the cell level and an increase in autophagy in these same cells. Therefore, it would be interesting to study the impact of RNase treatment of tumor supernatants highly concentrated in TLR7 ligands on reporter cell activity.

Although this first proposed mechanism remains a possibility, we believe that exocytosis of TLR7 ligands is probably more likely to be achieved *via* the secretion of extracellular vesicles. Different observations support this hypothesis: (i) the secretion of free RNA in a non-tumoral or tumoral context has been very rarely described, (ii) the presence of nucleases in the extracellular medium would rapidly degrade free RNA, which would strongly prevent TLR7 stimulation and autophagy induction, and necessarily implies conjugation with an antibody, as it was observed in the SLE model⁷¹⁴, (iii) numerous papers have described, in a non-tumoral or tumoral context, the secretion of extracellular vesicles containing notably non-coding RNAs, which can distinctively stimulate TLR7 and induce autophagy. Thus, we hypothesize that under basal or mild to moderate stress conditions, single-stranded non-coding RNAs, which have already been described separately as stimulating TLR7 and can induce autophagy in many non-tumor and tumor models, would be trapped in extracellular vesicles which would facilitate the transfer of TLR7 ligands from one tumor cell to another nearby one, the induction of autophagy TLR7-dependent as well as the chemoresistance of lung tumor cells⁷¹⁵⁻⁷¹⁸. This mechanism of chemoresistance transfer *via* secretion of extracellular vesicles that induce autophagy has already been described in tumor models, such as in NSCLC. Indeed, it was shown that chemoresistant cells to cisplatin secrete miR-425-3p, which promotes resistance to chemotherapies through an autophagy induction⁷¹⁹.

Optimization experiments for the isolation and extraction of extracellular vesicles are currently in progress to search for the potential presence of extracellular vesicles or exosomes containing TLR7 ligands in the TME. After these steps, it would also be interesting by BIAcore techniques to identify more precisely the nature of these TLR7 ligands and to search for the presence of other TLR ligands, such as TLR3 and TLR4, whose expression levels in lung tumor cells strongly correlate with the expression level of autophagy genes in lung tumor cells, and whose stimulation of these two receptors on these same cells has been shown to induce a complete autophagy flux.

It is also interesting to note that the autophagy level of lung tumor cells seems to negatively regulate the release of TLR7 ligands from living cells, as we have observed when the cells were treated with the autophagy inducer (**Figure D1.**). That observation is consistent with our hypothesis as it was already demonstrated that although autophagy has been shown to modulate the secretion of extracellular vesicles. Indeed, in hepatic stellate cells in a model of liver fibrosis or neuronal cells, autophagy has been shown to inhibit the release of pro-fibrogenic extracellular vesicles or prion-containing vesicles, respectively^{720,370}. It seems that autophagic vesicle secretion and macroautophagy are distinct mechanisms that appear to regulate each other to maintain cell homeostasis, which is consistent with the negative regulation of TLR7 ligands secretion by autophagy³⁶⁷. Interestingly, it has been shown in neuronal cells that inhibiting macroautophagy in these cells promotes the secretion of extracellular vesicles involving molecular actors of autophagy, such as LC3 and p62⁷²¹. As evoked previously, this mechanism could be a kind of regulatory loop of the autophagy level in lung tumor cells: when cells have a too high autophagy level, the release of TLR7 ligands in the TME, which can subsequently act in autocrine or paracrine to induce autophagy, is inhibited to prevent the induction of too high autophagy level with dramatic consequences for the cell, such as the induction of autophagy cell death.

3 What is the impact of autophagy on NSCLC tumor cell behavior, tumor progression and NSCLC patients?

3.1 Summary

After having demonstrated that autophagy is a dynamic phenomenon that can be finely modulated by conditions in the microenvironment and having identified a novel TLR7-mediated pathway for autophagy induction in lung tumor cells, we investigated the impact of autophagy on the malignant cell, tumor progression, and the NSCLC patient. Using two different approaches, either by autophagy gene expression or by expression of the autophagy protein LC3 in NSCLC patient tissues, we could determine that autophagy was associated with a poor prognosis for the patient. Using transcriptomic data from lung tumors, we were able to establish two clusters of patients according to the expression of autophagy genes. Patients with high expression of autophagy signature B expressed more genes associated with proliferation and fewer genes related to anti-tumor immunity. By MCP counter, we were able to establish that tumors with malignant cells strongly expressing autophagy genes are less infiltrated with different types of immune cells. This result was challenged by the failure to observe the same trend in NSCLC patients using a non-bioinformatics technique involving LC3 protein labeling on NSCLC patient tissue sections. Although the impact of autophagy level in lung tumor cells on immune cell density seems undetermined, its effect on the expression of molecules such as immune checkpoints was observed with our two approaches. *In silico* and *in vitro*, we demonstrated that autophagy correlates with PD-L1 expression and thus negatively impacts the anti-tumor immune response. We also

showed that the novel TLR7-mediated autophagy induction pathway in lung tumor cells promotes survival, resistance to platinum-salt based chemotherapies, and increased PD-L1 expression. These different biological observations were then translated into the clinic, where we demonstrated that autophagy is poorly prognostic in patients, that it is a predictive marker of patient response to platinum-salt based chemotherapies, and that it is a predictive marker of patient response to anti PD-1 immunotherapy.

3.2 Mechanisms underlying chemoresistance to chemotherapies following TLR7 stimulation

Although having demonstrated that TLR7-induced autophagy in NSCLC tumor cells promotes chemoresistance to platinum-salt based chemotherapies and ultimately is associated with a poorer response of NSCLC patients to chemotherapies, the underlying mechanism is still unknown. Regardless of TLR7-induced autophagy, platinum-salt based chemotherapies have been shown to induce a protective autophagy in lung tumor cells against induced stress and damage, which eventually promotes cell survival and limits chemotherapies effectiveness⁷¹⁰⁻⁷¹². Although autophagy is not the only mechanism by which cells can acquire resistance to chemotherapies, it plays a prominent role in the chemoresistance of lung tumor cells to chemotherapies. Since the precise intracellular mechanisms by which autophagy promotes cell survival under conditions of chemotherapy treatment are still undetermined at this time, it cannot be excluded that autophagy, *via* its canonical or non-canonical pathway, may act exclusively or in part to promote the establishment of one or more other well-described resistance mechanisms.

The mechanisms by which a tumor cell becomes resistant to chemotherapy are: reduced entry of chemotherapeutics into the cell and increased expulsion from the cell, increased metabolism involved in chemotherapy modification, target changes, compartmentalization of chemotherapy in the cell, increased DNA damage repair, and reduced apoptosis. Several hypotheses can be proposed to explain TLR7-induced chemoresistance through autophagy induction. One proposal is that the chemotherapy molecules may be trapped in the autophagosomes, thereby limiting the mortality usually observed with these chemotherapies. To the best of my knowledge, only one article demonstrated that mechanism in which autophagy induction in breast cancer cells promotes doxorubicin delivery to the lysosome and eventually prevents its cytotoxic action⁷²². Interestingly, this study showed that induction of a complete flux of autophagy was necessary for chemoresistance. As observed in our study, inhibition of the final phase of autophagy reverses this phenomenon, which could potentially suggest that TLR7-induced autophagy promotes chemoresistance by a similar mechanism.

Given the interrelationships between autophagy and cell death pathways, another explanation could be that autophagy degrades molecular actors involved in the activation of cell death or promotes the expression of molecules, limiting death

induction. Indeed, it has been established that autophagy induction could promote chemoresistance of tumor cells through the release of the anti-apoptotic protein BCL-2 released following the recruitment of BECLIN-1, initially complexed with BCL-2 and necessary for autophagy induction⁷²³. It is interesting to note that our team, in previous studies, showed that TLR7 stimulation promotes the expression of BCL-2 in stimulated lung tumor cells, revealing a potential role for autophagy as an anti-apoptotic process in this model⁶⁰². Similarly, while chemotherapies have been shown to promote P53 and, dependently or independently of this way, modulate autophagy in tumor cells, various studies described that autophagy blocking is responsible for an increase in P53-induced apoptosis^{467,724,725}. Therefore, tumor cells can, through autophagy, degrade P53-induced apoptosis and thus become more resistant to chemotherapeutic drugs. In addition, chemotherapies can also damage mitochondria and their genome, triggering the so-called intrinsic death of apoptosis. By promoting the recognition of damaged mitochondria and their elimination *via* a mechanism called mitophagy, autophagy has already been demonstrated to promote the survival and chemoresistance of tumor cells⁷²⁶. Among other mechanisms evoked in which autophagy seems to be involved in chemoresistance, autophagy, by promoting the activation of the p62/KEAP1/NRF2 pathway, allows the establishment of an antioxidant system that counteracts the induction of ROS induced by various chemotherapies, including those we used in our research work and this was demonstrated to promote chemoresistance in multiple tumor models and for different chemotherapies, including cisplatin^{727,728}.

3.3 Does chemoresistance following TLR7 stimulation specific to platinum-salt based chemotherapies?

In this research project, we demonstrated that TLR7 stimulation promotes chemoresistance to two anti-tumor chemotherapies, cisplatin and oxaliplatin, through an autophagy-dependent pathway. In contrast, TLR7 stimulation does not induce cytoprotective autophagy in these same taxol-treated cells. The difference in the effect of autophagy induced by TLR7 stimulation on chemotherapy resistance depending on the chemotherapy studied is exciting and should be further investigated. Several explanatory hypotheses can be evoked and could explain why tumor cells treated with cisplatin and oxaliplatin react similarly and in a totally different way to those treated with taxol.

3.3.1 Differences on target and signaling pathways involved?

Cisplatin and oxaliplatin act at the level of DNA and cause damage, whereas taxol acts at the level of cell architecture by preventing the depolymerization of microtubules, thereby inhibiting both replication and intracellular transport. Thus, induced autophagy will allow, as previously mentioned, to manage the stresses caused by DNA targeting agents. However, no mechanism to compensate for the deleterious action of taxol on cells has been described. The intracellular signaling pathways mobilized in lung tumor

cells following cisplatin/oxaliplatin versus taxol-induced stresses are also different: Treatment of cells with cisplatin/oxaliplatin leads to p53, p62 KEAP1 NRF2 signaling, and the DNA damage repair (DDR response) pathway. Taxol, on the other hand, does not induce these intracellular molecular pathways at all but instead induces endoplasmic reticulum stress associated with cell cycle blocking *via* p21 and completely independent of p53, as observed in breast tumor cells⁷²⁹.

Another possibility is that taxol-induced death must be mediated by the initiation of another cell death pathway for which induced autophagy would not have an inhibitory effect, which could explain why no chemoresistance is observed after treatment of cells with taxol. This hypothesis is fully consistent with our results, where we were able to show that cisplatin (data not shown) and oxaliplatin (**Supplementary Figure S3A., annex 1**) do cause cell death mainly by apoptosis. The same experiment with taxol will have to be performed to determine whether taxol induces apoptosis or other cell death. This hypothesis is reinforced by a study conducted on lung tumor cells other than A549 demonstrated that taxol could cause cell death *via* a mechanism distinct from apoptosis⁷³⁰. If taxol were found to induce apoptosis in our lung tumor cell model, the pathway by which cell death is mediated (intrinsic versus extrinsic) would need to be verified by western blot. Supposing cisplatin, oxaliplatin, and taxol induce cell death by the same intracellular pathway. In that case, the mechanisms discussed above that could explain the differences in resistance of lung tumor cells to cisplatin/oxaliplatin versus taxol should be further studied. The latter hypothesis is strongly favored because numerous examples in the literature have shown that one of the main mechanisms by which autophagy can promote cell survival is *via* induction of mitophagy⁷³¹.

3.3.2 Differences of chemotherapeutic drugs effects on autophagy?

Another explanation could be the different impacts of these chemotherapies on the autophagy mechanism. Indeed, although all three chemotherapies have been described to increase the autophagy level in treated cells, the causative processes remain undetermined. Indeed, as mentioned earlier in this manuscript, the increase in autophagy levels in cells may result either from induction of complete autophagy flux or from a blockage of the fusion of autophagosomes with lysosomes and, thus, their degradation. It is possible to imagine that cisplatin and oxaliplatin induce a complete flux of autophagy while taxol, by blocking the microtubule network, could prevent the migration of autophagosomes towards lysosomes and thus block their fusion and the degradation of autophagosomes, which is indeed the case^{710,732,733}. This difference may be sufficient to explain the chemoresistance observed in cisplatin- or oxaliplatin-treated cells and the absence of the same phenomenon in taxol-treated cells after TLR7 stimulation. Indeed, in this manuscript, we have demonstrated in lung tumor cells that the induction of a complete autophagy flux after TLR7 stimulation was necessary to promote the chemoresistance of these same cells to chemotherapies. We can easily think that the stimulation of TLR7 in lung tumor cells could boost the induction of autophagy influx, already induced following the activation of p53 in these same cells,

and thus promote chemoresistance to these same chemotherapies. On the other hand, in the context of treatment with the combination of TLR7 agonist and taxol, it can be thought that although TLR7 stimulation induces a complete autophagy flux in these cells, the action of taxol would inhibit the complete autophagy flux and thus chemoresistance would not be observed. This hypothesis should be studied in tumor cells treated with different chemotherapies by evaluating the colocalization profile of autophagosomes with lysosomes.

Another explanation that can be raised to explain the absence of an increase in the autophagy level following TLR7 stimulation in taxol-treated lung tumor cells, as well as the absence of TLR7-induced chemoresistance to taxol through autophagy, is that taxol, by acting at the level of microtubules and given its well-demonstrated capacity to block endolysosomal trafficking, could stop the trafficking and ultimately the activation of TLR7⁷³⁴. Although not described yet for TLR7, taxol-induced inhibition of the endolysosomal system has notably been shown to reduce the rate of EGFR trafficking in a lung tumor cell model⁷³⁵. Immunohistochemical analysis of this receptor will have to be carried out to specify the localization of TLR7 in these different conditions and to confirm or refute this hypothesis.

3.3.3 Differences in the intensity of cell stresses submitted to lung tumor cells?

Another possibility is that treatment with cisplatin or oxaliplatin causes mild to moderate stress in treated cells and a slight increase in autophagy levels. Although this mechanism had already been described in the lung tumor model, we observed the same thing in our study (**Figure 6E.**)⁷³⁶. The addition of TLR7 stimulation in these same cells could thus make it possible, by increasing the autophagy level as we observed (**Figure 6E.**), to manage this mild to moderate stress and therefore allow chemoresistance as we observed (**Figure 6D.**). On the other hand, taxol could cause too much stress to the cell, resulting in a huge increase in autophagy levels which cannot be increased anymore by additional stimulation of TLR7, as we observed (**Figure 6E.**). It is easily thinkable that in contrast to promoting chemoresistance, taxol-induced autophagy is too high and, therefore, can directly lead to autophagic cell death or indirectly by failing to cope with the excessive cell stress caused by taxol. In that case, TLR7 stimulation-induced autophagy will be unable to promote chemoresistance to taxol, as we observed (**Figure 6D.**). Interestingly, taxol has already been shown to induce autophagic cell death in a lung tumor cell model⁷³⁷.

It would be interesting to increase the concentrations of cisplatin and oxaliplatin, and thus the stresses imposed on the cells, to determine whether TLR7 stimulation can still increase autophagy in these treated cells compared to chemotherapies alone, as well as to promote chemoresistance. In support of this idea, it appears that cisplatin-induced chemoresistance is dose-dependent. Indeed, at low doses, cisplatin induces chemoresistance *via* the autophagy mechanism, but when concentrations increase, the stress imposed on the cells no longer allows the cell to trigger a compensatory autophagic response, and the cells die by necrosis⁷³⁸. In the meantime, it would be

wise to decrease the concentration of taxol submitted to the lung tumor cells to determine if the autophagy level of these cells is reduced and if, under these conditions, the stimulation of TLR7 could lead to an increase in the autophagy level of these taxol-treated cells, as well as to chemoresistance. Indeed, depending on the concentrations imposed on the cells and the cell model, paclitaxel may promote either cytoprotective autophagy or autophagic cell death^{711,737}.

Given their differences in target and mode of action, we believed that platinum-salt based chemotherapies, compared with taxol, could induce mitochondrial DNA damage, which eventually lead to mitophagy and by this way, prevent cell death from dying. Actually cisplatin has already been shown to promote survival and chemoresistance in many tumor models, through this mechanism⁷³⁹. Although not demonstrated for oxaliplatin, it seems very likely that, given the great structural similarity with cisplatin and the same mode of action, oxaliplatin could also promote mitophagy and, thus, chemoresistance to this same drug. However, taxol has also been shown to promote mitophagy in various tumor models, including lung tumor cells, which questions our hypothesis⁷⁴⁰. Although mitophagy is a mechanism by which the cell promotes its survival, an excess of mitophagy can conversely facilitate cell death *via* the induction of autophagic cell death⁵²⁵. Thus, it is conceivable that although all three chemotherapies induce mitophagy, the induction level in lung tumor cells differs depending on the chemotherapy. It is possible that in lung tumor cells, cisplatin and oxaliplatin induce mild to moderate stress that favors the induction of mitophagy that is tolerable for the cell. On the other hand, it is also possible that in these same cells, treatment with taxol may cause too much stress for the cell, which will respond through a strong induction of mitophagy, ultimately leading to cell death by autophagy. This hypothesis seems likely given that the autophagy level in the same cells treated with taxol is much higher than that observed in cells treated with platinum-salt based chemotherapies. Furthermore, taxol has been shown in lung tumor cells to promote autophagic cell death⁷³⁷.

3.4 Involvement of autophagy level intensity on the expression of PD-L1 by lung tumor cells?

In this manuscript, we also observed that the autophagy level impacts the expression of molecules that could modulate the interaction of lung tumor cells with immune cells and, ultimately, the anti-tumor immune response and the progression of NSCLC. Indeed, PD-L1 expression is positively correlated with the autophagy level of these cells, as also demonstrated by bioinformatics analysis.

In this research work, similarly as we observed for chemoresistance, we also demonstrated treatments with platinum-salt based chemotherapies or with TLR7 agonist alone increase PD-L1 expression. Combined together, these treatments are responsible for a greater increased expression of this molecule which is autophagy-dependent as an inhibition of both the initiation or the maturation steps prevents PD-

L1 expression increase following the treatment (**Figure 7D.**). As we observed for chemoresistance, treatment of lung tumor cells with taxol greatly increases PD-L1 expression, and that latter is not raised following TLR7 additional stimulation. As for chemoresistance, interestingly, the PD-L1 expression profile is very similar to the autophagy level profile within lung tumor cells in each cell condition (**Figure 6E.**). Therefore, the autophagy level within lung tumor cells seems critical for PD-L1 expression in these same cells. The involvement of autophagy was well demonstrated here by the drastic decrease in the expression of PD-L1 by the addition of autophagy inhibitors to the combinatorial treatment involving the TLR7 agonist and cisplatin or oxaliplatin. Different hypotheses could be proposed to explain the mechanisms by which autophagy could promote PD-L1 expression. The difficulty here is that both canonical and non-canonical functions of autophagy could be involved in this process.

3.4.1 Autophagy increases the expression of PD-L1 in malignant cells?

Very little information is available concerning the involvement of autophagy in the expression of PD-L1, and the conclusions vary according to the tumor model studied. Indeed, in a model of triple-negative breast cancer and prostate cancer, downregulation of PD-L1 was associated with autophagy degradation. Interestingly the authors of this article mentioned that autophagy was involved, but it implied a mechanism distinct from macroautophagy⁷⁴¹. Although in an NSCLC model, PD-L1 degradation was demonstrated to be mediated by the lysosome, and a recent study showed that autophagy facilitated PD-L1 delivery to the lysosomes, no direct link has been described between autophagy and PD-L1 degradation^{742,743}. In contrast, a recent study demonstrated that in lung tumor cell models, autophagy, by promoting the activation of STAT3 signaling, induced the expression of PD-L1⁵¹⁵. In gastric cancer, inhibition of autophagy has been shown to promote PD-L1 expression *via* p62 accumulation and stimulation of the NF- κ B⁷⁴⁴. Interestingly, it has been well described, both in non-tumor and tumor models, that PD-L1 expression can be increased by the stimulation of the NF- κ B pathway, which is activated notably following stimulation of TLR7^{745–748}. The MAPK pathway, which can be induced following TLR7 stimulation, has also been described as promoting PD-L1 expression, especially in lung cancer cells⁷⁴⁹.

Although cisplatin and taxol have been shown separately in various tumor models to modulate autophagy and facilitate PD-L1 expression in treated tumor cells, the possible link between the two has yet to be described^{750–753}. It is conceivable that the canonical autophagy pathway induced by these different chemotherapies could favor the degradation of proteins that inhibit the expression of PD-L1 or, on the contrary, facilitate the establishment of signaling pathways that promote its expression. Taxol, by inducing a more significant autophagic response than cisplatin or oxaliplatin, could lead to greater expression of PD-L1. To explain why TLR7 additional stimulation cannot lead to an increase in PD-L1 expression in taxol-treated lung tumor cells, we can suppose that, as evoked previously, taxol treatment is responsible for a great

increase of autophagy as well as PD-L1 expression, which reaches a ceiling and which both cannot be increased by TLR7 additional stimulation.

Another hypothesis by which TLR7 or chemotherapies could increase the expression of PD-L1 is the induction of inflammasome activation. Actually, given that inflammasome has been demonstrated to promote PD-L1 expression in various tumor and non-tumor models and all three chemotherapies have been shown to promote inflammasome development, we have hypothesized that this PD-L1 expression increase following treatment by chemotherapies could be mediated by inflammasome activation. Additional TLR7 stimulation in cisplatin- or oxaliplatin-treated cells could amplify that PD-L1 increase expression by further stimulation of the inflammasome or by its intrinsic signaling pathways. By contrast, as evoked previously, taxol could block TLR7 trafficking and activation, preventing the additional increase of PD-L1 expression. However, this hypothesis involving inflammasome seems unlikely given that autophagy has been shown to inhibit inflammasome activity in cells⁷⁵⁴

3.4.2 Autophagy facilitates trafficking of PD-L1 to the plasma membrane?

In contrast or in addition to our initial hypothesis, which consisted of determining the possible mechanisms by which canonical autophagy can increase PD-L1 protein expression in lung tumor cells treated with chemotherapies, alone or in combination with a synthetic TLR7 agonist, we can believe that non-canonical autophagy may be involved to facilitate the trafficking of PD-L1 to the plasma membrane, rather than to induce its de novo expression. Indeed, it has been shown that autophagy regulates the expression of proteins present at the plasma membrane, either positively by promoting its addressing to the plasma membrane or negatively by promoting its endocytosis. This first mechanism was observed in a murine fibroblast and renal epithelial cell model where, following a metabolic stress, induced autophagy facilitates the transfer of GLUT1 to the plasma membrane, a transporter facilitating the entry of glucose into the cell³⁷³. Conversely, in various models, and notably in lung tumor cells, autophagy has been described to facilitate EGFR endocytosis⁷⁵⁵.

The increased expression of PD-L1 following additional stimulation of TLR7 could either be a consequence of non-canonical autophagy and facilitated membrane trafficking of these two molecules and/or resulting from a de novo expression of these molecules. A limitation of this hypothesis is that since taxol inhibits microtubule depolymerization, this chemotherapy could inhibit non-canonical autophagy and trafficking of PD-L1 from the cytosol to the plasma membrane, which we did not observe in this research work (**Figure 7D.**)^{734,756}. Conversely, treatment of lung tumor cells with taxol induced increased expression of PD-L1 at the plasma membrane compared to the untreated condition. Taxol could either block the endocytosis of these molecules and thus promote their overexpression at the plasma membrane or block TLR7 trafficking, which would prevent an increased expression of PD-L1 after additional TLR7 stimulation in taxol-treated cells. The most likely hypothesis, given the fact that the membrane expression of PD-L1 is strongly reduced when autophagy is

inhibited (**Figure 7D.**) and given the autophagy level profile of cells treated with chemotherapies alone or in combination with the TLR7 agonist (**Figure 6E.**), is that the induction of an autophagy level threshold could facilitate the membrane addressing of PD-L1 by the non-canonical autophagy pathway.

To decide between the two hypotheses by which autophagy induced following a combination of platinum-salt based chemotherapies with a TLR7 agonist could facilitate the membrane expression of PD-L1 (de novo expression versus trafficking facilitated to the plasma membrane), we have compared the membrane expression of PD-L1 versus the global expression (membrane + cytosolic) by flow cytometry, following a combinatory treatment of cisplatin plus TLR7 agonist (**Figure D2.**)

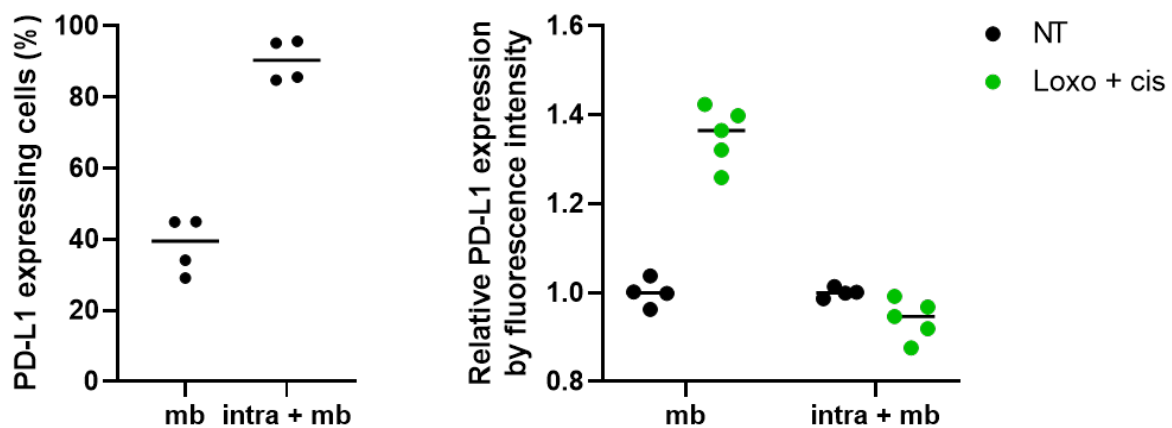


Figure D2. Comparison of PD-L1 expression at the plasma membrane with its global expression

At the left: Graph representing the PD-L1 positive A549 cells expression at the plasma membrane (mb) versus its global expression (intra + mb) in non-treated condition. On the right: Graph representing the relative expression of PD-L1 by fluorescence intensity at the plasma membrane versus its global expression in A549 treated or not (NT) with a combination of TLR7 synthetic agonist (Loxo, 1mM) and cisplatin (Cis, 50µM) (Loxo+ cis) for 24h. The experiment was performed twice in duplicates.

Two similar experiments were performed at 24h post-treatment. Although, we observed that around 40% of lung tumor cells express PD-L1 at the plasma membrane, almost all cells are positive intracellularly for PD-L1. Furthermore, for both experiments, the profile of PD-L1 expression increase at the plasma membrane versus global expression is similar. Indeed, in both experiments, following combinatory treatment, plasma membrane PD-L1 expression is increased whereas its global expression in treated cells is unchanged compared with the NT condition. These results, although preliminary and need to be confirmed, support the hypothesis that the induction of sufficient autophagy following TLR7 agonist and cisplatin treatment promotes PD-L1 trafficking to the plasma membrane.

3.5 Crosstalk between Autophagy, chemoresistance and immunogenic molecules expression in lung tumor?

Since in this research, we have shown that (i) autophagy is involved in chemoresistance of lung tumor cells (**Figure 6D.**) as well as the increased expression of PD-L1 (**Figure 7D.**), and (ii) increased expression of other molecules that can impact the efficacy of anti-tumor immune response, like B7H3, MHC-I and CD47 is associated with a raise in the cellular autophagy level (**Supplementary Figure 7A., annex 1**) we wanted to determine if a possible link between the autophagy level, the chemoresistance, and the expression of molecules that can impact the immune response could exist. This hypothesis was supported by the fact that in head and neck cancer, tumor cells that were resistant to death induced by platinum-salt based chemotherapies had an upregulated expression of PD-L1⁷⁵⁷.

For this purpose, expression profiles of MHC-I and PD-L1 in tumor cells chemosensitive (A549) or chemoresistant (CisR) to cisplatin were analyzed after the treatment of cells with the TLR7 agonist (Loxo, 1mM) alone or in combination with cisplatin (Cis, 50 μ M) (Loxo + cis). One experiment was performed for 48h post-treatment kinetics (**Figure D3.**).

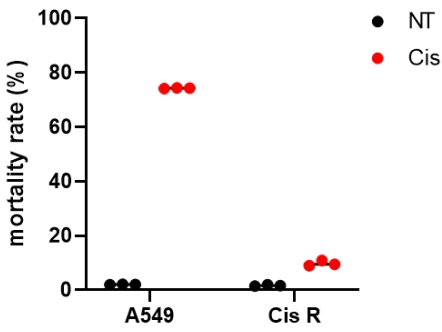
Before performing the experiment, chemoresistance capacity of the A549 CisR was evaluated by comparing the mortality rate of A549 chemosensitive versus A549 chemoresistance (CisR) treated by cisplatin for 48h. As compared with the A549 whose mortality rate is around 80%, the chemoresistant cells have a mortality rate very low, around 10%, therefore demonstrating their great capacity to resist to cisplatin (**Figure D3A.**).

