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NILTON DE FRANÇA JUNIOR

GALECTIN-9 BINDS TO TRAIL AGONIST RECEPTORS AND REGULATES TRAIL-INDUCED APOPTOSIS

CURITIBA

June/2021

NILTON DE FRANÇA JUNIOR

Galectin-9 binds to TRAIL agonist receptors and regulates TRAIL-induced apoptosis

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Pontifícia Universidade Católica do Paraná, como requisito à obtenção do título de Doutor em Ciências da Saúde.

Orientadora: Drª. Selene Elifio Esposito
Co-orientador: Dr. Olivier Micheau

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Galectina-9 se liga a receptores agonistas TRAIL e regula à apoptose induzida por TRAIL

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Programa de Pós Graduação em Ciências da Saúde

(PPGCS) Pontifícia Universidade Católica do Paraná

Prof. Dr. Christopher Benedict

La jolla Institute for Allergy and Immunology (LJI), San Diego,

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Université Claude Bernard, Lyon, França

Profª. Drª. Johanna Chluba

INSERM UMR1231

Université de Bourgogne, Dijon, França

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Galectin-9 binds to TRAIL agonist receptors and regulates TRAIL-induced apoptosis

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Dr. Olivier MICHEAU, Directeur de Recherche, InsermU1231	Directeur de thèse

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Pontifícia Universidade Católica do Paraná
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PUBLIC SESSION OF THESIS EXAMINATION FROM THE GRADUATE PROGRAM IN HEALTH SCIENCES, (DOCTORATE DEGREE), PONTIFICIAL CATHOLIC UNIVERSITY OF PARANA

On june 25, 2021 took place at the Center for Health Sciences the public session of thesis examination entitled, **"Galectin-9 binds to TRAIL agonist receptors and regulates TRAIL-induced apoptosis"** presented by **Nilton de França Junior**, candidate for a doctor degree in Health Science.

The Board of examiners was composed for the following members:

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In accordance with the program´s regulations, the Board of Examiners presented their evaluation, which were the following

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Prof. Dr. Olivier Micheau — Evaluation: approved
Profa. Dra. Andrea Novais Moreno Amaral — Evaluation: approved
Prof. Dr. Christopher Benedict — Evaluation: approved
Prof. Dr. Roger Chammas — Evaluation: approved
Prof. Dr. Serge Lebecque — Evaluation: approved
Profa. Dra. Johanna Chluba — Evaluation: approved

Final decision: Approved

Notes from the Examining Board:

The committee is convinced that the student has met all the requirements: he has given a very good presentation of his data and answered the committee's questions confidently.

Due to the COVID-19 pandemic, the defense occurred remotely. For this reason, the president of the evaluation committee signs these minutes on behalf of the other members.

Profa. Dra. Selene Elifio Esposito
President of the Examining Board

Prof. Dr. Cristina Pellegrino Baena
Coordinator of PPGCS PUCPR

Dedication

I dedicate this work
To my parents Nilton de França e Joraci de França
For the support they always gave me, for the incentive to go further and further.
I also dedicate to my advisors, Olivier and Selene, source of inspiration
Likewise, I dedicate to my wife, Amanda, example of wisdom.

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ABSTRACT

APO2L/TRAIL (TNF-related apoptosis-inducing ligand) arouses great interest in cancer therapy. This protein induces apoptosis in tumor cells through DR4 or DR5, two transmembrane glycoproteins that harbor N- and O-glycosylations, respectively. The glycosylation of DR4 or DR5 receptors is likely to allow unforeseen protein/protein interactions. For instance, galectins, owing to their ability to bind to oligosaccharides, can interact with glycoproteins, and are therefore potentially able to regulate TRAIL pro-apoptotic machinery in tumor cells. Given in addition that conventional chemotherapeutic drugs such as 5-fluorouracil (5FU), often restore or increase TRAIL-induced apoptosis, but that the molecular mechanism underlying this gain of function remains elusive, we investigated the possibility that galectins may link restoration of TRAIL sensitivity upon sequential chemotherapy. We provide evidence, here, that 5FU sensitizes tumor cells to TRAIL-induced apoptosis through its ability to induce the secretion of galectin-9 into the extracellular medium. Mechanistically, the binding of secreted galectin-9 with the extracellular domains of DR4 and/or DR5, increased apoptosis triggered by TRAIL. This gain of function and interaction was strictly related to the sugar moiety harbored byDR4 and DR5. Likewise, point mutations of the glycosylation sites or production of the receptors in prokaryotic cells not only abrogatedgalectin-3 and 9 binding, but also restoration of TRAIL sensitivity by 5FU. However, when the receptors are properly glycosylated, overexpression of galectins or addition of soluble recombinant of galectin-3 or -9 alone, is sufficient to increase tumor cell sensitivity to TRAIL-induced cell death. Overall, our results suggest that galectins are novel components of TRAIL's DISC whose regulation by 5FU helps explain, at least in part, how this conventional chemotherapy increases the therapeutic potential of TRAIL.

Keywords: Glycosylation, TRAIL, receptor, galectin

RESUMO

APO2L/TRAIL (ligante indutor de apoptose relacionado ao TNF) desperta grande interesse na terapia do câncer. Ao contrário da maioria dos tratamentos comumente usados clinicamente, esta proteína induz apoptose em células tumorais através de DR4 ou DR5, duas glicoproteínas transmembrana que abrigam N- e O- glicosilações, respectivamente. A glicosilação dos receptores DR4 ou DR5 permite que algumas moléculas, como as galectinas, reconheçam a sequência formada pelo oligossacarídeo e, portanto, se liguem a essa glicoproteína, podendo definir a via apoptótica da célula tumoral. Além disso, o 5-fluorouracil (5FU) é uma quimioterapia conhecida por restaurar ou aumentar a apoptose induzida por TRAIL, mas o mecanismo molecular subjacente a esse ganho de função permanece indefinido. Nós fornecemos evidências, de que 5FU sensibiliza as células tumorais à apoptose induzida por TRAIL por meio de sua capacidade de induzir a liberação de galectina-9 para o meio extracelular. Assim como no DISC (Sinal de Indução de Morte Complexo) componentes pró-apoptóticos, proteínas de ligação a carboidratos, como galectina-3 ou 9, por meio de interações diretas com receptores DR4 e DR5 são capazes de contribuir para apoptose induzida por TRAIL. Mecanisticamente, descobriu-se que a galectina-3 ou 9 interage especificamente com o domínio extracelular de DR4 e DR5, aumentando à apoptose desencadeada por TRAIL. A interação com DR4 ou DR5 foi estritamente relacionada ao estado de glicosilação dos receptores. A perda da glicosilação dos receptores DR4 e DR5 induzida por local dirigido a mutação ou produção em células procarióticas prejudicou a ligação da galectina-3 e 9. Os resultados obtidos também mostram que a superexpressão ou adição de recombinante solúvel de galectina-3 ou -9 por si só foi suficiente para aumentar a sensibilidade do TRAIL. Ao todo, nosso resultados revelam as galectinas como novos componentes TRAIL DISC, cuja regulação por 5FU, seja provavelmente responsáveis por sua capacidade de sinergia com TRAIL.

Palavras Chaves: Glicosilação, TRAIL, receptor, galectina.

RESUMÉ

APO2L/TRAIL (ligand inducteur d'apoptose lié au TNF) suscite un grand intérêt dans le domaine de l'oncologie. Contrairement à la plupart des traitements couramment utilisés en clinique, cette protéine induit l'apoptose dans les cellules tumorales par DR4 ou DR5, deux glycoprotéines transmembranaires qui hébergent des sites de N- et O-glycosylations, respectivement. La glycosylation des récepteurs DR4 ou DR5 est susceptible de permettre des interactions protéines/protéines imprévues. En raison de leur capacité à se lier aux oligosaccharides, les galectines peuvent, par exemple, interagir avec les glycoprotéines, et sont donc potentiellement capables de réguler la machinerie pro-apoptotique TRAIL dans les cellules tumorales. Étant donné en outre que les chimiothérapies conventionnelles, telles que le 5-fluorouracile (5FU), rétablissent ou augmentent souvent l'apoptose induite par TRAIL, sans que le mécanisme moléculaire sous-jacent à ce gain de fonction soit connu, nous avons cherché à savoir si les galectines pouvaient expliquer le rétablissement de la sensibilité des cellules tumorales à TRAIL au cours des chimiothérapies séquentielles. Nous apportons la preuve que le 5FU sensibilise les cellules tumorales à l'apoptose induite par TRAIL grâce à sa capacité à induire la sécrétion de la galectine-9 dans le milieu extracellulaire. D'un point de vue mécanistique, la liaison des galectines-3 ou -9 sécrétées avec les domaines extracellulaires des DR4 et/ou DR5, accroît l'apoptose déclenchée par TRAIL. Ce gain de fonction et d'interaction est strictement dépendant de l'état de glycosylation et donc des sucres présents sur DR4 et DR5. Ainsi, des mutations ponctuelles des sites de glycosylation ou de la production de récepteurs solubles dans les cellules procaryotes abrogent la liaison des galectines-3 et -9 aux récepteurs DR4 et DR5, ainsi que la restauration de la sensibilité à TRAIL par le 5FU. Cependant, lorsque les récepteurs sont correctement glycosylés, la surexpression des galectines ou l'ajout de versions recombinantes soluble de la galectine-3 ou -9, seules, suffisent à augmenter la sensibilité des cellules tumorales à la mort cellulaire induite par TRAIL. Dans l'ensemble, nos résultats suggèrent que les galectines sont de nouveaux composants du DISC de TRAIL dont la régulation par le 5FU permet d'expliquer, au moins en partie, comment cette chimiothérapie conventionnelle accroît le potentiel thérapeutique de TRAIL.

Mots clés: Glycosylation, TRAIL, récepteur, galectine.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5FU: 5-Fluorouracil

7-AAD: 7-Aminoactinomycin D

A1/BFL-1: Bcl-2-related protein A1

AAL: *Aleuria aurantia lectin*

AKT: Activated kinase protein

Apaf-1: Apoptotic protease activating factor- 1

ATCC: American type culture collection

Bad: Bcl-2 antagonist of cell death

Bak: Bcl-2 antagonist killer 1

Bax: Bcl-2 associated x protein

Bcl-2: B-cell lymphoma 2

Bcl-W: Bcl-2 like 2

Bcl-xL: Bcl-2-related gene X, long isoform

Bid: BH3 interacting domain death agonist

Bid: BH3-interacting domain death agonist

Bik: Bcl -2 interacting killer

Bim: Bcl-2 interacting mediator of cell death

Bmf: Bcl-2-modifying factor

Bok: Bcl-2 related ovarian killer

CARD: Caspase recruitment domain

CAS9: CRISPR associated protein 9

CerS6: Ceramide synthase 6

cFLIPL/S: Cellular Flice inhibitory protein long/short

cIAP: Cellular Inhibitor of apoptosis

cl-CD95L: Cleaved CD95 ligand

Con A: *Concanavalin A*

CRD: Carbohydrate recognition domain

CRD: Cysteine-rich domain

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

dATP: Deoxyadenosine triphosphate

DD: Death domain

DED: Death effector domain

DIABLO: Direct IAP binding protein with

DISC: Death-inducing signaling complex

DNA: Deoxyribonucleic acid

EDAR: Ectodysplasin A receptor

EDARADD: Ectodysplasin-A receptor-associated adapter protein

EGFR: Epidermal growth factor receptor

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

FADD: Fas-Associated death domain

FasL/CD95L: Fas ligand/CD95 ligand

FCS: Fetal calf serum

FdUMP: Fluorodeoxyuridine monophosphate

FdUTP: Fluorodeoxyuridine triphosphate

FITC: Fluorescein isothiocyanate

FUTP: Fluorouridine triphosphate

Gal-1: Galectin-1

Gal-3: Galectin-3

Gal-8: galectin-8

Gal-9: Galectin-9

GALNT14: UDP-N-acetyl-alpha-D-galactosamine:N- cetylgalactosaminyltransferase polipeptide 14

GBPs: Glycan-binding proteins

GC: Golgi complex

GLcNAC: N-acetylglycosamine

GMDS: GDP-mannose-4,6-dehydratase

GPI:Glycosyl-Phosphatidyl-Inositol

gRNA: Guide RNA

HCT116: Colorectal cancer cells

His-TRAIL: Polyhistidine trail

HRK: Harakiri

IZ-TRAIL: Isoleucine zipper

IκB: Inhibitor of NF-κB

JNK: c-Jun NH2 terminal Kinase

LGALS9: Gene galectin -9

LZ-TRAIL: Leucine zipper residues

MAL I: Maackia amurensis lectin I

MAL II: Maackia amurensis lectin II

Man: Mannose

MAPK: Mitogen-activated protein Kinase

Mcl1: Myeloid cell leukemia 1

MCMV: Mouse cytomegalovirus

MDA-MB-231: Triple-negative breast carcinoma

MGAT4: mannosyl (alpha-1,3) –glycoprotein beta-1,4-acetylglucosaminyltransferase

MOI: Multiplicity Of Infection

mRNA: Messenger ribonucleic acid

NF-κB: Nuclear factor-κB

NK: Natural killer

NOXA: Bcl-2-modifying factor

OPG: Osteoprotegerin

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate Buffer Saline

PHA: *Phaseolus vulgaris*

PI3K PhosphatidylInositol-3-Kinase

PNA: *Peanut agglutinin*

PTEN: Phosphatase and tensin homolog

PUMA: Bcl-2-modifying factor

PYD: Pyrin Domain

RCA: *Ricinus communis agglutinin*

rh-TRAIL: Version of recombinant human TRAIL

RIPK1: Receptor-interacting serine/threonine-protein kinase 1

RNA: Ribonucleic acid

siRNA: Interference RNA

Smac: Second mitochondrial activator of caspases

SNA: *Sambucus nigra allgutinin*

ST6Gal-I: ST6 beta-galactoside alpha-2,6-Sialyltransferase

TALEN: Transcription Activator

tBid: Truncated Bid

TNF: Tumor necrosis factor

TRADD: TNFR1-associated death domain protein

TRAIL-R1/DR4: TRAIL receptor 1/Death Receptor 4

TRAIL-R2/DR5: TRAIL receptor 2/Death Receptor 5

TRAIL-R3/DcR1: TRAIL receptor 3/Decoy Receptor 1

TRAIL-R4/DcR2: TRAIL receptor 4/Decoy Receptor 2

TRAIL: TNF-related apoptosis-inducing ligand

TS: Inhibit the action of thymidylate synthase

VAL: B cell lymphoma

VVL: *Vicia villosa lectin*

WGA: *Wheat germ agglutinin*

XIAP: X-linked Inhibitor of Apoptosis Protein

FOREWORD

This work was carried out as part of the fight against cancer and was made possible thanks to the financial support of the Brazilian Ministry of Research, Coordenação de aperfeiçoamento de pessoal de nível superior (CAPES), together with the Comitê Francês de Avaliação da Cooperação Universitária (Cofecub).

This project focused on the study and understanding of the molecular mechanisms involved in triggering programmed cell death through the interaction of TRAIL receptors with galectin-3 and -9. Cell death is a biological process used and studied by the scientific and medical community in oncology. A better understanding of the mechanisms that control cell death, and particularly the death of cancer cells, would allow us to consider new treatments or make existing treatments more effective and better tolerated by patients.

1. INTRODUCTION

1.1. CANCER

Currently, cancer represents the second-largest single cause of mortality worldwide. According to the World Health Organization (WHO), cancer was responsible for the death of 9.6 million people worldwide in 2019. By 2030 it is predicted to be the leading cause of death globally, affecting about 12 million people per year (Plummer et al., 2018). This increase can be explained in part by population growth and aging, and recent disease screening advances (Adami et al., 2019).

Cancer, or malignant tumor, is a generic name for a group of more than 100 diseases characterized by uncontrolled cell proliferation of abnormal cells, resulting from pleiotropic and multifactorial events (Parkin et al., 2002). Each tumor presents its characteristics making each cancer a unique disease (Baylin and Jones, 2016). During carcinogenesis, normal cells accumulate genetic and epigenetic changes leading to oncogenes' activation and inhibition of tumor suppressor genes (Beckman and Loeb, 2017; Gillies and Gatenby, 2007). At this stage, loss of homeostasis occurs as tumor cells can acquire the capacity to unlimited proliferation (immortality), insensitivity to antiproliferative mechanisms, invasion and metastasis production, stimulation of angiogenesis, and resistance to programmed cell death. Additional genomic instability and, to a lesser extent, tissue inflammation are two characteristics that favor the acquisition of other abilities. Modification of energy metabolism and the ability to prevent or resist immune system attacks are also proposed as cancer cell's inherent capabilities (Figure 1) (Hanahan and Weinberg, 2011).

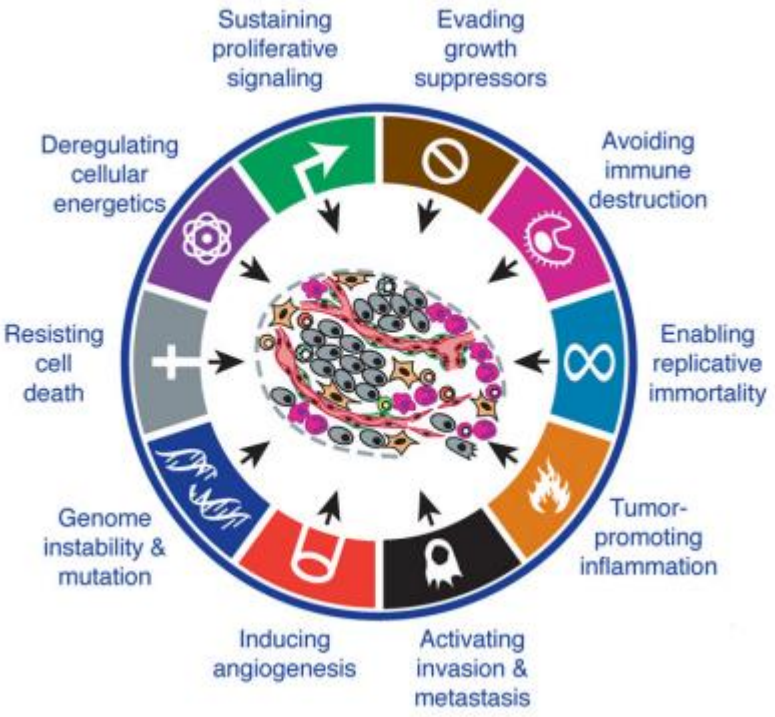


Figure 1: Essential ten capacities acquired by cancer cells during carcinogenesis. Essential ten capacities acquired by cancer cells during carcinogenesis. **Source:** Adapted from (Hanahan and Weinberg, 2011).

1.2. APOPTOSIS

Programmed cell death "apoptosis" is an essential process in maintaining body homeostasis (Degterev et al., 2003), resulting from the evolution of multicellular living beings. The death of one or more cells maintains the other's physiological function, leading to the organism's survival and development (Lockshin and Zakeri, 2001).

It is characterized by reduced cytoplasmic volume, bubble projections on the cell membrane, and phosphatidylserine positioning to the membrane's outer side (Feng et al., 2018). Characteristic changes in cell nuclei also occur, with the activation of endonucleases that fragment DNA (D'Arcy, 2019; Zhang and Xu, 2000). The resultant apoptotic bodies are then removed by phagocytes what contributes to the non-inflammatory profile of apoptosis (Elmore, 2007; Rathmell and Thompson, 2002). Two distinct pathways interconnected by molecular signaling can initiate apoptosis (Figure 2). The intrinsic pathway is usually activated through severe damage to DNA, hypoxia, or other cellular stresses, such as those caused by chemotherapy or ionizing radiation, and the extrinsic pathway triggers apoptosis through death receptors (DR) arranged on the cell surface. Activated DRs recruit intracellular adapter proteins,

leading to the activation of cysteine proteases, known as caspases.

In mammals, eight DR members have been identified (Table 1): tumor necrosis factor receptor 1 (TNFR1), CD95 (Fas), death receptor 3 (TRAIL-R3, DcR3), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, DR4), TRAIL-R2 (DR5), DR6, ectodysplasin A receptor (EDAR), and the p75 neurotrophin receptor (p75NTR) (Park, 2011). Their cognate ligands are cytokines belonging to the tumor necrosis factor (TNF) protein family (Guicciardi and Gores, 2009), most often referred to as death ligands.

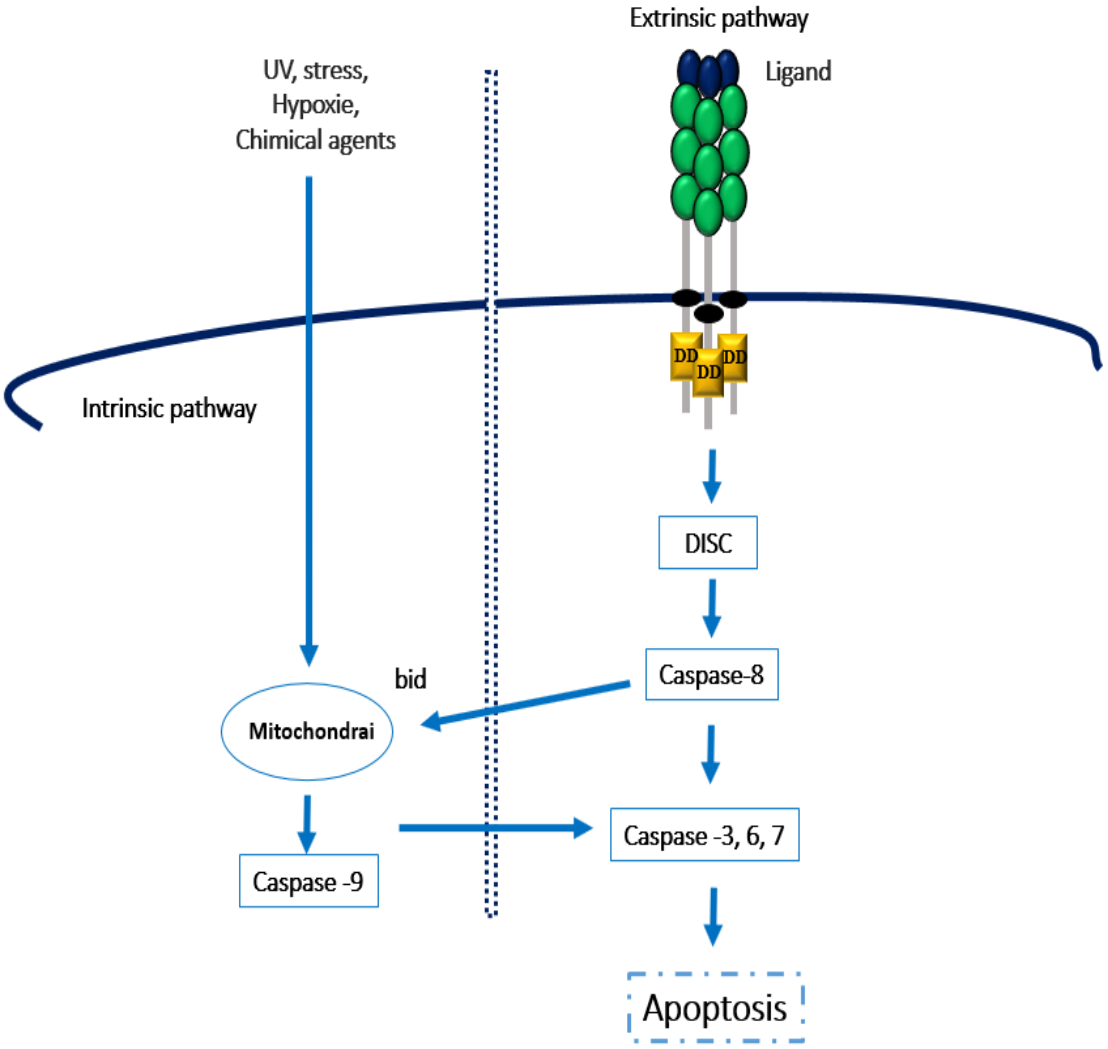


Figure 2: Simplified diagram of the intrinsic and extrinsic pathways of apoptosis. The intrinsic or mitochondrial pathway is activated after stress (chemical or physical) and is characterized by depolarization of the mitochondrial membrane, which also induces the cascade. The extrinsic pathway or the death domain receptor pathway is activated after binding of its ligand, which induces trimerization of death domain receptors and leads to activation of initiation caspases such as caspase-8 after assembly of the DISC complex (death-inducing signaling complex). Caspase-8, in turn, activates caspases effector (caspases-3, -6 and -7). **Source:** Adapted from (Fulda and Debatin, 2006).

Table1: *Members of the death receptors family and their cognate ligands.*

Death Receptor	Ligand	
	Name	**Abbreviation**
TNFR1	Tumor necrosis factor/ Lymphotoxin alpha	TNF/ LTα
Fas	Fas ligand	FasL
DR3	TNF-like protein 1	TL1A
DR4/DR5	TNF related apoptosis-inducing ligand	TRAIL
DR6	alpha-amyloid precursor protein	APP
EDAR	Ectodysplasin A	EDA
p75NTR	nerve growth factor/brain-derived neurotrophic factor/ neurotrophic factor 4,5	NGF/ BDNF/ NT4,5

Source: Adapted from (Lee et al., 2019).

1.3. TRAIL AND THE AGONIST RECEPTORS

The cytokine APO2L/TRAIL (TNF-related apoptosis-inducing ligand) induces apoptosis in cancer cells through DR4 or DR5 (MacFarlane et al., 1997; Pan et al., 1997b; Walczak et al., 1997). This promising antitumor compound has attracted much interest in oncology due to its ability to trigger selective cell death in a wide variety of cancers (Ashkenazi et al., 2008). TRAIL is naturally expressed on the surface of immune cells, particularly monocytes, dendritic cells, natural killer cells, and T cells, mRNA expression levels of TRAIL is strongly induced by inflammatory cytokines in a variety of interferon- or interleukin-2 (IL2) (Almasan and Ashkenazi, 2003; Smyth et al., 2001). Due to the restricted expression in the immune cells, TRAIL plays an essential role in tumor immune surveillance (Rossin et al., 2019).

TRAIL is a homotrimeric protein with an affinity to the cysteine-rich domain (CRD) of TNF family receptors. The activation of the agonist DR4 or DR5 by TRAIL leads to receptor trimerization and conformational changes that promote the recruitment of adapter proteins through homotypic death domain (DD) interactions (Anees et al., 2015), triggering pro-apoptotic signaling (Figure 3). On the other side of the spectrum, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and OPG receptors cannot induce

an apoptotic signal due to the lack of functional DD and, therefore, are called decoy receptors or TRAIL receptor antagonists. DcR1 has no intracellular domain and is anchored to the membrane through a GPI anchor, while DcR2 has a truncated and non-functional DD. OPG is a soluble protein that has a low affinity for TRAIL (Figure 3) and seems to be more involved in the activation of NF-kB (Nuclear Factor-kB) signaling (Anees et al., 2015). Unlike DR4 and DR5, which are usually expressed in tumor tissues (Ganten et al., 2009; Omran and Ata, 2014), antagonist receptors are present in normal tissues, only occasionally expressed in tumor cells (Daniels et al., 2005) .

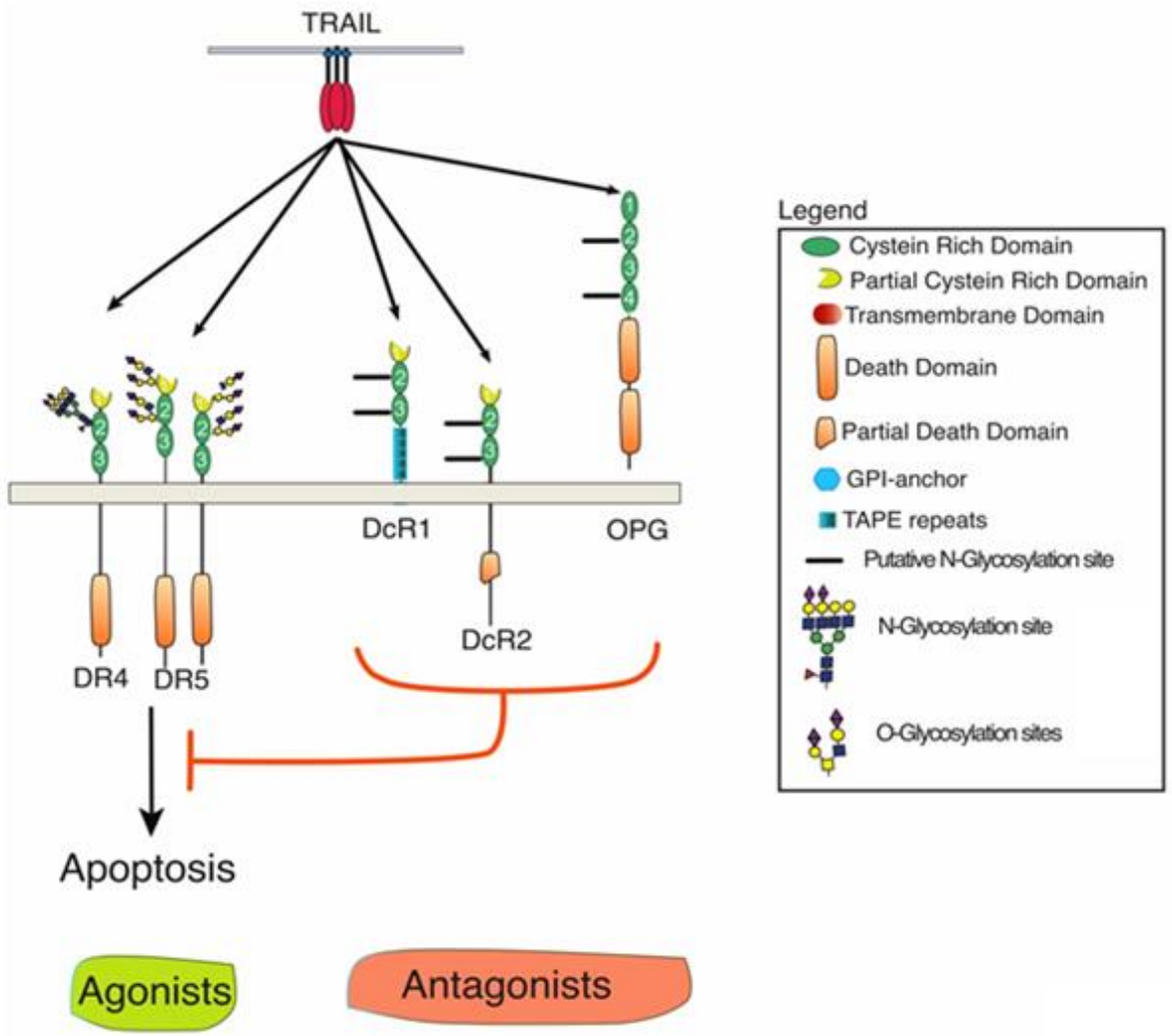


Figure 3: Schematic representation of TRAIL and its receptors. TRAIL and its agonist (DR4 and DR5) or antagonist (DcR1, Dcr2, or OPG) receptors are membrane-bound glycoproteins of the TNF superfamily. DR stands for Death Receptor, DcR for Decoy Receptor. Specific domains of putative and described O- and N-glycosylation sites are depicted in the legend. **Source:** (Micheau, 2018).

The DD protein superfamily includes about 100 members belonging to four subfamilies: death domain (DD), caspase recruitment domain (CARD), pyrin domain (PYD), and death effector domain (DED). Around 30 DD-containing proteins have been identified; however, only a small number of them, like FADD, TRADD, RIPK1, and EDARADD, have been identified to be consistently recruited to interact with members of the DR family (Sessler et al., 2013).

