Characterisation of SG33 Myxoma virus replication and oncolytic activity in pancreatic cancer models

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Résumé

Le cancer du pancréas (CP) est le septième cancer le plus meurtrier avec un taux de survie à cinq ans inférieur à 10%. La chirurgie reste le meilleur traitement mais n'est souvent pas possible à cause de l'évolution rapide de la maladie. D'autres traitements existent comme la radiothérapie et des combinaisons de chimiothérapies, mais celles-ci sont accompagnées de nombreux effets indésirables et un risque accru de résistance. De plus, ces protocoles thérapeutiques peuvent être inefficaces face aux complexités cellulaires et moléculaires des cancers pancréatiques et de leur microenvironnement tumoral. De ce fait des traitements alternatifs sont activement recherchés, tels que les thérapies ciblées, les immunothérapies et les thérapies géniques. Les virus oncolytiques (VO) sont une thérapie innovante particulièrement intéressante dans la médecine d'aujourd'hui. Cependant leur potentiel thérapeutique est limité par manque de stratégies pour le suivi et la quantification cinétique afin de déterminer leur efficacité au sein de cellules vivantes.

Lors de ces travaux nous montrons une approche pour imager à l'échelle de la cellule unique la réplication en temps-réel d'un virus oncolytique et son efficacité dans des cellules cancéreuses. Nous avons étudié le SG33, VO dérivant de la souche Lausanne Toulouse 1 (T1) du Myxoma virus (MYXV) utilisé comme contrôle. Nous avons équipé les génomes de SG33 et T1 avec le système ANCHOR avant d'infecter un panel de lignées. Le système ANCHOR est composé d'une protéine de fusion (OR-GFP) qui lie spécifiquement une séquence cible d'ADN courte et non-répétitive (ANCH) puis s'étale aux séquences voisines par oligomérisation. Son accumulation sur l'ADN viral créer un foci fluorescent. Nous avons trouvé que (i) l'ADN de SG33 et T1-ANCHOR peut être détecté et quantifié en temps réel, (ii) après infection les deux VOs ont une réplication périnucléaire avec des centres réplicatifs regroupés en fer à cheval et, (iii) SG33 réplique à des taux plus élevé par rapport à T1. Enfin, comme preuve de concept translationnelle nous avons déterminé l'efficacité oncolytique de SG33 dans des cellules cancéreuses primaires dérivées de l'adénocarcinome pancréatique (PDAC) aux niveaux populationnel et cellulaire. In vivo, SG33 se réplique dans des tumeurs expérimentales et inhibe la croissance tumorale. Ensemble, ces données démontrent une nouvelle stratégie de quantification du cycle infectieux de VOs en temps réel, avec un suivi de l'ADN viral avec une première évidence de stratégie théranostique

pour les patients PDAC. Cette technique permettrait de rationaliser l'utilisation de VOs chez des patients souffrant de maladies incurables.

Une autre classe thérapeutique innovante est les immunothérapies, efficaces dans de multiples cancers mais pas dans le PDAC. Ceci est principalement dû au fait que le cancer du pancréas est une tumeur 'froide' où l'environnement immunitaire est en manque et le peu de cellules immunes présentes sont plutôt de nature immunosuppressive. Les virus oncolytiques ont le potentiel d'induire la mort cellulaire via de multiples voies dont certaines considérées comme immunogènes. De plus, la libération d'antigènes associés aux tumeurs par la lyse de cellules tumorales agit en tant que vaccin par le recrutement de cellules immunes et le déclenchement d'une réponse immunitaire adaptative. L'utilisation de VOs dans le PDAC pourrait potentiellement la transformer en tumeur 'chaude'. Nos résultats suggèrent une capacité de SG33 à induire une mort immunogène dans des cellules primaires de PDAC permissives et nous avons étudié les voies de morts impliqués. Ces résultats nous permettent d'approfondir nos connaissances de l'activité oncolytique de SG33 et ouvre la possibilité d'exploiter son potentiel immunologique dans des tumeurs immunodéprimées telles que PDAC.

Summary

Pancreatic cancer (PC) is the seventh most deadly cancer with a survival rate at five years below 10%. Surgery remains the best treatment for PC but is most often incongruous due to the progressiveness of the disease. Other treatments such as radiotherapy and chemotherapy regimens are also available but are accompanied by numerous side effects and the risk of innate or acquired resistance. In addition, these treatment protocols could be inadequate when faced with the cellular and molecular complexity of pancreatic cancers and their tumour microenvironment (TME). Due to these shortcomings, other treatment options are being studied, including targeted therapies, immunotherapies and gene-based therapies. Oncolytic viruses (OV) are novel cancer gene therapies that are moving toward the forefront of modern medicines. However, their full therapeutic potential is hindered by the lack of convenient and reliable strategies to visualize and quantify OV growth kinetics and therapeutic efficacy in live cells.

Here, we present a first-in-class imaging approach for single-cell, real-time analysis of OV replication and efficacy in cancer cells. We selected SG33 as a prototypic, new OV that derives from wild-type Myxoma virus (MYXV) Lausanne Toulouse 1 (T1) that was used as control. We equipped SG33 and T1 genomes with the ANCHOR system and infected a panel of cell lines. The ANCHOR system is composed of a fusion protein (OR-GFP) that specifically binds to a short, non-repetitive DNA target sequence (ANCH) and spreads onto neighbouring sequences by protein oligomerization. Its accumulation on the tagged viral DNA results in the creation of fluorescent foci. We found that (i) SG33 and T1-ANCHOR DNA can be readily detected and quantified by live imaging, (ii) both OVs generate perinuclear replication foci after infection clustering into horse-shoe shape replication centres, and (iii) SG33 replicates to higher levels as compared to T1. Lastly, as a translational proof-of-concept, we benchmarked SG33 replication and oncolytic efficacy in primary cancer cells derived from pancreatic adenocarcinoma (PDAC) both at the population and the single-cell levels. In vivo, SG33 significantly replicates in experimental tumours to inhibit tumour growth. Collectively, we provide herein for the first time a novel strategy to quantify each step of OV infection in live cells and in real-time by tracking viral DNA and provide first evidence of theranostic strategies for PDAC patients. Thus,

this approach has the potential to rationalize the use of OV for the benefit of patients with incurable diseases.

Another coming-of-age therapy class are immunotherapies, having shown efficacy in many neoplasms, such as melanoma, but whose use has not shown as yet any benefits in PDAC. This is mainly due to the fact that pancreatic cancers are 'cold' tumours in which the immune environment is lacking and what few elements can be found are mostly of immunosuppressive nature. Oncolytic viruses have the potential of inducing cell death via multiple pathways, some of which are considered immunological and so dubbed immunogenic cell deaths (ICD). Furthermore, the liberation of tumour-associated antigens (TAAs) via tumour cell lysis acts as a vaccine, by recruiting immune cells and triggering an adaptive immunological response. The use of OVs in PDAC could therefore potentially turn it into an immunologically 'hot' tumour. Our results strongly suggest that SG33 is capable of inducing ICD in permissive PDAC primary cancer cells and studied the cell death pathways involved to this account. These results not only further our understanding of SG33's mechanism of oncolytic activity but also paves the way to exploiting its immunological potential in the immune-deprived tumours that are PDAC.

Glossary

3-MA	3-Methyladenine	BMDC	Bone marrow dendritic cell
4-1BB	cluster of differentiation antigen 137 co-stimulatory	BMI	Body mass index
5-FC	5-fluorocytosine	BRCA1/2	Breast cancer type 1/2
5-FU	5-fluorouracil	CA 19-9	Carbohydrate antigen 19-9
A549	Lung carcinoma epithelial cell line	CAF	Cancer-associated fibroblast
Ad	Adenovirus	CALR	Calreticulin
ADCC	Antibody-dependent cell-mediated cytotoxicity	CAR	Chimeric antigen receptor
ADEX	Aberrantly differentiated endocrine exocrine	CD3ζ	Cluster of differentiation antigen 3 zeta chain
ADP	Adenoviral death protein	CDA	Cytidine deaminase
AIF	Apoptosis-inducing factor	cDC	Conventional dendritic cell
ALL	Acute lymphoblastic leukaemia	CDK4/6	Cyclin dependent kinases 4/6
AMPK	AMP-activated protein kinase	CDKN2A	Cyclin-dependent kinase inhibitor 2A
ANXA1	Annexin A1	CEA	Carcinoembryonic antigen
APC	Antigen presenting cell	CEV	Cell-associated enveloped virions
Atg	Autophagy-related protein	cfDNA	Cell-free DNA
ATM	Ataxia telangiectasia mutated	cGAS	Cyclin GMP-AMP synthase
ATP	Adenosine triphosphate	CISH	Cytokine inducible SH-2-Containing Protein
BAK	Bcl-2 homologous antagonist killer	CLD18	Claudin-18
BAX	Bcl-2-associated X protein	CoT	Coeliac trunk
BCAP31	B-cell receptor-associated protein 31	Cre	Cre recombinase
BiTE/TriTE	Bi-/tri-specific T cell engager	CRISPR	Clustered regularly interspaced short palindromic

CT	Computed tomography	EUS-FNA	EUS-fine needle aspiration
CTC	Circulating tumour cells	FAMMM	Familial atypical multiple mole melanoma
ctDNA	Circulating tumour DNA	FAP	Fibroblast-activation protein α
CTL	Cytotoxic T lymphocytes	Fas-L/FasR	Fas ligand/Fas receptor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4	FBS	Fetal bovine serum
CXCL10	C-X-C motif chemokine ligand 10	FDA	Food and drug administration
DAMP	Danger-associated molecular pattern	FFA	Focus forming assay
DCK/UMK	Deoxycytidine kinase/uridine monophosphate kinase	FGF2	Fibroblast growth factor 2
DC	Dendritic cell	Flt3	Fms-like tyrosine kinase 3
DFS	Disease-free survival	FOLFIRINOX	Folinic acid, 5-fluorouracil, irinotecan, oxaliplatin
DMEM	Dulbecco's modified Eagle's medium	FPR1	Formyl peptide receptor 1
DNA/RNA	Deoxyribonucleic acid/ribonucleic acid	GATA6	GATA-binding factor 6
DPC4	Deleted in Pancreatic Cancer-4	gBRCAm	Germline BRCA-mutated
dsDAN/dsRNA	Double stranded DNA/double stranded RNA	GCV	Ganciclovir
EBV	Epstein–Barr virus	GM-CSF	Granulocyte-macrophage colony stimulating factor
ECM	Extracellular matrix	GNAS	Guanine Nucleotide binding protein, Alpha Stimulating
ECOG PS	Eastern Cooperative Oncology Group performance status	GSDMD	Gasdermin D
EEV	Extracellular enveloped virions	GSDME	Gasdermin E
EIF2S1/eIF2α	Eukaryotic translation initiation factor 2 subunit 1	H-1PV	Parvovirus H-1
EIFAK3/PERK	Eukaryotic initiation factor 2α-kinase 3	HA	Hyaluronic acid
EMT	Epithelial-mesenchymal transition	HBV	Hepatitis B virus
EpCAM	Epithelial cell adhesion molecule	HCC	Hepatocarcinoma
ER	Endoplasmic reticulum	HCV	Hepatitis C virus
EUS	Endoscopic ultrasound	HDI	Human development index

HeA	Hepatic artery	KO	Knock-out
HHV-8	Human Herpesvirus-8	KPC	Kras Pdx1-Cre mice
HMGB1	High mobility group box 1	KRAS	Kirsten rat sarcoma virus
HOS	Osteosarcoma cell line	LAMP1	Lysosomal-associated membrane protein 1
HPV	Human papillomavirus	LC3A/B	Microtubule-associated protein 1 light chain 3
HR	Hazard ratio	LPS	Lipopolysaccharide
HSP70	Heat shock protein 70	M1/M2	M1/M2 macrophage
HSV	Human simplex virus	mAB	Monoclonal antibody
hTERT	Human telomerase reverse transcriptase	MAPK	Mitogen-activated protein kinase
iCAF	Inflammatory cancer-associated fibroblast	MCN	Mucinous cystic neoplasm
ICD	Immunogenic cell death	MDACCC	MD Anderson Cancer Center Clinical
ICGC	International cancer genome consortium	MDSC	Myeloid-derived suppressor cell
ICI	Immune checkpoint inhibitor	meso	Mesothelin
ICTV	International committee on taxonomy of viruses	mFOLFIRINOX	Modified FOLFIRINOX
IEV	Intracellular enveloped virions	MHC	Major histocompatibility complex
IFN-γ	Inferferon-gamma	miRNA	MicroRNA
IFP	Interstitial fluid pressure	MLKL	Mixed lineage kinase domain-like protein
IL	Interleukin	MMP	Matrix metalloproteinase
IMV	Intracellular mature virions	MRI	Magnetic resonance imaging
IPMN	Intraductal papillary mucinous neoplasm	MSC	Mesenchymal stem cells
ISG	Interferon-stimulated genes	mTOR	Mammalian target of rapamycin
IV	Immature virions	MUC-1	Mucin-1
KC	Kras Cre mice	MVA	Modified Ankara virus
KDa	Kilo dalton	myoCAF	Myofibroblastic cancer-associated fibroblast

MYXV	Myxoma virus	PDO	Patient-derived organoid
NCCD	Nomenclature committee on cell death	PDX	Patient-derived xenograft
NCCN	National comprehensive cancer network	PEGPH20	Pegylated hyaluronidase
NDV	Newcastles' disease virus	PFA	Plaque assay analysis
NGS	Next generation sequencing	PI3K	Phosphatidylinositol 3-kinase
NIS	Sodium iodide symporter	PKB	Protein kinase B
NK	Natural killer	pNK	Peripheral natural killer cell
NPV	Negative predictive value	PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cell
ORF	Open reading frame	PRR	Pattern recognition receptor
OS	Overall survival	PSCA	Prostate stem cell antigen
P2RY2/P2RX7	Purinergic Receptor P2Y2/P2X7	Ptf1a	Pancreas transcription factor 1a
PAHs	Polycyclic aromatic hydrocarbon	PUFA	Polyunsaturated fatty acid
PAMG	PDAC molecular gradient	q-/RT-/PCR	Quantitative/reverse transcriptase/polymerase
PAMP	Pathogen-associated molecular patterns	QM-PDA	Quasi-mesenchymal pancreatic ductal
PanIN	Pancreatic intraepithelial neoplasia	RB	Retinoblastoma
PanNEN	Pancreatic neuroendocrine neoplasm	RIP	Receptor-interacting protein
PANX1	Pannexin 1	RIPK1	RIP kinase 1
PAR	Poly(ADP-ribose)	RK13	Rabbit kidney cell line
PARP-1	Poly(ADP-ribose) polymerase-1	ROBO-1	Roundabout homolog 1
PBMCs	Peripheral blood mononuclear cell	ROCK1	Rho-associated, coiled-coil-containing protein kinase 1
PC	Pancreatic cancer	ROR2	Receptor tyrosine kinase-like orphan receptor 2
PDAC	Pancreatic adenocarcinoma	S/G/M	Synthesis/gap/mitosis phase
pDC	Plasmacytoid dendritic cell	SBRT	Stereotactic body radiation therapy
PD-L1/PD-1	Programmed death-ligand 1/programmed cell death	scFV	Single-chain variable fragment

SCLC	Small cell lung cancer	TIR	Terminal inverted repeat
SFV	Semliki forest virus	TK	Thymidine kinase
siRNA	Small interfering RNA	TLR	Toll-like receptor
SMA	Superior mesenteric artery	TME	Tumour microenvironment
SMAD4	Sma mothers against decapentaplegia homologue	TMV	Tobacco mosaic virus
SMV	Superior mesenteric vein	TNF-α/TNFR	Tumour necrosis factor alpha/TNF receptor
SNAP25	Synaptosome Associated Protein 25	TNM	Tumour/lymph node/metastasis classification
SPARC	Secreted protein acidic and rich in cysteine	Tp53	Tumor protein P53
SPN	Solid pseudo-papillary neoplasm	TRAIL	TNF-related apoptosis-inducing ligand
SRV	Survivin	Treg	Regulatory T cell
sst2	Somatostatin receptor subtype 2	T-vec	Talimogene laherparepvec
STING	Stimulator of Interferon Genes	UPRT	Uracil phosphoribosyltransferase
SVN-2B	Survivin-2B	VAMP1	Vesicle associated membrane protein 1
TAA/TSA	Tumour-associated antigen/tumour-specific	VEGF/VEGFR	Vascular endothelial growth factor/VEGF receptor
TAM	Tumour associated macrophage	VGF	Vaccinia growth factor
TAN	Tumour associated neutrophil	VSV	Vesicular stomatitis virus
TAZ	WW domain-containing transcription regulator	VV	Vaccinia virus
TCGA	The cancer genome atlas	WES	Whole exome sequencing
TCR	T cell receptor	wt	Wild type
TEM	Transmission electron microscopy	YAP1	Yes-associated protein 1
TGF-β	Transforming growth factor beta	YE-NEO-001	Patient-specific neoantigen epitope
TGN	Trans-Golgi network	α-GT	(1,3)-galactosyltransferase
Th17	T helper 17	α-SMA	α-smooth muscle actin
TIL	Tumour-infiltrating lymphocyte	γCD	Cytosine deaminase

Introduction

Pancreatic cancer

Epidemiology

According to the 2020 GLOBOCAN statistics, pancreatic cancer (PC) is the 14th leading cause of cancer with an incidence of 495 773 new cases (Fig. 1) worldwide that year (2.6% of all cancers).[1] Incidence rate is slightly higher in men than in women with age standardised rates of 5.7 to 4.1 per 100 000 respectively in 2020.[2] Incidence also increases with age. Indeed, the average age at diagnosis is 71 years old and 80% of cases of pancreatic cancer are in patients of 60 years or older. Moreover, mortality rate increases with age, with nearly 90% of deaths being of patients 55 years and above.

PC is more common in countries of higher Human Development Index (HDI) with a trend matching the increase in risk factors, such as smoking, obesity and diabetes, and diverging diagnostic capacities. On the contrary, survival rates do not seem to vary significantly between developing and developed countries.

Although 14th in incidence rate, pancreatic cancer is the seventh cause of death by cancer with 466 003 deaths recorded in 2020 (Fig. 1). Once again, mortality rates are slightly higher in men (4.9 per 100 000) than in women (4.5 per 100 000). Given the steady rates of both incidence and mortality, it is estimated that pancreatic cancer will surpass breast cancer as the third cause of death by cancer by 2025. Furthermore, the World Health Organization estimates worldwide incidence and mortality rates to increase drastically by 2040 with an increase of 61.7% in the number of cases and 64.2% in mortality.

This continual increase is due on the one hand to diagnosis occurring at late stages of the disease due to non-specific symptoms and a lack of effective biomarkers. Additionally, treatment options are limited and often unsuccessful when faced with the cellular and

molecular heterogeneity, also owing to a high relapse rate. In fact pancreatic adenocarcinoma has the lowest five-year survival rate of 9%, all stages combined.[3]

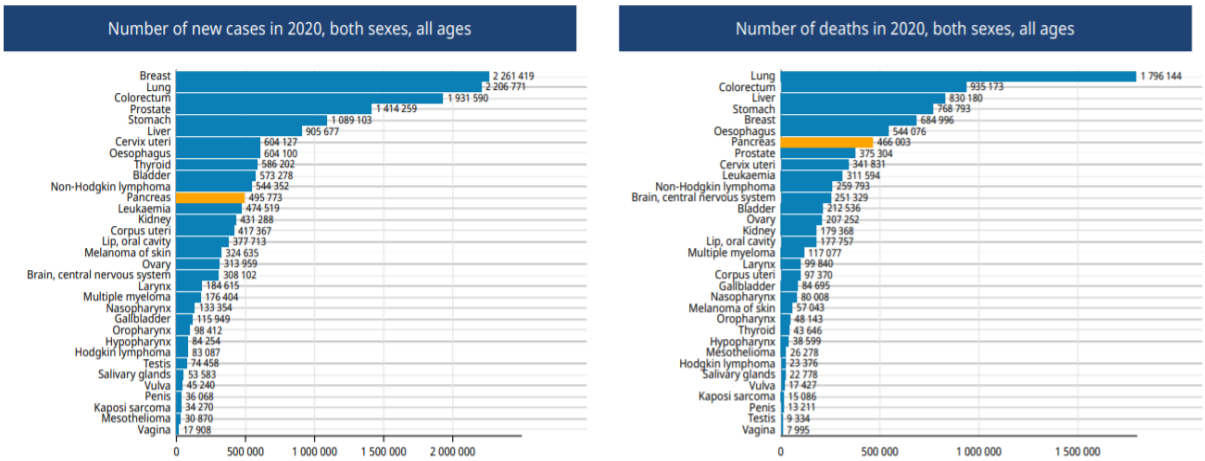

Figure 1: Incidence (left) and mortality (right) rates for pancreatic cancer in 2020 (GLOBOCAN)

Types of pancreatic cancer

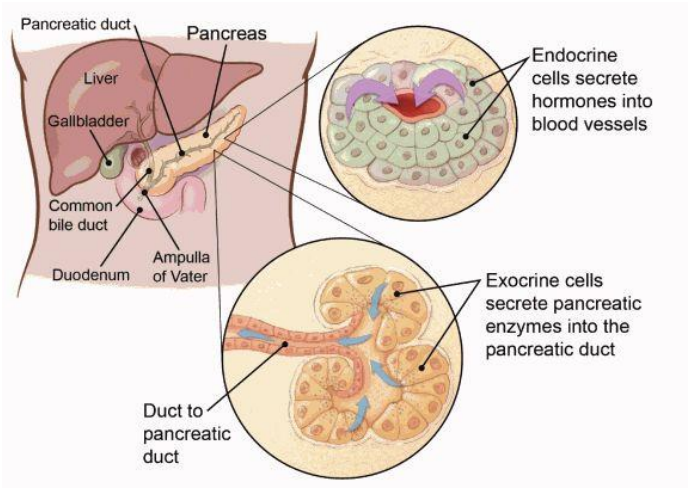

Figure 2: Anatomy of the pancreas (American cancer society)

The pancreas is an organ of the digestive tract with a dual function (Fig. 2). The endocrine component is made up of cells named islets of Langerhans that secrete the insulin or glucagon hormones in order to regulate blood sugar levels. The exocrine function of the pancreas is to

produce and release enzymes such as trypsin, chemotrypsin, amylase and lipase. These enzymes are discharged in the duodenum where they are essential for the break-down of lipids, carbohydrates and proteins.

Cancers of the pancreas can be divided into ductal and non-ductal; the latter make up only 5% of all pancreatic cancers. These include Pancreatic neuroendocrine neoplasms (PanNENs) with a five-year survival rate of 42%,[4] acinar cell carcinomas, pancreatoblastomas and solid pseudo-papillary neoplasm (SPN). Amongst the ductal pancreatic cancers is pancreatic ductal adenocarcinoma (PDAC) which is responsible for nine out of ten cases of PC and develop from exocrine cells. Other forms of ductal pancreatic cancers include adenosquamous carcinoma (1-4%), osteoclastic giant cell carcinoma, medullary carcinoma, and colloid carcinoma (1-3%). Colloid carcinoma presents a five-year survival rate >55%, far better than PDAC (<10%).[5]

Risk factors

The risk factors associated to developing PDAC are multiple with many still remaining unasserted. These risk factors can either be exogenous originating from the environment or behaviour and can therefore be modified. On the other hand, endogenous risk factors are innate to the patient and, for the most part, unchangeable.

- Exogenous risk factors:

The most common factor attributed to the high mortality rate is the elevated prevalence of smoking. Nearly a quarter of pancreatic cancers are caused by smoking tobacco and smokers have a 70% increased risk of developing PC.[6] Secondhand smoking has also revealed to increase the risk of developing PC with a hazard ratio (HR) of 1.54.[7] Even though cigarette smoking indubitably increases the risk of pancreatic cancer, the role in disease prognosis is still unclear.[8]

Although low or moderate alcohol consumption don’t seem to have a significant impact, heavy alcohol consumption (>24g/day) has been shown to increase the risk of PC by 15%.[9] Biological mechanisms have been proposed such as the development of chronic pancreatitis which then

leads to carcinogenesis, and the increase in inflammatory and fibrotic damage following the metabolism of alcohol.[10] A meta-analysis found an increase of nearly eight-fold in the risk of developing PC five years after diagnosis of chronic pancreatitis.[11] Chronic pancreatitis is not only caused by excessive alcohol consumption but can be also due to a genetic predisposition, autoimmune disorders or idiopathic.[12] A study determined the standardized incidence ratio of PC in patients with chronic pancreatitis to be 20.22, owing to the significant increased risk of developing pancreatic cancer.[13] Chronic pancreatitis is a fibro-inflammatory which leads to the failure of both endocrine and exocrine functions of the pancreas. Over time, the functional pancreas is replaced with fibrotic tissue and histological changes occur that resemble those found in pancreatic carcinogenesis.[14]

Obesity, being defined as a body mass index (BMI) of 30 or above, has been associated with a 50-60% increase in risk of developing PC.[15] A multicentre cohort study also demonstrated that obese patients have a worse prognosis compared to non-obese patients. Indeed, multivariable studies showed a 21% increase in relative risk of death for each BMI increase of 5 units.[16] Studies in murine models have shown that a restriction in calorie intact reduces pancreatic cancer progression. A mouse model constitutively expressing KRASG12D were either fed with normal chow, in which case 10% presented PanIN lesions after 3 months, or with high fat high calorie diet to become obese of which 45% presented precancerous lesions.[17]

Several epidemiological and meta- analyses found an increased risk of pancreatic cancer in patients with type 2 diabetes although the clear cause and effect link remains controversial. A study also found the inverse correlation of diabetes duration and risk of developing PC with a higher risk in patients of new on-set diabetes to those with long-term diabetes.[18] [19]

Exposure to certain chemicals, at the workplace for example, such as pesticides, asbestos, benzene, polycyclic aromatic hydrocarbons (PAHs) and chlorinated hydrocarbons have demonstrated a greater risk of PC. Several meta-analyses show the correlation between exposure to toxic substances and pancreatic cancer[20] [21], substances including cadmium[22] and selenium[23] intake.

Finally, epidemiological studies suggest that bacterial infections can increase PC through the increase in inflammation, rudimental in pancreatic carcinogenesis.[24] These infections include

Porphyromonas gingivalis, responsible for periodontal disease and Helicobacter pylori, which infects the stomach and gives rise to peptic ulcers.

- Endogenous risk factors:

As previously mentioned, there are discrepancies of pancreatic cancer incidence rates when comparing genders and age, with a slightly higher risk in males compared to females (1.39 males to 1 female) and an increase in patients with pancreatic cancer in older population (55+ years).

Ethnic disparities exist with African Americans being the most at risk when compared to other races, a cohort determined there was an increase in 42% when compared to the American Caucasian population.[25]

Hereditary pancreatic cancer can be found in 10 to 15% of PDAC cases, with a twofold increased risk when first-degree relatives suffer from pancreatic cancer.[26] Certain genetic syndromes have been uncovered to contribute to this risk: Peutz-Jeghers syndrome is caused by germ-line mutations in the *STK11* gene, which functions as a tumour suppressor by regulating cell cycle and is characterised as being a predisposition to cancer and accompanied with a lifelong risk of 36%.[27] [26] Hereditary pancreatitis is due to a mutation within the trypsinogen gene *PSSR1* and resembles chronic pancreatitis of other origins.[28] Hereditary pancreatitis yields a lifetime risk of 40-55% of developing PC.[29] Familial atypical multiple mole melanoma (FAMMM) syndrome is associated with a mutation in the *p16/CDKN2A* gene, another tumour suppressor that regulates cell cycle, and is found to increase the risk of melanoma and pancreatic cancer.[30] FAMMM increases the risk of pancreatic cancer up to 17%.[26] Hereditary breast–ovarian cancer caused by germline mutations of *BRCA-1* and *BRCA-2* genes, tumour suppressors responsible for repairing DNA, increases the risk of multiple malignancies including PC by 1.5-4%.[26] [31] Hereditary nonpolyposis colorectal cancer or Lynch syndrome caused by germline mutation in *MMR* genes or epigenetic silencing of *MSH2* also found in around 5% of colorectal cancers, with an increased risk of PC of 3%.[26] [32] In the majority of family history pancreatic cancer cases, a genetic syndrome or mutation is unidentified.

Clinical presentation

Symptoms

Symptoms of PDAC are non-specific although tumours located in the head of the pancreas, which are more frequent (70-80%) than those in the body or tail (20-30%), (Fig. 3) tend to be symptomatic - and therefore diagnosed - at an earlier stage.[33]

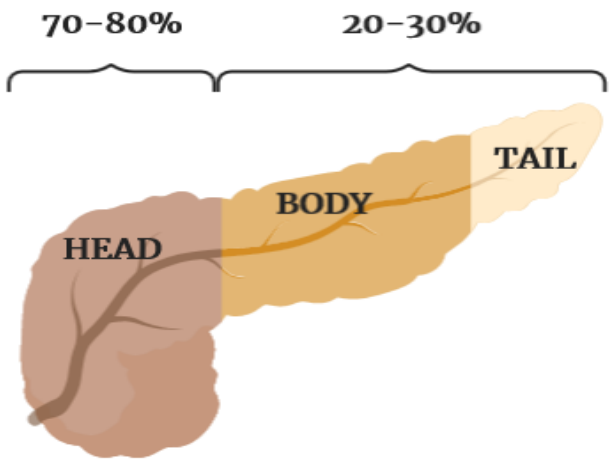

Figure 3: Tumour occurrence depending on localisation in the pancreas

The most common symptoms are fatigue, anorexia and weight loss, which are non-specific and appear late in the disease. Other symptoms include an intense abdominal pain and/or along the back and jaundice due to the obstruction of the common bile duct is obstructed by the tumour.

Up to 80% of patients will develop diabetes or be hyperglycaemic when diagnosed, either acting as a risk factor or caused by the neoplasm. New on-set diabetes is currently being studied as a new clinical biomarker for early detection of pancreatic cancer,[34] since it appears around 2 to 3 years prior to pancreatic cancer.[35]

Other less common symptoms include acute pancreatitis, thromboembolism, intestinal occlusion and other digestive problems.

Diagnosis

Diagnostic of pancreatic cancer is often late since clinical signs generally appear at a later stage of disease progression. Indeed, a mere 15-20% of patients are diagnosed at a time point where the tumour is operable. Additionally, there are no early biomarkers of PC, further hindering early diagnostic.

A diagnosis of pancreatic adenocarcinoma is obtained through imaging techniques, and more precisely using an abdominal computed tomography (CT) scan by dual-phase contrast which allows detection of liver and peritoneal metastases as well. Other imaging techniques that can be used in PC diagnosis include magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS).[36] EUS is also used to obtain biopsy samples by combining fine needle aspiration (EUS-FNA).

These biopsy samples are used for cytologic and histologic purposes and can indicate on the extent of tumour spread. Biopsy analyses are also indicated when in doubt of the malignancy, in diagnostic differential with chronic pancreatitis in its pseudo-tumour form or autoimmune pancreatitis, for example. It is also used to assess chemotherapy treatment in unresectable and non-metastatic tumours or neoadjuvant treatment in potentially resectable tumours. The accuracy of EUS-FNA ranges from 65 to 95% and the negative predictive value (NPV) from 50 to 70% with inconclusive results in 20% of cases.[37]

Searching for Kirsten rat sarcoma (*KRAS*) mutations, which occur in over 80% of PDAC, after EUS-FNA can help differentiate between benign and malignant lesions, increasing the NPV of cytopathological diagnosis from 67% to 88%. KRAS mutation assays are also used to differentiate PDAC from chronic pancreatitis and is increasingly investigated in liquid biopsies to monitor treatment management in patients as well as a marker of poor prognosis.[37]

The carbohydrate antigen 19-9 (CA 19-9) also called sialyl Lewis a (sLe^a) is currently the best validated biomarker,[38] synthesised by pancreatic cells and found in serum with a threshold of 37U/mL. As such, it has a high sensitivity (80%) and specificity (80-90%)[39] but an insufficient predictive positive value to diagnose PC. In some cases, patients of Lewis negative serotype will not produce the Lewis antigen necessary for CA19-9 biosynthesis and hence present a false negative result. On the other hand, false positives can arise in conditions such

as liver damage, pancreatitis and inflammation amongst others. The value of CA19-9 as a biomarker does rise when associated with risk factors, with an increase in risk of pancreatic cancer in patients with high CA19-9 within one year of diabetes mellitus diagnosis. Following CA19-9 serum levels and kinetics has proven to be a useful tool in surveillance as well as to judge prognosis, resectability, recurrence and therapeutic efficacy. It has been judged a supplemental tool to imaging techniques although its increase can precede imaging-identification of recurrence in resected patients.[40]

Other biomarkers are under investigation but are not yet validated for clinical use. Liquid biopsies are used to obtain circulating tumour cells (CTC), cell-free DNA (cfDNA) from which is extracted circulating tumour DNA (ctDNA), exosomes.[41]

Circulating tumour cells are found to be highly specific and the presence or number of circulating tumour cells has been found to be in correlation with clinical prognosis, with high CTC numbers correlating with metastatic disease and worse survival.[42] Current methods for apprehending and enhancing CTCs are still limited and causes the rarity of cells obtained.

Circulating tumour DNA originates from dying tumour cells through apoptosis or necrosis or can be secreted. The most commonly researched target in these ctDNA is the presence of mutated *KRAS* although entire ctDNA sequencing has also been studied, for example using next generation sequencing (NGS).[43] Similarly to CTCs, the low copies of ctDNA secreted during early stages of PC and its degradation makes it difficult to analyse using today's techniques and further studies are needed to forward ctDNA's use in early carcinogenesis.[41]

Genetic and protein compartments of exosomes have been studied as diagnostic tools in PDAC although, as with ctDNA, early detection is difficult. Exosomes are rich in microRNA (miRNA) which have the potential of being diagnostic biomarkers and elucidate on potential chemoresistance of tumours. For example, miR-155, an upregulated exosomal micro-RNA, has been identified as a marker of gemcitabine resistance in PC.[44] In 2015, a study on saliva samples from patients of PDAC found hsa-miR-21, hsa-miR-23a, hsa-miR-23b and miR-29c to be upregulated. Furthermore, in experimental models the detection of salivary miRNAs preceded that of systemic cancer cell markers, suggesting salivary miRNAs could hold a place as early diagnostic biomarkers.[45]

Although these innovative biomarkers are not yet used in routine, they are currently investigated in clinical trials. One such prospective study (NCT03821909) is exploring the diagnostic and prognostic value of CTCs and exosomes extracted from the portal venous blood obtained with EUS. Another current study (NCT04636788) investigates the use of liquid biopsy combined with EUS-FNA samples to find new exosomal small RNA biomarkers for pancreatic cancer treatment.

Anatomopathological classification

After histological confirmation of neoplasm, patients are categorised depending on disease progression. TNM classification has been established sorting pancreatic cancers on size of the primary tumour (T), the number of regional lymph nodes (N) and distant metastases (M) evaluation. Depending on the score in each category, patients are divided into diseases stages between I and IV.

Nowadays however, the most common classification used is according to the MD Anderson Cancer Center Clinical (MDACCC) radiologic staging of PC or National Comprehensive Cancer Network (NCCN). [33] PCs are divided into four groups depending on tumour vascular invasion, dictating patient survival prognosis and treatment options (Table 1).

Resectable tumours are those that have not spread from the pancreas, in contact with less than 180° of the superior mesenteric vein (SMV), no narrowing of the vein and no contact with the superior mesenteric artery (SMA), hepatic artery (HeA) or coeliac trunk (CoT). Borderline resectable tumours have a greater invasion or reduced the calibre of the SMV associated with less than 180° contact of the SMA, HeA and/or CoT. Locally advanced tumours have spread to the point that the mesenteric vein is obstructed beyond surgical reconstruction, as is the case with the hepatic artery. The prognostic of patients whose tumours have been deemed resectable will depend on the size and differentiation of the tumour as well as the spread throughout lymph nodes and the possibility of administering adjuvant chemotherapy.[46] For advanced tumours, prognosis will depend to patient intrinsic factors and metastatic tumoral volume. Indeed, the deterioration of patient's general condition and altered quality of life as

well as hepatic metastases, the number of metastatic sites and high CA 19-9 levels are negatively linked to survival.[47] [48]

Group	What does it mean?	Can surgery be done?	Why?
Resectable	Cancer has not spread outside the pancreas.	Yes, tumor can be removed completely with surgery.	Tumor has not grown into nearby arteries or veins.
Borderline resectable	It is hard to tell from imaging tests if the cancer has spread to nearby tissues.	It is unclear if the tumor can be removed with surgery.	Tumor may or may not have grown into nearby arteries and veins.
Locally advanced	There is cancer (metastasis) in nearby lymph nodes and tissues.	Tumor may or may not be removed with surgery. Lymph nodes may be resectable.	Tumor has grown into nearby lymph nodes and tissues.
Metastatic (unresectable)	Cancer has spread to distant parts of the body.	No, tumor cannot be removed with surgery.	Tumor has grown into surrounding tissues and has spread to distant parts of the body.

Table 1: NCCN Guidelines for Patients: Pancreatic Cancer 2019

PDAC biology

Pancreatic carcinogenesis and precancerous lesions

The evolution of pancreatic adenocarcinoma is still not fully elucidated but is caused by the dysplasia and disorderly proliferation of epithelial cells, undergoing precancerous lesions before forming a tumour. A timing model analysis of the evolution of pancreatic cancer and the associated genetic changes has revealed that it takes at least a decade from the original mutation to the apparition of the parental neoplastic cell of the primary tumour, another 6.8 years on average until metastatic apparition and an estimated two more years survival.[49] Studies in mice have elucidated different precancerous lesions, although the cell of origin (islet, acinar or ductal) remains debatable. These precancerous stages are either called pancreatic

intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) or mucinous cystic neoplasm (MCN) depending on the cellular origin of the lesion.

PanIN lesions are neoplasms that develop in intralobular ducts following acinar-ductular transformation and are separated into three grades depending on the epithelial cytology and architecture detected in immunohistochemistry (Fig. 4).[50] These lesions were demonstrated through the cre-recombinase mutation of pancreatic *KRAS* in genetically engineered mice (KC model). In humans, PanINs of grades 1 and 2 – also called low-grade PanIN – do not predict a development towards neoplasia but the high-grade dysplasia of PanIN 3[51] is defined as a carcinoma in situ.

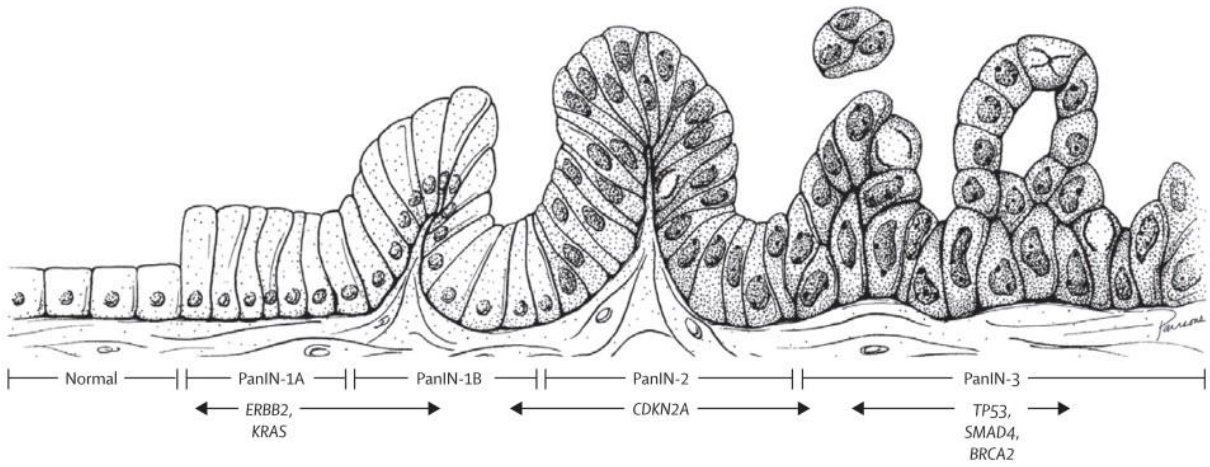

Figure 4: PanIN lesions classification (Hruban et al.)[52]

IPMN, along with MCN, are cyst forming lesions observed after breeding KC mice with models presenting other genetic mutations.[53] IPMN lesions are benign tumours of the duct epithelium that secrete mucin, used as an immunohistochemical marker for differentiation subtypes of lesions. They share similar genetic alterations with pancreatic cancer, such as *KRAS*, *p16/CDKN2A*, *SMAD4*, *TP53* and Guanine Nucleotide binding protein, Alpha Stimulating activity polypeptide (*GNAS*) gene mutations.[54] IPMN lesions are often multifocal.