Then we compared the PD-L1 expression profile of A549 chemosensitive versus chemoresistant. Interestingly, we observed that lung tumor cells naturally chemoresistant to cisplatin have a bigger proportion of cells positive for PD-L1 expression at the plasma membrane (around 20% for A549 versus 60% for CisR) (**Figure D3B.**). However, no difference on MHC-I expression level has been observed between chemosensitive versus chemoresistant to cisplatin lung tumor cells (**Figure D3C.**). Besides, we noted that profiles expression of PD-L1 and MHC-I seem fluctuate similarly, and seem differs according to the sensitivity of the latter to cisplatin and the treatment imposed on the cells. Actually, for both molecules, their expression in cisplatin-sensitive cells is increased following treatment with cisplatin alone and even more following combination with the TLR7 agonist. In contrast, for cisplatin-resistant cells, treatment with cisplatin alone and, to a lesser extent, with TLR7 agonist strongly increase PD-L1 and MHC-I expressions. Moreover, these expression levels are not increased by the combination of cisplatin and TLR7 agonist, compared to treatment with cisplatin alone (respectively **Figure D3B.** and **D3C.**).

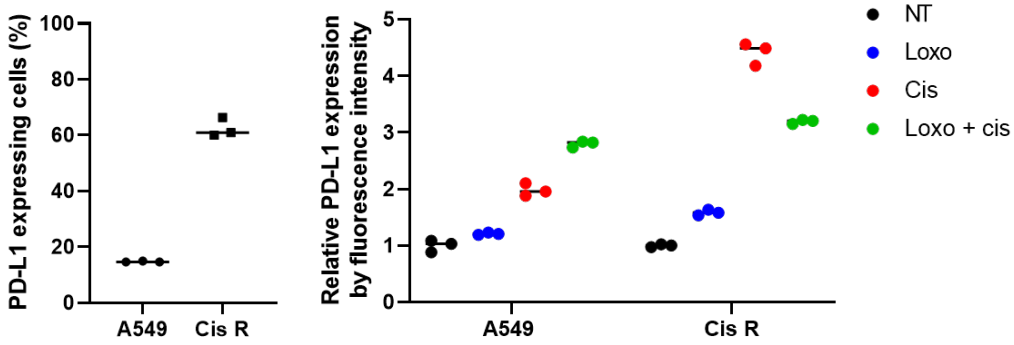
Given these preliminary data which suggest that cisplatin-resistant lung tumor cells are more susceptible to express these two molecules in cisplatin-treated cells and they do not require additional stimulation of TLR7 compared with chemosensitive cells, we

hypothesized that these chemoresistant cells have an higher autophagy basal level than the chemosensitive cells and that explain the differences observed between the two types of cells in their expression profiles of PD-L1 and MHC-I. To precise the actual involvement of autophagy in these increased expression of molecules after treatment with cisplatin plus TLR7 agonist, we treated chemoresistant cells with cisplatin alone or in combination with different autophagy inhibitors.

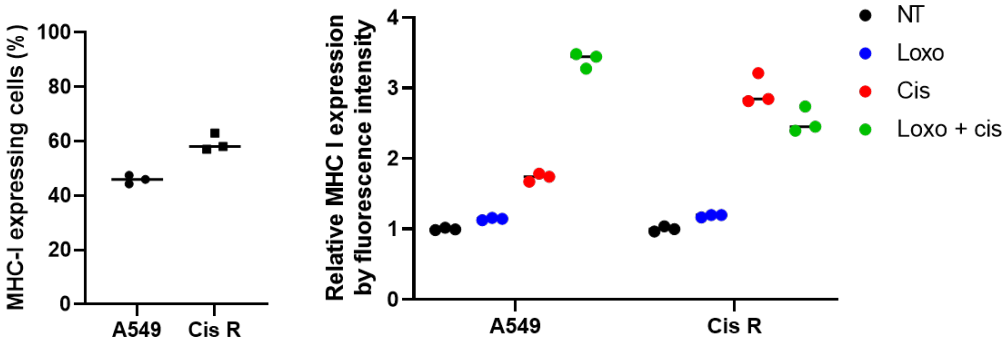
A.



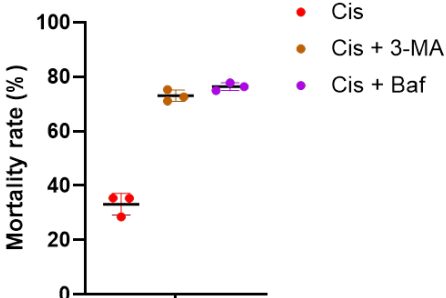
B.



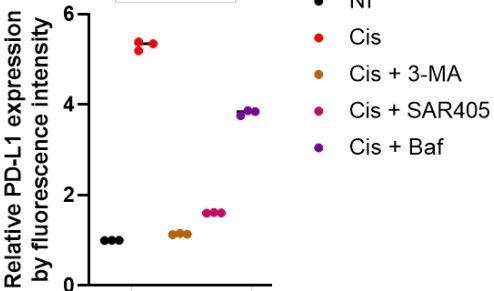
C.



D.



E.



F.

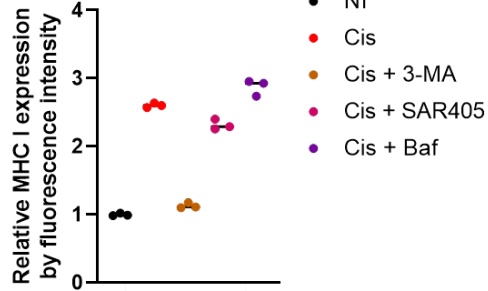


Figure D3. Comparison of PD-L1 and MHC-I expression profiles between A549 chemosensitive versus chemoresistant to cisplatin

A. Graph representing the mortality rate in A549 cells chemosensitive (A549) or chemoresistant to cisplatin (CisR) treated or not (NT) with cisplatin (Cis, 50 μ M) for 48h. **B.** At the left: Graph showing the PD-L1 positive A549 cells expression in non-treated condition whether cells are chemosensitive or chemoresistant to cisplatin (CisR). On the right: Graph representing the relative expression of PD-L1 by fluorescence intensity in A549 chemosensitive versus chemoresistant to cisplatin, treated or not (NT) with a TLR7 synthetic agonist (Loxo, 1mM), or with cisplatin (Cis, 50 μ M) alone or combined together (Loxo + cis) for 48h. **C.** Same analysis that B. but on MHC-I expression. **D.** Graph representing the mortality rate of A549 cells chemoresistant to cisplatin (CisR) treated or not (NT) with cisplatin (Cis, 50 μ M) alone or in combination with autophagy initiation inhibitor (3-MA, 10mM), or autophagy maturation repressor (Baf, 100nM) for 48h. **E.** Graph representing the relative expression of PD-L1 by fluorescence intensity in A549 chemoresistant to cisplatin, treated or not (NT) with cisplatin (Cis, 50 μ M), alone or combined with autophagy initiation inhibitor (3-MA, 10mM, and SAR405, 10 μ M), or autophagy maturation repressor (Baf, 100nM) for 48h. **F.** Same analysis that E. but on MHC-I expression. The experiment was performed one time in triplicates.

Interestingly, we observed that chemoresistant cells treated with inhibitors of autophagy initiation or maturation have a mortality rate increased compared with the cells treated with cisplatin alone (respectively around 75% for both inhibitors versus 45% without autophagy repressors), therefore demonstrating that chemoresistant cells are dependent on a complete autophagy flux to fulfill their resistance to cisplatin (**Figure D3D.**).

We also checked for these chemoresistant cells if autophagy impairment by same inhibitors could affect the increased expressions of PD-L1 and MHC-I in these treated cells. Interestingly, we observed that autophagy inhibition has a different impact on expression of these molecules following cisplatin treatment. Actually, PD-L1 increased expression following cisplatin treatment has been shown to be greatly reduced by inhibition of the autophagy inhibition (by 3-MA and SAR405) and to a lesser extent, by inhibition of the autophagy maturation (**Figure D3E.**). In contrast, even if the increased expression of MHC-I following cisplatin treatment is completely suppressed by 3-MA, other inhibitors including SAR405 have no impact on the expression of this molecule, rendering the analysis of autophagy involvement quite complicated (**Figure D3F.**).

Although these results need to be confirmed, this work raises the potential link between chemoresistance to cisplatin and the expression of molecules modulating the anti-tumor immune response, at least PD-L1, and a potential better response to immune checkpoint blockade immunotherapy. This would suggest that cisplatin-resistant NSCLC patients would also be more resistant to the anti-tumor immune response but potentially more sensitive to immune checkpoint blockade immunotherapy. It will be necessary to verify with other chemotherapies if the same expression profiles of these molecules are observed.

3.6 Platinum-salt based chemotherapies-induced autophagy promote chemoresistance and immunogenic molecules expression through STAT3 pathway?

One of the interesting potential molecular connexion between autophagy, chemoresistance, and immune checkpoint expression is through the activation of the STAT3 pathway (Figure D4.).

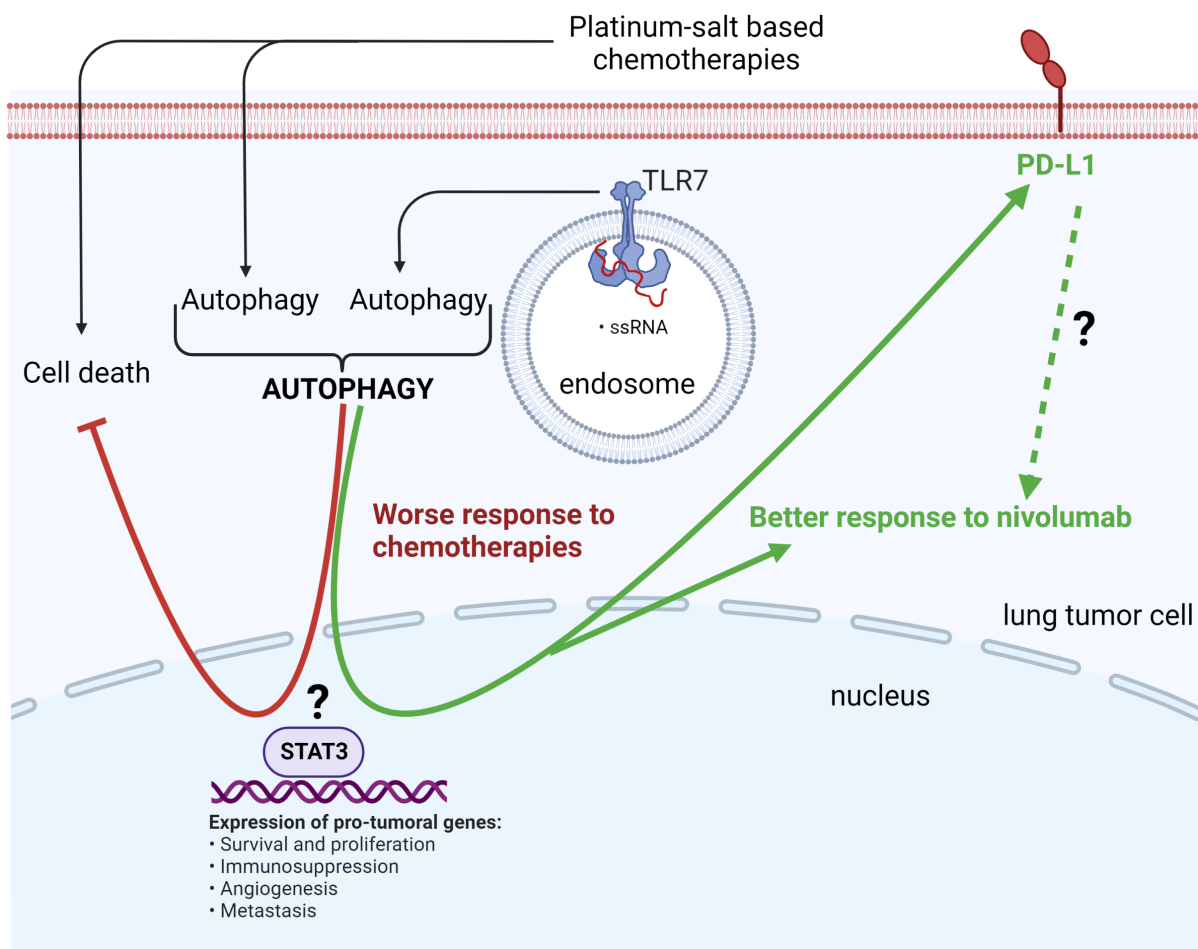


Figure D4. Synergy of autophagy level, resistance to platinum-salt based chemotherapies and increased response to nivolumab: Possible involvement of STAT3?

Indeed, autophagy has been shown to activate the STAT3 pathway following DNA damage and responds by repairing the genome⁷⁵⁸. Besides, the NF- κ B pathway, activated following TLR7 stimulation and able to induce autophagy, has been shown to promote the activation of the STAT3 pathway⁷⁵⁹. Furthermore, activation of this pathway has also been shown to promote chemoresistance to cisplatin and MHC-I expression in lung tumor cells^{760,761}. Similarly, STAT3 has also been shown to promote PD-L1 expression in lung cancer tumor cells, thereby inducing chemoresistance in these cells⁷⁶². Interestingly, it was shown that esophageal tumor cells could secrete exosomes containing PD-L1, and this molecule could, in turn, promote chemoresistance of the recipient cell *via* activation of the STAT3⁷⁶³. This mechanism could partly explain how, physiologically, chemoresistance transfer is mediated in the

context of anti-tumor chemotherapy and how autophagy is involved in this process. It is also interesting to add that STAT3 has been shown to inhibit autophagy in glioblastoma tumor cells⁷⁶⁴. This mechanism could explain in our model that, when autophagy is activated, the release of TLR7 ligands by lung tumor cells is reduced (**Figure D1.**).

Importantly, STAT3 is also a crucial signaling pathway for the cancer stem cells (CSC) homeostasis, a tumor cell subpopulation implicated in treatment resistance and relapse in the cancer patient⁷⁶⁵. Interestingly, we also observed in a preliminary experiment that the CD133 marker, which is a biomarker of CSCs, has an expression profile strictly similar to the MHC-I and PD-L1 expression profiles observed in cisplatin-sensitive A549 cells according to the treatment submitted to cells (**Figure D5.**).

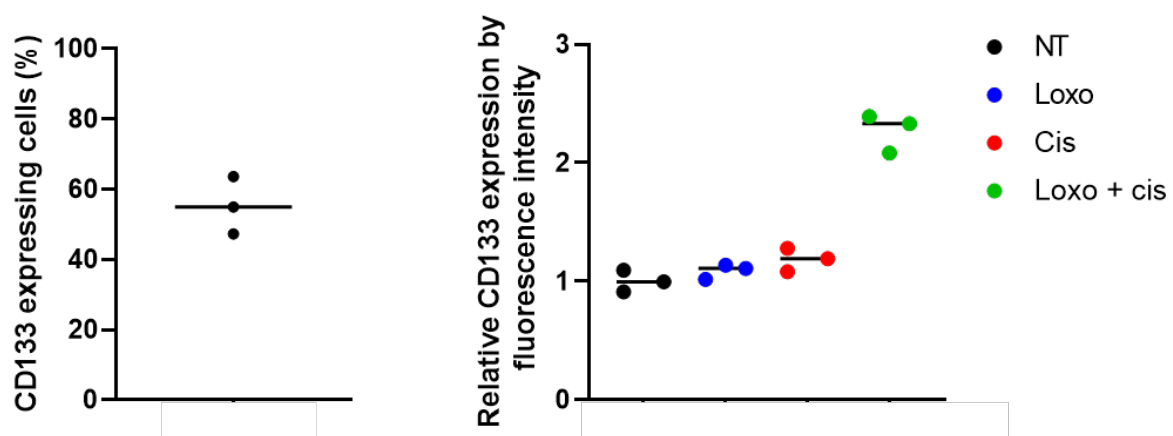


Figure D5. CD133 expression profile in A549 chemosensitive cells in diverse culture conditions

At the left: Graph showing the CD133 positive A549 cells expression in non-treated condition. On the right: Graph representing the relative expression of CD133 by fluorescence intensity in A549 chemosensitive cells, treated or not (NT) with a TLR7 synthetic agonist (Loxo, 1mM), or with cisplatin (Cis, 50 μ M) alone or combined together (Loxo + cis) for 48h. The experiment was performed one time in triplicates.

Although preliminary, this observation suggests that when lung tumor cells are subjected to sufficient autophagy induction, as observed with combinatory cisplatin treatment with the TLR7 agonist, the STAT3 signaling pathway could be activated, and lung tumor cells exhibit a CSC-like phenotype within they are resistant to cisplatin and oxaliplatin. As previously mentioned, the PD-L1 and MHC-I expressions are also increased in these cells. This hypothesis is supported by the fact that one study showed that inhibition of autophagy in lung tumor cells resulted in decreased CSC marker expression and reversed chemoresistance to cisplatin⁴⁶⁵.

Although STAT3 has also been shown to facilitate the chemoresistance of ovarian tumor cells to taxol⁷⁶⁶, we did not observed a similar resistance for Taxol in our model. One of the explanatory mechanisms could be, as mentioned above, that the dose of taxol is too high to allow STAT3 activation-induced to cope with and, therefore, to promote chemoresistance of these cells to this chemotherapy. On the other hand, taxol, *via* this mechanism, could promote the overexpression of PD-L1 and MHC-I.

3.7 Global point of view of interconnections between TLR7, autophagy, chemoresistance and PD-L1 expression

Given what we have demonstrated, and although it has to be confirmed by further experiments, we can easily imagine that along the course of chemotherapy treatment, the cell can release cellular RNA through a yet unknown mechanism. We speculate that it is mediated through extracellular vesicle release. In the recipient cell, the agonist will stimulate TLR7 and induce autophagy, which will then activate the STAT3 signaling pathway, allowing the overexpression of PD-L1 and MHC-I at the membrane. In addition, this pathway will also stimulate the release of exosomes containing PD-L1, which in turn will promote chemoresistance in the recipient cell *via* the activation of STAT3. These molecular mechanisms explain why the autophagy level in lung tumor cells is associated with a lower response of NSCLC patients to chemotherapy but associated with a better response of these patients to PD-1 targeted immunotherapy.

Our hypothesis is that in the basal state, cisplatin-resistant lung tumor cells have a higher autophagy level and activation of STAT3 than cisplatin-sensitive lung tumor cells and that overexpression could explain both the differences in sensitivity to cisplatin and the expressions of PD-L1 and MHC-I observed in chemoresistant cells. Based on this hypothesis, since cisplatin-resistant lung tumor cells have a higher autophagy level than sensitive lung tumor cells, the expression profiles of these two molecules are also different depending on their resistance to treatment. Indeed, in resistant lung tumor cells, treatment with cisplatin or TLR7 agonist, both of which have been shown to increase autophagy levels, is responsible for a more significant increase in autophagy levels and thus STAT3 activation than in cisplatin-sensitive lung tumor cells, which at the end lead to a more significant increase in expression of these two membrane proteins.

It is also suggested that treating lung tumor cells with taxol at the dose used in this manuscript results in much greater stress than cisplatin and oxaliplatin at the doses used, and that induces proportionally greater or lesser autophagic responses and STAT3 activation. In cisplatin- or oxaliplatin-treated lung tumor cells, the additional stimulation of TLR7 further increases the autophagy level and the activation of the STAT3 pathway, which results in both chemoresistance of tumor cells to these chemotherapies and increased expression of PD-L1 and MHC-I. In contrast, in taxol-treated lung tumor cells, the chemotherapy would result in a very high autophagy level and activation of STAT3 in treated tumor cells, which cannot be increased by further stimulation of TLR7.

3.8 Limitations of our study

It is essential to step back from the results demonstrated in this manuscript as some limitations to this project can be raised. Indeed, the results that we have shown both on the implication of autophagy induced by TLR7 stimulation on the chemoresistance

of NSCLC tumor cells and on the expression of molecules that can impact the recognition of these cells by the immune system have only been performed on a lung tumor line *in vitro* and must be reproduced on other cell lines to determine if the effects observed are not specific to this particular cell line. In addition, this A549 cell line is an adenocarcinoma type, so it will be necessary to verify that the effects observed are not specific to the adenocarcinoma type of NSCLC but are also on other histological types.

All the results observed *in vitro* will then have to be confirmed *in vivo*. Since autophagy seems to impact the expression of molecules at the membrane that can affect the anti-tumor immune response, we can think that potentially autophagy of these lung tumor cells allows the membrane expression of other molecules as well as the secretion of molecules, that we did not look for, but that can impact the immune infiltrate in the TME and the associated anti-tumor immune response. Thus, *in vivo*, it is possible by comparing in WT mice, tumor progression, chemoresistance, infiltrate, and anti-tumor immune response of tumors derived from autophagy-competent versus autophagy-deficient lung tumor cells, to determine the effective involvement of autophagy in that process.

In this manuscript, we evaluate the autophagy level in lung tumor cells from NSCLC patients by assessing the intensity of cytosolic labeling of the LC3 protein. In this study, we do not observe LC3 puncta within lung tumor cells of NSCLC patient's tissue slides. Therefore, we decided to determine the autophagy level within these cells by measuring the staining intensity (positive versus negative) of this autophagy protein. Although during the induction of the autophagy process, LC3 expression appears to be increased, expression of other autophagy proteins should be performed by the same experimental approach or by RT qPCR and transcriptomic analysis to confirm our results.

Another criticism that can be raised in this research work is that given the different autophagy inhibitors used, apart from SAR405, are not exclusively specific for autophagy, the results observed here could be a consequence of other pathways. Actually, given that all the autophagy inhibitors targeting the initiation can target the type III PI3K, which is specific to autophagy, they also can affect the type I PI3K, which mediates biological processes other than autophagy. However, given that all autophagy inhibitors, including SAR405, have been shown to inhibit chemoresistance or PD-L1 expression induced following TLR7 stimulation, we are confident that TLR7-mediated autophagy really impacts these processes (**Figure 6D and 7D.**). Furthermore, this result was confirmed by the absence of effects on TLR7-induced chemoresistance when specific type I PI3K or AKT inhibitors were added (**Supplementary figure S5C. and S7C.**). However, these results should be confirmed by using tumor cells genetically deficient for autophagy.

4 What is the impact of this new physiological way of autophagy induction on the tumor microenvironment and immune cells?

4.1 Impact of extracellular vesicles-containing TLR7 ligands on the other cells of the tumor microenvironment

In this study, we demonstrated that physiologically lung tumor cells could secrete TLR7 ligands, probably through extracellular vesicles release, and that *via* this mechanism, an autocrine or paracrine stimulation of TLR7 in neighboring malignant cells leading to an induction of autophagy could be observed (**Figure 3 and 4.**). Thus, in the context of treatment with platinum-salt based chemotherapies, this pathway could promote the spread of chemoresistance to the latter. It is important to note two things here.

First, extracellular vesicles seem highly unlikely to contain only TLR7 ligands. Extracellular vesicles must also include other molecules that could impact autophagy as well as other biological functions. This hypothesis is supported by the fact that elements, including TLR7 ligands, are present in the tumor microenvironment that stimulate the increase of autophagy levels in malignant cells. Since the inhibition of this receptor in lung tumor cells does not seem to be entirely sufficient to inhibit the increase in autophagy in these cells after incubation of the tumor supernatant, it appears that other molecules present in the tumor microenvironment are present and can cause an increase in autophagy in malignant cells (**Figure 4B.**).

The second point is that TLR7 is not exclusively expressed in lung tumor cells but also in other cell types in the microenvironment, notably the immune cells (**Figure 2C.**). Furthermore, the correlation of expression of this receptor with autophagy genes is very high in pan-immune cells (**Figure 2D.**). Thus, this mechanism, in addition to affecting the tumor cells directly, could also have an impact on the other cell types composing the tumor microenvironment and thus modulate tumor progression more or less positively depending on the ligand and/or the cell type studied (**Figure D6.**).

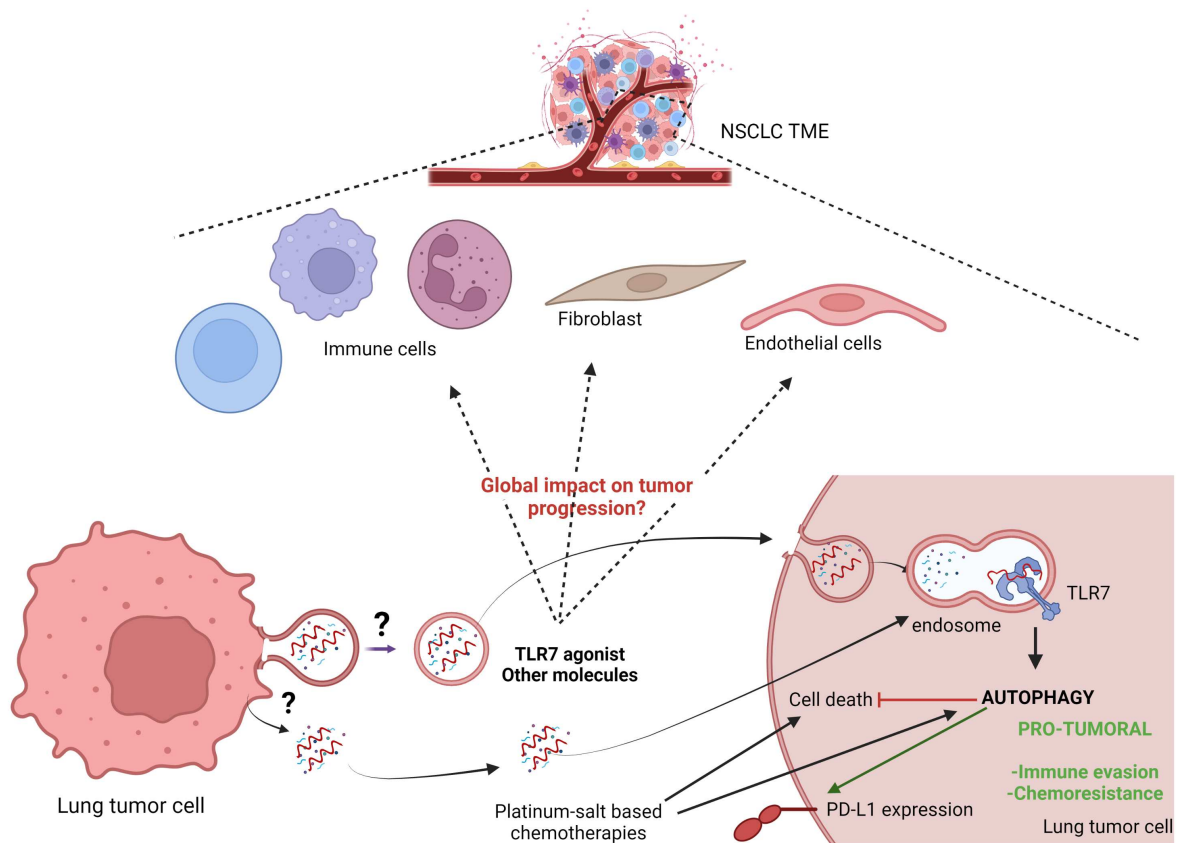


Figure D6. Potential impact of cargoes-containing vesicles on other cells composing the tumor microenvironment

In the context of tumors, the different cell types can secrete extracellular vesicles as a means for them to communicate with their neighbors within the tumor microenvironment. Thus, numerous examples have shown that tumor cells can also promote the secretion of extracellular vesicles to promote, in a non-exhaustive list, the transfer of chemoresistance from one cell to another during chemotherapy treatment to favor the secretion of pro-tumor molecules by fibroblasts, but also to modify the immune microenvironment and make it immunosuppressive⁷⁶⁷.

4.2 Impact of autophagy on immune infiltrate and immunogenic molecules expression in NSCLC tumor cells

It is interesting to note that depending on the approach, the impact of autophagy level within lung tumor cells on the immune infiltrate is different in this study. Actually, by transcriptomic analysis of gene expression and genes related to the various immune cell types composing the TME, and by the MCP counter, we could determine in bulk that NSCLC patients highly expressing autophagy genes are less infiltrated by immune cells types as compared with NSCLC patients weakly expressing autophagy genes. However, in one of the cohorts we used for our analysis, autophagy level on the tumor area did not demonstrate a significant difference in the immune infiltrate between autophagy low and autophagy high NSCLC patients, while a similar tendency, as

shown in genes expression, was observed. This analysis was only focused on the immune infiltrate density of general populations of immune cells, using broad markers like CD68, CD4, or CD8 to respectively discriminate macrophages, CD4 T lymphocytes, or CD8 T lymphocytes (**Supplementary figure S5B-D., annex 1**). The absence of other more discriminant markers and the lack of information concerning their subtypes or their state of activation render very complicated the analysis of lung tumor cells autophagy on the immune response.

Actually, admitting the density of each global population is not impacted by the autophagy status of lung tumor cells, it is easily thinkable that within each global population, the proportion of immunosuppressive or inactivated immune cells could be affected. That point is supported by the fact that we observed by transcriptomic analysis in bulk, that the lung tumor cells highly expressing autophagy are associated with an immune infiltrate more exhausted (specifically in T cells), expressing more inhibitory immune checkpoints than lung tumor cells weakly expressing autophagy genes. That result suggests that rather than impacting the density of immune cells, autophagy within lung tumor cells is more prone to affect the phenotype of infiltrated immune cells and lead to the increase of inhibitory molecules. By using more discriminant markers, it would be very interesting to determine the proportion of the phenotype status of each immune cell population between NSCLC patients who are highly autophagic and NSCLC patients who are weakly autophagic. Moreover, using our two approaches, we demonstrated that the autophagy level within lung tumor cells promotes the expression of inhibitory immune checkpoints like PD-L1 on these same cells, which also suggests that autophagy within lung tumor cells negatively impacts anti-tumor immune response.

