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Exploring the use of tolerogenic dendritic cells and pore-forming toxins in the context of oncolytic adenoviruses

Laura Moya Borrego

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FACULTAT DE MEDICINA I CIÈNCIES DE LA SALUT

PROGRAMA DE DOCTORAT EN BIOMEDICINA

**EXPLORING THE USE OF TOLEROGENIC DENDRITIC CELLS
AND PORE-FORMING TOXINS IN THE CONTEXT OF
ONCOLYTIC ADENOVIRUSES**

LAURA MOYA BORREGO

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*Exploració de l'ús de cèl·lules dendrítiques tolerogèniques i toxines
formadores de porus en el context dels adenovirus oncolítics*

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Memòria presentada per Laura Moya Borrego per optar al grau de Doctor/a per la
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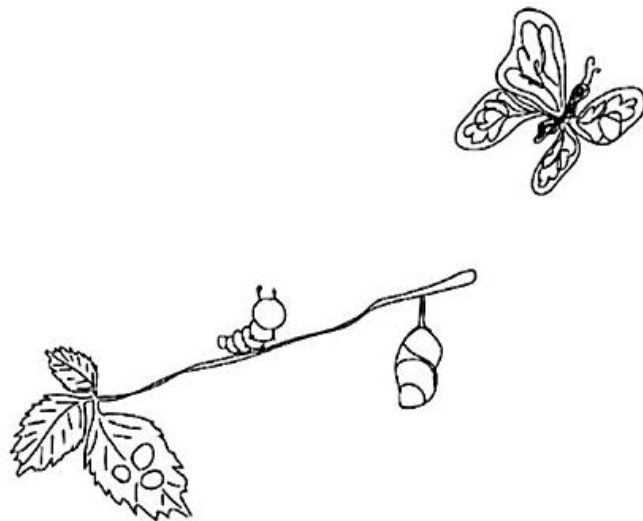
Si hem de ser fidels a algú, serem fidels a l'amor.
Estimarem el que fem, el que som, el que tenim. [...]
No permetrem que la por ens faci callar ni que els
condicionals ens esguerrin les il·lusions.

Obrirem el cor al món, perquè és la manera
d'aprendre a ser valents. Escoltant el que portem a
dins, respectant el que veiem a fora. Cuidant el que
és important perquè no ens torni a passar per alt. I
que diguin el que vulguin que si caiem, ens
tornarem a aixecar.

Quan arribi la nit i tornem a casa, potser estarem
sols però no deseparats. Dormirem al costat d'una
petita llum que espurneja: és la vida que batega.

– Gemma Ventura Farré, *És la vida que batega*

You do not just wake up and become the butterfly
growth is a process – rupi kaur



Al meu pare i a la meva mare

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ABBREVIATIONS

%	Percentage
#	Number
°C	Centigrade degrees
4-1BB	TNF ligand superfamily member 9
5-FC	5-fluorocytosine
α	Anti (flow cytometry antibodies)
Δ24	<i>Delta24</i> mutation, deletion of 24 bp in E1A protein
Ω	Ohm (electrical resistance unit)
μF	Microfaraday (electrical capacitance unit)
μg	Microgram
μL	Microliter
μM	Micromolar
μm	Micrometer
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACK	Ammonium-chloride potassium
ACT	Adoptive cell transfer
Ad	Adenovirus
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADP	Adenovirus death protein
AFP	Alpha-fetoprotein promoter
Ag	Antigen
AHR	Aryl hydrocarbon receptor
APC	Antigen-presenting cell
APM	Antigen presentation machinery
ATP	Adenosine triphosphate
Breg	Regulatory B cell
bp	Base pair
BsAb	Bispecific antibody
Bt	<i>Bacillus thuringiensis</i>
CAR	Chimeric antigen receptor/coxsackie adenovirus receptor
cDC	Conventional (mature) dendritic cell
CDX /CDXL	Cluster of differentiation protein # / its ligand
CE	Cell extract

cGAS	Cyclic GMP-AMP synthase
Cm	chloramphenicol
cm	centimeter
CMV	Cytomegalovirus
CO₂	Carbon dioxide
CPE	Cytopathic effect
CR	Complete response
CRT	Calreticulin
Cry	Crystal
CsCl	Cesium chloride
CTA	Cancer testis antigen
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXC	CXC chemokine subfamily
Cyt	Cytolytic
DAB	3,3'-Diaminobenzidine
DAMP	Damage-associated molecular pattern
DBP	DNA binding protein
DC	Dendritic cell
DIPG	Diffuse intrinsic pontine glioma
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double-stranded
E2F	Early region 2 binding factor
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMA	European medicines agency
ER	Endoplasmic reticulum
FAP	Fibroblast activation protein
FCM	Flow cytometry
FDA	Food and Drug Administration
FGF	Fibroblast growth factor

fmol	femtomole
Fw	Forward (primer/oligonucleotide)
<i>g</i>	Acceleration of gravity
<i>g</i>	gram
GC3	Guanine and cytosine at the codon third position
GITRL	Glucocorticoid-induced TNFR family-related gene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GV	Ganciclovir
h	hour
H₂O₂	Hydrogen peroxide
HA	Hyaluronic acid
HBsAg	Hepatitis B surface antigen
HEK293	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HMGB1	High-mobility group box 1 protein
HSG	Heparan sulfate glycosaminoglycan
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
HVR	Hypervariable regions
IC₅₀	Half maximal inhibitory concentration
ICD	Immunogenic cell death
ICI	Immune checkpoint inhibitor
ICS	Intracellular cell staining
iDC	Immature dendritic cell
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IM	Intramuscular
ImmTACs	Immune-mobilizing monoclonal T-cell receptors against cancer
Iono	Ionomycin
IRES	Internal ribosome entry sites
IRF3	Interferon regulatory factor 3