MCN lesions are the least common present in around 23% of cystic neoplasms and possesses a low risk of malignancy with only 13% of invasive carcinoma.[55] They are much more common in women than in men and are characterised by surrounding ovarian stroma. MCNs also present similar mutations to PDAC such as *KRAS* in low-grade dysplasia and *TP53*, *p16*, and *SMAD4*/ Deleted in Pancreatic Cancer-4 (*DPC4*) in high-grade dysplasia, although contain no *GNAS* mutations differentiating them from IPMN lesions.[54]

Surgical resection is indicated for all MCN lesions since they are mostly found in young patients and localised in the body or tail of the pancreas. Clinical management of IPMN lesions depends on the subtype as well as further examination of cystic fluid to determine whether surgical resection or surveillance is needed. Similarly, PanINs are vulnerable to surgery when they are classified as high-grade.[51]

Genetic aspects

Genetic mutations can be found in the majority of pancreatic tumours. Indeed, 97% of PC present genetic alterations, with 70-98% of patients presenting multiple gene modifications.[56] These genetic mutations impact protein functions and cell pathways which can either be suppressed and even totally annihilate or be enhanced up until being expressed constitutively. In pancreatic cancer the mutated suppressed genes are tumour suppressors, such as *p53*, *Smad4*, *CDKN2A* and *BRCA1*. Tumour suppressor genes are those involved in DNA damage repair, regulation of cell division, induction of cell death and suppression of metastasis, their dysregulation resulting in carcinogenesis and tumour progression. Inversely, the activating mutation found in PDAC occurs in a proto-oncogene, *KRAS*. Proto-oncogenes, when mutated, will provoke an increase in cell growth, proliferation and inhibition of apoptosis, causing the cell to be cancerous.[57]

The most common somatic mutation in pancreatic cancer (50-75%) occurs on chromosome 17p, affecting the *p53* gene, another tumour suppressor gene. Loss of p53 function can cause unbridled cell division, increased cell survival and genetic instability. Furthermore, loss of p53

will reduce levels of p21, produced through binding of p53 to DNA, which regulates the progression of cell cycle into S phase allowing for time to repair damaged DNA. Programmed cell death by p53 can also be lost through the downregulation of miR34a, a micro-RNA and direct transcriptional target of p53, and potential therapeutic strategy.[58] Further studies have shown *p53* mutation to aid in PDAC invasiveness and metastasis.[59] [60]

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) located on chromosome 9p codes for p16, a cell cycle regulator that inhibits cyclin dependent kinases 4 and 6 (CDK4 and CDK6) therefore blocking G1 to S phase transition. Germline mutations and deletion of *CDKN2A* are commonly found in multiple neoplasms including 49-98% of PDAC.[56] The p16 polymorphism was found to be associated with a shorter time to tumour progression.[61]

The third most common tumour suppressor gene mutated and deleted in pancreatic cancers (40-55%) is *DPC4*/*Smad4* located on chromosome 18q. Smad4 is a regulator of TGF-β and its loss reduces proapoptotic signalling and increasing G1/S transition, therefore resulting in inappropriate cell growth. Lower levels of Smad4 were found to correlate with the presence of lymph node metastases[62] and although there have been discrepancies with its potential as prognostic marker, a meta-analysis has found that patients with a loss of Smad4 expression had shorter overall survival.[63]

BRCA1/2 are involved the repairs of double strand breaks of DNA and therefore important in the G2/M progression of the cell cycle. *BRCA1/2* are found mutated in multiple cancers such as breast and ovarian cancers. Mutation of this gene results in a truncated protein with loss of function. *BRCA1/2* mutations are found in around 5-7% of PC in both sporadic and familial pancreatic cancers.[64] Patients with BRCA-associated PDAC were seen to have an improved overall survival (OS) when treated with platinum therapies with an OS in grade 3 patients of 48 months compared to 10 months without platinum exposure.[65] Furthermore, a recent phase 2 trial demonstrated a significant increase when combining gemcitabine and cisplatin for *BRAC1/2* mutated PC, with a two-year survival of over 30%.[66]

Conversely, the activating mutation of proto-oncogene *KRAS* on chromosome 12p is believed to be the most common initiating factor of PDAC (over 80%). The wild-type Ras protein is a small GTPase in the mitogen-activated protein kinase (MAPK) pathway, regulates signal

transduction from the extracellular environment to the cell's nucleus. These signals determine cell proliferation and differentiation as well as programmed cell death through apoptosis. *KRAS* mutation occurs early in pancreatic carcinogenesis and is found in early precancerous lesions such as PanINs and IPMNs.[67] [68] Studies have shown that *KRAS* mutations are linked to a worse prognosis[69] [70], and more particularly the differentiation of various *KRAS* mutations in prognosis, with the G12D mutation proposed as a prognostic tool.[71] Mutated *KRAS* is also currently being investigated as a potential biomarker for PDAC as well as a therapeutic target.[37]

Gruber et al. demonstrated the role of *KRAS* mutation in PDAC initiation in KRASG12D mice. Active transcriptional regulators yes-associated protein (YAP1) and WW domain-containing transcription regulator protein 1 (TAZ) brought on by the constitutively activated KRAS upregulate the JAK/STAT pathway ultimately facilitating acinar-ductal metaplasia and neoplastic progression.[72] Indeed, activated KRAS inhibits the Hippo signalling pathway which allows YAK/TAZ to translocate to the nucleus. However, the activation of YAP is seen to bypass that of KRAS making it a potential anti-PDAC therapeutic target, although the effectors and mechanisms of the Hippo pathway which allow YAP to be activated still remain incompletely elucidated.[73]

Another protooncogene found in PDAC is c-MYC (MYC) whose overexpression, due to gene amplification, promoter transactivation or post-transcriptional regulation, is found in up to 42% of advanced PDAC. Other than promoting cellular proliferation, MYC has an important role in cell differentiation and specification, with MYC being a crucial effector in the β-catenin pathway.[74] In pancreas-specific Cre recombinase driver mouse strains (Ptf1aCRE, Pdx1-Cre), the inactivation of MYC resulted in a significant decrease of pancreatic (principally exocrine) cell proliferation.[75] Separately from promoting tumour growth, MYC has been doted as being a 'metastasis drivers'. MYC's pro-metastatic activity is in part due to its recruitment of tumour associated macrophages (TAMs). Patient autopsy samples of high metastatic burden were 38-fold more abundant in circulating tumour cells and immune composition of the primary tumour showed an enrichment of Arg1+ macrophages – a marker of TAMs – when compared to low metastatic burden samples[76].

Molecular classifications

Due to the heterogeneity in genomic and molecular variations between pancreatic tumours and within the same tumour, it has been difficult to classify pancreatic adenocarcinomas. While genetic alterations are involved in carcinogenesis, the epigenetic and transcriptomic changes define the malignant tumour phenotype. These molecular classifications are sought to complement the histopathological categorisation used for diagnosis to better define therapeutic targets allowing for more precise and personalised medicine while avoiding non beneficial and aggressive treatments for patients.

- Genomic categorisation

Genomic studies separated tumours according to mutations and dysregulated signalling pathways, such as *KRAS* and DNA damage repair. An example is the ongoing clinical trials using PARP inhibitors in patients with germline mutations of *BRCA1/2* either as monotherapy or combined with chemotherapies such as platinum-based therapies.[77] Using genetic markers can be useful to orientate therapeutic strategies, however their use in treatment decisions have not yet been proven in the case of pancreatic cancer. Furthermore, when faced with such complex tumours that are PDAC where multiple genetic signatures are often found, targeting single genetic is insufficient.[78]

In a larger view, genomic aberrations such as deletions, amplifications, duplications and translocations can be used to group similar tumours. A study showed four subtypes of PDAC when grouping according to frequency and distribution of the genomic aberrations. The stable subtype contains less than 50 variations and a widespread aneuploidy advocating cell cycle and division defects. The locally rearranged subgroup contains significant focal event on one or two chromosomes and can be further divided into those with amplified focal regions and those with complex rearrangements. The scattered subtype has moderate chromosomal damage and less than 200 variations and finally the unstable category presents over 200 structural variations suggesting issues in DNA maintenance and a potential target for DNA-damaging agents.[79]

- Transcriptional classification

Using transcriptomic studies such as RNA sequencing or microarray signatures, Collisson et al. separated PDAC tumours into three subcategories depending on gene expression differentials: classical, quasi-mesenchymal (QM-PDA) and exocrine-like.[80] From primary resected PDAC, the tumour epithelium was micro-dissected from the stroma. The classical subtype is characterised as expressing the transcription factor GATA-binding factor 6 (GATA6) and is KRAS-dependent. The QM-PDA subtype showed high tumour grade and poor survival.

Virtual microdissection was used by Moffitt et al. in 2015 to digitally separate tumour, stroma and normal gene expression.[81] Two tumour specific subtypes were discerned from patient-derived xenografts: the basal-like category which exhibited faster tumour growth and from patients that seemed to benefit more from adjuvant therapy, and classical category which overlapped that described by Collisson et al. They further described two stromal subtypes which could be combined to the tumour-specific subtypes with particular biological and prognostic implications.

The International Cancer Genome Consortium (ICGC) then submitted four subtypes including pancreatic progenitor class which was similar to the Collisson and Moffitt classical group, and a squamous classification which closely resembles the Moffitt basal-like category. The final two include the aberrantly differentiated endocrine exocrine (ADEX) and the immunogenic subtypes, the latter characterised by infiltration of immune cells and immunosuppressive pathways.[82] The Cancer Genome Atlas (TCGA) furthered by demonstrating these two subtypes came from low neoplastic cell samples.[83]

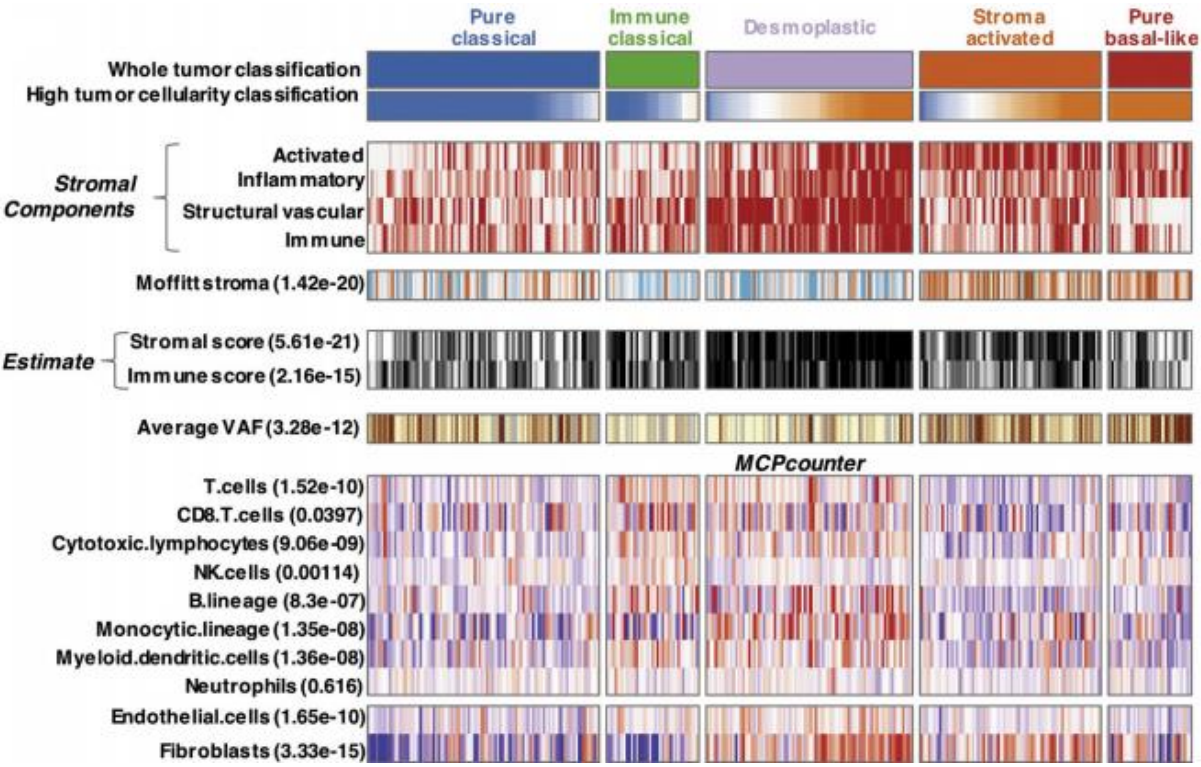

Figure 5: Characterizing the tumour microenvironment of PDA subtypes - Heatmap of the whole tumour and high tumour cellularity classifications and their association with genomically defined stromal characteristics including the identified stromal components, the stromal classification previously proposed by Moffitt et al. distinguishing an activated from a normal stroma (Adapted from Puleo et al.)[84]

Puleo et al. analysed 309 resected tumours and validated the basal-like and classical subtypes previously described. By including microenvironment signatures, five subtypes were overall established (Fig. 5).[84] Pure classical and pure basal-like tumours present low stromal signatures compared to the other three – immune classical, desmoplastic and stroma activated – that have high stromal content. These signatures not only associate transcriptomic data with immunohistochemistry findings, but also provide a prognostic factor. Pure basal-like tumours show a worse prognosis than when stroma is infiltrated, whereas conversely pure classical subtypes have a better prognosis than tumours with a higher stromal index. Overall classical subtypes have a better prognosis than basal-like tumours. A downfall of this study is that it focused on primary tumours and did not consider metastatic gene expression signatures or the differences between the two.

These studies tend to grossly separate tumours into either basal-like or classical tumours but no do not consider intratumoral heterogeneity. Indeed, single-cell RNA sequencing has shown the existence of both subtypes within the same tumour.[85] A PDAC molecular gradient (PAMG) was generated taking into account five histological groups along a continuum. This gradient has allowed to better characterise pancreatic cancer heterogeneity, generating a prognosis independently from disease stage, as well as predict potential treatment response to drugs. In the study, it was found that more aggressively characterised tumours were less responsive to mFOLFIRINOX.[86]

Epigenetic factors can also influence the expression of genes. This includes modifications of histones, non-coding RNAs and DNA methylation. Thus, using epigenetic signatures of tumours by using circulating DNA or primary culture organoids could further the use of transcriptomic markers to phenotype tumours for diagnosis, prognosis and therapeutic strategies.[87] Moreover, the expression levels of certain micro RNA (miRNA) shift during tumour development and progression, including an overexpression of miR-21, miR-34 and miR-155 found in PC.[88]

- Moving towards personalised medicine?

Defining the molecular classification of PDAC tumours serves to better understand the mechanisms of pancreatic carcinoma and better stratify patients for more efficient and targeted therapies in the optic of precision medicine. Through whole exome sequencing (WES) and RNA sequencing of PDAC biopsies, Aguirre et al. propose real-time genomic characterisation of tumours to bring forth potential therapeutic opportunities early in treatment care of patients with pancreatic cancer.[89]

Transcriptomic analyses of patient-derived organoids (PDOs) derived from PDAC patients whilst evaluating their sensitivity to chemotherapies has allowed a pharmacotyping of patients' tumours. This has the potential of evaluating on a patient-basis the response to available anti-PDAC therapies as well as agents investigated in clinical trials. Furthermore, using PDOs can

help overcome the low cellularity found with primary pancreatic cancer samples, facilitating molecular characterisation of patient tumour.[90]

The evolution and continuing progress in characterising the molecular and genomic complexity of pancreatic adenocarcinoma illustrates the inter- and intra-complexity of heterogeneous PDAC tumours. This in part explains the poor prognosis and low response of pancreatic cancer patients to current therapies. In addition, the complexity of PDAC tumours highlights the current research for novel treatments that can overcome these shortcomings as well as the development of combination protocols targeting multiple angles of pancreatic tumours.

The tumour microenvironment

The tumour microenvironment (TME) is constituted differently whether looking at a precancerous lesion or cancerous neoplasm and varies depending on tumour subtypes. A high abundance of cancer-associated fibroblasts (CAF) produces an extracellular matrix (ECM) rich in collagen and hyaluronic acid (Fig. 6), decreasing blood vessels and promoting hypoxia. This stromal matrix ranges from 25 to 80% of the solid tumour with a mean at 50%, creating a physical barrier for infiltrating immune cells and chemotherapy. Fibrosis and tissue tension created by the ECM is linked to a higher disease progression and a poor prognosis, but conversely, more aggressive tumours are usually undifferentiated with reduced stroma.[53]

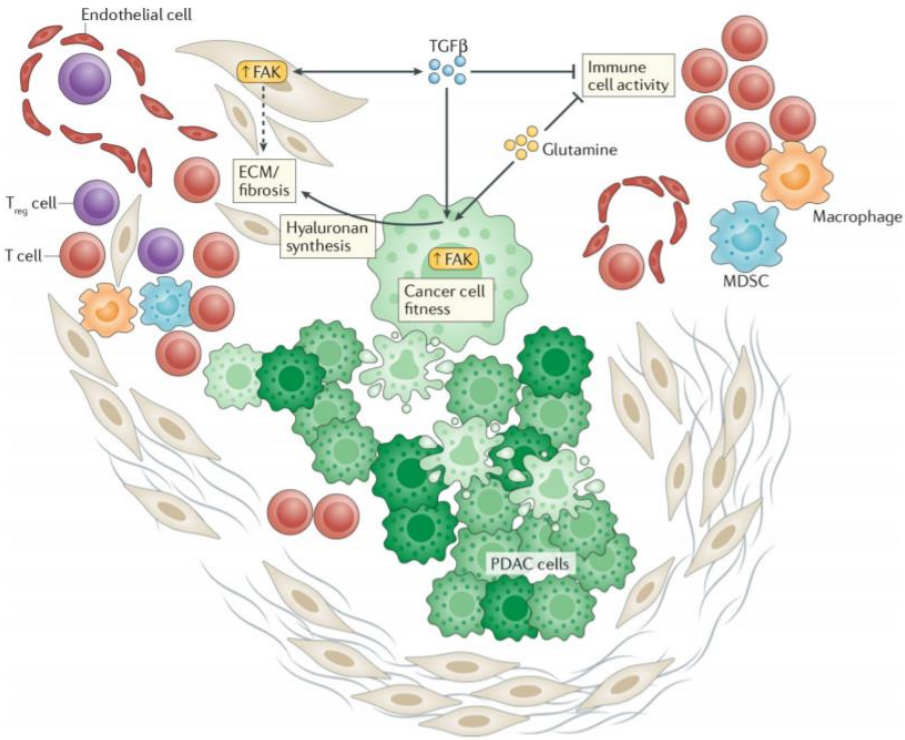

Figure 6: The PDAC tumour microenvironment (Ho et al.)[91]

One of the main types of cells in the TME and stroma are cancer-associated fibroblasts – making up between 15 and 85% of stromal cells[92] – which are derived either from stellate pancreatic cells, resident tissue fibroblasts, bone marrow-derived mesenchymal cells, adipocytes, pericytes or epithelial and endothelial cells having undergone epithelial-mesenchymal transition (EMT).[93]

Similarly to tumour cells, cancer-associated fibroblasts can be divided into subtypes which demonstrate high inter- and intra-tumoral heterogeneity. Binary classifications use the α-smooth muscle actin (α-SMA), fibroblast-activation protein α (FAP) to sort populations into αSMA-positive myofibroblastic CAFs (myoCAFs), and αSMA-negative/IL6-positive inflammatory CAFs (iCAFs).[94] A recent study using transcriptomic analyses of a wider scope of markers suggests a more complex categorisation of CAFs is necessary.[95]

Amongst their roles in the TME, CAFs produce extracellular matrix and growth factors that act on tumour development and progression, angiogenesis and contribute to the immunosuppressive nature of the TME. Furthermore, through remodelling the ECM, they are able to increase invasiveness and metastasis as well as drug resistance.[53] [96] Chemo- and

radiotherapy resistance is also achieved by CAFs through the release of exosomes containing chemoresistance factors, for example the factor Snail in line with gemcitabine resistance.[97]

CAFs have been the attended target of new therapies in order to strike not only the tumour cells but the whole TME. However, due to the heterogeneity of CAFs and incongruity on their multiple origins, targeting CAFs has proven to be difficult.[98] [92]

The TME of PDAC tumours is believed to be quiescent due to the immunosuppressive mechanisms that counteract anti-tumour immunity. Among the immune cells found are innate immune cells comprising of macrophages and myeloid-derived suppressor cells (MDSCs) (Fig. 6).

Through chemotactic agents, circular monocytes are recruited to the tumour to become tumour-associated macrophages (TAMs), although these can also originate from tissue residing macrophages.[99] Macrophages are one of the dominant stromal cell populations in PDAC tumours. TAMs are grossly divided into M1 and M2 macrophages depending on polarisation with M1 TAMs secreting pro-inflammatory substances, such as IL-1β, TNF-α, IL-6 or IL-12 and the capacity of triggering a Th1 mediated immune response, which have therefore primarily an antitumoral effect. Contrarily, M2 TAMs secrete anti-inflammatory cytokines including TGF-β1 and IL-10 which help tumour development.[100] Tumour-associated macrophages (TAM) create an immunosuppressive milieu through immunosuppressive chemokines and cytokines such as vascular endothelial growth factor (VEGF), contribute to tumour progression and metastasis by promoting epithelial to mesenchymal transition (EMT) and angiogenesis.[101] TAMs have also been shown to impact chemotherapy efficacy. Indeed, by increasing the activity of cytidine deaminase (CDA), an essential enzyme in gemcitabine metabolism, tumour-associated macrophages are able to induce chemoresistance.[102]

MDSCs are generally divided into polymorphonuclear (PMN-MDSC) similar to neutrophils, representing 80% of tumour-associated MDSCs, [101] and monocytic MDCS. MDSC infiltration in PDAC tumours is correlated with the progression of the disease.[103] In addition, they contribute to the TME by suppressing CD4+ and CD8+ T cell activity through increased expression of programmed death-ligand 1 (PD-L1), stimulation regulatory T cells (Tregs), block natural killer cell (NK cell) cytotoxicity and promote M2 conversion of TAMs.[104] [105]

Carstens et al. determined from patient PDAC samples that amongst T cells there are a majority of CD8+ T cells (47% of CD3+ cells) and CD4+ T cells (36% of CD3+ cells). It was previously thought that the TME had limited T cell infiltration, but studies have shown their effect to be dependent on spatial distribution, T cell subpopulation and simultaneous macrophage infiltration. For example, proximity of CD8+ cytotoxic T lymphocytes (CTL) to tumour cells correlates with patient survival.[106] In PDAC tumours CTLs are in a state of exhaustion where their effector function is impaired, they have shorter survival and increased expression of the checkpoint inhibitors programmed cell death 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[107] Using checkpoint inhibitors as monotherapy in PC has proven to be unsuccessful but are currently being investigated in combination protocols with other therapies.

The CD4+ T cell population can be further divided into helper Th1 cells that produce interferon γ (IFN-γ) increasing cellular type 1 immunity with the recruitment of CTLs, M1 macrophages, and NK cells. Oppositely can be found helper Th2 cells associated with disease progression and shorter overall survival in patients with resectable pancreatic adenocarcinoma.[108] A reciprocity between the two subtypes exists where each inhibits the differentiation of the other. Differentiation towards a certain subtype of T helper cells can also be induced by the other cells of the TME (CAFs, TAM, tumour cells and M1 macrophages, CTLs) implying the possibility of a shift from Th2 cells toward Th1 cells when acting upon these other actors.[104] T helper 17 (Th17) and T regulatory (Treg) cells are also involved in PDAC tumour regulation. Tregs can be found in precancerous IPMN lesions and the ratio of Tregs over CTLs (Treg/CD8+) cells is found to increase during tumour progression.[109]

Other immune cells within the PDAC tumour microenvironment include NK cells of reduced cytotoxicity and impaired anti-tumour activity as well as tumour-associated neutrophils (TANs) and B lymphocytes.

The desmoplastic compartment of the tumour microenvironment comprises extracellular matrix (ECM) and stromal cells and makes up 90% of the total tumour volume. [94] The ECM is made up of collagen I, III and IV, integrin, laminin, fibronectin, glycosaminoglycan, matrix metalloproteinase (MMP), and secreted protein acidic and rich in cysteine (SPARC). Secretion

of ECM enables tumour growth while decreasing penetration of anti-cancer drugs. In addition, PDAC tumours present low vascularity and therefore low tumour perfusion, further hindering access for antitumoral therapies.[110] Targeted therapies directed towards the degradation of ECM are currently being investigated.

Treatments

Surgery

Surgery for pancreatic cancer can either be for curative or palliative purposes. Only a fifth of PDAC patients are diagnosed at a stage where the tumour is resectable.

In the case of a resectable tumour, pancreaticoduodenectomy or Whipple's procedure is the most common protocol and aims to eliminate the tumour through the removal of the pancreatic head, parts of the duodenum, bile duct, gallbladder and sometimes part of the stomach. Other procedures include distal pancreatectomy with the removal of the pancreatic tail and often the spleen as well, or total pancreatectomy with the onset of diabetes and life-long insulin treatment. Surgery remains the only treatment considered as curative to this day, although relapse rates are high with over 80% of cases.

Palliative surgery is intended to relieve symptoms, for example caused by blockage of the bile duct, through a stent placement or bypass surgery. Because of the speed of disease progression and the risks involved, palliative surgery is not always considered especially in patients that are too weak.[111]

Neoadjuvant chemotherapy is administered before a surgical intervention and has multiple purposes. It allows to identify patients with aggressive tumours, induce a response to increase the success of resection, eventually treat micro-metastases and test the chemosensitivity of the tumour. Although not yet routine strategy, trials are underway to evaluate the benefits of neoadjuvant chemotherapy. For example the PANACHE-01 study is in phase 2 multicentric trial aimed to assess the safety and efficacy of administering FOLFOX or modified FOLFIRINOX protocol as neoadjuvant chemotherapy for resectable PDAC (NCT02959879).[112] The results have not yet been communicated.

Radiotherapy

Radiotherapy can be used in cases where the tumour is still at a local stage but for which surgery is not an option. For borderline resectable cancers, the goal is to shrink the tumour enough to then perform surgery. For locally advanced pancreatic cancers, however, neoplastic cells have spread to the blood vessels and organs surrounding the pancreas and surgery is not an option, radiotherapy and chemotherapy treatments are therefore used to slow the spread. Stereotactic body radiation therapy (SBRT) uses a linear accelerator to deliver radiation aimed at the tumour from outside the body. In certain cases, especially for locally advanced PC, radiotherapy is coupled with chemotherapy, known as chemoradiotherapy.

Using chemoradiotherapy to locally control the primary tumour was found to be suboptimal, with 50% of patients presenting local progression with associated obstructive symptoms and a negative impact on quality of life. SBRT when investigated as neoadjuvant therapy after three cycles of neoadjuvant chemotherapy led to 77% of patients receiving exploratory laparotomy leading to 56% undergoing surgical resection. This resulted in 97% having microscopically margin-negative resection and 9% of patients with complete pathologic response.[113]

Chemotherapy

There are several drugs available for the treatment of PC (Table 2) depending on disease progression, the results of tumour biopsy and patients' characteristics (age, well-being, co-morbidities etc.) but to this day pancreatic cancer remains incurable.

A study in 1997 compared gemcitabine to 5-fluorouracil (5-FU) in patients with advanced pancreatic cancer and found an increase in clinical benefits from 4.8% of patients under 5-FU to 23.8% of gemcitabine-treated patients and survival with 12 month survival rates of 2% and 18% for 5-FU and gemcitabine patients respectively.[114] As such, it was approved by the Food and Drug Administration (FDA) and has since been the treatment of reference for PDAC. In 2011 Conroy et al. compared gemcitabine treatment to that of the combination FOLFIRINOX comprised of Oxaliplatin, 5-FU, folic acid and Irinotecan. Results demonstrated the combination protocol increased overall survival and progression-free survival and was also accompanied by a reduction in adverse effects although grade 3 and 4 treatment-related adverse events and a

mortality was registered in both groups. OS increased from 6.8 months to 11.1 months and PFS was 3.3 months and 6.4 months in the gemcitabine and FOLFIRINOX groups respectively; at 6 months 31% of patients in the FOLFIRINOX groups had a decrease in quality of life compared to 64% in the gemcitabine group.[115] To further minimise these toxicities, a modified version of the regimen was investigated (mFOLFIRINOX). This modification consisted in using continuous intravenous infusion of 5-FU instead of a bolus injection and the administration of growth factors. The modified regimen showed an improved safety, with lower incidence of neutropenia and absence of febrile neutropenia originally due to bolus 5-FU, while maintaining the efficacy of the original protocol with an OS and PFS at 16.4 months and 9.9 months respectively.[116]

Molecule	Brand name	Mechanism of action	Adverse effects
Gemcitabine	Gemzar	Antimetabolite block RNA and DNA synthesis	Skin toxicity, oral inflammation
5-Fluorouracil	Adrucil	Antimetabolite block RNA and DNA synthesis	Cardiotoxicity, skin toxicity, oral inflammation
Capecitabine	Xeloda	5-FU precursor administered orally	Oral inflammation, indigestion, skin toxicity
Irinotecan	Camptosar	Antineoplastic topoisomerase I blocker causing DNA breaks	Indigestion, cardiotoxicity, hepatotoxicity
Oxaliplatin	Eloxatin	Inter-strand crosslinked DNA adducts causing DNA damage	Nephrotoxicity, ototoxicity, neurotoxicity
Cisplatin	Platinol-AQ	Inter-strand crosslinked DNA adducts causing DNA damage	Nephrotoxicity, ototoxicity, hematologic toxicity, indigestion
Paclitaxel	Taxol	Antimitotic block cells in metaphase	Peripheral neuropathies, reversible alopecia, allergies
Docetaxel	Taxotere	Antimitotic block cells in metaphase	Peripheral neuropathies, reversible alopecia, allergies

Table 2: Chemotherapy products used for the treatment of pancreatic ductal adenocarcinoma

Adjuvant chemotherapy treatment is indicated in all resectable tumours as chemotherapy insures beneficial survival independently of tumour stage. (Fig. 7) PRODIGE 24, a phase 3 trial comparing modified FOLFIRINOX to gemcitabine as adjuvant therapy in resectable pancreatic cancer demonstrated the improvement with a median overall survival of 54.4 months in the

mFOLFIRINOX group compared to 35.0 months in the gemcitabine arm.[117] The phase 3 ASPAC trial however, showed there was no benefit of using gemcitabine associated with nab-paclitaxel compared to gemcitabine alone.[118]

For borderline tumours, a chemotherapy induction treatment is preferred to surgery straight away with meta-analyses studies suggesting better survival and resection rate.[119] The analysis incorporated 24 reports presenting a resection rate of 67.8% and R0-resection (microscopically margin-negative resection) rate of 83.9% and concluded a median overall survival of 22.2 months and median progression-free survival of 18 months. This induction chemotherapy is achieved with either FOLFIRINOX or gemcitabine with nab-paclitaxel. A recent phase 3 trial (PREOPANC) showed an increase in overall survival of 35.2 months versus 19.8 months in the preoperative chemoradiotherapy and immediate surgery groups respectively for borderline resectable PDAC.[120]

Advanced pancreatic tumours regroup locally advanced and metastatic PDAC and take into account for the patients' daily living abilities throughout the disease (Fig. 7) according to the Eastern Cooperative Oncology Group performance status (ECOG PS).[121] In advanced tumours, induction chemotherapy by gemcitabine is the referenced treatment although studies have been done in attempt to increase the results obtained, either by modulating its' pharmacokinetics or by combining with other treatments. Combining gemcitabine with targeted therapies have not shown any significant improvement compared to gemcitabine, apart from Erlotinib which significantly improved survival, although only by less than a month.[122] However, combining gemcitabine to another chemotherapy, nab-paclitaxel, increased overall survival and progression-free survival in patients with advanced PC in a phase 3 trial (MPACT).[123] [124] Overall survival increased from 6.6 months to 8.7 months with the addition of nab-paclitaxel to gemcitabine. Recent studies have shown that FOLFIRINOX and gemcitabine combined to nab-paclitaxel present similar survival rates, with survival hazard ratio at 0.79[125] [126] although from a pharmacoeconomical point of view, FOLFIRINOX appears to be most cost-effective with an incremental cost-effectiveness of $547,480/ quality-adjusted life years gained over nab-paclitaxel + gemcitabine.[127]

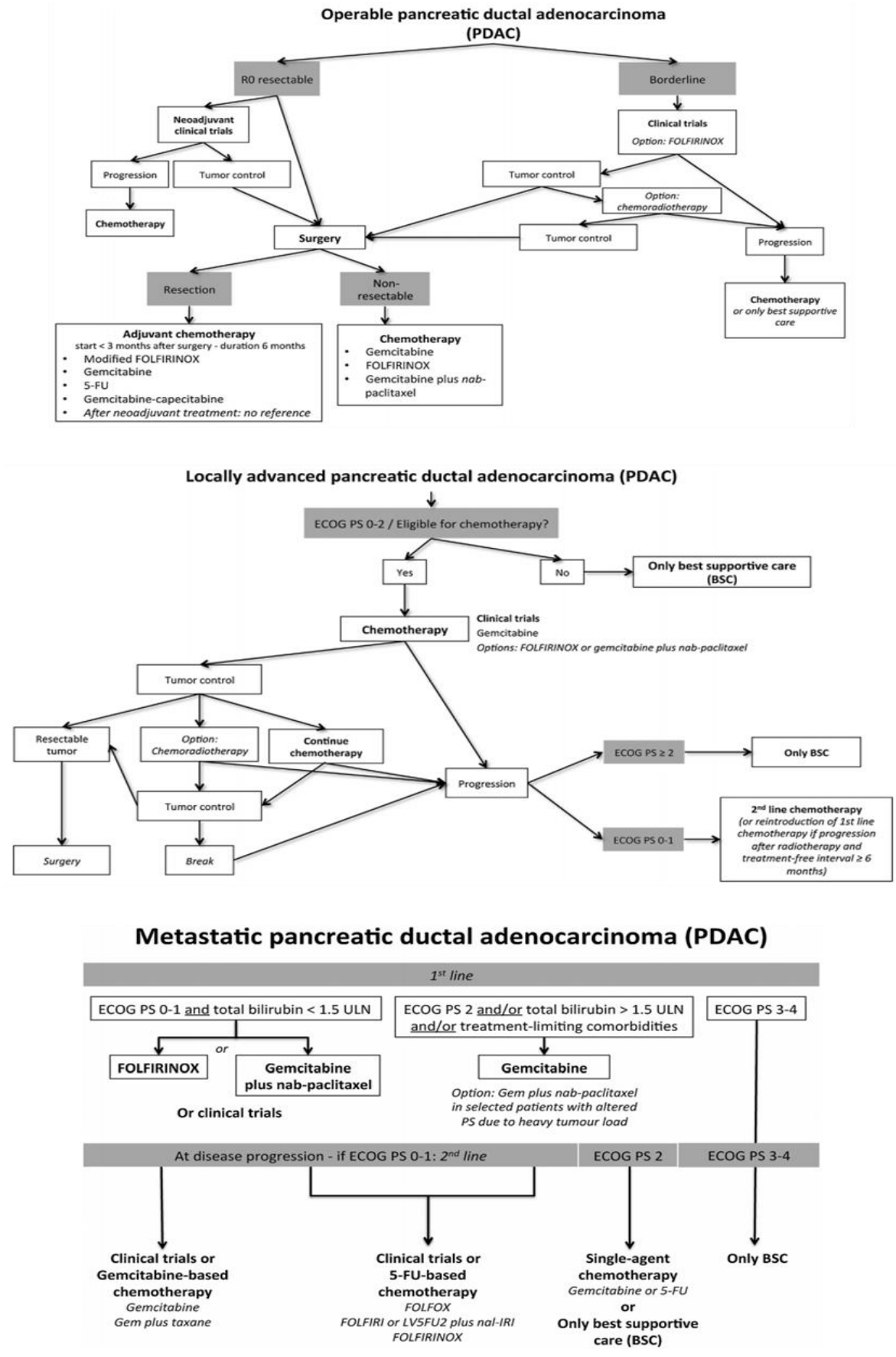

Figure 7: Algorithms for treatment approaches of operable (top), locally advanced (middle) and metastatic (bottom) pancreatic cancer (Neuzillet et al.)[33]

Looking at the PRODIGE and other current clinical trials, combinations with chemotherapy are being studied to the fullest. Other strategies have also arisen, for example by optimising drug delivery. Paclitaxel causes mitotic arrest in cancer cells but has very poor aqueous solubility. Several formulations were tested to allow for intravenous administration including polyoxyethylated castor oil solvent (Cremaphor). Unfortunately this formulation caused hypersensitivity which required patients to undergo premedication with steroids and antihistamines.[128] Nab-paclitaxel consists in the anticancer drug bound to albumin nanoparticles, ensuring improved pharmacology with a higher intratumoral accumulation of paclitaxel and fewer toxicities, such as neutropenia, when compared to the Cremaphor formulation. Another example is liposomal encapsulated irinotecan, currently investigated in a phase 3 trial (NAPOLI-3) as second-line treatment for patients with metastatic pancreatic cancer (NCT04083235).

Conventional therapies are limited when it comes to PDAC tumours. Although genetic mutations drive tumour initiation and progression, resistance mechanisms are more and more explained by tumour phenotype.[129] Furthermore the complex network that is the tumour microenvironment and the network of crosstalk between the actors associated with the hypovascularity and desmoplastic ECM leads to inefficient delivery and therefore dosing of chemotherapies in the tumour. In order to improve survival and reduce recurrence of PC, other treatment options are being investigated. Targeted therapies and immunotherapies are strategies that have worked in other tumours and are now being applied to pancreatic adenocarcinoma. In addition, gene therapies are being developed for genetic and neuromuscular disorders and in oncology.

Novel treatments

Targeted therapies

Contrarily to chemotherapies that have difficulty in differencing healthy and cancerous cells, targeted therapies are small-molecule inhibitors and monoclonal antibodies aimed towards

cancer cell surface receptors, growth factors or other proteins of dysregulated signalling pathways.[130]

As previously mentioned, *KRAS* is mutated in over 90% of PDAC tumours and therefore an exciting target. In the past *KRAS* oncogene has been dubbed an undruggable objective by targeted therapies since inhibiting all RAS isoforms would be deleterious to normal tissue as well and activated RAS signalling is achieved through protein-to-protein interactions lacking well-defined binding pockets.

New small molecules are being developed to target the mutations of *KRAS*. The FDA has recently approved an inhibitor of *KRAS* G12C (Sotorasib) in lung cancer, but this mutation is uncommon in PDAC tumours and the most frequent mutations, G12V and G12D, have yet to be targeted.[131]

Other directions include targeting downstream effectors of activated KRAS, namely the RAF-MEK-ERK and PI3K-AKT-mTOR pathways, by disrupting RAS membrane localisation or KRAS-Regulated Metabolic Targets. In addition, research is being done in developing anti-KRAS cancer vaccines to orientate the immune system against mutated *KRAS* expressing cells and using RNA interference to directly suppress KRAS expression.[132] Tipifarnib, a farnesyltransferase inhibitor disrupting RAS association at the plasma membrane, showed no increase in overall survival study when associated with gemcitabine compared to the chemotherapy alone.[133] A in vitro investigating the inhibition of ERK, an effector of KRAS signalling observed degradation of MYC and the triggering of a senescence-like phenotype. In addition, an increase in PI3K-AKT-mTOR signalling acted on sensitivity to ERK inhibitors. Finally, a mutant-KRAS peptide vaccine is currently in a phase 1 trial in combination with nivolumab and ipilimumab (NCT04117087).

Another example of targeted genetic mutations in PDAC are the *BRCA1/2* genes. Germinal mutations of these genes are believed to be more vulnerable to DNA damage and therefore more responsive to treatments inducing DNA breaks such as platinum therapy. Several retrospective studies have shown patients with *BRCA1/2* mutations to be more receptive to platinum-based therapies with improved survival in comparison to non-*BRCA1/2* mutated patients.[134] [135] [136] Wattenberg et al. found the objective response rate in mutated

patients to be 58% compared to 21% in the control group, and the real-world progression-free survival was 10.1 months and 6.9 months respectively.[134]

PARP inhibitors interfere with base excision repair and block PARP to the single-strand DNA breaks, preventing their repair and generating their accumulation. The PARP inhibitors investigated include Olaparib, Veliparib and Rucaparib with studies already demonstrating clinical benefits for patients with *BRCA1/2* mutations and other clinical trials of combination with chemotherapy are underway.[137] [138] [139] For example, the tumour response rate was 21.7% after Olaparib treatment and stable disease in 35% of patients.[137] A phase 3 trial (POLO) evaluated the administration of Olaparib as maintenance treatment following platinum-based therapy compared to platinum therapy alone in metastatic PDAC. The results showed an increase of progression-free survival with PFS of 7.4 months compared to 3.8 months in the control group, even though overall survival were not significantly different (18.9 months and 18.1 months respectively), surely owing to the use of platinum-based therapy in both arms.[140] This still warranted olaparib (Lynparza) to be approved by the FDA in 2019 for patients with germline *BRCA*-mutated (gBRCAm) metastatic PC.