In the literature, very little is known about the impact of tumor cell autophagy on the immune infiltrate in TME. Since autophagy is a mechanism that limits the activity of the inflammasome and the secretion of pro-inflammatory cytokines, autophagy in tumor cells naturally inhibits the development of an anti-tumor immune response. One paper demonstrated that autophagy in melanoma and colorectal cancer repressed the secretion of CCL5 and CXCL10 and, therefore, the recruitment of NK and T cells within the TME⁷⁶⁸. Intriguingly, while the majority of immune cell infiltration was not correlated with the level of LC3 in tumor cells, we observed a significant inverted correlation between autophagy and NK density as well as a tendency of a decrease of T cells density and CCL5 and CXCL10 gene expression in autophagy high patients. Another study demonstrated that autophagy is induced in lung tumor cells following radiotherapy, which eliminates dsDNA and triggers the activation of the cGAS STING pathway. Therefore, autophagy prevents the recognition and stimulation of the cGAS STING pathway, which eventually inhibits the implementation of an anti-tumor immune response⁷⁶⁹.

Finally, to the best of my knowledge, our study is the only one to demonstrate the importance of the level of autophagy in lung tumor cells on the expression of immunogenic molecules like MHC-I and PD-L1. Moreover, very few information is available concerning the involvement of autophagy in the expression of these

molecules, and the conclusions vary according to the tumor model studied. Indeed, in a model of triple-negative breast cancer and prostate cancer, down-regulation of PD-L1 was associated with degradation by autophagy. Interestingly, the authors of the paper mentioned that autophagy was involved, but it involved a distinct mechanism from macroautophagy⁷⁴¹. Concerning the link between autophagy in lung tumor cells and PD-L1, even though in an NSCLC model, PD-L1 degradation was demonstrated to be mediated by the lysosome and a recent study showed that autophagy *via* HIP1R facilitated PD-L1 delivery to the lysosomes, no direct link between autophagy and PD-L1 degradation have been described. In contrast, a recent study demonstrated that in lung tumor cell models, autophagy-induced PD-L1 expression^{742,743}. Interestingly, in a gastric cancer model, inhibition of autophagy has been shown to promote PD-L1 expression *via* p62 accumulation and stimulation of the NF- κ B pathway⁷⁷⁰. Very few studies have been performed on the impact of autophagy on MHC-I expression. Both in pancreatic cancer and dendritic cells, autophagy has been shown to degrade MHC-I^{771,772}. Although this was only demonstrated in one study where they showed that radiotherapy of lung tumor cells promoted autophagy and MHC-I expression, it appears that in NSCLC, autophagy positively modulates MHC-I expression⁷⁷³.

5 Impact of our research on clinics

5.1 Targeting TLR7-induced autophagy in NSCLC cancer: a promising strategy?

5.1.1 Non-specific therapeutic strategy and adverse effects

In this research work, we demonstrated that in the tumor microenvironment of NSCLC patients, natural ligands of TLR7 are present and could induce a complete autophagy influx in lung tumor cells, which could have significant biological and clinical consequences for the patient.

Since we have shown that autophagy in lung tumor cells promotes survival, proliferation, and, in the case of chemotherapy, chemoresistance as well as the expression of molecules that can impact the immune response, the question of the use of autophagy inhibitors in clinics to slow down tumor progression and better treat NSCLC patients arises but seems hazardous. Indeed, although autophagy is crucial for cancer cells and tumor progression, it is, first and foremost, an essential mechanism present in all cells and necessary for their homeostasis⁷⁷⁴. Thus, although inhibiting global autophagy could significantly impact tumor cell homeostasis, it would also have detrimental consequences on all cells composing the TME and the organism. Indeed, by inhibiting the autophagy of immune cells, the survival, activation, proliferation, and differentiation of each of the immune cell types involved in anti-tumor immunity, as well as immunity in general, would be very strongly damaged⁷⁷⁵. Thus, by limiting the control of immunity on tumor cells, this therapeutic strategy could potentially accelerate tumor progression and promote the development and occurrence of infections and cancers, without mentioning the high toxicity it would induce⁷⁷⁶. This non-specificity of

autophagy inhibition explains why, in the clinic, this therapeutic strategy of autophagy inhibition alone has demonstrated negligible therapeutic benefits in breast and pancreatic cancers^{777,778}. Although the increasingly used alternative, which consists in combining an autophagy inhibitor with a conventional anti-tumor therapy (radiation therapy, chemotherapies...) to boost the efficacy of the latter, has demonstrated very high therapeutic benefits in many tumor models without increasing toxicities, this therapeutic strategy fails to eliminate the entire tumor⁷⁷⁹.

Besides another therapeutic option that could be considered would be to inhibit TLR7. For the same reasons as above, this strategy seems risky. Indeed, TLR7, although expressed in lung tumor cells and acting as a pro-tumoral mechanism, is also expressed in other cells composing the TME and the organism. Indeed, TLR7 is also expressed by immune cells (macrophages, DC, and B cells) and is crucial in establishing an immune response, notably the anti-tumor one. Thus, inhibiting TLR7 could act as a double-edged sword: limiting the proliferation and survival of lung tumor cells while simultaneously inhibiting the anti-tumor immune response and thus promoting tumor progression. Since the function of TLR7 has mainly been studied in immune cells, no clinical or preclinical trials aimed at inhibiting it have been developed in the fight against cancer. On the other hand, different TLR and TLR7 antagonists have been designed and used in preclinical and clinical studies in inflammatory and autoimmune diseases, with encouraging first results⁷⁸⁰. Further studies are needed to determine whether the autophagy induction pathway following TLR7 stimulation demonstrated in this manuscript is a cancer cell-specific pathway that is not observed in normal cells. If the pathway described is specific to tumor cells, we do not believe it is, then a therapeutic strategy targeting this particular pathway would be interesting and feasible in clinics.

5.1.2 Targeting TLR7-induced autophagy to overcome chemoresistance to platinum-salt based chemotherapies

Therefore, one therapeutic strategy is to target TLR7 or autophagy in addition to conventional anti-tumor therapy⁵⁶⁰. In this research, we have demonstrated that autophagy induced by TLR7 stimulation promotes chemoresistance to two chemotherapies, cisplatin and oxaliplatin, and also promotes the expression of molecules that can impact the anti-tumor immune response. Thus, depending on the conventional anti-tumor treatment, the therapeutic strategy targeting TLR7 or autophagy could be different. Indeed, in the context of treatment with anti-tumor chemotherapies such as cisplatin or oxaliplatin, inhibition of the TLR7-mediated autophagy induction pathway could decrease chemoresistance and thus increase the efficacy of chemotherapies. On the other hand, given that the increase in autophagy level following a combination of cisplatin/oxaliplatin with the TLR7 agonist has been shown to promote PD-L1 and MHC-I expression in lung tumor cells, adding immune checkpoint blockade (ICB) immunotherapy to this TLR7 agonist plus chemotherapy treatment could boost the efficacy of the immunotherapy and be an exciting alternative.

5.1.3 Boosting TLR7-induced autophagy to promote autophagic cell death

In contrast to inhibition of the autophagy pathway, a strategy that is increasingly being developed in preclinical studies is to boost autophagy in tumor cells to induce autophagic cell death, which, in addition to promoting cell death by the treatment, allows the release of molecules that enable the establishment of an effective anti-tumor immune response. The various conventional anti-tumor therapies have been described as promoting the establishment of this autophagic cell death in many tumor models⁷⁸¹. This autophagic cell death has been described in the context of a too-high autophagy level in cells due to excessive or prolonged stress. From our study, it appears that taxol treatment of NSCLC patients would be more favorable than cisplatin and oxaliplatin, as neither chemoresistance was observed in lung tumor cells following the application of the chemotherapy nor after TLR7 stimulation. In addition, taxol treatment induced increased expression of PD-L1 and MHC-I. Thus, combining taxol and ICB immunotherapy could be very interesting in treating NSCLC, possibly by causing autophagic cell death, as previously observed⁷³⁷. Since TLR7 stimulation has not been shown to have any pro-tumor effect when treating lung tumor cells with taxol, it may even be possible to add a TLR7 agonist to stimulate immune cells and anti-tumor immunity. These different therapeutic strategies will have to be confirmed by co-culture experiments of lung tumor cells with immune cells and *in vivo* studies.

It is possible to imagine that the chemoresistance observed following TLR7 stimulation may be due to the low concentrations of cisplatin and oxaliplatin, which may induce a stress that the cell can handle. It would be interesting to know, by increasing the concentration of cisplatin or oxaliplatin to which the lung tumor cells are subjected, whether TLR7 stimulation in these cells still promotes chemoresistance. At higher concentrations, these chemotherapies may induce too much stress in the cells, which TLR7 stimulation enhances. The combination of TLR7 agonist with cisplatin/oxaliplatin at these concentrations could thus, instead of promoting chemoresistance, lead to cell death by autophagy due to an excessive increase in autophagy levels. This hypothesis is likely given that, on the one hand, this mechanism was observed in melanoma, where the combination of radiotherapy and TLR7 agonist led to autophagic cell death and an anti-tumor immune response, and on the other hand, tumor cells treated with high concentrations of cisplatin were shown to be no longer able to resist the latter, but on the contrary, lead to immunogenic cell death^{782,738}. Similarly, it is possible to imagine that by decreasing the concentration of taxol to which lung tumor cells are subjected, autophagic stress could be reduced. Thus, stimulation of TLR7 could lead to an increase in autophagy levels, which would not be detrimental to the cell, but on the contrary, could promote chemoresistance of these cells to taxol. This hypothesis is also supported by the fact that although the tumor model differs, taxol has been shown to induce cytoprotective autophagy, which promotes chemoresistance or autophagic cell death^{783,737}.

Thus, the therapeutic strategy of using high concentrations of chemotherapies combined with a TLR7 agonist in order to promote immunogenic cell death and an anti-

tumor immune response is very interesting. Besides, adding an ICB immunotherapy could be even more beneficial in limiting tumor progression. Although this therapeutic strategy of boosting autophagy and autophagic cell death by combining high concentration of chemotherapies and TLR7 agonist seems very interesting to limit NSCLC tumor progression, the use of high concentrations of chemotherapies could lead in parallel to very high toxicities. It would be interesting to determine if, at low chemotherapy concentrations, the addition of TLR7, TLR3, and TLR4 agonists, by increasing the concentration of these different agonists or combining them, could promote autophagic cell death in lung tumor cells. If it appears to be the case, this therapeutic strategy would limit tumor progression by inducing tumor cell death and release of pro-immune factors by the malignant cells, but also activation of immune cells by TLR ligands, both participating in the efficient elimination of tumor cells.

5.2 Using autophagy as a prognostic factor of responsiveness to chemotherapies and immunotherapy in NSCLC patients?

Given that it seems premature to target TLR7 or autophagy, this research allows us to consider the use of an autophagy level as a prognostic factor in NSCLC patients. Although our team has already shown that TLR7 expression could be used as a prognostic and predictive factor of response to chemotherapy in NSCLC patients, the autophagy level in these cells refines this prognostic and predictive factor (**Figures 6A.** and **6B.**). Indeed, we have shown in this work that the high expression of the autophagy level of lung tumor cells in NSCLC patients associated with a high expression of TLR7 was correlated with a worse survival than NSCLC patients whose tumor cells strongly express TLR7 but who have a lower autophagy level (**Figures 5B.** and **5C.**). The autophagy level of lung tumor cells is also both an independent prognostic factor for survival in NSCLC patients and a predictive factor for the response of these same patients to chemotherapy and ICB immunotherapy (**Figures 6 and 7G.**). The work of this research could thus be used in clinics and allows the use of the autophagy level of lung tumor cells as a prognostic factor and as an indication to adapt the treatments given to patients, which could ultimately improve the therapeutic benefit/risk ratio and thus the management of NSCLC patients.

6 Microbiome: a new physiological way of autophagy induction mediated by TLR7 that could impact the tumor development?

6.1 TLR7-induced autophagy: a new way by which microbiome could impact the autophagy level in tumor cells?

Although in this research project, we investigated the impact of TLR7 on the autophagy mechanism and tumor progression using specific synthetic agonists, TLR7 is first and foremost a single-stranded RNA receptor that can recognize single-stranded RNA of viral or cellular origin under physiological and pathophysiological conditions^{784–786}. Among single-stranded RNA viruses, two viruses have a tropism for the lung and lung cells: influenza virus (IAV) and respiratory syncytial virus (RSV), respectively, the causative agent of influenza and a virus responsible for severe lung disease in infants.

Although in this project we demonstrated the release of cell-derived single-stranded RNA from tumor cells into the tumor microenvironment of NSCLC patients, we investigated whether, during viral infection, natural TLR7 ligands of viral origin could be secreted into the tumor microenvironment. For that purpose, A549 cells were infected with either IAV or RSV, both with an MOI of 1, for 24 and 48h and then supernatants from these infection culture conditions were put on HEK-blue cells to measure the presence of TLR7 ligands within those supernatants. In the meantime, A549-GFP LC3 cells with infected in a similar way, to visualize the impact of these infections on autophagy (**Figure D7**).

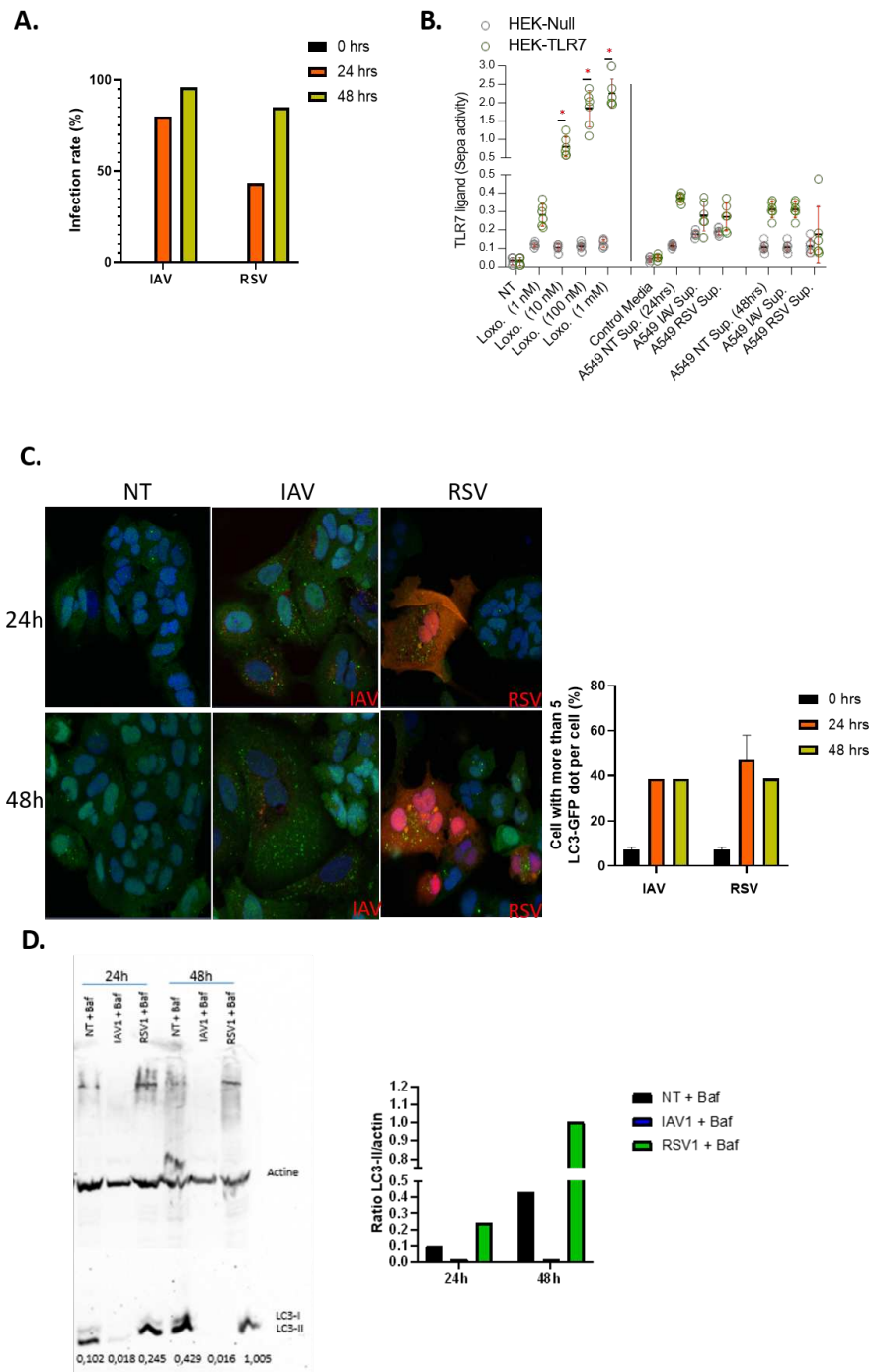


Figure D7. Investigation of the potential impact of viral TLR7 agonist on autophagy

A. Graph representing the infection rate of A549 infected with IAV or RSV, both with an MOI 1, measured by flow cytometry using antibodies targeting respectively the NP protein for IAV and the protein F for RSV, for 24 and 48h post-infection. This experiment is representative for three similar experiments we performed. **B.** Measurement of the presence of TLR7 viral ligands through the measurement of the Sepa activity of HEK-blue cells, following the treatment or not (NT) with different doses of TLR7 agonists (Loxo., 1nM, 10nM, 100nM, and 1mM), or after the incubation with a culture media alone (Control media), with a tumor supernatant from A549 cells non-infected (A549 NT Sup.) or with the supernatant of A549 infected for 24h with IAV and RSV, both with an MOI 1. The experiment was performed one time in triplicates. **C.** Example of images of A549 stably transfected with the GFP-LC3 construct (A549 GFP-LC3) infected or not (NT) with IAV or RSV, both of them coupled with mCherry for 24h and 48h, with an MOI 1. In the meantime, the quantification of the number of cells with more than 5

autophagosomes in each condition were measured and represented in a graph. The analysis of the images was performed with ZEN software, and the same person performed the quantification. The experiment was performed one time. D. Analysis of autophagy induction by western blot by studying the conversion of LC3-I (inactive form, 16kDa) to LC3-II (active form, 14kDa). Actin is also revealed and used here as a housekeeping gene (42kDa). A549 cells are either infected or not (NT) with IAV or RSV with an MOI of 1. 2h before cell lysate and western blot, cells were treated with an inhibitor of autophagy maturation, bafilomycin (Baf, 100nM). The LC3-II/actin ratio corresponding to autophagy induction is measured for each condition and plotted.

Before analysis on HEK-blue and A549-GFP LC3, the percentage of A549 infected at both 24 and 48h, for both viruses, was measured. We could observed that already at 24h post-infection, the percentage of infected cells with IAV is very high (around 75%) and is even more increased at 48h (around 90%). Concerning RSV, the percentage of A549 infected was around 40% at 24h and raise to 80% at 48h (**Figure D7A.**). After demonstrating that for both viruses and kinetics, the percentage of infection was fine, we focused on determining the presence of TLR7 viral agonist that could be released within the tumor microenvironment of NSCLC patients. For that, in one very preliminary experiment, we compared the HEK-blue activity incubated either with a tumor supernatant from cells non-infected or infected with the two viruses for 24 and 48h. Interestingly and unexpectedly, the activity of TLR7 reporter cells incubated with supernatants from the different infection conditions was identical to that of the control cells and the uninfected control condition. This observation contrasts with a study in which J Bitto et al. demonstrated that, upon infection with *Staphylococcus aureus*, membrane vesicles containing RNA formation are induced and released extracellularly. These ligands are then recognized by TLR7, which stimulates autophagy induction⁷⁸⁷. These results suggested that in NSCLC patients, the natural agonists of TLR7 present in the TME would be RNAs of cellular rather than viral origin (**Figure D7B.**). Those results should be confirmed by the reiteration of the experiment. Although no TLR7 ligand release following infection with the two viruses could have been observed in our model, we showed that both viruses could increase autophagy level of A549 upon infection with a similar kinetics. Actually, both viruses are responsible for an increase of the autophagy level in A549 at 24h post-infection, that cannot be raised at 48h post-infection (**Figure D7C.**). In order to determine if the increase of autophagy level after infection with both viruses results from a de novo induction of autophagy flux or a blocking of autophagosomes degradation, we performed a western blot. As explained in **figure 3D**, by measuring the ratio of LC3-II with actin in the different infection conditions of A549 infected as previously, and treated with bafilomycin for 2 hours, we observed in one preliminary experiment, for RSV, an increase of the ratio, meaning that this virus is able to induce a complete autophagy flux in malignant cells (**Figure D7D.**). Unfortunately, no bands were observed for IAV-treated cells in this experiment. These experiments should be reiterate to confirm or infirm the results observed. Besides it is important to note that in our model of infection, autophagy can be induced following various stresses caused by the viral infection, and the actual involvement of TLR7 in that process has yet to be determined. Further studies will need to be performed, including using lung tumor cells

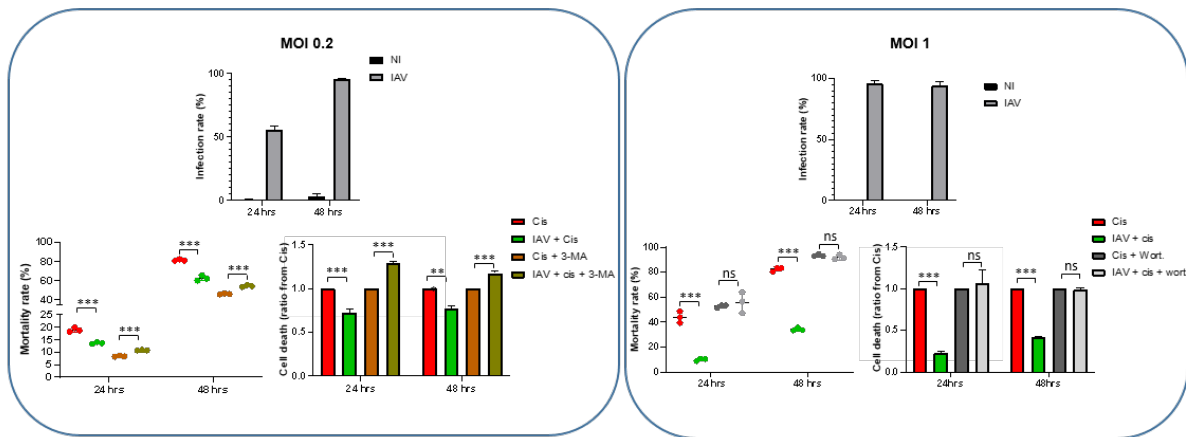
KO for TLR7, to determine the actual involvement of this receptor in increasing autophagy levels in lung malignant cells following infection by IAV and RSV.

6.2 Recognition of microorganisms by TLR7: a crucial factor impacting tumor progression through autophagy?

Since (i) TLRs are receptors mainly involved in pathogen recognition, (ii) TLRs have been shown to induce autophagy in both non-tumor and tumor models, (iii) and it promotes tumor progression by various mechanisms, the interest to study the consequences of viral infections on tumor progression, in particular *via* the modulation of the level of autophagy, is relevant and was the subject of a review I wrote (**Annex 2**).

Although the involvement of TLR recognition in autophagy induction has not been shown for any virus and autophagy induction in viral infection is multi-causative, all viruses have been described to elicit autophagy in normal and tumor cells for which they have a tropism. The autophagy induced by these different viruses can lead to the degradation of molecules that greatly modulate the behavior of the tumor cell and can thus promote tumor progression. Some viruses, by inducing a solid autophagy response in tumor cells, can also cause cell death by autophagy which is immunogenic and facilitates the establishment of an anti-tumor immune response. This autophagy cell death was observed notably during measles infection in melanoma and during Newcastle disease virus in glioma and lung tumors⁷⁸⁸⁻⁷⁹⁰. Finally, some viruses, and particularly persistent viruses, such as the Epstein-Barr virus and the human T-cell leukemia virus type 1, which must escape degradation by their host cells, can inhibit the autophagy process^{791,792}. Autophagy, by limiting inflammation and oxidative stress, two factors that promote the development of DNA damage, is a crucial mechanism for protecting cells from tumor transformation^{498,793}. Interestingly, these persistent viruses are all known to induce chronic inflammation and lead to the development of cancers⁷⁹⁴.

A.



B.

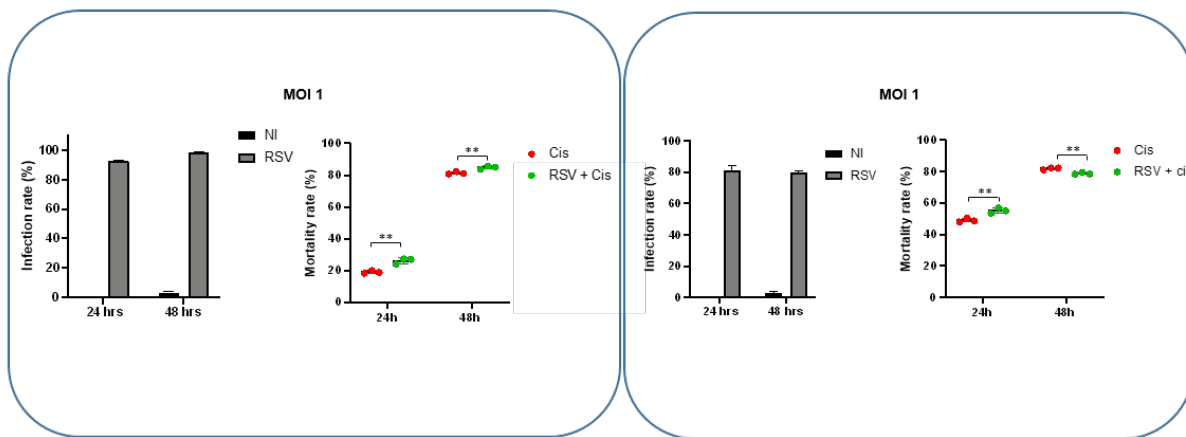


Figure D8. Impact of natural agonists of TLR7 and autophagy on chemoresistance of lung tumor cells to cisplatin

A. Two experiments in which A549 cells were treated or not (NT) with the cisplatin (Cis, 50 μ M), infected or not with IAV. Depending on the experiments, the autophagy inhibitor added and the MOI by which A549 cells are infected are different: At the left: MOI of 0.2 and 3-methyladenine (3-MA, 10mM), and on the right: MOI of 1 and wortmannin (wort, 100nM). The mortality rate, both at 24 and 48h, were evaluated and plotted. At the right of each graph containing the raw data, the cell death ratio from the NT condition was determined for each condition. The experiment was performed in triplicates. Student's t-test, ns: non-significant, ** $p < 0.01$ and *** $p < 0.001$. **B.** Two experiments in which A549 cells were treated or not (NT) with the cisplatin (Cis, 50 μ M), infected or not with RSV with an MOI of 1. The mortality rate, both at 24 and 48h, were evaluated and plotted. The experiment was performed in triplicates. Student's t-test ** $p < 0.01$.

In our NSCLC model, as demonstrated for the synthetic ligands, we observed that a natural TLR7 agonist, the IAV, could promote the chemoresistance of lung tumor cells to cisplatin (**Figure D8.**). We demonstrated that this chemoresistance is mediated by the increase in autophagy levels in infected tumor cells because the use of two different autophagy inhibitors of the initiation step completely inhibits the previously observed chemoresistance to cisplatin (**Figure D8A.**). RSV, another natural agonist, does not induce chemoresistance to cisplatin (**Figure D8B.**). Although both viruses can be naturally recognized by TLR7 and both cause an increase in autophagy levels in lung

tumor cells, only IAV, like the synthetic TLR7 agonists, promotes chemoresistance to cisplatin (**Figures D5.** and **D6.**). The mechanisms explaining this difference in resistance properties provided by these two viruses remain undetermined. Through further studies and the use of TLR7 KO cells, it will be necessary to determine the involvement of TLR7 in the increase of autophagy level observed in tumor cells for each of these viruses. Indeed, these viruses may stimulate other pathways and increase autophagy levels in infected cells.

Autophagy can be mediated by other receptors, notably immune receptors such as RIG-I, or by cellular stresses caused by the infection, such as oxidative stress. To the best of my knowledge, so far, no involvement of TLR7 in autophagy induction following infection by those viruses has been observed. It has been shown that IAV can induce autophagy by inhibiting the mTOR pathway after binding of the latter *via* its hemagglutinin to the plasma membrane⁷⁹⁵. RSV induces autophagy *via* ROS generation and AMPK activation⁷⁹⁶. Recently, a study demonstrated that an RSV viral protein, NS2, could bind Beclin-1, prevent its degradation and induce autophagy⁷⁹⁷.

Given the number of molecular actors that may be involved in the infection of cells and induce autophagy, it is very likely that this homeostatic mechanism, although not yet demonstrated, is generated by other means. Indeed, it is established that both IAV and RSV can be recognized by RNA sensors present in cells such as RIG-I and TLR7^{798–801}. These two receptors have been shown to induce autophagy^{301,299}. It is possible that the increase in autophagy observed in IAV-infected tumor cells is mainly mediated through the recognition of TLR7, whereas the increase in autophagy observed in RSV-infected cells is mediated through the activation of a different pathway, perhaps involving another molecular machinery, that ultimately does not increase the resistance of lung tumor cells to cisplatin.

Since many viruses have been shown, mainly in non-tumor models, to be able to hijack the autophagy process for their own needs and development, it is possible that these two viruses impact the autophagy process differently and that this is responsible for the differences in induced chemoresistance observed between these two viruses. In this manuscript, we demonstrated that complete autophagy flux following TLR7 stimulation was required for the chemoresistance of lung tumor cells to cisplatin and oxaliplatin. Although we did not precisely determine the induction of complete autophagy flux in lung tumor cells after IAV infection, we did demonstrate the latter after RSV infection of cells (**Figure D7D.**). Furthermore, although IAV, unlike RSV, has been shown in a non-tumor model to inhibit the terminal phase of autophagy, IAV promotes chemoresistance to cisplatin, unlike RSV⁸⁰². Thus, for both reasons, this hypothesis is not favored but will still need to be investigated by colocalization studies of autophagosomes with lysosomes *via* lysotracker or a GFP-RFP-LC3 construct, which allows visualization of autophagosomes in yellow and autolysosomes in red *via* quenching of the GFP molecule by lysosomal acidity.