Adapter protein Fas-associated with the death domain (FADD; Figure 4) presents a C-terminal DD that interacts with DRs, and an N-terminal DED, which activates and recruits other DED-containing proteins (Bodmer et al., 2000). The recruitment of FADD allows the binding of the primary caspases, caspase-8 and 10, resulting in the formation of the death-inducing signaling complex (DISC) (Scott et al., 2009). Once grouped in the DISC, the interaction between two procaspases-8 leads to the dimerization of their catalytic C-terminal domain sand the prodomain autoproteolysis in the aspartic acid residues 216, 374, and 384. The active caspase-8 dimer (p18-p10) is then released in the cytosol, cleaving effector caspases, thus, starting the caspase cascade recruitment (Hengartner, 2000). For caspase-10, the prodomain autoproteolysis interferes with aspartic acid residues 219 and 415 (Wang et al. 2001), which generates an active dimer of caspase-10 (p23-p12). Caspases-3, -6, and -7 are then dimerized, triggering the apoptotic process (Nuñez et al., 1998; Dickens et al., 2012). According to the activation level, caspase-8 can stimulate the intrinsic pathway, amplifying caspase activation through the mitochondria (Elmallah and Micheau, 2015; Li et al., 1998).

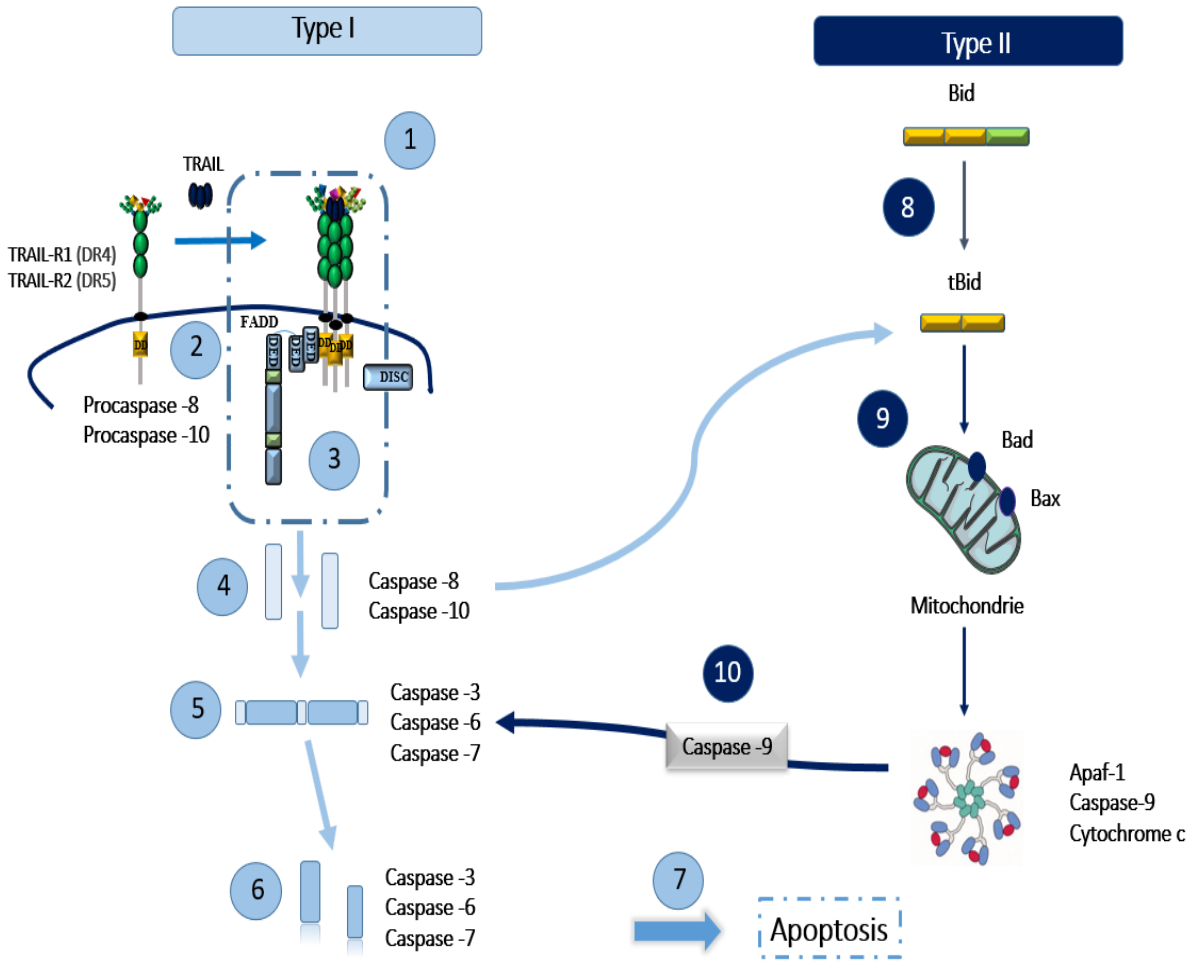


Figure 4: Type I and II apoptotic pathways. Binding of TRAIL to its agonistic receptors (DR4 and/or DR5) leads to its oligomerization (1) and subsequent recruitment of the FADD cytosolic adapter protein through DD homotypic interactions (2). Type I. FADD, in turn, allows the recruitment of initial pro-caspase-8/-10 through interactions with DED, leading to the formation of the so-called death-inducing signaling complex (3). In DISC, chains of caspase-8/-10 (4) are assembled, allowing the self-processing of caspase-8/10 and the release of its active fragments in the cytosol (5), where they activate, by proteolytic cleavage, effector caspases (6) to execute the apoptotic program (7). Type II Signal amplification via the intrinsic pathway is sometimes necessary when caspase-8 is not sufficiently activated. In this scenario, mitochondrial activation is induced by caspase-8/-10 through cleavage of the BH3 Bcl-2 family protein, Bid (8), whose truncated version (tBid) can translocate to mitochondria and induce an alteration in its outer membrane permeability through Bak and Bax interactions (9), allowing the release of cytochrome c, activation of caspase-9, another caspase initiator capable of activating caspase-3/-7 performer (10). **Source:** Adapted from (Elmallah and Micheau, 2015).

1.3.1. APOPTOSIS INTRINSIC PATHWAY ACTIVATION BY TRAIL

Cells sometimes require additional signal amplification through the mitochondrial pathway in order to undergo TRAIL-induced apoptosis. This phenomenon initially described for the Fas signaling pathway (Scaffidi et al., 1998) led to the classification of cells into two types, namely, type-I and type-II cells. In type-I cells, caspase-8 is activated and processed in the DISC in amounts sufficient to activate effector caspases and thus promote apoptosis directly. In contrast, in type II cells, the amounts of active caspase-8 generated in the DISC are limited. Therefore, cells initiate an amplification loop for further transduction of the apoptosis signal by the cleavage of the Bid protein (BH3 interacting domain death agonist), a member of the family Bcl-2 (Elmallah and Micheau, 2015) (Figure 4).

The truncated form of Bid protein (tBid) has a robust pro-apoptotic activity. It is found extensively on the outer mitochondrial membrane and can bind and inhibit Bcl-2 anti-apoptotic proteins(Li et al., 2008; Luo et al., 1998). All members of the Bcl-2 family are characterized by the presence of 1 to 4 conserved domains called the BH (Homology Bcl-2), numbered 1 to 4. These proteins can exert pro- or anti-apoptotic activity and are divided into three classes (Adams and Cory, 2018). The BH3-only family consists of eight members of apoptosis initiating proteins: BID (BH3-interacting domain death agonist), BAD (Bcl-2 antagonist of cell death), BIM (Bcl-2 interacting mediator of cell death), BIK (Bcl -2 interacting killer), BMF (Bcl-2-modifying factor), NOXA, PUMA (Bcl-2-modifying factor), and HRK (Harakiri), all of which promote apoptosis when overexpressed (Warren et al., 2019; Yip and Reed, 2008). The family of effector proteins includes Bax (Bcl-2 associated x protein), Bak (Bcl-2 antagonist killer 1), and Bok (Bcl-2 related ovarian killer).

When activated by tBid, effector proteins promote the mitochondrial outer membrane permeabilization (MOMP), resulting in the release of crucial pro-apoptotic proteins, such as cytochrome c. Cytochrome c induces the formation of a multiprotein complex, similar to DISC in function, which is the platform for the activation of caspase in the intrinsic apoptotic pathway (Roufayel, 2016). This complex, known as apoptosome, is composed of cytochrome c, factor 1 protease activator (Apaf-1), dATP, and pro-caspase-9 (Garrido et al., 2006). In the apoptosome, the apical caspase of the cascade of caspases (pro-caspase-9) becomes activated, leading to the onset of running caspases, pro-caspases-3, -6, and -7, which are essential to complete apoptosis (McIlwain et al., 2013).

The members of the anti-apoptotic Bcl-2 family, Bcl-xL (B-cell lymphoma, long isoform), Bcl-W (Bcl-2 like 2), Mcl1 (Myeloid cell leukemia 1), and A1/BFL-1 (Bcl-2-related protein A1), have four BH domains and similar three-dimensional structures (Strasser, 2005). The BH1, BH2, and BH3 domains interact to form a hydrophobic groove (Yin et al., 1994), allowing the bind of pro-apoptotic partners (Adams and Cory, 2007; Giam et al., 2008). Overexpression of any of these proteins results in resistance to multiple apoptotic stimuli showing some redundancy between them; it is unlikely that under physiological conditions, only one of these proteins is responsible for the survival of a population of cells (Delbridge and Strasser, 2015).

Like the Bcl-2 anti-apoptotic family, the IAPs (apoptosis protein inhibitors) are cell death regulators that, among other functions, bind caspases and interfere with apoptotic signaling through death receptors or intrinsic cell death pathways (Obexer and Ausserlechner, 2014). All IAPs share from one to three common structures, the so-called IAP baculovirus (BIR) repeating domains, which allow them to bind to caspases and other proteins. XIAP (X-linked inhibitor of apoptosis protein) is the most potent and best-defined member of the anti-apoptotic IAP family that directly neutralizes caspase-9 through its BIR3 domain and effector caspases-3 and -7 through its BIR2 domain (Galbán and Duckett, 2010). SMAC/Diablo, a natural XIAP inhibitor released by mitochondria in apoptotic cells, displaces bound caspases from XIAP domains BIR2/BIR3, thus reactivating the execution of cell death (Obexer and Ausserlechner, 2014).

1.3.2 TRAIL REGULATION MECHANISMS

Given TRAIL's ability to induce cell death, it is clear that this signaling must be precisely regulated to maintain the organism's homeostasis. Mutations found at domains such as DD (McDonald et al., 2001) or the ligand-binding site(Fisher et al., 2001) were observed in breast, head and neck cancers, and non-Hodgkin's lymphoma. All of them correlated with loss of receptor functionality (Bin et al., 2006; Lee et al., 2001). Interruptions in membrane traffic and the lack of exposition of receptors in the cell surface are also possible resistance mechanisms. Other studies reported that in breast cancer models, the receptor's internalization was the reason for TRAIL resistance. The blockade of clathrin-mediated endocytosis reversed the sensitivity to apoptosis measured by TRAIL (Austin et al., 2006; Zhang et al., 2009).

Additionally, the overexpression of decoy receptors, DcR1, or DcR2, leads to

apoptosis inhibition (LeBlanc and Ashkenazi, 2003; Pan et al., 1997a). TRAIL receptor antagonists are located at membrane sphingolipids and cholesterol-enriched microdomains, known as lipid rafts. Within lipid rafts, DcR1"kidnap" TRAIL and compete with DR4 and DR5 for ligand binding. DcR2 receptor acts in a more complex way as it inhibits the activation of caspase-8 without preventing the formation of DISC. Merino and colleagues demonstrated that DcR2 ectopic expression inhibited TRAIL-induced apoptosis by forming a heterocomplex with DR5, leading to inhibition of caspase-8 activation at the DISC (Merino et al., 2006).

Some evidence revealed that the overexpression of the anti-apoptotic protein c-FLIP (cellular inhibitory protein similar to FLICE) induces TRAIL resistance (Dolcet et al., 2005; Geserick et al., 2008). Three isoforms of c-FLIP expressed in humans have been described, a long variant, c-FLIP$_L$, and two short isoforms, c-FLIP$_R$, first isolated from the human Burkitt B-cell line of Raji lymphoma, and c-FLIP$_S$ (Djerbi et al., 2001; Golks et al., 2005; Irmler et al., 1997). c-FLIP is homologous to caspases and can prevent DISC formation (Irmler et al., 1997). Like c-FLIP, changes in the expression of Bcl-2 or XIAP members have often been observed in many types of cancer (Fulda et al., 2002).

Variations in the expression of Bcl-2-family proteins are found in several types of cancer. Bcl-2, Bcl-XL, or Mcl-1 overexpression prevents TRAIL-induced apoptosis in several tumor types, including prostate, breast, lung, and pancreatic tumor cells (Fulda and Debatin, 2004; Hari et al., 2015; Takahashi et al., 2013). In contrast, TRAIL-induced apoptosis can be impaired in HCT116 colorectal cancer cells deficient in pro-apoptotic proteins such as Bax (Deng et al., 2002; Gillissen et al., 2010). The Bax deficiency does not affect TRAIL-induced caspase-8 activation and subsequent Bid cleavage. However, it leads to partial processing of caspase-3 due to XIAP inhibition. The release of Smac/DIABLO from mitochondria through TRAIL-caspase-8-tBid-Bax cascade is essential to remove the XIAP inhibitory effect and allow apoptosis to continue (Deng et al., 2002). Besides, a Bax deficiency may be responsible for inefficiency in cytochrome c release after TRAIL stimulation, as demonstrated in Bax depleted embryonic mouse fibroblasts (Sinicrope et al., 2004).

1.4. TARGETING TRAIL TO TUMOR CELLS

In the past few decades, TRAIL has sparked a growing interest in oncology because of its ability to induce tumor cell death while sparing normal cells selectively. Thanks to this peculiarity, many recombinant molecules have been generated to optimize TRAIL properties as an effective antitumoral molecule.

Early versions of recombinant TRAIL included an exogenous sequence called *tag*, essential to ligand's oligomerization and activity. Different *tags* included polyhistidine tails (His-TRAIL) (Pitti et al., 1996), leucine zipper residues (LZ-TRAIL) (Rozanov et al., 2009; Walczak et al., 1997), isoleucine zipper (IZ-TRAIL) (Ganten et al., 2006), and FLAG (Schneider, 2000a). Nonetheless, these variants exhibited toxicity, especially for human liver and brain cells (Ganten et al., 2006; Nitsch et al., 2000), probably by the high degree of oligomerization of these ligands. Another possible explanation is the amount of zinc in the preparations, which affects the adequate TRAIL oligomerization (Hymowitz et al., 2000; Lawrence et al., 2001).

To overcome the presence of *tags*, an "unmarked" version of recombinant human TRAIL (rh-TRAIL), called dulanermin, was selected for clinical investigation(Herbst et al., 2010; Yee et al., 2007). Preliminary results showed patients good tolerance, with mild side effects. However, as the rh-TRAIL half-life is very short, around 30 minutes (Wu et al., 2001), it rarely leads to complete tumor eradication (Valldorf et al., 2016).

New generations of agonistic antibodies from TRAIL receptors are being evaluated, such as the APG350, which showed an extended half-life in mice and monkeys, showing promising activity in pancreatic cancer xenograft models (Gieffers et al., 2013). Another promising antibody is MEDI3039, a potent DR5 agonist in breast cancer cells in vitro and in vivo, but its effectiveness is not yet in clinical trials (Greer et al., 2019). Conventional chemotherapy uses several compounds, classified according to their function: alkylating agents, antimetabolites, intercalating agents, antimitotics, or topoisomerase inhibitors, as presented in Table 2.A treatment modality that combines two or more therapeutic agents is a cornerstone of cancer therapy. It enhances efficacy compared to monotherapy because it targets key pathways in a synergistic or additive manner (Mokhtari et al., 2017). This approach potentially reduces drug resistance while simultaneously providing therapeutic anti-cancer benefits, such as reducing tumor growth and metastatic potential, arresting mitotically active cells, and inducing apoptosis (Mierzwa et al., 2010).

Table 2: Chemotherapeutic compounds classified according to their function.

Agents	Action	Compound
Alkylating	Create covalent bonds at nucleic acids, impairing DNA replication and transcription.	Cisplatin Oxaliplatin Cyclophosphamide
Topoisomerase inhibitors	Prevents topoisomerase action, inducing DNA breakdown	Etoposide Irinotecan
Intercalating	Prevents DNA replication and transcription	Doxorubicin Actinomycin-D
Antimitotic	Interrupts the mitotic spindle	Paclitaxel/vincristine
Anti-metabolites	Inhibits incorporation of folic acid Inhibits thymidylate synthetase	Methotrexate FluoroUracil (5FU)

Source: The author

5-FU is an aromatic heterocyclic organic compound with a structure similar to that of the DNA (thymine) and RNA (uracil) pyrimidine molecules. It is a uracil analog with a fluorine atom at the C-5 position in place of hydrogen (RUTMAN et al., 1954)(Figure 5). Based on its structure, 5FU can bind to both RNA and DNA, interfering with nucleosides' metabolic synthesis, causing cytotoxicity and cell death. In mammalian cells, 5FU is converted into several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), acts inhibiting the action of thymidylate synthase (TS), fluorodeoxyuridine triphosphate (FdUTP), is incorrectly incorporated into DNA, and fluorouridine triphosphate (FUTP), that interrupt RNA synthesis (FUTP) (Wyatt and Wilson, 2009).

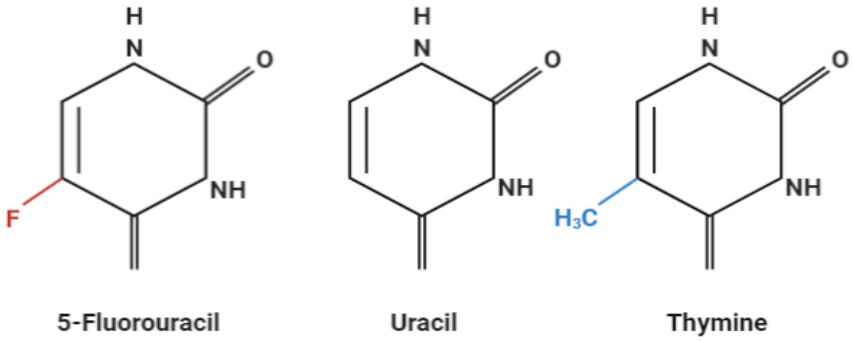


Figure 5: Chemical Structure of 5-FU, Uracil, and Thymine. **Source:** Adapted from (Van Laar et al., 1998).

The use of chemotherapeutic agents combined with TRAIL has proved to increases apoptosis activation *in vitro* and *in vivo* (Anan and Gores, 2005; Ciuleanu et al., 2016). The combined use of fluorouracil (5FU), cisplatin, and etoposide restored tumor cells' sensitivity to TRAIL-induced apoptosis. Treatment with 5FU increases the expression of DR4 and DR5, improves the recruitment of proteins of the DISC complex, and reduces c-Flip protein level (Ganten et al., 2004; Kondo et al., 2006; Morizot et al., 2011). In some cases, previous treatment with 5FU influenced the reduced expression of anti-apoptotic proteins that modulate the intrinsic and/or extrinsic pathways of apoptosis, such as Bcl-2, Bcl-xL, c-FLIP, or XIAP (Mühlethaler-Mottet et al., 2004; Nazim et al., 2017; Song et al., 2003). A study in primary cells showed that a treatment that combines 5FU and TRAIL does not exert toxicity in healthy hepatocytes (Meurette et al., 2006).

Specific agonist antibodies against DR4 or DR5 have been generated and some of them effectively induced apoptosis in a wide range of tumor cell lines, with no toxicity to normal cells (Lin et al., 2003; Pukac et al., 2005), either as a single agent or in combination with chemotherapeutic drugs. Apomab, TRA-8, and LBY135 showed inhibition of tumor cell growth in xenograft mice models (Adams et al., 2008; Lin et al., 2003). TRA-8 also induced cell death in primary human cervical and ovarian cancer (Estes et al., 2007; Kendrick et al., 2008). DS-8273a was tested in 16 advanced cancer patients enrolled in a phase I study. Treatment with DS-8273a resulted in a reduction of MDSCs (Myeloid-derived suppressor cells) without affecting myeloid or mature lymphoid cells (Dominguez et al., 2017). In leukemic and lymphoma cells, bortezomib was combined in vitro with HGSETR1 and HGS-ETR2, which resulted in synergistic effects on cell death (Georgakis et al., 2005; Smith et al., 2007). Bortezomib also sensitized a panel of non-small-cell lung carcinoma (NSCLC) cells to HGS-ETR1 and

HGS-ETR2, while cells were resistant to treatment with the antibodies alone (Luster et al., 2009). In primary cancer cells, doxorubicin has been shown to sensitize lymphoma cells to HGS-ETR1 and HGS-ETR2 *in vitro*, and breast cancer cells to TRA-8 *in vitro* as well as *in vivo*. However, despite promising results from tumor models in mice, the TRAIL death receptor targeting failed in clinical studies, showing no satisfactory antitumor efficacy. These disappointing results can be explained mainly by poor pharmacokinetics and the development of death receptor-induced apoptosis resistance (Brin et al., 2018; Ciuleanu et al., 2016; Dominguez et al., 2017; Wajant, 2019)

1.5. PROTEIN GLYCOSYLATION

Glycosylation is one of the most important post-translational modification processes determining the final protein structure and function. Unlike nucleic acids and polypeptides, oligosaccharide chains are often mounted on nonlinear branched structures by glycosyltransferases and glycosidases using specific sugar-donor substrates (Gabius et al., 2011; Reis et al., 2010). This process varies among species and seems to differ significantly among cell types (Brutschin and Brutschin, 2016). Carbohydrate units on the cellular surfaces are the first layer of interaction between cells and the extracellular matrix components (Taylor et al., 2015), and in general, carbohydrate residues act as signal molecules for the recognition, adhesion, immune response, cellular differentiation, and metastasis (Cohen, 2015; Li et al., 2008; Wu et al., 2007).

Oligosaccharides can form two types of glycosidic bonds with proteins: N-glycosylation and O-glycosylation (Potapenko et al., 2015). In N-glycosylation, an oligosaccharide chain is linked to the hydroxyl group of the asparagine side chain contained in the consensus sequence Asn-X-Ser/Thr, due to an N-glycosylamine bond. In rare cases, the sequence Asn-X-Cysteine is also used (Munkley et al., 2016; Potapenko et al., 2015). It is governed by a series of steps inside the endoplasmic reticulum (ER) and Golgi complex (GC). The first stage occurs in the ER membrane by incorporating an oligosaccharide nucleus, composed of three glucose (Glc), nine mannose (Man), and two N-acetyl glucosamine (GlcNAc) residues (Freeze and Aebi, 2005), into the protein-specific amino acid residue. The second stage involves a reorganization starting from the removal of Glc and Man residues followed by the

incorporation of other saccharides, depending on the protein's function (Pinho and Reis, 2015).

O-linked glycosylation is very common in secreted or plasma membrane glycoproteins (Reis et al., 2010). The first step is transferring the uridine diphosphate N-acetylgalactosamine (UDP-GalNAc) to the serine or threonine residues, catalyzed by enzymes of the UDP-N-acetylgalactosaminyl transferases (ppGalNAc-Ts) family. PpGalNAc-Ts control the first level of complexity of glycosylation, that is, O-glycan's locations and density. The second level of O-glycosylation complexity highlights the lengthening of the carbohydrate chains by other glycosyltransferases. After the first residue of N-acetylgalactosamine (GalNAc) is added, a galactose residue (Gal) is added. These carbohydrate residues can then be sialyzed by sialyltransferases, forming sialic acid antigens (Pinho and Reis, 2015; Reily et al., 2019).

1.5.2 DR4 AND DR5 Glycosylation

In 2007, Wagner and colleagues published the first article correlating TRAIL-DISC's arrangement with the glycosylation of TRAIL receptors (Figure 9).They demonstrated that cellular sensitivity to TRAIL is closely associated with elevated levels of N-acetylgalactosamine transferases (GALNT14), increasing the apoptotic potential of the DR5 receptor. It was found that inhibition of O-glycosyltransferase by siRNA impaired tumor cells' death after stimulation with TRAIL. In this same study, the authors performed mutagenesis, changing serines and threonines in alanine residues that prevented DR5 O-glycosylation and limited the apoptotic signal transduction capacity (Wagner et al., 2007).

In another study, Moriwaki and colleagues described that DR5 O-glycosylation is necessary to better aggregation and activation of the DISC complex. Besides, the deficiency of GDP-mannose-4,6-dehydratase (GMDS), a fundamental enzyme for fucosylation, can lead to TRAIL resistance in colorectal carcinoma cells (Moriwaki et al., 2011). Despite the evidence showing that O-glycosylation is necessary for the activation of apoptosis in tumor cells via the DR5 receptor, little data describe whether glycosylation is involved in regulatingDR4-induced apoptosis.

Our team recently demonstrated that the DR4 receptor is N-glycosylated and that this post-translational modification increases the receptor's ability to trigger apoptosis. Cells with mutations in N-glycosylation alter the distribution of DR4 and/or its arrangement on the cell surface and reduce apoptosis response through TRAIL

stimulation. The fact that DR4 is N-glycosylated while DR5 is O-glycosylated may provide potential clues for the differential promotion of apoptosis by these two receptors in some types of tumor cells (Figure 9) (Dufour et al., 2017). Also, proteins may undergo additional modifications, including sialylation, fucosylation, or terminal glycan branching. Recent studies have reported that terminal modifications have the potential to affect TRAIL receptor pro-apoptotic signaling (Moriwaki et al., 2011).

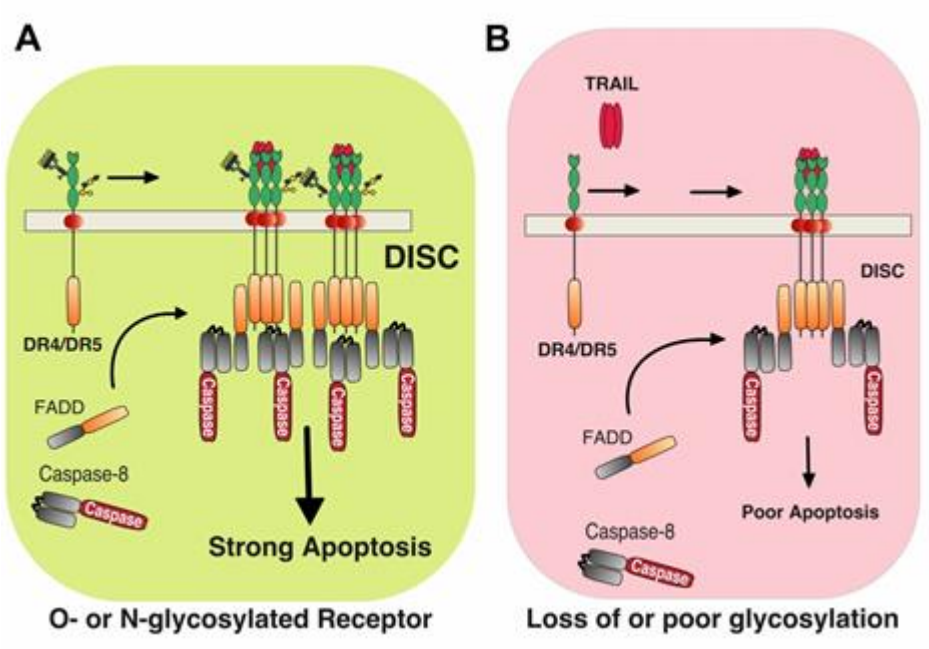


Figure 6: Schematic representation of TRAIL-induced DISC formation. TRAIL-induced apoptosis via DR4 and DR5 in tumor cells is closely associated with the glycosylation of their receptors. **(A**) The stimulation of DR4 or DR5 glycosylated by TRAIL induces the recruitment of the adapter protein FADD and caspase-8, thus forming the so-called TRAIL DISC (Death-Inducing Signaling Complex), where caspase-8 is processed, allowing the triggering of apoptosis. Carbohydrate transferases, including N-acetylgalactosaminyl, fucosyl- or sialyltransferases, as well as galectins, could act directly at the receptor level to regulate the formation and activation of TRAIL DISC. **(B)** In cells that exhibit low N-acetylgalactosaminyl transferase activity or express a non-glycosylated receptor, the binding of TRAIL to the receptors is not altered. However, DISC's formation and the processing of caspase-8 are restricted, impairing the ability of TRAIL to trigger. **Source:** (Micheau, 2018)**.**

1.6. Lectins

In nature, there is a diversity of glycan-binding proteins (GBPs) that selectively recognize specific carbohydrates, being responsible for diverse physiological and pathological processes (Mulloy and Linhardt, 2001; Taylor et al., 2015). Lectins are GBPs of non-immunological origin, without catalytic capacity, which presents two or more binding sites capable of interacting, reversibly, with carbohydrates, precipitating glycoconjugates and agglutinating cells (Ohtsubo and Marth, 2006; Sharon and Lis, 2004).

They are vastly distributed, found in viruses, bacteria, fungi, plants, and animals. They can be used, for example, as tools in the detection, isolation, and characterization of glycoconjugates involved in cellular processes, identifying changes on the cell surface during physiological and pathological events (Reily et al., 2019).

Lectins bind to their ligands through weak bonds such as hydrogen bridges, Van der Walls interaction, and hydrophobic interactions(del Carmen Fernandez-Alonso et al., 2013). The amino acid sequence analysis involved in the protein-carbohydrate recognition showed a polypeptide segment called the carbohydrate recognition domain or CRD (Park and Baenziger, 2004).Through this domain, lectins can be classified according to their structure and function (Loh et al., 2017; Vasta et al., 2011) (Table 3).

Table 3: Animal Lectins based on the structure of the carbohydrate-recognition domain (CRD)

Lectins	Specificity	Location	Role
S-type Lectins (galectins)	β-Galactosides	Cytoplasm/Extracellular membrane	Cell adhesion, cell migration, growth regulation, immune responses, apoptosis
C-type Lectins (Calcium-dependent)	(Mannose, Galactose, Fucose)	Extracellular, transmembrane	Recognition of pathogens, endocytosis, adhesion, immune-modulation
I-type	Sialic acid	Membrane	Regulation of myeloid cell inter-action, differentiation of myeloid cells, adhesion signaling
F-type	L-Fucose	Extracellular	Innate immunity
L-type	High-mannose Glycan	Intracellular, Golgi, Endoplasmic reticulum, extracellular membrane	Protein sorting, assisting secretion of specific glycoproteins
P-type	Man-6-P	Cytoplasm/Extracellular membrane	Endocytosis, intracellular targeting of lysosomal enzymes (lysosome biogenesis)
R-type	Mannose, Galactose,	Cytoplasm/Extracellular membrane	Glycoprotein homeostasis
Pentraxins	α-β/Galectosides	Cytoplasm/Extracellular membrane	Innate immunity
Tachylectin	GlcNAc MurNAc	Membrane	Innate immunity

Source: Adapted from (Loh et al., 2017; Vasta et al., 2011).

1.6.1 GALECTINS

Type S-type lectin or galectin family have highly conserved amino acid sequences (Barondes et al., 1994), with binding affinity for galactosides. Members of this family are widely distributed in nature, present in sponges, fungi, nematodes, insects, and vertebrates (Barondes et al., 1994; Leffler et al., 2002). Galectins remained conserved during evolution, and the presence of several galectins in a single species may indicate they diverged to specifically perform a variety of functions (Cooper, 2002).