Treatment approaches targeting the TME and in particular the tumour stroma is in ascent. An example is the use of pegylated hyaluronidase (PEGPH20). The hyaluronidase enzyme expressed degrades hyaluronic acid, decreasing the interstitial space viscosity and the tumour interstitial fluid pressure (IFP) facilitating the access of other drugs. Preclinical studies in KC and KPC mice showed a decrease in hyaluronic acid and interstitial fluid pressure after intravenous administration of PEGPH20. Following this, combination of PEGPH20 with gemcitabine compared to the chemotherapy alone revealed tumours to be highly vascularised. In addition, median survival of mice increased from 55.5 days for gemcitabine to 91.5 days for the combination.[141] Unfortunately, the median overall survival was not improved in a phase III trial combining PEGPH20 with gemcitabine or nab-paclitaxel in patients with metastatic PC.[142] It is, however, currently studied in a phase II trial in combination with pembrolizumab, an anti-PD-1 checkpoint inhibitor, (NCT03634332) for patients with hyaluronin-high (HA-high) metastatic PDAC.[143] This supersedes an in vitro study that demonstrated PEGPH20 treatment increased NK cell infiltration and activity of monoclonal antibodies, and could hence increase sensitivity to other immunotherapies.[144]

Immunotherapies

Immunotherapies have renewed anticancer treatments for several solid tumours as a means of activating the body's immune system against tumoral cells. Several strategies have risen to do so including monoclonal antibodies (mAb) that inhibit immune checkpoint including cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1) and programmed cell death protein ligand-1 (PD-L1). Other strategies include cancer vaccines that aim to target cytotoxic T lymphocytes (CTLs) against tumour-associated antigens (TAAs), and adoptive cell transfer (ACT) wherein a patient's immune cells are readministered following ex vivo modification to aim them towards T cell receptors (TCRs) or as CAR-T cells.[145]

- Immune checkpoint inhibitors (ICI)

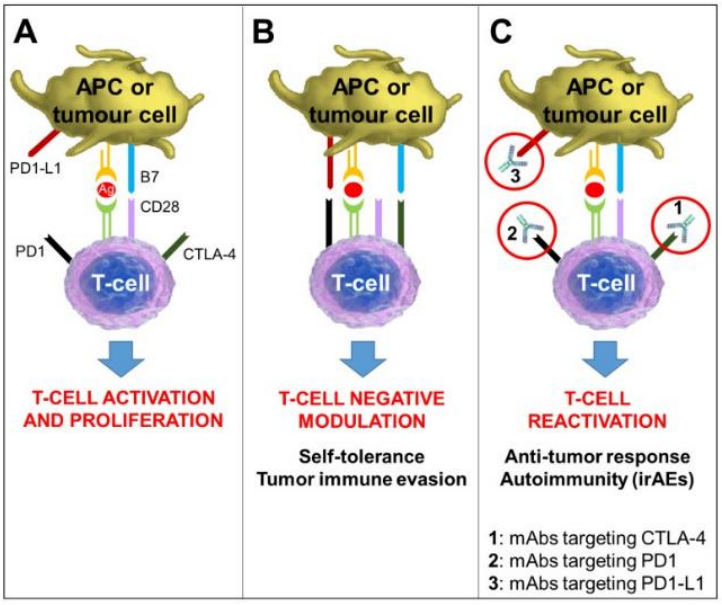

Figure 8: A) Normal T cell activation B) Tumour immune checkpoint modulation of T cells C) Restoration of T cell activation with immune checkpoint inhibitors (Prete et Salvatori)[146]

Blocking immune checkpoints to enable T cell activation through immune checkpoint inhibitors (Fig. 8) as monotherapy have failed to show significant improvements and even increased toxicity in multiple phase 1 and 2 trials for pancreatic adenocarcinoma.[147] [148]

Trials have undergone combining checkpoint inhibitors with chemotherapies such as ipilimumab, an anti-CTLA-4 mAb, with gemcitabine[149] or nivolumab, an anti-PD-1 mAb, with gemcitabine and nab-paclitaxel.[150] In both cases the addition of immune checkpoint inhibitor did not significantly impact response rates or overall survival. Nivolumab and ipilimumab are currently being investigated as combined treatment in metastatic PC (NCT04361162). There have been several studies reporting stable disease or partial responses in patients with Tremelimumab, an anti-CTLA-4 mAb,[151] and pembrolizumab, an anti-PD-L1 mAb,[152] respectively although these studies did not meet their primary endpoint efficacy criteria.

Winograd suggested the lack of efficacy of immune checkpoint inhibitors to be due to a lack of priming response of T cells and their efficacy can be restored through agonist αCD40 monoclonal antibodies with chemotherapy. Refractory tumours become highly sensitive to immune checkpoint inhibitors following αCD40 treatment which diminished the PD-1 phenotype of tumour-infiltrating lymphocytes.[153] Although minor progression has been seen using combinatory treatment protocols, further studies are warranted to improve the efficacy of checkpoint blockers for PDAC.

The exceptional long-term survivors of PDAC were linked to T cell immunity and neoantigens. These long-term survivors were not due to the quantity in neoantigens but rather the presence of high-quality and MUC16 neoantigens, therefore proposing tackling neoantigen-specific immunity as a way of treating tumours resistant to immune checkpoint blockade.[154] The immune deprived nature of the pancreatic cancer TME is a barrier to anticancer treatments including immunotherapies. CAFs not only participate in the formation of the fibrotic ECM creating a physical obstacle when reaching tumoral cells, but also secrete immunosuppressive molecules. For example, the secreted FAP protein has been shown to increase MDSCs and Treg cells, reduce NK cell cytotoxic activity[155] and its suppression increases the activity of immune checkpoint inhibitors.[156] [157] The heterogeneity of the stroma and the presence of immunosuppressive cells such as TAMs and MDSCs represent a challenge to tackle with immunotherapy treatments.[145]

- Tumour vaccines

Tumour vaccines consist in presenting tumour antigens to cytotoxic T lymphocytes thus triggering an adaptive immune response specifically targeted against the tumour. These vaccines can be produced from DNA, RNA, protein (antigen), dendritic cells or whole cancer cells. These antigens are either tumour-associated (TAAs) therefore expressed at a higher level on cancer cells but also present on normal cells, or tumour-specific (TSAs) in which case the antigens are exclusively found on neoplastic cells. The more specific these antigens are to the cancer cells, the safer the vaccine with TSAs presenting a significantly lower risk of self-tolerance and auto-immunity found with TAAs.[158] Neoantigens are patient-specific TSAs identified through next-generation sequencing and used for the generation of personalised cancer vaccines.[159]

Examples of peptide-based cancer vaccines include the KIF20A-66 and survivin-2B 80–88 (SVN-2B) peptides as TAAs. These peptides are human leucocyte antigen-A24-restricted antigens upregulated in PDAC. A phase 1/2 trial investigating the KIF20A-66 vaccine demonstrated better prognosis, with a disease control rate of 72% and mean survival time of 142 days, and induced peptide-specific response of CTLs in patients with metastatic pancreatic cancer.[160] Similarly a phase 1 trial of the SVN-2B peptide resulted in positive clinical and immunological responses in over 50% of patients.[161] Another example of peptide vaccine is the targeting of RAS. A 1996 phase 1/2 trial,[162] and more recently a 2001 phase 1/2 with intradermal injection[163] both demonstrated a specific immune response against the RAS peptide. Patients with resected PDAC however demonstrated no immunogenicity and no proven efficacy after KRAS vaccination.[164] Finally, OCV-C01 is a multipeptide cocktail vaccine containing KIF20A, vascular endothelial growth factor receptors (VEGFR) 1 and 2 antigenic peptides. A phase 2 study with this vaccine combined with gemcitabine resulted in an increase in disease-free survival (DFS) equal to 15.8 months compared to 13.5 months for control in patients with positive KIF20A expression compared to those without.[165]

Dendritic cells (DCs) are antigen-presenting cells (APCs) that can be activated with tumour antigens to subsequently promote T cell responses. To do so, these DCs migrate into draining lymph nodes which are readily present in the abdomen and peritoneum. Therefore using the

intraperitoneal route to administer DC vaccines could enhance the efficacy and inhibit metastases. In addition this technique could promote stronger therapeutic effects in patients with metastatic PC.[158] An example of DC vaccine uses autologous dendritic cells pulsed with three peptides, the human telomerase reverse transcriptase (hTERT, TERT572Y), the carcinoembryonic antigen (CEA; Cap1-6D), and survivin (SRV.A2). The vaccination was combined to the toll-like receptor (TLR)-3 agonist poly-ICLC and was found to induce tumour antigen-specific T cell population in patients with advanced PC.[166] When examining CTL responses following vaccination, Liang et al. demonstrated the significantly higher level of CTL responses with pulsed-DCs compared to unpulsed DCs after intraperitoneal injection in murine models.[167]

Whole cancer cell vaccines can be generated from either autologous or allogeneic cells. Algenpantucel-L is an allogeneic vaccine comprised of two PC cell lines, HAPa-1 and HAPa-2, which have been irradiated and genetically engineered. They have been transduced by a retrovirus to express the murine enzyme (1,3)-galactosyltransferase (α-GT) to activate a hyperacute rejection of the vaccine pancreatic cancer cell allograft. Naturally occurring human anti-alpha-gal antibodies bind to the murine epitope and induces antibody-dependent cell-mediated cytotoxicity (ADCC) towards these epitopes and consequently the endogenous pancreatic cancer cells. A phase 2 trial of Algenpantucel-L combined to gemcitabine and 5-FU improved both disease-free survival and overall survival[168] but the recently complete phase 3 study showed no improvement of survival in in patients with borderline resectable or locally advanced unresectable PDAC.[169]

A gene-based cancer vaccine is the GVAX vaccine, a DNA vaccine composed of autologous pancreatic cancer cells genetically modified to secrete the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) and irradiated to prevent further cell division. Latest results show that GVAX is safe and feasible to use as both adjuvant and neoadjuvant therapy with a better overall survival when combining GVAX to CRS-207 (6.1 months versus 3.9 months for GVAX alone), a second vaccine composed of live, attenuated *Listeria monocytogenes* expressing mesothelin delivered intravenously.[170] However, no improvement on patient survival when compared to standard chemotherapy[171] or by combining GVAX to immune checkpoint inhibitors such as Nivolumab[172] or Ipilimumab[173] has yet been shown but other combinations with immunotherapies are underway after signs of immunostimulation.

GI-4000 is a heat-killed recombinant *Saccharomyces cerevisiae* vaccine transfected with mutated forms of RAS so as to express these epitopes to stimulate dendritic cells. A phase 2 study involved administration of either gemcitabine or gemcitabine associated with GI-4000 in patients with mutations of *KRAS*. The group receiving the combination regimen presented higher median overall survival of 524 days compared to 444 days for the gemcitabine group. In addition, the GI-4000 group presented a significantly higher rate of mutation specific T cell response to RAS (47% compared to 8%).[174] Another heat-killed yeast vaccine is currently in phase 1 trial (NCT03552718) but this time genetically modified to express patient-specific neoantigen epitopes (YE-NEO-001).[175]

- Adoptive cell transfer

Adoptive cell transfer refers to the transfer of immune cells to a patient and, just as whole cancer cell vaccines, can either be autologous or allogeneic.

The most common example is the chimeric antigen receptor (CAR)-T cell therapy which involves obtaining T cells from a patient and genetically engineering them *ex vivo* to produce chimeric antigen receptors against tumour epitopes or antigens before reinjecting into the patient (Fig. 9). An interesting technique especially in haematological neoplasms, tisagenlecleucel (Kymriah) and axicabtagene ciloleucel (Yescarta) are EMA-approved therapies against B-cell acute lymphoblastic leukaemia (ALL) and large B-cell lymphoma respectively.

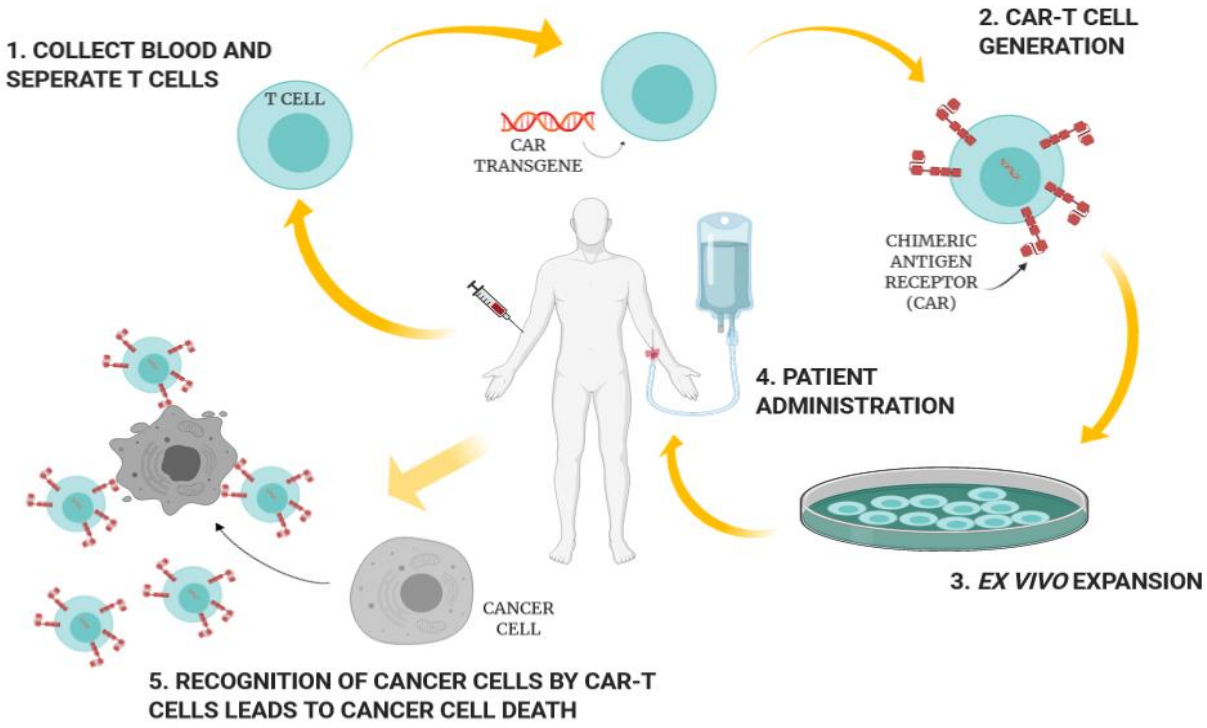

Figure 9: Construction of CAR T cells

The neoepitopes or antigens studied for pancreatic cancer CAR-T cells vary, those included in current or recently completed clinical trials are carcinoembryonic antigen (CEA), mesothelin (meso), mucin (MUC-1), KRAS (KRASG12V and KRASG12D), Claudin-18 (CLD18), CD133, receptor tyrosine kinase-like orphan receptor 2 (ROR2), epithelial cell adhesion molecule (EpCAM), CD70 and prostate stem cell antigen (PSCA). Preclinical studies focused on the mesothelin antigen has led to clinical trials of CAR-T cells. Anti-meso CAR-T cells were shown to have effective cytotoxic effect in vitro on pancreatic cancer cells and increased levels of TNF-α, IL-2, and IFN-γ immunogenic cytokines. These CAR-T cells managed to suppress the growth of PDX xenograft models of PDAC and resulted in an increase of CD4+ and CD8+ T cells both in peripheral blood and infiltrated in the tumour, as compared to mock treated mice.[176] More recently, studies involving CAR-T cells directed towards CEACAM7, which has a more restricted tissue expression compared to other CEA proteins, were cytotoxic for primary PDAC cultures. Moreover, anti-CEACAM7 CAR-T cells increased overall survival in a xenograft model compared to mutant CAR-T cell treatment, with three mice presenting complete regression of primary tumour and liver metastasis.[177]

An early phase I trial involves concomitant delivery of T cells, both armed with a murine-derived single chain antibody fragment (scFv), cluster of differentiation antigen 137 co-stimulatory domain (4-1BB) and cluster of differentiation antigen 3 zeta chain (CD3ζ) signalling domain transduced by lentivirus, targeting either mesothelin or CD19. On top of a mesothelin-targeted immune response (CARTmeso), this trial aims to deplete B cells, involved in the negative regulation of anti-tumour T cell immunity, with CD19-CAR T cells (CART19).[178] There are several disadvantages of using CAR-T cells which include the associated cost of production and the risks of under- or over-stimulation of the immune response depending on the neoepitopes expressed.

Other immune cells are also being investigated to express genetically-engineered CARs, including natural killer (NK), and macrophage (M) cells. Similarly to T lymphocytes, arming cytotoxic NK cells to target specific antigens (CAR-NK) has shown promising results in preclinical phases and in numerous clinical trials targeting both haematological and solid cancers. Only two clinical trials involve CAR-NK cells, both in phase 1/2 in China. The first is a bichimeric antigen receptor-natural killer cell produced with a lentivirus vector to express the Roundabout homolog 1 (ROBO-1) receptor, overexpressed in pancreatic tumours. ROBO-1 is a member of the axon guidance receptor family with a reported role in T cell chemotaxis modulation and tumour angiogenesis.[179] The other involves intravenous injection of peripheral NK cells (pNK) expressing a chimeric antigen specific for MUC-1 in patients with MUC-1 positive relapsed or regressive solid tumours such as PC.[180]

A recent phase I trial has begun involving CAR macrophages (CAR-M) for the treatment of solid tumours, including PC, overexpressing the HER2 receptor (CT-0508). The autologous macrophages in question are beforehand transduced using an adenoviral vector engineered to contain an anti-HER2 chimeric antigen and administered intravenously. Preclinical studies show the generation of a pro-inflammatory environment, an increase in anti-tumour T cell activity and recruitment of bystander immunosuppressive M2 macrophages into pro-inflammatory M1 macrophages.[181]

CAR cell therapy has encountered obstacles in its application to PDAC. Indeed, the dense stroma and abundance of immunosuppressive cytokines, such as IL-6, contribute to the suppressive function of cells, notably T cells.[182] Techniques in overcoming these obstacles are investigated, such as the use of heparanase to overcome the desmoplastic reaction of the TME, has improved antitumor activity and tumour infiltration of CAR-T cells in preclinical studies.[183] In addition to targeting tumour cells, CAR-T cells directed towards the FAP protein have been designed, although a preclinical study brought to light the associated cachexia and lethal bone toxicity.[184] Finally, after administration CAR-T cells can increase the expression of inhibitory PD-1 limiting their efficacy. To overcome this, CAR-T cells are being explored in combination with immune checkpoint inhibitors and in novel design strategies where PD-1 is genetically ablated[185] or PD-1/PD-L1 inhibitors are produced by the CAR-T cell.[186]

Gene therapies

Gene therapy uses genetic material to treat or cure diseases. These therapies are constituted in a transgene, which can be DNA, messenger RNA or interference RNA, and a vector, ranging from non-biological carriers to bacterial, viral and even yeast vectors. Gene therapy products include both gene therapy and cell therapies that utilise gene therapy to be modified (tumour vaccines and CAR-T cells) (Fig. 10). The generation of CAR cells uses retroviral vectors such as gammaretrovirus or a lentiviral vector to transduce the patient's T lymphocytes with the transgene encoding the engineered chimeric antigen receptor.

Other strategies of gene therapy are being developed in oncology and applied to pancreatic adenocarcinoma, such as gene silencing to inhibit oncogenes, chemosensitising agents and oncolytic viruses (Fig. 10). In addition, viruses have been exploited to assure the delivery of certain compounds to specific targets. In such cases the virus can act purely as a means of transport for the transgene in question, or can have in itself antitumoral capacities, as is the case with certain oncolytic viruses.

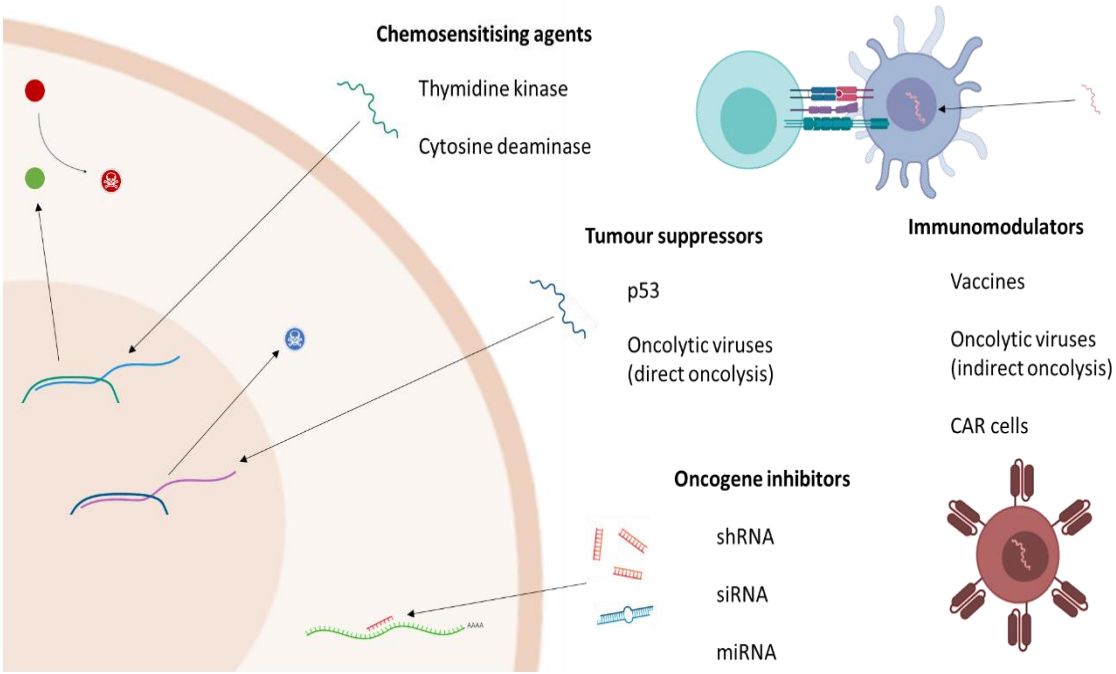

Figure 10: Applications of gene therapy in oncology

- Vaccines

GVAX, CRs-207 and GI-4000 are examples of tumour vaccines that utilise gene therapy to genetically modify the cells or microorganism in order to induce and enhance antitumour activity. Another gene therapy vaccine is the HyperAcute vaccine (algenpantucel-L) investigated in a phase 3 trial that ended in 2016 and is now in a long-term follow-up study over the next 15 years. (NCT03165188)[187]. The Hyperacute vaccine consists in intradermally-administered irradiated allogeneic pancreatic cancer cells transfected to express the murine gene encoding Alpha-(1,3) galactosyltransferase. Murine alpha-gal epitopes induce a hyperacute rejection of the cancer cell allograft resulting in the rapid activation of antibody-dependent cell-mediated cytotoxicity (ADCC) towards allograft cells and, in consequence, endogenous pancreatic cancer cells. The murine alpha-gal gene is transfected in allogeneic pancreatic cancer cells by means of a retroviral vector. The phase 3 trial did not show improvement in patients with borderline resectable or locally advanced unresectable PDAC compared to patients receiving the HyperAcute vaccine compared to standard of care consisting in chemotherapy (FOLFIRINOX or gemcitabine/nab-paclitaxel) followed by chemoradiation.[188]

- Gene silencing

Oncogene inhibitors are interference RNA used to silence genes associated with PC. siG12D-LODER is a small-interfering RNA (siRNA) against the mutated *KRAS* in a biodegradable polymeric matrix. This gene therapy is currently studied in a phase 2 trial combined to either gemcitabine and nab-paclitaxel or mFOLFIRINOX following a phase 1/2 trial demonstrating the tolerability and safety with an overall survival of 15.12 months and signs of stable disease as well as partial responses..[189]

Gene silencing can also be indirect for cell therapy. This is the case for the phase I/II trial of intravenous genetically modified tumour-infiltrating lymphocytes (TILs), in association with chemotherapy and recombinant IL2, indicated for gastrointestinal cancers (NCT04426669). The intracellular immune checkpoint – cytokine inducible SH-2-Containing Protein (CISH) – of these TILs has been inhibited using the CRISPR gene editing technique. CISH is an intrinsic signalling regulator of TCRs and so through its inhibition, could increase the anti-tumour activity of therapeutic T cells.[190] Preclinical studies have shown that *CISH* knockout (KO) enhanced T cell expansion and function but not maturation. In addition, CISH deletion upregulated PD-1 expression and when combined with anti-PD-1, slowed tumour growth in mice model significantly compared to anti-PD-1 alone.[191]

- Chemosensitisers

Chemosensitising agents potentialize antitumoral efficacy of chemotherapies. Typical examples include cytosine deaminase (yCD) from *Escherichia coli* that converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) which is then taken up by cellular enzymes and transformed into cytotoxic antimetabolites. Another is a mutant form of HSV-1 thymidine kinase (HSV-TK) which converts ganciclovir (GCV) to ganciclovir monophosphate, further converted to ganciclovir triphosphate by the cancer cells' enzymes. Ganciclovir triphosphate is found to delay proliferation processes in cancer cells, provoking apoptosis.[192]

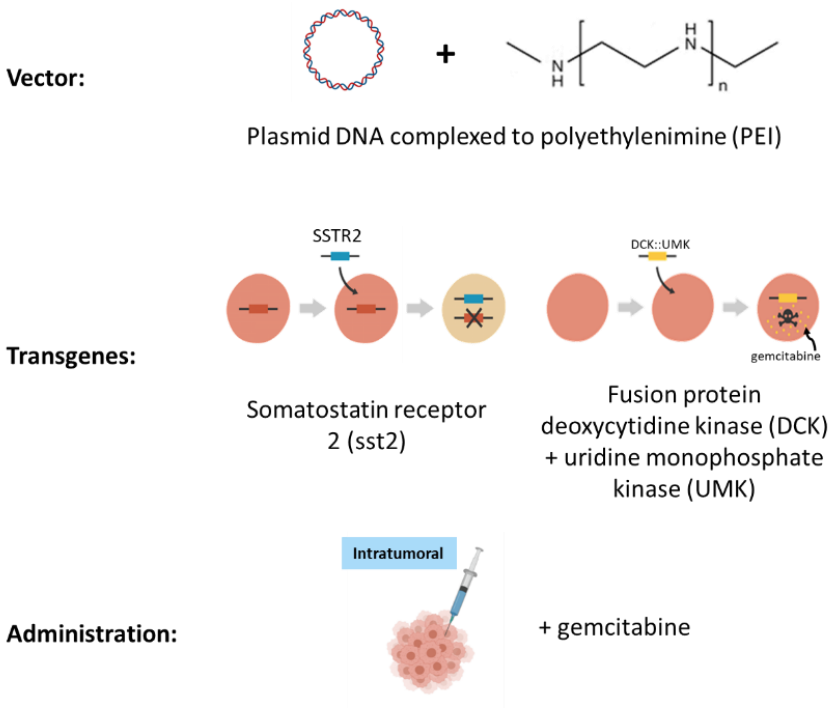

Figure 11: Construction of TherGAP chemosensitising agent

Following research from our team, a phase II trial combined the CYL-02 DNA plasmid and gemcitabine (TherGAP) by intratumoral injection, guided by endoscopy. CYL-02 plasmid encodes the mouse somatostatin receptor subtype 2 (sst2) and the fusion protein of human deoxycytidine kinase (DCK) and uridine monophosphate kinase (UMK) complexed to a synthetic polyethylenimine carrier (Fig. 11). Expression of the DCK::UMK fusion protein converts gemcitabine into its toxic metabolite. Expression of the sst2 protein – whose gene expression is often lost in pancreatic and colorectal cancers and negatively regulating multiple processes such as epithelial cell proliferation – is believed to induce both anti-oncogenic and local antitumor bystander effects. Combining the two allows for a lower dose of gemcitabine to cause tumour cell lysis. Results of phase I showed safety and feasibility with disease stability in patients and identification of biomarkers predictive of response to treatment.[193]

There are limits in the use of non-viral delivery including the low rate of genetic transfer into target cells. Furthermore, and similarly to most treatments, these therapies only target a single deficiency in tumoral cells. However, as previously described, PDAC tumours are both cellular and molecular heterogeneous and comprised of immunosuppressive TME which has warranted

the use of combinatorial protocols to increase efficacy and clinical response. Oncolytic viruses have the potential of both direct oncolysis, by targeting cancerous cells, and indirect effect by stimulating the immune system. Therefore, they are a promising category of treatment to overcome the limits presented by PC, either as monotherapy or combined with diverse other therapies.[194]

- Viral vectors

In addition to possible antineoplastic properties, viruses can be used as platforms expressing transgenes that are to be delivered to the tumour. These vectors can be either integrating or non-integrating depending on whether the transgene is incorporated into the genome of the host cell upon delivery.[195] The choice of vector will depend on various factors, such as the virus' tropism (dividing or non-dividing cells), the packaging capacity, the duration of transgene expression and the potential immune response triggered against the virus when delivered in vivo. Furthermore, viral vectors can either be replication deficient, replication competent or replication-conditional.[196]

A clinically-tested viral vector encoding an immunotherapy is TNFerade combined with 5-FU and radiation therapy for the treatment of unresectable locally advanced PDAC. TNFerade consists in a second-generation replication-deficient adenoviral vector transporting TNFα cDNA as transgene and injected directly into the tumour. Following a well-tolerated response and dose-dependent increase in the stabilization of treated tumours, phase III results however failed to be effective in prolonging survival in patients despite being safe[197].

Viruses

History

Viruses are sub-microscopic entities capable of infecting a wide range of organisms including animal and plant cells, other microorganisms such as bacteria and archaea. They are infectious agents that replicate strictly inside other cells or organisms. Viruses were first discovered in 1892 by Russian botanist Dmitri Ivanovski when identifying a disease of tobacco plants to be

caused by an extremely miniscule infectious agent but thought it to be unculturable bacterium.[198] Similarly, in 1989, a Dutch botanist and microbiologist Martinus Beijerinck found tobacco mosaic disease to be caused by an organism much smaller than bacteria, defining the term "virus",[199] although he was convinced this virus was in liquid form (*contagium vivum fluidum*). The particulate nature of viruses was exposed later, in 1935 when the tobacco mosaic virus (TMV) was first crystallised[200] and confirmed through electron microscopic images in 1939.[201]

Structure and classifications

Viruses differ between themselves when it comes to structure, replication, transmission and infectivity. Indeed, viral genome can either be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) in nature, single- or double-stranded, and either linear or circular. Genome size varies between two kilobases which code for two proteins for the *Circoviridae*, to two megabases coding for around 2500 proteins for the pandoraviruses. Certain viruses undergo genetic mutations, whether they are silent point mutations called antigenic drift, or larger recombinations or reassortments called antigenic shifts such as with the influenza virus.

The viral replication cycle in grossly divided into six steps: attachment, penetration or viral entry, uncoating, replication, assemble and release.[202] Viral replication occurs in the nucleus or the cytoplasm of the host cell depending on whether the virus possesses the machinery necessary for replication or needs to appropriate the cell's supply.

A viral particle or 'virion' is composed of genetic material associated with proteins (nucleoproteins) surrounded by a coat of protein named capsid, altogether forming the nucleocapsid. Depending on the morphology, viruses can be helical, icosahedral, prolate, enveloped – obtained from cell membranes - or complex. The latter have a capsid that is neither helical nor icosahedral and may possess additional structures, seen with certain bacteriophages.

Classification of viruses is accomplished by the concomitant use of the International Committee on Taxonomy of Viruses (ICTV) and Baltimore classifications since some viral species were unaccounted for in the ICTV classification.[203]

Since 2020, the ICTV classifies viruses according to the following sequence: Realm (*-viria*), Subrealm (*-vira*), Kingdom (*-virae*), Subkingdom (*-virites*), Phylum (*-viricota*), Subphylum (*-viricotina*), Class (*-viricetes*), Subclass (*-viricetidae*), Order (*-virales*), Suborder (*-virineae*), Family (*-viridae*), Subfamily (*-virinae*), Genus (*-virus*), Subgenus (*-virus*), Species.[204] The Baltimore classification categorises on the genetic material and the intermediates found throughout the viral replication cycle:

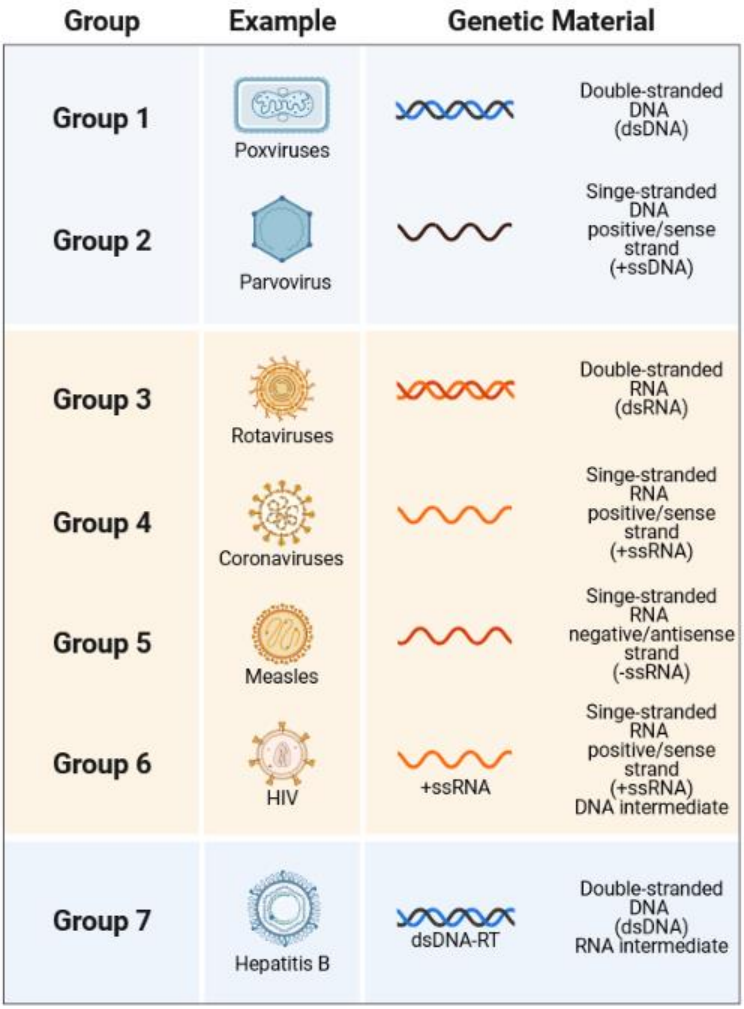

Table 3: Baltimore classification of viruses

Viruses in cancer

Although discovered just over a hundred years ago, viruses have been responsible for a plethora of pathologies, ranging from common human diseases such as the common cold,

influenza and chickenpox, to devastating epidemics and pandemics. In addition, certain strains of viruses were found guilty of inducing cancers. There are seven recognised human oncoviruses which include the Human papillomavirus (HPV) which causes cervical cancer, the Epstein–Barr virus (EBV) responsible for Burkitt lymphoma, Hepatitis B (HBV) and Hepatitis C (HCV) viruses linked to hepatocellular carcinoma (HCC), the Human T cell leukemia/lymphoma virus type 1 implicated in T cell lymphoma and finally the Human Herpesvirus-8 (HHV-8) associated with Kaposi sarcoma.[205] In the 1940/50's however, the biotechnology field of virotherapy arose in which viruses are used as therapeutics agents.

The idea of treating cancer patients with replicating viruses comes from observed clinical tumour regression after natural virus infections.[206] During the nineteenth century, patients suffering from cancer were found to be in clinical remission after having contracted an infectious disease. For example, remission periods were described in leukemic patients after simultaneously being infected with influenza, chickenpox or measles viruses.[207] Although research in virotherapy was promising in the mid-twentieth century, several limits to oncolytic viruses were encountered including uncontrolled infection or an induced immune response towards the virus. After a period of declined interest in OVs, renewed attention has been brought on these innovative therapies owing to the advances in technologies used to genetically modify viruses.

Oncolytic viruses

General points

Oncolytic viruses are defined as infecting cancer cells specifically to induce cell death while sparing healthy cells (Fig. 12). These viruses can be either naturally occurring or genetically modified and, contrary to other gene therapy products that use viral vectors, the virus is in itself an active drug reagent.[208]

Depending on the viral strain, the selectivity between cancerous and healthy cells can either be done at the entry level of the viral cycle or the replication stage. Indeed, certain viruses will

present a tropism of cancerous cells and therefore will only be able to enter in these cells, whereas other viruses are able to enter all cells but will only be able to replicate in tumour cells. Once inside the cancer host cell, OVs can bypass the cell's machinery to insure their replication. Through several mechanisms that are dependent on both the virus and the cell, OVs can induce cell death of the cancer cell (direct oncolysis). In certain cases, the lysis of these cells will liberate TAAs, TSAs and neoantigens that can activate the immune system towards an anti-tumour reaction (indirect oncolysis).

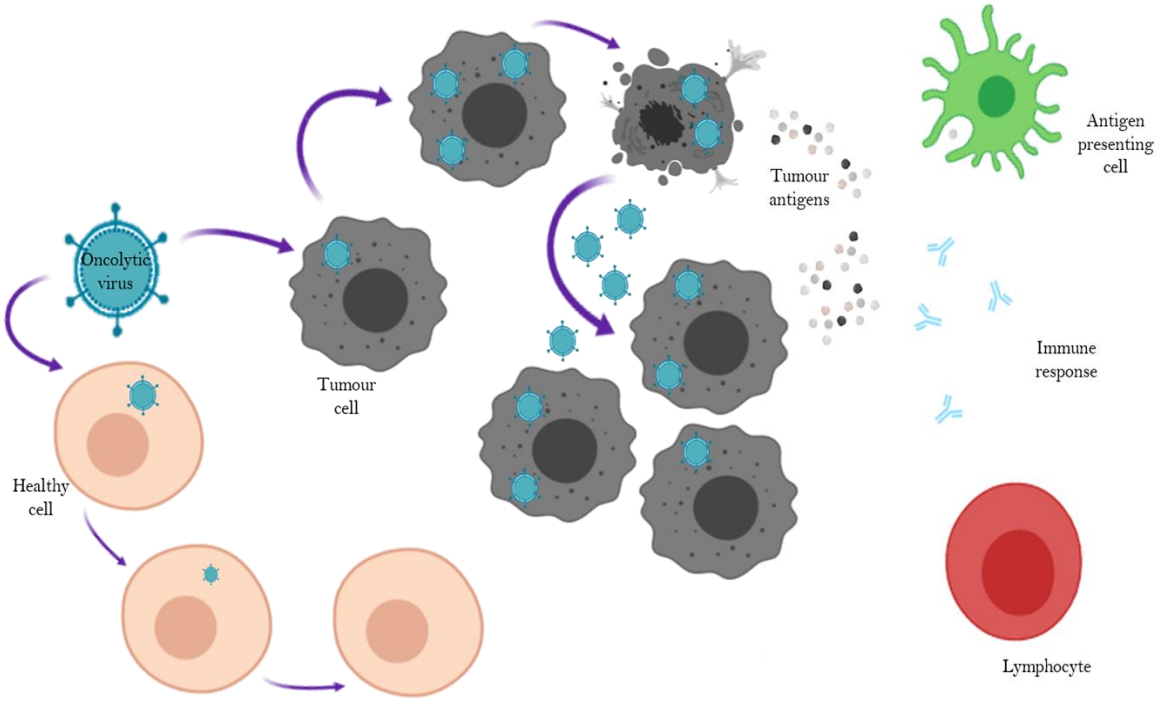

Figure 12: Oncolytic viruses' mechanism of action

Direct oncolysis

The targeting of cancer cells by oncolytic viruses can be done by exploiting their natural tropism. Indeed, most viruses utilise cell receptors for viral entry and some of these cell surface molecules are upregulated in tumour cells. For example, herpesviruses use cell surface receptors such as HVEM, nectin-1, and nectin-2 for infection, receptors that are found to be overly expressed in multiple tumour types, rendering these cells more susceptible to talimogene laherparepvec (T-vec).[209]

OVs are able to replicate in tumour cells since the cellular anti-viral pathways are downregulated. In normal cells viral replication is abrogated by activated antiviral cellular pathways such as type 1 interferon (IFN). Type 1 IFNs are expressed and secreted where they can bind to receptors as well as enter a positive feedback loop to amplify type 1 IFN expression. Through the IFN pathway, transcription factors are transported into the nucleus to allow for the expression of interferon-stimulated genes (*ISG*). These will consequentially turn the infected cell by reprogramming the physiology into an antiviral state. This state includes a reduced metabolism, activation of pro-apoptotic pathways, blocking cell cycle progression and disrupt angiogenesis to impede viral spread. Various antiviral effectors have been described that result from *ISG* expression and that can target different stages of the viral replication cycle.[210] For example, the myxovirus resistance 1 (Mx1) gene product will trap incoming viral components such as the nucleocapsid therefore hindering viral entry. Other IFN-induced antiviral effectors will target virus translation and replication as well as viral egress.