7 TLR: a new type of autophagy inducer in NSCLC?

In addition to the TLR7 encoding gene, other TLR encoding genes have been shown to strongly correlate with the autophagy genes in bulk (TCGA) and/or sorted cells (GSE). Depending on the cell type studied in the tumor microenvironment (TME), correlation levels between autophagy genes and TLR are different (**Figure 2D.**). Although the correlation levels between TLR encoding genes and autophagy genes vary according to the cell type of the TME, TLR expression and autophagy genes expression, seem strongly connected and not restrained to malignant cells. These results are consistent with the fact that in many models, both non-tumor cell types of the TME and tumor cells, TLRs have been shown to trigger autophagy.

Interestingly, we observed that TLR3 and TLR4 stimulation in lung tumor cell line induce an increase in autophagosomes number, which is consistent with a study where it was demonstrated that the stimulation of these two receptors promotes autophagy in NSCLC tumor cells with consequences on tumor development⁸⁰³ (**Figure D9.**). Given that TLR-encoding genes expression and autophagy genes expression seem quite connected in the different cell types composing the TME, it would be very interesting to investigate the impact of the other TLR ligands on autophagy induction and biological consequences in each cell of the TME.

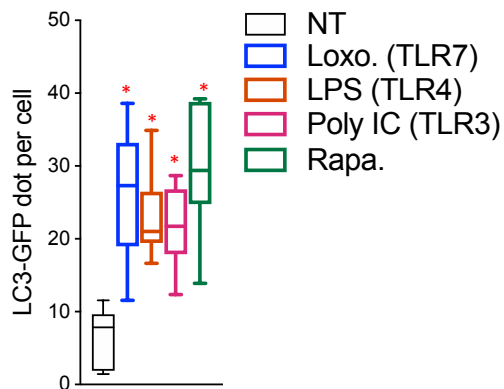


Figure D9. Effects of other TLR agonists on autophagy level of lung tumor cells

Graph representing the average LC3-GFP dot per cells in A549 GFP-LC3) treated or not (NT) with a synthetic TLR3 agonist, the Poly IC (1mM), or by TLR4 agonist, LPS (1mM) for 6h. As a positive control, cells were stimulated with TLR7 synthetic agonist (Loxo., 1mM). The analysis of the images was performed with ZEN software, and the same person performed the quantification. The experiment was performed three times in triplicates. Student's t-test, * $p < 0.05$.

The question about other TLR stimulation involvement in autophagy is all the more attractive about the fact that, although we have demonstrated the presence of natural ligands of TLR7 in the supernatant, the induction of autophagy observed in tumor cells

after incubation of the cells with supernatant is not totally inhibited when TLR7 is blocked by an antagonist, suggesting that molecular actors present in the TME other than TLR7 agonists could induce autophagy in these cells (**Figure 4B.**).

Further studies will be needed to determine the actual capacity of TLRs within each cell type composing the tumor microenvironment to induce autophagy. However, if it appears that the different cell types composing the tumor microenvironment express different TLR and these receptors can impact the autophagy process, a new pan of research will emerge about the study of consequences of TLR-induced autophagy in these different cells, notably during infection.

In addition to acute infection, it is now well established that tissues from which cancers derive, notably lungs, are not sterile organs. On the contrary, those tissues and organs are composed by a great diversity of microorganisms such as bacteria, viruses, and fungi which the set is called the microbiome and which is more and more described to be an emerging player in cancer progression^{804,805}. That impact of microorganisms in lung cancer progression is all the more interesting given that lungs are organs very exposed to the environment composed of a great diversity of microorganisms. It is interesting to add that for the same localization reasons, lungs are very exposed to external molecules present in the environment that humans can inhale and potentially lead to stresses within lung tumor cell or cell composing the tumor microenvironment as well as the cell death that can eventually lead to DAMP release and TLR-induced autophagy which potentially have an important impact on cancer progression.

Although preliminary, first studies seem to demonstrate that in various cancers, notably lung cancer, the microbiome is a key player that can impact either positively or negatively tumor progression, notably depending on cancer, its stage, or the microorganisms, by acting mainly on the immune system^{804,805}. Although very few data are available concerning microbiome impact on lung cancer, preliminary results demonstrate an emerging role of the microbiome in this latter. Actually, by using antibiotics, Cheng et al. demonstrated the importance of a commensal bacteria in promoting the host immune response against cancer⁸⁰⁶. Another study showed that, on the contrary, other bacteria, by acting on immunity, have been shown to promote lung tumor metastasis⁸⁰⁷. Although most studies aiming to evaluate the role of the microbiome in the efficacy of immunotherapy have been focused on the gut microbiota and not the local microbiome present in the microenvironment, preliminary results demonstrated that in various types of cancer, treatment of patients by antibiotics early before, during or shortly after administration of ICB negatively affect their efficiency, notably in lung cancer, therefore highlighting the importance of the microbiome in anti-cancer therapeutics effectiveness⁸⁰⁸. That result was recently confirmed by Derosa et al., who demonstrated that NSCLC patients previously treated with antibiotics have a worse outcome than NSCLC patients non-treated with antibiotics but both treated with ICB⁸⁰⁹. Interestingly it has been shown that the microbiome impacts cisplatin efficiency in a lung cancer model by promoting CD8 T cells cytotoxic function and impacting cancer cell sensitivity to apoptosis through the modulation of the pro-apoptotic molecule expression, BAX⁸¹⁰.

Although the mechanisms by which the microbiome performs all its effects impacting cancer progression and treatment efficiency are already unknown, TLR-induced autophagy could be another modulation way, whose importance should be investigated in the future.

8 Conclusion of my thesis works

During their life cycle, cells are subjected to various stresses that they must cope with to maintain their good functioning or survival. Autophagy is one of the main protective mechanisms developed by the cell along evolution. Since autophagy is a biological process in all cells, many studies have demonstrated that cancer cells depend on autophagy, which exerts pro-tumoral effects by different means, promoting survival, proliferation, resistance to therapies, metastasis development, and immune evasion. Therefore, it is not surprising that more and more studies are demonstrating that autophagy level is associated with a worse survival for cancer patients in various cancer types, and in the meantime, an increasing number of preclinical and clinical studies aim to target autophagy are ongoing. Despite these observations, very few data are available concerning mechanisms involved in autophagy regulation and the importance of these processes in lung progression. Therefore, my Ph.D. project had two objectives: the first one consisted in determining the molecular pathways by which, in a physiological context, the autophagy level in lung tumor cells can be modulated; the second was to assess the importance of the autophagy level in lung tumor cells on tumor progression by evaluating its possible involvement in various pro-tumoral mechanisms.

By bioinformatics analysis of public transcriptomic data on adenocarcinoma lung tumors and comparing autophagy gene expression between tumors and adjacent tissues, we discovered a new autophagy gene signature mainly expressed by tumor cells. This signature is associated with the expression of genes related to cell proliferation and immune checkpoint inhibitors and is inversely associated with genes related to immune infiltration. That study demonstrated a link between autophagy level in adenocarcinoma lung tumor cells and tumor cell proliferation and brought new evidence of a relationship between the autophagy level in malignant cells and their immune phenotype.

Furthermore, using a similar approach, we shed light that among the protein-encoding genes whose expression correlates the most with autophagy genes, the TLR7 (toll-like receptor) was one of the top, suggesting a potential link between autophagy and TLR7. That link was confirmed by *in vitro* and *ex vivo* experiments using NSCLC tissue slides and fresh tumors, where we demonstrated a new physiological way of autophagy induction in lung tumor patients. We observed that physiological TLR7 ligands are present in the tumor microenvironment and are responsible for a complete autophagy induction in lung tumor cells. Using different retrospective cohorts of lung cancer

patients and immunohistochemistry staining of autophagy, we then demonstrated that this new way of autophagy induction in lung tumor cells was associated with poorer patient survival and a poorer response to platinum-salt based chemotherapies. Interestingly, we also observed that autophagy induction by TLR7 increased the expression of the immune checkpoint PD-L1 in lung tumor cells. Importantly, using a retrospective cohort of NSCLC patients treated by immunotherapy (the nivolumab – an anti-PD1), we showed that the autophagy level of tumor cells favors patients' responsiveness to this immune checkpoint blockade, revealing a new link between autophagy and immunotherapies responses in lung tumors. This receptor, which recognizes single-stranded RNAs and can therefore recognize both endogenous and viral RNAs, opens up a new area of research, which consists of investigating the impact of infections by pathogens, as well as the release of single-stranded RNAs under pathophysiological conditions, on tumor progression. This further research could then be used to improve indications for cancer patients, including the recommendation of vaccinations to prevent the aggravation of tumor progression by certain pathogens.

The work performed during my Ph.D. allowed to understand more precisely the mechanisms involved in the autophagy modulation in lung tumor cells, as well as its consequences on tumor progression and anti-tumor therapeutics responsiveness. These results open a new area of research regarding the impact of autophagy in this disease and encourage to use autophagy as a predictive biomarker of tumor progression and response to therapies in NSCLC patients.

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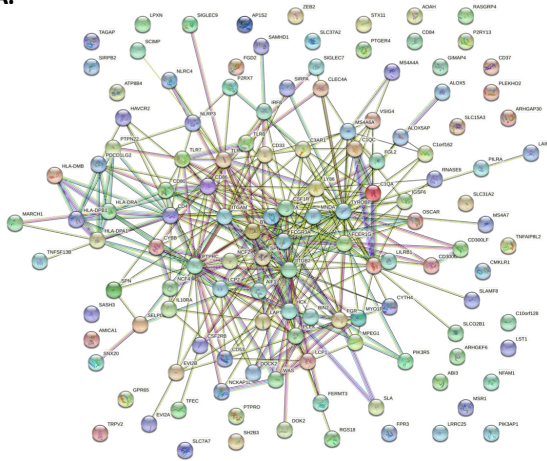
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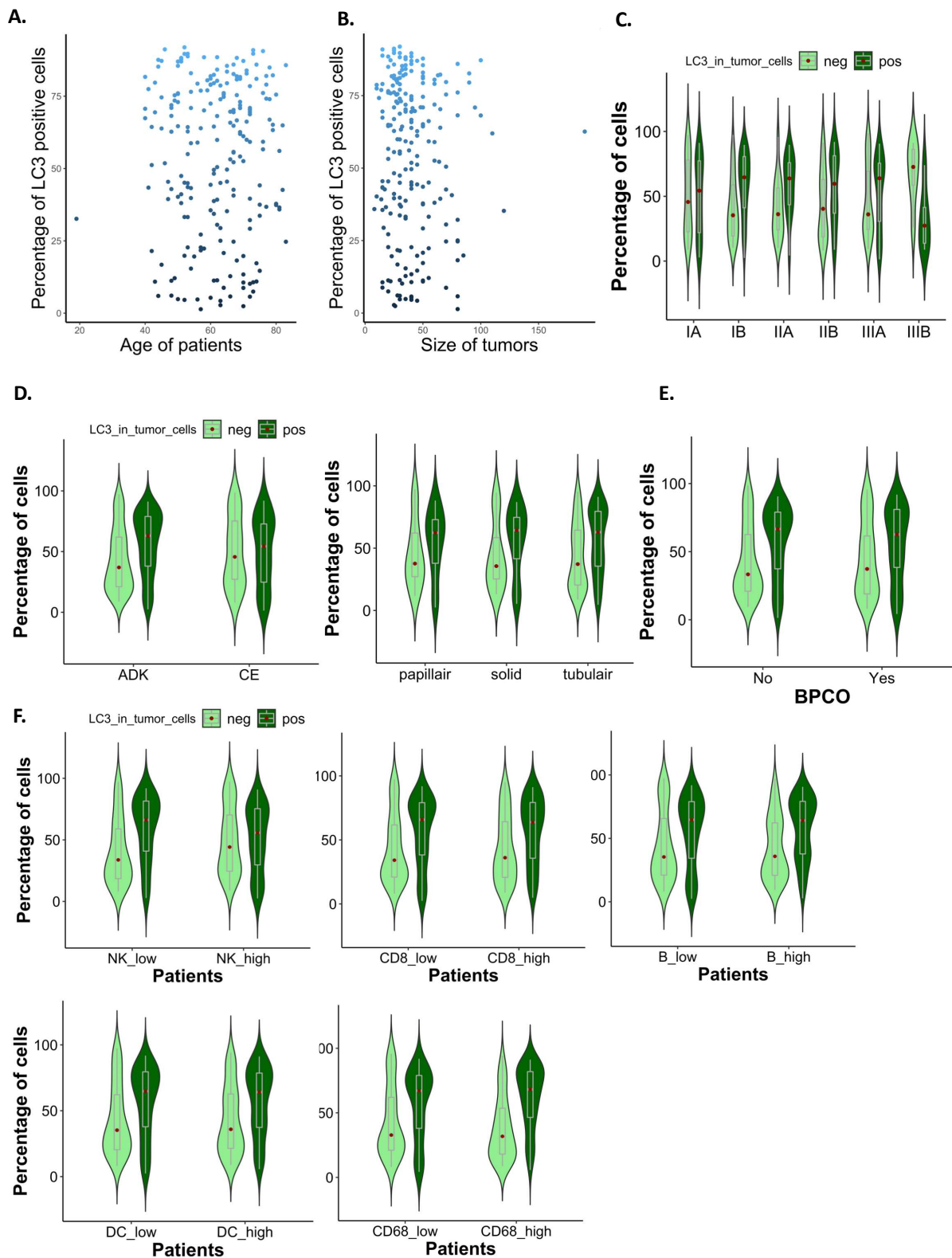
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Annex 1 – Supplemental figures for study 2

A.

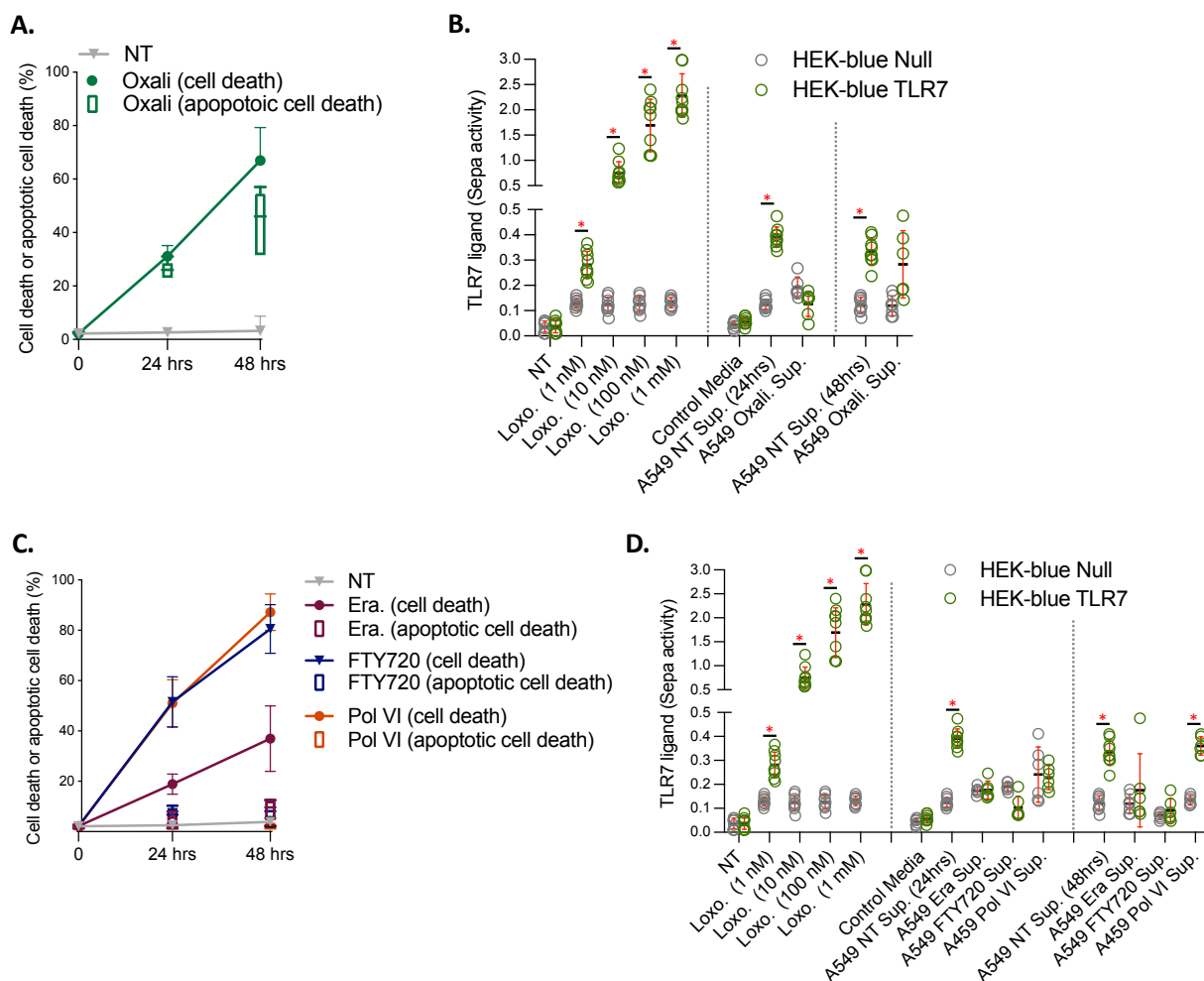


Supplementary Figure S1. Analysis of the functional protein association networks by STRING software. Association network of the genes involved in the blue module from WGCNA correlation analysis in TCGA-LUAD dataset with STRING.



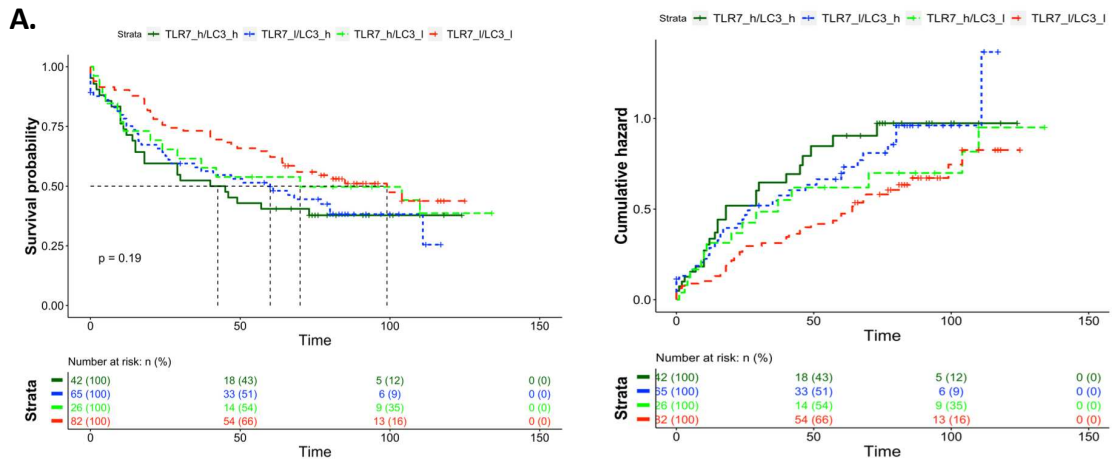
Supplementary Figure S2. Study of correlation between autophagy level within NSCLC tumor cells and clinical data

A, B. Evaluation of the percentage of lung tumor cells positive for LC3 in function of the age of the NSCLC patient (A) and the size of the tumor (B). **C-F.** Evaluation of the proportion of lung tumor cells positive or negative for LC3 in function of the stage of cancer (C), the phenotype of the tumor (D), the BPCO status (E) or, the immune cells infiltration (F). For immune infiltration, high versus low patients was defined as the median of density for each immune cell.



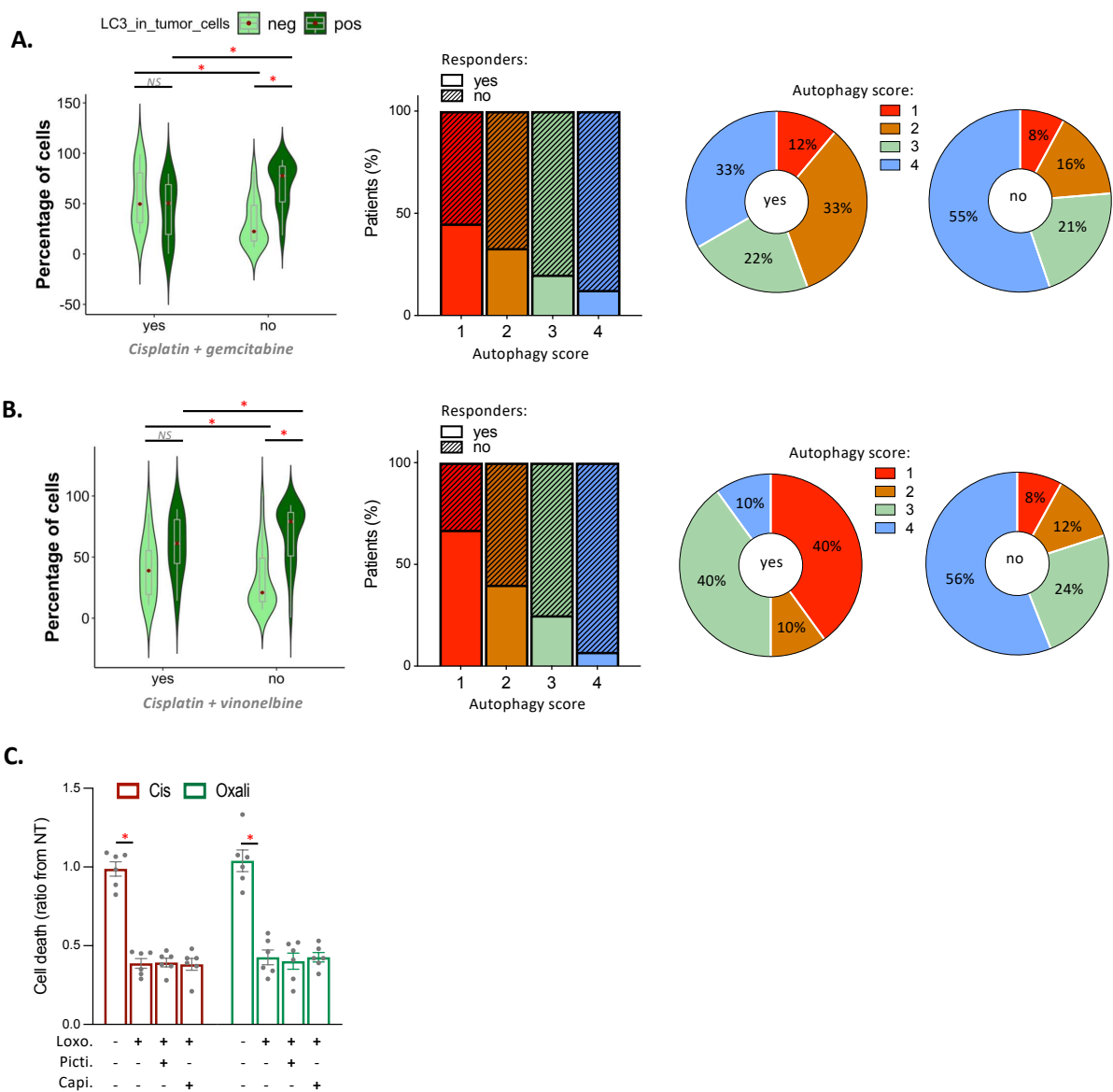
Supplementary Figure S3. Evaluation of the impact of apoptotic versus non-apoptotic cell death in the release of TLR7 physiological ligands in the tumor microenvironment

A. Graph representing the mortality rate (Live dead +) as well as the apoptotic rate (Annexin V+/ PI-) of A549 treated cells or not (NT) with oxaliplatin (Oxali., 200 μ M) for 24h and 48h. The experiment was performed three times in triplicates. **B.** Measurement of the abundance of TLR7 ligands through the measurement of the Sepa activity of HEK-blue TLR7 cells versus HEK-blue Null cells following the treatment or not (NT) with different doses of TLR7 agonists (Loxo., 1nM, 10nM, 100nM, and 1mM), or after the incubation with a culture medium alone (Control media), or with supernatant from A549 in culture for 24h (A549 NT Sup. (24hrs)) or 48h (A549 NT Sup. (48hrs)) or with supernatant from A549 treated with oxaliplatin (Oxali., 200 μ M) (A549 Oxali Sup.) for 24h and 48h. The experiment was performed three times in triplicates. **C.** Graph representing the percentage of dead (Live dead +) and apoptotic (Annexin V+/ PI-) lung tumor cells following treatment with the different cell death inducers: ferroptosis (erastin, era., 15 μ M), necroptosis (FTY720, 10 μ M) and pyroptosis (polyphillin VI, pol VI., 8 μ M) at 24 and 48h post-treatment. The experiment was performed three times in triplicates. **D.** Measurement of the abundance of TLR7 ligands through the measurement of the Sepa activity of HEK-TLR7 cells versus HEK-Null cells following the treatment or not (NT) with different doses of TLR7 agonists (Loxo., 1nM, 10nM, 100nM, and 1mM), or after the incubation with a culture medium alone (Control media), or with supernatant from A549 in culture for 24h (A549 NT Sup. (24hrs)) or 48h (A549 NT Sup. (48hrs)) or with supernatant from A549 treated with the different cell death inducers for 24h and 48h. The experiment was performed three times in triplicates. Student's t-test, *: p < 0.05.



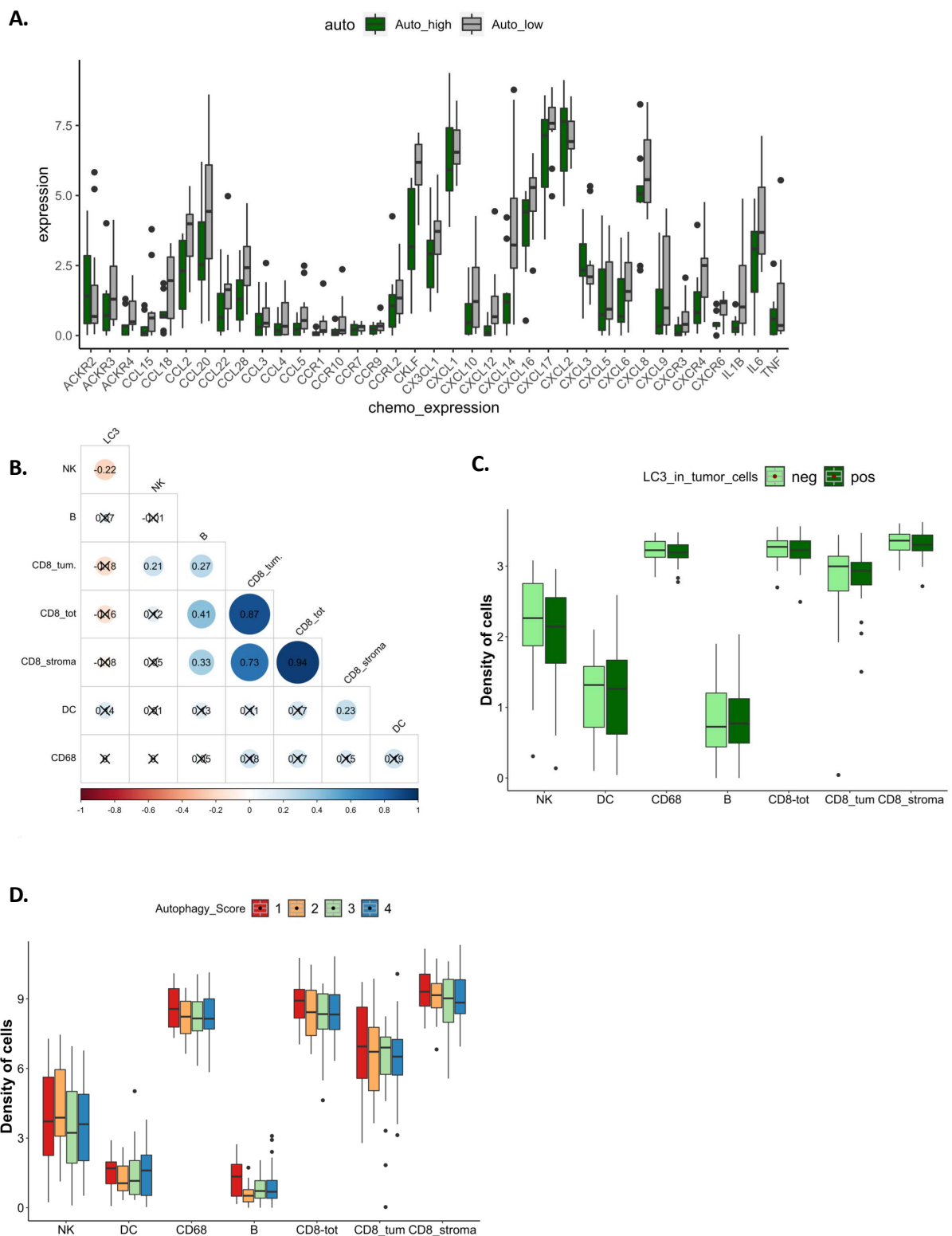
Supplementary Figure S4. Impact of TLR7 and LC3 expressions on NSCLC overall survival.

On the left : Kaplan Meier representing the survival probability of NSCLC patients over time based on the expression levels of TLR7 and LC3 on their lung tumor cells. Patients survival were compared between NSCLC patients composed mainly by tumor cells highly expressing TLR7 and LC3 (TLR7 high_LC3 high) and NSCLC patients composed mainly by tumor cells weakly expressing TLR7 and LC3 (TLR7 low_LC3 low). At the right : Kaplan Meier describing the cumulative hazard over time based on the expression levels of TLR7 and LC3 on their lung tumor cells.