They are often found in the cytoplasm, but depending on cell type and proliferative state, significant amounts of this protein can be detected in the nucleus and cell surface. Secreted galectins can interact with surface glycosylated partners (Brinchmann et al., 2018; Hughes, 2001), translating information encoded by glycans into immune cell activation, differentiation, and homeostasis (Rabinovich et al., 2007). Additionally, galectins may interact with intracellular ligands contributing to mRNA processing, immune regulation, and inflammation (Ilarregui et al., 2005).

The interactions with glycans can be highly complex because of the galectins multivalence and oligomerization and the multivalence of the ligands. They are then classified into three categories based on structural and binding profiles (Figure 10). Prototype galectins contain a single CRD and form non-covalent homodimers. Chimeric, have a CRD and an amino-terminal domain rich in proline, glycine, and tyrosine residues, which is sensitive to metalloproteinases and contributes to the oligomerization of these lectins. Tandem-Repeat galectins are unique polypeptides composed of two distinct CRDs connected by a peptide linker of 5 to 50 amino acid residues (Kamili et al., 2016).

To date, 15 galectins have been characterized, 11 of which are found in humans (Gitt et al., 1995; Sakthivel et al., 2015). Galectin-5 and -6 are expressed only in rats and mice, respectively, while galectins-11 and -15 are exclusively found in ruminants. Galectin transcripts may generate different isoforms. For example, at least seven different mRNAs have been identified for human galectin-8, differently expressed depending on the tissue (Bidon-Wagner and Le Pennec, 2002). It is the same for galectin-9, with three isoforms already identified (Demers et al., 2005).

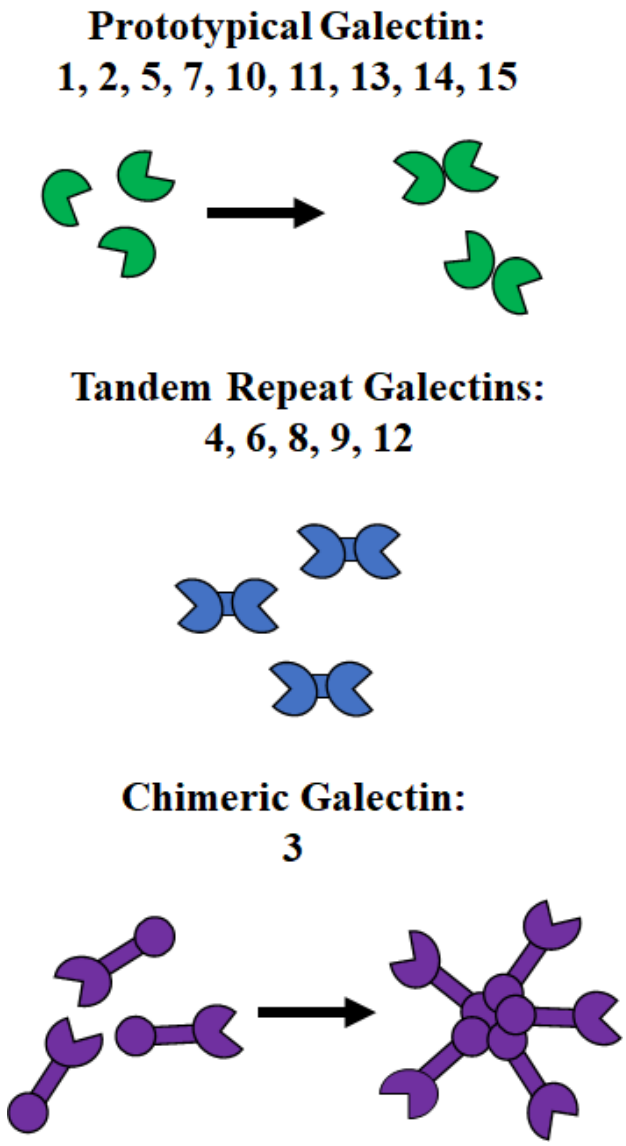


Figure 7: The three subtypes of galectins. Prototypical galectins have only one CRD, while the tandem repeat galectins have two CDRs. Galectin-3 is the only chimeric galectin found in mammals. **Source:** Adapted from (Boscher et al, 2011).

1.6.3. GALECTIN AND CANCER

Increasing evidence points out the roles of glycosylation in tumor progression stages. As tumor cells progress, the composition of glycans may change in parallel with changes in cell metabolism. This phenomenon includes incomplete synthesis and neo synthesis, which refers to abnormal glycosylation patterns (Kannagi et al., 2008).

Some tumors, such as melanoma, prostate, breast cancer, and ovarian cancer, show overexpression of galectins, which correlate with the stage of malignancy, aggressiveness, or metastatic potential (Blidner et al., 2015). For example, in endometrial tumors, galectin-1 was overexpressed compared to non-tumor endometrial cells, while silencing galectin-1 reduced tumor proliferation (Griffioen and Thijssen, 2014). In murine lung cancer, the autocrine secretion of galectin-1 caused tumor progression (Kuo et al., 2012). In human bladder cancer, increased expression of galectin-1 mRNA was correlated with tumor histological grade and advanced clinical stage. In this same study, the effects of galectin-3 were evaluated, with no correlation with tumor progression (Cindolo et al., 1999). Another study reported the activity of galectin-3 in tumor cells, depending on its location. Nuclear galectin-3 was correlated with antitumor responses (Califice et al., 2004); in contrast, the strong cytoplasmic expression of gal-3 was associated with tumor aggressiveness (Wang et al., 2018). Galectin-8 demonstrates different abilities to modulate migration, mainly in glioblastoma and colon cancer cells (Nagy et al., 2002). A similar scenario exists for galectin-9, which increases the adhesion of melanoma, colon cancer, in contrast, reduces the adhesion of melanoma cells, breast cancer, and the formation of colon cancer cell metastases (Hirashima et al., 2008; Irie et al., 2005).

Many glycans, glycosyltransferases, and glycosidases play critical roles in programmed cell death, preventing ligand-receptor interactions, which influence the formation of signaling complexes and modulating ligand secretion (Lichtenstein and Rabinovich, 2013; Wagner et al., 2007). TRAIL ligand promotes tumor cells' apoptosis through the DR5 death receptors, whose O-glycosylation status determines their sensitivity to the ligand (Wagner et al., 2007). In addition, N-glycosylation also plays an important regulatory role in DR4-mediated apoptosis, but not for DR5, which is devoid of N-glycans. In this context, defective apoptotic signaling by TRAIL receptors deficient in N-glycan was associated with less aggregation of the TRAIL receptor and reduced formation of DISC death-inducing signaling complex, but not with reduced TRAIL binding affinity (Dufour et al., 2017b). Gal-3 is, for example, capable of binding

directly to membrane receptors such as CD45 (transmembrane phosphatase) to induce apoptosis in lymphomas of the type T. Other studies have also shown that galectin-3 can induce or inhibit TRAIL-induced apoptosis (Mazurek et al., 2007). Until now, the underlying molecular mechanisms have been associated with the regulation of signaling downstream of TRAIL DISC and not at the level of the receptor itself. The sensitivity of tumor cells to apoptosis induced by TRAIL and the resistance of healthy cells can be explained in part by the profound changes in glycosylation. Galectin-3 can also interact with the intracellular FasL receptor domain to induce apoptosis (Dumic et al., 2006; Kageshita et al., 2002). Since CD95L is part of the TNF receptor family, it is interesting to question whether some galectins can also interact with the death of domain receptors for TRAIL and, more particularly for DR4, since it is N-glycosylated.

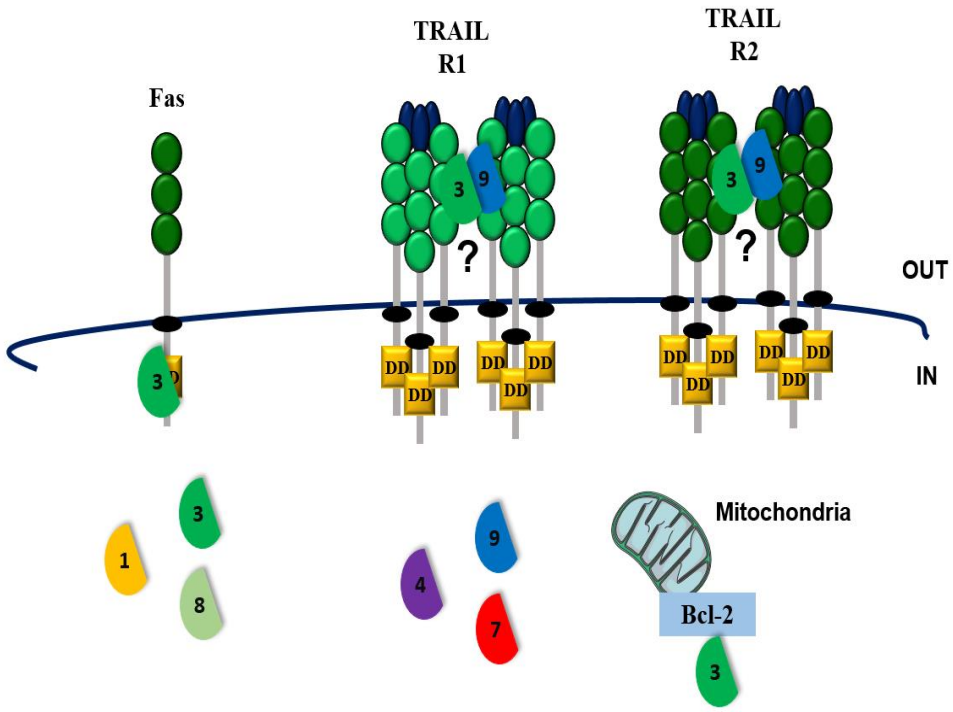


Figure 8: Interactions between galectins-3, -9, and TRAIL-R1/TRAIL-R2.

Source: The Author

2. JUSTIFICATION

Transduction of the apoptotic signal by death receptors involves different ligands of the TNF superfamily. Among these, TRAIL is the only real therapeutic interest since it effectively induces most tumor cells' death without destroying healthy ones. Therefore, it is valid to look for new strategies that potentialize TRAIL action and, mainly, reverse the resistance acquired by some tumor cells.

It is already known that some conventional chemotherapeutics sensitize tumor cells to apoptosis induced by TRAIL, but the mechanisms have not yet been completely elucidated. Considering that the importance of glycosylation for the proper functioning of the death receptors has already been described, we investigated whether chemotherapy could affect the receptor's glycosylation profile on the cell surface and stimulate their activation via galectins. The direct binding of galectin to glycosylated TRAIL receptors has not been widely studied; however, some evidence suggests that these interactions are eventually more constant and significant than expected.

3. GENERAL AIM

To investigate the interaction of galectin-3 and -9 with the extracellular domain of TRAIL receptors DR4 and DR5 and their contribution to TRAIL-induced apoptosis.

3.1. SPECIFICS AIMS

- To evaluate the ability of a conventional chemotherapeutic drug to sensitize tumor cells to TRAIL-induced apoptosis and to induce the release of galectin 3 and 9 to the extracellular medium;
- To analyze the modulation of TRAIL-induced apoptosis by galectin -3 and -9 expression;
- To analyze the modulation of TRAIL-induced apoptosis by exogenous galectin -3 and -9;
- To investigate the participation of galectin-3 and -9 as components of the TRAIL-DR4/5-DISC complex;

4. MATERIAL AND METHODS

4.1. CELL LINES AND VIRUSES

The human B Lymphoma cell line VAL and the triple-negative breast carcinoma cell line MDA-MB-231 were provided by Dr. Thierry Guillaudeux, Rennes (France), and Dr. Patrick Legembre, Rennes (France), respectively. HCT116 colon carcinoma cells were from the ATCC. TRAIL-receptor-deficient HCT116 and MDA-MB-231 (DKO) cells were generated using the TALEN approach as described by (Dufour et al., 2017b). HCT116 and MDA-MB-231 cells were cultured in DMEM medium (Dutscher, Brumath, France), supplemented with 10% fetal calf serum (Dutscher, Brumath, France). VAL cells were cultured in RPMI 1640 medium (Dutscher) containing ultra glutamine, 10% heat-inactivated calf serum (Dutscher, Brumath, France). Isogenic DKO (deficient for both DR4 and DR5) derivative MDA-MB-231 cells, reconstituted for DR4 or DR5 expression, and CRISPR/CAS9-mediated deletion of galectin-9 were grown in the presence or absence of puromycine (2.5 µg/mL) and blasticidin (10 µg/mL) from Fisher Scientific (Illkirch, France). Cells infected with the retroviral vector encoding full-length galectin-3 and -9 were either selected using puromycine as above or sorted by flow cytometry for GFP positivity. All these cell lines were grown in 5% CO2 at 37°C. Cells were stimulated or not with 5FU (1µg/mL) for 24 to 72h and treated with TRAIL (at the indicated concentration) for 6 to 24 hours before analysis (apoptosis, flow cytometry, Elisa, or Western blot). When indicated, recombinant galectins were added simultaneously with TRAIL.

4.1.2. CHEMICALS AND ANTIBODIES

For western blot analysis, the following antibodies were used. The anti-DR4 (Cat# AB16955) and anti-DR5 (Cat# AB16942) antibodies were purchased from Chemicon (Millipore, Molsheim, France). The anti-actin (C4) and anti-GAPDH (0411) antibodies were from Santa Cruz Biotechnology (Clinisciences, Montrouge, France). The anti-FADD (Cat# 610400) from Transduction Laboratories (BD biosciences, Le Pont de Claix, France). The anti-caspase-8 (clone 5F7) and anti-caspase-10 (clone 4C1) were from Medical & Biological Laboratories (Clinisciences, Nanterre, France). Anti-GAPDH (clone 0411) and anti-HSC70 (clone B-6) and Actin (clone I-9) antibodies were obtained from Santa Cruz Biotechnology (CliniSciences, Nanterre, France). The

mouse anti-galectin-9 (9S2-1) was from Biolegend (Ozyme, Saint-Cyr-l'Ecole, France). The rabbit anti-galectin-3 (Cat# 250503) and -9 (D9R4A) were from Abbiotec (CliniSciences, Nanterre, France) and Cell Signaling (Ozyme, Saint-Cyr-l'Ecole, France), respectively. Biotinylated lectins, *Aleuria Aurantia Lectin* (AAL, B-135), *Concanavalin A* (Con A, B-1005), *Maackia Amurensis* Lectin I (MAL I, B-1313), *Maackia Amurensis* Lectin II (MAL II, B-1265), Peanut *Agglutinin* (PNA, B-1155), *Ricinus Communis Agglutinin* I (RCA I, B-1085), *Sambucus Nigra Lectin* (SNA, B-1305), *Vicia Villosa Lectin* (VVL, B-1235), *Wheat Germ Agglutinin* (WGA, B-1025) were from Vector labs (CliniSciences, Nanterre, France). The lectin *Phaseolus vulgaris* (PHA-L, cat# L11270) was purchased from Thermo Fisher Scientific. Streptavidin-HRP (P0397) was from Dako (Les Ulis, France). Secondary antibodies: HRP-conjugated anti-rabbit was obtained from Jackson ImmunoResearch (Interchim, Montluçon, France), HRP-conjugated anti-mouse IgG1-, Ig2a- and Ig2b-specific antibodies were from Southern Biotech (Clinisciences, Nanterre, France). The following antibodies were used for Flow cytometry. Mouse anti-DR4 and anti-DR5 antibodies were from Covalab (Villeurbanne, France) (Dubuisson et al., 2019). Anti-galectin-9-APC (BLE348908) was from (Ozyme, Saint-Cyr-l'Ecole, France). *Phaseolus vulgaris* (red kidney bean) L-PHA- Alexa Fluor™ 488 Conjugate (10592893) was from Fisher Scientific. Other lectins are described above. The Alexa-488-coupled goat anti-mouse antibody was from Molecular Probes (Invitrogen, Cergy Pontoise, France). For apoptosis experiments, FITC-Annexin V (556420), PE-Annexin V (556422), APC-Annexin V (550475) and 7AAD (559925) were from (BD Biosciences, France). Puromycin (cat# ant-pr-1) was purchased from Invitrogen (Toulouse, France). Measurement of galectin-9 in the supernatants was performed using the following Elisa Kit (cat # SEA309Hu, Costar, Cambridge, MA, USA).

4.1.3. PLASMID CONSTRUCTIONS

pMIGR-Gal3 vectors (OM1329 and OM1330) were kindly provided by Dr. Heisterkamp (Children Hospital of Los Angeles, CA). pMIGR-Galectin-9 (OM1480) retroviral vector and pRSET-Galectin-9 were obtained from a pUC57 vector encoding the full-length sequence of LGALS9 (OM334, obtained from Genscript) digested with BamHI and EcoRI and subcloned into pMIGR (El Fajoui et al., 2011) or after a BglII-EcoRI restriction digest, to obtain pMIGR-Gal-9 (OM1480) and pREST-Galectin-9 (OM1361). pRSET-Galectin-3 (OM1360) was also obtained as above from a pUC57 vector encoding the full-length sequence of LGALS3 (OM333, obtained from Genscript). Retroviral vectors encoding full-length DR4, DR5, or DR4-156A (N-glyc-deficient) were described in (Dufour et al., 2017b). Lentiviral CRISPR vectors encoding gRNAs targeting LGALS9 were generated as described by (Ran et al., 2013). using pLenti-CRISPR V2 (Addgene 52961) and the following primers: 5'- CAC CGA GTC CAG CTG TCC CCT TTT C-3' and 5'- AAA CGA AAA GGG GAC AGC TGG ACT c-3' to generate OM 1221; 5'-CAC CGG AAC GGA AGC TGG GGG CCC GAG G -3' and 5'- AAA CCC TCG GGC CCC CAG CTT CCG TTC-3' to generate OM 1479. Sequences of all constructs were confirmed by sequencing.

4.1.4. RECOMBINANT PROTEIN PRODUCTION

His-h TRAIL, galectin-3, and galectin-9 were produced as described previously (Schneider, 2000). The insoluble fraction of galectin-3 and -9 was solubilized with guanidine (6M) for one hour at 30°C, the cell extracts were centrifuged at 27000 g and the supernatant was loaded onto a Nickel (Ni-NTA) column. Soluble galectins were eluted with glycine and the sample was neutralized by the addition of Tris-base (1M) prior to dialysis in PBS containing β-mercaptoethanol.

4.1.5 GENE DELETION OR DOWNREGULATION

For siRNAs experiments, HCT116 cells were transfected either with a GAL3 siRNA (L-010606-00-0005) or an ON-TARGETplus non-targeting pool D-001810-10-20 (Fisher Scientific, France) as follows. Cells ($5x10^5$) were added in each well of a 6-well plate and allowed to adhere overnight at 37°C. The following day, cells were transfected with indicated siRNA (1 nM) using INTERFERin, according to the provider's protocol (Polyplus, Strasbourg, France), and analyzed 72 h after transfection for expression by Western blotting and apoptosis. For CRISPR/CAS9 experiments, MDA-MB-231-DKO cells were infected with a lentiviral CRISPR vector encoding a gRNA encompassing the 5'UTR and the exon 1 or a gRNA located in exon 2 of LGALS9, OM1221 (pLenti-CRISPR-LGALS9-H4) or OM1479 (pLenti-CRISPR-LGALS9-G4), respectively. Briefly, Lenti-X™ 293T cells (Ozyme) were seeded in 10 cm2 dishes and co-transfected with 1 µg/mL pVSV-G, 10 µg/mL psPAX2 (Addgene #12260), and 10 µg/mL OM1221 or OM1479 using polyethyleneimine (PEI, Cliniscience) according to the manufacturer's instructions. Viral particles were generated as described previously (Morgenstern and Land, 1990) and cells were transduced for 16 h in 6-well plates in the presence of polybrene (Sigma-Aldrich, Lyon, France) (8 µg/mL). Cells were then washed in phosphate-buffered saline, harvested, plated in a complete medium containing puromycin (2.5 µg/mL), blasticidin (10 µg/mL), or both. Serial clonal dilutions or populations were next amplified for at least 14 to 20 days before the analysis of galectin-9-deficiency by PCR and Western blot.

4.1.6. EVALUATION OF APOPTOSIS BY ANNEXIN-V LABELING

Apoptosis was determined by detecting phosphatidylserine externalization after co-labeling with Annexin V-FITC and 7-AAD. Analyzes were performed using a flow cytometer (FACS Calibur, France). Apoptosis is presented as the percentage of positively staining cells compared to untreated cells as a control. Each experiment was performed independently at least three times. Briefly, $1x10^5$ cells were cultured in 24-well plates overnight in complete medium. The following day, cells were trypsinized, centrifuged at 1500 rpm for 5 min, washed in PBS (1X), centrifuged at 1500 rpm for 5 min, and Annexin-V and 7-AAD staining was performed according to the manufacturer's instructions. The acquisition included a minimum of 10,000 cells.

4.1.7. CHEMOTHERAPY AND TRAIL TREATMENTS

For sequential treatments, cells were cultured in 24-well plates ($1x10^5$) or 75 cm^2 flasks ($5x10^5$) overnight in complete medium. The next day, cells were treated or not with 5FU (1 µg/mL) in a medium containing 1% FBS for 24, 48, and 72 hours. After treatment with 5FU (Sigma-Aldrich, Lyon, France), his-TRAIL at concentrations of (200; 500 and 1000 ng/mL) was added or not for 6 h.

4.1.8 ANALYSIS OF TRAIL RECEPTOR EXPRESSION BY FACS

Cells were incubated with indicated antibodies or control mouse IgG1 at 10 µg/ml for 30 minutes at 4 °C, followed by goat anti-mouse secondary antibody Alexa-488 or Alexa-680 for 30 minutes at 4° C. Antibodies and conjugates were diluted in PBS (1X) containing 3% BSA. After each incubation, the cells were washed three times with PBS (1X). Surface staining was analyzed on a FACS calibur flow cytometer.

4.1.9. LECTIN EXPRESSION ANALYSIS ON THE CELL SURFACE BEFORE AND AFTER 5FU TREATMENTS

Cells were incubated with the indicated lectins at 10µg/ml for 30 minutes at 4°C, followed by Alexa-680 anti-steptavidin secondary antibody (Invitrogen S21378 – Lot 57591A) for 30 minutes at 4 °C. Antibodies and conjugates were diluted in PBS (1x) containing 3% BSA. After each incubation, cells were washed three times with PBS (1x). Surface staining was analyzed by FACS calibur flow cytometer.

4.1.10. ANALYSIS OF MDA-MB-231 EDITED FOR GALECTIN-9 AND CORRESPONDING TRAIL RECEPTOR AFTER CELL PERMEABILIZATION

Cells were permeabilized or not with 1% saponin for 15 minutes and then incubated with APC-conjugated anti-Galectin-9 or APC control, mouse IgG1 at 10 µg/mL for 30 minutes. Antibodies and conjugates were diluted in PBS (1X) containing 3% BSA). After each incubation, cells were washed three times with ice-cold PBS (1x). Surface staining was analyzed on the FACS calibur flow cytometer.

4.1.11. DETECTION OF GALECTIN-9 IN THE SUPERNATANTS BY ELISA

Determination of secreted galectin-9 in the supernatant of MDA-MB-231 WT, HCT116 WT, and VAL WT cells was performed after treatment of the cells with 5FU (1 µg/mL) for 24; 48 or 72 h. Supernatants (10 mL) were collected and concentrated (1000 µL) in the Speed Vac (Thermo ScientificTM), for 6 hours in high centrifugation. An enzyme-linked immunosorbent assay (ELISA) kit for galectin-9 (SEA309Hu, Lot L190205427, Costar, Cambridge, MA, USA) was used to detect galectin-9 in the supernatant. In summary, supernatants and standards were added in triplicates (100 µL per well) to each well of the ELISA plate. The plate was sealed and incubated for 1 h at 37 °C. Soon after, the wells were washed (300 µl PBS-Tween three times), blocked, and emptied and the biotinylated detection antibody was added for 1 h at 37 °C, followed by 100 µL avidin peroxidase for 30 min. TMB substrate was added to each well. After 10 min at 37 °C, 50 µL stop solution was added and absorbance was measured at a wavelength of 450 nm. This kit allows for the detection of Gal-9 with a dynamic range of 7.8 to 500 pg/mL.

4.1.12. IMMUNOPRECIPITATIONS

For TRAIL DISC analysis $8x10^7$ cells were stimulated in 1 mL of complete medium with 5 µg His-TRAIL for 15 to 60 minutes at 37°C. Cells were then washed with PBS before lysis in NP40 lysis buffer containing a protease inhibitor cocktail. Cell lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich) for 1 hour, then the DISC was immunoprecipitated overnight with protein G-coated beads (Amersham Biosciences, Les Ullis, France) at 4°C in the presence of an anti-His antibody (clone AD1.1.10) from AbD serotec (Bio-rad, Marnes-la-Coquette, France)(Morlé et al., 2015). Beads were washed four times with NP40 lysis buffer, and then immunoprecipitated complexes were eluted in loading buffer (63 mM Tris-HCl pH 6.8, 2 % SDS, 0.03 % phenol red, 10% glycerol, 100 mM DTT), then boiled for 5 min before analysis by western blot.

4.1.13. PREPARATION OF CELL LYSATES

Cells were harvested by centrifugation at 1500 rpm for 5 minutes at 4°C, washed once with ice-cold PBS, and lysates were prepared by resuspending the resulting cell pellets in 50 µL lysis buffer per 5x106 cells (1% of NP40, 20mM Tris-HCl pH 7.5, 150mM NaCl and 10% glycerol) supplemented with complete protease inhibitor cocktail (Roche Diagnostic, France) according to the manufacturer's instructions. After 20 minutes of incubation on ice, the lysates were centrifugated at 20000 g for 15 minutes at 4°C to remove cell debris and transferred to a fresh tube.

4.1.14. WESTERN BLOTTING

Immunoprecipitates or cell lysates were separated by SDS-PAGE then transferred to PVDF or nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.05% Tween 20 (PBST) and 5% powdered milk. For immunoblots aiming at detecting lectin binding profiles, membranes were saturated with PBS containing 0.05% Tween 20 and 5% BSA. Immunoblots were incubated overnight with a specific primary antibody or biotinylated lectins, washed four times in PBST then incubated for 1 hour with an HRP-conjugated secondary antibody. Blots were developed using the Covalight Xtra ECL enhanced chemiluminescence reagent (ref. 00118075) according to the manufacturer's protocol (Covalab, France).

4.1.15. BINDING STUDIES

Binding data processing was performed with ForBio Data Analysis Software version 7.1.0.36 with Savitsky-Golay filtering to reduce noise. Association and dissociation data were fit globally (single-phase exponential decay function) in Prism version 5.0a software (GraphPad Software, San Diego, CA.).

4.1.16. STATISTICAL ANALYSIS

Statistical analysis was performed using the Student's t-test. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software, San Diego, CA.). P values *<0.05 and **<0.01 were considered significant.

5. RESULTS

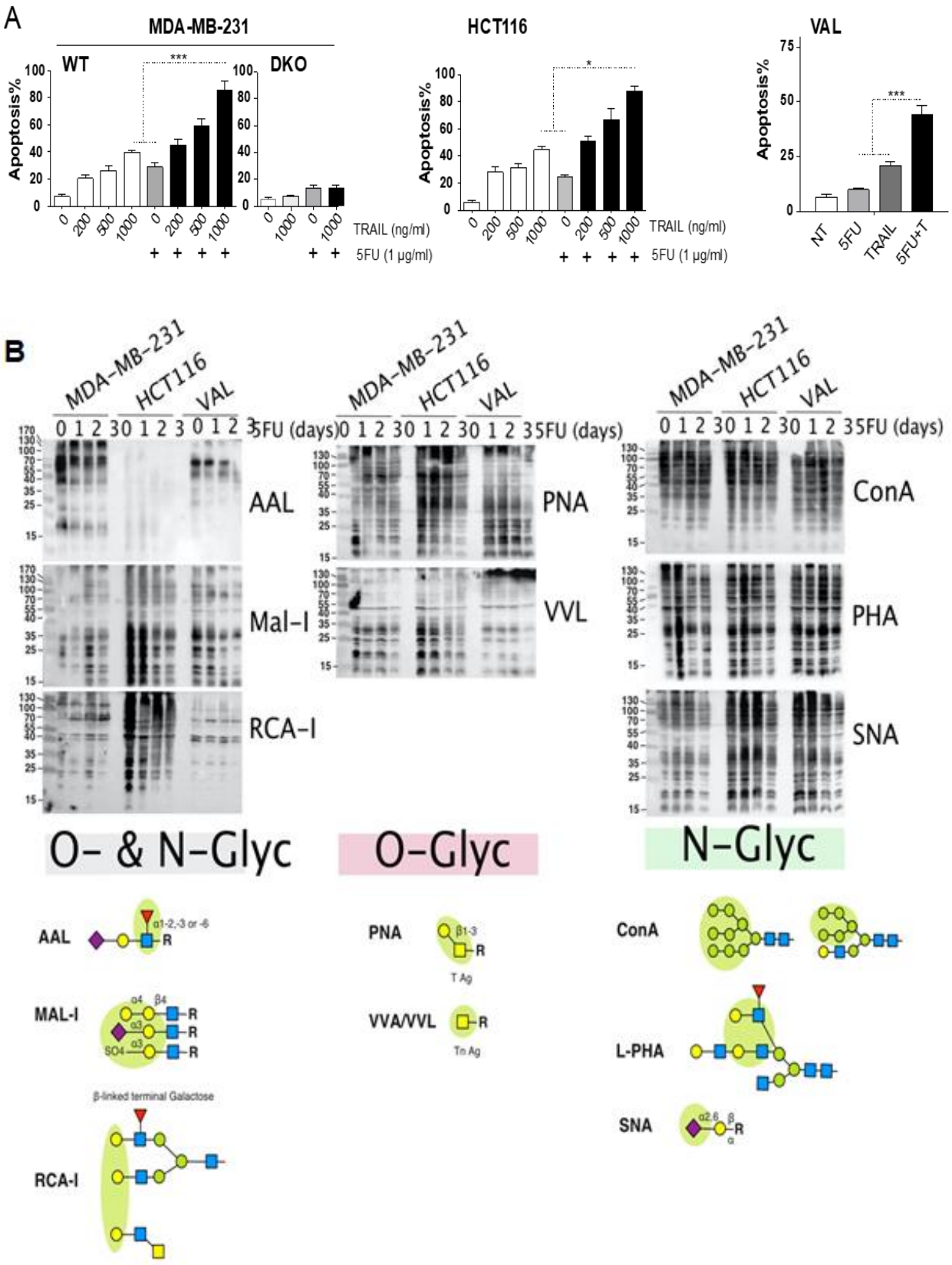
5.1. 5FU-MEDIATED SENSITIZATION OF TUMOR CELLS TO TRAIL-INDUCED CELL DEATH IS ASSOCIATED WITH ALTERATIONS IN GLYCOSYLATION

To investigate if conventional chemotherapeutic drugs such as 5FU could contribute to restoring or sensitizing tumor cells to apoptosis induced by TRAIL, the triple-negative breast cancer MDA-MB-231, the colon carcinoma HCT116, and the B cell lymphoma VAL cell lines were treated sequentially with 5FU and then stimulated with TRAIL (Figure 9A). As expected, all of them showed little sensitivity to TRAIL-induced apoptosis after 72 hours, even when treated with high concentrations (Figure 9A). Pre-treatment with 5FU, at a concentration that induces less than 20% of apoptosis, significantly increased its susceptibility to TRAIL-induced cell death, reaching up to 80% of apoptosis in HCT116 and MDA-MB-231 cells and almost 50% in lymphoma cell line B. The VAL cell line is highly resistant, known to express high levels of c-FLIP and DcR2, a TRAIL receptor antagonist at the steady-state level (Jacquemin et al., 2012).