Cancer cells, however, commonly have a defective IFN response pathway, either because of defective IFN receptors or by low expression or defective phosphorylation of downstream proteins, allowing proliferative and survival advantages as well as non-responding to antiproliferative and antiangiogenic signalling. Viruses are therefore not blocked to replicate in these cells as they are in normal cells.[211]

To replicate viruses are capable of seizing control of the cell's machinery and manipulate the translation control pathways for their own needs.[212] Through carcinogenesis cells deregulate biological processes identified as 'hallmarks of cancer' which include sustained proliferation, replicative immunity, induced angiogenesis, invasion and metastasis.[213] [214] These attributes help tumour malignancy and progression and are exploited by viruses which make cancer cells more susceptible to viral infections.

For example, the tumour suppressor genes *p53* and retinoblastoma (*RB*), frequently mutated and disrupted in cancer, are gatekeeper genes to control viral infections. Certain viruses, such as HCV and HPV have mechanisms that attenuate these genes[215] whereas other viruses including reovirus and myxoma virus have been shown to preferentially replicate in cells lacking

functional p53, ataxia telangiectasia mutated (ATM) or RB.[216] Furthermore, p53 and RB regulate antiviral response pathways in addition to their tumour suppressor functions, further lowering a cancer's cell defence against viral replication.[217]

By blocking the cell's antiviral response and promoting virus replication, cancer cells evolve a high sensitivity to viral infections. During replication, viruses will affect cellular pathways to their own benefit and will trigger cell lysis and liberate virions that will propagate and infect surrounding tumour cells. The parvovirus H-1 (H-1PV) is currently studied in PDAC and glioblastoma, tumours in which the apoptotic pathway is dysregulated. H-1PV is able to redirect tumour cell death through the cathepsin-mediated pathway, additionally deemed to be immunogenic.[218] [219] Bcl-xl, an antiapoptotic protein is overexpressed in certain cancers, conferring chemoresistance but is exploited by Newcastles' disease virus (NDV). Indeed, Bcl-xl overexpressing cells assured an increased replication and fusogenicity of cells amongst them, compared to control cells. Oncolysis was still mediated by apoptosis, most likely through alternative pathways, but was delayed suggesting NDV utilises the antiapoptotic state of cells to further its oncolytic activity.[220]

Indirect oncolysis

Although used as single-agent therapies on tumour cells, oncolytic viruses are able to act on the immune system, engaging an antitumoral response involving the priming of T cells by tumour antigens, the infiltration and circumventing of immunosuppressive pressure and the indirect oncolysis of tumour cells. Through tumour cell lysis, oncolytic viruses are able to liberate TAAs and TSAs that can be taken up by APCs and presented to T cells (known as T cell priming). Moreover, oncolytic viruses act as in situ vaccines with the simultaneous release of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), known as immunogenic cell death, contributing to the pro-immune milieu. In some cases, the magnitude of T cell response is insufficient to provide an adequate antitumoral immune response, particularly in heterogeneous tumours such as PDAC. This can be overcome by genetically-engineering antigens to be expressed by the OV, although this necessitates prior knowledge of tumour antigens whereas in situ OV vaccination does not.[221]

The TME can also be targeted by OVs. For example, Vaccinia virus (VV) and Vesicular stomatitis virus (VSV) also cause cell death in uninfected cells. In the case of VSV this is due to a loss of blood flow inside the tumour, induced by the recruitment of neutrophils, with activated 'rigid' neutrophils blocking the fragile tumour vasculature, and causing intra-tumoral cell death.[222] In addition, VEGF contributes to the immunosuppressive phenotype of the TME and its inhibition enhances immune infiltration as well as systemic delivery of therapeutics.[223] These findings warrant the combination of OVs with antiangiogenic inhibitors in a sequential protocol, blocking VEGF after OV delivery.[224]

Furthermore, CAFs also display an increased sensitivity to oncolytic virus infection.[225] This can be seen, for example, with tumour cells co-cultured with CAFs and infected by VSV or VV, presenting higher viral replication than in tumour cells alone. Indeed, fibroblast growth factor 2 (FGF2) produced by cancer-associated fibroblasts is increased in tumours and impedes antiviral responses, increasing viral infectivity. Interestingly, an engineered Maraba virus to encode the human *FGF2* gene increased the reduction in tumour burden when compared to parental strain, showing the possible advantages of reciprocal TME crosstalk to oncolytic virotherapy.

T cell infiltration within the tumour is a limiting step, especially in tumours such as PDAC, and more so in desmoplastic PDAC, that present a physical stromal barrier. A method to circumvent this is with viruses' ability to attract neutrophils which produce inflammatory mediators and cytotoxic proteases that degrade ECM.[222] VSV is, in addition, able to infect CAFs promoted by the secretion of transforming growth factor beta (TGF-β) by tumour cells, further disrupting production of extracellular matrix.[226] OVs can further disrupt the stromal environment to increase CTL infiltration by being genetically engineered to express ECM-degrading enzymes. Such is the case of VCN-01, a replication-competent adenovirus encoding the human glycosylphosphatidylinositol-anchored PH20 hyaluronidase. Preclinical studies have shown safety and selectivity of the virus and significant antitumour activity when administered both systemically and intratumorally, with reduced tumour growth and increased survival. Additionally, the hyaluronic acid content of treated tumours was notably decreased, increasing OV efficacy and endorsing the possibility of combination with chemotherapies. The results of a phase 1 clinical trial are yet to be presented.[227]

Although the tumour environment has proven to play a role in oncolytic virus replication, the full extent of its influence and the actors in play can be improved by understanding fully the activities of the different TME elements as well as deciphering the complex crosstalk between them. The identification of new actors and targets could not only shed light on influencers of OV therapy but also help identify patients that would most likely benefit from virotherapy and pave the way to treatment combinations.[217]

Combinations are currently studied notably with immune checkpoint inhibitors since after OV infection and recruitment of immune cells, immune checkpoints are eventually triggered to limit inflammation. Therefore, through the induction of tumour responses to OV infection, turning them from 'cold' to 'hot' increases their susceptibility to checkpoint blockade immunotherapies.[228] Examples of combinations currently studied in PDAC clinical trials include the phase 1b with reovirus (Reolysin) and pembrolizumab showed viral immunomodulation and is examined further in a phase 2 study.[229] A thymidine kinase deleted vaccinia virus expressing the GM-CSF factor (pexastimogene devacirepvec) is studied combined with ipilimumab in a phase 1 trial targeting multiple solid tumours.[230] In addition, preclinical studies are ongoing with a plethora of OVs and ICIs, such as a novel recombinant myxoma virus (vPD1) designed to secrete a soluble form of PD-1 from host cells following viral infection and replication showing an increase in tumour eradication in melanoma murine model.[231] The next step in OV combination is to evaluate the benefits when associated with adoptive T cell treatments.[221]

Viral tropism

The use of viruses as oncolytic actors is due to their preferred infectivity of tumour cells over normal cells. This selectivity is due to the virus' tropism, which can be naturally inherent to the viral strain, or engineered. There are several manners in which viral tropism has been modified so as to increase selectivity of neoplastic cells.

One way of moulding OV tropism is by surrounding the virus with a polymer cloak and the addition of a target for a specific receptor. An example is the EGF polymer-coated adenovirus

by Morrison et al. used to reduce the wide range tropism of adenoviruses in ovarian cancer.[232]

Tropism can be directed by using engineered antibodies. Watkins et al. bound the single-chain variable fragment (scFv) of the antibody to an adenoviral fibre protein and EGF, facilitating the targeting of EGFR-expressing cells by the virus.[233] More recently, the scFv fragment saw incorporated in the envelope of the human simplex virus (HSV) linked to glycoproteins used by HSV for cell entry. This concoction targeted cell entry and subsequent oncolysis to specific cells, allowing for the systemic delivery of engineered HSV.[234]

PeptiCRAd is an adenovirus vaccine platform transporting tumour MHC-I-restricted peptides although not encoded within the viral genome. These epitopes are adsorbed onto the viral capsid, omitting the need for chemical or genetic modification of the virus and with the possibility of targeting multiple antigenic entities. All the while retaining its infectivity, PeptiCRAd showed efficient antitumour activity and expansion of an epitope-specific CD8+ T cell population.[235]

Viral genes can be put under the control of modified promoters to influence viral behaviour. Promoters that are abundantly activated in tumour cells are used to increase viral replication in these cells while sparing healthy cells. An example is the use of the cyclin E promoter to target adenoviral replication to tumoral cells.[236] Moreover, promoters that are downregulated in tumour cells are also used but for gene regulation since they allow for a lower level of infection all the while retaining oncolytic activity, measuring up to a safer viral profile.[237]

Specificity of oncolytic viruses to infect tumour cells can be controlled through viral gene regulations by microRNAs. MicroRNA levels vary between healthy and cancerous cells, therefore by placing critical viral genes under the control of miRNAs that are weakly expressed in tumour cells, the OV will not be able to replicate in healthy cells with normal levels of the miRNA. For example, vaccinia virus' *B5R* gene codes for a glycoprotein involved in virus morphogenesis, trafficking, and dissemination. To increase specificity of VV to tumour cells, B5R deletion was tested, but resulted in decreased oncolytic activity. The insertion of a complementary target sequence for miRNA let-7a, that has lower expression in many kinds of

cancer cells than in normal cells, in the promoter region of B5R. This allowed the vaccinia virus to selectively infect tumour cells and induce oncolysis while sparing normal cells.[237]

Engineering viruses

Many methods have been developed to increase oncolytic viruses' tumoral delivery and oncolytic activity. To improve OVs requires a good understanding of the molecular interactions between the virus, the tumour and the immune system as well as the advantages between one route of administration versus the others. These characteristics are also used to first and foremost define the virus chosen according to the pathological setting.

Specificity of tumoral cells can also be increased through gene deletion. Thymidine kinase (TK) is an enzyme encoded by vaccinia virus (VV) and is essential for viral replication in the cytoplasm of host cells, assuring phosphorylation of thymidine before incorporation in newly synthesised DNA. By the deletion of this gene, VV replication becomes dependent on the cell's TK, so is no longer possible to assure viral replication in healthy cells but is possible in tumoral cells that present high levels of thymidine kinase.[238] Another way of increasing specificity is used with adenoviruses by deleting genes involved in inhibiting apoptosis. The E1B-19KD adenoviral protein is a Bcl2 homologue that blocks TNF-α mediated apoptosis induction allowing efficient viral replication. Therefore, in the presence of TNF-α, replication of E1B-19 kDa mutant adenovirus in normal cells was reduced secondary to rapid apoptosis induction whereas TNF-α had limited effects on virus replication in tumour cells due to blocks at multiple levels in apoptosis pathways (e.g., p53 mutation, Bcl-2 overexpression).[239]

Gene deletion can also be used to increase oncolytic efficiency. For example adenoviruses were engineered to better infect mesenchymal stem cells (MSC), increasing shielded delivery and subsequent infection of tumour cells.[240] Another example is the HSV JS1 strain (the backbone of T-VEC) with deleted genes that interact with PCNA or downregulate PKR allowing for viral replication. Since tumour cells undergo aberrant division and frequently downregulate PKR, this engineered herpesvirus can effectively replicate.[241]

Modifications of OVs can be achieved through gene insertion paving the way to using them as vectors. A common example is by inserting a suicide gene within the viral genome such as E. coli cytidine deaminase or HSV's thymidine kinase. Arming these OVs with suicide genes in addition to the viruses' ability to induce cell death, increases the efficiency of direct tumour cell lysis. Other genes that have been inserted to increase efficiency include those that express cell death molecules. For example, TNF-related apoptosis-inducing ligand (TRAIL) has been associated with both apoptosis and necroptosis and expressed by modified adenoviruses.[242] [243]

Gene insertion is also used as a means of increasing antitumour immunity. OVs such as T-vec, adenoviruses and VV have been engineered to express GM-CSF in order to increase recruitment of APCs, presentation of tumour antigens and proliferation and maturation of immune cells.[244] Genes coding for cytokines, and other immune stimulatory molecules have also been studied as additions to viral genomes to promote the stimulation of the immune system and establishment of an adaptive antitumour response. These include heat shock protein 70 (HSP70), which allows for more peptides to be seen by APCs, and interleukin 12 (IL-12), involved in T cell differentiation.[245] [246]

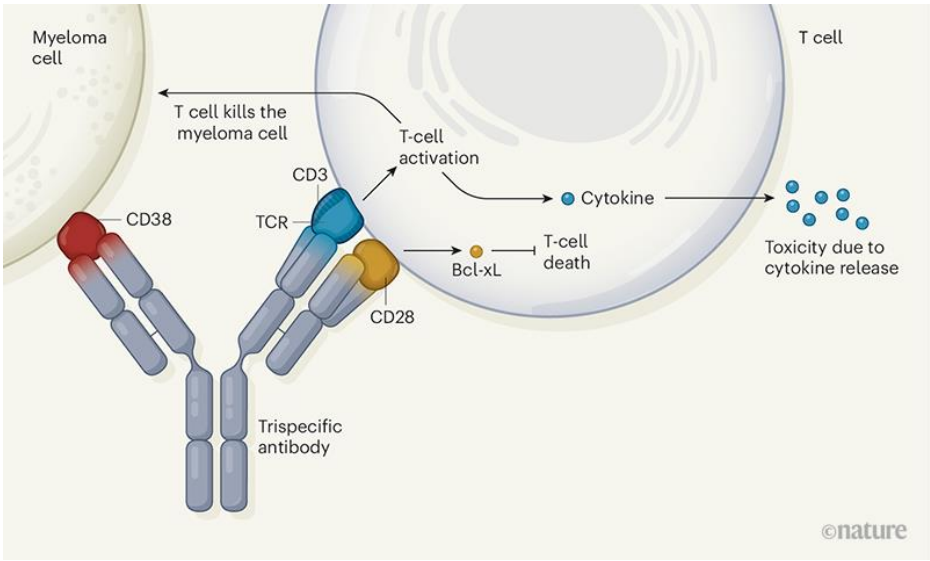

Figure 13: Example of a trispecific antibody (TriTE) (Garfall et June)[247]

More recent immunotherapeutic transgenes include bi- and tri-specific T cell engager (BiTE and TriTE). These are recombinant proteins that possess two or three single-chain fragment variables (scFvs) creating a link between T cells and tumour cells (Fig. 13).[248] The vaccinia virus has been used to encode a BiTE recognising CD3 receptor on T cells and the tumour cell surface antigen EphA2. The modified VV induce T cell activation and additional lysis of bystander tumour cells.[249]

The development of multi-armed OVs with complex transgenes has brought to light the necessity of regulating gene expression dynamics that may not follow those of viral replication. For instance, the optimal expression of immunotherapies would be once maximal viral spread is achieved, to avoid a premature activation of the immune response. For such cases, viruses including poxviruses that are tightly regulated with gene expression divided into early, intermediate and late, are of particular interest.[221]

In addition of increasing the safety or efficacy of oncolytic viruses, genetic modifications can be used to potentialize concomitant radiotherapy. The sodium-iodide symporter (NIS) gene causes the infected cells to express this cotransporter and accumulate iodine. It is used in combination with radioiodine to treat thyroid cancers.[250] In addition, the NIS transporter has been applied to the theranostic field of imaging OV distribution, replication and elimination using a gamma camera applied to adenoviruses, vaccinia virus and measles virus.[251] [252] [253]

Genetic engineering of oncolytic viruses has helped develop this therapy as competent anti-cancer treatment options by increasing both their safety and efficacy. However the balance between the two is important to keep in mind. For example, the deletion of three genes in vaccinia virus has led to better tumour selectivity and accordingly improved safety, but also weakened oncolytic potency in ovarian carcinomatosis models.[254] This illustrates the importance of selecting the correct oncolytic virus and modifications in specific pathophysiology settings.

Viruses studied as clinical cancer treatments

A variety of viruses have been doted with oncolytic properties and are currently being investigated as potential therapies for diverse solid tumours (Table 4).[246] These include the herpes simplex virus (HSV), adenoviruses (Ad) and vaccinia virus (VV) as well as reovirus and parvovirus, amongst others.

	DNA	RNA	
		Positive strand	Negative strand
Single-stranded	- Parvovirus (MVM, LuIII, H-1PV)	- PVSRIPO derived from Polio virus - Coxsackie virus - Toca 511 derived from amphotropic murine leukemia virus - Zika virus	- Measles virus (MeV) - Influenza virus - Vesicular stomatitis virus (VSV) - Newcastle virus (NDV) - Mumps virus - Maraba virus
Double-stranded	- Poxvirus (VACV, MYXV) - Herpes Simplex virus (HSV) - Adenovirus (Ad)	- Reovirus	

Table 4: Oncolytic viruses currently investigated

Adenoviruses have been extensively studied and modified as both an oncolytic virus and a vector for other gene therapies due to their high transfection efficiency and low risk of insertional mutagenesis. Out of the 57 serotypes of Ad the most used as virotherapeutic is serotype 5 (Ad5) and has been widely investigated through modifications to increase both specificity and efficacy, such is the case of ONYX-015 and Oncorine H101.[255]

Multiple oncolytic adenoviruses are currently studied in clinical trials, but the first OV in clinical trials is the ONYX-015 strain an adenovirus engineered to remove the *E1B* gene to allow for replication in p53-mutated tumour cells. Clinical trials combining ONYX-015 with chemotherapy in head and neck cancer showed it to be safe and cancer selective, but the therapeutic effects and biodistribution were limited after systemic injection.[256] Oncorine (H101) similar to ONYX-015 also presents a deletion of the *E1B* gene to preferentially infect cancer cells.

Oncorine was approved by the Chinese State Food and Drug Administration in 2005 for nasopharyngeal cancer.[257]

Another Ad in clinical trials is Enadenotucirev (ColoAd1), a chimeric Ad11p/Ad3 chimeric replication-selective adenovirus with a deletion in the Ad11p *E3*-region and *E4*-domain in addition to a partial *E2B* substitution by the Ad3 *E2B* genes. Designated an orphan medicine in 2015 by the EMA for ovarian cancer, the vaccine OV is currently investigated in colorectal, epithelial and head and neck cancer, amongst others.[258] [259] Enadenotucirev has been shown to be active against a range of human carcinoma cell lines, achieving lysis more rapidly than the wild-type adenoviral strains while activity in normal cells is attenuated.[260]

HSV was one of the first viruses to be studied as an anticancer therapy. Mutant forms of HSV-1 including G207, HSV1716, NV1020 and Talimogene laherparepvec lack viral genes making them unable to replicate in terminally differentiated and non-dividing cells and as such improving their safety. These mutant forms of HSV have been studied in glioblastoma multiforme, melanoma and squamous carcinoma amongst others.[261] [262] [263]

Talimogene laherparepvec (T-vec) is a second generation HSV based on the JS1 strain and genetically modified to express GM-CSF. T-vec successfully completed a phase 3 trial in 2013 for melanoma and obtained FDA followed by EMA approval in 2015 (Imlygic), making it the first clinically approved oncolytic virus in the western world. T-vec is now being studied in early-stage clinical trials for pancreatic cancer, soft-tissue sarcoma, and head and neck squamous-cell carcinoma.[264] [265]

Vaccinia virus is a naturally oncolytic virus due to its sensitivity to type 1 interferon. Many characteristics makes it interesting as an anti-cancer agent, including its short life cycle and its ability to replicate even in hypoxic conditions.[266] Furthermore, VV does not have a specific receptor for viral entry, allowing its potential use in multiple tumours, and replicates in the cytoplasm of host cells avoiding the risk of genomic integration.[267] Certain deletions have been tested to increase specificity to tumour cells, including the *TK* gene (similarly to HSV) and vaccinia growth factors (VGF).[268] Moreover, many genes have been inserted within the VV genome, for example JX-594 a *TK*-deleted VV encoding the GM-CSF gene (Pexa-Vec) which was

investigated in a phase 3 clinical trial for advanced hepatocellular carcinoma associated with sorafenib (NCT02562755). The trial however failed an interim futility analysis, deemed unlikely to meet primary overall survival objective, and recruitment was prematurely stopped. Since VV is derived from the vaccinal strain to treat smallpox, an issue regarding its oncolytic potential is the recognition by the immune system. Breitbach et al. however, have been able to demonstrate an effective infection and expression of transgene in tumour cells after systemic administration of the JX-594 strain.[269]

Oncolytic viruses in pancreatic cancer

Given the poor efficiency of current treatments for PDAC, oncolytic virotherapy is seen as a promising field as a therapy option. Several different viruses are currently being studied, with some being investigated in clinical trials.[265]

Although serotype 5 of adenoviruses are commonly used in anticancer treatment research, their natural tropism of respiratory tissue makes them inadequate in gastrointestinal neoplasms. Whilst techniques have evolved to expand and modify viral tropism, a number of serotypes including Ad12, Ad40, Ad41 and Ad52 present a natural tropism for gastrointestinal tissues.[270]

ONYX-015, a mutated adenovirus that replicates in p53 deficient cells failed to demonstrate an effective response in a phase 1 trial as monotherapy and limited responses in phase 2 when administered through EUS guiding and combined to gemcitabine. Additionally, viral replication within the tumour was undetectable and all patients developed neutralising antibodies.[271] To increase therapeutic effectiveness, the OV was engineered to express uracil phosphoribosyltransferase (UPRT) to overcome 5-fluorouracil resistance. [272] The additional *UPRT* gene resulted in an increase in the sensitivity of various pancreatic cancer cells to 5-FU, but in vivo the resulting regression of intraperitoneal pancreatic tumours was achieved by a high dose of adenovirus which produced adverse events such as severe diarrhoea and dehydration.[273]

The Ad5 serotype was modified to express the yeast cytidine deaminase and mutant HSV thymidine kinase fusion protein in addition to the adenoviral death protein (ADP). The ADP may enhance spread and oncolytic activity of replication-competent adenoviruses. Ad5-yCD/mutTK(SR39)rep-ADP has demonstrated to improve radiotherapy efficacy in PDAC in vitro models[274] and has begun a phase 2 trial in combination with chemotherapy in Korea (NCT04739046).

Other modified adenoviruses in investigation include VCN-01 which expresses the PH20 hyaluronidase, which showed intratumoral replication after systemic administration and an increase in immune infiltration in a recently completed a phase 1 trial.[275] Finally, a phase 1/2 trial of LOAd703, an adenovirus encoding TMZ-CD40L and 4-1BBL immunostimulatory transgenes has shown interesting immune and antitumoral responses. In fact, the most common adverse effects have been of grade 1 and 2 with a single a single grade 3 event with the highest dose tested; MDSCs were reduced in 8/13 patients, effector memory T cells and tumour antigen specific T cells were increased in 10/13 patients; and 6 patients have had partial responses.[276]

After its success in the treatment of advanced melanoma, talimogene laherparepvec (T-vec) has also been applied to the treatment of unresectable pancreatic adenocarcinoma in a phase 1 study underway (NCT03086642) despite a previous phase 1 trial concluding with no objective response.[277] A spontaneously mutated strain of HSV, HF10, which was the subject of a phase 1 trial in Japan, was well tolerated and showed no signs of viral shedding as well as reduced CA19-9 levels and signs of immune infiltration.[278] Similarly, OxienX010, a HSV1, and OH2, a HSV2 HG52 strain, both encoding human GM-CASF are investigated in phase 1 trials.

Myb34.5 is a genetically altered, replication-conditional recombinant HSV with deletions in the viral *ICP6* gene, to ensure preferential replication in actively dividing cells. In addition, the HSV γ_1*34.5* gene expression is regulated by the cellular B-myb promoter, further restricting viral replication and effect to tumour cells. Preclinical studies of the Myb34.5 strain showed effective cytopathic and antitumoral effects in PDAC-derived cells along with an increased effectiveness of chemotherapy. Indeed, Myb34.5 showed viral replication and oncolytic properties in both in vitro and in vivo PDAC models with significant antitumoral efficacy when combined with

standard of care gemcitabine, surpassing that of gemcitabine alone.[279] In 2015, the FDA and European Commission designed Myb34.5 an orphan drug for pancreatic cancer.

Given the high mutation of KRAS in PDAC, reoviruses were chosen as candidates due to their ability to replicate only in cells with an activated RAS pathway. Indeed, preclinical studies showed reovirus to replicate in PDAC cell lines and inhibited tumour growth in murine xenografts.[280] A completed phase 2 trial has failed to show an increase in progression-free survival when pelareorep (Reolysin) was administered alone or combined to paclitaxel and carboplatin.[281] Another phase 2 compared pelareorep with gemcitabine to the chemotherapy alone and once again showed no significant difference despite being well tolerated and detecting tumoral viral replication with concomitant increase in immune checkpoint PD-L1.[282] Following this result, a phase 1 study combining the reovirus with pembrolizumab (anti-PD-1) showed encouraging efficacy and the combination is now in phase 2.[229] Assuredly, 10/11 patients achieved disease control, with tumour biopsies showing viral replication and T-cell receptor sequencing revealed the apparition of new T-cell clones during treatment.

Rodent protoparvovirus (H-1PV) has a natural tropism for human cancer cells and since it does not naturally occur in humans, there is no pre-existing immunity to H-1PV. Preclinical studies have shown increased effective antitumoral and clinical effects in second line after gemcitabine, the correlation of H-1PV replication with Smad4 expression[283] and its immunostimulatory potential.[284] These immunostimulatory traits in PDAC models include the enhanced capacity for stimulating natural killer cells to release antitumoral cytokines and chemokines,[285] as well as the induction of a Th1 orientated immune signature in human peripheral blood mononuclear cells (PBMCs) co-culture, with increase of IFN-γ and TNF-α production.[286] The results of the phase 1/2 trial (ParvOryx02) have yet to be published, although the Principal Investigator has reported the treatment to be tolerable with patients showing prolonged survival times and pleasing immunologic signatures at the Oncolytic Virus Immunotherapy Summit and International Oncolytic Virus Conference in 2019. [272]

Replication competent vaccinia virus strains such as GLV-1h68 and GLV-1h151 both showed efficacy in preclinical models of PDAC with an enhanced effect when combined with chemotherapies or radiotherapy respectively.[287] [288] Several VV strains are under clinical investigation, including an attenuated virus (GL-ONC1) as neoadjuvant therapy[289] and a p53-expressing Modified Ankara virus (MVA) in combination with pembrolizumab after failure of previous therapies.[290] Vaccinia virus has also been actively used as an antitumour vaccine platform. An example is the PANVAC-FV vaccine composed of fowlpox virus encoding for CEA and MUC-1 (Falimarev/PANVAC-F) and a vaccinia virus encoding the same TAAs in addition to TRICOM, triad of T-cell co-stimulatory molecules, B7.1, ICAM-1 and LFA-3 (Inalimarev/PANVAC-V). This vaccine strategy is currently in a phase 1 trial in combination with recombinant human GM-CSF (NCT00669734).

Although promising results have been shown in preclinical studies, the clinical trials involving OVs have been in majority of early phase and those completed have not shown conclusive results. The poor representation of pancreatic TME and heterogeneity in research models remains a limiting factor in the discrepancy of results found in preclinical and clinical studies.[291] Another challenge found with gene therapies including oncolytic viruses is the systemic delivery for targeting both the primary tumour and metastases.

Pancreatic cancers continue to pose challenges to OVs. Strategies are evolving in combatting the tumour microenvironment by degrading ECM with hyaluronidase or the use of vitamin D in conjunction.[292] Other obstacles include the derelict immune environment and heterogeneity of potential molecular targets, although oncolytic viruses are increasingly being tested as immunostimulatory agents or in their compatibility with immunotherapies. Finally, viral pathogenicity in humans can limit their use in clinical settings, which is the case with vaccinia virus, adenoviruses associated with pre-existing immunity, or the risk of viral shedding with HSV. These issues are avoided with non-pathogenic viruses that present a tropism for human tumour cells, such as H-1PV.

Another example is myxoma virus, a poxvirus with lagomorphs as natural hosts and a natural tropism for human transformed cells. In addition, MYXV allows the ability to insert and express multiple therapeutic transgenes without compromising virus replication and production.

Finally, a method of systemic delivery through ex vivo virotherapy, where adsorption of the virus to isolated leukocytes is carried out before reinjecting the infected cells has been tested in MYXV-resistant myeloma cells. this technique allowed systemic delivery and infection of virus resistant tumour cells with subsequent therapeutic effect in a preclinical model.[293]

There have not yet been any clinical trials studying MYXV as potential anticancer virotherapeutic although it is extensively studied in several neoplasms including small cell lung cancer, ovarian cancer, glioblastoma, gallbladder cancer, melanoma, hematologic malignancies and pancreatic cancer.[294]

Myxoma virus

Myxomatosis

The myxoma virus is transmitted in the mouths of biting arthropods such as the mosquito or the flea, by direct contact or through contaminated fomites. It has as evolutionary hosts the North American brush rabbit (*Sylvilagus californicus*) and the South American tapeti (*Sylvilagus brasiliensis*), in which it causes a benign cutaneous fibroma infection. In the European rabbit (*Oryctolagus cuniculus*) however, MYXV causes myxomatosis, a lethal systemic infection with a mortality rate close to 100%.

As such, in 1950, the myxoma virus was released in Australia as a means to control the population of European rabbits. It was demonstrated that the original highly lethal strain which killed hosts rapidly, adapted, the resulting attenuated strains had longer infectious periods and lesser fatality rates. In addition to this, genetic resistance developed within the rabbit population, proving this method of pest control to be ineffective.[295] As a result of the virus-host arms race, myxoma virus has been extensively studied, in particular to define viral virulence and immunomodulatory factors.

Similarly, in 1952 the South American strain of MYXV was introduced in France to control the wild rabbit population and subsequently spread throughout Europe, infecting domestic rabbits as well. The derived strain was henceforth called Lausanne or T1 myxoma virus and prompted the development of a vaccine to protect rabbits against developing myxomatosis.[296]

Vaccines

No treatment exists for myxomatosis in rabbits so prevention by vaccination is available in many countries. Live attenuated viruses are used as vaccines and are either heterologous in the case of Shope fibroma virus (SFV) or strains of attenuated myxoma virus. Amongst the homologous vaccines are the Borghi and SG33 strains. Borghi virus is obtained from the Californian strain MSD attenuated by inoculation on embryonated chicken eggs (161 passages) followed by serial passages on rabbit kidney (RK13) cell culture (40 passages), until complete loss of pathogenicity. Another homologous vaccine is the SG33 strain, originating in Toulouse, derived from the Lausanne strain and attenuated by serial passages on RK13 cell culture at 33°C.[297] The SG33 viral strain has also demonstrated a vaccinal utility to immunise sheep against bluetongue disease.[298]

Viral structure

The myxoma virus (MYXV) is a Poxviridae from the genus *leporipoxvirus* and Chordopoxvirus subfamily. It is a large brick-shaped virus (Fig. 14) measuring around 250nm x 300nm x 200nm. Viral particles, or virions, are enveloped by one or more envelopes containing host-derived lipids and self-synthesized glycolipids. The genome is composed of a single molecule of non-segmented, linear, double-stranded DNA of 161.774kbp, with an A-T content of 56.4%. It encodes 171 open reading frames (ORFs), twelve of which are present in duplicate as they are situated within the terminal inverted repeats (TIRs) sequences. The ends of the DNA strand are in covalently closed hairpin loop structures.

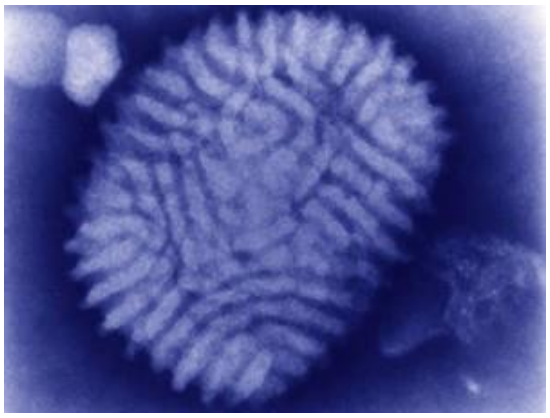

Figure 14: Myxoma virus, TEM. Credit: David Gregory & Debbie Marshall. Attribution 4.0 International (CC BY 4.0)

In general, housekeeping (replication, transcription, translation and virion assembly) and structural proteins of MYXV are encoded by genes found in the centre of the viral genome whereas immunomodulatory or host range motifs are closer to the termini of the DNA sequence. A significant number of these genes, particularly those found in the core of the Myxoma virus genome, are conserved among leporipoxviruses and orthopoxviruses.

As such, the functions of many of these sequences were deduced or predicted by homology with known genes within the highly studied Vaccinia virus and Shope fibroma virus genomes. Certain viral proteins, either cell-associated or secreted, encoded by the Myxoma virus genome have been described as contributing or potentially contributing to the downregulation of immune and inflammatory responses. Furthermore, MYXV is speculated to be able to modulate antiviral responses such as regulating apoptosis of infected cells.[299] The poxvirus ability to inhibit apoptosis is linked with the downregulation of the inhibitory properties of interferon.

Replication cycle

The viral replication cycle lasts around 16h, occurring in the cytoplasm of host cells and is grossly divided into two: the early and late phases, the latter being where virion structures are observable or virus morphogenesis.

Viral entry has not been completely elucidated but it would seem that there are no specific cell membrane receptors needed. Instead, MYXV uses unspecific glycosaminoglycans to bind and enter cells. The permissiveness of cells to poxviruses is not determined by cell entry, since they

are capable of penetrating both permissive and restrictive cells, but rather on the capacity of the virus to replicate once intracellular.[300] Similarly to Vaccinia virus, it has been suggested that myxoma virus also uses the endocytic pathway as a means of cell entry. On the other hand and contrary to Vaccinia virus, MYXV cell entry is decreased when infection occurs at temperatures lower than 37°C and in acidic environment (pH<7.4).[301]

Poxviridae encode the machinery necessary for their replication and transcription, encoding viral DNA and RNA polymerases, but depend on their host to provide certain transcription factors.[300] Virus replication and the beginning of virion assembly occurs in cytoplasmic replication factories. Along the viral replication cycle, MYXV viral particles undergo different intermediate forms including crescent, immature virions (IV), intracellular mature virions (IMV), intracellular enveloped virions (IEV), cell-associated enveloped virions (CEV) and extracellular enveloped virions (EEV).[302] [303]

During viral egress, the IV mature and are enveloped (IMV), then acquire a second envelope by intracellular organelles (IEV) such as from the trans-Golgi network (TGN) or early exosomes, are transported to the cell surface where the outer layer of this obtained envelope fuses with the cell's plasma membrane. Here, viruses are released (EEV) or retained at the cell surface (CEV). Sometimes virions are released in vacuoles creating an additional layer to the viral envelopes. Although IEV and EEV are both infectious, virions that have obtained a third layer to their envelope are not. Another form of viral egress was found through the formation of microvilli by actin fibres (Fig. 15).

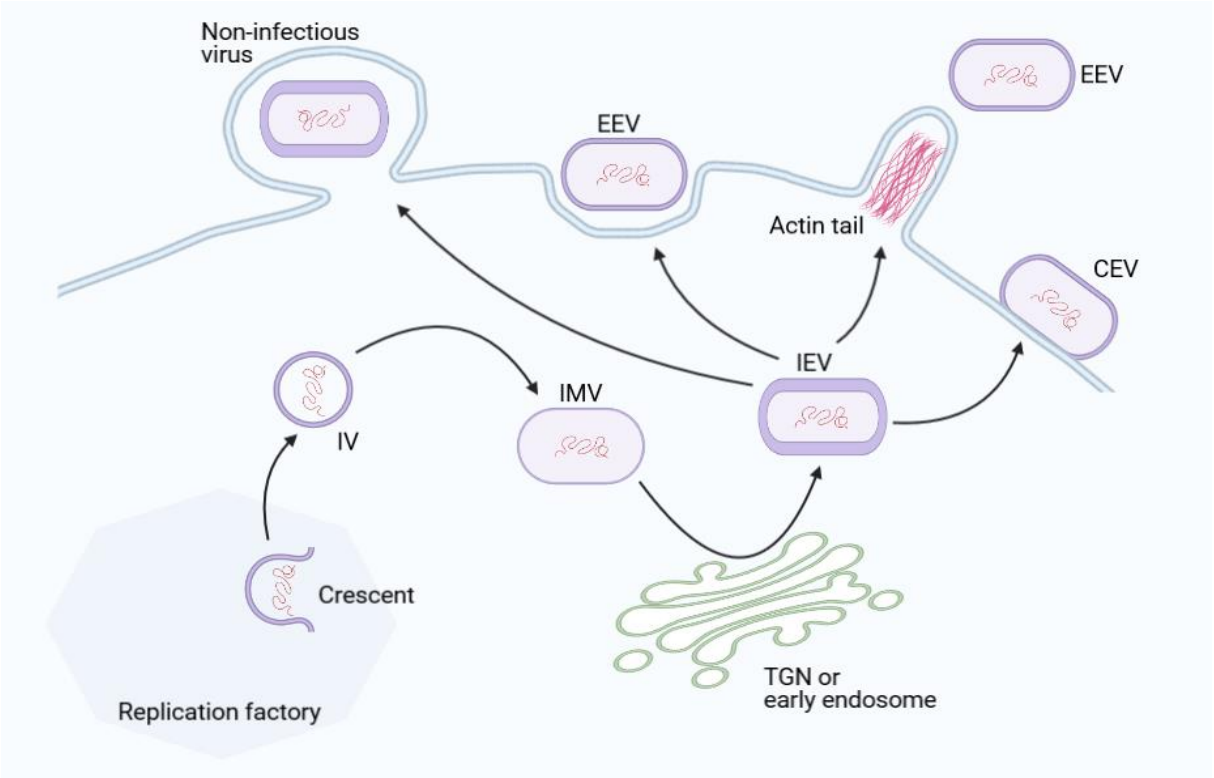

Figure 15: Myxoma virus egress of infected cell

Cellular determinants of infection

Although non-pathogenic for humans, myxoma virus displays the capacity of replicating in transformed human cells and further induces cell death, revealing its potential as an oncolytic virus. Indeed, similarly to reovirus, myxoma virus preferentially infects cells with dysfunctional tumour suppressors p53, ATM and Rb.[216]

The viral M-T5 is an ankyrin repeat protein that has been shown to regulate MYXV's ability to propagate in tumour cells. M-T5 interacts with cullin-1, a cell cycle regulatory protein. This interaction allows the virus to overcome the G0/G1 cell cycle arrest to protect from innate host antiviral responses triggered at this cell cycle checkpoint.[304] In addition, M-T5 interacts with Akt-1/protein kinase B (PKB). Akt is a serin/threonine kinase that plays a central role in maintaining balance between cell survival, proliferation and cell death. Many tumours, including PDAC present a high level of Akt, these tumour cells were divided according to their level of Akt and categorised according to sensitivity to wild type (wt) myxoma virus and M-T5 deleted virus. Type 1 cells have high endogenous levels of Akt whereas type 2 cells have low

endogenous levels and type 3 cells have no detectable Akt. Therefore, type 1 cells are sensitive to both strains, type 3 cells are resistant to both, and type 2 cells were only sensitive to wtMYXV through Akt induction by M-T5.[305] Rapamycin, which interacts with the mammalian target of rapamycin (mTOR), increases activation of rapamycin and therefore enhances infection of myxoma virus. Furthermore, rapamycin restored permissivity of type 2 cells to *M-T5* deleted myxoma virus.[306]

Viral proteins M-T5, M13, M11, and Serp2 are regulators of cell death to avoid a too fast clearance of infected cells and consequentially, the virus. Serp2 which inhibits apoptosis is related to cowpox CrmA that blocks caspases 1, 8 and granzyme B, M11L blocks pro-apoptotic signals as well as the release of cytochrome C and M13 and M-T5 avoid activation of caspase 3.[307] [308] [309] [310]

Through analogy with similar proteins, in particular those of Vaccinia virus, the tropism and cell death regulatory roles of some myxoma viral proteins have been elucidated, although many are still to be determined as well as their part in viral infection and oncolytic activity.

Myxoma virus and PDAC

In 2008, Woo et al. demonstrated for the first time myxoma virus' ability to enter and replicate in PDAC cell lines as well as their oncolysis in a dose-dependent manner. In addition, the oncolytic potential differed depending on the cell line studied and that the sensitivity to myxoma virus was proportionate to endogenous activated Akt levels and inversely proportionate to gemcitabine sensitivity.[311] Since upregulation of activated Akt is involved in gemcitabine resistance, these data suggest not only a predictiveness to Myxoma permissivity depending on basal cellular Akt levels, but the utility of MYXV in gemcitabine resistant PDAC.

Building on these results, Wennier et al. evaluated myxoma virus treatment alone or combined to gemcitabine in both in vitro and in vivo models of pancreatic cancer. In addition to human

pancreatic cancer cell lines, they demonstrated the infectivity of MYXV in murine PDAC cell lines with varying viral titers among the cell lines 4 days post-infection.[312]

Regarding cotreatment with gemcitabine, a simultaneous administration of both resulted in a decrease of MYXV early gene expression suggesting the necessity of sequential administrations. In a human PDAC cell line presenting a relatively high resistance to gemcitabine, the treatment by gemcitabine prior to myxoma virus induced significantly lower cell viability as compared to treatments alone whereas the administration of gemcitabine after MYXV did not have such a robust effect. In the murine gemcitabine-sensitive cell model, on the other hand, presented a significantly higher decrease in cell viability when treated with the virus first, whilst the administration of gemcitabine first showed no difference when compared to single therapies.