Supplementary Figure S5. Impact of autophagy level in lung tumor cells on the resistance to neoadjuvant chemotherapies combinations in NSCLC patients.

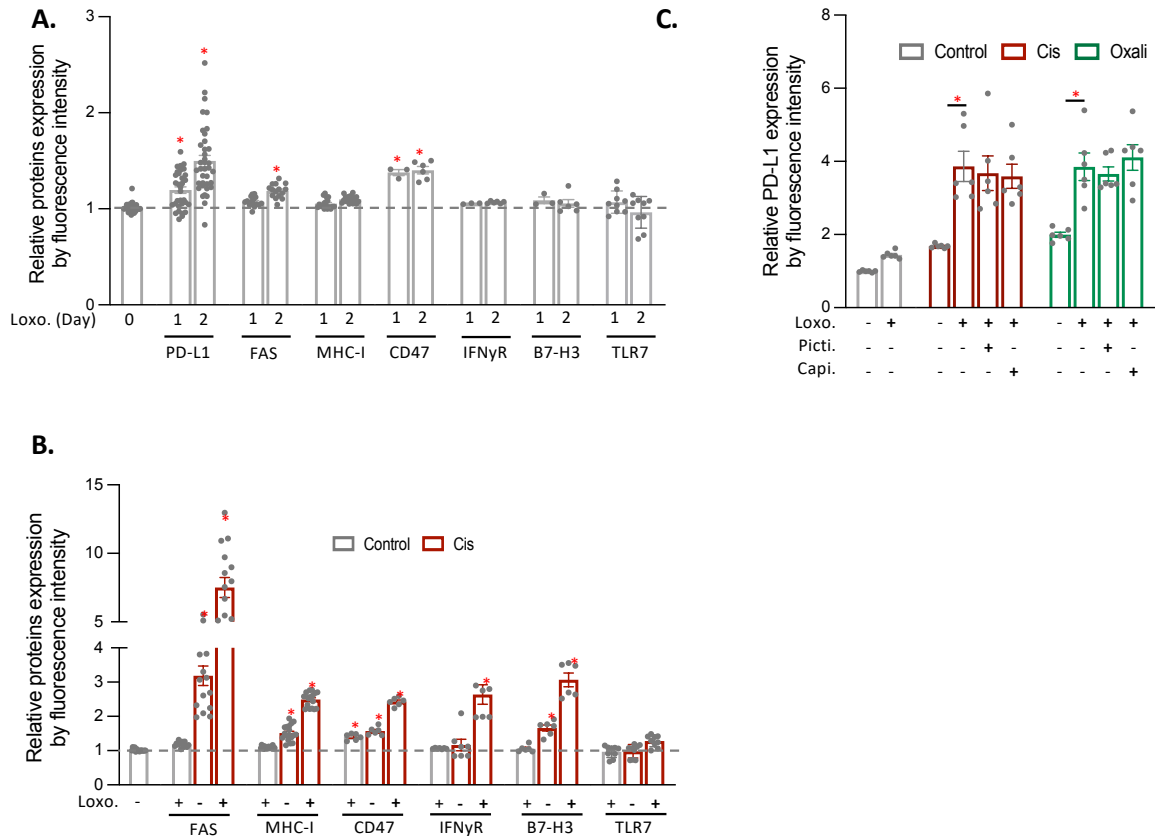
A. At the left: Graph representing the percentage of LC3 positive versus LC3 negative lung tumor cells in NSCLC patients treated with neoadjuvant chemotherapies in function of their responsiveness to the combination of cisplatin plus gemcitabine. In the middle: Distribution of the proportion of NSCLC patients who respond (yes) versus do not respond to the chemotherapies combination (no) in function of their autophagy score. On the right: Proportion of the different autophagy scores of NSCLC patients between responders (yes) versus non-responders to the chemotherapies combination (no). **B.** At the left: Graph representing the percentage of LC3 positive versus LC3 negative lung tumor cells in NSCLC patients treated with neoadjuvant chemotherapies in function of their responsiveness to the combination of cisplatin plus vinorelbine. In the middle: Distribution of the proportion of NSCLC patients who respond (yes) versus do not respond to the chemotherapies combination (no) in function of their autophagy score. On the right: Proportion of the different autophagy scores of NSCLC patients between responders (yes) versus non-responders to the chemotherapies combination (no). **C.** Graph showing the mortality ratio of the different combination treatments from that of chemotherapy alone for each chemotherapy at 48 hours post-treatment. In addition to treatment with cisplatin (Cis, 50 μ M), or oxaliplatin (200 μ M), cells were treated or not with a synthetic TLR7 agonist (Loxo., 1mM) alone or with either an inhibitor of a pan AKT inhibitors (Capivasertib, Capi., 8nM), or a pan type 1 PI3K inhibitor (Pictilisib, picti., 3nM), both targeting the type 1 PI3K. The experiment was performed at least three times in triplicates. Student's t-test, * : $p < 0.05$.



Supplementary Figure S6. Effect of autophagy level in lung tumor cells on the infiltration of immune cells in NSCLC patients.

A. Study of correlation between genes expression coding for cytokines, chemokines, and their receptors and the autophagy level of tumor cells composing the NSCLC (based on the autophagy gene expression signature B). **B.** Study of correlation between the LC3 expression in NSCLC tumor cells and the infiltration within the tumor of the main immune cell types (NK, CD8 T cells, B cells, DC, and macrophages (CD68)) using our cohort 1. From the more correlated in blue to the more inversely correlated in red. Cross means no significant correlation **C.**

Measurement of the density of each immune cell type infiltrated in function of whether the NSCLC patients are LC3 low versus LC3 high. **D.** Measurement of the density of each immune cell type infiltrated in function of the autophagy score of the NSCLC patients.



Supplementary Figure S7. Effect of TLR7 synthetic agonist alone or in combination with cisplatin on the expression of molecules impacting the immune phenotype of the lung tumor cells.

A. Graph describing the ratio of relative proteins expression by fluorescence intensity measured by flow cytometry of various molecules in TLR7 synthetic agonist-treated A549 cells (Loxo., 1mM) from the value observed in non-treated cells, for 24h and 48h. The experiment was performed at least three times in triplicates. **B.** Graph representing the ratio of relative proteins expression by fluorescence intensity measured by flow cytometry of various molecules in TLR7 synthetic agonist-treated A549 cells alone (Loxo. 1mM), or in combination with cisplatin (Cis., 50 μ M) (Loxo + cis), from the value observed in non-treated cells, at 48h post-treatment. The experiment was performed at least three times in triplicates. **C.** Graph representing the ratio of the relative PD-L1 expression by fluorescence intensity measured by flow cytometry of the different combination treatments from that of cisplatin alone at 48 hours post-treatment. In addition to treatment with cisplatin (Cis, 50 μ M), cells were treated or not with a synthetic TLR7 agonist (Loxo., 1mM) alone or in combination with either an inhibitor of a pan AKT inhibitors (Capivasertib, Capi., 8nM), or a pan type 1 PI3K inhibitor (Pictilisib, picti., 3nM), both targeting the type 1 PI3K. The experiment was performed at least three times in triplicates. Student's t-test, * : $p < 0.05$.

Annex 2 – Revues



Autophagy Modulation by Viral Infections Influences Tumor Development

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Autophagy is a self-degradative process important for balancing cellular homeostasis at critical times in development and/or in response to nutrient stress. This is particularly relevant in tumor model in which autophagy has been demonstrated to have an important impact on tumor behavior. In one hand, autophagy limits tumor transformation of precancerous cells in early stage, and in the other hand, it favors the survival, proliferation, metastasis, and resistance to antitumor therapies in more advanced tumors. This catabolic machinery can be induced by an important variety of extra- and intracellular stimuli. For instance, viral infection has often been associated to autophagic modulation, and the role of autophagy in virus replication differs according to the virus studied. In the context of tumor development, virus-modulated autophagy can have an important impact on tumor cells' fate. Extensive analyses have shed light on the molecular and/or functional complex mechanisms by which virus-modulated autophagy influences precancerous or tumor cell development. This review includes an overview of discoveries describing the repercussions of an autophagy perturbation during viral infections on tumor behavior.

Keywords: tumor development and progression, autophagy, viral infection, oncolytic virus, oncogenic virus, immunity, tumor resistance, tumorigenesis

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AUTOPHAGY

Autophagy is a general term defining a catabolic mechanism present in all eukaryotic cells that leads to three different intracellular routes to the degradation of substrates present in the cell by the lysosome. It includes chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. CMA is the way by which cells degrade intracellular proteins by their translocation to the lysosome through the interaction of chaperone recognizing universal motif in the sequence of CMA substrate (KFERQ motif) and the lysosome protein LAMP-2A (1). Microautophagy involves a direct engulfment of cargos through the invagination of the lysosome membrane or through the direct entry of cytoplasmic materials to the multivesicular bodies of late endosomes (1). Macroautophagy, referred hereafter as autophagy, is a multistep process consisting of the formation of phagophore that elongates, engulfs targeted proteins or organelles in a double-membrane vesicle called autophagosome, and finally fuses with lysosome (**Figure 1**) (2). This process is orchestrated by more than 30 autophagic proteins (Atg), organized in complex.

Autophagic induction is modulated by two protein complexes: the ULK1/2 (unc51-like autophagy activating kinase) and the Beclin-1/PI3KC3 (class III phosphatidylinositol 3-kinase) complexes. Once activated, these complexes recruit other proteins involved in elongation and formation of autophagosomes, including the two conjugated systems comprising Atg12-Atg5-Atg16L and LC3. After completion, the mature autophagosome fuses with lysosome to form autolysosome, wherein the sequestered materials and organelles are degraded by lysosomal enzymes (**Figure 1**). Even if autophagy has often been considered as a non-selective mechanism, many studies highlighted an important role for autophagy in selective materials and/or organelles recycling, including mitophagy, which selectively targets damaged mitochondria to autophagosome, or xenophagy, which permits the selective degradation of pathogens and/or pathogens' elements through autophagy (2).

Since its discovery by Christian Deduve in 1963, our knowledges on autophagy and its role in physiology has greatly

been increased. Autophagy acts as a quality control mechanism by degrading and recycling damaged or old proteins and organelles. Given its important role in homeostasis, it is not surprising that a defect of autophagy has been associated with various pathologies, including neurodegenerative diseases, infection susceptibility, aging, metabolic disorders, and cancer. In cancer, autophagy seems acting as a double-edged sword: in one hand, autophagy limits the tumorigenesis of precancerous cells, and in another hand, it serves as an important survival mechanism for established tumors. The role of autophagy in cancer will be detailed in the different parts of this review and is illustrated in **Figure 2**.

AUTOPHAGY AND VIRUSES

As an intracellular parasite, a virus's behavior is closely linked to its capacity to prevent and/or subvert cellular antiviral responses. Given that viral infection and replication cause cellular stresses,

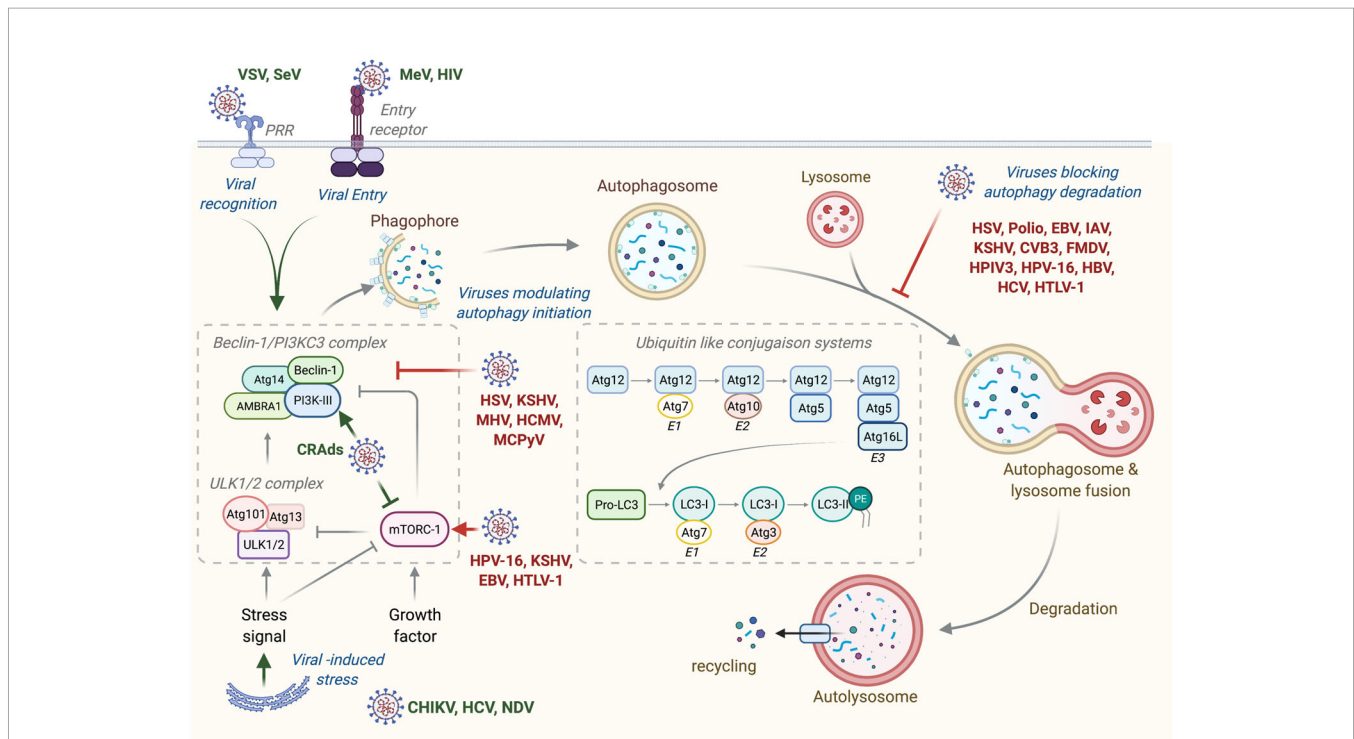


FIGURE 1 | Autophagy machinery and its modulation by viruses. Beclin-1/PI3KC3 complex activation, which is regulated by different mechanisms including the ULK1/2 complex and mTORC1 complex, results in the induction of an autophagic vesicle, which is characterized by a double-membrane, named autophagosome. Two ubiquitin-like systems are essential for autophagosome formation. In the first, autophagy-related gene-12 (Atg12) is conjugated to Atg5, together forming a complex with Atg16L1, which decorates the outer membrane of the phagophore. Microtubule-associated protein 1 light chain-3 (LC3, also known as Atg8) constitutes the second ubiquitin-like system and conjugates phosphatidylethanolamine (PE) at the outer and inner autophagosomal membrane. Unlike the Atg12/Atg5/Atg16L1 complex that is recycled by the protease Atg4, the LC3-PE (referred to LC3-II) remains associated with the inner membrane of autophagosome. The incorporation of phospholipid into the autophagosome membrane is essential for its elongation and regulates the membrane transport system. Autophagosome maturation is characterized by the formation of an autolysosome, the product of fusion with the lysosome. Viruses activate or inhibit autophagy at several steps, as indicated on the figure. VSV, Vesicular Stomatitis Virus; SeV, Sendai Virus; MeV, Measles Virus; HIV, Human Immunodeficiency Virus; CRAds, Conditionally Replicating Adenoviruses; HSV, Herpes Simplex Virus; KSHV, Kaposi's Sarcoma-associated Herpesvirus; MHV, Mouse Hepatitis Virus; HCMV, Human Cytomegalovirus; MCPyV, Merkel Cell Polyomavirus; HPV-16, Human Papillomavirus 16; EBV, Epstein-Barr Virus; HTLV-1, Human T cell Leukemia/Lymphoma Virus type 1; CHIKV, Chikungunya Virus; HCV, Hepatitis C Virus; NDV, Newcastle Disease Virus; Polio, Poliovirus; IAV, Influenza A Virus; CVB3, Coxsackievirus B3; FMDV, Foot and Mouth Disease Virus; HPIV3, Human Parainfluenza Virus 3; HBV, Hepatitis B Virus.

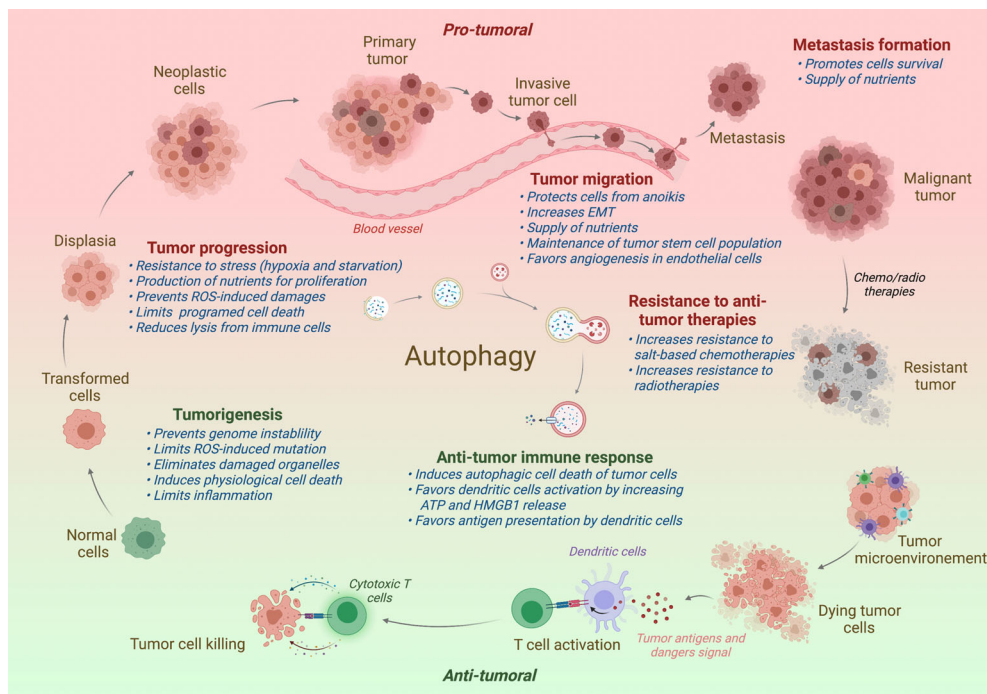


FIGURE 2 | Impact of autophagy on tumor progression. In normal cells, autophagy restricts the tumorigenesis by limiting genome instability and strong inflammation. However, when the tumor is established, autophagy has been shown to promote tumor development, including the tumor progression, migration, and the formation of metastasis. Autophagy has also been associated to a better resistance to antitumor radio- or chemotherapies. In contrast to support tumor, autophagy favors the antitumor immune responses, promoting the immunogenic cell death and the presentation of tumor antigens to the adaptive immune cells. Protumor effects of autophagy are written in red and antitumor effects in green. Effect of autophagy (blue) is indicated at different steps of tumor development. ROS, Radical oxygen species; EMT, Epithelial-Mesenchymal Transition.

autophagy is frequently associated with a by-product of infection. However, viral infection-induced autophagy is not a passive process, and an increasing number of studies have shown that autophagy has both antiviral and proviral capacities for the replication of a broad range of viruses (3).

Viral Infections Modulate Autophagy

Any single step of viral life cycle, including virus binding, entry and recognition, membrane fusion, exposure of viral component, and replication or even antiviral responses, may modulate autophagy (Figure 1) (4–6).

The first evidence demonstrating that virions binding could induce autophagy has been observed following measles virus (MeV) infection (7). CD46, a complement family receptor, serves as a binding and entry receptor for vaccinal or attenuated strains of MeV (8, 9). The team of Mathias Faure demonstrated that MeV/CD46 binding leads to an induction of autophagy through a direct interaction of CD46 with the Beclin-1/PI3KC3 using a scaffold protein named Golgi associated PDZ and coiled coil motif-containing (GOPC) (7). Further investigations showed that MeV also activates autophagy by targeting an autophagy-associated protein called immunity-associated GTPase family M (IRGM) (10, 11). Human immunodeficiency virus type 1 (HIV-1) can also activate autophagy in uninfected CD4 T cells through the fusogenic activity of envelope glycoproteins gp120 and gp41

(called Env) (12). While specific pathway has not been really elucidated, it is important to note that the signaling activity of CD4 and CXCR4 are not associated with autophagy induction in infected cells, in contrast to the fusogenic activity of Env proteins (12).

In addition to binding and membrane fusion, a broad range of studies have demonstrated an induction of autophagy following the virus recognition. The delivery of viral elements into the cytosol can lead to the induction of autophagy through the activation of pattern recognition receptor (PRR). Among PRR, intracellular endosomal Toll-like receptors (TLRs) are important sensors of viral components, including single-stranded (ss)RNA (e.g., TLR7 and TLR8), double-stranded (ds)RNA (e.g., TLR3), and DNA with CpG site (e.g., TLR9). The majority of TLRs recruit the adaptor myeloid differentiation primary response protein MyD88, whereas TLR3 and TLR4 recruit the TIR domain-containing adaptor molecule 1 (TRIF). In all cases, TLR stimulation leads to the activation of nuclear factor- κ B (NF- κ B) for the production of inflammatory cytokines and interferon (IFN) production (13). TLR stimulation also activates autophagy, presumably by promoting the binding of both MyD88 or TRIF to Beclin-1 (14). The role of TLR-induced autophagy in virus remains to be determined, but several studies have shown an important impact of this process on antiviral immune responses (4). For instance, plasmacytoid dendritic cells

(pDCs) deficient for Atg5 exhibited a reduced TLR-dependent production of IFNs after infection with vesicular stomatitis virus (VSV), sendai virus (SeV), or herpes virus type 1 (HSV-1) (15). The RIG-I like receptors (RLRs) retinoic acid-inducible gene I (RIG-I), which senses dsRNA in the cytosol, also induces autophagy following sendai virus infection (16). RIG-I-mediated autophagy is regulated by the translocation of beclin-1 into the mitochondria dependently of the mitochondrial antiviral signaling protein (MAVS) and tumor necrosis factor receptor-associated factor (TRAF) 6 (16). Protein Kinase R (PKR), which recognizes dsRNA, also triggers autophagy by the phosphorylation of the eukaryotic translation initiation factor (eIF) 2 (17). During HSV-1 infection, the cytosolic DNA sensor cyclic GMP_AMP (cGAMP) synthase (cGAS), which recognizes dsDNA, triggers autophagy through the activation of the initiation complex, releasing Rubicon (an autophagy inhibitor) from the beclin-1/PI3KC3 complex (18, 19).

Viral replication causes drastic modifications to cell homeostasis, which often enhance autophagy. As a good example, chikungunya virus (CHIKV) replication induces autophagy through the synergy of both endoplasmic reticulum (ER) and oxidative stresses (8). CHIKV proteins accumulation promotes ER stress and unfold protein responses (UPR) *via* the activation of inositol-requiring Ser/Thr protein kinase/endonuclease (IRE) 1a. CHIKV also increases the production of reactive oxygen species (ROS) and reactive nitrogen species (NOS), which stimulates autophagy by activating AMP-activated protein kinase (AMPK)-mediated inhibition of mammalian target of rapamycin complex 1 (mTORC1) (20). Similar observation has been reported during HCV infection, whose replication enhances ER and UPR responses that lead to autophagy induction (21).

Autophagy During Viral Infection: A Double-Edged Sword

Due to its role in cytosolic materials clearance, autophagy can degrade viral components, including viral particles or proteins or even host factors required for the replication of the virus. Indeed, autophagy is considered as an important part of innate antiviral responses, especially in non-immune cells. However, this virus-specific autophagic degradation, also known as virophagy, can be subverted by many viruses.

The first example of virophagy has been observed during Sindbis virus (SINV) infection, in which Beclin-1 and Atg5 protect against viral infection and finally against SINV-mediated encephalitis (22). Mechanistically, SAMD-specific E3 ubiquitin protein ligase 1 (SMU1) and Fanconi anemia group C protein (FANCC) are both required for the delivery of SINV capsid protein into the autophagosome, through the interaction of the autophagy receptor p62 with SINV proteins (23, 24). SMU1 and FANCC are also involved in the targeting of herpes simplex virus -1 (HSV-1) proteins for autophagic degradation (24). During poliovirus infection, galectin 8 restricts viral infection through the initiation of autophagic degradation of the viral RNA genome (25). Autophagic degradation of the viral non-structural protein A5 (NS5A) of hepatitis C virus (HCV)

has been observed in infected cell through the interaction of NS5A with an ER transmembrane protein, SHISA5 (26). Human immunodeficiency virus 1 (HIV-1) replication can also be perturbed by autophagic degradation. Histone deacetylase 6 (HDAC6), in complex with the HIV restriction factor APOBEC3G, is responsible for the degradation of the virion infectivity factor Vif by autophagy (27). In addition to eliminate Vif factor, autophagy also selectively degrades both the transactivator Tat, involved in viral transcription, and the restriction factor tripartite motif-containing protein 5 α (TRIM5 α), respectively, in HIV-infected CD4⁺ T cells and Langerhans cells (28, 29).

Autophagy can also influence the immune antiviral responses. In infected plasmacytoid dendritic (pDC) cells, autophagy favors the transport of viral genome of vesicular stomatitis virus (VSV) from the cytosol to TLR7-containing endosome, enhancing the type I interferon (IFN) production (15). Similarly, in mouse models of murine norovirus (MNV) infection, production of IFN γ -mediated antiviral responses require the autophagy elongation complex Atg12/Atg5/Atg16L1 (30). Importantly, the antiviral adaptive immune response can also be modulated by autophagy, mainly by promoting viral antigen presentation on class II major histocompatibility complex (MHC). That mechanism has been notably observed for Epstein-Barr virus (EBV), SINV, or HSV (4, 31).

While autophagy can restrict viral infection, some persisting viruses have developed several strategies to escape from the autophagic degradation, or even manipulate autophagy for their own benefit. The inhibition of autophagy induction has been noticed for a broad range of viral infections mainly by targeting Beclin-1. Indeed, HSV-encoded neurovirulence factor ICP34.5, viral homologues of Bcl-2, ORF16, or M11—respectively expressed by Kaposi's sarcoma associated herpesvirus (KSHV) and murine γ -herpes virus (MHV) 68—and human cytomegalovirus (HCMV)-encoded TRS1 and IRS1 are all viral proteins capable to bind Beclin-1 and inhibit autophagy (32–35). mTOR pathway, an important negative regulator of autophagy initiation complex, can also be targeted by viruses to inhibit autophagy. A good example is the KSHV G protein-coupled receptor (vGPCR), which stimulates mTOR pathway and therefore inhibits autophagy in cells during KSHV infection (36).

In addition to target the initiation step of autophagy, some viral proteins also prevent the fusion of autophagosomes with lysosomes. HSV-1 encodes the TANK-binding kinase (TBK1) protein to prevent autophagic degradation, by regulating the autophagosome maturation through the phosphorylation of the autophagy receptors p62 and optineurin (37). Similarly, the K7 protein of KSHV promotes rubicon/beclin-1 interaction and inhibits the activity of PI3KC3, which leads to the blocking of the fusion of autophagosomes with lysosomes (38). In most of the cases, viruses with the ability to block autophagy maturation step can use autophagic vacuoles as support membranes for their own replication. The first example of virus that has been described to manipulate autophagy to enhance its replication is

the poliovirus, for which autophagy inhibition decreases infection efficiency, whereas the autophagy inducer rapamycin increases it (39). Other picornaviruses, such as coxsackievirus B3 (CVB3) and foot-and-mouth disease virus (FMDV), also use autophagy to replicate (40, 41). HCV induces the formation of autophagosomes but blocks its fusion with the lysosome, which favors viral replication and virion production (42). Like K7 protein of KHSV, HCV-encoded NS4B induces rubicon expression, leading to the inhibition of autophagy maturation (43). Influenza virus (IAV) and human parainfluenza virus type 3 (HPIV3) can also trigger the accumulation of autophagosomes for viral replication, respectively, *via* the interaction of the viral protein M2 or M with LC3 (44, 45).

Indeed, manipulation of autophagy by many viruses confers double advantages for viruses: (i) it permits to avoid their degradation by inhibiting the autophagy influx, and (ii) it supplies to the viruses a double-membrane vacuole that could serve as a support for their replication.

ONCOGENIC VIRUSES MODULATE AUTOPHAGY TO FAVOR TUMORIGENESIS

Oncogenic Viruses and Tumorigenesis

Cancer is a multifactorial disease, combining genetic predispositions and environmental factors. Among all the elements promoting the cancer development, viruses can be directly linked with the formation of tumor. It is now well admitted that 15% of human cancers are caused by viruses (46). So far, only seven viruses have been demonstrated to have an oncogenic activity: Epstein-Barr virus (EBV), hepatitis B and C viruses (HBV and HCV), human papilloma virus (HPV), human T lymphotropic virus type 1 (HTLV-1), Kaposi sarcoma-associated herpes virus (KSHV), and Merkel cell polyomavirus (MCPyV). Despite being very different in terms of tropism or viral structure and cycle, they share one characteristic: they are all responsible for persistent infections, criteria that seem to be essential to lead to virus-mediated oncogenesis. In order to persist and proliferate, viruses have to maintain their genome inside infected cell, prevent apoptosis of host cell while favoring its replication and escaping from the recognition of antiviral immune cells (47). To do that, oncogenic viruses encode proteins, called oncoproteins, that target and inactivate tumor suppressor proteins, such as retinoblastoma protein (pRb) or tumor protein 53 (p53) (48–52). p53 is a key transcription factor activated under stress responses, and it is responsible for cell cycle arrest or apoptosis induction. Similarly, pRb blocks cell cycle progression in G1 phase under stress conditions. HBV-encoded hepatitis B virus X (HBx), HTLV-1-encoded latency-associated nuclear antigen (LANA), and MCPyV-encoded large T antigen are all viral proteins that target and inhibit both p53 and pRb (52–55). Oncogenic viruses can also express proteins that target specifically p53 [such as E6 protein of HPV, latent membrane protein 1 (LMP1) of EBV, or NS5A protein of HCV] or pRb [including

core protein of HCV, Epstein Barr virus latent 3C (EBNA3C) of EBV or E7 protein of HPV] (56, 57). Dysregulation of several cell cycle components, such as cyclins and cyclin dependent kinases (CDK), are also observed following oncogenic virus infection to bypass cell cycle checkpoints and pursue an infinite proliferation of infected cell. For example, E6 and E7 proteins of HPV have inhibitory activities on the CDK inhibitors p21 or p27 (58–60). Direct integration of viral genomic materials into the host genome may also modify expression of tumorigenesis-leading genes (61). Other studies demonstrated that oncogenic viruses have also developed strategy to escape from antiviral immune responses, inhibiting type I IFN response or cytotoxic activities of CD8⁺ T cells and natural killer (NK) cells (62–75).