We then speculated that 5FU could induce qualitative variations in protein glycosylation. Changes in N- and O-glycosylation during 5FU stimulation were analyzed by immunoblot from cell extracts prepared after 24, 48, or 72 hours of stimulation with 5FU (1 µg/mL), using a panel of lectins (Figure 9B). Staining intensities were quantified using Image J software (9C). Specifically, the early increase of PNA and VVL staining on day one, followed by a decrease on days 2 and 3, shows that 5FU probably alters the O-glycosylation profile in the three cell lines (Figure 9B). Likewise, albeit not observed in VAL cells, an increase in MAL-I staining was also observed in the extracts obtained from MDA-MB-231 and HCT116 cells, suggesting that 5FU likely induces an increase in O-glycosylation. Consistent with that, immunoblot changes in N-glycosylation were also detected in MDA-MB-231 and HCT116 cells, as demonstrated by the early increase in L-PHA staining. This lectin, which binds specifically to terminal galactose, N-acetylglucosamine, and mannose residues, is reminiscent of complex N-glycans. However, in VAL cells, the increase in L-PHA staining was found to occur slightly later and to a much lower extent than MDA-MB-231 and HCT116 cells (Figure 9B). High-mannose moieties were also observed in VAL cells, as demonstrated with ConA staining. Finally, changes in sialylation and fucosylation were also found after 5FU stimulation, as reflected by variations in SNA

and AAL staining. SNA binds to sialic acids bound to galactoses at position α2-6 and α2-3, while AAL displays a high affinity for fucose residues at α1-6 or 1-4 position and N-acetylactosamine structures.

To determine whether the qualitative changes described above correspond to glycosylated membrane-bound proteins, lectins binding on intact cells was analyzed by flow cytometry after a 72h treatment with 5FU (Figure 9D). Results showed that 5FU induces an increase in the cellular surface glycosylation evidenced by the staining with WGA, a lectin with affinity for N-acetylglucosamine and sialic acid residues, found in both N- and O-glycosylated proteins, and L-PHA, that exhibits high specificity for N-glycosylated proteins. Staining with the other lectins were either inconsistent or unchanged.


A
MDA-MB-231
WT
DKO
HCT116
VAL
Apoptosis%
TRAIL (ng/ml)
5FU (1 μg/ml)
NT
5FU
TRAIL
5FU+T
B
MDA-MB-231
HCT116
VAL
5FU (days)
AAL
Mal-I
RCA-I
PNA
VVL
ConA
PHA
SNA
O- & N-Glyc
O-Glyc
N-Glyc
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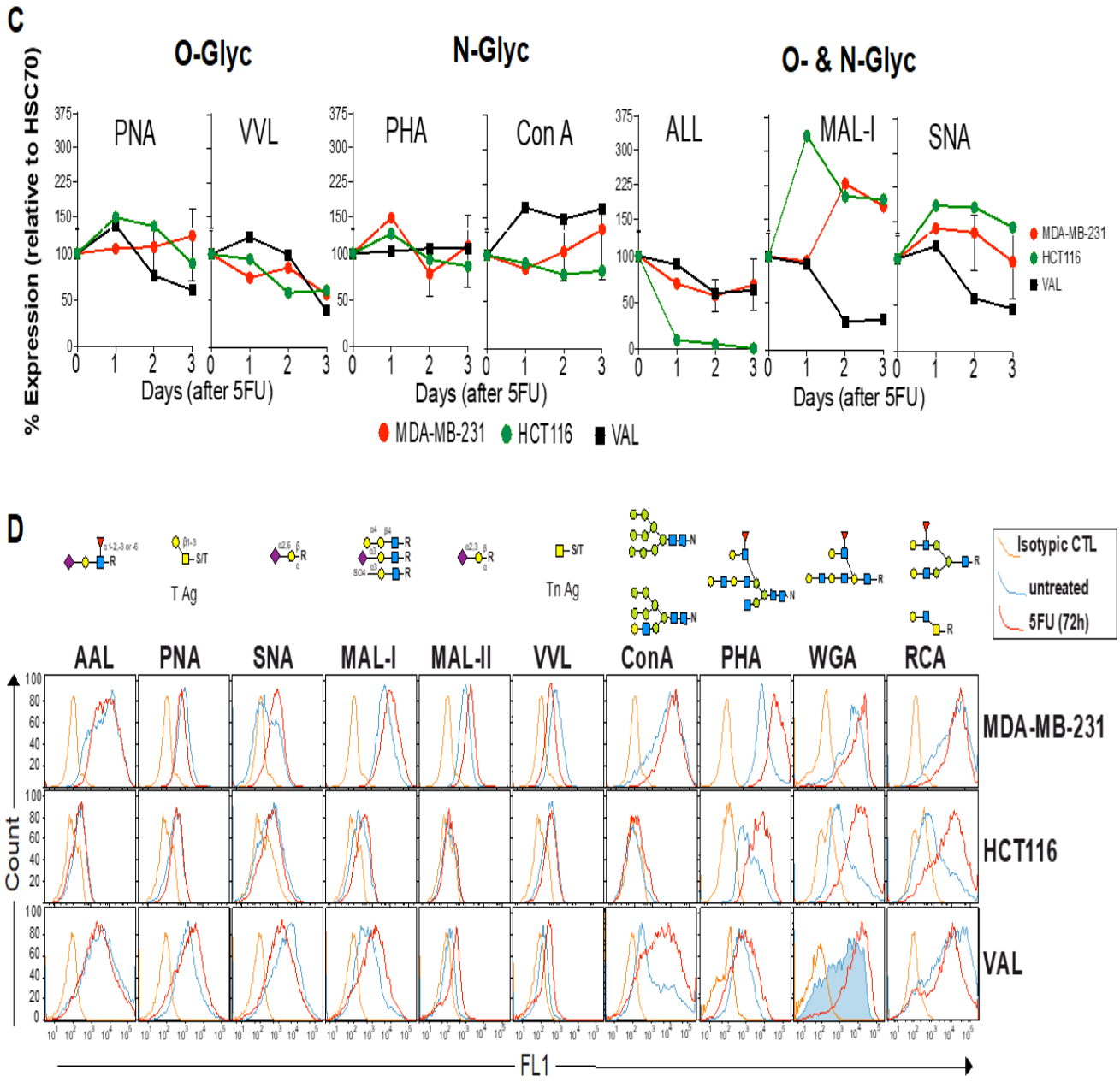

Figure 9: Alteration of glycosylation by 5FU induces apoptosis.**A.**MDA-MB-231, HCT116 and VAL cells were treated or not for 72 h with 1 µg/mL 5FU and stimulated or not with increasing TRAIL concentrations for an additional 6 hours, except VAL and MDA-MB-231 TRAIL-receptor-deficient (DKO) cells which were stimulated with 1 µg/mL TRAIL. Apoptosis was monitored by flow cytometry after Annexin V and 7AAD staining. **B.** Alteration of glycosylation by 5FU analyzed by immunobot. Indicated cells were stimulated with 1 µg/mL 5FU for 24, 48 or 72 hours and cell extracts were analyzed by immunoblot using a panel of lectins recognizing O- and N-glycosylated proteins. Lower part: sugar moieties recognized by each lectin are highlighted in green in each corresponding illustrated glycotype. **C.** Staining intensities were quantified using Image J.**D**. Cells were stimulated with 5FU during 72 has above and lectin binding was assessed by flow cytometry as indicated in the material and method. Isotypic control and specific stainings in unstimulated or stimulated cells are shown in the inlet box. **Source:** The author

5.2. 5FU INDUCES GALECTIN-3 AND -9 SECRETION

Given that 5FU may be involved in changes in glycosylation, including TRAIL DR4/DR5 receptors, we questioned whether 5FU could alter expression levels of galectin or its subcellular location. To answer this question, we first assessed by immunoblot the impact of 5FU on the expression levels of galectins -3 and 9 during treatment (24, 48, and 72 hours). The results observed in MDA-MB-231 cells using NP40, a mild detergent, reveal the loss of galectin-3 and -9 in the soluble fraction and the accumulation in the insoluble fraction in a time-dependent manner after stimulation (Figure 10). A similar result was observed in VAL cells (Figure 10A). However, this loss in the NP40soluble fraction was not associated with a concomitant accumulation in the insoluble.

We assume after secretion galectins may be able to bind to transmembrane proteins and be detectable in the extracellular medium. To answer this question, we monitor the galectin content in the conditioned medium of cells treated with 5FU for 72 hours by flow cytometry and ELISA. Consistent with this hypothesis, and although to a limited extent, using two different anti-galectin-9 antibodies, we were able to detect an increase in the staining of galectin-9 on the cell surface after 5FU stimulation in MDA-MB-231, HCT116 cells, and VAL (Figure 10C and D). In line with this finding, analysis of galectin-9 secretion by ELISA indicated that 5FU stimulation induces significant galectin secretion by the three studied cell lines (Figure 10E). These results together provide evidence that 5FU not only alters the glycosylation status of tumor cells but also induces the release of galectin-9 and probably other galectins in the extracellular compartment.

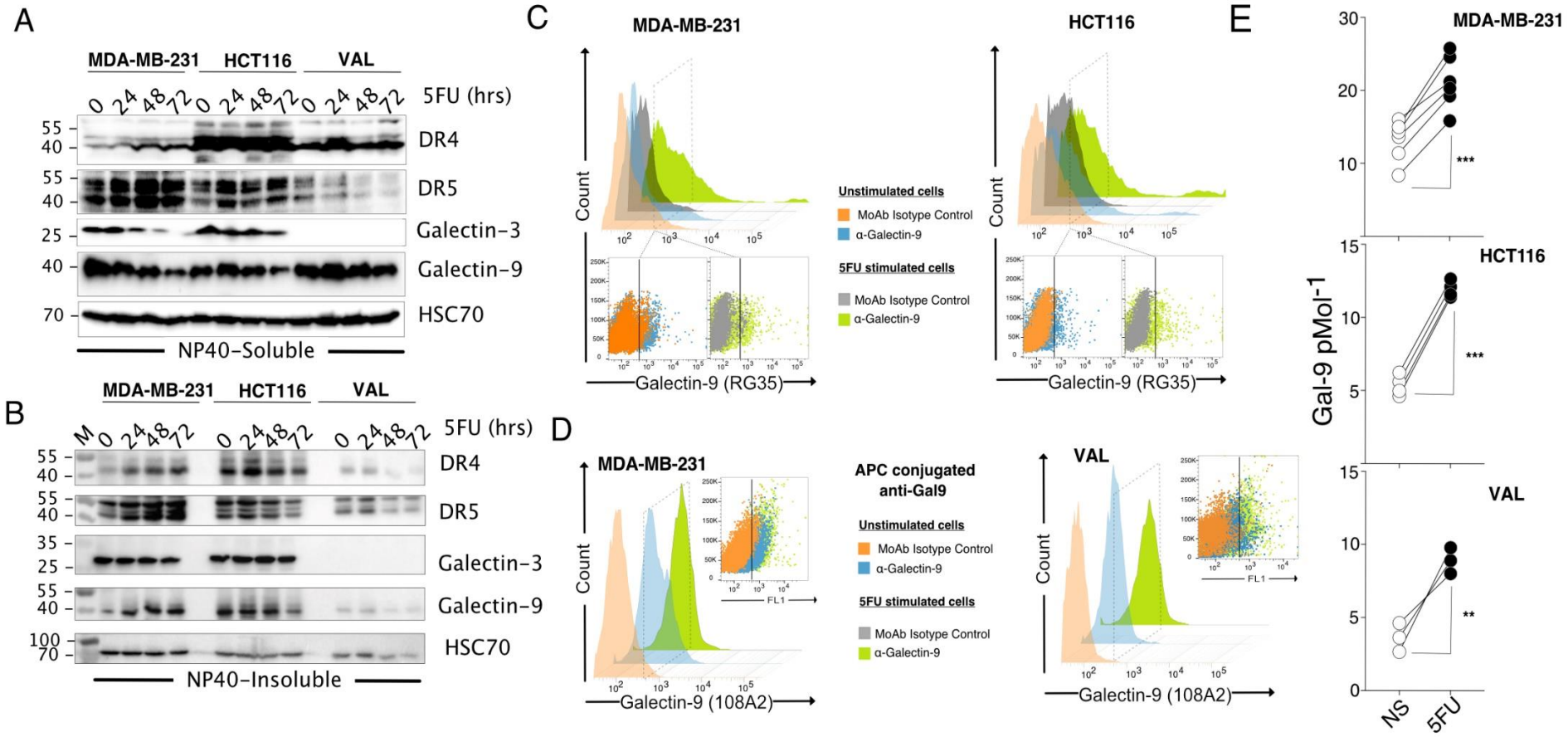

Figure 10: 5FU induces galectin-9 secretion. **A,B** Indicated cells were stimulated with 1 µg/mL 5FU for 24, 48 or 72 hours and cell extracts were analyzed by immunoblot for DR4, DR5, galectin-3 and -9 staining from NP40 soluble or insoluble fractions. HSC70 served here as loading control. **C,D** Analysis of galectin-9 membrane expression after 5FU stimulation. Indicated cells were stained with two commercial Galectin-9 antibodies 72 h after 5FU stimulation. **E** Galectin-9 secretion in cells stimulated for 72 h with 5FU was estimated by ELISA from corresponding conditioned media. **Source:** The author.

5.3. GALECTIN-3 INCREASES TRAIL-INDUCED APOPTOSIS

To investigate the contribution of galectin-3 in TRAIL induced apoptosis, we first inhibited its expression in HCT116 cells using interference RNA (siRNA) and compared it with control (non-galectin-3 siRNA targeting). The reduction in galectin-3 expression, as assessed by RT-qPCR and immunoblotting, was around 70% (Figure 11A), with a 50% reduction in the apoptosis rate. Consistent with these results, overexpression of galectin-3 in the MDA-MB-231 cell line, on the contrary, enhanced TRAIL-induced apoptosis (Figure 11B and C), regardless of its phosphorylation status. Notably, the addition of exogenous galectin-3 together with TRAIL was sufficient to recapitulate the gain of function demonstrated above in MDA-MB-231 cells (Figure 11D), suggesting that 5FU-mediated secretion of galectin-3 is likely to account for the increased sensitivity of the tumor cells to TRAIL-induced cell death.

Since 5FU was found to modify glycosylation and induce the secretion of galectins, we speculated that this change in subcellular localization is likely to account for the gain of function, probably through direct interaction galectin-3 with DR4 or DR5. In agreement with this hypothesis, immunoprecipitation and DISC-forming proteins' detection showed that galectin-3 overexpression leads to an increase in its recruitment in the TRAIL-DISC complex (Figure 11E). Concomitant with this increase in galectin-3 recruitment, DR4, but not DR5, was also more prevalent in the complex, particularly in galectin-3 overexpressing cells compared to control cells (Figure 11E). As expected, overexpression of galectin-3 in MDA-MB-231 cells did not further increase TRAIL-induced cell death after 5FU stimulation (Figure 11F), probably because 5FU is alone able to induce the secretion of galectins, and the amount of secreted galectin is sufficient to induce the increase in apoptosis following TRAIL stimulation.

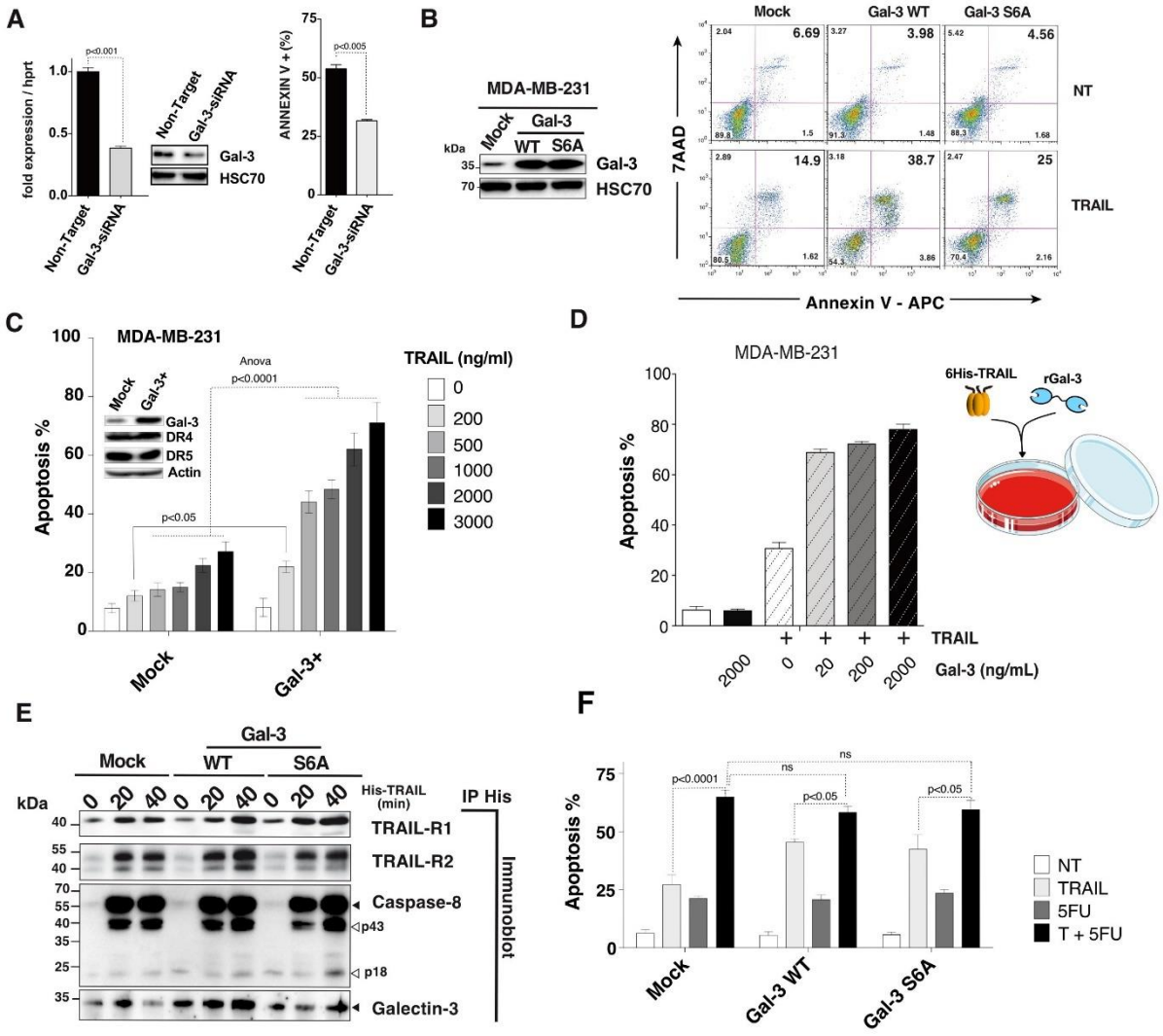

Figure 11: Galectin-3 increases TRAIL-induced apoptosis. **A** Evaluation of galectin-3 protein expression by western blot in the HCT116 line, after siRNA. HSC70 was used here as a loading control. **B** Evaluation of overexpression of galectin-3 and DR4/DR5 receptors by western blot in HCT116, after the approach of overexpression by lentivirus. Actin was used here as a loading control. Sensitivity of Gal-3 siRNA cell derivatives indicated for TRAIL-induced apoptosis. **C** Analysis of apoptosis induced by TRAIL at different concentrations in MOCK cells and cells overexpressing gal-3. **D** lustration of the experimental scenario and TRAIL-induced apoptosis (1000 ng/ml) and increasing concentrations of recombinant galectin-3. **E** Immunoprecipitation and detection of DISC-forming proteins in MDA-MB-231 cells, whether or not overexpressing wild galectin-3. **F** Sensitivity of cell derivatives indicated for apoptosis induced by 5FU in combination with TRAIL. **Source:** The author

5.4 GALECTIN-9 ENHANCES TRAIL-INDUCED APOPTOSIS

In order to understand how galectin-3 and -9 are likely to interfere with TRAIL-DISC assembly and apoptosis we further investigated the potential interactions of these carbohydrate-binding proteins with DR4 and DR5 by co-immuno-pull-down. For this, recombinant versions of the extracellular domain of DR4 or DR5 fused to the human Fc chain were produced in HEK 293cells with preservation of their glycosylation status. As shown in figure 12A, DR4 and DR5, as well as the unique mouse TRAIL receptor (mDR5), were able to pull-down galectin-9, but not galectin-3 or galectin-4 (Figure 12A and B). Importantly to note that the interaction of galectin-9 with the receptors used in this experiment, was strictly dependent on glycosylation. The production of the DR5 receptor in prokaryotic cells (Bacteria), unable to glycosylate nascent proteins, as well as the mutation of mDR5 (Figure 12C) or DR4 (Figure 12D) in N-glycosylation sites, severely impaired the interaction of this galectin with these receptors.

To further investigate how galectin-9 regulates TRAIL-induced cell death, we took advantage of the MDA-MB-231 isogenic cell lines, generated previously by Dufour et al. (2017), to generate cells overexpressing or not galectin-9. These cells express either the WT or non-glycosylable form of DR4 (N156A). Cells overexpressing galectin-9were found to be more sensitive to TRAIL-induced cell death as compared to the parental counterpart or isogenic derivatives (Figure 12I), except for those expressing the non-glycosylable DR4 (DKO-^{rec}DR4-N156A). Noteworthy, the lack of gain of function of galectin-9 was tightly associated with the loss of DR4 glycosylation, but not a loss of DR4 expression (data not shown). Likewise, the sensitivity of the MDA-MB-231-DKO-^{rec}DR4-N156A isogenic cells expressing or not galectin-9 was comparable and clearly weaker than the parental cells expressing both DR4 and DR5, or even that of MDA-MB-231-DKO reconstituted with WT form of DR4 (Figure 12I). Importantly, similar to galectin-3, recombinant galectin-9, added simultaneously with TRAIL, also enhanced, in a dose-dependent manner, the sensitivity of MDA-MB-231cells to apoptosis induced by this cytokine (Figure 12J), clearly demonstrating that galectins can directly interact with and regulate TRAIL receptor signal transduction

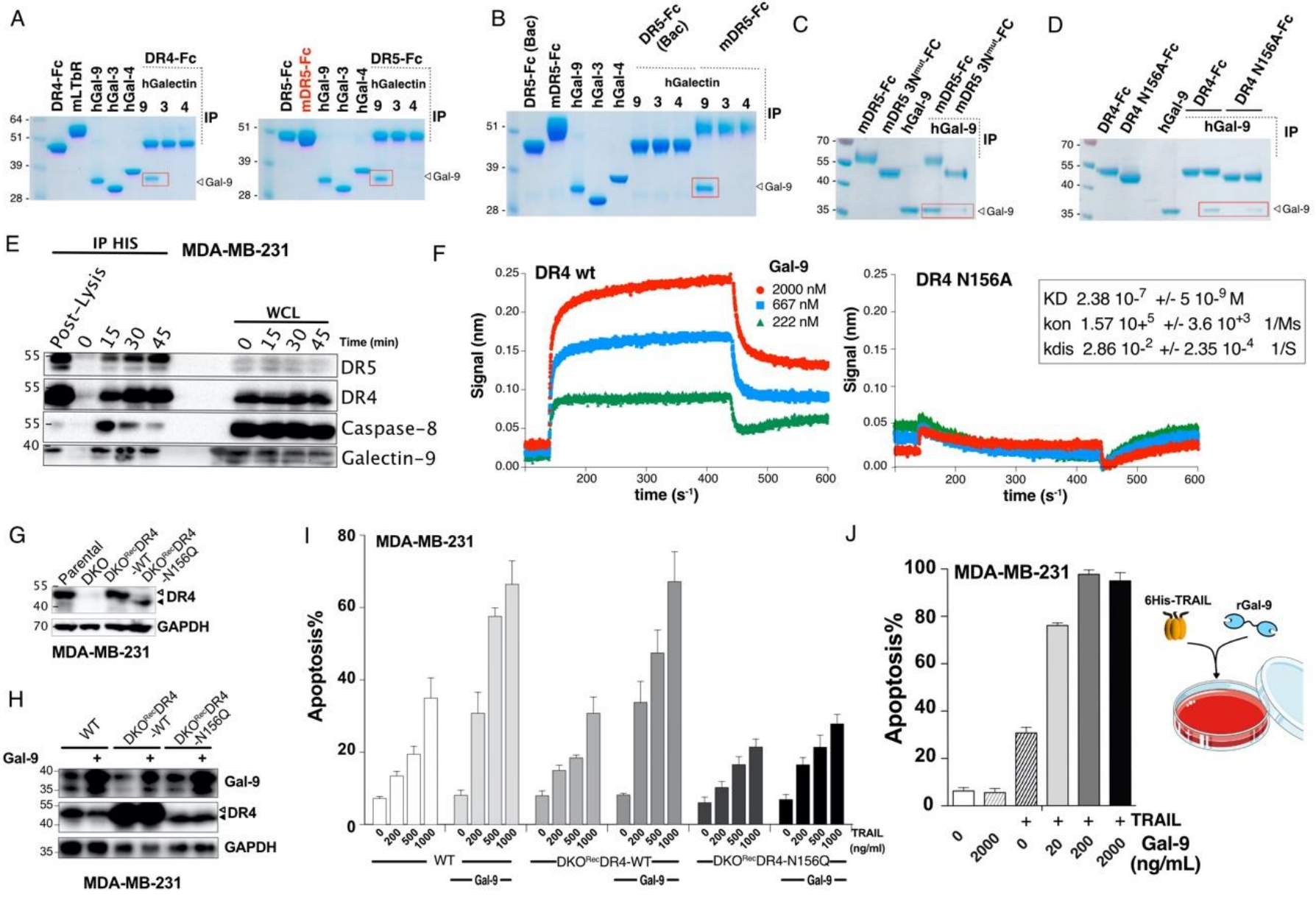
A
DR4-Fc
mLTbR
hGal-9
hGal-3
hGal-4
DR4-Fc
hGalectin
9 3 4
IP
Gal-9
DR5-Fc
mDR5-Fc
hGal-9
hGal-3
hGal-4
DR5-Fc
hGalectin
9 3 4
IP
Gal-9
B
DR5-Fc (Bac)
mDR5-Fc
hGal-9
hGal-3
hGal-4
DR5-Fc (Bac)
mDR5-Fc
hGalectin
9 3 4 9 3 4
IP
Gal-9
C
mDR5-Fc
mDR5 3Nmut-FC
hGal-9
mDR5-Fc
mDR5 3Nmut-FC
hGal-9
IP
Gal-9
D
DR4-Fc
DR4 N156A-Fc
hGal-9
DR4-Fc
DR4 N156A-Fc
hGal-9
IP
Gal-9
E
IP HIS
MDA-MB-231
Post-Lysis
0 15 30 45
WCL
0 15 30 45
Time (min)
DR5
DR4
Caspase-8
Galectin-9
F
DR4 wt
Gal-9
2000 nM
667 nM
222 nM
Signal (nm)
time (s-1)
DR4 N156A
KD 2.38 10-7 +/- 5 10-9 M
kon 1.57 10+5 +/- 3.6 10+3 1/Ms
kdis 2.86 10-2 +/- 2.35 10-4 1/S
G
Parental
DKO
DKORecDR4 -WT
DKORecDR4 -N156Q
DR4
GAPDH
MDA-MB-231
H
WT
DKORecDR4 -WT
DKORecDR4 -N156Q
Gal-9
Gal-9
DR4
GAPDH
MDA-MB-231
I
MDA-MB-231
Apoptosis%
TRAIL (ng/ml)
WT
DKORecDR4-WT
DKORecDR4-N156Q
Gal-9
J
MDA-MB-231
Apoptosis%
6His-TRAIL
rGal-9
TRAIL
Gal-9 (ng/mL)

Figure 12: Galectin-9 enhances TRAIL-induced apoptosis. **A** Recombinant galectin-3, -4, -9 as well as DR4 and DR5 fused to Ig Fc (DR4-, DR5-Fc) or immuno-pulled downs of indicated combinations were analyzed by Coomassie blue. **B** Similar experiment was performed using a version of DR5-Fc produced in bacteria (non-glycosylated) and compared to the unique mouse agonist TRAIL receptor (mDR5). **C-D** Comparison of galectin-9 pull-down by glycosylated (mDR5 or DR4) or their corresponding non-glycosylable mutated versions, mDR5-3Nmut and DR4-N156A, respectively. **E** TRAIL DISC analysis showing time dependent pull-down of DR4, DR5 and galectin-9. **F** Galectin-9 affinity for glycosylated or non-glycosylated DR4, analyzed by SPR**. G-H** Analysis of DR4 and Galectin-9 expression in MDA-MB-231 parental and isogenic cell lacking TRAIL receptor expression (DKO) and DKO cells reconstituted for DR4 or DR4-N156A expression and derivatives overexpressing galectin-9. GADPH was used here as a loading control. **I** Sensitivity of indicated isogenic cell derivatives to apoptosis induced by increasing concentrations of TRAIL. **J** llustration of the experimental setting and apoptosis induced by TRAIL (1000 ng/ml) and increasing concentrations of recombinant galectin-9. **Source:** The author

We next questioned whether the loss of galectin-9 expression would impair or compromise the efficacy of the sequential 5FU/TRAIL treatment in our cells. To address this question, we used the CRISPR/CAS9 approach to edit galectin-9 in MDA-MB-231-DKO cells. Next, we reconstituted either DR4 or DR5, using a retroviral approach. Galectin-9 deficiency was monitored by flow cytometry after membrane permeabilization (Figure 13A).

Given that the knockout of galectin-9 in MDA-MB-231 cells did not alter the expression of DR4 and DR5 receptors, we next evaluated its sensitivity to TRAIL-induced cell death. The loss of galectin-9 was associated, regardless of the receptor, with a loss of sensitivity to TRAIL-induced apoptosis (Figure 13B). It is important to emphasize that the rescue of the loss of function of galectin-9 by the addition of recombinant galectin-9 in the culture medium restored the sensitivity of TRAIL. The deletion of galectin-9, however, only partially compromised the synergy of the sequential 5FU/TRAIL treatment, regardless of the receptor considered (Figure 13C). Loss of galectin-9 was associated, regardless of the receptor, with a loss of sensitivity to TRAIL-induced apoptosis (Figure 13B). Importantly, the rescue of the loss of function of galectin-9 by the addition of recombinant galectin-9 in the culture medium restored TRAIL sensitivity. Galectin-9 edit, however, only partially compromised the synergy of the sequential 5FU/TRAIL treatment, regardless of the considered receptor (Figure 13C).