In an immunodeprived murine intraperitoneal dissemination model of the human PDAC cells, myxoma virus but not gemcitabine improved survival. The regimen of gemcitabine followed by myxoma virus was also tested and showed a further increase in survival compared to single treatments with long term survivors. Unfortunately for comparison, however, the virus followed by gemcitabine combination was not investigated in this model.

The same study was done with murine PDAC cells in an immunocompetent model this time. Myxoma virus as monotherapy, contrarily to gemcitabine alone, significantly prolonged survival. Similarly to the in vitro studies, the gemcitabine before myxoma virus sequence was similar to the mock but the virus followed by gemcitabine regimen was significantly improved compared to the other conditions, with 100% of mice presented as long-term survivors.

These results show the benefits of combining gemcitabine to myxoma virus for better therapeutic effect and how optimal sequential treatment administration is dependent on the cell type. In addition these results propose an increase in oncolytic activity when the immune system is intact, suggesting an antitumoral immunostimulatory potential of myxoma virus.

Myxoma virus is a novel platform as anticancer therapy and has not been fully elucidated with regards to its potential as oncolytic virus particularly in pancreatic adenocarcinoma. Indeed, the scarce investigations in this context limits our knowledge on its efficacy and safety or how derivative strains and modifications could increase this potential. In addition, the methodologies regarding viral investigations are further restrictive with regards to

understanding OV activity. Indeed, research involving oncolytic viruses depend largely on endpoint analyses, such as quantitative titer of viral genome copies, or indirect exploration of intracellular viral activity with labelled proteins. These methods are inadequate to fully comprehend real-time viral infection and replication of tumours.

The attenuated nature of the SG33 strain compared to Lausanne strain of myxoma virus has shown to be useful for the vaccination of European rabbits against myxomatosis. The effect on virus oncolytic activity has not, however, been tested. The mutations in virulence and immunomodulator genes has the potential to benefit the antitumoral manoeuvre of myxoma virus.

SG33

Comparison with Myxoma virus

When compared to the parent strain, SG33 presents a deletion of 13.5Kb.[296] The deletion spans from gene *M151* to the right terminal inverted repeats (TIRs), involving genes linked to the virulence of myxoma virus (Table 5). The *M151R* and *M001R* genes are partially deleted with in-frame fusion of their open reading frames (ORFs) resulting in truncated and inactive proteins. Whereas *M152R* to *M002R* genes are completely deleted which leads to the absence of the protein or remain as a single copy with the left TIRs (*M002L*, *M003.1L*, *M003.2L*, *M004L*, *M005L*, *M006L*, *M007L*, *M008L*, *M008.1L*).

MYXV ORF	Position on the genome (Lausanne)	SG33	Relevant matches/ homologs	Putative function/structure
M151R	146684-147685	Partially deleted - truncated inactive protein	Human leukocyte elastase inhibitor (sp P30740)/VAC WRSPI-2 (sp P15059)	Serpin/SERP-2
M152R	147688-148488	Deleted - protein absent	Human leupin (gi 2118384)	Serpin-like/SERP-3/Zinc finger/RNA binding
M153R	148526-149146	Deleted - protein absent	SPV C7L (sp P32225)/murine herpesvirus 68 IE1 homolog (gi 2317971)	Herpesvirus IE1-like zinc ring finger protein
M154R	149883-149239	Deleted - protein absent	VAC M2L (sp P21092)	Nonessential gene
M156R	149997-150305	Deleted - protein absent	SPV C8L (sp P32224)/VAC K3L (sp P20639)	Interferon resistance/eIF2a homolog/S1 RNA binding
M008.1R	150313-151422	Deleted - protein copy absent	Bovine plasminogen activator inhibitor (sp P13909)	Secreted serpin
M008R	151400-152947	Deleted - protein copy absent	SPV C4L (sp P32228)/VAC A55R (sp P21073)	Kelch ring canal protein homolog
M007R	152998-153789	Deleted - protein copy absent	SPV C6L (sp P32226)/VAC B8R (sp P21004)/VACV B8R	IFNg receptor homolog, a chain
M006R	153826-155355	Deleted - protein copy absent	SPV C4L (sp P32228)/VAC A55R (sp P21073)	Kelch ring canal protein homolog
M005R	155391-156842	Deleted - protein copy absent	VAC B4R (sp P21001)/human ankyrin (sp Q01485)	Ankyrin-like host range (7 ankyrin repeats)/Apoptosis regulator; RNAse3 domain
M004.1R	156862-157134	Deleted - protein copy absent	SPV C2L (sp P32230)	
M004R	157138-157851	Deleted - protein copy absent	CAP T4 (sp P18385)/VAC B9R (sp P21005)	ER-localized apoptosis regulator/RDEL; helix-hoop-helix
M003.2R	158084-158425	Deleted - protein copy absent	CAP T3C (sp P18388)	
M003.1R	158495-158950	Deleted - protein copy absent	CAP T3A (gi 74384)/VAC B15R (sp P21089)	
M002R	159129-160109	Deleted - protein copy absent	MPV TNF-R homolog (gi2738073)/VAC B28R homolog (gi 439102)	Soluble TNF receptor homolog
M001R	160189-160971	Partially deleted - truncated inactive protein	VAC 35K major secreted protein B29R/C23L (sp P21090)	Secreted chemokine binding protein

Table 5: Deleted genes in the SG33 viral genome compared to wild type Myxoma virus

Among these deleted proteins is the anti-apoptotic factor encoded by M151R, a serpin (serine protease inhibitor) which targets the interleukin 1β converting enzyme. We can also find immune modulators such as those coded by genes *M152R* an atypical serpin, *M153R* which is linked to MHCI and Fas-CD95 down-regulation, *M154R* which has 50% homology with the NFκB-inhibiting M2L vaccinia virus gene, and M156R representing a structural homology with the eukaryotic translation initiation factor eIF2α.

In addition to the deletion, other differences were observed between the genomes of the Lausanne and SG33 strains. Whilst the majority of SG33 genes are 100% identical to those found in the Lausanne strain, the sequence between *M135R* to *M001R* is only 75 to 91% identical. *M138* and *M139* sequences were shown, however, to be 100% identical to the Californian MSD strain.[313] Other discrepancies within the genome sequences have been demonstrated, such as intergenic mutations or amino acid changes. Such is the case within the M011L and M143R-encoded proteins, both of which having been characterised as apoptosis regulators. Although some have been shown not to come to fruition, the consequences on protein activity for the majority of these amino acid discrepancies have yet to be defined.

Thesis objectives

Pancreatic cancer is still considered incurable, in part due to the lack of effective treatments. An interesting innovative therapy is the use of oncolytic viruses. The non-pathogenic nature of myxoma virus in humans as well as the capacity for genetic modification, the ability to produce long-lived infections in human tumour cells and the absence of pre-existing neutralising antibodies makes it an optimal candidate as such. The vaccinal strain of MYXV SG33 possess all these characteristics and renders myxoma virus even safer for European rabbits, the one known susceptible host. The general goal of this thesis was therefore to determine the antitumoral oncolytic activity of SG33 in pancreatic adenocarcinoma.

The lack of efficient methods to minutiously characterise viral replication and propagation hinders our full comprehension of oncolytic viral infectivity in tumour cells. Overcoming this technical obstacle could help characterise OV pharmacodynamics in real-time within tumour models and consequently its oncolytic activity. To do so, we used a novel strategy, the ANCHOR system, which allows the visualisation and quantification of viral replication in live cells and in real-time. With this technique, we analysed the infectivity of SG33 and compared it to the parent T1 strain. Next, we analysed the oncolytic activity of SG33 in primary PDAC cancer cell lines and investigated the therapeutic use of SG33-ANCHOR in determining viral replication in a murine vivo model.

The second part of the thesis was devoted to determining the immune implication of SG33 oncolysis. In this regard we investigated the immunogenic cell death (ICD) induced by SG33 in permissive PDAC primary cells. The ICD of SG33 was further characterised by determining which mechanisms of death induced by the virus were implicated. Finally we demonstrated in an immunocompetent ex vivo murine model, the effect of SG33 tumour cell lysis on the stimulation of dendritic cells.

This work has the potential to rationalize the use of OV, and more particularly SG33, for the benefit of patients suffering of pancreatic adenocarcinoma, a disease still incurable to this day. We also aimed to further our knowledge on SG33's mechanism of action by investigating its oncolytic activity, paving the way to exploit its immunological potential in immune-deprived tumours such as pancreatic cancer.

Part 1: A novel imaging approach for single-cell real-time analysis of oncolytic virus replication and efficacy in cancers

Introduction

Determining the potential of a virus as an antitumour therapy is more difficult than conventional compounds. Indeed the pharmacodynamics are different and vary depending on the virus studied. The study of viruses, and more precisely oncolytic viruses, revolves around limited methods that are either direct or indirect.

Direct methods of investigating viruses include transmission electron microscopy (TEM). Although TEM allows the visualisation of viral particles within cells, it is extremely time consuming, requiring extensive and tedious sample preparation.[314] The use of a microtome for sectioning and the necessary fixation can introduce artifacts or damage samples. Another direct method often used for viral studies are fluorescent reporter proteins associated to early/late viral protein expression. For example, Wennier et al. utilise two different fluorescent reporter proteins to follow early and late myxoma virus protein expression in PDAC cell lines.[312] While these reporter compounds help discern the expression of particular viral genes, it is difficult to discern the early stages of viral cycle such as the beginning of viral genome replication.

Indirect methods include plaque assay analysis (PFA) which measures the cytolysis capacity or focus forming assay (FFA) using immunostaining to detect viral antigens in infected cells. Both of these study the infectious capacity of the virus. Other indirect techniques include quantitative (q-) and real-time (RT-) polymerase chain reaction (PCR) to detect viral genetic material, or ELISA to detect viral proteins.[315] These indirect are end-point analyses that necessitate fixed or lysed samples therefore abrogating their use in real-time.

To better follow viral replication and propagation in live models of tumour cells, the ANCHOR system (NeoVirTech) was developed and incorporated within the viral genome of SG33.

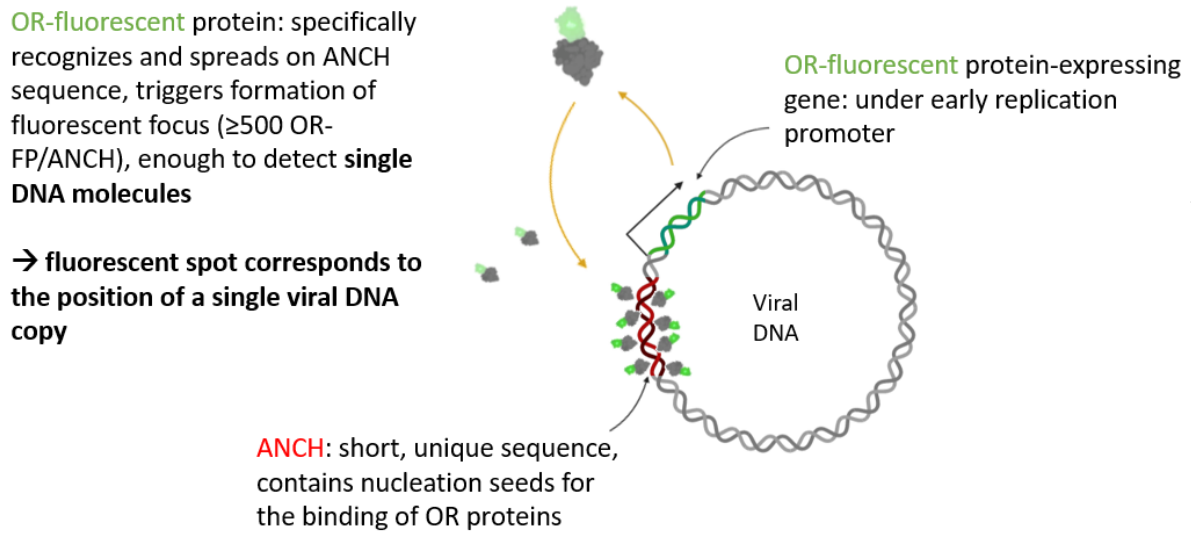

Figure 16: The ANCHOR system

The ANCHOR system is composed of two entities. The first is the ANCH sequence which is short and non-repetitive, inserted in the genome of the virus. The sequence is inserted upstream of the *OR-GFP* gene and presents nucleation sites recognised by the fusion protein. The second sequence codes for a fusion protein composed of 'OR' a protein of bacterial origin which specifically recognises the ANCH sequence. The second part of the fusion protein expresses GFP fluorescence. The accumulation of the OR-FP (up to 500 OR-FP/ANCH) forms a fluorescent foci detectable and quantifiable by microscopy.

In this work we used SG33 equipped with the ANCHOR system to follow replication and propagation in live models of PDAC. This technique will help determine the permissivity of primary pancreatic cancer derived cell lines to SG33, in comparison with wild type myxoma virus, and establish its antitumoral activity. These characteristics were determined in a variety of models, at both in vitro and in vivo levels.

RESEARCH ARTICLES

HGT

A Novel Imaging Approach for Single-Cell Real-Time Analysis of Oncolytic Virus Replication and Efficacy in Cancer Cells

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Oncolytic viruses (OVs) are novel cancer gene therapies that are moving toward the forefront of modern medicines. However, their full therapeutic potential is hindered by the lack of convenient and reliable strategies to visualize and quantify OV growth kinetics and therapeutic efficacy in live cells. In this study, we present an innovative imaging approach for single-cell real-time analysis of OV replication and efficacy in cancer cells. We selected SG33 as a prototypic new OV that derives from wild-type Myxoma virus (MYXV). Lausanne Toulouse 1 (T1) was used as control. We equipped SG33 and T1 genomes with the ANCHOR system and infected a panel of cell lines. The ANCHOR system is composed of a fusion protein (OR-GFP) that specifically binds to a short nonrepetitive DNA target sequence (ANCH) and spreads onto neighboring sequences by protein oligomerization. Its accumulation on the tagged viral DNA results in the creation of fluorescent foci. We found that (1) SG33 and T1-ANCHOR DNA can be readily detected and quantified by live imaging, (2) both OVs generate perinuclear replication foci after infection clustering into horse-shoe shape replication centers, and (3) SG33 replicates to higher levels as compared with T1. Lastly, as a translational proof of concept, we benchmarked SG33 replication and oncolytic efficacy in primary cancer cells derived from pancreatic adenocarcinoma (PDAC) both at the population and at the single-cell levels. *In vivo*, SG33 significantly replicates in experimental tumors to inhibit tumor growth. Collectively, we provide herein for the first time a novel strategy to quantify each step of OV infection in live cells and in real time by tracking viral DNA and provide first evidence of theranostic strategies for PDAC patients. Thus, this approach has the potential to rationalize the use of OVs for the benefit of patients with incurable diseases.

Keywords: oncolytic virus, ANCHOR system, live imaging, pancreatic cancer

INTRODUCTION

SINCE THE DAWN OF TIME, viruses have been responsible of pandemics, epidemics, and a variety of infectious diseases. They were first discovered at the end of the 19th century, accountable for multiple pathologies yet unidentified. Since it was reported that a 42-year-old female patient with myelogenous leukemia underwent tumor remission after an influenza infection, the attempt to use viruses to eliminate tumors has never stopped.[1]

Employing therapeutic oncolytic viruses (OVs) against tumor cells has grown exponentially since the 1950s/60s, with the first OV derived from Herpes Simplex Virus 1 (HSV-1, Imlygic©)[2] having received its product license in 2015 for advanced melanoma; multiple other viral oncolytic strains are currently investigated in (pre)clinical studies for the treatment of diverse neoplasms.[3] With OV finally reaching patients' bed, several limitations and challenges remain to be tackled to broader the use of such innovative therapies in oncology programs.

One of the main hurdles so far in virotherapy programs is to achieve real-time noninvasive monitoring of viral replication, propagation, and oncolysis in live cells to address OV biodistribution, pharmacokinetics, and to identify potential detrimental side effects after infection of normal cells. Thus, novel technological approaches are needed for the in-depth understanding of OV biology and therapeutic efficacy.

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Methods studying viral infection and replication include enzyme-linked immunosorbent assay (ELISA) and antigen-specific immunofluorescence, quantitative PCR (qPCR), and reverse transcriptase-qPCR,[4] and transmission electron microscopy[5]—a laborious and cumbersome technique. These are usually end-point analyses that necessitate fixed or lysed biological samples and are unable to discern all the various stages of a viral life cycle. Furthermore, these techniques provide at best multiple timepoint analyses and, consequently, cannot be used in longitudinal studies.

Nowadays, fluorescent reporter proteins associated with early or late viral promoters are the most common method used in preclinical programs to track viral infection.[6] For *in vivo* preclinical and clinical studies, the sodium iodide symporter (NIS) expressed by viral genomes provides noninvasive imaging information on viral infection in cancer models[7] and NCT03456908. A limit to this technique, however, is the necessity of administering a radiomarker (125I) to ensure detection. Collectively, these methods provide an understanding of the viral replication cycle and viral progeny assembly but are missing early steps in viral infection or replication.

Viral oncolytic activity, often simultaneously studied in OV research, is often defined through cell viability assays such as tetrazolium colorimetric assays (MTT/MTS), which prove to have the same end-point analyses limits.

To follow viral infection and propagation in real time to address every step of the viral cycle would be essential to gain basic knowledge on OV behavior and efficacy in cancer cells. This, in turn, will help accelerate the progress of virotherapy toward the clinic by providing priceless biodistribution and pharmacokinetic data.

The ANCHOR system is a novel technique based on genetic engineering that allows real-time monitoring of DNA localization. In brief, the ANCHOR system is composed of a fusion protein (OR-GFP) that specifically binds to a short nonrepetitive DNA target sequence (ANCH) and spreads onto neighboring sequences by protein oligomerization. Its accumulation results in the creation of a fluorescent focus that allows DNA tracking in live cells. In recent studies, the ANCHOR system was successfully used for the real-time visualization and quantification of human cytomegalovirus replication in living cells,[8] the *in vivo* labeling of adenovirus DNA,[9] to trace baculovirus infection[10] and to shed light on critical early steps characterizing HIV-1 infection.[11]

During this study, we developed for the first time in the field an OV genome equipped with the ANCHOR system to detect and quantify viral DNA and oncolytic activity in live cells. We selected prototypic Myxoma virus (MYXV) as a large enveloped DNA virus that is pathogenic for lagomorphs but not for humans. MYXV has already demonstrated oncolytic potential against diverse tumoral cells, while remaining innocuous for healthy cells.[12] In this study, we characterize for the first time the oncolytic properties of the vaccinal strain SG33, derived from parental MYXV. Using the ANCHOR system, we found that SG33 surpasses MYXV replication and we precisely defined SG33 viral cycle in model cells, both at single-cell and population levels.

Pancreatic adenocarcinoma (PDAC) is a disease with no cure that will soon rank second worldwide in death related to cancer.[13,14] PDACs are heterogeneous by essence, both at the molecular and cellular levels, and such heterogeneity strongly contributes to resistance to therapy.[14–16]

Several groups including ours demonstrated that OVs have therapeutic potential for this disease,[17,18] but to our knowledge, the importance of interpatient tumor heterogeneity on virotherapy efficacy has been scarcely explored so far. As a proof of concept, we characterized the efficacy of replication and oncolysis of SG33 in primary samples from patients with PDAC, both *in vitro* and *in vivo* in experimental models of tumors. We found that SG33 spares normal pancreatic cells and replicates in PDAC primary cultures. *In vivo*, SG33 infects and spreads into tumors to inhibit cancer growth. Collectively, our study represents a significant step toward precision medicine for virotherapy of cancer.

MATERIALS AND METHODS

Cell lines and culture conditions

Experimental procedures performed on mice were approved by the ethical committee of INSERM CREFRE US006 animal facility and authorized by the French Ministry of Research: APAFIS#3600-2015121608386111v3.

RK13 (rabbit kidney cells), Hela (human cervix cancer cells), and WM266-4 (human melanoma cancer cells) were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific, Illkirch, France) 4.5 g/L glucose (for MiaPACA2) containing 10% fetal bovine serum (FBS; ThermoFisher Scientific), 100 IU/mL penicillin (ThermoFisher Scientific), 100 μg/mL streptomycin (ThermoFisher Scientific), and 250 ng/mL fungizone (ThermoFisher Scientific), 2 mM glutamine (Sigma), and 100 IU/mL plasmocin (Invivogen) (hereafter referred to as complete medium). Pancreatic cancer patient-derived primary cells PDAC012T, PDAC087T, and PDAC072T were obtained and cultured as previously described.[19] Cells were incubated at 37°C with 5% CO_2.

Viruses cloning, viral production, and titration

SG33-ANCHOR and T1-ANCHOR were obtained by homologous recombination of *lacZ* gene from the SG33-LacZ and T1-LacZ viruses with the donor plasmid pVmyxlac-ANCHORGFP-GPT. SG33-LacZ and T1-LacZ were derived from the SG33-attenuated vaccine strain and Lausanne-like Toulouse 1 strain (T1) of MYXV, respectively.[20] In these recombinant viruses, the *lacZ* gene under the control of the poxvirus late p11 promoter is inserted between the M009L and M010L MYXV genes.

The donor plasmid pVmyxlac-ANCHORGFP-GPT includes the *lacZ* sequence in which three sequences were inserted through appropriate restriction sites; the ANCHOR system is composed of a sequence coding for a fusion protein ORGFP and a nonrepetitive DNA target sequence ANCH, and finally the EcoGPT selection cassette. ORGFP and EcoGPT genes were each under the control of a poxviral promoter p7.5. In the SG33-ANCH virus, the ORGFP expression cassette is lacking to generate a nonfluorescent viral DNA genome.

MYXV-ANCHOR recombinant viruses were selected by resistance to mycophenolic acid[21] and GFP positive criteria by fluorescence microscopy. MYXVs were produced in RK13 cells. In brief, RK13 cells were infected until 80% of cytopathic effect. The supernatant and cell lysate were harvested, and were either frozen and thawed three times (crude virus), or purified through two 36% sucrose cushion as previously described.[22] MYXV quantification was performed by plaque assay and the virus titer was determined as plaque forming units (PFUs).[23]

Real-time viral replication analysis at the cell population level

Cells were seeded in 96-well plates (Corning, Boulogne-Billancourt, France) at 10×10^3 cells per well in 100 μL of complete medium. After 24 h, cells were washed with phosphate buffered saline (PBS) and infected at corresponding multiplicity of infection (MOI) during 1 h at 37°C in 100 μL of phenol-free complete (see above) culture medium, so as to avoid viral green fluorescence quenching. Infection medium was then replaced with phenol-free complete culture medium. After infection, cells were imaged in real time at 37°C, 5% CO_2 with the IncuCyte Zoom (Sartorius). Images were acquired at 10× magnification every 3 h using brightfield and green filters (Ex: 440–480 nm, Em: 504–544 nm) to determine cell confluence and viral replication, respectively, and were analyzed using the Zoom2016B software.

Real-time viral cytolytic activity assay

Cells were seeded in 96-well plates (Corning) at 10×10^3 cells per well in 100 μL of complete medium. After 24 h, cells were washed with PBS and infected with SG33-ANCH lacking the ORGFP protein at corresponding MOIs during 1 h at 37°C in 100 μL of phenol-free complete culture medium. Infection medium was then replaced with phenol-free complete culture medium supplemented with 250 nM of cytotox green (Ex: 491 nm, Em: 509 nm), a reagent for real-time quantification of cell death (Sartorius). Cells treated by 0.5 μM of Staurosporine were used as a positive control. After infection, cells were imaged in real time at 37°C, 5% CO_2 with the IncuCyte Zoom (Sartorius). Images were acquired at 10× every 3 h using brightfield and green filters (Ex: 440–480 nm, Em: 504–544 nm) to determine cell confluence and cell death, respectively, and were analyzed using the Zoom2016B software.

Viral genome replication quantitation

Cells were seeded in 12-well plates (Corning) at 100×10^3 cells per well in 1 mL of complete medium. After 24 h, cells were washed with PBS and infected at corresponding MOIs during 1 h at 37°C in 200 μL of phenol-free complete (see above) culture medium. Infection medium was then replaced with phenol-free complete culture medium. Viral replication was measured with the IncuCyte Zoom (Sartorius) at 10× using a green filter (Ex: 440–480 nm, Em: 504–544 nm) and analyzed using the Zoom2016B software.

Cells were recovered and DNA was extracted using the One-4-All Genomic DNA Mini-Prep kit (Bio Basic) following manufacturer's instructions. Samples were diluted in sterile water before viral DNA was quantified by qPCR, as described previously,[24] using QuantiNova Probe directed toward the M071lq gene of MYXV with the LightCycler 96 (Roche, Boulogne-Billancourt, France) system. Data were analyzed using the LightCycler 480 1.5 software.

Viral DNA quantification in live cells

Cells were seeded and infected in 96-well plates as previously described. After 48 to 72 h of infection, cells were fixed with formalin (Sigma) according to manufacturer's instructions and imaged at 10× magnification using the CellInsight CX7 High-Content Screening (Cell Insight CX7 HCS; ThermoFisher, Illkirch, France), with light emitting diode (LED) 10× (numerical aperture, NA, 0.30) nonconfocal with the following light sources (Ex/Em, in nm): 438/480 (blue), 485/521 (green), and 549/600 (red). Number of cells was determined after nuclei staining with Hoechst 33342 (ThermoFisher). Fluorescence intensity was quantified using HCS Studio software for integrated data collection and analysis (ThermoFisher).

Single-cell analysis of viral infection

Cells were seeded in 96-well plates (PerkinElmer) at 10×10^3 cells per well in 100 μL of complete medium. After 24 h, cells were washed with PBS and infected at corresponding MOIs during 1 h at 37°C in 100 μL of phenol-free medium. Infection medium was then replaced with 100 μL of phenol-free complete culture medium. Forty-five whole-well images were acquired every 15 min for 25 h using the Operetta CLS High-Content Analysis System (PerkinElmer), with LED 5× Air (NA 0.4) confocal with eGFP filter (Ex: 460–490 nm, Em: 500–550 nm). Images were exported and analyzed using the Columbus program (PerkinElmer).

Viral propagation assay in three-dimensional spheroid model

RK13 and PDAC087T cells were seeded in 96-well ultralow attachment bottom-rounded plates (Corning) at 5.10^3 cells per well and centrifuged 400 *g* for 10 min. After 5 to 7 days of culture, spheroids were formed and infected at corresponding PFU of SG33-ANCHOR (10×10^3 to 500×10^3) and incubated at 37°C, 5% CO_2. Four

(RK13 cells) or 7 (PDAC087T cells) days later, spheroids were imaged using the Operetta CLS High-Content Analysis System (PerkinElmer), with LED 5×Air (NA 0.16) non-confocal with brightfield and eGFP filter (Ex: 460–490 nm, Em: 500–550 nm). Images were acquired using Harmony 4.9, exported and analyzed using the Columbus (Perkin-Elmer) 2.8.2 program. PDAC087T cells were also imaged using the LSM 880 AxioObserver (Zeiss), laser 10×plnApo (0.45NA) confocal with brightfield, and FITC filter (Ex: 470/40 nm, Em: 525/50 nm). Images were acquired using Zeiss black 2.3 and analyzed using Zeiss blue 2.3.

Experimental tumors

PDAC087T cells were engrafted subcutaneously into the flank of SCID CB17 mice (2×10^6 cells per mouse, $n=5$ mice per group). Two weeks later, when tumors became palpable, 5×10^5 PFUs of SG33 were diluted in 50 μL of PBS and injected intratumorally. Control tumors received 50 μL of PBS (placebo). Tumor size was measured using a caliper and monitored using the Aixplorer echography system. Mice were killed 2 weeks after virus injection and tumors were collected and imaged for fluorescence using the Ivis Spectrum (PerkinElmer). Tumors were sampled and minced in liquid nitrogen, and centrifugated for 20 min at 11,000 g and 4°C. Cleared supernatants were used to infect RK13 cells, and viral replication was analyzed as previously described.

Quantification and statistical analysis

Unless otherwise indicated, data represent mean ± standard error of the mean of at least three biological replicates. Statistical significance was calculated by unpaired student test with Welch's correction, using Graphpad Prism 8.0 software (Graphpad). $p<0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.005$.

RESULTS

We generated the MYXV Lausanne T1 and SG33 viral genomes equipped with ANCHOR-GFP molecular beacon (Material and Methods and Fig. 1A, B) to address viral replication in real time in live cells. RK13, which are highly permissive to MYXV, were infected with MOI = 0.1 to MOI = 5 of either Lausanne T1 or SG33. SG33-

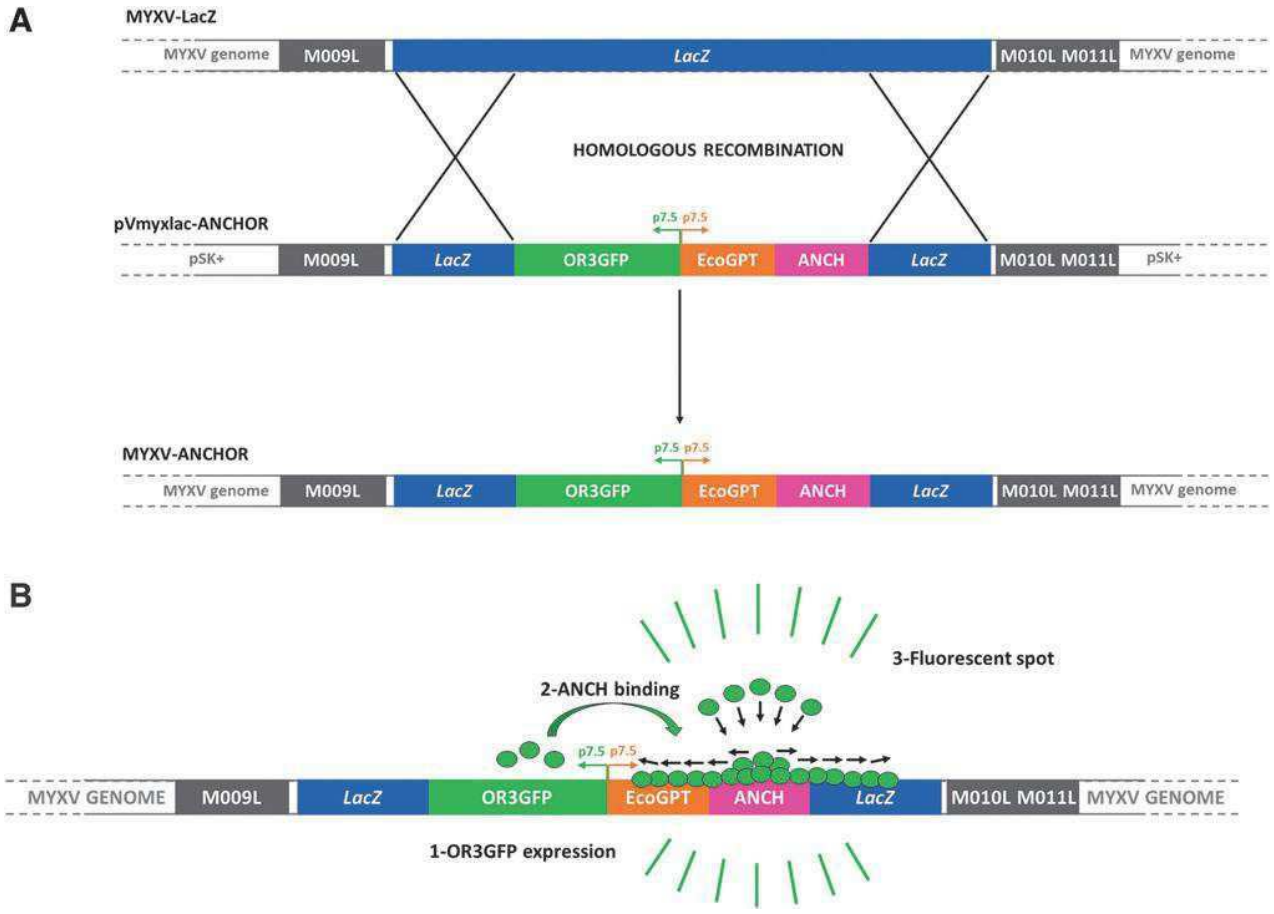


Fig. 1. MYXV-ANCHOR construction. **(A)** MYXV-ANCHOR virus was obtained thanks to the homologous recombination of the LacZ gene from MYXV-LacZ recombinant virus and the donor plasmid pVmyxlac-ANCHOR. **(B)** The ANCHOR system is composed of an ANCH DNA target sequence that specifically binds dimers of OR protein through nucleation sites. In this construction OR3 is fused to GFP. Once expressed, ORGFP dimers spread on the DNA and are recruited to form a large metastable nucleoprotein complex. The accumulation of this complex on ANCH sequence forms a fluorescent spot that is easily detected by fluorescence microscopy.

ANCHOR infection resulted in significantly larger viral plaques and perinuclear viral replisomes characteristic of Poxvirus infection (Fig. 2A, top panel), as compared with cells infected with T1-ANCHOR (Fig. 2A, bottom panel).

Forty-eight hours later, replication level in infected cells was quantified by live imaging. RK13 cells infected with vaccinal SG33 demonstrated higher viral genome replication, as compared with cells infected with parental T1 Lausanne strain (Fig. 2B), with a 2- to 19-fold increase in fluorescence intensity, and the highest difference in replication was measured when using the lowest dose of virus for infection. These data demonstrate that in model cells, SG33 replicates to a larger extent than parental T1 Lausanne MYXV strain. Additional data demonstrated that SG33 genome replication was readily detected after infection of WM266-4 (melanoma) and Hela (cervix) cancer cells (Supplementary Fig. S1A).

To confirm the correlation between fluorescence intensity and viral genome replication, we infected RK13 cells with increasing levels of SG33-ANCHOR (MOI 0.1, 1, 5) and measured total fluorescence intensity directly after infection, and 48 h postinfection using the Incucyte Zoom system (Supplementary Fig. S1B). Similarly, we quantified viral genome copies in these RK13 cells at both timepoints. We found a strong correlation between the two approaches at both timepoints (Fig. 2C), with $R^2 = 0.99$ at $T = 0$ and $R^2 = 0.97$ at $T = 48$ h postinfection, demonstrating that ANCHOR and qPCR share the same performance for viral genome quantification.

We next performed time lapse studies to better characterize the viral cycle of SG33 in permissive cells (Fig. 2D and Supplementary Movie S1). Using high-definition microscopy and three-dimensional (3D) surface plots of viral replication foci, we detected viral input ($T = 0$, Fig. 2D) that progressively disaggregate to form viral DNA factories in infected cells ($T = 180$, Fig. 2D).

We next performed longer noninvasive viral genome tracking studies to determine cell-to-cell viral propagation and virus-induced cell death. Thus, RK13 cells were infected with MOI = 1 and MOI = 10 of SG33-ANCHOR. Uninfected RK13 cells were used as control to address cell autofluorescence. SG33-ANCHOR genomes were readily detectable in living cells, at the single-cell level, either as viral input (Fig. 2E top left panel) or viral DNA factories (Fig. 2E top right). Time monitoring indicates that SG33 reaches full replication 202 ± 19 min and 202 ± 10 min after infection with MOI = 1 and MOI = 10, respectively (Fig. 2F). Remarkably, we confirm that SG33 MYXV traffics from cell to cell using cytoplasmic bridges[25] (Fig. 2E, bottom left panel). Notably, we identified that higher dose of virus significantly accelerates viral propagation from cell to cell (390 ± 29 min vs. 638 ± 36 min after infection with MOI = 10 and MOI = 1 of SG33, respectively). We show that SG33-ANCHOR is lytic (Fig. 2D, bottom right panel), and that once infected, RK13 cells die within 563 ± 31 min (MOI = 1) and 598 ± 28 min (MOI = 10), respectively.

Next, we aimed to monitor SG33 viral spread and oncolytic activity at the cell population level. Thus, RK13 cells were infected with increasing MOI of SG33-ANCHOR. Figure 3A and Supplementary Movie S2 depict viral progression as imaged noninvasively using the Incucyte Zoom. We detected and quantified viral replication down to MOI = 0.01 of SG33-ANCHOR, which increased dose dependently to reach a peak of replication as early as 24 h after infection with MOI = 10 of virus, and after 60 h of infection of cells with MOI = 1 of virus (Fig. 3B). Linear regression analysis indicates a positive correlation between the viral dose and the increase in viral replication. In parallel, we analyzed cell confluence and found that SG33 replication correlates with dose-dependent inhibition of cell proliferation (Fig. 3C).

To determine whether the decrease in cell confluence was due to virus-induced cell death, we performed the same experiment with SG33-ANCH virus lacking the ORGFP protein, and a green fluorescent cytotoxicity marker to characterize cell death (Fig. 3D). Cells treated with staurosporine were used as control. Figure 3E shows that SG33 increases cytolysis in RK13 cells in a dose-dependent manner. Collectively, we demonstrate here for the first time the oncolytic potential of SG33-ANCHOR in live infected cells.

Three-dimensional cell culture systems are becoming increasingly important in preclinical cancer research, as they recapitulate microtumors, metastases, and the tumor microenvironment much better than monolayer culture systems could. Although 3D tumor cell cultures are increasingly employed in virotherapy research,[26] live monitoring of OV replication in spheroids has not be achieved so far. Accordingly, we generated spheroids from RK13 cells. Five days later, 3D cultures were infected with increasing PFUs of SG33-ANCHOR (10×10^3 to 500×10^3). Cultures were monitored noninvasively for GFP fluorescence using the Operetta microscope 4 days postinfection. As shown in Fig. 3H, we identified a dose–response increase in fluorescence demonstrating infection of 3D models by SG33-ANCHOR. Remarkably, we found that SG33 infection strongly alters spheroids structure and increases cancer cell death, as monitored by detection of cellular debris.

As a pilot study of applying SG33-ANCHOR as oncolytic virotherapy in PDAC, we selected a panel of patient-derived primary cells of basal-like and classical PDAC subtypes.[19] We first addressed SG33-ANCHOR specificity and found that the virus failed to replicate into and spared normal pancreatic HPDE cells (Fig. 4A and Supplementary Fig. S1C).

We next addressed SG33-ANCHOR replication at the single-cell level and quantified viral DNA content in infected PDAC primary cell lines by fluorescence using the CellInsight CX7 HCS platform. As shown in Fig. 4B, we identified that PDAC012T primary cells poorly replicate SG33-ANCHOR; in contrast, we identified a dose-dependent increase in SG33 viral DNA production in PDAC087T cells, and, to a lesser extent, in PDAC072T cells.