Oncogenic Viruses and Autophagy Inhibition

In precancerous cells, autophagy limits the formation of ROS, damages of DNA, and inflammation with the consequence of limiting tumorigenesis. This observation has been established in a lot of studies using different cancer models, where a decrease of autophagy activity has been observed in precancerous cells compared with non-tumor cells. Depletion of key autophagy genes has been associated with an increase of tumorigenesis in several tissues, leading to classify some autophagy genes as tumor suppressor genes. The first autophagy gene described to be involved in tumor formation is Beclin-1, for which a monoallelic depletion has been observed in various cancers (e.g., breast, ovarian, prostate, hepatocarcinoma, or lymphoma). Depletion of UVRAG in breast, colon, gastric, and prostate cancers also increases tumorigenesis (76–79). Similarly, knockout of both conjugated systems (Atg5/Atg7 or Atg3) favors tumorigenesis in liver tumor murine model, due to the accumulation of ROS in cells (80).

An increased range of studies highlighted that oncogenic viruses are able to modulate autophagy to persist in infected cells and/or favor the virus-induced tumorigenesis [extensively reviewed elsewhere (81–83)]. For instance, HPV inhibits autophagy during several steps of viral cycle. Cellular entry of type 16 HPV inhibits autophagy through the activation of autophagy-suppressor PI3K/akt/mTOR pathway, induced by the binding of viral proteins L1 and L2 to heparan sulfate proteoglycans (HSPGs) expressed on cell membrane (84). HPV-encoded E5 protein, by inhibiting p53, alters the transcription level of several autophagy genes involved in the formation of autophagosome (Beclin 1, atg5, atg7...), whereas E6 and E7 viral proteins inhibit the autophagy maturation step, observable by an accumulation of p62 (85, 86). In HPV-infected precancerous tissues and primary human keratinocytes, Mattosio *et al.* have observed an accumulation of the autophagy receptor p62 in the cytosol of infected cells, suggesting an inhibition of autophagy in those cells (86). Similarly, in patients with oropharyngeal and oral cavity squamous cell carcinoma (SSC), HPV-positive tumors exhibit a lower level of LC3B as compared with HPV negative tumors, demonstrating that infection represses autophagy (87). Importantly, HPV-encoded E6 and E7 proteins inhibit

autophagy maturation in anal tumor mice model, and this inhibition is associated with a greater susceptibility to HPV-induced anal carcinogenesis (88). Altogether, those studies strongly suggest that inhibition of autophagy during HPV infection actively participate in normal cells transformation.

HBV and HCV, the causative agents of hepatocellular carcinoma (HCC), have developed mechanisms to inhibit autophagy maturation, leading to the accumulation of autophagosomes inside infected cells and favoring the replication of viruses and the tumorigenesis (89–91). HBV-encoded oncogenic protein HBx prevents autophagosome maturation possibly by repressing the v-ATPase activity, and therefore by impairing the fusion of autophagosome with lysosome (92). This study highlights that HBx-mediated disruption of autophagic degradation may be critical for the development of HBV-associated HCC. Importantly, downregulation of autophagy in HBV-associated HCC patient specimens is inversely correlated with the expression of the microRNA-224 (miR-224), factor that promotes liver tumorigenesis. Moreover, the same study shows that miR-224 is preferentially recruited and degraded by autophagy, suggesting that HBx-mediated autophagy inhibition could promote HBV-associated tumorigenesis through the accumulation of miR-224 (93). While HBx protein has been shown to perturb the autophagic influx, another study observes an induction of HBx-mediated autophagy in HBV infected cells, which promotes the degradation of tumor necrosis factor receptor superfamily member 10B (TNFS10, called also TRAIL) and prevents the TNFS10-mediated antiviral immunity (94). Moreover, HBx-mediated autophagy has also been demonstrated to play an important role in NF κ B-dependent production of pro-inflammatory cytokines, such as interleukin (IL)-6 or IL-8 that are two predictors of HCC progression in patients suffering from chronic hepatitis infection (95, 96). As previously described, HCV-encoded NS4B protein induces rubicon expression, leading to the inhibition of autophagosomes maturation (97, 98). Autophagy inhibition leads to an accumulation of the autophagy receptor p62 in HCV-associated HCC patients. Recent report also observed that an elevated expression of clusterin (CLU) in tumor tissues of HCV-HCC patients increases autophagy genes and upregulated p62 (99). Importantly, upregulation and phosphorylation of p62 activate the transcription factor Nrf2, involved in the metabolic reprogramming that promotes malignancy of HCV-positive HCC (100).

Viral cycles of KSHV and EBV, which belong to the herpesvirus family, are divided into two phases : a latency and a lytic phase (101, 102). Oncogenic properties of these viruses are linked with the latency phase, with a cell proliferation promoted and a cell survival increased. Interestingly, it has been shown that during this phase, autophagy is mainly inhibited. For KSHV infection, autophagy inhibition is led by both the viral FLICE-like proteins FLIP (vFLIP) through the repression of the conjugated system LC3 and LANA viral protein by preventing the downstream autophagy genes expression related to p53 (52, 103, 104). Autophagy is also inhibited during the lytic phase of

KSHV infection. Expression of viral G-protein-coupled receptors (vGPCR) promotes the autophagy repressor pathway PI3K/AKT/mTOR as well as the degradation of the autophagic protein ATG14L, and viral bcl2 (vBcl2) represses Beclin-1 activation (105–107). Moreover, as previously mentioned, K7 protein of KSHV leads to the inhibition of autophagy maturation (108). The autophagy-associated protein p62 can also interfere with the lytic phase of KSHV, as an inhibition of exportin 1 (XPO1) induced retention of p62, which enhanced expression of innate immune-related genes (e.g., IRF7, ISG15, IFIT1, IFIT2, and IFIT3) that leads to a reduction of KSHV lytic replication (109). While further investigations are required to decipher the relation linking autophagy inhibition and oncogenesis in KSHV infected cells, one study showed that KSHV-mediated subversion of autophagy perturbs senescence and facilitates the proliferation of infected cells, two processes involved in cell tumorigenesis (110). Similarly, in EBV-infected cell, latency protein LMP-2 promotes mTOR pathway and inhibits autophagy (111). Recent study also demonstrates that EBV infection blocks the autophagic flux to favor its replication through the activation of two viral Bcl-2 homologue proteins, BHRF1 and BALF1 (112, 113). This impairment of complete autophagy leads to an accumulation of p62 in the nucleus of EBV-transformed lymphoblastic cell lines (LCLs) that promotes oxidative stress and limits the efficiency of DNA damage response, processes that could enhance virus-mediated oncogenesis (114). Recent investigation also suggested that autophagy induction, mediated by the upregulation of CXCR4 in EBV-associated gastric carcinoma (EBVaGC), protects tumor cells from apoptosis and promotes replication of EBV (115). Interestingly, publications converge to show that modulation of autophagy in EBV-infected tumor cells supports the replication of viruses and favors persistent latent infection, two processes that could facilitate tumorigenesis.

HTLV-1 also encodes an oncoprotein, called Tax-1, that modulates autophagy by acting both on autophagy initiation and maturation steps (116). In HTLV-1 transformed T or in Tax-immortalized CD4 memory T cells, Tax-1 facilitates autophagic initiation by activating I κ B kinase complex, which subsequently recruits and activates the autophagy initiation complex Beclin-1/PI3KC3 (117). Tax-1 also blocks the fusion of autophagosomes with lysosomes, leading to an accumulation of autophagic vacuoles in the cytosol that promotes HTLV-1 production (118). It is interesting to note that another study has observed that HTLV-1-encoded basic leucine-zipper factor (HBZ) promotes the activity of mTOR pathway, which could be an additional autophagic suppressor mechanism in infected cells (119). These results indicate a critical role of HTLV-1-deregulated autophagy in promoting survival and transformation of T cells infected by the virus.

MCPyV, the causative agent of Merkel cell carcinoma (MCC), reduces autophagy level in infected cells dependently of the viral oncogenic large antigen (LT-ag) protein. LT-ag favors the expression of miR-30a-3p, miR-30a-5p, and miR-375, which target and repress the level of expression of key autophagy genes (*Beclin-1*, *atg7*, and *p62*) (120). In MCC tumor, low

expression of ATG7 and p62 are correlated with MCPyV-positive tumor, suggesting the importance of autophagy evasion in MCPyV-associated tumorigenesis. Moreover, Torin-1 treatment (an mTORC1 inhibitor) induces cell death of MCC, which can be reduced by autophagy inhibitors, suggesting that MCPyV oncoproteins suppress autophagy to protect cancer cells from cell death (120).

ONCOLYTIC VIRUSES ENHANCE AUTOPHAGY TO INCREASE TUMOR CELL DEATH AND IMMUNOGENICITY

Oncolytic viruses (OVs) have recently emerged as a promising cancer therapeutic approach with great potential for the treatment of a broad spectrum of cancer, especially tumors that have acquired drug resistance to the first-line chemotherapeutics. OVs have been selected or designed to specifically target and kill cancer cells, with low replication capacity in normal tissues (121, 122). The mechanisms of tumor selectivity are multiple, including the expression of viral genome through the cancer-specific gene overexpression involved in the transcriptional element, the specific expression of virus receptor by the cancer cells, the overactivity of metabolic capacity in tumor cells (which is required for virus replication) but also tumor-specific defects of antiviral immunity [extensively reviewed elsewhere (123)]. Several interesting OVs are under investigation in preclinical and clinical studies, including coxsackievirus, adenoviruses (AdV), herpes simplex virus (HSV), Marada virus, Measles virus (MeV), Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), parvovirus, and vaccinia virus (123). Direct lysis of tumor cells along with indirect induction of antitumoral immunity against specific tumor antigens are the two ways by which OVs limit tumor progression.

Oncolytic Viruses and Autophagy-Dependent Cell Death

Paradoxically, although autophagy is well recognized as a cell survival process that promotes tumor development, it can also participate in a caspase-independent form of programmed cell death. Due to the important role of autophagy in cell survival and the close relationship between autophagy and other types of cell death (e.g., apoptosis or necrosis), classification of autophagy as type of programmed cell death has been largely discussed and debated during the last decade [extensively reviewed elsewhere (124)]. However, it is now admitted that autophagy-dependent cell death (ADCDC) is a type of cell death that requires autophagy and/or autophagy components and for which the inhibition of autophagy machinery and/or components, genetically or chemically, prevents cell death (125). ADCDC has been firstly described to mediate physiological cell death *in vivo*, during the developmental program of *D. melanogaster* (126), but autophagy also appears to be involved in the death of many cancer cells in response to several therapies, especially in tumors with the lack

of crucial apoptotic modulators (e.g., BAX and BAK or caspases) [extensively reviewed elsewhere (127)].

Oncolytic viruses destroy tumor cells by inducing different types of cell death. For example, parvovirus and NDV exert oncolytic activities by triggering apoptotic pathways in many cancers, while vaccinia virus leads to programmed necrotic cell death in ovarian and colon infected tumor cells (128–131). In addition, some oncolytic viruses are also able to induce tumor cell lysis through autophagy induction (**Figure 3**) (132).

A number of different conditionally replicating adenoviruses (CRAds) have been shown to induce autophagy in tumor cells (133). In nude mice subcutaneously transfected with glioma tumor cells, adenoviruses expressing the adenovirus early (E) 1A gene (which leads to viral replication) under the control of the human telomerase reverse transcriptase promoter (hTERT-Ad) kill telomerase-positive tumor cells by inducing autophagic cell death, notably by inhibiting the mTORC1 activity in infected cells (134). A more recent study showed that OBP-31, an oncolytic adenovirus that is derived from hTERT-Ad, induces the autophagic cell death of human glioma tumor cells through an E2F1-mir7-EGRF pathway (135). Similarly, CRAds, which use the survivin promoter, enhance autophagy in glioma infected cells and favor the elimination of tumor cells *via* a beclin-1 dependent mechanism (136). Delta-24-RGD, an oncolytic adenovirus that selectively replicates in cancer cells with an abnormal Rb pathway, induces an autophagy-dependent cell death of brain tumor stem cells and improves the survival of glioma-bearing mice (137). *In vitro* experiments also showed that wild-type or E1-deficient adenoviruses induce autophagy in lung, cervical, and colon cancer cells and that autophagy induction correlates with an increase of viral replication and tumor cell death (138).

The oncolytic HSV-1 strain RH2, which lacks the $\gamma 34.5$ gene, induces the cell death of squamous cell carcinoma (SSC). While no significant changes were observed using caspase inhibitors, the cytotoxicity of RH2 infection was inhibited when infected tumors cells were treated with autophagy inhibitors (3-methyladenine and bafilomycin), demonstrating that HSV-1/RH2 induces autophagic cell death in SCC cells (139). In gastric carcinoma, an excessive endoplasmic reticulum stress was observed in Newcastle disease virus (NVD)-infected cancer cells, triggering autophagy and cell death (140). Similarly, Meng et al. (2012) reported that infection of malignant U251 cells with NDV boosts the formation of autophagosomes, which facilitates the replication of the virus (141). Interestingly, pharmacological modulation of autophagy was investigated in order to enhance the oncolytic potential of NDV strain FMW (NDV/FMW) in drug-resistant lung tumor cells (A549 resistant to cisplatin or paclitaxel). Combination of NDV with chloroquine or rapamycin significantly promoted the oncolytic efficiency of NDV/FMW in lung cancer bearing mice (142). Edmonton strain of MeV (MeV-Edm) exploits selective autophagy to increase its replication in non-small-cell lung cancer (NSCLC) by targeting the degradation of mitochondria *via* autophagy (mitophagy), which results in a decrease of innate immune response by limiting the production of RIG-I like

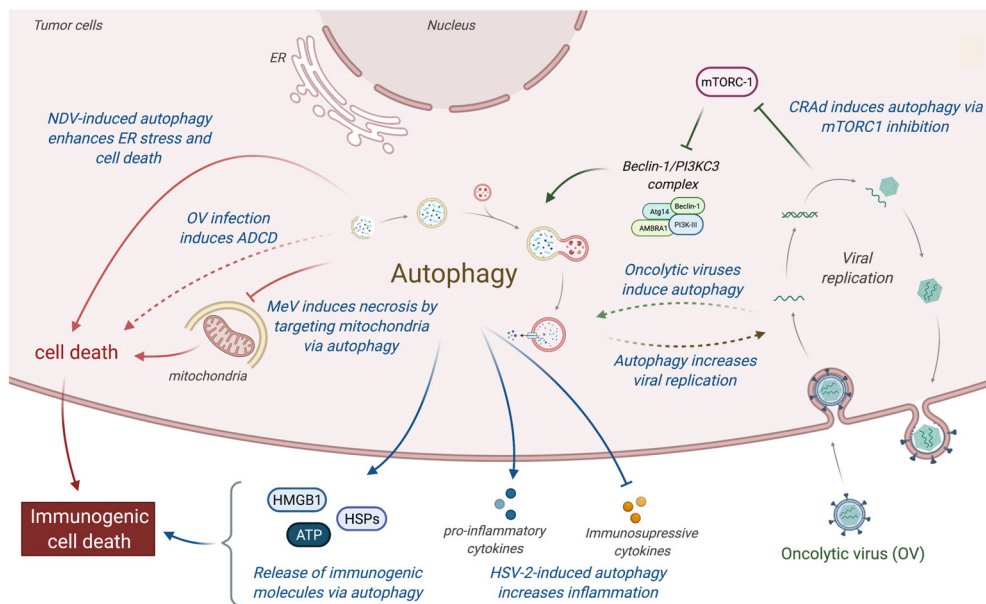


FIGURE 3 | Relations between oncolytic viruses and autophagy in tumor. Oncolytic viruses induce an immunogenic cell death by inducing autophagy, which favors the cell death of tumor-infected cells and the release of immunogenic molecules (e.g., HMGB1, ATP, or HSPs proteins). More specifically, CARds replication induces autophagy by an inhibition of mTORC1, which enhances the induction of cell death and the release of immunogenic molecules. MeV induces mitophagy to eliminate mitochondria, resulting to a decrease of apoptosis and an increase of necrosis, a more immunogenic cell death. NDV infection increases ER stress, resulting to an induction of autophagic cell death and the secretion of immunogenic molecules. In addition, HSV-2 infection can also perturb the tumor microenvironment by favoring the expression of pro-inflammatory molecules (e.g., TNF α , IL-1 β ; and GM-CSF) and limiting the production of immunosuppressive molecules (e.g., IL-10 and TGF β). CRAds, Conditionally Replicating Adenoviruses; MeV, Measles Virus; NDV, Newcastle Disease Virus; HSV-2, Herpes Simplex Virus 2; ADCD, autophagy-dependent cell death.

receptor (RLR)-dependent type-I interferon production (143). Another study showed that the persistent viral replication mediated by MeV-Edm-induced mitophagy prevents apoptosis but at the end leads to necrotic cell death (144), suggesting that MeV-induced autophagy could switch from apoptosis to a more immunogenic cell death in NSCLCs following the infection.

An attractive strategy among virus-based oncolytic system is to design viral vectors that express pro-autophagic genes, in which gene-virotherapy approach significantly enhances tumor cell death by activating autophagy, especially in tumors that have acquired resistance for apoptosis. Several studies have investigated the therapeutic effect of engineered recombinant OV expressing *beclin-1* gene. For example, the oncolytic adenovirus expressing *beclin-1* (SG511-BECN) infection induces a significant autophagic cell death in a variety of leukemic cell lines and primary leukemic blast (145). Interestingly, SG511-BECN induces cell death of both acute and chronic myeloid leukemia, but has less cytotoxicity in normal cells. In murine leukemia model, SG511-BECN prolongs mice survival and decreases the xenograft tumor size by inducing autophagic cell death (145). Recently, oncolytic vaccinia virus that expresses Beclin-1 (OVV-BECN) was also tested for its *in vitro* and *in vivo* oncolytic activity in blood

cancer (146). OVV-BECN induces a significant autophagic cell death in both wild-type leukemia and multiple myeloma cells lines and has a greater antitumor activity compared with the wild-type vaccinia virus, demonstrating a favorable therapeutic effect of autophagy in vaccinia-based treatment of blood cancers.

Oncolytic Viruses and Autophagy-Mediated Immunogenic Cell Death

Besides their direct killing potential, oncolytic viruses require the activation of immune responses to be long-lasting effective against cancer. Most of the OVs, including HSV-1, MeV, adenoviruses, or NDV, have been described to induce an immunogenic cell death (ICD), which is critical for their virotherapy efficacy (147). ICD is a type of cell death that is sufficient to induce an adaptive immune response against exogenous or endogenous antigens expressed by dying cells and elicited by the presence of danger associated molecular patterns (DAMP). While pathways of ICD induction could be multiple, it is often related to endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) production (148). The main hallmarks of ICD that have been described so far include ecto-expression of calreticulin (which is normally expressed in ER), ATP release, high-mobility group box (HMGB) 1 release in

different redox states but also the activation of annexin 1, the secretion of type I IFN and IL-1 β , or the exposure of F-actin and heat shock protein (HSP-70 and HSP90) (147). ICD leads to the activation of adaptive immune cells, including dendritic cells (DC) and T lymphocytes (LT). OVs are one of the most described ICD inducers, and infected tumor dying cells are often associated with the presence of danger signals (ATP and HMGB1 release or CRT exposure), the secretion of pro-inflammatory cytokines, as well as the release of tumor associated antigens (TAA), which lead to a strong activation of antitumor immune responses (149–153).

Pathways of ICD induction vary a lot between viruses, but mounting evidences indicate that autophagy plays a critical role in that induction. One of the first examples highlighting the importance of autophagy in ICD has been observed during embryonic development. During embryonic cavitation, embryonic bodies (EBs) derived from cells deficient for autophagy genes (*atg5* or *beclin-1*) fail to cavitate. This defect is due to the persistence of cells corps during the embryonic development, which is the result of the absence of “eat-me” signal (exposure of phosphatidylserine) and the poor release of ATP in autophagy-deficient EBs (154). In tumor model, Michaud et al. reported that autophagy is required for the immunogenicity of chemotherapy-induced cell death, as autophagy-deficient dying cells fail to release ATP and subsequently to attract DC and LT into the tumor bed in colon carcinoma mice model (155). Similarly, it has been observed that radiotherapy of lung and colon cancer elicited an anticancer immune response that was dependent on autophagy-induced ATP release from dying cells, release that is required for a dense recruitment of lymphocytes into the tumor site (156). In epithelial and glioblastoma cancer cells, autophagy regulates the passive HMGB1 release from dying cells and active HMGB1 secretion (157, 158). Strikingly, the close correlation between autophagy induction and ICD properties observed during antitumor cytotoxic agents treatment in blood cancers supports the relationship between autophagy and ICD (158). In addition to favoring the exposition of DAMP from dying cells, autophagy can also promote antigen presentation from cancer cells to DCs and subsequently to T cells, a necessary step for the implementation of a robust antitumor immune response. Indeed, autophagy promotes the presentation of antigens not only by major histocompatibility complex (MHC) class II but also by MHC class I, as observed for endogenous viral antigens during HSV-1 infection or during influenza infection of tumor cells, and for cross-presentation of TAA from uninfected tumor cells (159, 160).

Oncolytic adenoviruses have been shown to induce an autophagy-dependent ICD, leading to the release of a great number of DAMP molecules and TAAs (137). In prostate cancer cells, the combination of Ad5/3 fiber-modified oncolytic adenovirus armed with granulocyte macrophage colony-stimulating factor (Ad5/3-D24-GMCSF) with low-dose of temozolomide (a chemotherapeutic agent) results in the increase of intracellular level of autophagy in tumor dying cells and favors immunogenic cell death, as indicated by elevated calreticulin exposure, ATP secretion, and HMGB1 release (161).

A more recent report demonstrated that oncolytic adenovirus-induced autophagy is critical for the processing and the presentation of TAAs incorporated into viral capsid protein on MHC class II, suggesting that the combination of adenoviruses with autophagy inducers may enhance the antitumor immune responses (162). NDV infection also induces a strong ICD in lung and glioblastoma cancer cells, observable by the elevated exposure of calreticulin, the release of HMGB1, ATP, and HSP79/90, and the induction of a long-term tumor-specific immune response (163, 164). A recent study has shed light on the important role of autophagy in NDV-mediated ICD in lung cancer, as the depletion of autophagy-related genes, in contrast to inhibition of apoptosis or necrosis, significantly inhibits the induction of ICD determinants by NDV infection (165). In addition to increase the DAMP or TAAs expression by tumor dying cells, autophagy can also modify the tumor microenvironment. Using the herpes simplex virus type 2 (HSV-2)-based oncolytic virus Δ PK, one report has demonstrated that autophagy promotes the release of proinflammatory cytokines (TNF α , IL-1 β ; and GM-CSF) through the TLR2 activation, and that contributes to the inhibition of tumor immunosuppressive microenvironment in melanoma cells (166).

OTHER CORRELATIONS BETWEEN VIRAL INFECTION, AUTOPHAGY, AND TUMOR PROGRESSION

In addition to favoring the tumorigenesis and the immunogenic cell death, modulation of autophagy by viral infections could also influence other steps of tumor development, including tumor cell survival, proliferation, migration, and resistance to antitumor therapies, as well as immune response efficiency.

Tumor Cell Survival

When the tumor is established, the impact of autophagy on tumor growth and/or clinical features of patients remains unclear and seems to be greatly influenced by the type and/or the stage of the disease. However, several studies described that autophagy has pro-tumor effects, promoting the tumor cell survival, the proliferation, the migration, and the resistance to radio- and chemotherapies (Figure 2) (167). One of the most obvious involvement of autophagy in tumor growth is the fact that autophagy could recycle non-essential cytoplasmic elements to support tumor cell survival. Indeed, the more the tumor grows, the more the cells are moved away from the vascular system, leading cells under hypoxic and/or starvation condition (168). Both starvation and hypoxia have been demonstrated to induce a strong autophagy response in tumor cells, and this autophagy acts as recycling mechanism to promote stress tolerance and to supply metabolic precursors for the tumor cell survival (169, 169). Interestingly, some common oncogenic gene mutations (e.g., *ras*) have been shown to increase autophagy in order to maintain tumor cell survival (170).

Autophagy could also limit the induction of programmed cell death, including apoptosis, by the selective removal of damaged organelles (e.g., damaged mitochondria) or by the selective elimination of the pro-apoptotic signal transduction [extensively reviewed elsewhere (171)]. Interestingly, several viruses have been observed to promote survival of infected cells by repressing apoptosis through an autophagy-dependent mechanism (**Figure 4**) (172–179). In human and mouse neuroblastoma cells, wild-type rabies virus limits apoptosis in infected cells by inducing a complete autophagic influx (179). The overexpression of HBV protein HBx and HCV protein NS5A can both reduce the starvation-induced cell death through the activation of autophagy and the inhibition of mitochondrial apoptosis, leading to the tumor cell survival of respectively advanced HCC and hepatoblastoma cells (174, 175). Similarly, the Tax protein of human T-cell leukemia virus type 1 (HTLV-1) favors the resistance of astrogloma infected cells for FASL-mediated and TRAIL-mediated apoptosis by increasing autophagy (176). In lung tumor, NDV-induced autophagy promotes viral replication and tumor cell survival by preventing cancer cells from caspases-dependent apoptosis, recruiting p62-mediated autophagy to control cytochrome c release from mitochondria (180).

Contribution of autophagy in tumor survival has also been illustrated by the fact that autophagy could prevent lysis of tumor

cells from antitumor cytotoxic immune cells. For instance, autophagy has been reported to play an important role in hypoxia-induced resistance of lung tumor cells to cytolytic T lymphocyte-mediated lysis (181). Indeed, inhibition of Atg5 or Beclin-1 reduce the phosphorylation of STAT3, an important transcription factor involved in tumor survival and resistance to T cell cytotoxicity. Similarly, Viry *et al.* revealed that hypoxia-induced autophagy is responsible for the degradation of granzyme B in tumor cells delivered by NK cells, leading to their resistance to NK-mediated cell death (182). Interestingly, similar STAT3 modulation has been reported in infected cells (183). Given the fact that these proteins are also responsible for autophagy-mediated resistance to starvation in tumor, it will be very interesting to investigate the impact of virus-induced autophagy on the resistance to antitumor immunity.

Metastasis Formation

Autophagy has also been associated with metastasis formation. Actually, the role of autophagy in metastasis occurrence is controversial (184). In one hand, autophagy has been shown to limit metastasis of some tumor cells. For instance, autophagy decreases the glioblastoma tumor cells migration and invasion, reversing the epithelial-mesenchymal transition (EMT) (185). Similarly, autophagy has been associated with the selective degradation of MET/HGF receptor tyrosine kinase, two kinases

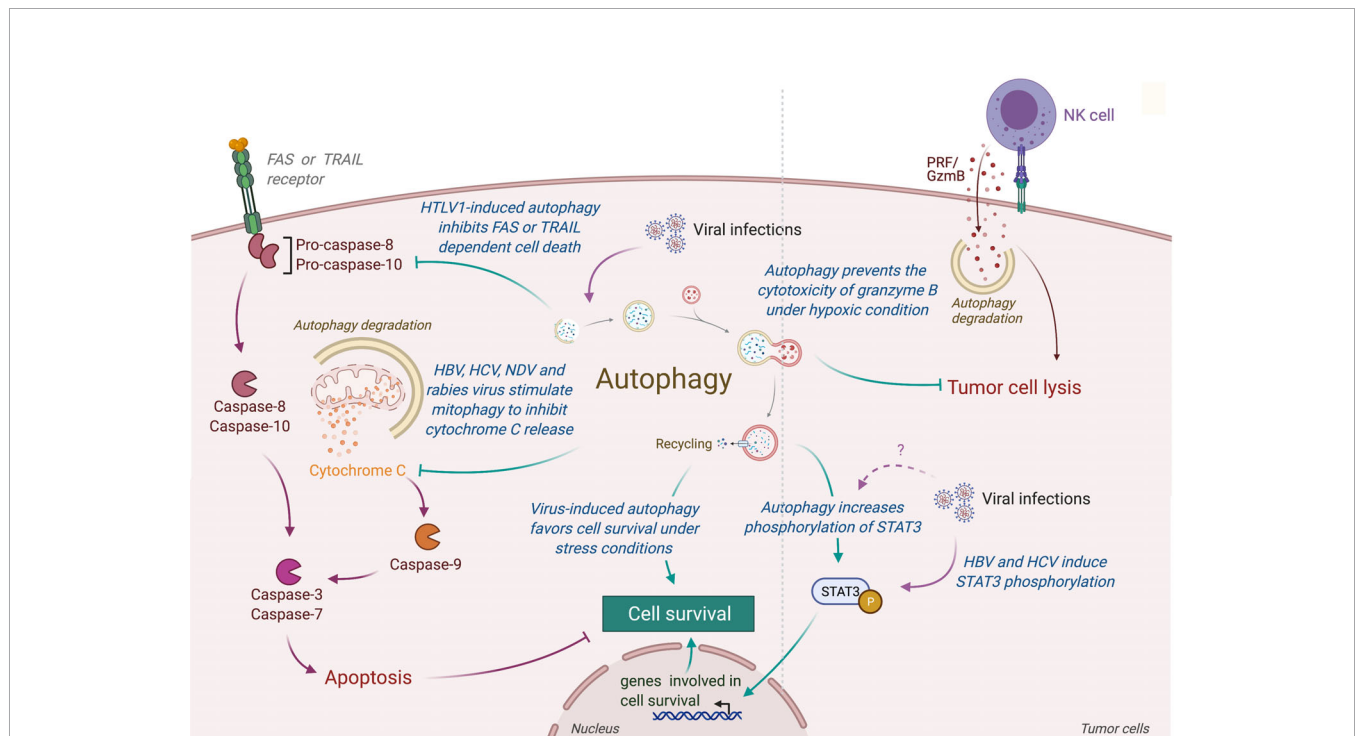


FIGURE 4 | Role of virus-induced autophagy in tumor cell survival. Viral infections can induce autophagy in tumor cells, leading to a protection of tumor cells from stress-induced or immune cells-induced cell death. In one hand, autophagy protects tumor infected cells from stress conditions (e.g., starvation or hypoxia) by limiting the accumulation of damaged organelles or by increasing the expression of genes involved in cell survival (e.g., via the phosphorylation of STAT3). In another hand, virus-induced autophagy limits the induction of apoptosis, protecting tumor cells from death receptor- or mitochondria-mediated cell death. Autophagy also takes part in the resistance of infected tumor cells from immune cell lysis by targeting and neutralizing granzyme B activity. HTLV-1, Human T cell Leukemia/lymphoma Virus type 1; HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; NDV, Newcastle Disease Virus; PRF/GzmB, Perforin/Granzyme B.

involved in cell invasion, therefore inhibiting cancer cell lines mobility (186). In the other hand, autophagy has been described to be essential for the resistance to cell-detachment-induced apoptosis, called anoikis (187). For example, the group of Jian Fan demonstrated that the inhibition of autophagy suppresses pulmonary metastasis of hepatocellular carcinoma in mice through impairing anoikis resistance (188). Emerging evidence show that autophagy not only enhances the survival of disseminating tumor cells but also promotes the survival and the maintenance of a stem-like subpopulation of tumor cells that drives invasion, treatment resistance, and cancer recurrence (189). In addition, several autophagy substrates have been shown to be involved in the regulation of the epithelial-mesenchymal transition (EMT), tumor cell migration, and invasion [extensively reviewed elsewhere (190)].