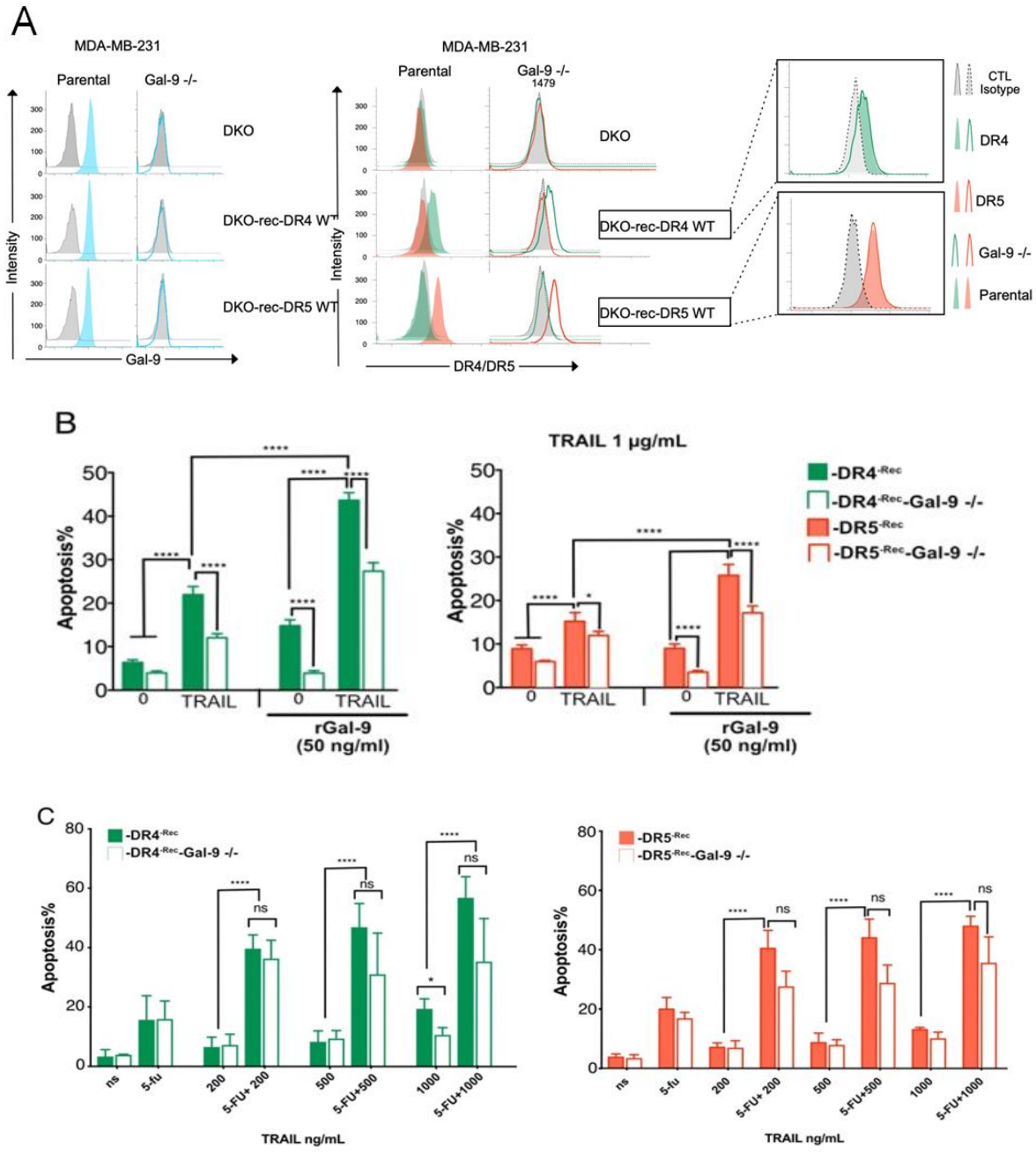

Figure 13: Galectin-9 gene editing impairs TRAIL-induced apoptosis. **A** Analysis of MDA-MB-231 cells edited for galectin-9 and corresponding TRAIL receptor isogenic precursors were analyzed by flow cytometry for galectin-9 expression after cell permeabilization. **B** Sensitivity of these cells, with the exception of the TRAIL receptor DKO isogenic cell line was assessed by flow cytometry for annexin V and 7AAD staining after stimulation with increasing concentrations of TRAIL in the presence or absence of recombinant galectin-9 as indicated. **C** Cells, expressing or not DR4, DR5, and/or galectin-9 were analyzed as in (B) after sequential treatment for 72 h with 5FU (1 µg/mL) followed or not by a 6 h TRAIL stimulation at the indicated concentration. **Source:** The author.

7. DISCUSSION

APO2/TRAIL ligand and its agonist receptors have been studied for cancer treatment for over 20 years (Micheau, Shirley and Dufour 2013). TRAIL receptors 1 and 2 share structural homology (Özören and El-Deiry, 2003). Both receptors recruit FADD and caspase-8, triggering apoptosis through the formation of the DISC complex (Ashkenazi, 2002; Kischkel et al., 2000). However, some tumor cells can escape TRAIL's cytotoxic action by developing resistance mechanisms(Trivedi and Mishra, 2015; Zhang and Fang, 2005). In the present work, we demonstrated that the chemotherapeutic 5FU sensitized the tumor cells to TRAIL-induced apoptosis due to its ability to release galectin-9 that interact directly with TRAIL agonist receptors. The loss of DR4 and DR5 glycosylation caused by site-directed mutation or production in prokaryotic cells impaired galectin-9 binding. Altogether, our results reveal galectins as new components of the TRAIL-DISC complex, whose regulation by 5FU is probably responsible for synergy with TRAIL.

We initially studied the combined effects of 5FU and TRAIL in apoptosis on cells previously resistant to TRAIL. Increased apoptosis was found in MDA-MB-231 triple-negative breast cancer, colon carcinoma HCT116, B cell lymphoma VAL cell lines when compared to cells treated with TRAIL alone (Figure 9A). As previously reported, 5FUcan reduce the expression of c-FLIP and XIAP (Ganten et al., 2004; Kondo et al., 2006; Morizot et al., 2011), and can also promote the alteration in the Bcl-2 proteins balance in favor of pro-apoptotic family members (Mühlethaler-Mottet et al., 2004; Song et al., 2003). However, until now, it remains unknown how 5 FU favors the formation of DISC and the recruitment of caspase-8 (Byun et al., 2018; Galligan et al., 2005; Shirley et al., 2011).

We assessed whether treatment with the 5FU could induce qualitative variations in cellular glycoconjugates. In this approach, we used a panel of specific lectins for different ligands that together could show whether the changes caused by 5FU treatment would affect both N- and O-type glycosylations. For all cell types, we verified an increase in the staining density for N-type, while only in the VAL cells were alterations in the O-glycosylation noticed, highlighting the growth after 72h of stimulation. The most evident changes occurred for the stainings with PHA and SNA, which specifically recognize N-glycosylated units.

Since the accumulation of sialyl antigen is a common modification in tumor cells (Pinho and Reis, 2015; Trinchera et al., 2017), we hypothesized that 5FU could remove sialic acid units, facilitating the binding of lectins that specifically recognize galactose or N-acetylgalactosamine units. It would allow cells to be recognized by macrophages through

galactose receptors, therefore degraded (Lu and Gu, 2015). Even not investigated in this study, we could also assume that the observed accumulation of N- and O-glycosylated proteins in the detergent soluble protein fraction may result from the stimulation of increased expression of enzymes acting in glycosylation.

Aiming to better investigate the role of surface glycosylations and assuming the roles of galectins in the activation of several physiological events (Wang et al., 2019), we questioned whether 5FU could alter the levels of galectin expression or its subcellular location. Although it is unclear how galectins are secreted, their retention on the cell surface requires glycosylated plasma membrane receptors (Stewart et al., 2017), forming a network, resulting in cascade signaling events (Brewer et al., 2002; Hsu and Liu, 2002).Our results showed that treatment with 5FU increased the amount of galectin-9 on the cell surface of the three cell lines tested, as detected by flow cytometry and later confirmed by ELISA.

Given that that 5FU induces galectin-9 secretion and galectin-3 has been described to confer resistance or to facilitate TRAIL-induced cell death, depending on its subcellular location or phosphorylation state (Mazurek et al., 2011, 2007; Seyrek et al., 2019), we defined a series of experiments to evaluate the effects of galectin-3 expression in 5FU/TRAIL-treated cells. Interestingly, when HCT116 cells (susceptible to TRAIL) are transfected with siRNA targeting Gal-3, TRAIL-induced apoptosis was reduced by a factor of 2. Consistently with these results, we demonstrate that MDA-MB-231 tumor cells transfected with overexpression galectin-3 are more likely to undergo TRAIL-induced apoptosis, regardless of their phosphorylation state. In line with these results, the overexpression of wild-type galectin-3 (WT) or its non-phosphorylable version (S6A) sensitizes MDA-MB-231 cells TRAIL-induced apoptosis. However, overexpression of galectin-3 does not influence the 5FU-induced mortality rate, nor apoptosis induced by the 5FU TRAIL combination.

It is worth noting that the mechanism of apoptotic activity by galectin-3 is not fully understood (Mazurek et al., 2012), and the interaction of galectins with TRAIL receptors has been rarely documented (Pan et al., 1998). However, there is evidence that galectin-3 phosphorylation increases TRAIL-induced apoptosis (Lee et al., 2003), implying that the effect of galectin-3, in this case, occurs in the cytosol. Also, galectin-3 interacts with several molecular components of apoptosis regulatory pathways. Galectin-3 is complexed with the Fas receptor (CD95), whose engagement is known to induce apoptosis (Takenaka et al., 2004). Galectin-3 also causes activation of mitochondrial apoptosis events, including cytochrome c release and activation of caspase-3, but not caspase-8 (Nakahara et al.,

2005). However, to date, no study has verified the presence of galectin-3 with the DISC assembly.

Galectin-3 overexpression in MDA-MB-231 cells showed that this lectin can be recruited into the complex and that its overexpression can lead to an increase in TRAIL DISC recruitment. Notably, TRAIL DISC contains more DR4 receptors in the MDA-MB-231cells that express galectin-3 than in control cells. The recruitment of DR5, however, is relatively similar in all cell models tested. These results suggest that galectin-3 can increase TRAIL-induced apoptosis, promoting the recruitment of the DR4 receptor to DISC. Since galectin-3 appears to contribute to the sensitization of cells resistant to TRAIL, acting proximally within the DISC, we hypothesized that its deregulation in sensitive cells could inhibit the pro-apoptotic signaling of TRAIL.

We also investigated the protein-protein interactions of DR4 and DR5 receptors with galectin-3 and -9. Furthermore, we generated cell lines deficient and overexpressing galectin-9 to analyze this galectin's relative contribution by triggering apoptosis by the TRAIL ligand. Our results show that the presence of galectin-9 was essential for better TRAIL-induced apoptosis. Therefore, the arrangement and grouping of TRAIL receptors on the cell surface after TRAIL stimulation may represent a crucial step to induce efficient activation of caspase-8, demonstrating that the presence of galectin will probably have a critical regulatory role in the process.

Our results on the potentiation of TRAIL activity by galectin-9 give continuity to this hypothesis. The non-glycosylated version of DR4 overexpressing galectin-9 cannot induce apoptosis after stimulation with TRAIL, despite this galectin's presence. Notably, these results are in line with the functional tests performed with the recombinant extracellular domain of the DR4 and DR5 receptor, fused to the Fc chain. Galectin-9 interacted with the glycosylated receptors. Its binding was drastically reduced or hampered by the lack of glycosylation. These results suggest that DR4 glycosylation is crucial for the formation of DISC after TRAIL stimulation; the direct interaction between galectin-9 and the receptor is necessary for the effectiveness of apoptosis. In line with these results, the regulatory function associated with DR4 N-glycosylation is essential for apoptosis induced by TRAIL (Dufour et al., 2017b).

We also evaluated the participation of galectin-9 in the receptor aggregation and activation within the DISC complex in breast cancer cells. Surprisingly, galectin-9 was found at DISC, along with the recruitment of caspase-8 and DR4 and DR5 receptors.

Likewise, the results generated by the SPR protein-protein interaction analysis revealed that the affinity of the glycosylated Fc-fused-receptor to galectin-9 is an important

factor, being drastically reduced when the recombinant receptor was produced with a point mutation preventing N-glycosylation. In addition, we evaluated the addition of exogenous galectins followed by TRAIL treatment. Galectin -3 and -9 were able to restore apoptotic sensitivity to TRAIL, but the addition of galectins alone could not induce apoptosis in breast cancer cells.

By contrast, the activities demonstrated with recombinant proteins added *in cellulo* may not represent the endogenous protein's functions. Additional studies are needed to definitively establish the apoptosis-inducing functions of endogenous galectins in pathological processes. The cell surface receptors responsible for galectin's actions also continue to be clarified(Pace et al., 1999; Stillman et al., 2006). As mentioned earlier, galectins do not have specific individual receptors; galectin-3 and -9 have been shown to bind to many different glycoproteins, regulating critical biological processes during the development of the organism (Díaz-Alvarez and Ortega, 2017; Wada et al., 1997). Despite the vast repertoire of recognition of different ligands, recently, a study with blackberry lectin (Morus nigra, Morniga-G), demonstrated that this lectin induced the cell death of Tn-positive leukemic cells by means of O-glycosylation concomitant, caspase and TRAIL/DR5 dependent pathways (Poiroux et al., 2019).

In consonance with this result, we demonstrate the ability of galectin-3or -9 to bind to glycosylated extracellular domains of the DR4 and DR5 receptors, increasing apoptosis induced by TRAIL. Knowing that 5FU is responsible for restoring tumor cells' sensitivity through its ability to release galectins in the extracellular compartment, we speculated that the knockout for galectin-9 could interfere with the restoration of sensitivity induced by 5FU. As expected, the 5FU TRAIL combination could not sensitize cells to apoptosis due to the lack of galectin-9. It is worth mentioning that cells expressing theDR4 receptor were more sensitive than cells expressing DR5. The reasons for this selective involvement are still unknown. Still, recent data suggest that DR5 can also exhibit pro-tumorigenic potential and contribute to stress-induced cell death of the endoplasmic reticulum, regardless of its binding to TRAIL (Dufour et al., 2017). The fact that DR4 is N-glycosylated while DR5 is O-glycosylated provides significant clues for the differential promotion of apoptosis by DR4 and DR5 in some types of tumor cells(MacFarlane et al., 2005; Micheau, 2018). In this sense, it would be interesting to generate cells deficient for O-glycosylation and to resume testing against gene edition of galectin-3 or-9with 5FU treatments in these cells, in order to better clarify the role of O- versus N-glycosylation in regulating TRAIL or non-apoptotic signal transduction.

In summary, our results demonstrate that the chemotherapeutic agent 5FU induces the release of galectin-9 from tumor cells to the extracellular environment. The release of endogenous galectin allows interaction with the extracellular domain of DR4 and DR5, contributing to increased TRAIL-induced apoptosis. The interaction with DR4 or DR5 was strictly related to the state of glycosylation of the receptors. The mutation of the N-glycosylation sites of DR4 impaired the binding of galectin-9, even after overexpression of this galectin in breast cancer cells. Likewise, overexpression or addition of soluble recombinant of galectin-3 or galectin-9 alone was sufficient to increase the sensitivity of TRAIL, while its deletion impaired TRAIL-induced apoptosis.

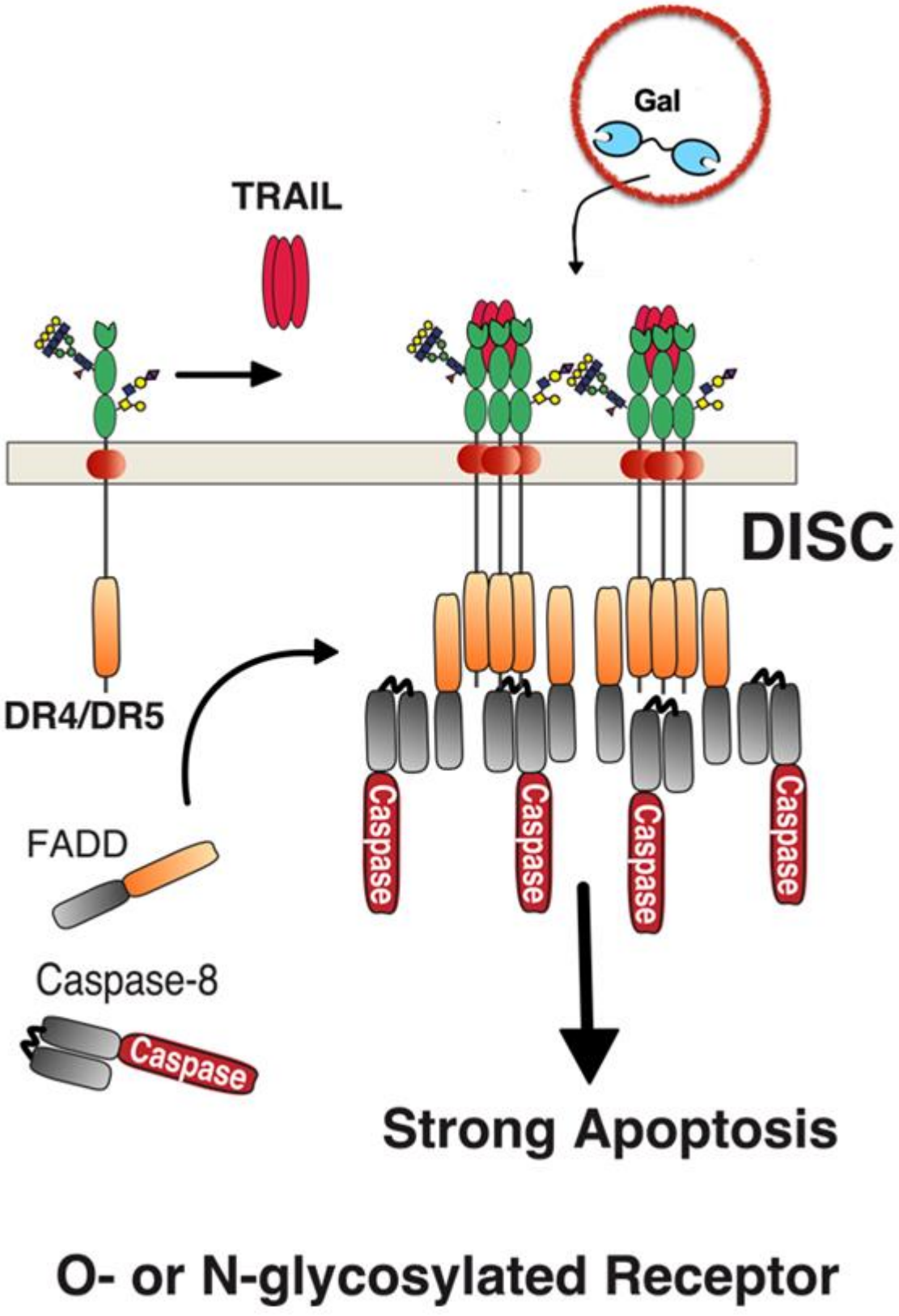

Figure 14: TRAIL-induced apoptosis via DR4 and DR5 in tumor cells is closely associated with the glycosylation of their receptors. The stimulation of DR4 or DR5 glycosylated by TRAIL induces a better oligomerization of receptors in the presence of galectin, allowing the recruitment of the adapter protein FADD and caspase-8, thus forming the so-called TRAIL DISC (Death-Inducing Signaling Complex), where caspase-8 is processed, allowing triggering of apoptosis. **Source:** Adapted from (Micheau, 2018)**.**

6. CONCLUSIONS

Regulation of APO2L/TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis by carbohydrate-binding proteins at the membrane level has, to date, never been found to occur in a direct manner. We provide, here, strong evidence that galectins can directly interact with TRAIL receptors and increase their pro-apoptotic activity. The binding of galectin-9 to DR4 or DR5, as expected, was found to require proper glycosylation of the receptors. While our immune-pull down experiments fail to show binding of galectin-3 to the recombinant receptors, either DR4 or DR5, our functional study suggests that in vivo, both galectin-9 and galectin-3 are likely to bind to these receptors, as both carbohydrate-binding proteins were able to increase cell sensitivity to TRAIL-induced cell death. However, given that all transmembrane glycoproteins are potentially able to interact with galectins, it cannot be excluded that additional partners maybe required for interaction with DR4 or DR5. Notwithstanding, and remarkably, the finding that conventional chemotherapeutic drugs such as 5FU may sensitize tumor cells to TRAIL-induced apoptosis due to their ability to induce galectin release to the extracellular compartment is sustained by our finding that galectins such as galectin-3 and -9 can interact directly with fully glycosylated DR4, and increase its pro-apoptotic potential. While further studies will be required to extend our findings to additional conventional chemotherapeutic drugs and understand how they induce the secretion of galectins, our results uncover galectins as novel TRAIL signaling regulators, whose sugar specificity allow them not only to bind TRAIL receptors, but also any glycosylated protein within their neighborhood, extending the field of investigations to be explored.

7. FUTURE PERSPECTIVES

As next steps, the knockout for galectin-3 and the study of the involvement of other galectins that may be related to the pro-apoptotic process induced by TRAIL will be carried out. In addition, in the coming months, we expect to investigated is the participation of other chemotherapeutic agents, which may be involved in the sensitization of tumor cells by increasing TRAIL-induced apoptosis

8. STUDY LIMITATIONS

The present study has limitations: The regulation of glycosylation after treatment with the chemotherapeutic agent 5FU was inconclusive in this study. Although the results obtained by staining with lectins directed to the recognition of N- and O-glycosylation present differences between the control and cells treated with 5FU, additional studies must be performed. In this case, the participation of enzymes responsible for the glycosylation process could be investigated, for example, overexpression of enzymes involved in the glycosylation of DR4 and DR5, in particular, ST6Gal-I (ST6 beta-galactoside alpha-2,6-sialyltransferase) or MGAT4 (mannosyl (alpha-1,3) -glycoprotein beta-1,4-Nacetylglucosaminyltransferase). These enzymes are respectively involved in protein glycosylation. Another point to be raised, although the extracellular location of galectins has been well established, many issues surrounding unconventional galectin secretion still need to be resolved. There is evidence that these are secreted by direct translocation and using a vesicle-based pathway, but it is not clear how these separate pathways relate to each other. Studies on secretion regulation often depend on drugs that can have multiple effects, therefore, few conclusions about the regulation mechanism can be safely drawn. Therefore, there is a clear need for more work to investigate the mechanism and regulation of galectin secretion regulated by chemotherapy treatment.

9. SUPPLEMENTARY ACTIVITIES

9.1. FINANCING AND COLLABORATIONS OF THIS RESEARCH

This work was carried out with the support of CAPES/COFECUB (COMITÊ FRANCÊS DE AVALIAÇÃO DA COOPERAÇÃO UNIVERSITÁRIA COM O BRASIL), Doctorate Scholarship Abroad No. 16/2015 with CAPES Support process 88887.142643/2017-00, Project No. 88887.130187/2017-01.

This thesis project involves a co-tutela agreement between the Pontifícia Universidade Católica do Paraná (PUC/PR) e a Université de Bourgogne (UNB).

9.1.2 PhD SECONDMENT

The second was held at the National University of Ireland Galway, during the period of 6 months (1st June 2019 - 31st November 2019) for the purpose of collaborative research and sharing of advice and experiences.

This visit was made possible by the Marie Skłodowska-Curie research and innovation team, funded by the European Commission Exchange Project (RISE), DISCOVER (MSCA-RISE project number 777995).

Our joint aim during my visit was to identify the mechanism underlying the functions of TNF receptors DR5- and/or DR4 in ER stress-induced apoptosis.

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ANNEX I - CO-AUTHOR ARTICLE

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Neutral Sphingomyelinase 2 Heightens Anti-Melanoma Immune Responses and Anti–PD-1 Therapy Efficacy

Anne Montfort[1,2], Florie Bertrand[1,2], Julia Rochotte[1,2,3], Julia Gilhodes[4], Thomas Filleron[4], Jean Milhès[1,2], Carine Dufau[1,2,3], Caroline Imbert[1,2], Joëlle Riond[1,2], Marie Tosolini[1], Christopher J. Clarke[5], Florent Dufour[6,7], Andrei A. Constantinescu[6,7], Nilton De França Junior[6,7], Virginie Garcia[1,2], Michel Record[1,8], Pierre Cordelier[1], Pierre Brousset[1,4], Philippe Rochaix[1,4], Sandrine Silvente-Poirot[1,8], Nicole Therville[1], Nathalie Andrieu-Abadie[1,2], Thierry Levade[1,2,3,9], Yusuf A. Hannun[5], Hervé Benoist[1,2,3], Nicolas Meyer[1,4], Olivier Micheau[6,7], Céline Colacios[1,2,3], and Bruno Ségui[1,2,3]

ABSTRACT

Dysregulation of lipid metabolism affects the behavior of cancer cells, but how this happens is not completely understood. Neutral sphingomyelinase 2 (nSMase2), encoded by *SMPD3*, catalyzes the breakdown of sphingomyelin to produce the anti-oncometabolite ceramide. We found that this enzyme was often downregulated in human metastatic melanoma, likely contributing to immune escape. Overexpression of nSMase2 in mouse melanoma reduced tumor growth in syngeneic wild-type but not CD8-deficient mice. In wild-type mice, nSMase2-overexpressing tumors showed accumulation of both ceramide and $CD8^+$ tumor-infiltrating lymphocytes, and this was associated with increased level of transcripts encoding IFNγ and CXCL9. Overexpressing the catalytically inactive nSMase2 failed to alter tumor growth, indicating that the deleterious effect nSMase2 has on melanoma growth depends on its enzymatic activity. *In vitro*, small extracellular vesicles from melanoma cells overexpressing wild-type nSMase2 augmented the expression of IL12, CXCL9, and CCL19 by bone marrow–derived dendritic cells, suggesting that melanoma nSMase2 triggers T helper 1 (Th1) polarization in the earliest stages of the immune response. Most importantly, overexpression of wild-type nSMase2 increased anti–PD-1 efficacy in murine models of melanoma and breast cancer, and this was associated with an enhanced Th1 response. Therefore, increasing *SMPD3* expression in melanoma may serve as an original therapeutic strategy to potentiate Th1 polarization and $CD8^+$ T-cell–dependent immune responses and overcome resistance to anti–PD-1.

Introduction

Sphingolipids (SL) are essential structural and signaling molecules able to modulate cell growth, differentiation, migration, and death as well as cancer progression (1). Ceramide is an important SL that behaves as an anti-oncometabolite (2). It can be generated by hydrolysis of sphingomyelin (SM) as a consequence of sphingomyelinase (SMase) activation (2). Several SMases have been described so far including neutral, alkaline, and acid SMases (3).

Among neutral SMases, neutral SMase 2 (nSMase2), which is encoded by *SMPD3* (4), is activated by an array of stimuli, including proinflammatory cytokines such as IL1β (5) and TNFα (6). Anthracyclines also increase transcription of *SMPD3* in MCF-7 breast cancer cells, a phenomenon able to promote cell growth arrest and cell death (7). In addition, overexpression of nSMase2 inhibits the growth of the F4328 mouse osteosarcoma cell line (8). Although the role played by nSMase2 in cancer progression remains to be fully addressed, inactivating *SMPD3* mutations are observed in some patients with acute myeloid or lymphoid leukemia (8), and *SMPD3* expression is downregulated in some human and mouse mammary tumors via epigenetic mechanisms (9).

Our group reported alterations of SL metabolism in melanoma (10–17). For instance, downregulation of *SGMS1*, the gene encoding sphingomyelin synthase 1, is associated with SL metabolism reprogramming in melanoma cells and a worse prognosis in patients with advanced melanoma (14). Other researchers have shown that β-galactosylceramidase (GALC) plays an oncogenic role in melanoma by limiting the expression of nSMase2 and ceramide levels (18). Conversely, GALC downregulation induces nSMase2 upregulation, with a subsequent increase of intracellular ceramide, and inhibits the tumorigenic activity of mouse and human melanoma cells (18).

Melanoma is a highly immunogenic cancer, the progression of which is associated with immune escape mechanisms. MAbs inhibiting immune checkpoints such as CTLA-4 and PD-1 have demonstrated significant efficacy in the treatment of metastatic melanoma, yielding high response rate and long-lasting tumor control. Despite promising results, about 40% of patients do not have therapeutic responses, and a significant proportion of responders experience

[1]INSERM UMR 1037, Cancer Research Center of Toulouse (CRCT), Toulouse, France. [2]Equipe Labellisée Fondation ARC pour la recherche sur le cancer, Toulouse, France. [3]Université Toulouse III - Paul Sabatier, Toulouse, France. [4]Institut Universitaire du Cancer (IUCT-O), Toulouse, France. [5]Stony Brook Cancer Center, and Department of Medicine, Stony Brook University, New York, New York. [6]INSERM, UMR1231, Laboratoire d'Excellence LipSTIC, Dijon, France. [7]UFR Sciences de Santé, Université Bourgogne Franche-Comté (UBFC), Dijon, France. [8]Team "Cholesterol Metabolism and Therapeutic Innovations," Cancer Research Center of Toulouse (CRCT), UMR1037 Inserm/Université Toulouse III - Paul Sabatier/ERL5294 CNRS, Toulouse, France. [9]Laboratoire de Biochimie, Institut Fédératif de Biologie, CHU Purpan, Toulouse, France.

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A. Montfort, F. Bertrand, and J. Rochotte share co-first authorship of this article.

C. Colacios and B. Ségui contributed equally as co-senior authors of this article.

Corresponding Author: Bruno Ségui, INSERM UMR1037, Cancer Research Center of Toulouse, 2 Avenue Hubert Curien, Toulouse Cedex 1 31037, France. Phone: 33582741621; E-mail: bruno.segui@inserm.fr

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tumor relapse within 2 years following treatment induction (19). To the best of our knowledge, whether melanoma nSMase2 influences immune responses and the response of tumors to immune checkpoint inhibitors (ICI) is currently unknown.

Herein, we investigated the role of nSMase2 in melanoma progression and its influence on the response of tumors to anti–PD-1 therapy. We found that *SMPD3* was frequently downregulated and mutated in patients with melanoma, likely contributing to immune escape mechanisms. We went on to demonstrate that correcting the expression of nSMase2 in mouse melanoma enhanced Th1 and $CD8^+$ T-cell–dependent immune responses and potentiated the efficacy of ICI therapy *in vivo*.

Materials and Methods

SMPD3 expression, mutations, and DNA methylation in human melanoma

SMPD3 expression in patients with primary and metastatic melanoma was evaluated using the Oncomine (20) and The Cancer Genome Atlas (TCGA) melanoma (21) databases; each dataset was evaluated separately (**Fig. 1**). TCGA genomic and clinical data were downloaded from the UCSC cancer genome browser project (http://www.genome.ucsc.edu/). Among the 422 patients with melanoma from the TCGA dataset, the analysis population consisted in 342 patients with distant metastasis for whom there were overlapping RNA sequencing and clinical data (Supplementary Data File S1). No other inclusion/exclusion criteria were applied for this analysis. Differentially expressed genes (DEG) in human metastatic melanoma samples exhibiting high ($n = 68$) versus low ($n = 68$) *SMPD3* expression were identified according to a fold-change (FC) threshold of 2 and a P value < 0.05 adjusted with the Benjamini–Hochberg procedure. Voom transformation and DEG analysis were performed with R packages egdeR and limma, respectively. The top 200 genes upregulated (FC > 7.75) in human metastatic melanoma samples exhibiting high *SMPD3* expression level group were selected for pathway analysis. This was performed with Autocompare ZE based on the Zelen exact test for P value estimation (ref. 22; available at https://sites.google.com/site/fredsoftwares/products/autocompare-ze) using C5 database from the

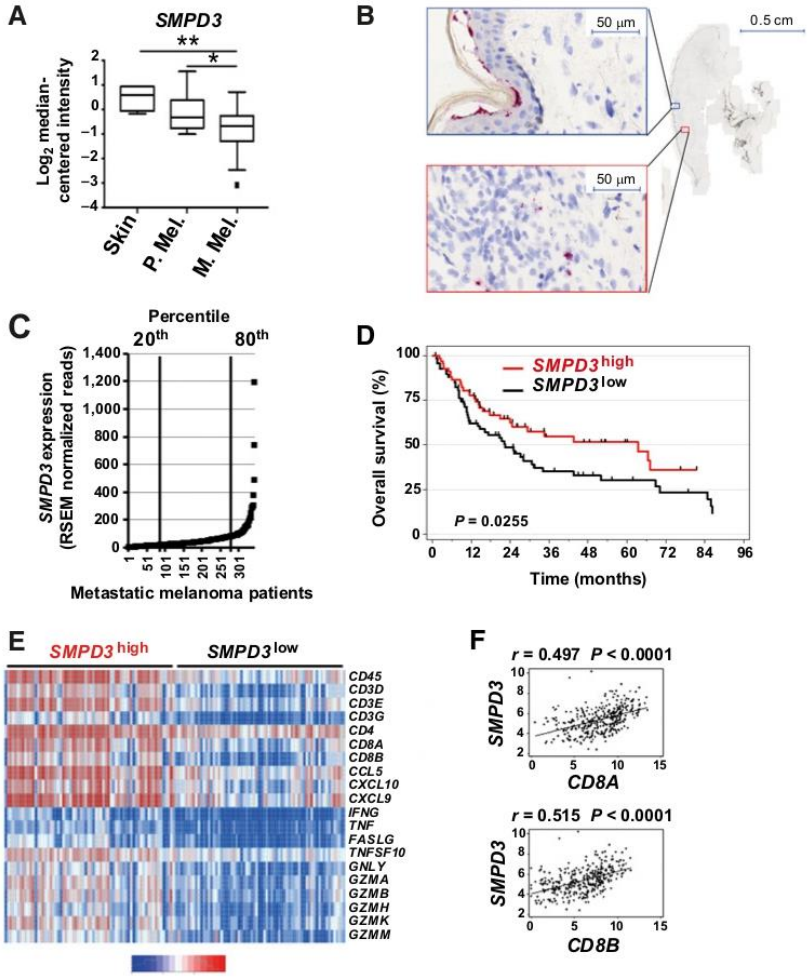

Figure 1.