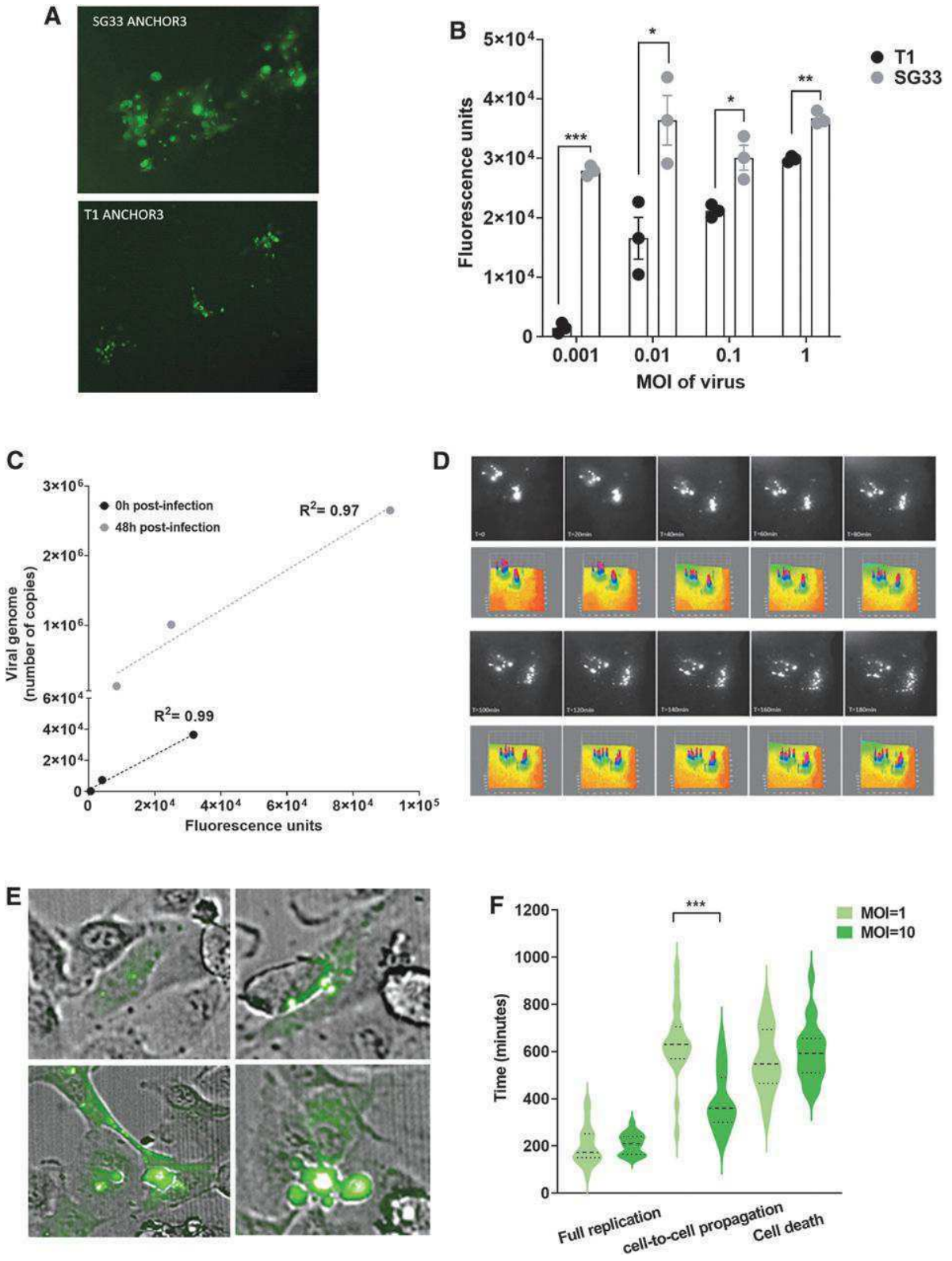


Fig. 2. Analysis of SG33-ANCHOR viral cycle in model cells. **(A)** RK13 cells were infected with MOI = 0.25 of SG33-ANCHOR (*top panel*) or Lausanne T1-ANCHOR (*bottom panel*) viruses and viral factories were detected 72 h later by direct fluorescence analysis in live cells using a Zeiss AXiovert Z1 fluorescence microscope. **(B)** RK13 cells were infected with the indicated MOI of SG33-ANCHOR or Lausanne T1-ANCHOR viruses. Viral replication was quantified as fluorescence units 72 h later using the Thermo CX7 imaging and dedicated software. Results are mean ± s.e.m. of three independent experiments performed in duplicate. $*p<0.05$; $**p<0.01$; $***p<0.005$. **(C)** RK13 cells were infected with increasing MOI of SG33-ANCHOR. Representation of the correlation between fluorescence detected using the Incucyte Zoom software and viral genome copies quantified by qPCR directly after (T0) and 48 h (T48) post-infection. Results are representative of two independent experiments. **(D)** RK13 cells were infected with MOI = 0.25 of SG33-ANCHOR and imaged every 20 min for 180 min. 3D surface plots of viral replication foci were produced with Fiji Software. RK13 cells were infected with MOI = 1 or MOI ± 10 of SG33-ANCHOR and imaged every 15 min by confocal microscopy using the Operetta CLS High-Content Analysis System. **(E)** Representative micrographs of viral infection steps. **(F)** Results are mean ± s.e.m. of 100 infected cells per group. $***p<0.005$.

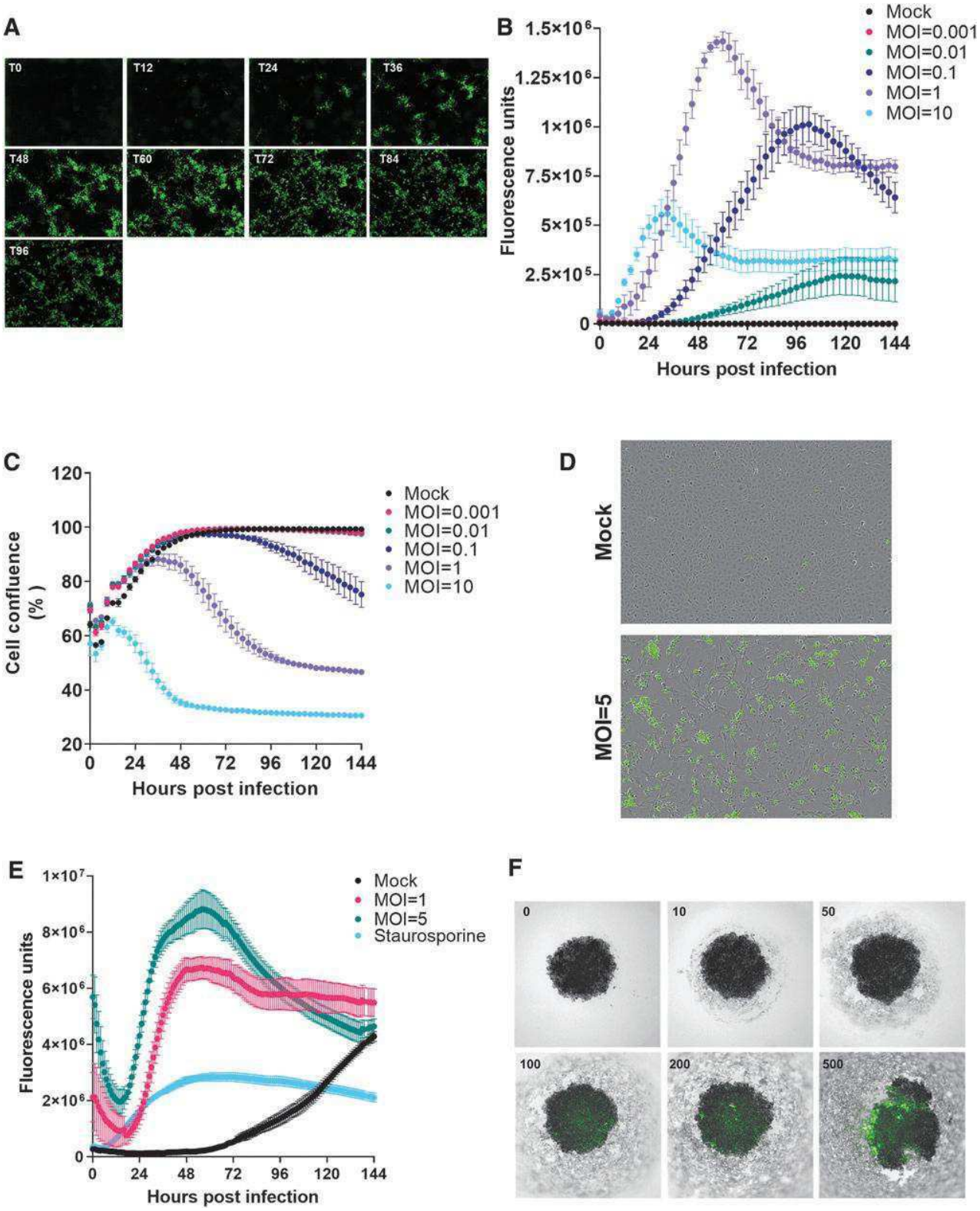


Fig. 3. SG33 cytolytic activity in model cells. RK13 cells were infected with SG33-ANCHOR and imaged every 3 h using the Incucyte Zoom. **(A)** Representative micrographs of RK13 cells infected with MOI = 1 of SG33-ANCHOR at the indicated time. **(B)** SG33-ANCHOR replication was measured by integrated fluorescence intensity at the indicated time. Results are mean ± s.e.m. of five independent experiments performed in triplicate. **(C)** Cell confluence was quantified noninvasively at the indicated time using the Incucyte Zoom software. Results are mean ± s.e.m. of five independent experiments performed in triplicate. RK13 cells were infected with SG33-ANCH, incubated cell death marker cytotox green imaged every hour using the Incucyte Zoom. **(D)** Representative micrographs of cell death in RK13 cells non-infected (*top*) or infected with MOI = 5 of SG33-ANCH (*bottom*) for 48 h. **(E)** Cell death was measured by fluorescence at the indicated time. Results are mean ± s.e.m. and representative of four independent experiments performed in triplicate. **(F)** RK13 spheroids were generated and infected by SG33-ANCHOR at the indicated dose. Operetta microscopy was used to image spheroids that were non-infected (0) or infected with a range of SG33-ANCHOR p.f.u. (10–500). Results are representative of two independent experiments.

Next, we monitored SG33-ANCHOR replication in PDAC012T, PDAC087T, and PDAC072T live cultures at the population level (Fig. 4C–E). PDAC cells were infected with increasing MOI of SG33-ANCHOR, ranging from MOI = 0.1 to MOI = 10, and viral replication and cell death were monitored noninvasively. We found that SG33-ANCHOR replicates in all primary cancer cells tested, although at different levels. In PDAC087T, SG33-ANCHOR replication is higher than that of PDAC012T and PDAC072T. Interestingly, SG33-ANCHOR is oncolytic for PDAC072T cells, despite low replication levels, and PDAC087T cells, while PDAC012T cells resisted to SG33-ANCHOR infection (Fig. 4F). These data indicate for the first time that SG33-ANCHOR replicates specifically in human cancer cells and is oncolytic for PDAC primary cells but could also be affected by cellular heterogeneity found in the tumors.

Next, we determined whether viral replication and progression could be followed in 3D cultures of PDAC primary cells. For this, we formed tumoroids of PDAC087T cells and infected them with 500×10^3 PFU of SG33-ANCHOR, with noninfected tumoroids as control. Seven days later, viral replication was detected and, similarly to what was seen in RK13 spheroids, SG33-ANCHOR altered spheroid composition with a significant increase of cellular debris (Fig. 4G). In addition, viral particles can be detected in detached dying/dead cells, as seen by fluorescent signals in cell debris around the spheroid. Overall, we managed to detect SG33 replication and propagation within 3D tumor models, to induce cancer cell death.

We next investigated whether SG33-ANCHOR replication and antitumoral activity could be characterized in experimental tumors, *in vivo*. We selected PDAC087T cells that were engrafted subcutaneously in athymic mice. When tumors were palpable, 5×10^5 PFUs of SG33-ANCHOR were injected intratumorally. Control tumors received PBS. Tumor size was measured using a caliper. We found that SG33-ANCHOR infection impairs tumor burden (Fig. 5A) and delays tumor growth ($-38\% \pm 6\%$, $p < 0.001$, Fig. 5B). We performed echography analyses at the end of the infection protocol and before autopsy and measured a mean 25% decrease in tumor size in the SG33-ANCHOR–treated tumor group, as compared with tumors receiving placebo (Fig. 5C). Tumors were sampled and analyzed for fluorescence using the Ivis Spectrum. Results in Fig. 5D illustrate the presence of infected cells within tumors treated with SG33-ANCHOR.

Lastly, tumors from both groups were homogenized, and cell-free supernatant was used to infect reporter RK13 cells to quantify tumor viral load. We found that cells infected with cell-free supernatant from SG33-ANCHOR–treated tumors developed infection, as compared with cells treated with cell-free supernatant from control tumors (Fig. 5E, F). Collectively, these data demonstrate for the first time that SG33 is oncolytic for PDAC cells and that the ANCHOR system allows for rapid characterization of cancer cell susceptibility to OV infection and lysis, both *in vitro* and *in vivo*

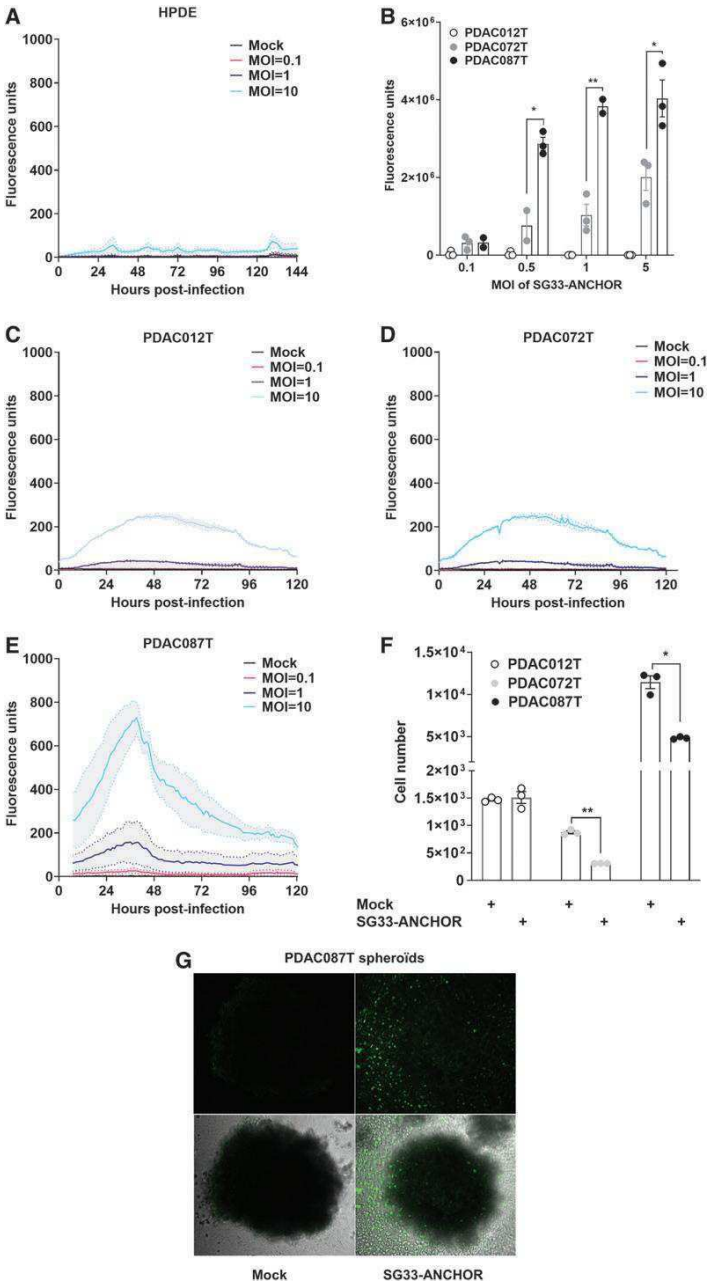
DISCUSSION

OVs are innovative therapies that could be extremely beneficial within the treatment plan of patients with cancer. For this, however, a deep understanding of viral infection, replication, and spread as well as defining the oncolytic activity of these viruses are prerequisites before their use in a clinical context. The ANCHOR system is a novel tool of genetic engineering that allows for the noninvasive detection of DNA in live cells. In this study, we generated for the first time OV genomes equipped with the ANCHOR system to quantify OV replication and oncolytic activity in live cells, at single-cell and populations levels.

In proof-of-concept studies, we confirmed the infectious and lytic capacity of SG33, a new vaccinal strain of MYXV,[20,27] and demonstrate that SG33 outranks parental T1 Lausanne, in a dose-dependent manner. We also monitored the precise timing for SG33 to replicate, expanse to neighboring cells, notably confirming propagation using cytoplasmic bridges[25] to induce cell death, and demonstrated viral spreading within 2D and 3D cellular models. Undoubtedly, such discoveries were largely facilitated by the ANCHOR system, which demonstrate equivalent performance with end-point qPCR for viral DNA genome.

As stated before, the lytic potential of SG33 was previously shown[27]; in this study, we demonstrate that the ANCHOR system does not hamper the replication and lysis capacities of this virus, paving the way for future theranostic strategies. However, we believe there is

Fig. 4. Characterization of SG33 replication and oncolytic activity in primary cells derived from pancreatic cancer. **(A)** HPDE normal pancreatic cells were infected with SG33-ANCHOR at the indicated MOI and imaged every 3 h using the Incucyte Zoom. Viral replication was measured by fluorescence at the indicated time using the Incucyte Zoom software. Results are mean ± s.e.m. of five independent experiments performed in triplicate. **(B)** Human PDAC primary cells were infected at the indicated dose of SG33-ANCHOR and SG33 DNA replication was quantified as mean viral DNA content per infected cells and expressed as fluorescence units. Results are mean ± s.e.m. of three independent experiments performed in duplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$. Primary cancer cell lines PDAC012T **(C)**, PDAC072T **(D)**, and PDAC087T **(E)** were infected by SG33-ANCHOR at the indicated dose. Viral replication was quantified noninvasively using the Incucyte Zoom replication. Results are mean ± s.e.m. of three independent experiments performed in triplicate. **(F)** Cell number was quantified 72 h following infection of primary cells with MOI = 5 of SG33-ANCHOR. Results are mean ± s.e.m. of three independent experiments. $***p < 0.005$. **(G)** PDAC087T spheroids were generated and infected by SG33-ANCHOR at the indicated dose. Confocal microscopy was used to image spheroids that were noninfected (*left panels*) or infected with 500.10^3 p.f.u. of SG33-ANCHOR (*right panels*). *Top:* fluorescence only. *Bottom:* merged florescence and brightfield. Results are representative of two independent experiments.



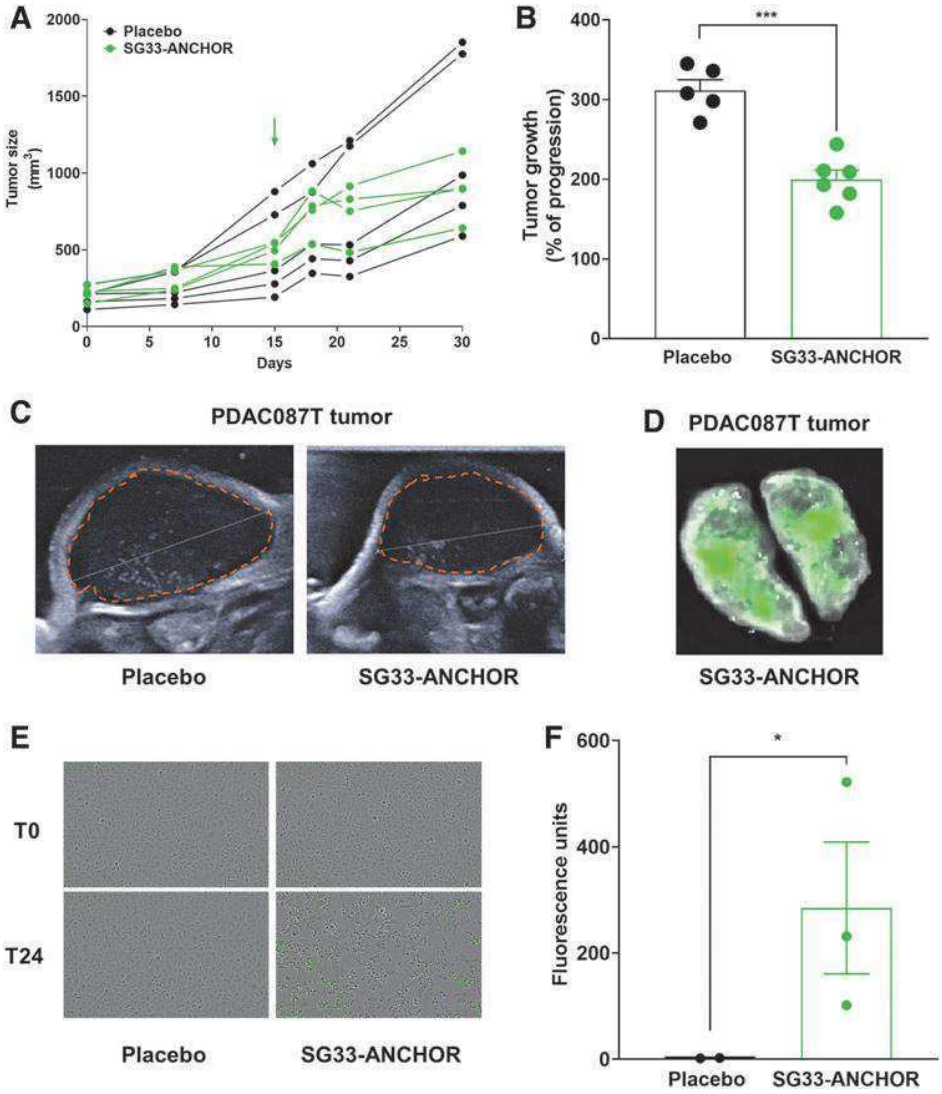


Fig. 5. SG33-ANCHOR antitumoral efficacy and tracking in experimental pancreatic tumors. Experimental pancreatic tumors were generated following engraftment of PDAC087T cells in athymic mice. 5x105 p.f.u. of SG33-ANCHOR were injected intratumorally. Control mice received PBS (placebo, $n=5$ mice per group). **(A)** Tumor size monitoring in untreated and SG33-treated mice at the indicated times. The *green arrow* represents time of injection. **(B)** Tumor size was measured at day = 0 and day = 15 following SG33-ANCHOR treatment. Results are represented as mean ± s.e.m of tumor progression with 5 mice per group. ***$p<0.005$. **(C)** Representative echography at day = 15 following infection of untreated tumors (*left panel*) and tumors that received SG33-ANCHOR (*right panel*). **(D)** At day = 15, tumors injected with SG33-ANCHOR were sampled and imaged for fluorescence using the Ivis spectrum. Tumors were minced and lysed to recover virus. Tumors receiving PBS were used as control. Reporter RK13 cells were infected with cell-free supernatant from control tumors receiving placebo, or tumors infected by SG33-ANCHOR and viral replication was imaged **(E)** and quantified **(F)** using the Incucyte Zoom. Results are represented as mean ± s.e.m of three cell-free supernatant.

still room for improvement, notably using concomitant live detection of viral proteins fused to fluorescent proteins, when analyzing spheroid or whole tumor infection in animals.

We then demonstrated for the first time that SG33 has oncolytic potential for PDAC, a disease with no cure, that is eligible for virotherapy.[18] We infected primary human PDAC cells that recapitulate tumor phenotype and genotype diversity and defined different profiles of viral replication and cytopathic effects. Indeed, PDAC087T cells displayed the highest level of viral replication and subsequent cell death; PDAC012T and PDAC072T cells, however, showed comparable levels of SG33 replication but only PDAC072T cells were significantly lysed by the virus. Notably, we found that SG33-ANCHOR successfully infected and replicated into PDAC tumoroids, to induce cell death.

Hence, we demonstrated the antitumor capacity of SG33-ANCHOR *in vivo* since treated experimental tumors presented significant delays in tumor growth when compared with control tumors. As our study demonstrates heterogeneity in virotherapy efficacy in PDAC primary cells, it remains essential to identify cellular and molecular

factors that govern SG33 efficacy in PDAC cells to accelerate clinical transfer. Importantly, SG33 originates from the *in vivo* recombination between a wild-type South American (T1 Lausanne) strain and a California MSD-derived strain, and carries a large deletion in the right open reading frame of its genome.[20] In greater details, SG33 genome lacks one copy of the viral m-t5 ankyrin coding sequence that coordinates with Akt cellular signaling to establish a new cellular environment more favorable for virus replication.[28] As this protein is partially lost in SG33, we obtained preliminary results indicating that AKT status is not fully predictive of PDAC cell infection and lysis by SG33 (data not shown).

We speculate that complemental high-throughput screening studies conveyed with the ANCHOR system following protocols established during this study are in reach to identify the molecular mechanisms involved in PDAC cells permissiveness to SG33 viral infection and oncolysis. Together with transcriptomic investigations, this will help define a molecular signature predictive of SG33 efficacy for patient stratification, and to devise new combinations to improve the therapeutic index of virotherapy.

To date, the human sodium/iodide symporter (hNIS) is the most prevalent reporter transgene to generate precise insights into the kinetics of gene expression, viral biodistribution, toxicity, and therapeutic outcomes in the field of virotherapy. hNIS promotes the accumulation of radiotracers at the site of transgene expression,[29] both in preclinical models and in patients.[30] Yet, this reporter system has several disadvantages, such as a limited intracellular retention capacity of radiotracers in hNIS-infected cells and potentially dangerous radiotracer uptake by both malignant and normal tissues. These include the thyroid gland and the digestive tract.[31]

During this study, we demonstrate that ANCHOR ''all-in-one system'' allows for OV detection in tumors, up to 15 days after infection, without hindering viral replication or spread, and without the need of any supplemental tracers. Remarkably, the ANCHOR system is compatible with tumor viral load tittering *ex vivo*, which is mandatory to address the viral pharmacokinetics and to adapt treatment in patients. However, we failed to detect SG33 growth in tumors in live animals using GFP-labeled viral genomes, probably because of high-background autofluorescence due to animal hair bulbs and chlorophyll in food. Studies are currently undergoing to generate novel SG33 genomes equipped with red fluorescent proteins such as Santaka to allow for noninvasive in depth tissue fluorescent imaging of the virus.

CONCLUSION

Several oncolytic strains of viruses are now developed for the therapy of hematologic and solid tumors.[32] For all of them, novel strategies for the noninvasive monitoring of OV biodistribution and efficacy are key to overcome the current limitations of oncolytic therapy. Poxviruses such as MYXVs may take the stage as they are nonpathogenic for humans, are large virus with DNA genomes that can be equipped with therapeutic genes of interest, can be administered locally and in the blood to target distant metastasis, and can induce an immune response against the tumor.[33]

By nature, SG33 holds the promise of increased specificity toward cancer cells and greater mobilization of immune cells, as already demonstrated for rabbit and ovine bone marrow-derived dendritic cells.[34] In this study, we demonstrate not only that SG33 replicates to higher levels than MYXV T1 and that SG33 is oncolytic for primary cancer cells, but also that the ANCHOR system provides sufficient specificity and efficacy necessary for longitudinal monitoring of SG33 viral infection in complex models without hampering viral fitness.

Considering that SG33 is a clinical-grade veterinary vaccine with limited manufacturing constraints that could be easily repurposed for human health, our study advocates for additional preclinical investigations to accelerate the clinical transfer of SG33-ANCHOR for the benefit of PDAC patients.

AUTHORS' CONTRIBUTIONS

Conceptualization was carried out by P.C., S.B., F.G., C.C., L.B., and N.D.; investigations were done by L.Q., S.T., S.K.-G., A.R., C.Q.-F., and H.L.; supervision was performed by P.C., S.B., and F.G.; writing—original draft—was by P.C. and L.Q.; writing—review and editing—was carried out by S.B., N.D., and F.G.; funding acquisition was taken care of P.C., S.B., and N.D.; and data analysis was carried out by P.C., S.B., F.G., L.Q., and A.R.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Movie S1
Supplementary Movie S2

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Discussion

This study has taken advantage of a novel technique allowing the visualisation and quantification of viral infection in real time conditions to investigate the oncolytic potential of SG33 in PDAC models.

We first confirmed the use of the ANCHOR system to quantify viral genome and replication. In permissive rabbit kidney cells (RK13) we managed to demonstrate SG33 replicates at higher levels than parental T1 Lausanne strain of MYXV, exemplifying SG33 as a more potent prospective anticancer treatment of pancreatic cancer. From there we sought to define the viral replication cycle precisely in live cells, first through the development of DNA replication factories then by quantifying the time needed between the beginning of viral replication, maximum replication, viral propagation and cell death. With this precise quantification, we found a difference in viral cycle depending on viral input quantity, with a higher input leading to shorter cell-to-cell propagation. When investigating at the population level, SG33 replication in RK13 cells was found to be dose-dependent which was synonymous with a decrease in cell proliferation and augmentation of cell death in both 2D and 3D spheroid models.

When applying these non-invasive real-time approaches to a panel of primary PDAC cell lines, we found variations in viral replication and oncolysis that were cell dependent which raised three different profiles: permissive cells that actively replicated SG33 leading to cell death, resistant cells with no significant viral replication or cell lysis, and finally cells that presented viral replication but not as significant oncolysis. Furthermore, in permissive PDAC cells, we found it possible to detect viral replication in both 3D spheroid models and in subcutaneous tumours, suggesting the ANCHOR system allows for viral replication monitoring in both in vitro and in vivo models.

The possibility of following SG33 in live models paves the way for the development of theranostic platforms. Indeed, SG33-ANCHOR could be used to detect and treat micrometastases following systemic administration. Furthermore, infection of patient tumour samples ex vivo would help to quickly determine their susceptibility to virotherapy. Prior to the clinical and theranostic stage, it is important to fully extrapolate SG33's uses and applications. To do so, one should investigate the optimal route of delivery and pharmacokinetics involved

following SG33 administration as well as consider the virus' involvement within the tumour microenvironment and the impact of PDAC heterogeneity and the complex crosstalk on its activity.

Before arriving at these larger scale investigations, it is important to elucidate completely SG33's antitumoral activity in tumour cells and tackle the issue of heterogeneity as seen in primary derived PDAC cell lines. One of the profiles identified shows a lack of viral replication and cell death indicating a resistance in early stages of viral cycle. This blockage could be identified, and subsequently alleviated, by determining potential antiviral pathways present within these cells but absent in permissive cells, through transcriptomic analyses of these cell lines before and after infection. Another profile seen was low replication of SG33 within the cells but active oncolysis. In this case, the cells are more susceptible to virus-induced cell death, which could be detrimental if the rapid oncolysis hampers SG33's ability to effectively replicate and spread within the population. Therefore determining oncolytic mechanisms triggered by SG33 is important. Furthermore, this could also unveil potential combinations with other antitumoral compounds, in particular immunotherapies to help combat tumours on several fronts.

To determine the immunostimulatory potential of SG33 oncolysis of PDAC cells, we next analysed the immunogenic cell deaths that follow viral infection of permissive PDAC cells.

Part 2: Immunogenic cell death in SG33 oncolytic activity

Immunogenic cell death

Myxoma virus and Immunogenic cell death

Several oncolytic viruses have been attributed the ability to induce an immune stimulation following lysis of tumour cells. Examples include the measles virus that has been shown to induce apoptotic cell death in mesothelioma cells, leading to the phagocytosis by dendritic cells and subsequent priming of CD8+ T cells.[316] DC maturation and cross-presentation to CD8+ T cells was also observed in a melanoma model infected with reovirus.[317] Finally, a 2020 study characterised immunogenic cell death (ICD) of wild type adenovirus, semliki forest virus (SFV) and vaccinia virus in osteosarcoma (HOS) and lung carcinoma epithelial (A549) cells. Ad-infected cells underwent multiple ICD-associated cell death mechanisms including necroptosis, pyroptosis and autophagy, whilst SFV induced highly immunogenic apoptosis. VV immunogenic tumour cytolysis was achieved through necroptosis and autophagy,[318] although interestingly a previous study found VV-induced necroptosis in ovarian cancer cells was independent to TNFα signalling, suggesting an alternative to autocrine cytokine release and stimulation.[319]

Although similarities between poxviruses have been used to clarify myxoma virus' activity based on the extensively studied VV, disparities between the two are noted, for example in terms of tropism.[300] [320] These differences could be expanded to include oncolytic activity and immunostimulatory potential, which warrants the need for further study of MYXV as a potential immunogenic OV. Immunogenic cell death analysis in Myxoma virus has been limited. Recently, MYXV infection of small-cell lung cancer (SCLC) led to the release of the ICD marker ATP. In addition, the intratumoral delivery of myxoma virus in an immunocompetent murine model of SCLC induced significant necrosis predicted to release tumour-associated antigens, and resulted in immune cell infiltration and tumour growth inhibition. This immunogenic response was also accompanied by rapid MYXV clearance.[321] These results support the

importance of an intact immune system suggested by Wennier et al. in PDAC cell models. Indeed, survival benefits were noted when comparing immunosuppressed and immunocompetent PDAC murine models infected with myxoma virus, with 60% of long-term survivors in the latter. The benefit of an intact host immune system was additionally observed when sequentially combining MYXV and gemcitabine. [312]

The presence of an immune system with MYXV virotherapy also presents an obstacle. For example, myxoma virus was found to induce an early antiviral response in immunocompetent melanoma[322] and glioma[323] models. The rapid clearance of the virus seen in the SCLC immunocompetent murine model compared to immunodeficient models,[321] suggests that tumour-infiltrating immune cells target virally-infected cells therefore restricting the duration of viral presence in the tumour and in consequence hinder the virotherapy efficacy. In such a case, the attenuated phenotype of the SG33 strain, probably caused by deletion of the region including immune modulators, can be a solution to improve tumour-based virus half-life. In addition, the deletion of certain MYXV genes copies, such as *M152R* an anti-apoptotic factor and *M154R* homologous to VV's NFκB inhibitor, could potentiate SG33's ability of inducing immunogenic cell deaths in tumour cells.

ICD markers

The Nomenclature Committee on Cell Death (NCCD) has proposed to define any regulated cell death that induces an adaptive immune response in immunocompetent hosts as Immunogenic Cell Death (ICD). This adaptive immune response can be triggered by endogenous or cellular antigens, or exogenous antigens such as those expressed through viral infections. Apart from viral causes, other triggers of ICD include treatments by certain chemotherapies (Anthracyclines for example), radiotherapy and photodynamic therapy.[324], [325] Stimulation of the immune system is achieved through the recognition of danger-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs), warning the immune system of a state of danger by activating antigen presenting cells such as DCs and macrophages. According to the recommendations of the NCCD updated in 2018, there are six DAMPS mechanistically linked to ICD.

- Calreticulin:

Calreticulin (CALR) translocation from the endoplasmic reticulum (ER) to the outer leaflet of the plasma membrane. CALR is originally involved in the maintenance of calcium homeostasis within the ER and its translocation to the membrane occurs within the early stages of immunogenic cell death. CALR translocation is preceded by the phosphorylation of eukaryotic translation initiation factor 2 subunit alpha (EIF2S1 or eIF2α) by eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3 or PERK) alongside a protein synthesis block and associated with ER stress. Equally paving the way is the cleavage of B-cell receptor-associated protein 31 (BCAP31), BAX and BAK, dependent on caspase 8, and the anterograde transport of calreticulin towards the plasma membrane involving the Golgi apparatus with vesicle-associated membrane protein 1 (VAMP1) and synaptosomal-associated protein 25 (SNAP25). CALR exposed at the plasma membrane will act as an "eat me" signal for immune cells such as Macrophages, dendritic cells (DC) and neutrophils, necessary for antigen cross-presentation to cytotoxic T cells. Furthermore, cell surface exposure of CALR allows for priming of Th17 cells. This "eat me" signal is antagonised by CD47.

- ATP:

Release of adenosine triphosphate (ATP) via autophagy-dependent accumulation in autolysosomes, lysosomal-associated membrane protein 1 (LAMP1) translocation to the plasma membrane, rho-associated, coiled-coil-containing protein kinase 1 (ROCK1)-dependent cell blebbing and opening of pannexin 1 (PANX1) channels. Released ATP is a chemotactic agent, a "find me" signal for macrophages, neutrophils and DC precursors, by binding to purinergic receptor P2Y G-protein coupled (P2RY2). Extracellular ATP also activates the canonical inflammasome by binding to P2RX7 receptor, thus inducing immunostimulatory effects.

- HMGB1:

High mobility group box 1 (HMGB1) released within the extracellular milieu will bind to Toll-like receptors 2 and 4 eliciting pro-inflammatory effects. Upon binding to dendritic cells' TLR4,

HMGB1 stimulates antigen processing and cross-presentation although has no impact on DC maturation. HMGB1 can be either pro-inflammatory or anti-inflammatory, however, depending on its oxidation state.

- Other markers:

Similarly to ATP and HMGB1, annexin A1 (ANXA1) is released during ICD and will stimulate the formyl peptide receptor 1 (FPR1) found on phagocytic cells, adding on to the chemotaxis effect.

Detection of endogenous double-stranded (ds) RNA by TLR3 or dsDNA by cGAS in malignant cells results in the production of type 1 interferon (IFN). IFN1 can contribute to an autocrine/paracrine loop in surrounding tumoral cells leading to their expression of IFN-stimulated genes (*ISG*s) such as C-X-C motif chemokine ligand 10 (CXCL10), a T cell chemoattractant. The release of dsRNA and dsDNA by cancer cells also leads to their uptake by immune cells and the induction of a type 1 interferon response via multiple TLRs and the cGAS-STING pathway.

Cell deaths involved

As previously mentioned, ICD is characterised as being a consequence of programmed cell deaths. As so, multiple cell lysis pathways have been linked to the stimulation of an immune response.

- Apoptosis:

Apoptosis (Fig. 1), known as 'cell suicide', is induced by a cascade of caspases either through external stimuli – known as the extrinsic apoptotic pathway – or through intracellular stress for the intrinsic apoptotic pathway. Firstly, the extrinsic pathway follows the binding of TNF-α or Fas-L to their respective receptors, TNFR or FasR which induces the cleavage and subsequent activation of caspase 8. The intrinsic pathway is triggered by cell stress, releasing cytochrome c from mitochondria which leads to cleavage and activation of caspase 9. Both pathways

ultimately conclude with the cleavage and activation of effector caspases 3, 6 and 7 which degrade intracellular proteins executing the cell death protocol.