Viral infections and virus-related molecules are also known to promote metastasis formation. EBV has been shown to encode several mature miRNAs, where some of them have been demonstrated to promote tumor development by targeting virus-infected host genes or self-viral genes. Several studies demonstrated that these miRNA could promote EMT, migration and metastasis of the nasopharyngeal carcinoma (NPC) cells (191–195). HPV16 E6 protein expression has been shown to increase actin polymerization through the degradation of Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1) protein, facilitating the migration of the cervical cancer cell and the development of metastasis (196). Viral proteins E7 of HPV16 and E1 of HCV have been shown to inactivate the tumor metastasis repressor Nm23-H1 in human keratinocyte HaCaT cell line and HCC cells line, respectively, increasing metastasis formation (197, 198). HSV2, HBV, or HCMV related-proteins or miRNA have also been demonstrated to promote metastasis development (199–201). Interestingly, HCMV-mediated promigratory signal requires the activation of Ras homolog family member A (RhoA), a protein involved in cell migration that can be modulated by autophagy (202). A recent report showed that under HBV infection, HBx-induced autophagy promotes the expression of long non-coding RNA activated by transforming growth factor (TGF)- β (lncRNA-ATB) and TGF- β , two key actors for the migration and invasion capabilities of liver cells (203).

While the role of autophagy in virus-induced metastasis needs to be further analyzed to understand the actual function of this mechanism in tumor development, it is interesting to notice the increasing number of evidences that report a strong correlation between viruses capable to modulate autophagy and their capacity to increase tumor cell migration and metastasis development.

Resistance to Antitumor Therapies

Another aspect by which autophagy favors the tumor development concern its impact on the resistance to antitumor treatments. In various tumor models (e.g., ovarian, colon, or osteosarcoma tumors), autophagy has been demonstrated to be an important actor of chemoresistance, leading to an increase of the survival of tumor cells under treatment of salt-based chemotherapies (204–208). Autophagy has also been observed

to be an important promoter of resistance to radiotherapy in various tumor models, including lung, glioma, pancreatic, and colorectal cancer (209–212). Interestingly, in order to overcome this resistance, several therapeutic approaches consisting in combining chemotherapy and radiotherapy with autophagy inhibitors have emerged with very promising results (213, 214).

Viruses or viral proteins have also been linked with the resistance to different antitumor therapies (215–220). While mechanisms involved in viruses-mediated resistance to therapies differ with the type of viral infection, the main strategy consists in inhibiting apoptotic cell death in infected tumor cells. EBV miR BART20-5p has been observed to favor chemoresistance to 5-FU and docetaxel in gastric cancer by targeting the pro-apoptotic BCL2-associated agonist of cell death (BAD) expression (221). EBV, though the viral LMP1 protein, has been demonstrated to decrease the expression of the two pro-apoptotic factors PDCD4 and FasL, leading to a chemoresistance to cisplatin in nasopharyngeal carcinoma cells (222). EBV has also been shown to induce chemoresistance to 5-FU in gastric cancer cells by decreasing the cleavage of PARP and caspase 3 and increasing the anti-apoptotic Bcl2 expression (223). In nasopharyngeal carcinoma, EBV-encoded miR-BART4 and miR-BART8-3p favor resistance to radiotherapy by inhibiting apoptosis (224, 225). EBV latent viral protein LMP1 has also been demonstrated to induce radioresistance by preventing DNA Damage Response (DDR) through the phosphorylation of AMPK (thr¹⁷²) and inhibiting therefore its interaction with DNA-dependent protein kinase (DNA-PK), required for the DDR (226). While autophagy has not been investigated in this study, it is interesting to note that AMPK activity is an important regulator of autophagy, and AMPK-induced autophagy could help to the EBV induced radioresistance. Similarly, a recent study demonstrated that LMP1 induces autophagy *via* the binding of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) to beclin-1 (227). This autophagy stimulation has been shown to enhance the resistance of LMP1-positive nasopharyngeal carcinoma (NPC) cells against irradiation by protecting tumor cells from apoptosis. M. Antonioli et al. also demonstrated that HPV infection in oropharyngeal squamous cell carcinoma sensitize tumor cells to cisplatin-induced apoptosis by inhibiting autophagy, supporting the idea that modulation of autophagy during acute or latent infections could impact the resistance of tumor cells to antitumor therapies (228).

Antitumor Immune Responses

Type I interferon (IFN-I) is an important class of pro-inflammatory cytokines produced in response to viral infection and other environmental stresses. While IFN-I is very important for efficient virus clearance, emerging studies have shown that these proteins are also a very important driver for antitumor immunity, promoting the efficiency of immune cells to eliminate tumor [extensively reviewed elsewhere (229)]. Many intracellular pathways can recognize viral components and lead to the production of IFN-I, including the stimulation of RIG-I like receptor (RLR) and mitochondria antiviral signaling protein (MAVS), present in the outer membrane of mitochondria.

Autophagy, notably through the targeting of mitochondria (mitophagy), is able to regulate RLR-mediated IFN-I production (230). Several viruses induce mitophagy to impair the type-I interferon production, including the SARS-Coronavirus, the IAV and parainfluenza virus or MeV (Figure 5) (231–234). Damaged mitochondria can also release danger signals, including the reactive oxygen species (ROS) and mitochondrial DNA, which in return can activate inflammasome and the release of pro-inflammatory cytokines that could perturb tumor immunity (235, 236). Interestingly, some viruses, including IAV and MeV, have been also shown to induce mitophagy to prevent NLRP3-mediated inflammation (237, 238). Autophagy could also prevent virus-induced IFN-I production by other mechanisms. Induction of autophagy by HPV16 E7 protein is responsible for the autophagic degradation of STING protein, which impairs IFN-I production and induces important deregulation of tumor immunity and promotion of Head and Neck Squamous cell carcinoma (239). Similarly, Bluetongue virus (BTV), an orbivirus targeting ruminants, impairs IFN-I pathway by preventing STAT1 phosphorylation and by degrading STAT2 through autophagy (240). Inhibition of autophagy maturation in HCV-infected hepatocytes, through the expression of Rubicon, was recently associated with an increase of expression of type I IFN-related genes and an upregulation of HCV replication (241). Other pro-inflammatory cytokines,

important for tumor immunity, could also be regulated by virus-induced autophagy. As a good example, MCMV M45 protein induces the autophagic degradation of two cellular signaling proteins involved in nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B)-mediated cytokine production, the NF- κ B essential modulator (NEMO), and the receptor-interacting protein kinase 1 (RIPK1) (242, 243).

In addition to limit pro-inflammatory cytokine production, viruses have elaborated strategies to escape from the cytotoxic immune cells. To avoid the recognition and elimination of infected cells by cytotoxic lymphocytes, many viruses decrease the major histocompatibility (MHC) class I antigen presentation pathway [extensively reviewed elsewhere (244)]. Similar strategy has also been observed in many cancer cells. The decrease of MHC-I expression has also been observed in dendritic cell (DC) infected with HIV, MCMV, or varicella zoster virus, and it has been associated with a reduction of their capacity to stimulate T cell proliferation (245). Interestingly, autophagy has been associated to a decrease of MHC-I expression on DC by facilitating the endocytosis and the degradation of MHC-I (246). A recent study demonstrated that autophagy regulates the expression of MHC-I in pancreatic tumor cells, as the inhibition of the autophagy machinery increased MHC-I expression on tumor cells, enhanced antitumor T cell response, and reduced tumor growth in mice model (247).

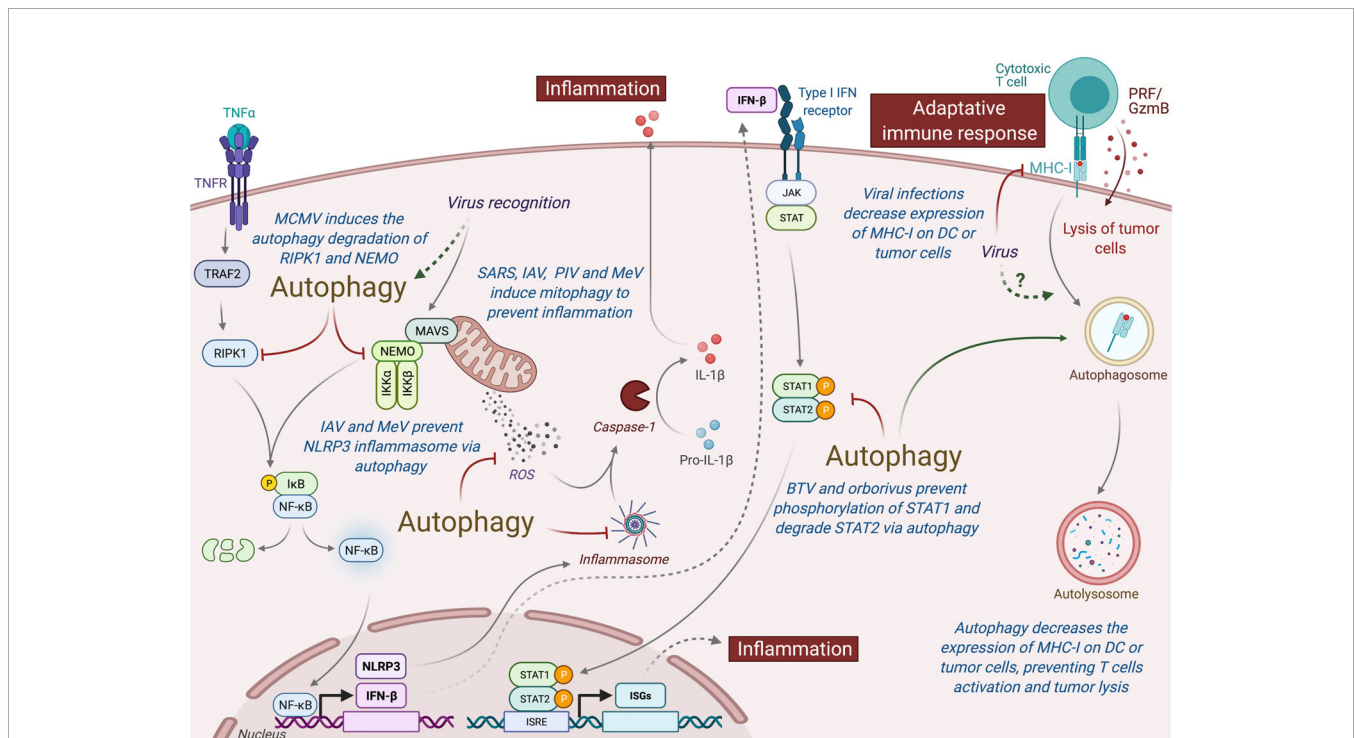


FIGURE 5 | Autophagy induced under viral infection can perturb antitumor immunity. Autophagy can interfere with innate and adaptive immunity and impact the antitumor immune responses. Viruses-induced autophagy perturbs type I IFN pathway by limiting NF- κ B activation and STAT1/STAT2 phosphorylation. Several viruses (e.g., SARS, IAV, PIV, and MeV) can also modulate mitochondria activity via mitophagy and prevent inflammation by inhibiting the release of ROS or by preventing the formation of inflammasome. Viral infections or autophagy can also modulate the expression MHC-I by tumor cells or DC, limiting the action of antitumor cytotoxic T cells. MCMC, Murine Cytomegalovirus; IAV, Influenza A Virus; MeV, Measles Virus; SARS, SARS-Coronavirus; PIV, Parainfluenza Virus; BTV, Bluetongue virus; MHC-I, Major Histocompatibility Complex I; PRF/GzmB, Perforin/Granzyme B, DC, dendritic cells.

CONCLUSION

Since several years, investigations on autophagy process shed light many biological pathways to an autophagy-dependent modulation. To understand the role of autophagy in host cells, it is important to consider the physiologic context, as autophagy could have distinct functions according to the biological model and/or the stage of the disease. During the initial step of tumor development, cells accumulate damages that disturb key molecules involved in cell cycle and promote tumorigenesis. Both tumor suppressor genes and oncogenes are implicated in autophagy regulation, linking autophagy directly to cancer occurrence. Recent discoveries showed that oncogenic viruses, which lead to the transformation of precancerous cells to malignant tumor, inhibit autophagy by targeting specifically autophagy molecules. Inhibiting autophagy in infected cells favors the accumulation of damaged organelles and/or proteins in host cells and therefore promotes the accumulation of stress components and genetic mutations.

In another hand, autophagy induced in more advanced tumor is more prone to help tumor growth. As a cellular degradative process, autophagy helps proliferative tumor cells for their nutrient supply and has an important prosurvival function, favoring tumor cell resistance to several therapies. Autophagy may also promote metastasis by protecting detached and stressed tumor cells as they travel through blood vessels and establish new colonies at distant sites. Importantly, many viruses capable to

modulate autophagy have similar impact on tumor behavior, and while the direct link between autophagy and tumor development has not been well established in all studies, strong evidences have suggested an important connection between autophagy and virus-mediated tumor modifications. However, further investigations are needed to clarify autophagy's modulation by viral infections and tumor development. Considering the role of autophagy in aberrant cell physiology, the understanding of these molecular processes is crucial for the development of new therapeutics against cancers and potentially other proliferative diseases.

AUTHOR CONTRIBUTIONS

LL and P-EJ performed similar bibliography. LL and P-EJ equally contributed to write the manuscript. P-EJ performed figures and supervised this work. IC, SS and MA helps authors during the conception and correction of the manuscript. All authors contributed to the article and approved the submitted version.

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> L'autophagie est un processus métabolique important pour maintenir l'homéostasie cellulaire à des moments critiques du développement et/ou en réponse à un stress environnemental. Cela est particulièrement pertinent dans le cas des cancers, pour lesquels il a été montré que l'autophagie a un impact important sur leur survie et sur la croissance tumorale. D'une part, elle limite la transformation cancéreuse des cellules précancéreuses à un stade précoce, mais, d'autre part, elle favorise la survie et la prolifération cellulaires, les métastases et la résistance aux thérapies anti-tumorales dans les tumeurs plus avancées. L'autophagie peut être induite par une grande variété de stimulus extracellulaires et intracellulaires. Les infections virales ont souvent été associées à une modulation de l'autophagie, dont l'impact sur la réplication virale ou la survie des cellules infectées diffère selon le modèle étudié. Dans un contexte tumoral, certains mécanismes moléculaires complexes par lesquels la modulation de l'autophagie par les virus peut influencer le développement des cellules précancéreuses ou cancéreuses ont été révélés. Cette revue présente les découvertes récentes concernant les répercussions d'une perturbation de l'autophagie par l'infection virale sur la survie et la progression des tumeurs cancéreuses. <

L'autophagie : un mécanisme clé de l'homéostasie cellulaire

L'autophagie, un mot qui se réfère littéralement au fait de « se manger soi-même », définit un mécanisme catabolique de dégradation et de recyclage présent dans toutes les cellules eucaryotes. Ce processus peut

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L'autophagie modulée par les virus

Un rôle dans la progression tumorale

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être activé afin de cibler certaines molécules ou organelles défectueuses présentes dans le cytosol et de les délivrer aux lysosomes pour qu'ils les dégradent. Les composés issus de cette dégradation pourront être réutilisés par la cellule pour son fonctionnement. L'autophagie est donc un mécanisme crucial pour le maintien de l'homéostasie cellulaire. Elle permet aux cellules de survivre à des stress environnementaux, tels que le manque de nutriments ou l'hypoxie. Trois différents types d'autophagie existent selon la machinerie moléculaire mise en jeu : la macroautophagie, la microautophagie et l'autophagie par l'intermédiaire des chaperonnes [1]. La macroautophagie (que l'on appellera autophagie par la suite) est la plus étudiée ; son importance a été mise en évidence dans des contextes physiologiques mais aussi physiopathologiques. Elle comporte de multiples étapes, et débute par la formation d'une membrane, appelée phagophore (dont l'origine cellulaire peut varier selon les modèles étudiés), qui va peu à peu s'étendre pour former les autophagosomes, des vésicules à double membrane qui capturent des composants intracellulaires altérés (protéines ou organelles). Les autophagosomes fusionnent ensuite avec des endosomes et des lysosomes, ce qui entraîne la dégradation des éléments emprisonnés et le recyclage dans le cytosol des métabolites ainsi produits (→) (Figure 1).

Bien que l'autophagie ait longtemps été considérée comme un processus de dégradation non sélectif, de nombreuses études ont montré qu'il pouvait être très spécifique grâce, notamment, à l'activité de protéines intermédiaires solubles permettant la liaison des protéines ou des organelles altérées au phagophore en

(→) Voir aussi le numéro thématique **Autophagie**, m/s n° 3, mars 2017

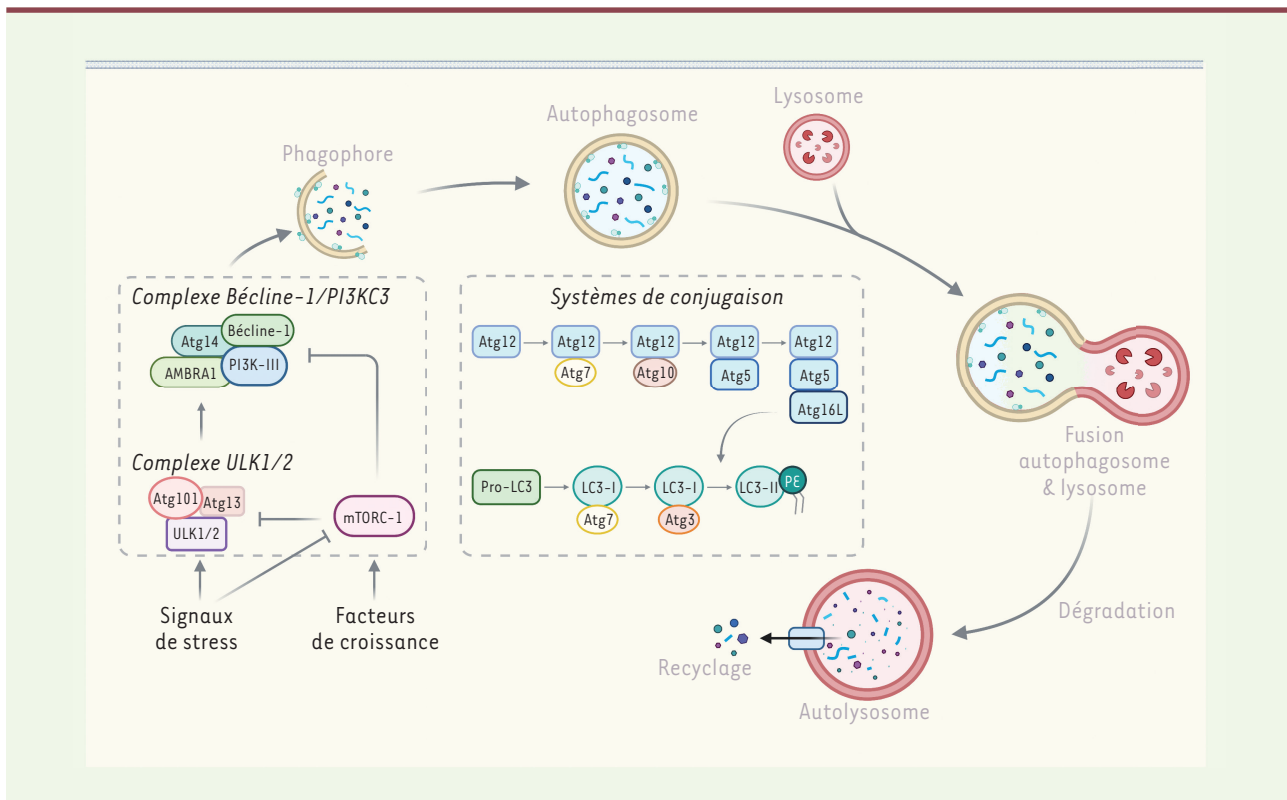


Figure 1. Le mécanisme de l'autophagie. Le complexe d'initiation des autophagosomes, formé de la bécline-1 et de VSP (*vacuolar protein sorting*) 34 (un membre de la famille de la phosphatidylinositol 3-kinase [PI3K]) joue un rôle crucial dans la formation et l'élongation du phagophore. L'activation de ce complexe, qui est inhibée ou stimulée par différents partenaires, produit, sur les membranes du phagophore, des groupements phosphatidylinositol 3-phosphate (PI(3)P) permettant le recrutement de protéines nécessaires à l'apport de membranes au phagophore en croissance. L'élongation de ce dernier est assurée par le système de conjugaison constitué des protéines autophagiques (ATG) ATG12 et ATG5 à l'origine (avec ATG7 et ATG10) du complexe macromoléculaire ATG12-ATG5-ATG16 permettant la formation et le recrutement de LC3-II, formé de la conjugaison de LC3 (*microtubule-associated protein 1A/1B-light chain 3*) à la phosphatidyléthanolamine, responsable de l'élongation de l'autophagosome. La fermeture de la vésicule s'achève avec la libération des protéines ATG, et seul LC3-II lié à la membrane interne reste confiné à l'intérieur. La maturation de l'autophagosome est sous le contrôle de protéines facilitant les fusions vésiculaires, et la dégradation par les enzymes des lysosomes (figure créée avec BioRender.com).

formation (p62/SQSTM1 [*sequestosome 1*], NDP52 [*nuclear dot protein of 52 kDa*], OPTN [*optineurin*], NBR1 [*next to BRCA1 gene 1*]). C'est ainsi que la notion d'autophagie sélective a peu à peu émergé, et que sont apparues des désignations particulières selon les substrats ciblés : mitophagie, RE-phagie, ou encore virophagie, pour qualifier l'autophagie ciblant respectivement les mitochondries, le réticulum endoplasmique (RE), ou les composants viraux [1].

Depuis la mise en évidence de l'autophagie par le médecin biochimiste belge Christian de Duve en 1963¹, nos connaissances sur son mécanisme et sur son rôle physiologique ont beaucoup progressé. Elle est présente en permanence dans toutes les cellules eucaryotes, où elle agit comme un mécanisme de « contrôle qualité » en dégradant et en recyclant les protéines ou les organelles défectueux afin de maintenir l'homéostasie cellulaire. Il n'est donc pas très étonnant qu'un défaut

d'autophagie soit associé, non seulement au vieillissement, mais aussi à diverses maladies humaines : maladies neurodégénératives, désordres métaboliques, susceptibilité aux infections, ou encore survenue et développement de cancers [2].

Autophagie et cancer

Rôle protecteur de l'autophagie contre la survenue de cancers

En raison de sa fonction homéostatique, de nombreuses recherches ont été réalisées pour déterminer l'impact de l'autophagie sur la survenue et le développement de cancers, mais cet impact reste difficile à évaluer dans ce contexte et semble très dépendant de l'environnement tumoral étudié. Plusieurs études s'accordent

¹ Prix Nobel de physiologie ou médecine en 1974.

néanmoins pour définir l'autophagie comme un mécanisme suppresseur de tumeur dans les cellules saines, empêchant la survenue de cellules précancéreuses capables de devenir des tumeurs malignes [3]. En limitant la présence de mitochondries endommagées (ou d'autres organites défectueux), l'autophagie limite en effet l'apparition d'espèces réactives de l'oxygène pouvant causer des dommages à l'ADN cellulaire et entraîner la transformation des cellules saines en cellules cancéreuses. De nombreux patients souffrant de cancer présentent d'ailleurs des mutations de certains gènes impliqués dans l'autophagie. Le premier gène à avoir été considéré comme un gène suppresseur de tumeur est celui codant la bécline-1, dont la déplétion par mutation mono-allélique est observée dans de nombreuses tumeurs [4]. Depuis, l'altération de l'expression d'autres gènes (*Bif-1* [*Bax-interacting factor 1*] ou *UVRAG* [*UV radiation resistance-associated gene*]) favorisant la mise en place de tumeurs a été observée dans les cellules tumorales. Des études réalisées *in vitro* ou dans des modèles murins ont également montré que l'inhibition du processus autophagique favorise la survenue de cancer, notamment de tumeurs solides, ce type de tumorigenèse étant associé à une production excessive d'espèces réactives de l'oxygène dans les cellules [5].

Rôle de l'autophagie sur la progression tumorale

Bien que l'autophagie puisse limiter la survenue de cancers, elle semble avoir un rôle opposé lorsque que la tumeur est établie, une forte activité autophagique des cellules cancéreuses pouvant être de mauvais pronostic. L'autophagie favorise en effet la survie des cellules tumorales en condition de stress, notamment dans les tumeurs solides dont les cellules sont soumises à l'hypoxie ou à un manque de nutriments, surtout lorsque la tumeur devient volumineuse, la néo-vascularisation étant alors plus faible au centre de la tumeur [6]. Carence nutritive et hypoxie induisent une forte réponse autophagique, ce qui permet la dégradation de composants cellulaires non essentiels afin de fournir les précurseurs métaboliques indispensables à la survie et à la prolifération des cellules cancéreuses. L'autophagie favorise également le développement de métastases en permettant à la cellule tumorale de résister au signal de mort cellulaire induit par sa perte d'adhérence à la matrice extracellulaire (anoïkis), mais également en favorisant le maintien des cellules dans leur nouvelle niche, où se développera une tumeur secondaire [7].

L'autophagie a également un rôle important dans la résistance des cellules tumorales aux traitements anti-cancéreux conventionnels, tels que la chimiothérapie et la radiothérapie [8]. Certaines approches thérapeutiques consistent ainsi à combiner la chimiothérapie ou la radiothérapie et un inhibiteur de l'autophagie afin d'améliorer l'efficacité de ces traitements, avec des premiers résultats prometteurs [9, 10].

D'autres mécanismes expliquent le rôle protecteur de l'autophagie dans les tumeurs : le maintien des cellules souches cancéreuses, des cellules impliquées dans la résistance aux thérapies anti-cancéreuses, le développement de métastases, et la récurrence des cancers [11] ; l'échappement des cellules tumorales à la lyse par les cellules immunitaires (par les lymphocytes T cytotoxiques et les cellules NK [*natural*

killer]), en induisant, dans les cellules cancéreuses, la dégradation des granzymes, des protéases que ces cellules de l'immunité produisent afin de les détruire [12].

Autophagie et virus

Une infection virale module l'autophagie

De nombreux facteurs peuvent stimuler le processus d'autophagie : la carence en nutriments ou en énergie, les stress du réticulum endoplasmique ou le stress oxydant, certaines cytokines, mais aussi l'infection par des microorganismes pathogènes. En effet, pour les virus par exemple, on sait aujourd'hui que de très nombreuses étapes de leur cycle répliatif modulent l'activité autophagique de la cellule qu'ils infectent [13]. Leur simple reconnaissance par leur récepteur d'entrée peut être suffisante pour induire la formation d'autophagosomes. C'est notamment le cas pour le virus de la rougeole qui se lie à CD46² [14]. La reconnaissance des virus par les récepteurs de l'immunité innée peut également être à l'origine d'une stimulation de l'autophagie. Les récepteurs *Toll-like* (TLR), qui reconnaissent de nombreux composants protéiques ou nucléiques exprimés par les microorganismes pathogènes, ou les récepteurs *RIG-I* (*retinoic acid-inducible gene-1*)-like (RLR), qui reconnaissent l'ADN double brin cytosolique, sont ainsi à l'origine de la formation d'autophagosomes dans les cellules infectées [15]. Les virus peuvent également moduler la réponse autophagique des cellules qu'ils infectent par des mécanismes indirects. Les stress résultant de leur réplication (stress du réticulum endoplasmique ou stress oxydant) ou la production de cytokines pro-inflammatoires (interleukine [IL]-6, IL-1 β , interféron [IFN]- γ) ou anti-virales (IFN de type I), qui sont sécrétées en réponse à l'infection pour induire une réponse antivirale, sont autant de processus qui stimulent l'autophagie [16].