Low expression of *SMPD3* in patients with melanoma is associated with worse prognosis and low immune gene signatures. **A,** *SMPD3* expression analysis in normal human skin ($n = 4$), primary (P. Mel., $n = 14$), and metastatic (M. Mel., $n = 40$) melanoma samples from the Oncomine database. *, $P < 0.05$; **, $P < 0.01$. **B,** *SMPD3* expression analyzed by ISH on a skin sample from a patient with advanced melanoma. Pictures are representative of staining carried out on samples from five patients. **C,** *SMPD3* expression analysis in tumor biopsies from patients with metastatic melanoma (TCGA melanoma cohort; $n = 342$). RSEM, RNA-Seq by Expectation Maximization. **D,** Overall survival in patients with metastatic melanoma from the TCGA melanoma cohort, exhibiting tumors with high (>80th percentile; $n = 68$) or low (<20th percentile; $n = 68$) *SMPD3* expression in melanoma samples. **E,** Heatmap depicting the differential expression of a selected set of genes related to immune responses in melanoma biopsies (TCGA) with high ($SMPD3^{high}$) or low ($SMPD3^{low}$) *SMPD3* expression. Genes were clustered using a Euclidean distant matrix and average linkage clustering. **F,** Correlation analyses of *SMPD3* expression with the indicated genes (TCGA).

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Broad Institute (http://software.broadinstitute.org/gsea/msigdb/collections.jsp; ref. 23). Date of origin for computation of overall survival was the date of specimen procurement. Survival rates were estimated using the Kaplan–Meier method, and comparison between groups (low expression vs. high expression) was performed using the log-rank test. The strength of relationship between continuous covariates was assessed using Spearman rank correlation coefficient. DNA methylation data, obtained with the Infinium HumanMethylation450K BeadChip technology, were available for 233 patients out of the 342 patients with metastatic melanoma and downloaded from TCGA website. *SMPD3* mutation analysis in human melanoma was assessed on cBioportal (http://www.cbioportal.org/; refs. 24, 25). The characterization of mutations predicted to be potentially damaging for nSMase activity was assessed by polyphen2 (http://genetics.bwh.harvard.edu/pph2/).

Cell lines

B16K1 is a genetically modified cell line kindly provided by Dr. Anne Françoise Tilkin Mariamé in 2010 (26, 27). To generate the B16K1 cell line, B16F10 cells were engineered to stably express the MHC class I molecule H-2Kb (27). B16K1 cells were cultured in DMEM medium (Gibco, #61965-026) containing 10% heat-inactivated FCS and were authenticated in February 2012 by the Leibniz Institute DSMZ GmbH. To guarantee cell line authenticity, the B16K1 cell line was used within 10 passages following thawing and tested for the expression of melanocyte-lineage proteins such as tyrosinase-related protein 2 (TRP2). Medium was changed every 2 to 3 days. 4T1 cells were from the ATCC (CRL-2539) and kindly provided by Dr. F. Cabon (CRCT) in 2015; they were not further authenticated, used within a maximum of 10 passages from thawing, and cultured in DMEM medium containing 10% FCS. Yumm 1.7 cells were kindly provided by Dr. M. Bosenberg (Yale University School of Medicine, New Haven, CT) in 2014. Cells were generated from tumors of $Braf^{V600E}Cdkn2A^{-/-}Pten^{-/-}$ mice and were described elsewhere (28, 29). Yumm 1.7 cells were cultured in Opti-MEM (Gibco, #31985-047) supplemented with 5% FCS. Cell lines were tested for the absence of mycoplasma contamination by PCR using the GoTaq G2 DNA polymerase Kit (Promega, #M7845) and the primers: GCT GTG TGC CTA ATA CAT GCA T; ACC ATC TGT CAC TCT GTT AAC CTC.

Cell transfection

B16K1 cells were transfected (Superfect reagent, QIAGEN, #301305) with a plasmid (pEF6-V5-TOPO, Invitrogen, #46-0705) containing the cDNA encoding mouse nSMase2, which was kindly provided by Dr. Y. Hannun and was previously described (30). These transfected cells produce a V5-tagged nSMase2. Transfected cells were selected for their resistance to blasticidin (7 μg/mL; Invivogen, #ant-bl-1). Resistant cells were cultured in DMEM containing 10% FCS and 7 μg/mL blasticidin, and analyzed by Western blot for the detection of the V5 tag (see below for Western Blot protocol). Two cell populations were selected: B16K1 nSMase2^{high} and B16K1 nSMase2^{low}, which overexpressed or not V5-tagged nSMase2, respectively. Mock-transfected B16K1 cells were obtained by transfecting a plasmid conferring resistance to blasticidin. nSMase enzyme activity was also assessed to determine nSMase2 overexpression (see below for protocol).

Mice

Wild-type (WT) C57BL/6 mice were from Janvier Laboratories. CD8α-deficient C57BL/6 mice were a gift from Prof. J. van Meerwijk (INSERM U1043, Toulouse, France). For each experimental condition, 6- to 12-week-old female mice were used. Mice were maintained in specific pathogen–free conditions at the CRCT animal facility (US006 CREFRE - Inserm/UPS), which is accredited by the French Ministry of Agriculture to perform experiments on live mice (accreditation number A-31 55508). All *in vivo* experimental protocols were conducted with the approved of the local ethic committee, an Institutional Animal Care and Use Committee, and in accordance with the French and European regulations on care and protection of Laboratory Animals.

In vitro bone marrow–derived dendritic cell culture

To generate mouse dendritic cell (DC) *in vitro*, bone marrow–derived cells (BMDC) from C57BL/6 mice were harvested and cultured in complete RPMI, which was RPMI (Gibco, #61870-010) supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL; Sigma, #P0781), 50 μmol/L β-mercaptoethanol (Gibco, #31350-010), and 20 ng/mL GMCSF (Peprotech, #315-03-100UG) at 37°C with 5% CO_2. At day 3, nonadherent cells were removed, and new complete RPMI was added with half of it changed every 2 to 3 days. After at least 7 days of culture, DC differentiation was analyzed by FACS as described below. DCs were cultured for 24 hours in the presence or absence of 5 to 10 μg/mL small extracellular vesicles (sEV), purified as described below.

sEV purification

sEV purification was performed as previously described from the conditioned medium of B16K1 nSMase 2 WT and catalytically inactive (C.I.) cells (31). Briefly, cells were cultured in DMEM 10% sEV-free FCS (prepared after overnight centrifugation at 100,000 × *g*, 4°C). The medium was collected after 3 days of culture, and sEVs were isolated by differential ultracentrifugation. Briefly, the culture medium was centrifuged at 300 × *g* for 10 minutes. The supernatant was then sequentially centrifuged at 2,000 x *g* for 20 minutes, at 10,000 × *g* for 45 minutes, and at 110,000 × *g* for 70 minutes at 4°C to sediment sEVs. The sEV pellet was then washed with PBS, and further centrifuged at 110,000 × *g* for 70 minutes at 4°C. The resultant sEV pellet was resuspended in PBS for *in vitro* culture and FACS analysis or in Western blot cell lysis buffer (see protocols below).

Western blot analysis

Cells and sEVs were lysed in a buffer containing 50 mmol/L HEPES (Sigma, #H3375), pH 7.5, 150 mmol/L NaCl (Sigma, # S-9625), 10% glycerol (Sigma, #G-7893), 1% Triton X-100 (Sigma, #X100), 1 mmol/L $NaVO_4$ (Sigma, #S-6508), 10 mmol/L β-glycerophosphate (Sigma, #G9891), 50 mmol/L NaF (Sigma, #S-7920), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, #P-3075), 10 μg/mL leupeptin (Sigma, #L2884), 2 μg/mL pepstatin A (Sigma, #P-5318), and 10 μg/mL aprotinin (Sigma, #A6279) for 30 minutes on ice and sonicated. Equal amounts of proteins were separated in a 7.5% to 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech, GE Healthcare, #10600002). Proteins were detected using anti-V5 (Invitrogen, #477701A) and anti–β-actin (Cell Signaling Technology), anti-mouse TRP2 (Santa Cruz Biotechnology, #sc-10451), anti-mouse TSG101 (Abcam, #ab83), anti-mouse HSP70 (Cell Signaling Technology, #4872), and anti-mouse, anti-goat, or anti-rabbit horseradish peroxidase–conjugated IgG (Cell Signaling Technology). Staining was revealed using an ECL detection system (Clarity Western ECL substrate, Bio-Rad, #170-5060) and visualized by film exposure (Genesee Scientific, #30-101) or Chemidoc Imaging systems (Bio-Rad).

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In vitro PD-L1 expression

B16K1 cells (0.3×10^5) expressing high or low levels of nSMase2 were plated in 6-well plates and treated with vehicle [PBS (Eurobio Scientific, #CSP1PBS01-01)], 0.1% BSA (Euromedex, #1035-70-C), 50 ng/mL recombinant murine TNF (Peprotech, #315-01A), and 100 U of recombinant murine IFNγ (Peprotech, #315-05) alone or combined in DMEM 10% FCS for 72 hours. Cells were detached using Versene solution (Gibco, #15040-033), PD-L1 expression (BD Biosciences, MIH5) was assessed by flow cytometry on an LSRFortessa X-20 (BD Biosciences), and data were analyzed with the FlowJow10 software.

mRNA ISH

Biopsies of melanoma tumors were performed on five patients between 2014 and 2017. All patients provided informed written consent. No particular inclusion/exclusion criteria were applied. The protocol was approved by "CPP du Sud-Ouest et Outre-Mer IV" (Limoges, France) committee. Patient studies were performed in accordance to the Declaration of Helsinki. ISH for *SMPD3* mRNA was performed on formalin-fixed paraffin-embedded cutaneous tumor sections (and surrounding healthy tissue) using a specific probe from RNA ScopeVR (Advanced Cell Diagnostics, #490279) and the Ventana Discovery–automated ISH slide staining systems according to the manufacturer's instructions.

Cloning WT and C.I. V5-nSMase2 in pMSCV-Puro

Retroviral expression vectors encoding WT or mutant (D428A) mouse nSMase-2 were obtained by cloning the product of the partial BamHI and PmeI digestion of pEF6-V5-His (Clontech, Takara, K1062-1) donor expression vectors encoding WT and C.I. mouse nSMase-2 into pMSCV-Puro (Clontech, #634401; ref. 32) linearized with BglII and HpaI.

Retrovirus production and cell transduction

The generation of retroviruses has been described previously (33). Viral particles of WT and C.I. (D428A) nSMase-2 derived from pMSCV-Puro vectors or control vector (pMSCV-Puro empty vector) were produced to transduce 1 to 3×10^6 mouse B16K1, Yumm1.7, and 4T1 for 16 hours in 6-well plates in the presence of Polybrene (8 μg/mL, Santa Cruz Biotechnology, #134220) in DMEM 10% FCS. Cells were then washed in PBS, harvested, plated in DMEM 10% FCS containing puromycin (2.5 μg/mL, Gibco, #12122530), and incubated for 3 days before amplification and subsequent analysis of the polyclonal populations (puromycin selection, enzyme activity assay, and Western blot for detection of the V5 Tag as described in the sections above and below). B16K1 and 4T1 cells overexpressing the WT or C.I. form of nSMase2 are mentioned in the text as B16K1 nSMase2 WT or C.I. and 4T1 nSMase2 WT or C.I.; the Yumm1.7 transduced with the vector encoding WT nSMase2 or control vector are mentioned in the text as Yumm nSMase2 WT or control.

In vitro B16 melanoma cell proliferation and clonogenicity

To study cell proliferation, B16 cells were cultured in DMEM medium containing 0% or 10% FCS. Cells were counted at the indicated times using a cell counter (Beckman). Melanoma cells were grown on soft agar (clonogenicity assay) or as spheroids. For the growth on soft agar, 6-well plates were precoated with 2 mL DMEM 4.5 g/L glucose containing 10% FCS and 0.6% agar (Euromedex, #EU0031). DMEM (2 mL) containing 10% FCS, 0.3% agar, and 10,000 B16K1 nSMase2^{high} or nSMase2^{low} cells were overlaid onto the precoated wells. After a 2-week incubation at 37°C in 5% CO_2 atmosphere, cell colonies were visualized by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining (500 μg/mL, Euromedex, #4022-B) at 0.5 mg/mL diluted DMEM 10% FCS and incubated at 37°C for 1 hour before washing with PBS and imaging (phase-contrast pictures, Olympus CKX41, Olympus Life Science). For spheroid growth, 96-well plates were precoated with 50 μL 1% agarose (Lonza, #50004). Three thousand B16K1 nSMase2^{high} or nSMase 2^{low} cells were overlaid onto the precoated wells in DMEM 4.5 g/L glucose (100 μL) containing 10% FCS. After a 12-day incubation at 37°C in 5% CO_2 atmosphere, spheroids were imaged using the Olympus CKX41 microscope (phase-contrast pictures).

Neutral SMase activity measurement

Cellular and tumor nSMase activities were assayed as described previously (34) using [choline-methyl-^{14}C]SM (100,000 dpm/assay) as substrate. Briefly, B16K1, Yumm1.7, and 4T1 cells were resuspended in a buffer containing 0.1% Triton X100 (Sigma, #X100), 20 mmol/L HEPES (pH 7.4; Sigma, #H3375), 10 mmol/L $MgCl_2$ (Euromedex, 2189-A), 2 mmol/L EDTA (Sigma, #E-5134), 5 mmol/L DTT (Euromedex, #EU0006-A), 0.1 mmol/L Na_3VO_4 (Sigma, #S-6508), 0.1 mmol/L Na_2MoO_4 (Sigma, #331058), 10 mmol/L β-glycerophosphate (Sigma, G9891), 750 μmol/L ATP (Sigma, #FLAAS), 10 μmol/L leupeptine (Sigma, #L2884), and 10 μmol/L pepstatin-A (Sigma, #P-5318) and sonicated. For tumors, 1 mL of the above-mentioned buffer was used to homogenize 100 mg of tumor extract using the FastPrep 24 technology (MP Biomedical) with ceramic beads (MP Biomedicals, #6913100). nSMase activity assay was performed by mixing the protein extract with [choline-methyl-^{14}C]SM [100,000 dpm/assay; PerkinElmer, NEC6630 (IOUC)] resuspended in a buffer containing 20 mmol/L HEPES (pH 7.4) and 1 mmol/L $MgCl_2$ as substrate (v/v) for 2 hour at 37°C. The generated [^{14}C] phosphorylcholine is then separated by Floch technique by adding chloroform/methanol (2:1, v/v) solution and counting of the radioactive aqueous phase by scintillation on the Tri Carb 2910 TR (Perkin Elmer) using the liquid scintillation cocktail Ultima Gold (Perkin Elmer, #6013329).

SL analysis from tumors

Tumors were collected and homogenized in 20 mm Tris buffer with protease inhibitors (Roche, #11873580001) using glass beads (Sigma Aldrich, #G8772) in the FastPREP-24 Classic instrument (MP Biomedicals). Lipids were initially extracted from 0.5 to 1 mg of tumor cell lysate by addition of 2 mL 2:3 70% isopropanol:ethyl acetate and submitted for analysis at Stony Brook Lipidomics core. Lipids were further extracted by addition of 2 mL 2:3 70% isopropanol:ethyl acetate and centrifugated for 5 minutes (1,000 x *g*). Organic phases were dried down and reconstituted in 100 μL mobile phase B (0.2% formic acid, 1 mmol/L ammonium formate in methanol). SL analysis was carried out by tandem high-performance liquid chromatography: mass spectrometry on a Thermo Finnigan TSQ 7000 triple quadrupole mass (35). Ceramide peaks were identified by comparison with known standards, and the area under the curve was measured. Absolute lipid levels were determined using a standard curve of known lipid amounts using standards. Results from mass spectrometry analysis were normalized to total protein concentration as determined by Bradford assay (Bio-Rad, #500-0006).

RNA isolation and qRT-PCR

For RNA isolation from tumors 12 days after B16K1 cell injection, tumors were collected and dissociated using a tissue homogenizer (Precellys, Bertin) using at 6,500 rpm for 2 cycles of 30 seconds in vials containing ceramic beads (MP Biomedicals, #6913100). RNA was purified using the RNeasy Midi Kit (QIAGEN, #75144). cDNA

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from total RNA was prepared with the SuperScript II Reverse Transcriptase using 1 μg of RNA from each sample (Thermo Fisher Scientific, #18064022). qPCR was performed using the SYBR Green Master Mix (Takara, #RR420L) and primers for transcripts encoding murine β-actin (QT01136772), hypoxanthine-guanine phosphoribosyltransferase (HPRT; QT00166768), CXCL9 (QT00097062), IL12p40 (QT00153643), CCL19 (QT02532173), CCL2 (QT00167832), CXCL11 (QT00265041), CXCL10 (QT00093436), CCL5 (QT01747165), TNF (QT00104006), CCL17 (QT00131572), CCL22 (QT00108031), and IL1β (QT01048355; QIAGEN, QuantiTech Primer Assay). Assays were run on the StepOne Plus instrument (Thermo Fisher Scientific), and gene expression for all cytokines and chemokines tested was calculated according to the formula $2^{-\Delta Ct}$. The mean Ct value for the expression level of HPRT and β-actin was used as reference.

Confocal microscopy analysis

B16K1 cells were cultured on glass coverslips for 24 hours and fixed in PBS paraformaldehyde. Cells were stained with anti-giantin (Invitrogen, #BS-13356R) and anti-V5 (Invitrogen, #2F11F7) antibodies and dye-coupled secondary antibodies [goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, #A11008); goat anti-mouse IgG2a Alexa Fluor 568 (Invitrogen, #A-21134)] and analyzed by confocal microscopy (Zeiss, LSM510).

In vivo tumorigenesis of B16K1 cells

B16K1 cells (3×10^5) expressing WT or C.I. V5-tagged nSMase2 were intradermally injected in WT or CD8α-deficient mice. Tumor volume was calculated using a caliper at the indicated days with the formula: Tumor volume $= 0.52 \times \text{length} \times \text{width}^2$. For some experiments, mice received i.p. injection of anti–PD-1 therapy (200 μg; BioXcell; clone RMP1-14), anti–CTLA-4 (200 μg first cycle then 100 μg; BioXcell; clone 9H10), or vehicle (PBS) on days 6, 10, and 13 after tumor cell injection.

Tumor immune infiltrate analysis

One million B16K1 cells overexpressing or not a WT or C.I. V5-tagged nSMase2 were intradermally and bilaterally injected in WT mice. Tumors were weighed and digested with the mouse Tumor Dissociation Kit (Miltenyi, #130-096-730) before flow cytometry analysis.

Flow cytometry analyses

For the analysis of sEVs by flow cytometry, 4 μL of latex beads (Invitrogen, #A37304) diluted in 1 mL PBS were precoated with 5 μg of sEVs during 16 hours at 4°C under rotation. Following BSA (Euromedex, #1035-70-C) saturation and washing, beads were incubated with an anti-mouse CD63 (BioLegend, NVG-2).

To analyze for the *in vitro* maturation of DCs, cells were cultured with or without 10 μg/mL sEVs during 24 hours and then stained with the following antibodies: anti-CD11c (eBioscience, N418), anti-CD80 (eBioscience, 16-10A1), anti-CD86 (BB Biosciences, PO3), anti–MHC-I (BD Biosciences, AF6-88.5), and anti–MHC-II (eBioscience, M5/114.15.2).

For the analysis of the tumor immune infiltrate, cells were stained with antibodies or MHC-I/TRP2 dextramers (APC-conjugated H-2Kb/SVYDFFVWL, Immudex) and live-dead reagents (Invitrogen, #L34TIL analysis). To assess for IFNγ and TNF production by tumor-infiltrating lymphocytes (TIL), cells from the dissociated tumors were incubated for 4 hours in the presence of 1X cell stimulation cocktail (eBioscience, #00-4970-93) and 1X Protein transport inhibitor (eBioscience, #00-4980-03) prior to cytometry analysis. Antibodies used in this study were as follows: anti-mouse CD45 (BD Biosciences, 30-F11), anti-mouse Thy1.1 (BioLegend, H12), anti-mouse CD8 (BioLegend, 53-6.7), anti-mouse CD4 (BD Bioscience, GK1.5), anti-mouse FoxP3 (eBioscience, FJK-16s), anti-mouse CD11c (eBioscience, N418), anti–PD-L1 (BD Bioscience, MIH5), anti-IFNγ (BD Biosciences, XMG1.2), anti-TNF (BD Biosciences, MP6-XT22), anti-Granzyme B (BioLegend, GB11), anti–TIM-3 (eBioscience, J43), anti–CTLA-4 (eBioscience, UC10), and anti-TCRβ (BD Bioscience, H57–597). For the staining of intracellular targets, cells were first stained for membrane markers, then fixed and permeabilized using the "Foxp3 fixation/permeabilization staining buffer set" (eBioscience, #00-5523-00) before staining for Foxp3 or TNF and IFNγ. Samples were acquired using an LSRFortessa X-20 (BD Biosciences), and data were analyzed with the Diva or FloJow 10 softwares. Gating strategies are depicted in Supplementary Data File S2.

Statistical analysis

Each experiment was designed to use the minimum number of mice or samples required to obtain informative results and sufficient material for subsequent studies. No specific statistical tests were used to predetermine the sample size. For animal experimentation, we used at least 5 mice per group, and experiments were typically performed twice, unless otherwise stated in the figure legends. Statistical significance of difference between groups was evaluated using the GraphPad Prism 7 software. Briefly, we tested whether the values come from a Gaussian distribution using a D'Agostino–Pearson omnibus normality test. When passing the normality test, a Student *t* test was used. Otherwise, a Mann–Whitney *U* test was used. For statistical significance of animal survival, the log-rank test was used. Differences were considered to be statistically significant when $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). *In vivo* and *in vitro* experiments were monitored in a nonblinded fashion, and no method of randomization was used. In tumorigenesis experiments, mice for which no tumor was observed during 10 days after the inoculation of cancer cells were excluded. For the analysis of TILs, tumors in which the number of cells was too small were excluded from the analysis.

Results

SMPD3 expression in human melanoma biopsies correlates with a $CD8^+$ T-cell gene signature

Analysis from the Oncomine database indicated that low levels of *SMPD3* transcripts are found in human metastatic melanoma as compared with primary tumors and normal skin (**Fig. 1A**), suggesting that nSMase2 downregulation is likely associated with melanoma progression. ISH experiments on skin sections from patients with advanced melanoma using RNAscope technology revealed robust and low *SMPD3* expression in the tumor-adjacent stratum granulosum and cutaneous melanoma, respectively (**Fig. 1B**; Supplementary Fig. S1A–S1D). Moreover, a majority of tumor biopsies from the TCGA cohort of patients with advanced melanoma expressed *SMPD3* at low levels (**Fig. 1C**). Because gene hypermethylation has been shown to downregulate *SMPD3* expression in solid tumors (9), we analyzed the methylation status of *SMPD3* in the TCGA melanoma cohort. This analysis identified one CpG island located within the promoter core (73 pb downstream of the transcription start site) for which the methylation level (>0.27) inversely correlated to gene expression (Supplementary Fig. S1E and S1F). This suggests that, at least in some patients, DNA methylation of the promoter region may contribute to

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SMPD3 downregulation in melanoma. The clinical outcome for patients with metastatic melanoma exhibiting high and low *SMPD3* expression was analyzed. Low *SMPD3* expression was significantly associated with shortened overall survival (**Fig. 1D**), further suggesting that *SMPD3* downregulation is associated with a worse prognosis for patients with melanoma.

Focusing on the TCGA melanoma cohort, we examined which gene signatures were associated to high or low levels of *SMPD3* expression in tumor samples. A high *SMPD3* expression was mostly associated with signatures related to "Immune system process" and "Lymphocyte activation" according to Gene ontology classification (Supplementary Table S1). Further analysis showed that high *SMPD3* expression was associated with high expression of *CD3G, CD3D*, and *CD3E*, which reflect TILs (**Fig. 1E**; **Table 1**). Among T-cell–associated genes, we found that *CD8A, CD8B*, and *CD4* were enriched in melanoma samples expressing *SMPD3* at high levels. Moreover, various Th1-related genes such as *IL12, CXCL10*, and *TBX21* as well as genes related to cell-mediated cytotoxicity were expressed at higher levels in melanoma samples exhibiting high *SMPD3* expression (**Fig. 1E**; **Table 1**). Accordingly, *SMPD3* expression significantly correlated with the expression of a set of genes, which likely reflect $CD8^+$ T-cell infiltration (**Fig. 1F**; **Table 1**). The expression level of genes encoding other known SMases was not associated with a gene signature of $CD8^+$ TILs in patients with metastatic melanoma except for *SMPD2*, which poorly, yet significantly, correlated with *CD8B* (**Table 1**). In contrast, *SMPD4* was negatively correlated with T-cell–related genes (**Table 1**).

Thus, expression of *SMPD3* (but not the expression of other SMases) is associated with a $CD8^+$ T-cell gene signature in human melanoma samples. We hypothesize that this may contribute to the improved overall survival seen for patients with high *SMPD3* expression.

nSMase2 expression impairs melanoma growth by enhancing $CD8^+$ T-cell responses

To assess the impact of *SMPD3* expression on melanoma growth and $CD8^+$ T-cell–mediated antitumor responses, we conducted studies using the B16K1 mouse melanoma cell line, which exhibits low endogenous nSMase activity (Supplementary Fig. S2A) and overexpresses MHC class I (26, 36). We first generated B16K1 melanoma cell lines with or without nSMase2 overexpression (**Fig. 2A**; Supplementary Fig. S2B). The overexpressed enzyme was mainly localized at the plasma membrane (**Fig. 2B**), and high expression of nSMase2 led to robust increases in cellular nSMase activity (Supplementary Fig. S2A) and ceramide levels (**Fig. 2C**), affecting neither two- nor three-dimensional cell growth nor clonogenicity *in vitro* (Supplementary Fig. S2C and S2D).

Upon intradermal injection of B16K1 cells in C57BL/6 mice, nSMase2 overexpression, as evaluated by Western blot (Supplementary Fig. S2E, top plot, and S2F), triggered an intratumor increase in nSMase activity (Supplementary Fig. S2E, bottom plot) and elevation of ceramide levels (**Fig. 2D**). More precisely, high nSMase2 expression led to increased levels of the C12, C14, C16, C18:1, C22:1, and C24:1 ceramides species in tumors (**Fig. 2D**). Of note, nSMase2 overexpression was also linked to a significant increase in intratumor sphingosine levels, but it did not significantly affect levels of SM and sphingosine 1-phosphate (S1P; Supplementary Fig. S2G). In addition, high nSMase2 expression was associated with a strong decrease in B16K1 tumor growth in WT mice, as compared with tumors expressing low levels of the enzyme (**Fig. 2E**).

Because nSMase2 overexpression did not decrease *in vitro* proliferation of B16K1 cells but impaired tumor growth *in vivo*, we hypothesized that high nSMase2 expression in melanoma cells could trigger a remodeling of the tumor microenvironment. Building upon the data obtained using human samples from the TCGA dataset, we analyzed the immune response in mice engrafted with B16K1 melanoma cells expressing nSMase2 at low or high levels. Leukocytes ($CD45^+$) and T cells ($Thy1^+$) frequencies were significantly increased in melanomas that expressed nSMase2 at high levels (**Fig. 2F**). Among T cells, the proportion of $CD8^+$ TILs was 3-fold higher in tumors expressing nSMase2 at high levels than in tumors expressing low levels of the enzyme (**Fig. 2F**).

To determine whether this increase in $CD8^+$ TILs may be important in mediating the deleterious impact of nSMase2 on melanoma growth, $nSMase2^{high}$ and $nSMase2^{low}$ B16K1 cells were engrafted in CD8α-deficient mice. Importantly, high nSMase2 expression failed to impair B16K1 melanoma growth in CD8α-deficient mice (**Fig. 2G**).

Collectively, these data indicate that nSMase2 overexpression in mouse melanoma enhances $CD8^+$ T-cell–dependent immunity, which consequently impairs tumor growth.

nSMase2 enzyme activity is required for enhancing T-cell–dependent anti-melanoma immune responses

Our analysis of the *SMPD3* nucleotide sequence in human melanoma from four independent studies identified mutations in the coding sequence, ranging from 2.5% to 20% mutation frequency depending on the study (37–40). The highest mutation frequency was observed in desmoplastic melanoma, whereas the lowest was in uveal melanoma (**Fig. 3A**). Most of the mutations were missense mutations, and half of them affected residues in the catalytic domain (**Fig. 3B**). Moreover, 12 mutations were predicted to be potentially damaging for nSMase activity (HumDiv score > 0.85) according to PolyPhen-2 analysis (**Fig. 3B**).

Table 1. Correlation of *SMPD1–4* expression with immune-related genes.

		IFNG	*STAT1*	*IL12A*	*IL12B*	*IRF1*	*TBX21*	*CD8A*	*CD8B*	*CXCL9*
SMPD1	Correlation	−0.032	0.0295	−0.103	−0.021	0.0287	0.0376	−0.001	0.0214	−0.032
	P value	0.553	0.586	0.055	0.689	0.597	0.488	0.975	0.693	0.544
SMPD2	Correlation	0.048	0.019	**−0.231**	0.061	0.067	0.102	0.074	**0.110**	0.012
	P value	0.379	0.728	<0.001	0.259	0.213	0.059	0.172	0.041	0.818
SMPD3	Correlation	**0.459**	**0.312**	**0.125**	**0.499**	**0.502**	**0.538**	**0.497**	**0.515**	**0.502**
	P value	<0.001	<0.001	<0.001	0.0204	<0.001	<0.001	<0.001	<0.001	<0.001
SMPD4	Correlation	**−0.151**	**−0.240**	−0.066	−0.104	**−0.142**	−0.080	**−0.135**	**−0.119**	**−0.211**
	P value	0.005	<0.001	0.218	0.054	0.008	0.135	0.012	0.027	<0.001

Note: The strength of relationship between genes in melanoma samples using Spearman rank correlation coefficient. Significant correlations are highlighted in bold.