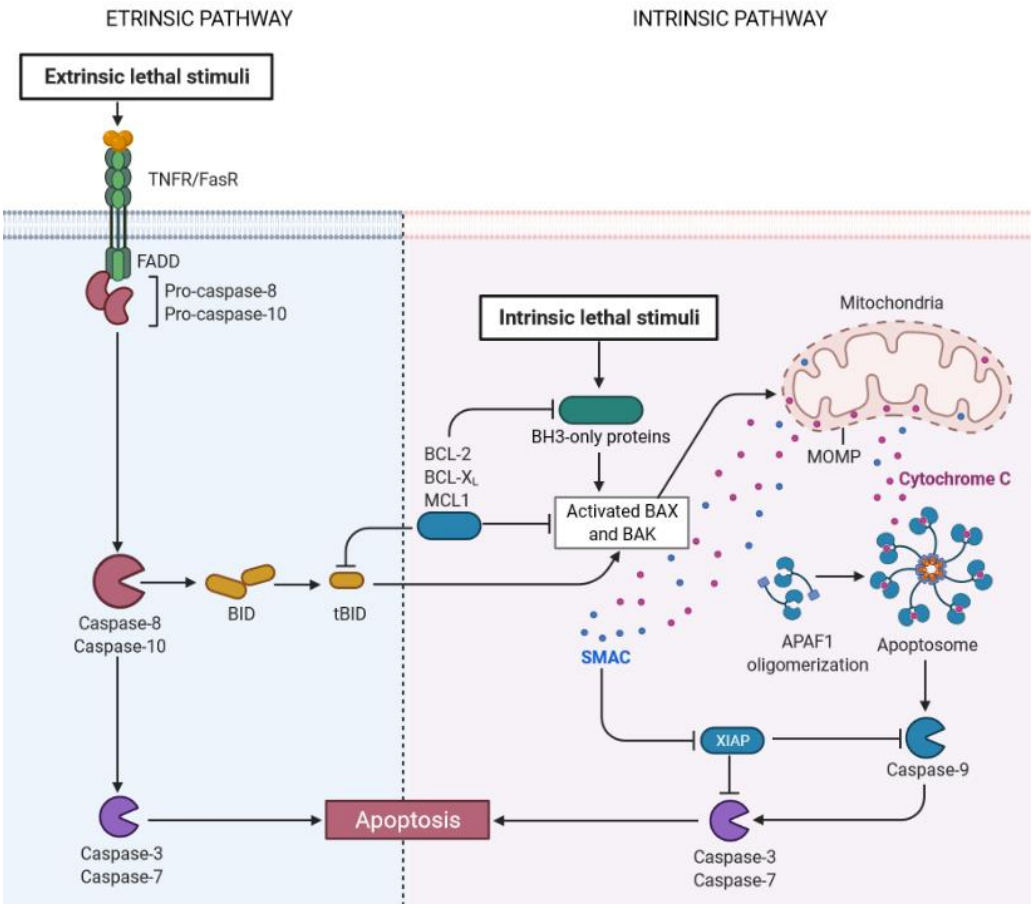


Figure 1: Apoptotic cell death

Through the release of DAMPs, immunogenic apoptosis has been shown to induce the maturation of dendritic cells and the activation of an antitumoral adaptive immune response.[326] On the opposite hand, caspases 3 and 8 have been shown to reduce the immunogenicity of cell deaths. For example, caspase 3 increases the phagocytic uptake of dying cells and anti-inflammatory signals through increased exposure of phosphatidylserines.[327] In addition, caspase 3 increases immunosuppressant prostaglandin E2 secretion from lytic cells[320] [321] and inhibits type 1 IFN signalling of mitochondrial DNA release.[330]

- Necroptosis:

Necroptosis (Fig. 2) can be triggered due to cell stress, damage or infection. The activation of the necroptotic route is determined on whether cells express receptor-interacting protein kinase 3 (RIPK3) and the inhibition of caspase 8. Indeed, when caspase 8 is expressed, necroptosis is blocked and cells are rerouted towards the extrinsic apoptosis pathway.[331] In the absence of caspase 8, ubiquitination of RIPK1 (also known as RIP) ensures its activation through phosphorylation, ensuing the recruitment of RIPK3 and the phosphorylation of Mixed Lineage Kinase domain Like pseudokinase (MLKL) forming the necrosome.[332] Thus proceeds the generation of pores in the plasma membrane, the swelling of intracellular organelles and the secretion of proinflammatory signals, such as highly immunogenic DAMPs and cytokines through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The triggering of necroptosis has been proposed as an alternative in cancer-based treatments to overcome apoptosis resistance.[333]

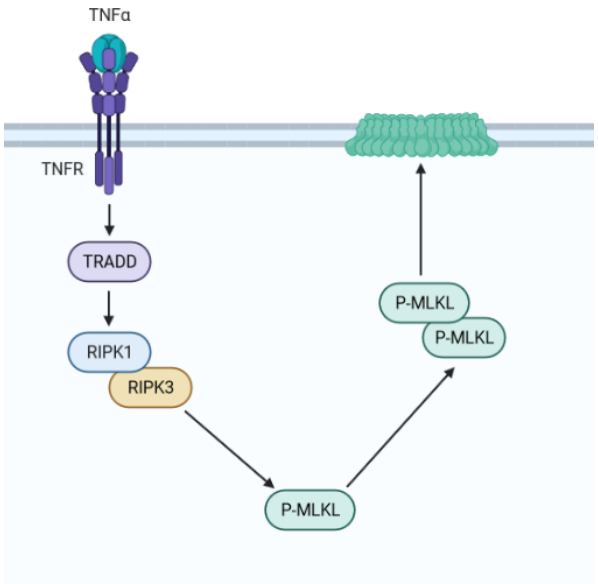


Figure 2: Necroptosis cell death

- Autophagy:

Autophagy (Fig. 3), regulated by kinases such as mTOR and AMPK, involves the inhibitory phosphorylation of ULK kinases. These kinases form a complex with conserved autophagy-

related proteins (Atg) and Beclin-1 (Atg6).[334] This complex is transported to the phagophore, the autophagosome initiation site, where it helps activate downstream autophagy elements. Phosphatidylinositol 3-kinase (PI3K) type III contributes to the initiation of autophagic isolation membranes by promoting the invagination of plasma membrane at sites rich in phosphatidylinositol-3-phosphate (PI3P). The expansion and closing of these isolation membranes depend on the Atg5-Atg13 pathways and microtubule-associated protein 1 light chain 3 (LC3) translocation from the cytosol and sustained lipidation.[335] The catabolic process that ensues will degrade cytoplasmic proteins and cellular organelles found within the autolysosomes formed through the fusion of autophagosomes and lysosomes, and the release of the degradation products includes the release of DAMPs and neoantigens. Indeed, autophagy has been reported to contribute to the presentation of tumour antigens through both major histocompatibility complex (MHC) I and II.[336] The role of autophagic processes in cancer is incongruous since autophagy has also been shown to promote tumorigenesis and progression, therefore further studies elucidating the properties of autophagy is warranted.[337]

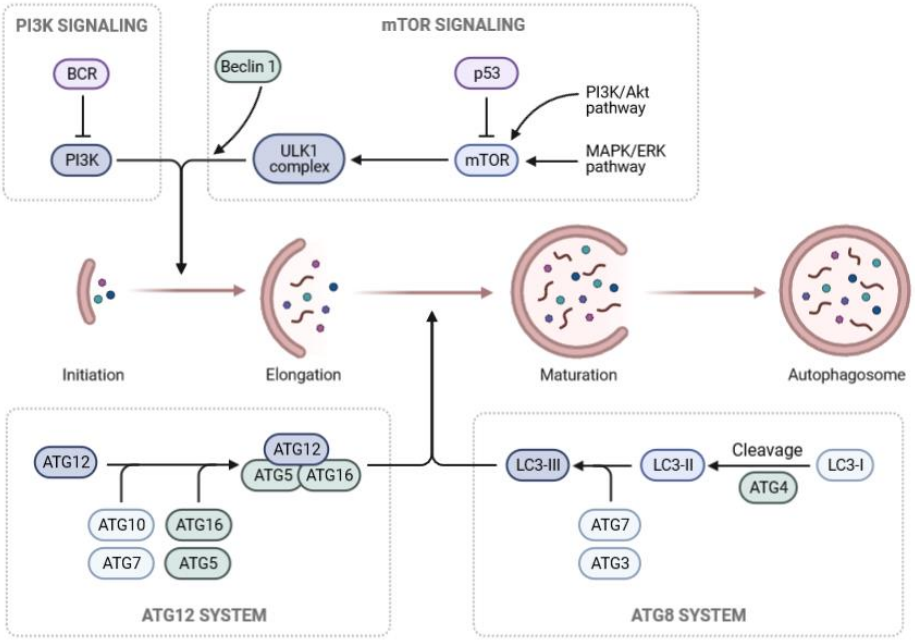


Figure 3: Autophagy pathway

- Pyroptosis:

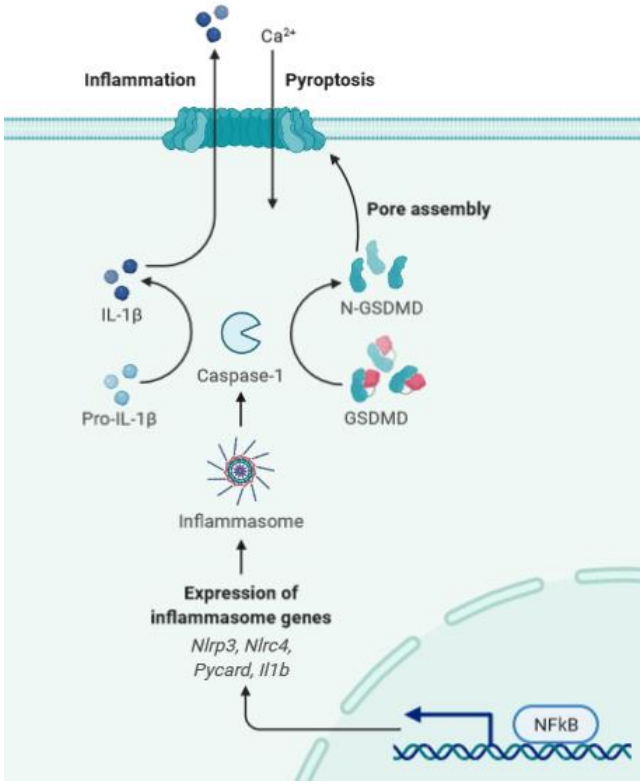


Figure 4: Canonical pyroptosis pathway

Canonical pyroptosis (Fig. 4) involves the formation of pores in the plasma membrane through the cleavage and activation of gasdermin D (GSDMD) protein mediated by caspase 1 following the inflammasome pathway. Caspase 1 is also found to activate interleukin (IL) 1β and IL18, contributors of inflammation during pyroptosis. If GSDMD is lacking, caspase 1 will redirect via the apoptosis route by activating caspases 3 and 9 of the intrinsic apoptotic pathway. In addition, in certain cases caspase 8 can induce pyroptosis by itself cleaving gasdermin D. Noncanonical pyroptosis also involves GSDMD but through the activation of caspases 4, 5 and 11 after binding to gram-negative bacterial lipopolysaccharide (LPS). Finally, the pyroptotic cell death pathway can also be achieved through gasdermin E (GSDME) activated by caspases 3 and 8 of the extrinsic apoptotic pathway. Pyroptosis leads to the activation and release of pro-inflammatory signals IL-1β and IL-18 as well as DAMPs such as HMGB1 and LDH which amplifies inflammation and the recruitment of immune cells.[338]

- Other immunogenic cell deaths:

Other regulated cell death pathways that participate to ICD include ferroptosis and parthanatos. Ferroptosis is triggered by iron- and reactive oxygen species (ROS) oxidising polyunsaturated fatty acids (PUFA), leading to the accumulation of lipid peroxides. For ferroptosis to occur, the glutathione-dependant antioxidant defence is ineffective against the accumulation of toxic PUFA. Parthanatos, or PARP-1 dependant cell death, is due to the presence of DNA damage and genotoxic stress, recognised by poly(ADP-ribose) polymerase-1 (PARP-1) which leads to the accumulation of poly(ADP-ribose) (PAR) and nuclear translocation from mitochondria of apoptosis-inducing factor (AIF). This results in DNA fragmentation and cell death.[339]

Encompassing the immune system

Following tumour cell death DAMPs and TAAs are released and bind to PRRs on macrophages, dendritic cells and natural killer cells.[340] Certain motifs such as secreted ATP will promote immune cell migration acting as a 'find me' signal. Macrophages engulf and degrade dying cells, enabled by 'eat me' signals such as membrane exposure of CALR, allowing the resulting antigens to be presented by antigen-presenting cells (APCs) such as DCs.[341] On the contrary, healthy cells present 'don't eat me' signals, such as CD47 whose upregulation can be exploited by tumour cells to evade the immune system.[342]

These activated DCs then travel to secondary immune locations, for example lymphoid organs, where they mature and come in contact with naïve T cells. T cell activation requires the cross-presentation of tumour antigens presented by the dendritic cells towards the CMHI or CMHII of CD8+ and CD4+ T cells respectively. Additionally a co-stimulatory signal is needed to efficiently activate T cells. This signal is ensured by co-stimulatory receptors found on DC cells like CD80 and CD86. Next, signals delivered by DCs including IL-12 and type I interferon contribute to the polarisation and differentiation and final step of the priming of effector T cells.[343] These tumour-directed T cells produce IFN-γ promoting anti-cancer activity.

The immune-deficient and immunosuppressive nature of pancreatic cancer is supplemented by the immunomodulatory nature of tumour cells. These mechanisms include the up-regulation of immune checkpoints (PD-L1, CTLA4), the downregulation of T cell co-stimulation signals and the recruitment of immunoregulating cells such as myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages. Some immunotherapies have shown promising results, such as GM-CSF and tumour vaccines, with increased overall survival (OS) of patients with resected pancreatic cancer when combined with chemoradiation. However to date there are no immunotherapies approved as standard-of-care for pancreatic cancer.[344] Although PDAC have been thought as being deprived of immune populations, Balachandran et al. have illustrated the immune heterogeneity found in pancreatic tumours is not only due to the quantity but also the quality of tumoral neoantigens. This heterogeneity would not only dictate the response to immunotherapies, but also prognosis, with neoantigen-specific immunity acquired during primary tumour development associated with decreased relapse and prolonged survival.[154]

To evaluate SG33 as an entity capable of inducing an antitumour immune response, it is important to understand the mechanisms behind viral oncolysis and the impact it has on the immune system. This could potentially help us understand the heterogeneity in cell lysis seen among primary PDAC cell lines and open the possibility of using immunotherapies in combination so as to increase SG33 antitumoral activity and alleviate certain obstacles in oncolysis.

Materials and methods

Cell line and culture conditions

Pancreatic cancer patient-derived primary cells PDAC087T were obtained as previously described;[345] Cells were incubated at 37°C with 5% CO_2 and cultured in Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific, Illkirch, France) 4.5g/L glucose containing 10% fetal bovine serum (FBS; ThermoFisher Scientific), 100 IU/mL penicillin (ThermoFisher

Scientific), 100µg/mL streptomycin (ThermoFisher Scientific), and 250ng/mL fungizone (ThermoFisher Scientific), 2mM glutamine (Sigma), and 100 IU/mL plasmocin (Invivogen) (hereafter referred to as complete medium).

Virus and viral replication

SG33-ANCHOR was obtained as previously described.[346] All the multiplicity of infection (MOI) used in this study is based on PFU/cell determined with RK13 cells. All infections were done at an MOI of 1.

Cells were seeded in 96-well plates (Corning) at 10×10^3 cells per well in 100µL of complete medium. After 24h, cells were washed with PBS and infected with SG33-ANCHOR during 1h at 37°C in 100µL of phenol-free complete culture medium. Infection medium was then replaced with phenol-free complete culture medium supplemented with the corresponding inhibitors. Viral replication was measured with the IncuCyte Zoom (Sartorius) at 10× using a green filter (Ex: 440–480nm, Em: 504–544nm) and analysed using the Zoom2018B software.

Calreticulin (CRT) exposure

Cells were seeded in 60mm dishes (Corning) at 320×10^3 cells per dish in 3mL of complete medium. After 24h, cells were washed with PBS and infected with SG33-ANCHOR during 1h at 37°C in 200µL of phenol-free complete culture medium. Infection medium was then replaced with phenol-free complete culture medium. Cells treated by 0.5µM of Idarubicin were used as a positive control. Cells were collected at 6-, 24- and 48-hours post-infection, fixed with 100µL formalin (Sigma) according to manufacturer's instructions and 90% cold methanol, before being stained with a conjugated anti-calreticulin antibody (Cell Signaling 77344). Cells were analysed using the MACSQuant VYB flow cytometer (Miltenyi Biotec) with the V2 (525/50 nm) channel to detect viral replication (GFP) and the Y3 (661/20 nm) channel to detect CALR.

Extracellular ATP

Cells were seeded in 96-well plates (Corning) at 10×10^3 cells per well in 100µL of complete medium. After 24h, cells were washed with PBS and infected with SG33-ANCHOR during 1h at 37°C in 100µL of phenol-free complete culture medium. Infection medium was then replaced with phenol-free complete culture medium. Cells treated by 0.5µM of Idarubicin were used as a positive control. Supernatants were collected at 6-, 24- and 48-hours post-infection and extracellular ATP was measured with an ATP detection kit (CellTiter-Glo Luminescent Assay, Promega) according to manufacturer's instructions. Luminescence was measured with a Clariostar plate reader.

HMGB1 release

Cells were seeded in 96-well plates (Corning) at 10×10^3 cells per well in 100µL of complete medium. After 24h, cells were washed with PBS and infected with SG33-ANCHOR during 1h at 37°C in 100µL of phenol-free complete culture medium. Infection medium was then replaced with OptiMEM culture medium supplemented with 2% fetal bovine serum (FBS; ThermoFisher Scientific), 100 IU/mL penicillin (ThermoFisher Scientific) and 100µg/mL streptomycin (ThermoFisher Scientific). Cells treated by 0.5µM of Idarubicin were used as a positive control. Supernatants were collected at 6-, 24- and 48-hours post-infection and HMGB1 was measured with ELISA kit (IBL International) according to manufacturer's instructions. Optical densities were measured at 450nm (Reference wavelength 600-650nm) using a Clariostar plate reader.

Cell death pathways inhibitor treatments

Cells were seeded and infected as previously mentioned. Culture or infection medium was replaced with phenol-free complete culture medium supplemented in pharmacological inhibitors. Necrostatin-1 (Cell Singaling 17802) a RIPK1 inhibitor was used at a final concentration of 10µM. Caspase inhibitors Z-DEVD-fmk (Calbiochem 264155), Z-IETD-fmk (Calbiochem 218840), Ac-LEHD-fmk (Calbiochem 218728) and Y-VAD-fmk (Calbiochem 218746)

which selectively block caspases 3, 8, 9 and 1 respectively were used at final concentrations of 10µM each. Chloroquine diphosphate blocks autolysosome formation and was used at a final concentration of 12µM.

Cell proliferation analysis

Cells were seeded in 60mm dishes (Corning) at 320×10^3 cells per dish in 3mL of complete medium. After 24h, cells were washed with PBS and infected with SG33-ANCHOR during 1h at 37°C in 200µL of phenol-free complete culture medium. Infection medium was then replaced with phenol-free complete culture medium supplemented with the corresponding inhibitors. Cells were harvested at 48 hours post-infection washed in PBS and quantified using the Cell Counter (Beckman Coulter).

Western blotting of cell death markers

Cells were seeded in 100mm dishes (Corning) at 800×10^3 cells per dish in 10mL of complete medium. After 24h, cells were washed with PBS and infected with SG33-ANCHOR during 1h at 37°C in 500µL of phenol-free complete culture medium. Cells treated by 0.5µM of Idarubicin or 0.5µM of Staurosporine were used as a positive control. Cells were harvested at 6-, 24- and 48-hours post-infection washed in PBS and lysed in RIPA buffer (Bio Basic). Proteins in lysates were denatured by heating at 95°C for 5mins. Fifty micrograms of proteins were separated using 10% Tris SDS-PAGE gel (BioRad), electrotransferred to a nitrocellulose membrane and probed with antibodies. Antibodies were rabbit monoclonal and include anti-caspase 3 (Cell Signaling 14220), anti-PARP (Cell Signaling 9532), anti-RIP (Cell Signaling 3493), anti-MLKL (Cell Signaling 14993), anti-caspase 1 (Cell Signaling 3866), anti-gasdermin D (Cell Signaling 97558), anti-LC3A/B (Cell Signaling 12741) and anti-beclin-1 (Cell Signalling 3495). Immunocomplexes were detected by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling 7074). Specific protein bands were visualized using ECL Western Blotting Substrate (BioRad) and images were obtained by using ChemiDoc XRS+ (BioRad). Rabbit monoclonal anti-GAPDH (Cell signaling 5174) antibody was used to detect GAPDH which was used as a loading control.

Quantification and statistical analysis

Unless otherwise indicated, data represent mean±standard error of the mean of at least three biological replicates. Statistical significance was calculated by unpaired Student's test with Welch's correction, using Graphpad Prism 8.0 software (Graphpad). $p<0.05$ was considered statistically significant. *$p<0.05$.

Results

Immunogenic cell death in permissive primary pancreatic cancer cells

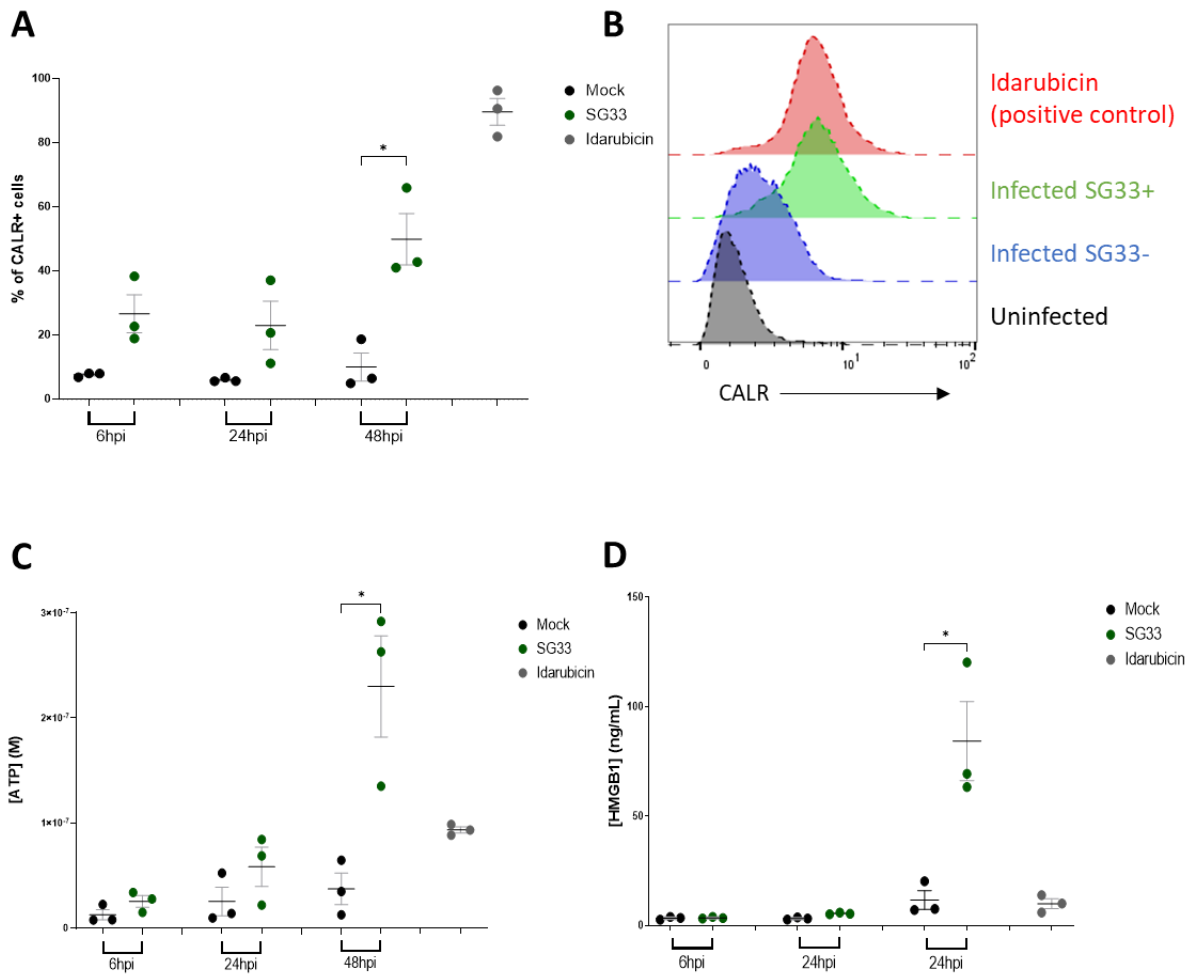


Figure 5: Detection of ICD-related DAMPs following SG33 oncolysis. (A) PDAC087T cells were infected at MOI 1 of SG33-ANCHOR and plasma membrane calreticulin exposure was detected at 6-, 24- and 48- hours post-infection. Results are mean±s.e.m. of three independent experiments performed in triplicate. ***(B)*** *Infected PDAC087T cells at 24 hours post-infection separated in actively viral-replicating (green curve) and non-replicating (blue curve) populations.* ***(C)*** *Quantification of ATP in supernatant of non-infected (mock) and infected PDAC087T cells at 6-, 24- and 48- hours post-infection. Results are mean±s.e.m. of three independent experiments performed in triplicate. *p<0.005.* ***(D)*** *Dosing of secreted HMGB1 in supernatant of non-infected (mock) and infected PDAC087T cells at 6-, 24- and 48- hours post-infection. Results are mean±s.e.m. of three independent experiments performed in duplicate.*

During this study we investigated the immunogenic cell death of SG33 oncolysis. To do so, we selected a primary human pancreatic cancer cell line, PDAC087T, which we previously showed

to be permissive to SG33 viral replication and cell lysis. Following SG33 infection of PDAC087T cells we detected markers of immunogenic cell death (ICD) at 6-, 24- and 48-hours post-infection. The three DAMPs associated with immunogenic potential of almost all ICD triggers consist in the translocation of calreticulin to the outer plasma membrane and the release of ATP and HMGB1 in the extracellular milieu. In this effect, we quantified membrane calreticulin and ATP and HMGB1 in the extracellular milieu of non-infected and cells infected with SG33. The antineoplastic Idarubicin was used as positive control.

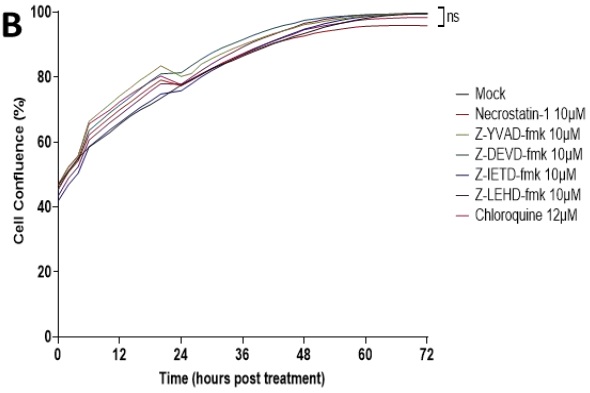
Our results show an increase an increase in CALR plasma exposure as early as 6 hours post-infection with over triple the amount of CALR+ cells in the infected population when compared to non-infected cells (7.62 ± 0.39 % 26.63 ± 5.93 % for uninfected and infected cells respectively at 6hpi). At 48 hours post-infection the difference is significative with nearly 50% of infected cells as CALR+ compared to less than 10% in non-infected (Fig. 5A). Furthermore, in the infected population we separated the cells actively replicating the virus (green curve) from the cells displaying no viral replication (blue curve) using the GFP emitted through the ANCHOR system (Fig. 5B). By comparing the mean fluorescence intensity of calreticulin, we found that the increase in CALR signal is present in the SG33-replicating cells with only a slight increase in fluorescence intensity in cells without SG33 replication. The quantification of ATP in the supernatant of infected cells (Fig. 5C) shows a later increase than that seen with CALR exposure. Indeed, extracellular ATP of infected cells when compared to non-infected cells increases at 48 hours post-infection with 37nM of released ATP compared to 230nM of released ATP for uninfected cells and infected respectively. Similarly to ATP, our results demonstrate that HMGB1 is secreted after CALR exposure. Indeed, at 48 hours post-infection we can see a significant increase in supernatant HMGB1, with an average of 11ng/mL for uninfected cells and 84ng/mL for infected cells, which is not the case at 6- and 24- hours post-infection (Fig. 5D).

Taken together, our results demonstrate that SG33 is capable of releasing immunogenic signals during oncolysis of permissive PDAC cells.

A

Cell death	Markers	Inhibitors
Apoptosis	Caspase 3, PARP	Z-DEVD-fmk
Extrinsic pathway	Caspase 8	Z-IETD-fmk
Intrinsic pathway	Caspase 9	Ac-LEHD-fmk
Necroptosis	P-RIP/RIP P-MLKL/MLKL	Nectrostatin-1
Pyroptosis	Caspase 1 Gasdermin D	Z-YVAD-fmk
Autophagy	LC3A/B I and II Beclin-1	Chloroquine diphosphate

B



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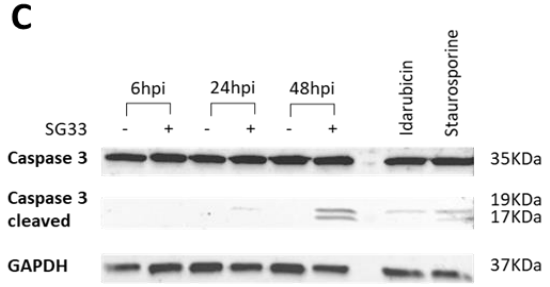

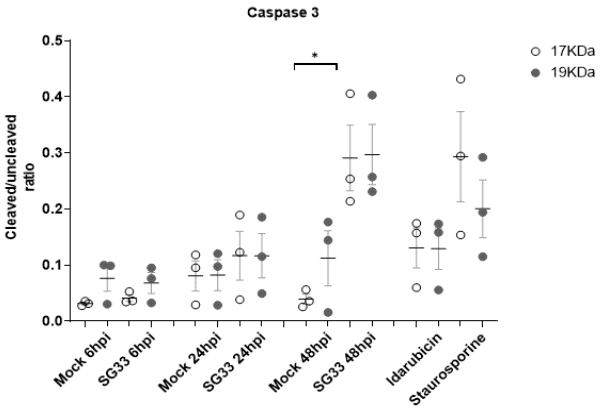

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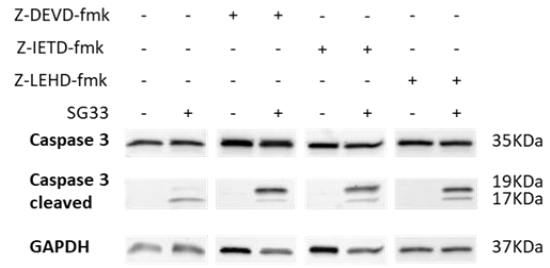
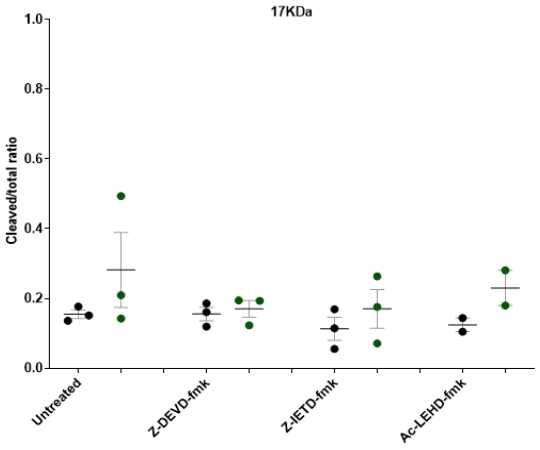
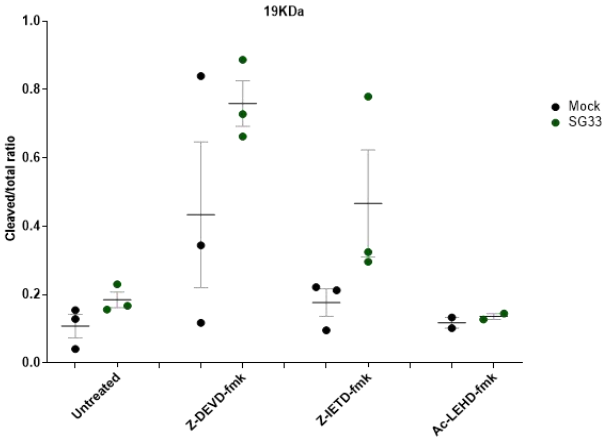






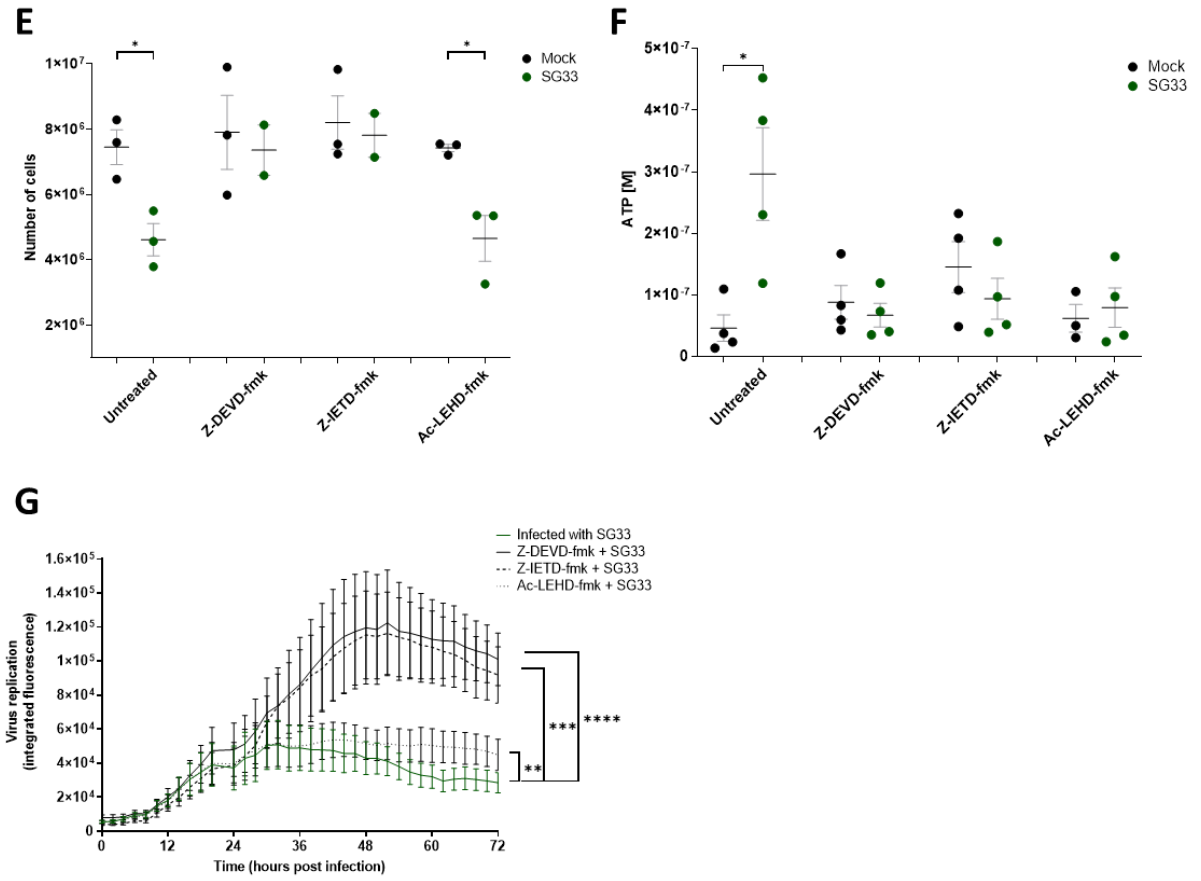


Figure 6: Mechanisms of SG33 apoptotic cell death in permissive PDAC cells. ***(A)*** *Summary table of the cell death pathways with the markers investigated and inhibitors used for each.* ***(B)*** *Determination of the dose of inhibitors to use so as not to impact the proliferation of uninfected cells.* ***(C)*** *Western blot analysis of caspase 3 and cleaved caspase 3 of uninfected (mock) and infected cells at 6-, 24- and 48- hours post-infection. Treatment by Idarubicin and Staurosporine for 24 hours were used as positive controls of ICD and apoptosis respectively. Results are mean±s.e.m. of three independent experiments.* ***(D)*** *Analysis of uncleaved and cleaved caspase 3 in uninfected and infected cells collected after 48 hours and either untreated or treated with Z-DEVD-fmk, Z-IETD-fmk or Z-LEHD-fmk. Results are mean±s.e.m. of three independent experiments.* ***(E)*** *Cell proliferation determined by the number of cells in uninfected and infected populations either untreated or treated by corresponding caspase inhibitors. Results are mean±s.e.m. of three independent experiments.* ***(F)*** *Quantification of ATP in supernatant of non-infected and infected PDAC087T cells untreated or treated with the corresponding pharmaceutical inhibitors. Results are mean±s.e.m. of four independent experiments performed in triplicate.* ***(G)*** *Viral replication was measured by integrated fluorescence intensity at the indicated time. Results are mean±s.e.m. of three independent experiments.*

Apoptotic cell death in SG33 oncolysis

ICD can be induced through multiple regulated cell death pathways. To better understand the mechanisms involved in SG33 oncolysis and their involvement in the triggering of ICD markers, we focused our investigation on four common regulated cell deaths: apoptosis, necroptosis, pyroptosis and autophagy. To evaluate the impact of these cell deaths in SG33 we looked at molecular markers associated to the pathway (Fig. 6A) and used pharmacological inhibitors to

block each pathway specifically, at doses that would not impact the proliferation of uninfected cells (Fig. 6B).

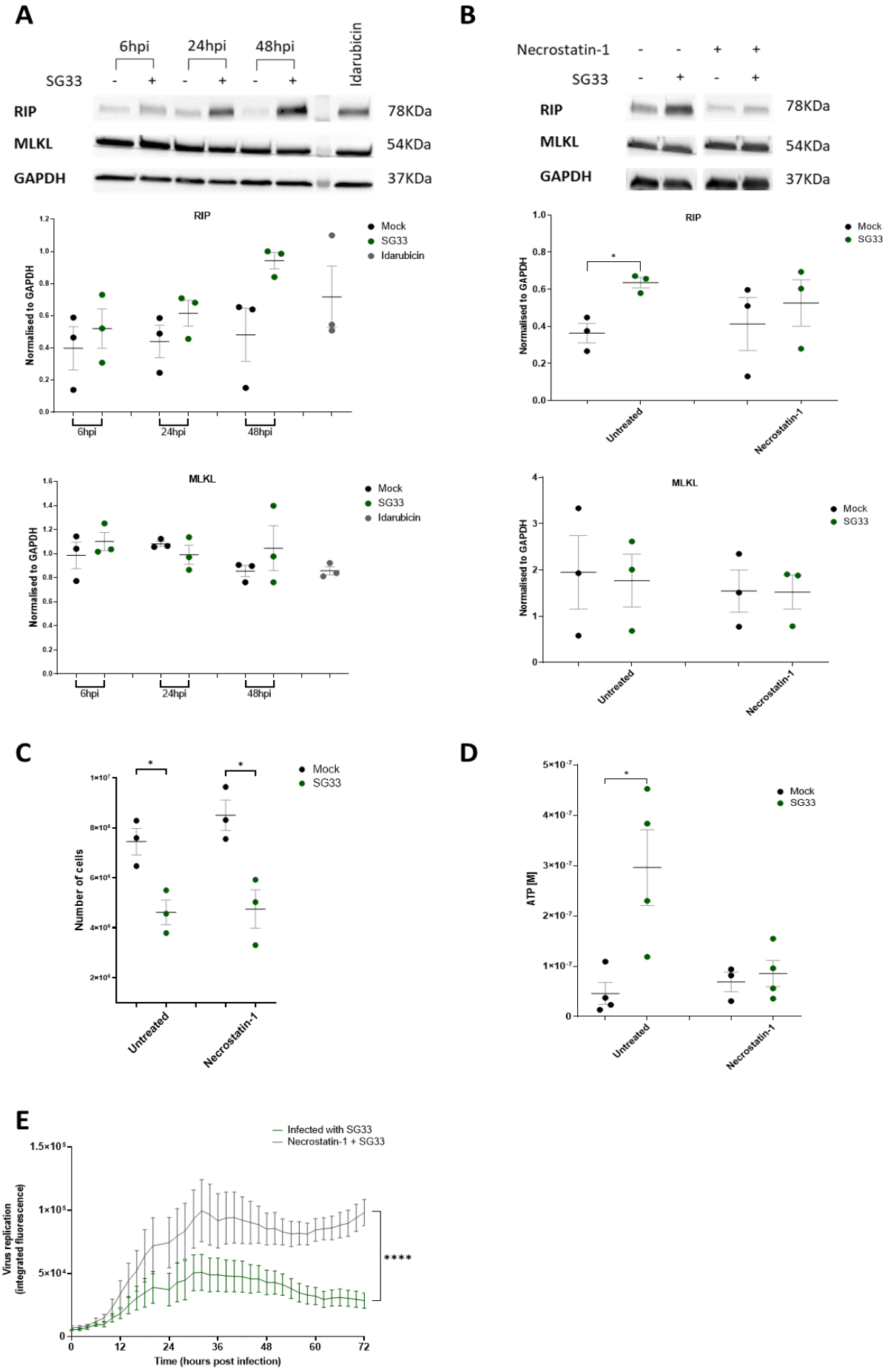
Apoptosis was detected through the cleavage of caspase 3 by Western blotting. The results show an increase in cleaved caspase 3 24 hours post-infection and significantly at 48 hours post-infection (Fig. 6C) with a seven-fold increase of 17KDa cleaved subunit for infected cells compared to non-infected cells. When treated with a caspase 3 inhibitor, Z-DEVD-fmk, there is an accumulation of the 19KDa cleaved subunit and a reduction of the 17KDa after SG33 infection. Similar results are seen with the caspase 8 inhibitor Z-IETD-fmk and at a lesser extent, the caspase 9 inhibitor Z-LEHD-fmk (Fig. 6D). When investigating the antiproliferative activity of SG33, there is a reduction in the number of cells between the uninfected and infected populations of 40%. This same reduction is seen when the populations are treated with the caspase 9 inhibitor. However, when caspase 3 or caspase 8 is inhibited, there is no significant difference in the number of cells between uninfected and infected cells (Fig. 6E). When quantifying the release of ATP in the extracellular medium between uninfected and infected populations, the treatment with all three caspase inhibitors causes a reduction in the release of ATP seen after SG33 infection in untreated cells (Fig. 6F). Finally, the treatment with caspase 3 and caspase 8 inhibitors, Z-DEVD-fmk and Z-IETD-fmk, resulted in an increase in viral replication within the infected populations was seen by the increase of total integrated fluorescence, with nearly a three-fold increase of integrated fluorescence at 48 hours post-infection with either inhibitors. The caspase 9 inhibitor Z-LEHD- also resulted in an increase in viral replication but to a lesser extent than the other two inhibitors (Fig. 6G) with a 1.2 fold increase of integrated fluorescence at 48 hours post infection. Together these results point towards SG33 oncolysis mediated by the extrinsic apoptotic pathway.