L'autophagie participe à la réponse anti-virale

L'autophagie, en recyclant les composants cytosoliques défectueux, peut également capturer et dégrader des constituants viraux présents dans le cytoplasme : particules ou protéines virales, mais également composants de la cellule nécessaires à la réplication des génomes viraux. Ce mécanisme de dégradation spécifique, appelé virophagie, attribue à l'autophagie un rôle important dans l'immunité antivirale, notamment dans les cellules non immunitaires. La première description de la virophagie a été réalisée dans le cadre de l'infec-

² La molécule CD46 (*membrane cofactor protein* ou MCP) est une protéine contrôlant l'activation du complément.

tion par le virus Sindbis³ : l'autophagie permet la dégradation d'une protéine de capsid de ce virus, limitant ainsi sa propagation, donc la survenue de la maladie [17]. Ce mécanisme de dégradation spécifique a ensuite été décrit pour des infections par d'autres virus, notamment le virus de l'immunodéficience humaine de type 1 (VIH-1) [18].

L'autophagie peut également servir de relai pour la mise en place de réponses immunitaires antivirales efficaces, un rôle découvert dans le contexte de l'infection par le virus de la stomatite vésiculaire (VSV). L'autophagie favorise en effet le transport des antigènes viraux du cytosol vers des vésicules riches en TLR7, ce qui permet aux cellules infectées de reconnaître plus efficacement les virus et favorise la mise en place d'une réponse anti-virale adéquate [19]. L'autophagie participe également à la mise en place des réponses immunitaires adaptatives, notamment contre le virus Sindbis ou les Herpesvirus (dont le virus d'Épstein-Barr, EBV). Elle favorise la présentation des antigènes viraux en association avec les molécules du complexe majeur d'histocompatibilité de type II [20].

L'autophagie participe donc efficacement à la réponse antivirale. Néanmoins, la coévolution entre les virus et leurs cellules hôtes a permis à certains virus de développer des mécanismes permettant d'inhiber l'autophagie, voire de se servir des vésicules produites au cours du processus afin de favoriser leur réplication [21]. Certains virus empêchent le déclenchement de l'autophagie, ce qui leur permet de persister dans la cellule qu'ils ont infectée. De nombreuses protéines virales ciblent ainsi tout particulièrement la bécline-1, une protéine essentielle pour enclencher le mécanisme d'autophagie. C'est le cas notamment de HSV (*Herpes simplex virus*), KHSV (*Kaposi's sarcoma-associated herpesvirus*), MHV (*mouse hepatitis virus*) ou HCMV (*human cytomegalovirus*). Certains virus (comme KHSV) peuvent également stimuler la voie de signalisation impliquant mTOR (*mammalian target of rapamycin*), un répresseur majeur de l'autophagie [22].

Une autre stratégie adoptée par les virus pour se protéger de la dégradation autophagique est le blocage de la fusion des autophagosomes avec les lysosomes. Cette stratégie a un double avantage pour les virus : non seulement elle les protège de la dégradation par les lysosomes, mais aussi elle leur fournit de très nombreuses vacuoles à double membrane, idéales pour leur réplication. Le premier virus pour lequel cette stratégie a été décrite est le virus de la poliomyélite [23]. Depuis, de nombreux virus se sont révélés capables de bloquer la phase de maturation de l'autophagie afin de bénéficier des vésicules à double membrane comme plateformes pour leur réplication.

Relations entre virus, autophagie et développement tumoral

Virus oncogéniques et autophagie

Bien que le cancer soit une maladie multifactorielle, combinant des prédispositions génétiques et des facteurs environnementaux, il est désormais établi qu'environ 15 % des cancers chez l'homme ont pour origine une infection virale, en particulier par les virus EBV (*Épstein-*

Barr virus), HBV ou HCV (*hepatitis B or C viruses*), HPV (*human papillomavirus*), HTLV-1 (*human T lymphotropic virus of type 1*), KHSV, ou MCPyV (*Merkel cell polyomavirus*) [24]. Bien que différents, aussi bien en termes de structure et de tropisme cellulaire que de cycle viral, ces virus sont tous à l'origine d'une infection chronique, un critère qui semble essentiel à leur pouvoir oncogénique.

Afin de se maintenir dans la cellule infectée, ces virus ont tous développé des mécanismes moléculaires pour stabiliser leur génome dans la cellule qui les héberge, empêcher sa mort, et échapper à la reconnaissance et à la lyse par les cellules immunitaires [24]. Des protéines impliquées dans le contrôle du cycle cellulaire, telles que p53 et pRb (protéine du rétinoblastome), sont parmi les cibles communes des oncoprotéines codées par le génome de ces virus. La dérégulation induite par ces virus apporte aux cellules infectées une capacité de prolifération, alors incontrôlée, qui facilite le développement de la tumeur. L'intégration du génome viral dans celui de la cellule hôte modifie également l'expression de gènes qui codent des protéines participant à la régulation de l'homéostasie cellulaire, ce qui peut contribuer à la transformation d'une cellule normale en une cellule cancéreuse. Les virus oncogéniques ont ainsi développé des stratégies permettant l'inhibition de la phase d'initiation de l'autophagie, favorisant à la fois leur persistance dans la cellule hôte, mais également la tumorigenèse des cellules infectées. Le virus HPV de type 16, par exemple, inhibe l'autophagie de différentes manières : ses protéines, telles que HPV16 E5, E6 et E7, activent le répresseur de l'autophagie mTOR, inhibent le déclenchement de l'autophagie, et empêchent la fusion des autophagosomes avec les lysosomes [25]. Chez l'homme, on a ainsi montré que l'infection de cellules tumorales par HPV (notamment chez des patients atteints de cancer anal) diminuait leur activité autophagique ; dans des modèles murins, l'inhibition de l'autophagie induite par le virus augmente la survenue de tumeurs [26].

Les virus HBV et HCV, à l'origine du cancer du foie, ont également développé des mécanismes inhibant le processus d'autophagie, notamment la phase de dégradation, ce qui favorise leur réplication [27]. Chez des patients atteints d'un cancer hépatique, cette inhibition de l'autophagie dans les cellules tumorales infectées par HBV permet, entre autres, d'augmenter le niveau d'expression de miR-224, un micro-ARN qui favorise le développement du cancer. De même, la protéine non structurale NS4B du virus HCV inhibe la phase terminale du processus autophagique. Ceci conduit à l'accumulation de la protéine autophagique p62, ce qui

³ Le virus Sindbis appartient au genre Alphavirus et à la famille des Togaviridae. Il est transmis par des moustiques *Culex* et provoque une fièvre, des arthralgies, un exanthème et des maux de tête.

stimule la voie dépendant du facteur de transcription Nrf2 (*nuclear factor [erythroid-derived 2]-like 2*) et favorise la tumorigenèse des cellules hépatiques infectées par le virus.

Le cycle des virus KHSV et EBV se décompose en deux phases : une phase de latence et une phase lytique. Leurs propriétés oncogéniques sont liées à la phase de latence, caractérisée par une diminution importante de l'activité autophagique de la cellule infectée. KHSV inhibe le processus en ciblant différentes molécules régulatrices et modulatrices de la cascade autophagique. La subversion de l'autophagie induite par le virus perturbe la sénescence cellulaire, ce qui favorise la prolifération des cellules infectées, deux conditions qui participent à la tumorigenèse cellulaire [28]. Néanmoins, le possible lien entre inhibition de l'autophagie et oncogénèse des cellules infectées reste à démontrer. Le virus EBV inhibe l'autophagie en activant le répresseur mTOR [29]. Cette perturbation est ainsi à l'origine d'une accumulation de p62 dans le noyau cellulaire, avec pour conséquences, un stress oxydant et une efficacité réduite de la réponse aux dommages de l'ADN, deux facteurs pouvant favoriser l'oncogénèse. Le virus HTLV-1, par l'action de sa protéine Tax-1, et le virus MCPyV réduisent, quant à eux, l'activité autophagique dans les cellules infectées en stimulant l'expression de plusieurs micro-ARN, favorisant l'apparition de cellules pré-cancéreuses [30].

Virus oncolytiques et autophagie

Certains virus sont à l'origine d'une régression tumorale. Ces virus, dits oncolytiques, ont été testés pour le traitement de certains cancers, notamment dans les cas de résistance acquise aux traitements conventionnels. Ces virus induisent une réponse anti-cancéreuse durable au cours du temps, car en plus d'infecter préférentiellement les cellules tumorales et de les lyser, ils provoquent la libération de molécules capables de stimuler la réponse immunitaire anti-tumorale [31]. Cette approche thérapeutique est particulièrement intéressante pour le traitement des tumeurs dites « froides », qui sont peu infiltrées par des cellules immunitaires [57] (→).

(→) Voir la Synthèse de J. Pol et al., *m/s* n° 2, février 2013, page 165

Virus oncolytiques et mort autophagique immunogène

Les virus oncolytiques exploitent différentes voies de mort cellulaire pour induire la lyse des cellules qu'ils infectent. Les parvovirus ou le virus NDV (*Newcastle disease virus*) induisent une apoptose dans de très nombreux cancers [32]. D'autres virus, comme les virus de la vaccine ou le virus de la variole, induisent préférentiellement une nécrose, notamment dans les cancers du côlon et de l'ovaire ou dans le mélanome [33]. Certains autres virus oncolytiques conduisent à une mort cellulaire qui dépend du processus autophagique.

Bien que l'autophagie ait été décrite comme un mécanisme par lequel la cellule compense les différents stress qu'elle subit afin de survivre dans des conditions défavorables, un excès d'autophagie peut lui être néfaste et conduire à sa mort. L'implication de l'autophagie dans la mort cellulaire programmée a été décrite pour la première fois dans une étude où des cellules ont été soumises à une carence nutritive importante et prolongée. Les auteurs ont remarqué la présence d'un

nombre de vésicules autophagiques plus important dans les cellules mourantes que dans les cellules vivantes, suggérant qu'un stress prolongé serait responsable d'une hyperactivité autophagique associée à une consommation excessive de matériel cytosolique [34]. L'existence même d'un tel processus, appelé mort cellulaire dépendant de l'autophagie (dont l'abréviation en anglais est ADCD, pour *autophagy-dependent cell death*), fut longtemps contestée. L'impact fonctionnel de l'autophagie dans des cellules mourantes n'est en effet pas facilement démontrable : l'autophagie pourrait être aussi bien une réponse compensatoire de la cellule stressée, essayant de survivre, qu'un mécanisme qui agit directement, ou indirectement, sur l'induction d'une voie de mort cellulaire. Mais depuis ces premières descriptions, de très nombreuses études ont montré l'implication de l'autophagie dans l'induction de voies de mort cellulaire, soit directement en dégradant des inhibiteurs de ces voies (comme K-ras pour l'apoptose, la ferritine pour la ferroptose, la catalase pour la nécrose), soit indirectement, par l'utilisation des vacuoles autophagiques comme plateformes d'assemblage des complexes de mort cellulaire [34]. Toutes ces interrelations entre autophagie et voies de mort cellulaire ont contraint les chercheurs à préciser ce que recouvre le terme ADCD, aujourd'hui défini comme un type de mort cellulaire qui requiert l'action de composants moléculaires de la machinerie autophagique, et dont l'inhibition, génétique ou chimique, empêche la mort de la cellule [35].

Certains virus oncolytiques peuvent ainsi induire une ADCD. C'est notamment le cas des adénovirus, du virus NDV, du virus de la rougeole et du virus HSV dans de nombreux types de cancers [36]. Aujourd'hui, les thérapies fondées sur l'utilisation de virus oncolytiques visent à déclencher une ADCD afin de détruire plus efficacement les cellules tumorales. La stratégie la plus couramment utilisée pour stimuler l'ADCD consiste en l'ajout, dans le génome viral, d'un transgène qui permet la surexpression de la bécline-1 dans la cellule infectée. Cette stratégie a été appliquée au virus de la vaccine ou à des adénovirus, chez la souris, avec des résultats prometteurs dans les leucémies ou les myélomes, pour lesquels une diminution de la masse tumorale a été observée. L'implication de l'autophagie dans ce processus a été démontrée par sa disparition lorsqu'on inactive les gènes de l'autophagie [34].

Autophagie et mort cellulaire immunogène

Malgré leur capacité intrinsèque de lyse des cellules cancéreuses qu'ils infectent, les virus oncolytiques nécessitent l'induction d'une réponse immunitaire

anti-tumorale pour avoir un réel impact dans le temps sur le développement des cancers. Cette capacité d'induire une réponse immunitaire à la suite de la lyse des cellules infectées est appelée mort cellulaire immunogène. Elle est caractérisée par l'exposition, à la surface des cellules mourantes, de la calréticuline (un signal « *eat me* », en français, « mange-moi ») et par la libération d'un cocktail de molécules, telles que des cytokines, des signaux de dangers (ATP, HMGB1 [*high mobility group box 1*], etc.), ou des antigènes. Tous ces éléments qui favorisent le recrutement et l'activation des cellules immunitaires dans le microenvironnement tumoral sont présents dans la majorité des cancers dont les cellules ont été lysées par des virus oncolytiques. Bien que les mécanismes impliqués dans la mise en place d'une réponse immunogène par les virus oncolytiques ne soient pas totalement élucidés, il semble que l'autophagie des cellules mourantes y contribue. L'équipe dirigée par Beth Levine (1960-2020)⁴ a montré, dans un article princeps, que des cellules déficientes pour le mécanisme d'autophagie et mourantes ne sont pas reconnues et éliminées efficacement par les cellules immunitaires, contrairement aux cellules compétentes pour l'autophagie. Cet échappement immunitaire des cellules déficientes pour l'autophagie provient de leur incapacité d'exprimer à leur surface des signaux « *eat me* » de reconnaissance et de sécréter efficacement des molécules immunogènes [37]. Cette découverte sera ensuite confirmée dans différents modèles tumoraux, pour lesquels l'autophagie est requise pour la libération de molécules immunogènes après la lyse des cellules. L'équipe dirigée par Guido Kroemer a par exemple montré que seules les tumeurs compétentes pour l'autophagie sont capables d'attirer des cellules immunitaires et d'induire une réponse immunitaire anti-tumorale après traitement par différents composés cytolytiques [38]. Dans les cancers de la prostate, les adénovirus favorisent ainsi la libération d'ATP et d'HMGB1 par un mécanisme qui dépend de l'autophagie [39]. De même, dans les cancers du poumon, dans le glioblastome ou dans le mélanome, les virus NVD ou HSV induisent une mort immunogène *via* leur action sur l'autophagie des cellules qu'ils infectent, en augmentant notamment la sécrétion d'ATP, d'HMGB1 et d'HSP (*heat shock protein*) 79/90, mais aussi de certaines cytokines pro-inflammatoires : TNF- α (*tumor necrosis factor α*), IL-1 β et GM-CSF (*granulocyte macrophage colony-stimulating factor*) [40]. Certains adénovirus sont également capables, en induisant une autophagie, de favoriser la présentation d'antigènes tumoraux *via* les molécules du complexe majeur d'histocompatibilité, augmentant ainsi l'activation de l'immunité anti-tumorale [41].

L'induction de l'autophagie dans des cellules cancéreuses infectées par des virus oncolytiques est donc impliquée dans la lyse directe des cellules tumorales, mais elle participe également à la mise en place d'une réponse immunitaire efficace limitant le développement tumoral. De nombreuses études sont actuellement en cours en vue d'une utilisation thérapeutique de virus oncolytiques recombinants capables d'induire plus efficacement le processus autophagique dans les cel-

lules tumorales (en utilisant, par exemple, des adénovirus ou le virus de la vaccine exprimant la bécline-1) [42, 43].

Concordance de l'impact des infections virales et de l'autophagie sur la progression tumorale

Un nombre de plus en plus important d'études montrent l'impact des infections virales sur l'évolution des cancers. En plus des virus oncogéniques et oncolytiques, qui ont un rôle important dans la transformation tumorale et la lyse des cellules cancéreuses, d'autres virus peuvent participer à la progression tumorale, en influençant notamment la survie des cellules infectées, l'échappement immunitaire, l'angiogenèse, le développement de métastases ou la résistance aux traitements anti-cancéreux. Les mécanismes impliqués sont vraisemblablement divers. Néanmoins, de plus en plus de travaux révèlent l'existence d'une relation entre modification de l'autophagie par les virus et devenir des tumeurs.

Survie des tumeurs et modification de l'immunité anti-tumorale

De nombreux virus favorisent la survie des cellules qu'ils infectent en inhibant, notamment, l'apoptose ou d'autres voies de mort cellulaire programmée. Même si différents mécanismes peuvent être impliqués, l'autophagie semble jouer un rôle important. L'induction de l'autophagie dans les cellules infectées limite, par exemple, leur apoptose en réprimant l'expression des protéines pro-apoptotiques *via* la séquestration ou la dégradation des mitochondries, un processus notamment décrit dans le cas de cellules tumorales infectées. Dans des tumeurs pulmonaires, l'infection par le virus NDV induit en effet un mécanisme d'autophagie qui séquestre le cytochrome c (un facteur pro-apoptotique sécrété par les mitochondries) et empêche ainsi la mort des cellules tumorales [44]. Dans des cancers du cerveau, l'infection par le virus HTLV-1 induit une autophagie protectrice capable d'inhiber la mort cellulaire apoptotique induite par les récepteurs de mort, comme FAS-L (le ligand de la protéine Fas) et TRAIL (*tumor-necrosis-factor-related apoptosis-inducing ligand*) [45]. Plus récemment, il a été montré que l'induction d'une autophagie par le virus HBV favorisait la survie des cellules infectées et le développement du cancer du foie [46].

L'autophagie induite par une infection virale peut protéger les cellules cancéreuses non seulement de la mort cellulaire, mais également de l'effet de stress environnementaux ou d'attaques par le système immunitaire.

⁴ Beth Levine a découvert le rôle de la bécline-1 dans l'autophagie en 1998.

En plus de limiter l'apoptose, la séquestration/dégradation sélective des mitochondries *via* l'autophagie permet de diminuer les réponses aux stress et la réponse immunitaire en limitant notamment la production d'espèces réactives de l'oxygène et la formation du complexe de l'inflammasome responsable de la production de cytokines pro-inflammatoires. L'autophagie induite par les virus peut également diminuer la production de cytokines en dégradant directement des facteurs cellulaires impliqués dans la réponse inflammatoire. L'infection par le virus HPV16 dans les cancers oro-pharyngés en fournit un exemple. En effet, dans les cellules tumorales infectées, l'autophagie induite par ce virus favorise la dégradation de STING (*stimulator of interferon genes*), un senseur cellulaire impliqué dans la production de cytokines inflammatoires en réponse aux virus [47]. La voie NF- κ B (*nuclear factor- κ B*) est également une cible de l'autophagie, avec pour conséquence, la perturbation de la réponse des cellules immunitaires du microenvironnement tumoral [48].

Les cellules cancéreuses sont également protégées de l'action des cellules immunitaires cytotoxiques par l'induction de l'autophagie, qui dans des conditions d'hypoxie, permet la capture et la dégradation des granzymes B produits par les lymphocytes T cytotoxiques et les cellules NK (*natural killer*) [49].

De même, dans les cancers du poumon, le déficit en protéines autophagiques réduit la phosphorylation de STAT3, un facteur de transcription important pour la résistance des tumeurs à la lyse par les lymphocytes T cytotoxiques [50]. Étant donné que de nombreux virus induisent l'activation de STAT3 dans les cellules infectées afin de favoriser la survie des tumeurs, il serait intéressant d'analyser le rôle de l'autophagie dans ce contexte.

Une autre stratégie favorisant l'échappement des cellules infectées par un virus à la lyse par les lymphocytes T cytotoxiques repose sur la réduction de l'expression des molécules du complexe majeur d'histocompatibilité de classe I (CMH-I) par les cellules infectées (par le virus VIH, le virus de la grippe ou le virus de la varicelle) ou par les cellules tumorales. Le rôle de l'autophagie dans ce processus n'est cependant pas clairement établi, bien qu'elle favorise l'endocytose des molécules du CMH-I et leur dégradation, et que son inhibition conduise à une présentation antigénique accrue, à la mise en place d'une réponse immunitaire plus importante et à une croissance tumorale fortement diminuée dans un modèle de cancer du pancréas [51].

Formation de métastases et résistance aux traitements anti-cancéreux

Les infections virales favorisent également l'angiogenèse, une étape majeure pour le développement des tumeurs, la dissémination des cellules tumorales dans l'organisme et le développement de métastases. Ce processus repose sur la production accrue de facteurs angiogéniques, comme le VEGF (*vascular endothelial growth factor*), et de facteurs chimio-attractants. Le virus HCV, en stimulant la production de CCL20 (*C-C motif chemokine ligand 20*), favorise la migration et l'invasion de cellules endothéliales dans la tumeur [52]. Les virus EBV, HPV, HSV2, HBV ou HCMV stimulent également l'angiogenèse et la formation de métastases de différents cancers. Comme l'infection virale,

l'autophagie est un acteur important de la formation de métastases : elle favorise la survie des cellules tumorales lors de la transition épithélio-mésenchymateuse, ainsi que leur migration. Bien que le lien entre virus et induction autophagique dans le cadre du développement de métastases ne soit pas encore établi, une étude récente montre que, dans le cancer hépatique, l'infection par le virus HBV, *via* l'induction d'une autophagie, favorise l'expression du TGF- β (*transforming growth factor β*) et du lncRNA-ATB (*long noncoding RNA activated by TGF- β*), deux molécules impliquées dans la migration et l'invasion des cellules tumorales [53].

Les infections virales des cellules cancéreuses ont été associées à leur résistance à différents traitements anti-cancéreux, en inhibant l'apoptose induite par ces traitements, comme c'est le cas par exemple du virus EBV pour la chimiothérapie [54], ou des virus HPV16 et EBV pour la radiothérapie [55]. L'infection par le virus EBV, en activant la voie Akt (ou protéine kinase B), conduit également à une résistance des lymphocytes B au rituximab, un anticorps monoclonal dirigé contre ces cellules et qui induit leur lyse [56]. L'implication de l'autophagie dans les mécanismes de la résistance aux traitements anti-cancéreux associée aux infections virales reste cependant à déterminer. Notons néanmoins qu'autophagie et infection virale ont des effets similaires sur la résistance aux traitements, l'un modulant l'autre et tous deux inhibant principalement l'apoptose.

Conclusion

Selon le type et le stade de la maladie cancéreuse, le degré d'activité autophagique des cellules tumorales peut avoir différentes conséquences sur ces cellules. Au cours de l'étape initiale du développement tumoral, les cellules accumulent des dommages qui affectent les molécules impliquées dans le cycle cellulaire et favorisent le processus de tumorigenèse. Par ailleurs, les gènes suppresseurs de tumeurs et les oncogènes participent au contrôle de l'autophagie, reliant ainsi l'autophagie à l'apparition du cancer. Les virus oncogènes, qui conduisent à la transformation de cellules pré-cancéreuses en cellules cancéreuses, inhibent l'autophagie (Figure 2). Dans les cellules infectées, cette inhibition favorise l'accumulation d'organelles ou de protéines endommagées, à l'origine de molécules de stress pour la cellule et de mutations géniques. En revanche, plusieurs virus oncolytiques induisent un processus d'autophagie qui permet la reconnaissance des cellules tumorales mourantes par les cellules de l'immunité et leur élimination. À

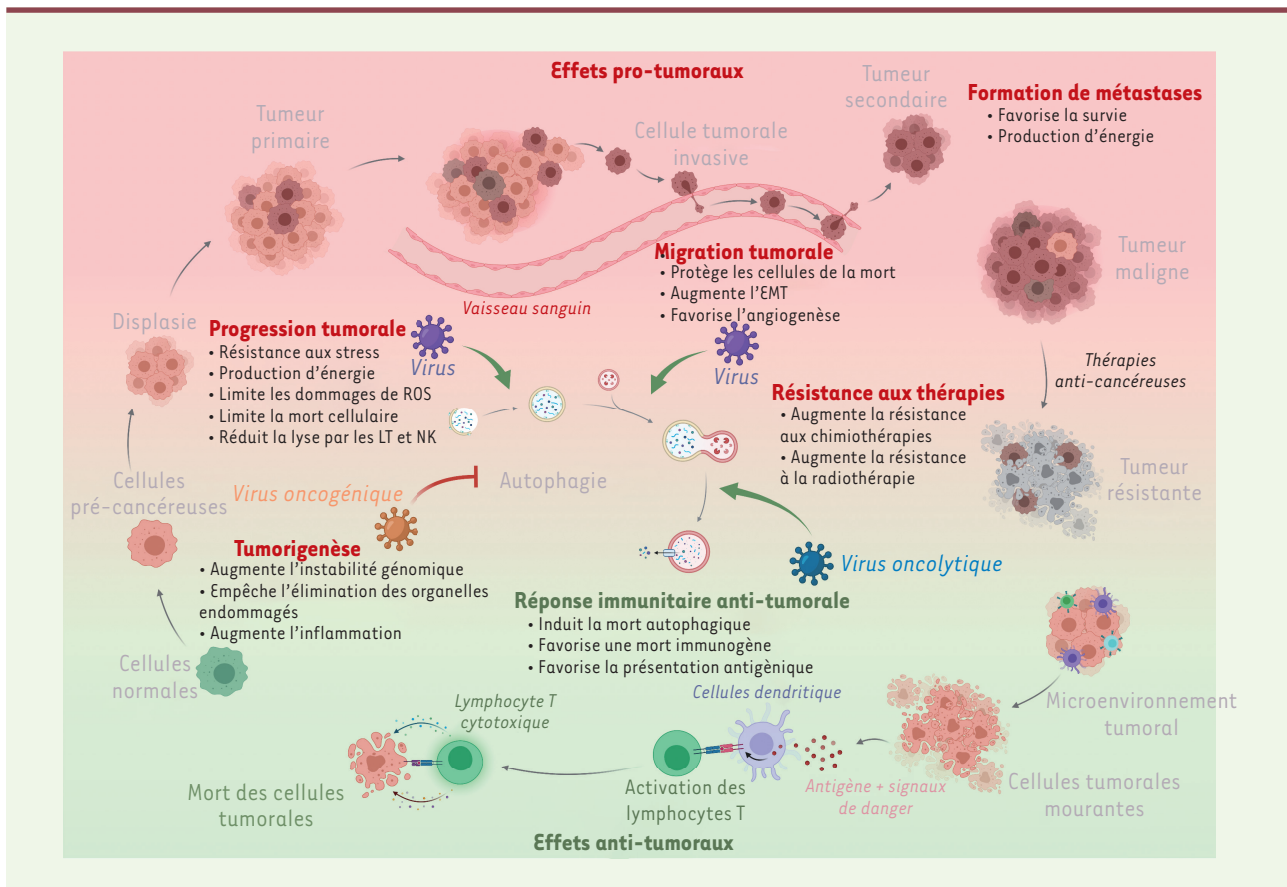


Figure 2. Impact de la modulation de l'autophagie par les virus dans un contexte tumoral. Les infections virales activent (flèche verte) ou inhibent (flèche rouge) le processus d'autophagie dans les cellules tumorales. L'impact de ces modifications sur le développement de la tumeur est indiqué : les titres en vert correspondent à un effet anti-tumoral, et les titres en rouge à un effet pro-tumoral. LT : lymphocyte T ; NK : cellule *natural killer* ; EMT : *epithelial-mesenchymal transition* ; ROS : espèces réactives de l'oxygène (figure créée avec BioRender.com).


un stade plus avancé de la maladie, l'autophagie est susceptible de favoriser la croissance de la tumeur (Figure 2) en apportant aux cellules en train de proliférer les nutriments nécessaires. L'autophagie joue un rôle important dans la survie des cellules cancéreuses en favorisant notamment leur résistance à plusieurs types de thérapies. Elle peut également participer à la formation de métastases en protégeant de l'anoïkose, lors de leur migration dans les vaisseaux sanguins, les cellules tumorales qui se sont détachées de la tumeur primaire et qui iront établir de nouvelles colonies à distance. En modulant le processus autophagique, de nombreux virus vont ainsi agir sur le développement tumoral.

La relation entre l'autophagie et le développement tumoral n'est pas établie. En revanche, l'existence d'un lien important entre l'autophagie et la perturbation du développement des tumeurs induite par les infections virales a été suggérée. Des études supplémentaires sont nécessaires pour comprendre les mécanismes reliant les infections virales et l'autophagie dans le contexte du développement tumoral. Cela pourrait déboucher, à terme, sur de nouvelles thérapies anticancéreuses et contre d'autres maladies marquées par une prolifération cellulaire. ♦

SUMMARY

Autophagy modulation by viruses: An important role in tumor progression

Autophagy is an important process for cellular homeostasis at critical steps of development or in response to environmental stress. In the context of cancers, autophagy has a significant impact on tumor occurrence and tumor cell growth. On the one hand, autophagy limits the transformation of precancerous cells into cancer cells at an early stage. However, on the other hand, it promotes cell survival, cell proliferation, metastasis and resistance to anti-tumor therapies in more advanced tumors. Autophagy can be induced by a variety of extracellular and intracellular stimulus. Viral infections have often been associated with a modulation of autophagy, with variable impacts on viral replication and on the survival of infected cells depending on the model studied. In a tumor context, the modulation of autophagy induced by the viral infection of tumor cells seems to have a significant impact on tumor progression. The aim of this

review article is to present recent findings regarding the consequences of autophagy disturbance by viral infections on tumor behavior. 

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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TIRÉS À PART

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