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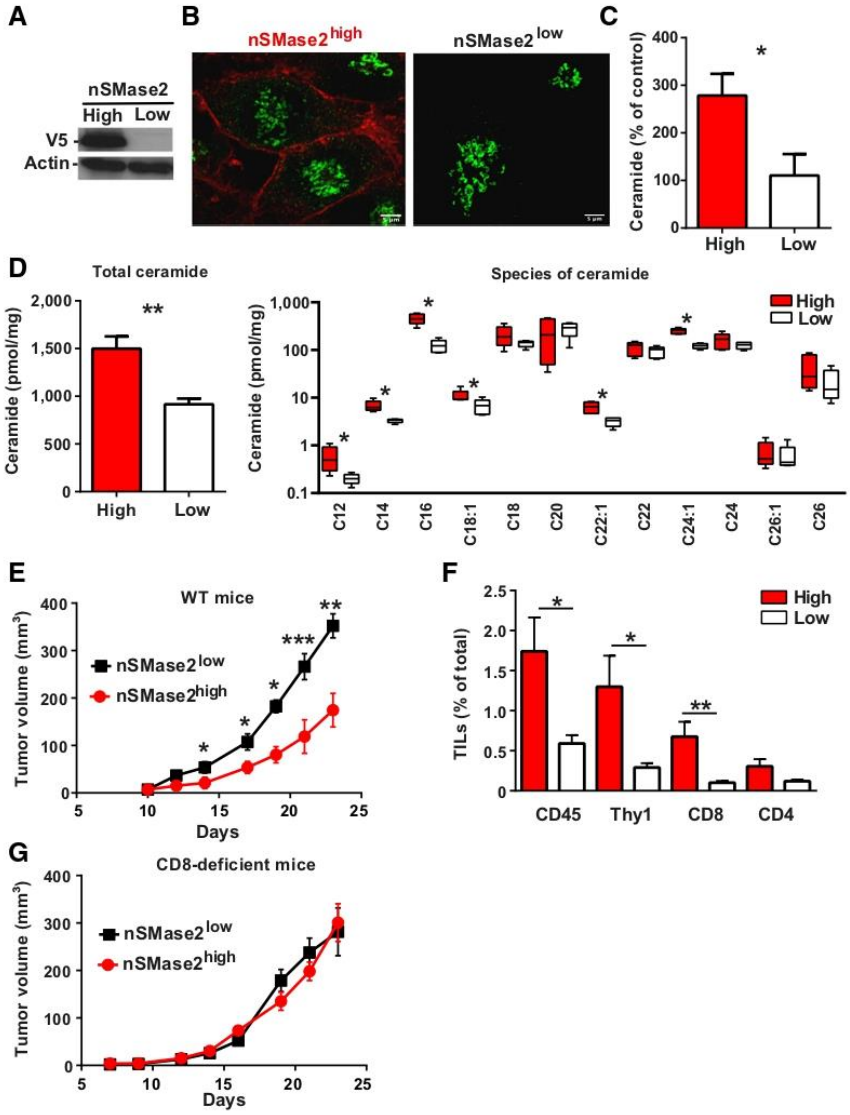

Figure 2.

nSMase2 expression in mouse melanoma enhances CD8$^+$ T-cell–dependent immune responses. **A,** B16K1 cells transfected to overexpress V5-tagged nSMase2 (nSMase2^{high}) or not (nSMase2^{low}) were analyzed by Western blot using anti-V5 and anti–β-actin antibodies. **B,** Confocal microscopy analysis showing V5-tagged nSMase2 (red staining) and giantin (green staining) localization in B16K1 cells expressing nSMase2 at low or high level. **C,** Intracellular ceramide levels in B16K1 nSMase2^{high} and B16K1 nSMase2^{low} cells. Data are expressed as the percentage of values obtained as compared with mock-transfected B16K1cells. Values are mean ± SEM of four independent experiments. **D–G,** C57BL/6 WT (**D–F**) and CD8α-deficient (**G**) mice were intradermally and bilaterally (**D**, **E**, and **G**) or monolaterally (**F**) injected with B16K1cells expressing high (nSMase2^{high}; red bars, Tukey boxes and curves) or low (nSMase2^{low}; black and white bars, Tukey boxes and curves) levels of nSMase2. **D,** At day 12, WT mice were sacrificed and the levels of total as well as of subspecies of ceramide were analyzed by mass spectrometry ($n = 5$ tumors/mice per group, Student t test and Kruskal–Wallis). **E,** Tumor volumes in WT mice at the indicated days ($n = 4$–5 mice per group). **F,** Tumor-infiltrating leukocytes were analyzed using flow cytometry at day 12 following tumor graft ($n = 5$–6 mice per group). **G,** Growth of B16K1 nSMase2^{high} and nSMase2^{low} tumor in CD8α-deficient mice. Data are mean ± SEM of five mice per group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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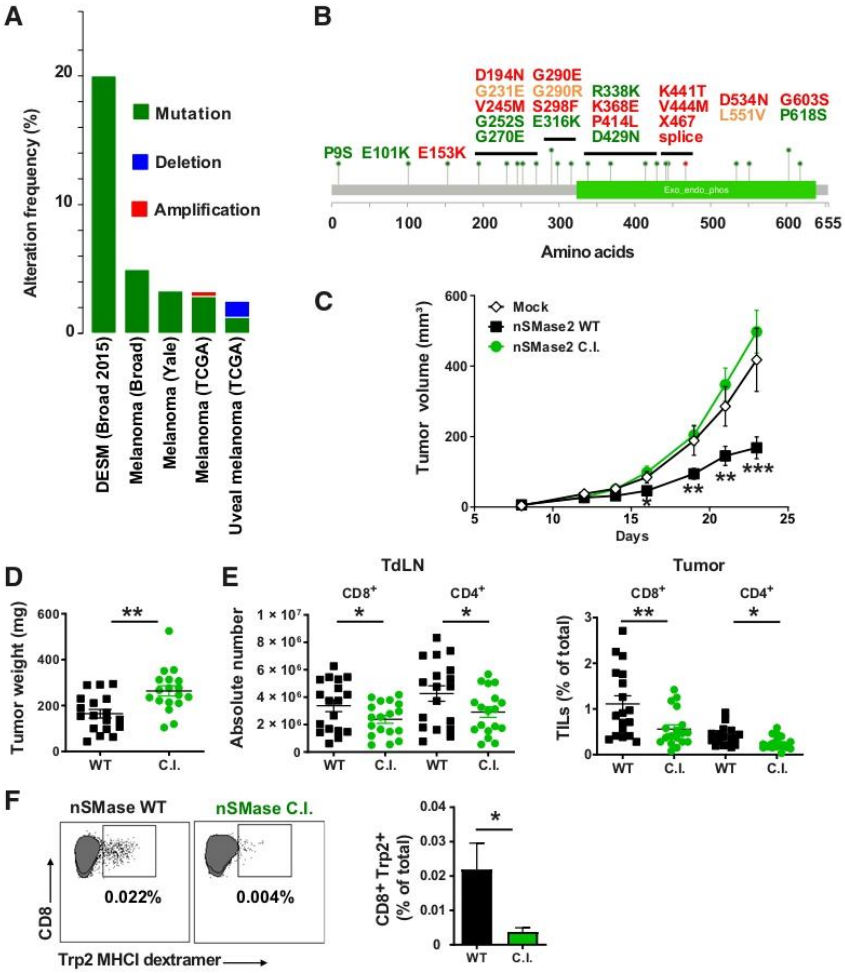

Figure 3.

Catalytic activity is required for nSMase2 to suppress melanoma growth and stimulate immune responses. **A,** Frequency of mutations and copy-number alterations in human melanoma samples from the indicated studies (www.cbioportal.org). **B,** Localization of missense (green dots) and splice (red dot) mutations on nSMase2 amino acid sequence from the studies depicted in **A**. The catalytic site corresponds to the green box. Mutations are predicted to be benign (green), possibly damaging (orange), or probably damaging (red; http://genetics.bwh.harvard.edu/pph2/). **C,** B16K1 cells expressing or not (mock) the WT or C.I. nSMase2 were intradermally injected in C57BL/6 WT mice, and tumor volumes were determined at the indicated days. Data are mean ± SEM of four mice per group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **D–F,** B16K1 cells expressing the WT or C.I. nSMase2 were intradermally and bilaterally injected in WT mice, and 12 days later, tumor-draining lymph nodes (TdLN) and tumors were collected. Tumors were weighed (**D**), and T-cell content was analyzed by flow cytometry in TdLNs (**E**, left plot) and tumors (**E**, right plot). Data are mean ± SEM of 18 mice per groups pooled from three independent experiments (**D** and **E**). **F,** CD8$^+$ T cells specific for Trp2 peptides were quantified using dextramer technology. Representative staining and proportion of total Trp2-specific CD8$^+$ T cells are depicted. Numbers are mean ± SEM of six mice per group (*, $P < 0.05$; **, $P < 0.01$).

We then evaluated whether a single missense mutation (D428A) in the catalytic domain of murine nSMase2, which potently impairs enzyme activity (41), had an impact on CD8$^+$ T-cell–dependent immune responses and melanoma growth in mice. B16K1 cells were transduced with a retroviral vector encoding either the WT or C.I. form of the V5-tagged nSMase2 (Supplementary Fig. S3A). This approach resulted in a significant increase in nSMase activity in WT nSMase2–expressing cells compared with mock-transduced cells (Supplementary Fig. S3B) but not in the ones expressing C.I. nSMase2. There were no differences in proliferation capacity *in vitro* (Supplementary Fig. S3C) or subcellular localization (Supplementary Fig. S3D) between the cells transduced with WT nSMase2 and those transduced with C.I. nSMase2. However, nSMase activity in WT nSMase2–transduced B16K1 cells, triggered by the retroviral LTR promoter, is less than

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the activity observed in nSMase2^{high} B16K1 cells transfected with the pEF6-V5_TOPO plasmid, which has a strong promoter (EF-1α; Supplementary Fig. S3E). The *in vivo* growth of tumors retrovirally transduced to express WT nSMase2 was reduced (by more than 50%) as compared with mock-transduced B16K1 tumors, but overexpressing the C.I. nSMase2 failed to alter tumor growth (**Fig. 3C**). This indicates that the deleterious effect nSMase2 has on melanoma growth depends on its enzymatic activity.

Next, the immune response was analyzed at day 12 post-B16K1 cell injection, at the inflection point of growth curves. At this time point, WT nSMase2 tumor weight was already significantly reduced compared with C.I. nSMase2 tumor weight (**Fig. 3D**). In accordance with results displayed in **Fig. 2**, tumors expressing WT nSMase2 had higher levels of total CD4$^+$ and CD8$^+$ T cells in both tumor-draining lymph nodes and tumors compared with tumors expressing C.I. nSMase2 (**Fig. 3E**). We next analyzed the infiltration of tumors by CD8$^+$ T cells specific for TRP2, a differentiation antigen of melanocytic cells. We observed higher proportions of TRP2-specific CD8$^+$ T cells in tumors expressing WT nSMase2 than in those expressing the C.I. enzyme (**Fig. 3F**).

Together, these data indicate that the catalytic activity of nSMase2 is required for enhancing T-cell–dependent immune responses toward melanoma cells.

nSMase2 enhances the expression of Th1-related cytokines

To get insights into the molecular mechanisms by which melanoma nSMase2 enhances CD8$^+$ T-cell–dependent immune responses, we first evaluated the impact nSMase2 has on the expression of Th1-related cytokines in tumors. We observed that the levels of mRNA encoding CXCL9 and IFNγ were significantly increased upon WT nSMase2 expression in melanoma tumors as compared with the ones expressing C.I. nSMase2 (**Fig. 4A**). Consistent with this, *SMPD3* expression was significantly correlated with the expression of *IFNG* and *CXCL9* genes in human melanoma biopsies (**Fig. 4B**).

nSMase2 facilitates the budding of exosomes, a subset of sEVs (42), and this likely contributes to the modulation of the anti-melanoma immune response (43–45). Accordingly, we purified and analyzed the molecular composition of sEVs secreted by B16K1 cell expressing WT and C.I. nSMase2 (Supplementary Fig. S4). The total amount of secreted sEVs (Supplementary Fig. S4A), the ultrastructural morphology of sEVs (Supplementary Fig. S4B), and the expression of in sEV markers (CD63, TSG101, and HSP70) and melanoma antigens (MC1R and TRP2; Supplementary Fig. S4C–S4E) were similar for both cell types. Considering that DCs efficiently take up sEVs, a phenomenon that can influence their phenotype (43), the ability of the sEVs to facilitate DC maturation *in vitro* was examined. The DC surface maturation markers CD80, CD86, MHC-I, and MHC-II were similarly upregulated upon incubation with sEVs from B16K1 expressing either WT or C.I. nSMase2 (Supplementary Fig. S4F). However, sEVs from B16K1-expressing WT nSMase2 greatly enhanced BMDC intracellular levels of mRNA encoding some Th1-related cytokines, namely IL12, CXCL9, and CCL19 (**Fig. 4C** and **D**). No significant changes were observed in the induction of other Th1-related cytokines such as CCL5, CXCL11, CXCL10, or TNF in BMDCs in response to sEVs from B16K1 nSMase2 WT or C.I. (**Fig. 4E**, top plots). Finally, we did not observe differences in the expression of transcripts encoding the myeloid chemoattractant CCL2, the regulatory T cell (Treg) chemoattractants CCL17 and CCL22, or the proinflammatory cytokine IL1β (**Fig. 4E**, bottom plots).

Collectively, the data indicate that melanoma nSMase2 enhances the expression of a set of Th1-related cytokines and chemokines in murine and human tumors, which likely facilitates the establishment of a CD8$^+$ T-cell–dependent immune response against melanoma.

nSMase2 expression in cancer cells enhances the efficacy of anti–PD-1 therapy in mice

nSMase2 overexpression significantly reduced B16K1 tumor growth in a CD8$^+$ T-cell–dependent manner but failed to trigger complete tumor rejection. Thus, we evaluated the impact of melanoma nSMase2 on the response to anti–PD-1 therapy, which is a gold standard in melanoma immunotherapy (19). We first evaluated the therapeutic activity of anti–PD-1 toward melanoma tumors expressing nSMase2 at low and high levels (**Fig. 5A**). Although anti–PD-1 therapy significantly delayed the growth of nSMase2^{low} melanoma (**Fig. 5B** and **C**), all tumors relapsed, presumably due to immune escape mechanisms and, consequently, all mice died within 40 days post-B16K1 injection (**Fig. 5D**). nSMase2 overexpression alone delayed melanoma growth (**Fig. 5B** and **C**) and significantly increased the overall survival (**Fig. 5D**). Strikingly, the therapeutic efficacy of anti–PD-1 therapy was dramatically enhanced by nSMase2 overexpression (**Fig. 5B** and **C**). In the group of mice injected with B16K1 nSMase2^{high} cells and anti–PD-1, all mice survived (**Fig. 5D**). Two months after the first tumor challenge, parental B16K1 melanoma cell reinjection in the surviving mice did not lead to tumor development and, as a consequence, did not compromise overall survival. This demonstrates that animals were totally vaccinated toward B16K1 melanoma cells (**Fig. 5D**).

We obtained similar results using the B16K1 tumor model comparing cells expressing WT or C.I nSMase2. WT nSMase2 significantly promoted the therapeutic efficacy of not only anti–PD-1 therapy but also anti–CTLA-4 therapy, whereas C.I. nSMase2 failed to do so (Supplementary Fig. S5A–S5C). Therefore, nSMase2 enzyme activity was required for enhancing ICI therapeutic efficacy. In this model, the effect obtained upon anti–PD-1 treatment was weaker than the one observed in **Fig. 5**. This difference might be related to the fact that B16K1 cells retrovirally transduced with WT nSMase2 exhibited significantly less nSMase activity than the B16K1-transfected cells (nSMase2^{high}; Supplementary Fig. S3E).

We confirmed the deleterious impact nSMase2 has on melanoma growth using another model, the Yumm 1.7 melanoma cells transduced with a control retroviral vector or a vector encoding for WT nSMase2 (Supplementary Fig. S6A). Overexpression of nSMase2 did not impair the *in vitro* proliferation of Yumm 1.7 cells despite increased nSMase activity, as compared with mock-infected cells (Supplementary Fig. S6A). However, nSMase2 overexpression not only dramatically impaired the growth of Yumm 1.7 tumors in immunocompetent mice, but also significantly reduced their resistance to anti–PD-1 therapy (Supplementary Fig. S6B).

To extend our observations to another cancer model and in a different mouse strain (BALB/c), we performed similar experiments using 4T1 breast cancer cells. Overexpressing WT nSMase2 in 4T1 cells significantly increased nSMase activity but did not compromise *in vitro* cell proliferation as compared with 4T1 cells overexpressing the C.I. nSMase2 (Supplementary Fig. S6C). However, WT nSMase2 slightly reduced the growth of 4T1 tumors in immunocompetent mice (Supplementary Fig. S6D). Anti–PD-1 therapy further reduced tumor growth in 4T1 tumors that overexpressed WT nSMase2, whereas C.I. nSMase2–overexpressing 4T1 tumors were totally resistant to anti–PD-1 therapy under our experimental conditions (Supplementary Fig. S6D).

Taken together, these data indicate that expression of WT nSMase2 in cancer cells enhances the efficacy of ICI in preclinical models.

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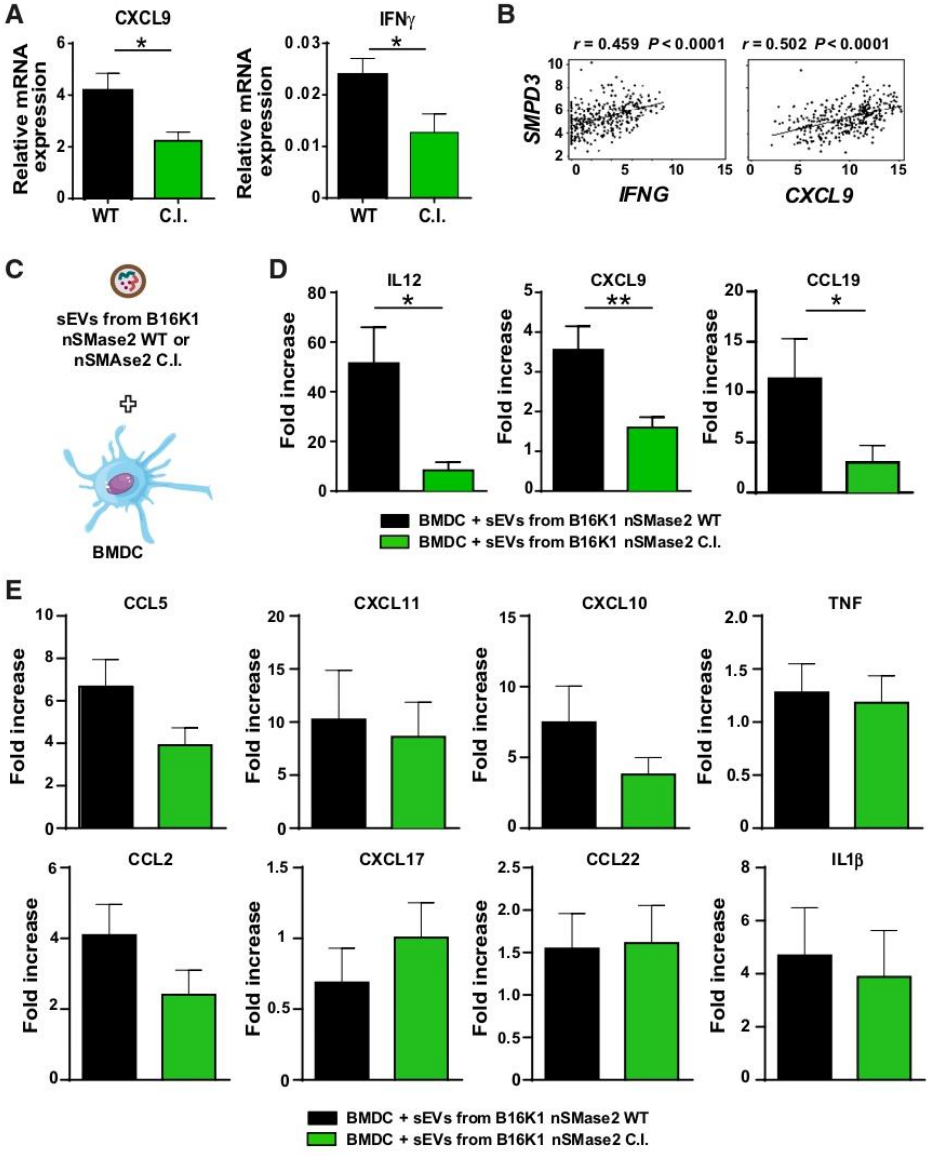

Figure 4.

Melanoma nSMase2 enhances Th1-related gene expression in melanoma tumors and sEV-treated BMDCs. **A,** C57BL/6 mice were injected with B16K1 cells expressing the WT or C.I. form of nSMase2 as described in **Fig. 3**. At day 12, tumors were harvested, RNAs were purified, and transcripts encoding CXCL9 and IFNγ were analyzed by RT-qPCR. Data are mean ± SEM of eight tumors per group (*, $P < 0.05$). **B,** Correlation analyses of *SMPD3* expression with the expression of genes coding for *IFNG* and *CXCL9* (TCGA melanoma dataset). **C–E,** BMDCs were incubated with or without 5 to 10 μg/mL sEVs (pooled results) from B16K1 expressing either WT or C.I nSMase2 (**C**). After 24 hours, expression of the indicated transcripts by BMDCs was analyzed (**D** and **E**). Data are mean ± SEM of five to nine independent experiments carried out on BMDCs treated with two independent sEV preparations and depicted as the fold increase of expression as compared with untreated control BMDCs (*, $P < 0.05$; **, $P < 0.01$).

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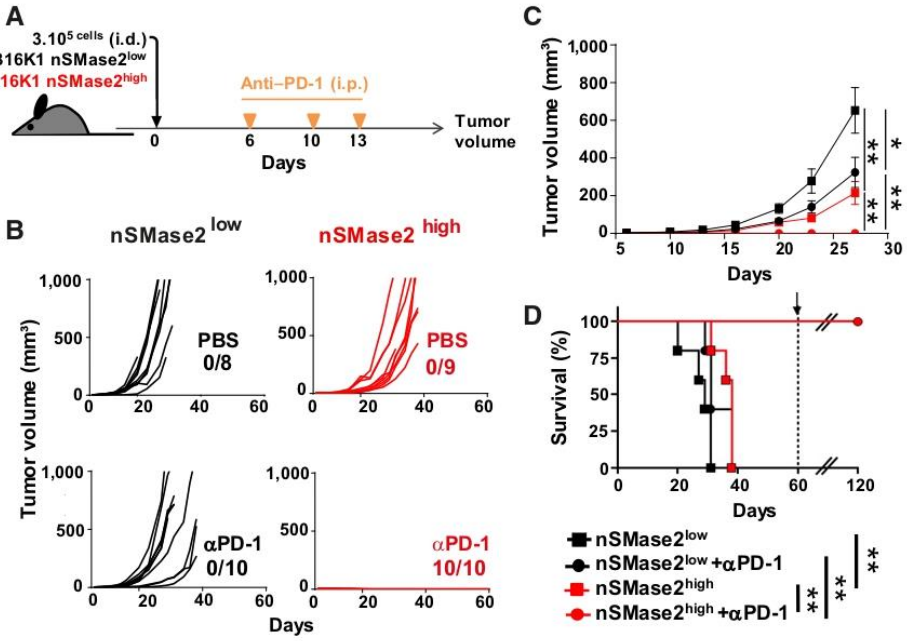

Figure 5.

nSMase 2 potentiates the efficacy of anti–PD-1 therapy *in vivo*. **A–D,** WT mice were intradermally and bilaterally injected with B16K1 expressing high (nSMase2^{high}) or low (nSMase2^{low}) levels of nSMase2 and received i.p. injection of anti–PD-1 (αPD-1; 200 μg) or vehicle (PBS) at days 6, 10, and 13 (5 mice per group, 8–10 tumors; **A**). Individual tumor curves are depicted. Inset, numbers indicate the number of total regressions out of the total number of tumors (**B**). Overall tumor growth analysis in each group. Values are mean ± SEM of 8 to 10 tumors per group (*, $P < 0.05$; **, $P < 0.01$; *t* test; **C**). Overall survival was determined for each group. At day 60, surviving mice were challenged with a second parental B16K1 injection (arrow). Mice did not develop tumors and survived (**D**; *, $P < 0.05$; **, $P < 0.01$; log-rank test).

nSMase2 expression enhances Th1 responses after anti–PD-1 therapy *in vivo*

To understand how melanoma nSMase2 enhanced the response to anti–PD-1 therapy, we monitored the immune response upon nSMase2 expression with or without anti–PD-1 therapy. nSMase2 overexpression increased the proportion of CD8$^+$ T cells in tumors (**Fig. 6A**) and decreased the proportion of CD4$^+$Foxp3$^+$ cells (i.e., Tregs) among Thy1$^+$ TILs (**Fig. 6B** and **C**), irrespective of anti–PD-1 therapy. In addition, nSMase2 overexpression increased the proportion of CD4$^+$ and CD8$^+$ TILs producing IFNγ and TNF, and this was further enhanced upon anti–PD-1 therapy (**Fig. 6D** and **E**). Of note, the proportion of CD8$^+$ TILs expressing granzyme B was significantly increased in B16K1 nSMase2^{high} tumors under anti–PD-1 therapy (**Fig. 6E**). Considering the key role of IFNγ and TNF on PD-L1 expression (36, 46, 47), which can be a predictive marker of response to anti–PD-1 therapy, we next analyzed the expression of PD-L1 on macrophages (CD11b$^+$F4/80$^+$ cells), CD45$^-$ cells, and CD11c$^+$ DCs in tumors. We found that both nSMase2 overexpression and anti–PD-1 therapy increased PD-L1 expression on macrophages and CD45$^-$ cells. Combining nSMase2 expression and anti–PD-1 therapy further increased the expression of PD-L1, especially on CD45$^-$ cells (Supplementary Fig. S7A). The expression of PD-L1 on DCs was however increased only upon anti–PD-1 therapy in tumors expressing high levels of nSMase2 (Supplementary Fig. S7A). Of note, the upregulation of PD-L1 was unlikely a direct consequence of nSMase2 overexpression in melanoma cells because PD-L1 was not upregulated in nSMase2-overexpressing cells as compared with control cells *in vitro* (Supplementary Fig. S7B). Moreover, PD-L1 was equally upregulated on B16K1 cells expressing high or low levels of nSMase2 when treated with IFNγ alone or in combination with TNF *in vitro* (Supplementary Fig. S7B).

Finally, we observed no major impact of nSMase2 overexpression and/or anti–PD-1 treatment on the expression of TIM-3 and PD-1 on CD4$^+$ and CD8$^+$ TILs. We only observed a slight decrease in the percentage of PD-1$^+$ and PD-1$^+$TIM-3$^+$ CD4$^+$ TILs in nSMase2^{high} tumors (Supplementary Fig S7C). We also observed a significant increase in CTLA-4 expression on CD8$^+$ TILs from nSMase2^{high} tumors as compared with nSMase2^{low} tumors, a phenomenon that was abrogated upon anti–PD-1 treatment (Supplementary Fig S7C).

Collectively, the data indicate that melanoma nSMase2 enhances Th1 responses upon anti–PD-1 therapy, thus facilitating tumor rejection.

Discussion

The present study provides the evidence that (i) *SMPD3* is expressed at low levels in most human metastatic melanoma samples and (ii) low *SMPD3* expression is associated with shortened overall survival in patients. High *SMPD3* expression was associated with "Immune system process" and "Lymphocyte activation" gene signatures, and

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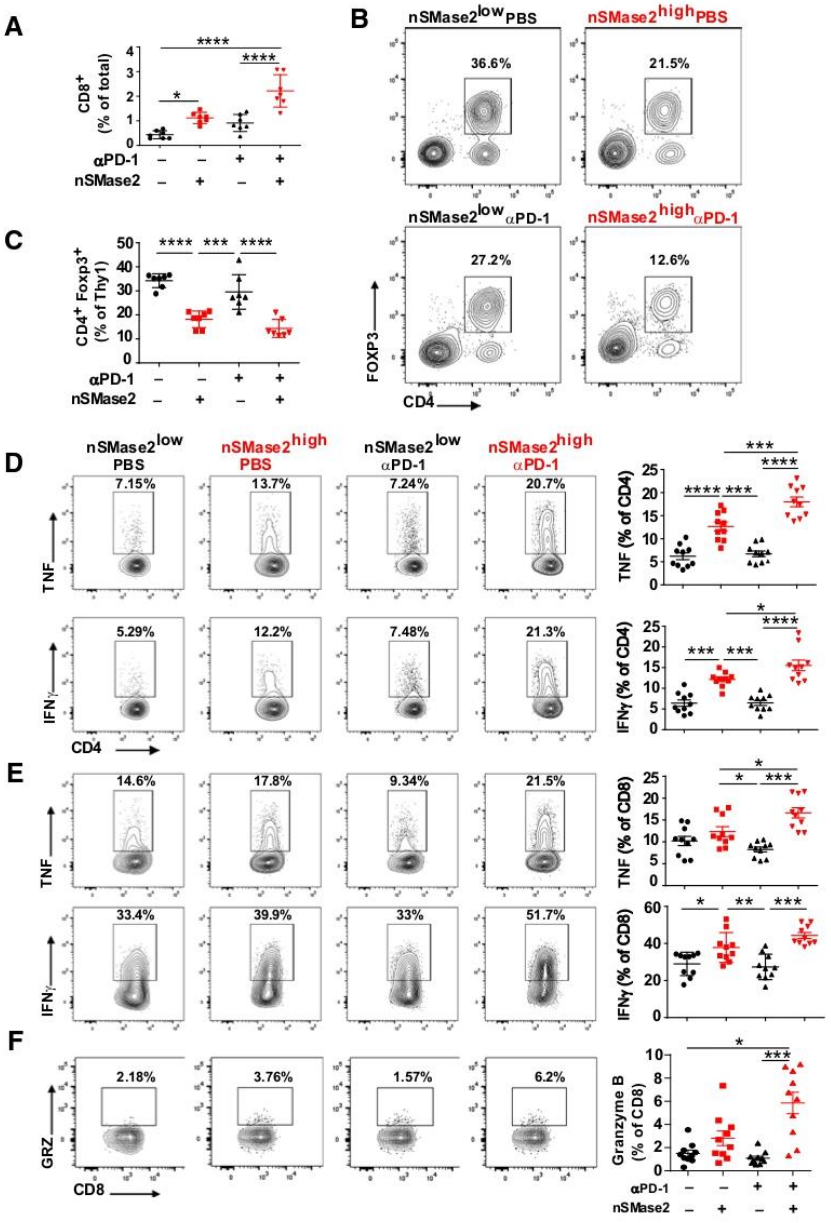

Figure 6.

nSMase2 potentiates the PD-1–dependent immune response in mouse melanoma tumors. **A–D,** nSMase2^{high} or nSMase2^{low} B16KI cells were bilaterally and intradermally grafted to C57BL/6 WT mice. Mice were then treated with 200 μg of anti–PD-1 or vehicle at day 7 prior to tumor immune infiltrate analysis by flow cytometry at day 10. The two tumors from each mouse were pooled prior to immune cell infiltration analysis. **A–C,** Infiltration of tumors by CD8$^+$ T cells (**A**) and CD4$^+$FoxP3$^+$ regulatory T cells (**B** and **C**; $n = 7$ mice group). **D** and **E,** IFNγ and TNF production by tumor-infiltrating CD4$^+$ (**D**) and CD8$^+$ (**E**) T cells following 4-hour phorbol myristate acetate (PMA)/ionomycin incubation in the presence of a Golgi transport blocker. **F,** Alternatively, proportion of granzyme B$^+$ cells was determined among CD8$^+$ TILs ($n = 10$ mice per group). Statistical analyses: one-way ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

melanoma samples expressing *SMPD3* at high levels exhibited a gene signature of TILs. In agreement with the data obtained from human melanoma databases, high nSMase2 expression increased CD8$^+$ TIL frequency and the CD8$^+$ T-cell–dependent immune response to decrease melanoma growth in WT mice but not in mice lacking CD8$^+$ T cells. This demonstrates that nSMase2 antitumorigenic properties are fully dependent on its ability to stimulate adaptive immunity. Collectively, our data reveal that *SMPD3* downregulation or mutation facilitates melanoma progression by contributing to melanoma immune escape.