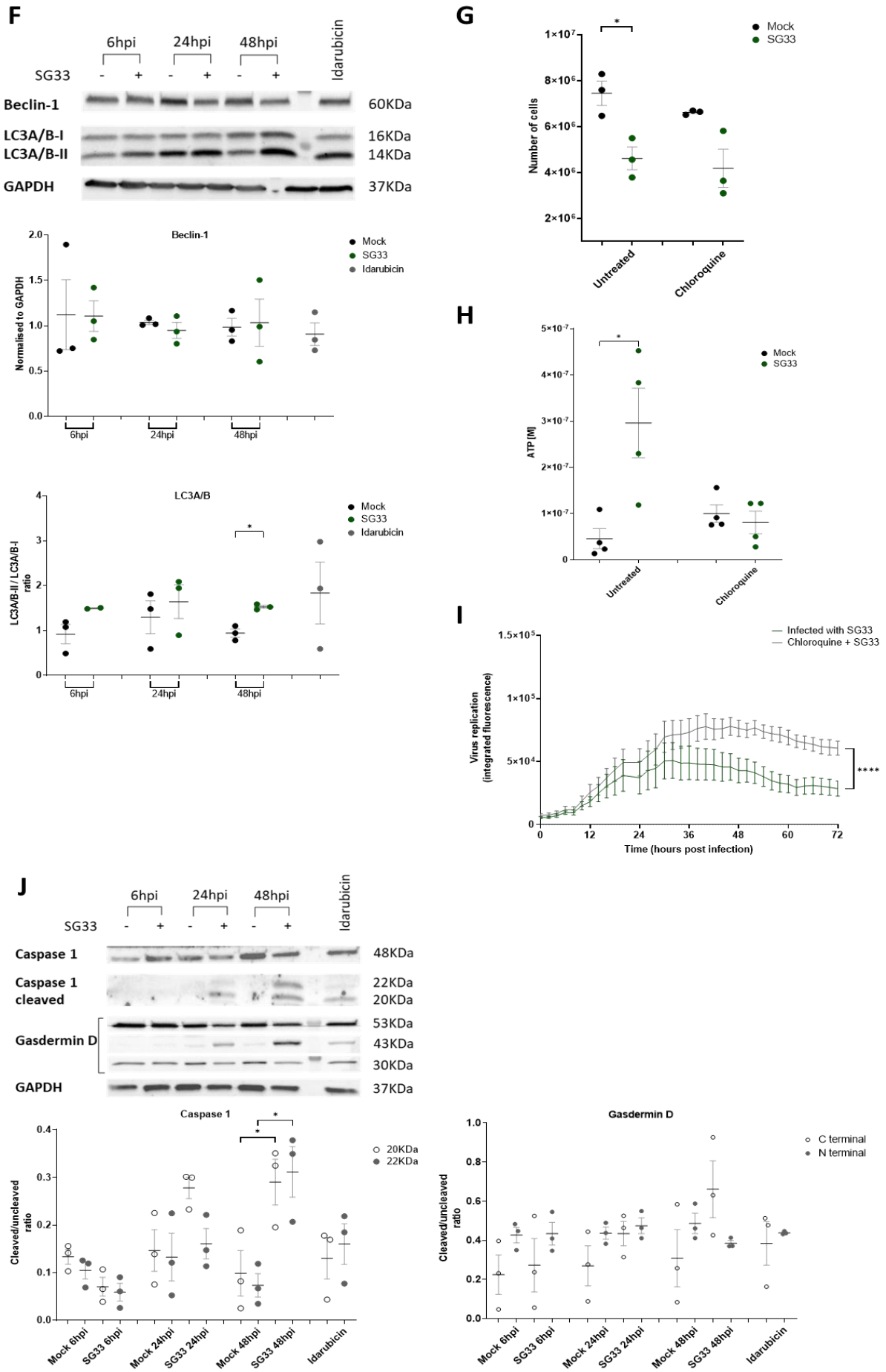
Other cell deaths in SG33 oncolysis

To detect necroptotic cell death we analysed the presence of RIP and MLKL proteins through western blotting. We found an increase in RIP at 24- and 48-hours post-infection between uninfected and infected cells, with the difference at 48hpi showing a two-fold increase in protein. However, there were no notable differences in the level of MLKL between uninfected

and infected cells at any timepoints (Fig. 7A). The difference observed in RIP expression between the populations was abolished after treatment by RIPK1 inhibitor, necrostatin-1, whilst MLKL remained unchanged (Fig. 7B). Necrostatin-1 treatment had no effect on the antiproliferative activity of SG33 as seen in Fig. 7C, with over 50% less cells in the infected population compared to uninfected. However it did block the release of ATP induced by the virus, since there is no longer a significant difference between uninfected and infected cells when treated by necrostatin-1 (Fig. 7D). Additionally, necrostatin-1 treatment significantly increases viral replication in the permissive cell population, as seen by the increase in total integrated fluorescence which is two-fold at 48 hours post_infection (Fig. 7E). Despite an upregulation of early actors in the pathway, our results show that necroptosis does not participate in SG33 oncolysis.

Autophagy was analysed by protein detection of beclin-1 and of unlipidated LC3A/B-I and lipidated LC3A/B-II. There were no differences in beclin-1 levels over time between uninfected and infected cells, the ratio of LC3A/B-II over LC3A/B-I increased following infection, with a significant 1.6 fold difference compared to uninfected cells at 48 hours post-infection (Fig.7F). The autophagic inhibitor used in this study, chloroquine, does not impact either autophagic markers (data not shown), in consistence with its downstream mechanism of action. Treatment with chloroquine only slightly impacted SG33-induced antiproliferation, with the difference in number of cells without and with infection of just less than 40% (Fig. 7G). Chloroquine treatment also hindered ATP release in the extracellular milieu (Fig. 7H) and increased total viral replication (Fig. 7I) of infected cells with a 1.8-fold increase at 48 hours port-infection. Together our results show that SG33 oncolysis is not dependent on autophagy despite signs of increased autophagosome structures.


A
6hpi
24hpi
48hpi
Idarubicin
SG33
RIP
MLKL
GAPDH
78KDa
54KDa
37KDa
Normalised to GAPDH
Mock
SG33
Idarubicin
B
Necrostatin-1
Untreated
Necrostatin-1
C
Number of cells
D
ATP [M]
E
Infected with SG33
Necrostatin-1 + SG33
Virus replication (integrated fluorescence)
Time (hours post infection)
****


F
6hpi
24hpi
48hpi
Idarubicin
SG33
Beclin-1
60KDa
LC3A/B-I
LC3A/B-II
16KDa
14KDa
GAPDH
37KDa
Beclin-1
Mock
SG33
Idarubicin
Normalised to GAPDH
LC3A/B
LC3A/B-II / LC3A/B-I ratio
G
Mock
SG33
Number of cells
Untreated
Chloroquine
H
ATP [M]
I
Infected with SG33
Chloroquine + SG33
Virus replication (integrated fluorescence)
Time (hours post infection)
J
Caspase 1
48KDa
Caspase 1 cleaved
22KDa
20KDa
Gasdermin D
53KDa
43KDa
30KDa
GAPDH
37KDa
Caspase 1
20KDa
22KDa
Cleaved/uncleaved ratio
Mock 6hpi
SG33 6hpi
Mock 24hpi
SG33 24hpi
Mock 48hpi
SG33 48hpi
Idarubicin
Gasdermin D
C terminal
N terminal

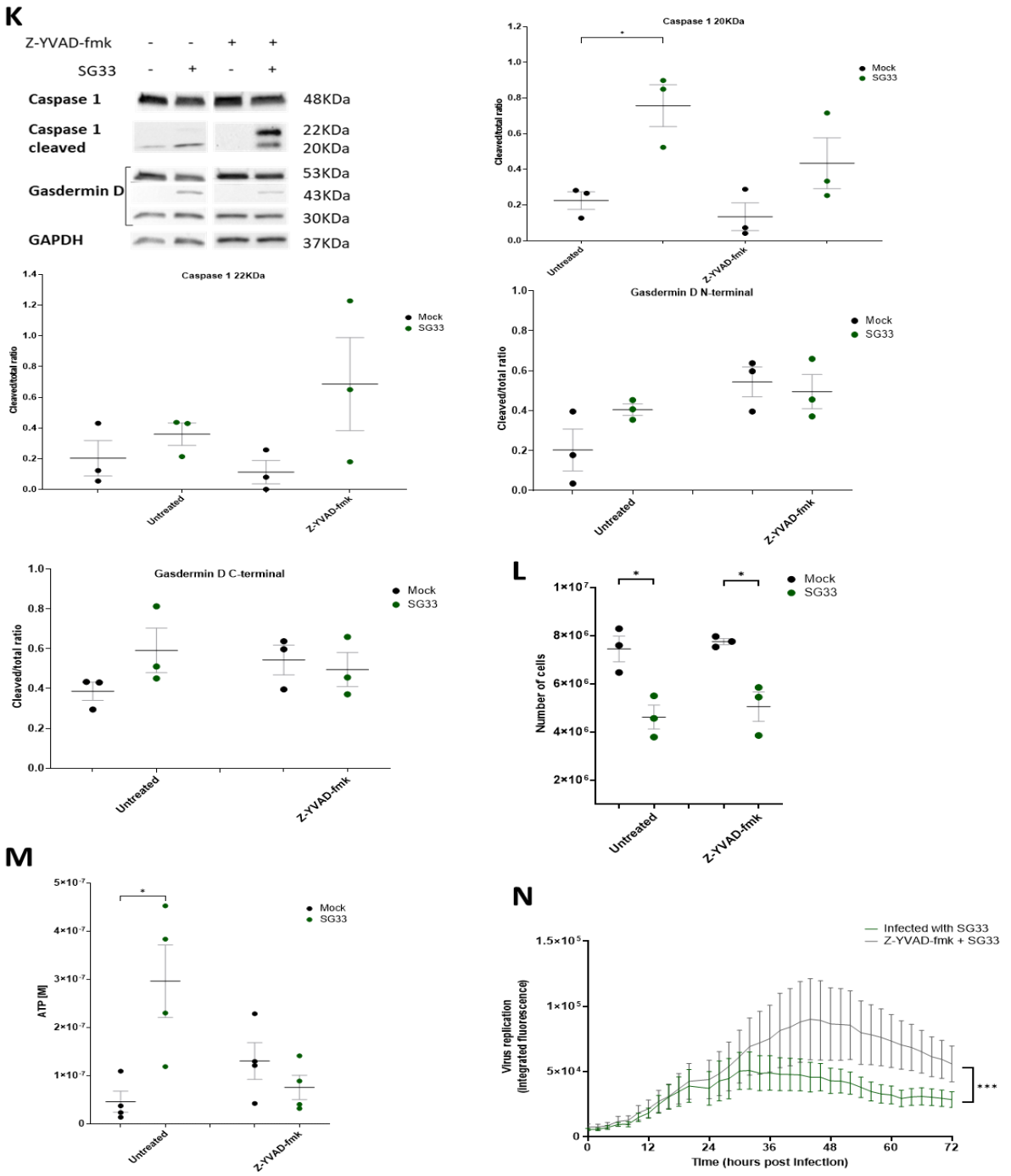


Figure 7: Mechanisms of non-apoptotic cell deaths in SG33 oncolysis of permissive PDAC cells. Western blot analysis of RIP and MLKL ***(A)****, Beclin-1, LC3A/B-I and LC3A/B-II* ***(F)****, caspase 1 and cleaved caspase 1 as well as gasdermin D and cleaved gasdermin D* ***(J)*** *of uninfected (mock) and infected cells at 6-, 24- and 48- hours post-infection. Treatment by Idarubicin for 24 hours was used as positive control of ICD. Results are mean±s.e.m. of three independent experiments. Analysis of RIP and MLKL* ***(B)****, caspase 1 and cleaved caspase 1 as well as gasdermin D and cleaved gasdermin D* ***(K)*** *in uninfected and infected cells collected after 48 hours and either untreated or treated with necrostatin-1 or Z-YVAD-fmk respectively. Results are mean±s.e.m. of three independent experiments.* ***(C) (G) (L)*** *Cell proliferation determined by the number of cells in uninfected and infected populations either untreated or treated by corresponding inhibitors. Results are mean±s.e.m. of three independent experiments.* ***(D) (H) (M)*** *Quantification of ATP in supernatant of non-infected and infected cells untreated or treated with the corresponding pharmaceutical inhibitors. Results are mean±s.e.m. of four independent experiments performed in triplicate.* ***(E) (I) (N)*** *Viral replication was measured by integrated fluorescence intensity at the indicated time. Results are mean±s.e.m. of three independent experiments.*

The final immunogenic cell death studied, pyroptosis, was analysed by the detection of caspase 1 and gasdermin D cleavage by western blot. Caspase 1 cleavage increased due to SG33 infection, with both cleaved subunits at 22KDa and 20KDa being significantly elevated with a 6-fold increase of either in infected cells compared to non-infected cells at 48 hours post-infection. Similarly, the cleaved 43KDa subunit of gasdermin D increases in the infected cell population as compared to uninfected cells, although this two-fold mean difference is not statistically significant. On the other hand, the cleaved 30KDa subunit of gasdermin D remains unchanged between infected and uninfected cells at all timepoints (Fig. 7J). The blockage of caspase 1 activation by Z-VAD-fmk reduced the amount of the 20KDa cleaved caspase 1 in infected cells, while the 22KDa increased. In addition, the noted increase of cleaved 43KDa gasdermin D in infected cells was hindered by Z-VAD-fmk while 30KDa cleaved gasdermin D remained unaffected (Fig. 7K). The caspase 1 inhibitor did not impact the proliferation tendency seen in fig. 7L as the number of cells in uninfected and infected populations persisted with a difference of 40% regardless of treatment. As with the other inhibitors, Z-YVAD-fmk reduced the amount of ATP released in the infected population (Fig. 7L) and increased the replication of SG33, with a two-fold increase of integrated fluorescence at 48 hours post-infection. Similarly to necroptosis, although we found an upregulation of early pathway actors, our results suggest that pyroptosis does not participate in SG33 oncolysis.

Discussion

In this second part of the thesis, we managed to evince SG33's ability of inducing immunogenic cell death of permissive primary pancreatic cancer cell line PDAC087T, as evidenced by the upregulation or release of ICD-associated DAMPs. The slight increase of CALR mean fluorescence intensity observed in the non-replicating cells could be due to bystander effect of infected cells on the surrounding cells. To further our understanding of immunogenic oncolytic activity, we examined four common cell deaths described to be the source of these ICD events: apoptosis, necroptosis, autophagy and pyroptosis.

Following a caspase cascade either stimulated by extrinsic (caspase 8) or intrinsic (caspase 9) pathways, procaspase 3 is first cleaved into 19KDa subunits, forming heterodomains (P19/P12

complexes) and subsequent removal of a prodomain to generate the mature P17/P12 complex translocated into the nucleus.[347] Our results showed that SG33 infection caused an increase in the amount of both the 19KDa and 17KDa subunits. Treatment of infected cells with caspase 3 inhibitor Z-DEVD-fmk, caspase 8 inhibitor Z-IETD-fmk or caspase 9 inhibitor Ac-LEHD-fmk all reduced the level of 17KDa caspase 3 subunit, with caspases 3 and 8 inhibitors resulting in an accumulation of the 19KDa subunit. We found that the blockage of either caspase 3 or caspase 8 impeded the antiproliferative effect of SG33 on PDAC087T cells suggesting an extrinsic apoptotic pathway as mechanism of SG33 oncolysis. This effect was not seen when cells were treated with caspase 9 inhibitor, ruling out the intrinsic pathway of SG33-triggered cell death in these tumoral cells. The lack of cell death following infection and subsequent treatment by Z-DEVD-fmk or Z-IETD-fmk can explain the significant increase in viral replication, only slightly increased by Ac-LEHD-fmk. In correspondence with these results, both caspase 3 and caspase 8 blockade hinders the release of ATP initially triggered by SG33, suggesting the extrinsic apoptotic pathway induced by the virus is in part responsible for the release of ICD DAMPs described earlier.

Necroptosis requires the subsequent accumulation and activation of RIP and MLKL to trigger the formation of necrosomes within the plasma membrane. Our findings show an accumulation of the RIP protein over time after infection by SG33 but no variations in MLKL protein levels or effect of necrostatin-1 on the antiproliferative activity of SG33. This result is consistent with the known crosstalk between apoptosis and necroptosis. Indeed, for the necroptotic pathway to be achieved, caspase 8 inhibition is required. As previously shown, SG33 tumour cell death is mediated by the extrinsic apoptotic pathway which requires activation of caspase 8. Caspase 8 not only plays a role in apoptotic cell death but also regulates necroptosis, by cleaving and thereupon inactivating RIP kinase 1, an enzyme necessary for the activation of RIP and ensuing activation of MLKL.[331] Therefore the accumulation of RIP suggests an upstream activation of necroptosis which is blocked by the activity of caspase 8. In order to confirm this, we would have to look at the phosphorylated (and therefore activated) forms of RIP and MLKL and expect no changes in these protein levels except when treated with caspase 8 inhibitor Z-IETD-fmk. Extrinsic apoptosis and necroptosis both share common receptor signalling compounds which are TNFα or FasL which points to the triggering of TNFα expression following SG33, which in consequence activates these pathways via an autocrine loop (Fig. 9). Similar results with TNFα

have been found with vaccinia virus, but in this case necroptotic cell death pathway is successfully achieved as a consequence of VV caspase inhibitor protein B13R/Spi-2.[348] The MYXV homolog of VV Spi-2, M-151R is partially deleted within the SG33 genome, leading to a truncated inactive form of the protein. This could explain the difference in cell death pathways triggered by vaccinia virus and SG33.

Although Beclin-1, a marker of early stages of autophagy, levels do not vary between infected and non-infected populations, the ratio of LC3A/B-II over LC3A/B-I increases over time in infected cells. The lipidation of LC3A/B-I into LC3A/B-II is essential for mature autophagosome formation,[349] our results therefore suggest an increase in the generation of autophagic vacuoles following SG33 infection. The decrease in number of cells after infection is not as significant when treated with chloroquine, a downstream inhibitor of autophagy, suggesting an induction of autophagy by SG33 in PDAC087T cells but not as significant as extrinsic apoptosis. Rapamycin is a specific inhibitor of mTOR and an inducer of autophagy which has been studied in association with myxoma virus, notably in glioma[323] and melanoma[322] models where the combination not only increased MYXV antitumoral activity but also potentialized the immunostimulatory effects in murine models with intact immune systems. This suggests autophagy plays a role in SG33-mediated tumour cell death. We used chloroquine as an inhibitor of autophagy, which blocks the fusion of autophagosomes and lysosomes, a phenomenon downstream to LC3A/B-II integration.[350] In consequence we were not able to use this inhibitor to hinder and therefore confirm the increase in autophagic markers following SG33 infection. The next step is to corroborate our results by using an upstream inhibitor of autophagy, such as 3-Methyladenine (3-MA) which blocks autophagosome formation by inhibiting types I and III Phosphatidylinositol 3-kinases (PI3K),[351] or by confirming chloroquine blockage of autophagolysosome formation with microscopy. Further studies will then be necessary to elucidate the actors and mechanisms involved in SG33-mediated autophagy.

Similarly to caspase 3, caspase 1 is cleaved sequentially in 22KDa and 20KDa subunits to be active. SG33 infection of PDAC087T cells resulted in an increase of both cleaved subunits of caspase 1 as well as an increase in the cleavage of gasdermin D (GSDMD) into the 43KDa subunit, although this latter increase is not statistically significant. However, the 30KDa cleaved subunit of gasdermin D does not differ between uninfected and infected cell populations.

Gasdermin D is an effector of both caspases 1 and 3. In the case of caspase 1, GSDMD is cleaved to liberate the 30KDa domain responsible for the subsequent trigger of pyroptosis. On the other hand, caspase 3 cleavage of gasdermin D results in the 43KDa subunit, inhibiting pore formation.[352] Our results thus suggests that despite increased caspase 1 activation, GSDMD is cleaved and subsequently inactivated by caspase 3. Concomitant with the absence of Z-YVAD-fmk effect of SG33 antiproliferative effect, canonical pyroptosis does not seem to be triggered by SG33 infection. Caspase 8 is also known to cleave gasdermin D leading to pyroptosis, although this pathway is most likely unemployed due to caspase 3 inactivation of GSDMD.[353] We confirmed these results by comparing gasdermin D levels in uninfected and infected cells either untreated or treated with caspase 3 and caspase 8 inhibitors (Fig. 8) and found the 43KDa subunit to be significantly decreased in the treated cells. Interestingly, our results show caspase 1 inhibition reduces the cleavage of gasdermin D into the 43KDa subunit. To further elucidate the complex crosstalk between pyroptosis and apoptosis, it would be interesting to analyse other actors of this cell death pathway such as IL-1β and IL-18 that are also activated by caspase 1, caspases 4 and 5 that can also cleave GSDMD as well as components of non-canonical pyroptosis such as gasdermin B and E.

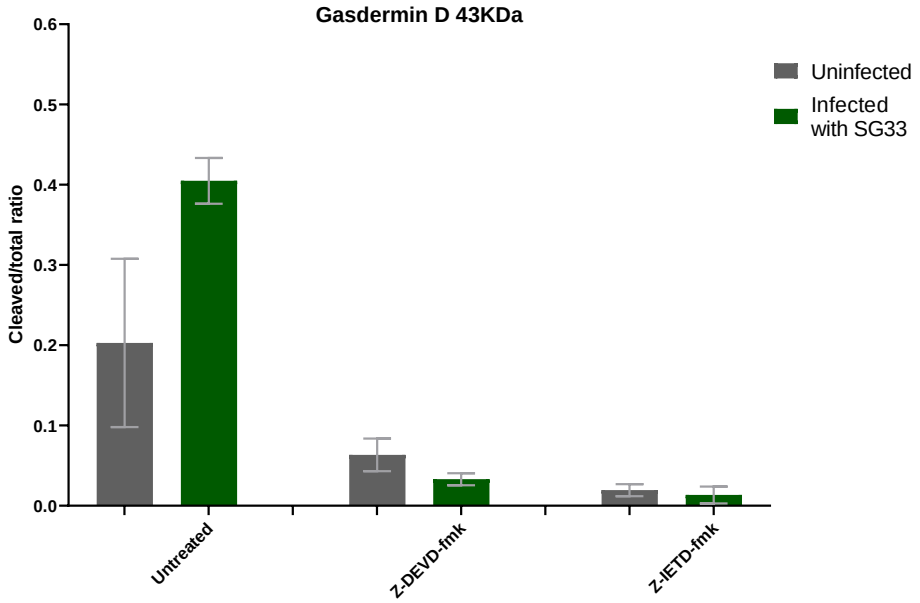


Figure 8: Analysis of gasdermin D and cleaved gasdermin D in uninfected and infected cells collected after 48 hours and either untreated or treated with Z-DEVD-fmk, Z-IETD-fmk. Results are mean±s.e.m. of three independent experiments.

All inhibitors tested in this study reduced the release of ATP in infected cells. It would be necessary to examine the effect of these inhibitors on the release of HMGB1 and plasma translocation of CALR in order to better clarify the ICD cell deaths of SG33 oncolysis. If these confirm our results, we could imagine that although they are not responsible for SG33 provoked cell death, these pathways play a role in the release of immunostimulatory signals following infection. Whether this effect is separate or due to the intertwined natures of these pathways remains to be found.

In conclusion, from our results we hypothesize that SG33 oncolysis of permissive PDAC087T cells is the result of extrinsic apoptosis triggered by TNFα expression of infected cells. Furthermore, in apoptotic-resistant cells, necroptosis could be induced through the same autocrine cytokine-release stimulation (Fig. 9). To verify our working hypothesis we will need to compare transcriptomic data from uninfected and infected cells, particularly transcriptomic data involved in TNFα expression, and regulated cell death pathways.

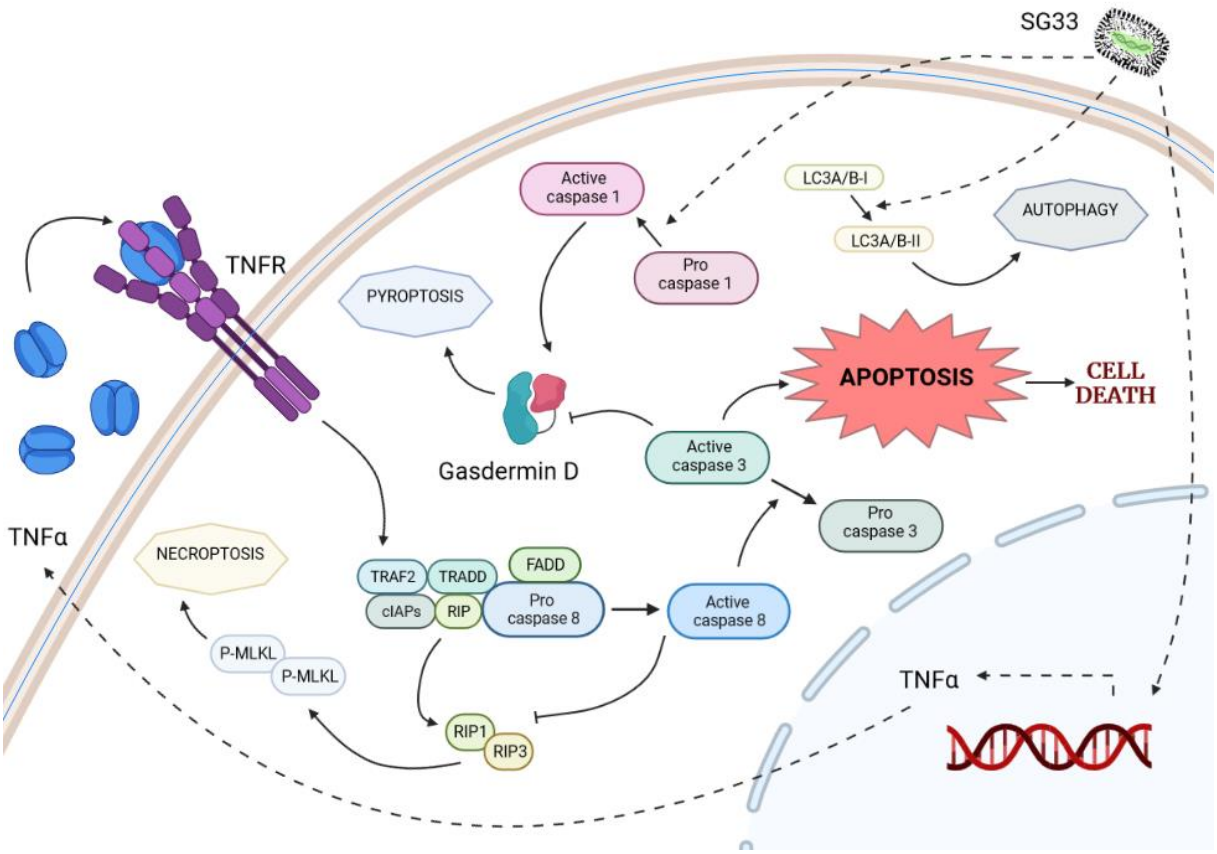


Figure 9: Proposed mechanism of SG33 oncolysis in permissive PDAC087T cells

Further studies are warranted, however, to confirm the roles of autophagy and pyroptosis and determine the molecular crosstalk involved between the different cell deaths and the actors involved. In addition, supplemental studies are needed to elucidate the immunogenic impact of the cell deaths triggered by SG33 as well as uncover other potential cell deaths. For example, when analysing the poly (ADP-ribose) polymerase (PARP) protein, we found that SG33 infection increases the cleavage (therefore activation) of PARP, and that this increase in cleavage was not impacted by any of the pharmacological inhibitors used (Fig. 10)

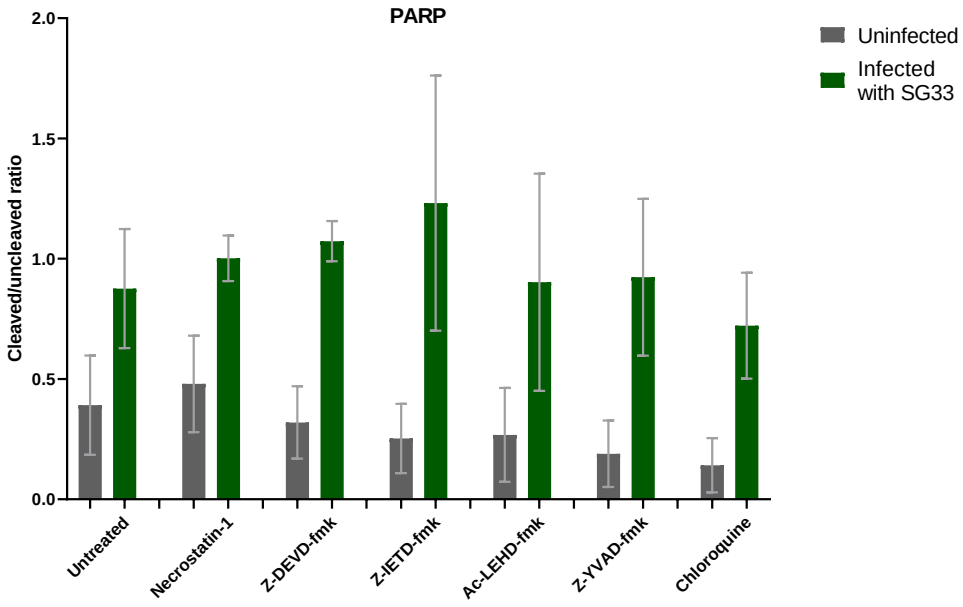


Figure 10: Analysis of PARP and cleaved PARP in uninfected and infected cells collected after 48 hours and either untreated or treated with the corresponding inhibitor. Results are mean±s.e.m. of three independent experiments.

This result would suggest a role of parthanatos, a mechanism triggered by excessive oxidative damage to DNA which leads to over-expression of PARP. Determining the role of this cell death in SG33 oncolysis can be done with similar studies as we've undertaken for the other cell death pathways. In this case, parthanatos can be blocked using a PARP inhibitor, such as Olaparib, currently studied as targeted therapy in PDAC treatment.

General discussion

By using the ANCHOR system we can quickly determine the infectivity of SG33 in tumour samples. The use in clinic of this strategy will help not only follow viral dissemination within the primary tumour, but could also help detect distant metastases. The interference of the GFP protein used to detect viral replication in our studies poses an issue in live models due to high interference. We are currently developing SG33-ANCHOR virus with the fusion protein comprised of santaka fluorescing at 561 nm and believed to give better results in vivo. In order to characterise the viral replication and spread in populations, we are working with bioinformaticians that model SG33 activity in different tumour conditions. The modelisation of SG33 will help us determine the doses and intervals between administrations to optimise SG33 antitumoral activity depending on the PDAC tumour at hand.

Determining the cell deaths and the molecular mechanisms involved in SG33 oncolysis could help enlighten the oncolytic heterogeneity we found in PDAC primary cell lines in the first part of the thesis. Indeed, our results show that tumour cell sensitivity to oncolytic viruses do not only depend on active viral replication, but also on the virus' ability to induce cell death.

PDAC tumours are found to be dysregulated in a variety of cellular pathways, including apoptosis[354] and more precisely the extrinsic pathway.[355] This includes the use of decoy receptors which leads to decrease binding of death ligands (such as FasL) to their receptors (Fas), or a structurally-similar molecule to caspase 8 c-FLIP but without caspase activity. Both of these result in a decrease in death receptor signalling. Cells can also become resistant to extrinsic apoptosis through upregulation of a death receptor adaptor TRAF2 which inhibits the TNF receptor and instead activates NF-κB signalling. Comparing such apoptotic dysregulations and sensitivities to SG33 oncolysis of PDAC tumours will complete our understanding of determinants of viral efficacy. For example, if TRAF2 is found upregulated in SG33 resistant cells, by silencing expression, for example using siRNA directed against TRAF2, we should restore the virus-triggered apoptotic cell death. In this case, we could imagine arming the virus with anti-TRAF2 compounds, by inserting the sequence within the viral genome to be expressed within the tumour cells. This shows the benefits of using OV since they can be used as genetic

platforms to encode and subsequently express the intended molecule. In addition, poxviruses replication is highly conserved and well known [293] as well as SG33's genomic sequence.[296] With the innovative tools for genetic engineering available today, it is possible to position the sequence in question under a late promoter, the poxviral late (p11) promoter for example. To do so, deciphering the molecular mechanisms involved within the virally-induced apoptotic pathway as well as the crosstalks between cell deaths is essential. In particular, it is important to address the role we believe TNFα has in SG33 oncolysis, since TNFα also has a central role in antiviral defences.

Our results point towards extrinsic apoptosis as SG33 oncolysis mechanism in PDAC087T cells. The first step would be to investigate the cell deaths triggered in other permissive cell lines. For example, PDAC072T were cells that presented low viral replication but were sensitive to oncolysis. By comparing PDAC cells in their susceptibility of triggering and completing apoptotic or other cell death pathways could help us comprehend the disparities found between virus replication and oncolytic activity among these cell lines. In order to better understand the cellular genes needed for SG33 replication and oncolysis, our team is conducting a CRISPR screening of the permissive PDAC087T cells. Comparing SG33's ability to replicate and induce cell death in CRISPRised cells, in addition to the transcriptomic analyses of the cells before and after infection could reveal the cellular determinants involved in SG33's viral cycle. Ultimately, this work can help stratify patients that would most benefit from virotherapy or devise treatments to improve viral oncolysis in patients' tumours.

Conversely to OV oncolysis resistance, another obstacle found with oncolytic viruses is the balance between cell survival - to ensure correct replication of the virus - and cell death allowing the dissemination and infection of other tumour cells. Indeed, it is possible that cells that poorly replicate SG33 are killed too quickly and therefore unable to ensure the correct propagation of OV within the population. Hence, early triggered apoptosis in these infected cells is a way for the population as a whole to combat oncolytic virus infection. In this case, inhibiting apoptosis would allow the correct replication of SG33. Evading apoptosis to ensure correct replication is a common trait amongst viruses. Within the viral genes deleted from the SG33 genome is *M151R*, a serpin (serine protease inhibitor) with anti-apoptotic functions by inhibiting the interleukin-1β-converting enzyme. Restoring this sequence or the homolog found in vaccinia virus, *B13R/Spi-2* within the SG33 sequence could delay the onset of apoptosis, allowing the

time needed for SG33 to replicate correctly. In this case, we predict oncolysis would be redirected towards necroptosis, as it is with VV.[348] As stated beforehand, the possibility of engineering SG33 to express transgenes at selective timepoints within the viral cycle makes it a promising therapeutic tool. As such, SG33 would able to begin replication, but expresses the natural viral antiapoptotic agent before precarious cell death.

Studying cell death pathways triggered or blocked in SG33 oncolytic-resistant PDAC cells can allow us to determine whether the immunogenic potential of SG33 oncolysis is also cell dependent. Overcoming SG33 oncolysis resistance could not only improve the antitumour capacity but also increase the immunogenic signals released and immunostimulatory effects after infection. To establish this, however, it is primordial to first understand the immunostimulatory potential and mechanisms of SG33 alone.

Studies ex vivo and in vivo are necessary to acknowledge the impact of the immunogenic cell death seen at the cell population level in immunocompetent organisms. Indeed, the cytolytic properties found in cellular models are poor prognostic indicators of in vivo antitumoral activity, particularly in regard to impact of the immune system.[206] To do so and to avoid the cross-reaction of using human PDAC tumours in immunocompetent mice, we have selected primary murine pancreatic cancer cells R211F1; preliminary results show this model to be permissive to SG33 oncolysis which leads to an increase in ICD markers. To fully understand the extent of SG33 oncolysis on the immune system is twofold:

First is to evaluate the activation of APCs and their capacity to prime T cells towards an antitumoral state. This can be done by retrieving and differentiating bone marrow dendritic cells (BMDCs) from immunocompetent mice and placing them in contact with SG33-lysed PC cells. The medium used to differentiate BMDCs is important, however, since typical supplements such as IL-4 can reduce type I interferon responses in dendritic cells. Indeed primary results using IL-4 and GM-CSF to differentiate BMDCs resulted in low cell viability and an increase in plasmacytoid DCs (pDCs) compared to conventional DCs (cDCs) when in contact with SG33 viral particles. Further studies to examine dendritic cell stimulation following SG33 oncolysis will necessitate the differentiation of BMDCs using Fms-like tyrosine kinase 3 (Flt3) ligand.

Secondly, it is crucial to evaluate the recruitment of immune cells to the tumour. This can be done in immunocompetent mice engrafted with murine pancreatic cancer cells after administration of SG33 either intratumorally or intravenously. In this model it is also possible to evaluate different routes of administration of the virus as well as the efficacy of combining SG33 oncolytic virotherapy to immunotherapies with the aim to increase antitumor activity.[356]

Conclusion

The work of this thesis contributes to the development of oncolytic virotherapy as novel treatment for pancreatic adenocarcinoma, a currently incurable neoplasm associated with late diagnosis and poor prognosis. We put to light the use of the ANCHOR system as a means of monitoring viral replication and propagation in a variety of PDAC tumour models and in real time. The oncolytic virus studied, SG33, is a vaccinal attenuated strain of myxoma virus, which we showed to replicate at higher levels than the parental strain. We further managed to describe SG33 replication and oncolytic activity in PDAC primary cell lines while being ineffective in normal pancreatic cells. We further described the immunogenic potential of SG33 by tracking DAMPs associated with immunogenic cell death and investigated the oncolytic mechanisms that could be involved.

Further research is needed to completely elucidate SG33 oncolytic activity, and the molecular mechanisms involved in PDAC tumour permissivity. This work is the basis for future studies in murine models, in order to determine the role of the immune system and pharmacokinetics associated with the possible administration routes. Using the ANCHOR system, will be able to monitor SG33 in vivo, tracking antitumour activity and potential dissemination to distant metastases. Finally, the results obtained in our studies will contribute to the preclinical information required to the elaboration of clinical studies and potential combination protocols.

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Annex: Scientific communications

Publications:

- *A Novel Imaging Approach for Single-Cell Real-Time Analysis of Oncolytic Virus Replication and Efficacy in Cancer Cells*, Quillien et al. – Paper published in journal Human Gene Therapy (2021)

- *Pancreatic cancer and the drugs of tomorrow*, Quillien et al. – Review in progress

Congress and webinars:

- CRCT retreat – Oral presentation 'My thesis in 180s' (2019)

- Toulouse Onco-Week (TOW) congress – Poster (best scientific poster award) *First-in-class imaging approach for single-cell, real-time analysis of oncolytic virus replication and efficacy in cancer cells* (2020)

- Competition Société Française de Thérapie Cellulaire et Génique (SFTCG) – Presentation (Runner-up award*) First-in-class imaging approach for single-cell, real-time analysis of oncolytic virus replication and efficacy in cancer cells* (2020)

- *The Role of Preclinical Imaging in Viral and Bacterial Research* PerkinElmer Webinar (2020)

- Looking toward the Future of Cell & Gene Therapies PerkinElmer Webinar (2020)

- Médecine Génomique et Oncogénomique Société Française de Médecine Preédictive et Personnalisée (SFMPP) congress (2020)

- Webinar SFTCG – Oral presentation *Nouvelle approche d'imagerie pour l'analyse en cellule unique et en temps-réel de la réplication et de l'efficacité d'un virus oncolytique dans les cellules cancéreuses pancréatiques* (2021)

- *Combination in Immunotherapy of Cancer and Overcoming Resistance, from molecular aspects to clinical issues* – Symposium organiser (2021)

Résumé

Le cancer du pancréas (CP) est le septième cancer le plus meurtrier avec un taux de survie à cinq ans inférieur à 10%. La chirurgie reste le meilleur traitement mais n'est souvent pas possible à cause de l'évolution rapide de la maladie. D'autres traitements existent comme la radiothérapie et des combinaisons de chimiothérapies, mais celles-ci sont accompagnées de nombreux effets indésirables et un risque accru de résistance. De plus, ces protocoles thérapeutiques peuvent être inefficaces face aux complexités cellulaires et moléculaires des cancers pancréatiques et de leur microenvironnement tumoral. De ce fait des traitements alternatifs sont activement recherchés, tels que les thérapies ciblées, les immunothérapies et les thérapies géniques. Les virus oncolytiques (VO) sont une thérapie innovante particulièrement intéressante dans la médecine d'aujourd'hui. Cependant leur potentiel thérapeutique est limité par manque de stratégies pour le suivi et la quantification cinétique afin de déterminer leur efficacité au sein de cellules vivantes.

Lors de ces travaux nous montrons une approche pour imager à l'échelle de la cellule unique la réplication en temps-réel d'un virus oncolytique et son efficacité dans des cellules cancéreuses. Nous avons étudié le SG33, VO dérivant de la souche Lausanne Toulouse 1 (T1) du Myxoma virus (MYXV) utilisé comme contrôle. Nous avons équipé les génomes de SG33 et T1 avec le système ANCHOR avant d'infecter un panel de lignées. Le système ANCHOR est composé d'une protéine de fusion (OR-GFP) qui lie spécifiquement une séquence cible d'ADN courte et non-répétitive (ANCH) puis s'étale aux séquences voisines par oligomérisation. Son accumulation sur l'ADN viral créer un foci fluorescent. Nous avons trouvé que (i) l'ADN de SG33 et T1-ANCHOR peut être détecté et quantifié en temps réel, (ii) après infection les deux VOs ont une réplication périnucléaire avec des centres réplicatifs regroupés en fer à cheval et, (iii) SG33 réplique à des taux plus élevé par rapport à T1. Enfin, comme preuve de concept translationnelle nous avons déterminé l'efficacité oncolytique de SG33 dans des cellules cancéreuses primaires dérivées de l'adénocarcinome pancréatique (PDAC) aux niveaux populationnel et cellulaire. In vivo, SG33 se réplique dans des tumeurs expérimentales et inhibe la croissance tumorale. Ensemble, ces données démontrent une nouvelle stratégie de quantification du cycle infectieux de VOs en temps réel, avec un suivi de l'ADN viral avec une première évidence de stratégie théranostique pour les patients PDAC. Cette technique permettrait de rationaliser l'utilisation de VOs chez des patients souffrant de maladies incurables.

Une autre classe thérapeutique innovante est les immunothérapies, efficaces dans de multiples cancers mais pas dans le PDAC. Ceci est principalement dû au fait que le cancer du pancréas est une tumeur 'froide' où l'environnement immunitaire est en manque et le peu de cellules immunes présentes sont plutôt de nature immunosuppressive. Les virus oncolytiques ont le potentiel d'induire la mort cellulaire via de multiples voies dont certaines considérées comme immunogènes. De plus, la libération d'antigènes associés aux tumeurs par la lyse de cellules tumorales agit en tant que vaccin par le recrutement de cellules immunes et le déclenchement d'une réponse immunitaire adaptative. L'utilisation de VOs dans le PDAC pourrait potentiellement la transformer en tumeur 'chaude'. Nos résultats suggèrent une capacité de SG33 à induire une mort immunogène dans des cellules primaires de PDAC permissives et nous avons étudié les voies de morts impliqués. Ces résultats nous permettent d'approfondir nos connaissances de l'activité oncolytique de SG33 et ouvre la possibilité d'exploiter son potentiel immunologique dans des tumeurs immunodéprimées telles que PDAC.