The expression of genes encoding other SMase isoforms did not correlate with immune-related gene signatures in human melanoma. Thus, the distinctive biological properties of nSMase2 in melanoma do not extend to the other SMases, presumably due to different subcellular localization and/or biochemical properties as well as different roles in cell signaling (48). One should note however that acid SMase likely

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modulates melanoma progression. Indeed, the growth of B16F1 melanoma cells is potentiated in acid SMase–deficient mice, indicating that the acid SMase–dependent pathway plays some critical role in the melanoma microenvironment (49), possibly by modulating tumor angiogenesis rather than the anti-melanoma immune response (50). In contrast, acid SMase deficiency in the host impaired melanoma cell metastasis. Mechanistically, acid SMase secretion by platelets has been proposed to trigger the activation of α5β1 integrins on B16F10 melanoma cells, thereby promoting melanoma cell extravasation to the lungs (51). Furthermore, acid SMase expression in human and mouse melanoma cells enhanced the proteasomal degradation of the microphthalmia-associated transcription factor (MITF; ref. 52). Consequently, acid SMase may limit the expression of key proteins involved in melanoma progression, which are regulated by MITF (52). More recently, melanoma acid SMase was shown to enhance the anti-melanoma immune response in mice, yet the molecular mechanisms remain to be established (53). We show that in human melanoma, the expression of *SMPD1*, the gene encoding acid SMase, did not correlate with the expression of diverse immune-related genes such as *CD8A* and *CD8B*, arguing against a role of acid SMase in $CD8^+$ T-cell–dependent immune responses in human melanoma.

The mechanisms by which nSMase2 facilitates the $CD8^+$ T-cell–dependent immune response likely rely on the alteration of intratumoral SL content because expression of a C.I. nSMase2 mutant had no effect on B16K1 tumor growth. Indeed, intratumoral ceramide and sphingosine content was increased in WT nSMase2–overexpressing melanoma tumors. Sphingosine is also a substrate of sphingosine kinases, which produce S1P, itself a critical mediator of lymphocyte trafficking (54). However, given that the levels of intratumor S1P remained unchanged upon nSMase2 overexpression, it is unlikely that S1P directly mediates the nSMase2-triggered increase in $CD8^+$ TILs. Another interesting hypothesis is that ceramide, which exhibits structural similarities to Lipid A, the biologically active core of lipopolysaccharide (55), may mimic pathogen-associated molecular patterns, facilitating DC maturation and ultimately priming the adaptive immune response. Recently, the administration of nanoliposome-loaded C6 ceramide in mice was shown to impair hepatocarcinoma growth by increasing M1 tumor-associated macrophage polarization and $CD8^+$ T-cell activation (56). While our study was in progress, nSMase2 expression was found to be inversely correlated with GALC during melanoma progression (18). This indicates that SM metabolism is tightly connected with that of glycosphingolipids, which are known to modulate the immune responses (57). Whether and how nSMase2 is a key player in mediating SM and glycosphingolipid relationships for modulating immune responses in melanoma remains to be investigated.

Under our experimental conditions, melanoma nSMase2 not only facilitated the Th1 polarization of TILs but also reduced the proportion of Tregs in tumors. Given that sEVs produced by B16K1 expressing WT nSMase2 promoted the expression of IL12, CXCL9, and CCL19 by DCs *in vitro*, this phenomenon might be implicated in the promotion of Th1 polarization *in vivo* and indicates that melanoma nSMase2 may trigger this polarization in the earliest stages of the immune response. Whereas nSMase2 overexpression in mouse melanoma significantly delayed melanoma growth, all mice died within 40 days after melanoma cell injection, strongly suggesting melanoma immune escape. This is likely related to the increased expression of PD-L1 on both $CD11b^+F4/80^+$ macrophages and $CD45^-$ cells in B16K1 tumors overexpressing nSMase2. The latter phenomenon is likely the direct consequence of the nSMase2-dependent increased production of IFNγ and TNF in tumors (36, 46, 47). As a matter of fact, whereas anti–PD-1 therapy had limited therapeutic effects toward B16K1 melanoma, it greatly suppressed the growth of tumors overexpressing WT nSMase2. These observations demonstrate that melanoma nSMase2 enhances the therapeutic response to anti–PD-1 therapy. In addition, this enhanced therapeutic effect triggered by nSMase2 expression was also observed in Yumm 1.7 melanoma and 4T1 breast cancer cell lines, which are both resistant to anti–PD-1 therapy.

We previously demonstrated that increased expression of sphingosine kinase 1 by melanoma cells potently impairs $CD8^+$ T-cell–dependent immune responses and response to ICI (15). Our present study showing the positive impact *SMPD3* that has on antimelanoma immune responses further argues that targeting SL metabolism may represent an original therapeutic strategy to overcome resistance of melanoma to anti–PD-1 therapy. In addition, *SMPD3* expression in melanoma samples may serve as a novel biomarker to predict the clinical response to immunotherapy. This is currently being evaluated in a prospective clinical trial (IMMUSPHINX: NCT03627026) conducted in our institute and others, and the primary objective of this clinical trial is to evaluate whether SL metabolites and/or SL-metabolizing enzymes are putative biomarkers to predict response/resistance in patients with advanced melanoma treated with anti–PD-1 therapy alone or in combination with anti–CTLA-4 therapy.

Authors' Disclosures

A. Montfort reports grants from Ligue contre le Cancer Régionale Midi-Pyrénées, Fondation de France, Ligue Nationale contre le Cancer, Rotary Toulouse Clubs, Fondation ARC, Cancéropôle Grand Sud-Ouest, Fondation Toulouse Cancer Santé, Prestige Grant award (Marie Curie Actions and Campus France), and TRANSCAN research program during the conduct of the study, as well as a patent for WO2017134116A1 pending. F. Bertrand reports a patent for WO2017134116A1 pending. V. Garcia reports grants from Ligue contre le Cancer Régionale Midi-Pyrénées, Fondation de France, Ligue Nationale contre le Cancer, Rotary Toulouse Clubs, Fondation ARC, Cancéropôle Grand Sud-Ouest, Fondation Toulouse Cancer Santé, Prestige Grant award (Marie Curie Actions and Campus France), and TRANSCAN research program during the conduct of the study. N. Andrieu-Abadie reports grants from INSERM, Paul Sabatier University Toulouse III, Ligue Nationale contre le Cancer, Ligue Régionale contre le Cancer (Midi-Pyrénées), INSERM Transfert, Cancéropole Grand Sud-Ouest, Rotary Toulouse Clubs, Fondation Toulouse Cancer Santé, Fondation ARC, ERA-NET Transcan-2, and Fondation de France during the conduct of the study, as well as a patent for WO2017134116A1 pending. T. Levade reports grants from INSERM, Paul Sabatier University, Toulouse III, Ligue Nationale contre le Cancer, Ligue Regionale contre le Cancer (Midi-Pyrénées), INSERM Transfert, Canceropole Grand Sud-Ouest, Rotary Toulouse Clubs, Fondation Toulouse Cancer Sante, Fondation ARC, ERA-NET Transcan-2, and Fondation de France during the conduct of the study, as well as a patent for WO2017134116A1 pending. N. Meyer reports grants and personal fees from Bristol-Myers Squibb and MSD, and personal fees from Roche, Novartis, Pierre Fabre, Sanofi, Sun Pharma, and AbbVie outside the submitted work. O. Micheau reports grants from ANR, CAPES-COFECUB, and INCa during the conduct of the study. C. Colacios reports grants from INSERM, Paul Sabatier University, Toulouse III, Ligue Nationale contre le Cancer, Ligue Regionale contre le Cancer (Midi-Pyrénées), INSERM Transfert, Cancéropôle Grand Sud-Ouest, Rotary Toulouse Clubs, Fondation Toulouse Cancer Santé, Fondation ARC, ERA-NET Transcan-2, and Fondation de France during the conduct of the study, as well as a patent for WO2017134116A1 pending. B. Ségui reports grants from INSERM, Paul Sabatier University, Toulouse III, Ligue Nationale contre le Cancer, Ligue Régionale contre le Cancer (Midi-Pyrénées), INSERM Transfert, Cancéropôle Grand Sud-Ouest, Rotary Toulouse Clubs, Fondation Toulouse Cancer Santé, Fondation ARC, ERA-NET Transcan-2, and Fondation de France during the conduct of the study; grants and personal fees from Bristol-Myers Squibb outside the submitted work; and a patent for WO2017134116A1 pending. No disclosures were reported by the other authors.

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Authors' Contributions

A. Montfort: Conceptualization, formal analysis, investigation, writing–original draft, writing–review and editing. **F. Bertrand:** Conceptualization, formal analysis, investigation. **J. Rochotte:** Conceptualization, formal analysis, investigation. **J. Gilhodes:** Formal analysis, methodology, writing–original draft. **T. Filleron:** Validation, methodology. **J. Milhès:** Investigation. **C. Dufau:** Investigation. **C. Imbert:** Investigation. **J. Riond:** Investigation. **M. Tosolini:** Investigation, methodology. **C.J. Clarke:** Investigation, writing–review and editing. **F. Dufour:** Investigation. **A.A. Constantinescu:** Investigation. **N. De França Junior:** Investigation. **V. Garcia:** Investigation. **M. Record:** Methodology. **P. Cordelier:** Methodology. **P. Brousset:** Methodology. **P. Rochaix:** Methodology. **S. Silvente-Poirot:** Methodology. **N. Therville:** Investigation. **N. Andrieu-Abadie:** Writing–original draft, writing–review and editing. **T. Levade:** Writing–original draft, writing–review and editing. **Y.A. Hannun:** Writing–original draft, writing–review and editing. **H. Benoist:** Writing–original draft. **N. Meyer:** Methodology, writing–original draft, writing–review and editing. **O. Micheau:** Investigation, methodology, writing–original draft. **C. Colacios:** Conceptualization, formal analysis, supervision, validation, investigation, writing–original draft, writing–review and editing. **B. Ségui:** Conceptualization, formal analysis, supervision, funding acquisition, validation, investigation, writing–original draft, writing–review and editing.

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Cancer Immunology Research

Neutral Sphingomyelinase 2 Heightens Anti-Melanoma Immune Responses and Anti−PD-1 Therapy Efficacy

Anne Montfort, Florie Bertrand, Julia Rochotte, et al.

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ANNEX II - INTERNATIONAL THESIS AGREEMENT IN PORTUGUESE VERSION

CONVÊNIO DE COTUTELA INTERNACIONAL DE TESE

Preâmbulo

Considerando:

Pela Université Bourgogne Franche-Comté:
- **O Código da Educação;**
- **O decreto de 26 de maio de 2016, fixação do quadro nacional para a formação e modalidades levando à emissão de doutorado nacional, MEN;**
- **Os estatutos da Université Bourgogne Franche-Comté; a seguir designados UBFC, sediada na 32 Avenue de l'Observatoire 25000 Besançon, representada pelo Senhor Nicolas CHAILLET, Presidente.**

ENTRE

A Université de Bourgogne, sediada na Esplanade Erasme, 21000 Dijon, representada pela Senhor Alain BONNIN, Presidente;

E

A Pontifícia Universidade Católica do Paraná (doravante referida como PUCPR), instituição de educação superior sem fins lucrativos, neste ato representada por seu Reitor, Prof. Waldemiro Gremski, e pelo Presidente da Associação Paranaense de Cultura - APC, mantenedora da PUCPR, Délcio Afonso Balestrin, ambas com sede na Rua Imaculada Conceição, 1155, CEP 80215-901, Prado Velho, Curitiba, Brasil, inscritas no CNPJ 76.659.820/0003-13 e 76.659.820/0001-51, respectivamente.

decidem o que segue a respeito da preparação de uma tese em cotutela por:

Nilton de França Junior

AB
KC

JURÍDICO Marcela Grupo Marista

TÍTULO I - MODALIDADES ADMINISTRATIVAS

ARTIGO 1: Dados do Doutorando;

A presente tese será preparada a partir do ano letivo 2018/2019, por:

Nilton de FRANÇA JUNIOR,

***Nascido em* 04/08/1984 em Curitiba, Paraná, Brasil.**

ARTIGO 2: Matrícula e taxas de matrícula

A matrícula do Doutorando para a tese em cotutela inicia-se durante o ano universitário de 2018/2019. A duração prevista dos trabalhos de pesquisa é fixada em 3 anos, segundo a regulamentação em vigor na França, a contar da data da primeira matrícula de tese na Université Bourgogne Franche-Comté. A defesa da tese está prevista para o ano de 2020. Esta duração só poderá ser prolongada a título excepcional após visto favorável dos dois estabelecimentos e por proposição dos orientadores da tese. Esta solicitação deve ocorrer 6 meses antes da data prevista para conclusão da tese e ser objeto de uma alteração.

O Doutorando deverá ser matriculado nos dois estabelecimentos. As taxas de matrícula serão recebidas pela Université de Bourgogne, para 2018/2019 e pela Pontifícia Universidade Católica do Paraná, para 2018/2019 e 2019/2020.

ARTIGO 3: Modalidades especificas

3.1 - Previdência social e Segura de Responsabilidade civil

O doutorando se compromete a contratar um plano de previdência social e uma apólice de seguro para cobrir riscos de responsabilidade civil para todo o período de formação do doutorado, serviços esses que devem valer tanto na França como no Brasil.
O Sr Nilton de França Junior estará coberto por um plano de seguridade social e de responsabilidade civil privada. Será necessário apresentar os comprovantes de ditos serviços no ato da matrícula.

3.2 - Alojamento

Durante sua estadía na França o doutorando permanecerá no seguinte endereço:

49 Rue Jeannin, 21000 Dijon,

Durante sua estadia no Brasil o doutorando permanecerá no seguinte endereço:

Rua Imaculada Conceição, 1155, Curitiba, Paraná, Brasil, CEP 80215-901.

AB KC

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

TÍTULO II - MODALIDADES PEDAGÓGICAS

ARTIGO 4: Trabalhos de pesquisa

Os trabalhos de pesquisa tratarão do tema: « Investigação das vias de morte induzidas pela saliva de Amblyomma sculptum em células tumorais e TRAIL. »

Os orientadores são:

- **na UBFC :**
  Sr Olivier MICHEAU
  Titulo: Directeur de Recherches 2 (DR2) HDR
  Laboratório: INSERM, UMR 1231 , Laboratório de Lipídeos, Nutrição e Câncer.

- **na PUCPR :**
  Srta Selene ELIFIO ESPOSITO
  Titulo: Professora titular
  Laboratório: Laboratório Experimental Multiusuário.

Os dois orientadores cientes do presente convênio, se comprometem a orientar a preparação da tese em cotutela de maneira conjunta e integral.

O trabalho de pesquisa será realizado alternadamente em ambas as instituições, quando necessário, seguindo um cronograma co-decidido a cada ano pelos dois supervisores.

ARTIGO 5: Atividades de formação

Dentro UBFC, o estudante poderá se beneficiar do treinamento oferecido pelo Pôlo de Pesquisa, facilitando seu rastreamento de carreira, cursos de formação oferecidos pela escola de pós-graduação que permite estabelecer a formação através da investigação, formação técnica e científica oferecida pela Unidade de Pesquisa.

Dentro PUCPR, para obter o título de Doutor pela PUCPR, o doutorando deverá completar os módulos de ensino conforme descritos a seguir:

- **disciplinas de formação, pertencentes ao núcleo obrigatório;**
- **disciplinas eletivas, pertencentes ao núcleo de concentração;**
- **disciplinas do núcleo instrumental;**

ARTIGO 6: Redação da tese

A tese será redigida em Inglês;
A redação deverá ser complementada por um resumo substancial em francês e português.

ARTIGO 7: Defesa da tese

A tese deverá ser defendida uma única vez no Brasil. A defesa será reconhecida por ambas as universidades.
O idioma utilizado na defesa da tese poderá ser português ou inglês.
Os relatores serão nomeados conjuntamente pelas duas universidades em conformidade com as normas de cada uma : 2 relatores externos à UBFC e à PUCPR.

AS
KC

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

ARTIGO 8: Júri

A autorização para defesa da tese será acordada conjuntamente pelos dois estabelecimentos, após visto favorável de suas instâncias competentes. A banca da tese será designada pelas autoridades legais do estabelecimento onde a tese será defendida, após acordo do estabelecimento co-signatário.
A composição do júri tese deve cumprir, na medida do possível, os regulamentos de ambos os países e permitir, tanto quanto possível, uma representação equilibrada de mulheres e homens. Ela será composta equilibradamente por representantes científicos dos dois países e terá ao menos quatro membros. Ela terá:

- **ao menos a metade de personalidades exteriores aos dois estabelecimentos,**
- **ao menos a metade de professores ou equivalentes (pesquisadores).**

O número de membros da banca não poderá exceder à sete. A composição da banca deverá levar em conta as possibilidades de financiamento das despesas de deslocamento e de hospedagem de todos os membros.
O Presidente da banca deverá ser um professor titular ou equivalente. Ele será designado de acordo com o regulamento da universidade em que a tese será defendida e não deve ser nenhum dos orientadores da tese.

ARTIGO 9: Diplomas

A ata de defesa será redigida na língua do país onde ela será defendida. Em seguida, ela será traduzida em francês. Ela deverá permitir apreciar as aptidões do candidato ao expor seus trabalhos e o domínio que tem do seu tema de pesquisa.
Após o pronunciamento da banca, as instituições universitárias – Universidade de Bourgogne Franche-Comté,(UBFC), concederá o Título de Doutor em Bioquímica e Biologia Molecular e a Pontifícia Universidade Católica do Paraná (PUCPR) concederá o título de Doutor em Ciências da Saúde.

ARTIGO 10: Proteção da propriedade intelectual e publicação

A proteção do objeto da tese, assim como das publicações e da utilização dos resultados de pesquisa comuns às duas universidades, deverão ser assegurados de acordo com os procedimentos específicos de cada país envolvido na cotutela.

ARTIGO 11: Entrega, relatório e reprodução das teses

O Doutorando deverá concordar com as normas em vigor nos dois países para o depósito, a descrição e a reprodução de teses.

AB
KC

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

TÍTULO III - DISPOSIÇÕES FINAIS

ARTIGO 12: Adendo

O doutorando e seus orientadores, cientes do presente convênio, se comprometem a respeitá-lo; se durante a preparação do doutorado, modificações ou adaptações se fizerem necessárias (prorrogação do prazo, programa de pesquisa), um adendo deverá ser conjuntamente acordado por ambas as universidades signatárias do presente convênio de cotutela.

O presente convênio é estabelecido em 4 cópias originais;

Assinaturas com carimbos dos estabelecimentos:

Curitiba, de 2018.

La Directrice de thèse / *Orientador de tese,*
Prof.ª Selene ELIFIO ESPOSITO

Université Bourgogne Franche-Comté

Le Directeur de thèse /
Orientador de tese,
M. Olivier MICHEAU

Le Directeur du LNC /
Diretor da LNC,
M. Laurent LAGROST

Le Directeur de l'Ecole Doctorale ES /
Responsável da Escola Doutoral ES,
M. Thierry RIGAUD

14 JUIN 2018
Le Président de l'UBFC,
Presidente de UBFC,
Monsieur Nicolas CHAILLET

31 MAI 2018
Le Président de l'Université de Bourgogne,
Presidente da Université de Bourgogne,
Monsieur Alain BONNIN

Le Recteur de PUCPR,
Reitor da PUCPR,
Senhor Waldemiro GREMSKI

Lino Alfonso Jungbluth
CPF 454.515.249-04

Pedro João Wolter
Procurador
CPF: 077.560.709-68

Le Président de L´Associação Paranaense de Cultura
Délcio Afonso BALESTRIN

Le Doctorant / *A Doutorando*
Nilton de FRANÇA JUNIOR

Le Directeur de l'internationalisation/
Diretor da Internacionalização
Dr. Marcelo Távora MIRA

ANNEX III - INTERNATIONAL THESIS AGREEMENT, FRENCH VERSION

CONVENTION DE COTUTELLE INTERNATIONALE DE THESE

Préambule

Vu:

pour l'Université Bourgogne Franche-Comté :

- **Le Code de l'Education ;**
- **L'arrêté du 25 mai 2016 fixant le cadre national de la formation et les modalités conduisant à la délivrance du diplôme national de doctorat, MEN ;**
- **Les Statuts de l'Université Bourgogne Franche-Comté; ci-après dénommée UBFC, sise au 32 avenue de l'Observatoire 25000 Besançon, représentée par son président, Monsieur Nicolas CHAILLET.**

ENTRE

L'Université de Bourgogne, sise Esplanade Erasme, 21000 Dijon, représentée par Monsieur Alain BONNIN, Président,

ET

La Pontifícia Universidade Católica do Paraná, établissement à but non lucratif, ici représentée par son Recteur, Prof. Dr. Waldemiro Gremski, et par le Président de l'Associação Paranaense de Cultura – APC, entreteneur de la PUCPR, Mr. Délcio Afonso Balestrin, les deux avec leurs sièges au-près de la Rue Imaculada Conceição, 1155 – Prado Velho, Curitiba, Paraná, Brésil 80215-901 avec leurs respectifs numéros de référence fiscaux 76.659.820/0003-13 et 76.659.820/0001-51.

arrêtent les dispositions suivantes concernant la préparation d'une thèse en cotutelle par:

Nilton de França Junior

TITRE I – MODALITÉS ADMINISTRATIVES

ARTICLE 1: Renseignements sur le Doctorant

Cette thèse est préparée à partir de l'année universitaire 2018/2019, par :

Nilton de França Junior,

né le 04/08/1984 à Curitiba (Paraná, Brésil), de nationalité brésilienne.

ARTICLE 2: Inscription et droits d'inscriptions;

L'inscription du Doctorant pour une thèse en cotutelle prend effet pendant l'année universitaire 2018/2019. La durée prévisionnelle des travaux de recherche est fixée à 3 ans à compter de la date de la première inscription en thèse à l'Université Bourgogne Franche-Comté. La soutenance de la thèse est prévue au cours de l'année 2020. Cette durée ne pourra être prolongée qu'à titre exceptionnel après avis favorable des deux établissements et sur proposition des directeurs de thèse. Cette demande doit intervenir 6 mois avant la date prévisionnelle de fin de thèse.

Le doctorant devra être inscrit dans les deux établissements. Les droits d'inscription seront perçus par l'Université de Bourgogne, pour 2018/2019. Les droits d'inscription seront perçus par l'Université Pontificale Catholique du Paraná, pour 2018/2019 et 2019/2020.

ARTICLE 3: Modalités particulières

3.1 - Sécurité Sociale et Assurance Responsabilité civile;

Le doctorant s'engage à souscrire une couverture sociale et une assurance responsabilité civile durant toute la durée de ses études doctorales, le couvrant tant en France qu'en Brésil.
Monsieur Nilton de França Junior est affiliée au régime général de sécurité sociale et souscrit à une Assurance Responsabilité Civile privée.
Les justificatifs correspondants seront produits au moment de l'inscription.

3.2 - Hébergement

Lors de son séjour en France, le doctorant résidera à l'adresse suivante:

49 Rue Jeannin, 21000 Dijon

Lors de son séjour en Brésil, le doctorant résidera à l'adresse suivante :

Rua Imaculada Conceição, 1155, Curitiba, Paraná, Brasil, CEP 80215-901.

AB

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

TITRE II – MODALITÉS PÉDAGOGIQUES

ARTICLE 4: Travaux de recherche

Les travaux de recherche porteront sur : « Recherche sur les voies de la mort induite par la salive Amblyomma dans les cellules tumorales et TRAIL. »

Les directeurs de recherche seront:

- **à l'UBFC:**
  Monsieur / Olivier MICHEAU
  Titre et qualité: Directeur de Recherches 2 (DR2) HDR
  Laboratoire: INSERM, UMR 1231 , Laboratoire Lipides Nutrition Cancer.

- **à la PUCPR:**
  Mademoiselle : Selene ELIFIO ESPOSITO
  Titre et qualité : Professeur titulaire
  Laboratoire : Laboratoire Expérimental Multi-utilisateur.

Les deux directeurs de thèse, qui ont pris connaissance de cette convention, s'engagent à exercer conjointement et pleinement l'encadrement de cette thèse en cotutelle.

Les travaux de recherche seront effectués en alternance dans les deux établissements selon un calendrier élaboré chaque année, en tant que besoin, conjointement par les deux directeurs de thèse.

ARTICLE 5: Activités de formations

Au sein d'UBFC, le doctorant peut bénéficier à la fois de formations proposées par le Pôle Recherche facilitant sa poursuite de carrière, de formations proposées par son école doctorale permettant d'asseoir sa formation par la recherche, et de formations techniques et scientifiques proposées par l'Unité de Recherche.

Au sein de PUCPR, pour obtenir le titre de Docteur de la PUCPR, le doctorant devra valider les modules d'enseignement décrits ci-dessous:

- **disciplines d'éducation, appartenant au cours obligatoires**
- **disciplines au choix, appartenant aux cours de concentration,**
- **disciplines des cours fondamentaux**

ARTICLE 6 : Rédaction de la thèse

Le mémoire de thèse sera rédigé en Anglais. La rédaction devra être complétée par un résumé substantiel en langue française et portugaise.

ARTICLE 7 : Soutenance de la thèse

La thèse donnera lieu à une soutenance unique au Brésil. La soutenance sera reconnue par les deux établissements.
La langue utilisée pour la soutenance sera le Portugais ou Anglais.
Les rapporteurs seront désignés conjointement par les deux établissements et conformément aux règles des deux établissements : 2 rapporteurs extérieurs à l'UBFC et de la PUCPR.

AS

Nilton de FRANÇA JUNIOR

UBFC

JURIDICO Marcela Grupo Marista

ARTICLE 8 : Jury

L'autorisation de soutenance de la thèse est accordée conjointement par les deux établissements, après avis favorable de leurs instances compétentes. Le jury de la thèse est désigné par les autorités légales de l'établissement où la thèse sera soutenue, après accord de l'établissement cosignataire.
La composition du jury de soutenance doit respecter, dans la mesure du possible, la réglementation en vigueur des deux pays et permettre, autant que possible, une représentation équilibrée des femmes et des hommes. Il est composé sur la base d'une proportion équilibrée par des représentants scientifiques des deux pays et comprend au moins quatre membres. Il comprend :

- **au moins la moitié de personnalités extérieures aux deux établissements.**
- **au moins la moitié de professeurs ou assimilés (directeurs de recherche).**

Le nombre des membres du jury ne peut excéder sept. La composition du jury devra prendre en compte les possibilités de financement des frais de déplacement et d'hébergement de tous les membres.
Le Président du Jury doit être un Professeur ou équivalent. Il sera désigné selon La réglementation de l'Université où la thèse sera soutenue et ne devra pas être l'un des co-directeurs de thèse.

ARTICLE 9: Diplômes

Le rapport de soutenance sera établi dans la langue du pays où la thèse sera soutenue. Il sera ensuite traduit en français. Il devra permettre d'apprécier les aptitudes du candidat à exposer ses travaux et la maîtrise qu'il a de son sujet de recherche.
Après admission prononcée par le jury, l'UBFC décernera le grade de « Docteur de la Communauté d'Université et d'Etablissements - Université Bourgogne Franche-Comté, *dans* Biochimie Biologie Moléculaire et l'établissement PUCPR cocontractant décernera le titre de Docteur en Science de la Santé.

ARTICLE 10 : Protection intellectuelle et Publication

La protection du sujet de thèse ainsi que les publications, l'exploitation et la protection des résultats de recherche communs aux deux laboratoires d'accueil du doctorant doivent être assurées conformément aux procédures spécifiques à chaque pays impliqué dans la cotutelle.

ARTICLE 11: Dépôt, signalement et reproduction des thèses

Le doctorant devra se conformer aux règles en vigueur dans les deux pays pour le dépôt, le signalement et la reproduction des thèses.

Nilton de FRANÇA JUNIOR

JURÍDICO Marcela Grupo Marista

TITRE III – DISPOSITIONS FINALES

ARTICLE 12 : Avenant

Le doctorant et les directeurs de thèse ayant pris connaissance de cette convention s'engagent à en respecter les termes ; en cas de modifications ou d'adaptations rendues nécessaires (durée, programme de recherche) durant la préparation du doctorat, un avenant devra être adopté conjointement par les deux établissements signataires de la présente convention de cotutelle.

La présente convention est établie en 4 exemplaires originaux,

Visa des autorités pédagogiques de chaque université /
Visto das autoridades pedagógicas de cada universidade

A Pontifícia Universidade Católica do Paraná

La Directrice de thèse / *Orientador de tese,*
Prof.ª Selene ELIFIO ESPOSITO

Université Bourgogne Franche-Comté

Le Directeur de thèse /
Orientador de tese,
M. Olivier MICHEAU

Le Directeur du LNC /
Diretor da LNC,
M. Laurent LAGROST

Le Directeur de l'Ecole Doctorale ES /
Responsável da Escola Doutoral ES,
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Monsieur Nicolas CHAILLET

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Presidente da Université de Bourgogne,
Monsieur Alain BONNIN

Le Recteur de PUCPR,
Reitor da PUCPR,
Senhor Waldemiro GREMSKI
Lino Afonso Jungbluth
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Pedro João Wolter
Procurador
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Le Président de L´Associação Paranaense de Cultura
Délcio Afonso BALESTRIN

Le Doctorant / *A Doutorando*
Nilton de FRANÇA JUNIOR

Le Directeur de l'internationalisation/
Diretor da Internacionalização
Dr. Marcelo Távora MIRA

ANNEX IV - LETTER OF INVITATION, PhD SENCONDEMENT.

DISCOVER

OÉ Gaillimh
NUI Galway

Nilton De Franca,
INSERM, UMR1231
Dijon
30th April 2019

RE: Letter of invitation

Dear Nilton De Franca,

On behalf of the National University of Ireland Galway I wish to invite you visit our University for a period of 6 months (1st June 2019 – 31st Nov 2019) for the purpose of collaborative research and sharing of advice and experiences which would be of benefit to our research team/students.

This visit is made possible by the European Commission funded Marie Skłodowska-Curie Research and Innovation Staff Exchange (RISE) project, DISCOVER (MSCA-RISE project number 777995). In the RISE programme, you will remain an employee of your home institution (INSERM) and will return at the end of the visit.

Your visit will bring valuable expertise in Molecular and cellular biology, especially in the field of the molecular pathways regulating the interaction between the immune system and cancer cells. During your visit, my laboratory will benefit from your expertise in genetics, protein biochemistry and cell death assays to progress our project funded by the European Union (H2020 DISCOVER RISE project) aiming at understanding the role of tumour necrosis factor receptors (TNF) and how they can be utilized to eradicate cancer cells. will help be valuable for the RISE project. Our joint aim during your visit is to identify the mechanistic underlying the functions of the TNF receptors DR5- and/ or DR4 in ER-stress induced apoptosis.

NUI Galway will provide you with the necessary required office space, support and access to all University facilities. In addition, you will be introduced to and collaborate with our colleagues across the higher education sector in Ireland.

Your travel and accommodation expenses (2,000 Euro per month) will be provided for as detailed in the DISCOVER RISE grant agreement.

I look forward to your joining us in Galway.

Yours Sincerely,

Eva Szegezdi

Eva Szegezdi, Ph.D.
Research Lecturer in Cancer Biology
PI Cancer Niche Group
Director of Blood Cancer Biobank Ireland Apoptosis Research Centre
National University of Ireland
Galway
T: + 353 (0)91 495037
Email: eva.szegezdi@nuigalway.ie