IT	Intratumoral
IV	Intravenous
kb	kilobase
kDa	kilodalton
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
L	Liter
LAG-3	Lymphocyte activation gene 3
LC₅₀	Lethal concentration 50%
LTR	Left inverted terminal repeat
MAGE	Melanoma-associated antigen
MDSC	Myeloid-derived suppressor cells
MerTK	MER tyrosine kinase
MHC	Major histocompatibility complex
MigDC	Migratory dendritic cell
min	Minute
mL	Milliliter
MLP	Major late promoter
MLTU	Major late transcription unit
mm³	Cubic millimeter
MMP	Matrix metalloproteases
mRNA	Messenger ribonucleic acid
miRNA	Micro RNA
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MYC	Myelocytomatosis oncogene
MyD88	Myeloid differentiation primary response 88
MV	Measles virus
NAbs	Neutralizing antibodies
NaCl	Sodium chloride
NaH₂PO₄	Monosodium phosphate
NaOH	Sodium hydroxide
NDV	Newcastle disease virus
NFκB	Nuclear factor kappa B
ng	nanogram
NK	Natural killer

NKG2A	NK group 2 family of receptor A
NOD-scid	Non-obese diabetic
NSG	Nod Scid Gamma
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma-1
OAd	Oncolytic adenovirus
OD	Optical density
OV	Oncolytic virus
OVA	Ovalbumin
P2RY2	Purinergic receptor P2Y2
p53	Tumor protein p53
pA	Polyadenylation sequence
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death ligand 1
PES	Phenazine ethosulfate
PFP	Pore-forming protein
PFT	Pore-forming toxin
pg	picogram
PMA	Phorbol-myristate-acetate
polyA	Polyadenylation
PRR	Pattern recognition receptor
PS	Parasporin
qPCR	quantitative PCR or real-time PCR
RA	Rheumatoid arthritis
Rb	Retinoblastoma
RCD	Regulated cell death
RIG-I	Retinoic acid-inducible gene I
RITR	Right inverted terminal repeat
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (medium)
RT	Room temperature
Rv	Reverse (primer/oligonucleotide)
SA	Splicing acceptor
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scFv	Single-chain variable fragment
Scid	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	second
SEM	Standard deviation of the mean
shRNA	Short hairpin RNA
siRNA	Small-interfering RNA
SRP	Signal recognition particle
ss	Single-stranded
ssDBP	single-stranded DNA binding protein
Sp-1	Specificity protein 1
STING	Stimulatory of interferon genes
strep	streptomycin
T1D	Type 1 diabetes
TAA	Tumor-associated antigens
TAM	Tumor-associated macrophages
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TDLN	Tumor-draining lymph node
TGF-β	Transforming growth factor-beta
TIGIT	T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif domain
Th	T helper
TIL	Tumor-infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin-domain-containing 3
TIME	Tumor immune microenvironment
TIMP	Tissue inhibitor of metalloproteinases

TLR	Toll-like receptor
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-alpha
tolDC	Tolerogenic dendritic cell
TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
Treg	Regulatory T cell
tRNA	Transfer RNA
TSA	Tumor-specific antigens
TU	Transducing unit
V	Volt or volume
VA	Viral-associated
VEC	Vascular endothelial cell
VEGF	Vascular endothelial growth factor
VIP	Vegetative insecticidal protein
VitD3	Vitamin D3
vp	Viral particle
VSV	Vesicular stomatitis virus
wt	Wild-type

SUMMARY

Oncolytic viruses (OVs), engineered to infect preferentially and selectively replicate within cancer cells while sparing healthy tissues, have regained interest in oncology for their potential to restore host anti-tumor immunity. OVs have been evaluated in clinical settings as standalone therapies or in combination with chemotherapy, radiotherapy, and immunotherapy. Among them, oncolytic adenoviruses (OAdS) are well-studied and commonly employed as OVs for their powerful oncolysis capacity and immune response stimulation. However, their therapeutic efficacy as anticancer agents remains unsatisfactory due to the limited intratumoral virus spreading and subsequent antiviral immune responses. This doctoral thesis addresses these challenges in two distinct chapters.

Viral epitopes are often more immunogenic than tumor epitopes, leading to predominant antiviral immune responses following OAd administration, which can undermine or even mask antitumoral immune responses. To counteract this, we hypothesized that tolerogenic dendritic cells (tolDCs) exposed to OAd-derived epitopes could induce specific viral tolerance, thereby shifting the balance from viral to tumor antigen adaptive immune responses. Our findings revealed that IL-10-induced tolDCs primarily belonged to the CD11c⁺CD11b⁺ cDC2 subset, exhibited an anti-inflammatory cytokine profile, and demonstrated a reduced ability to drive allogeneic CD8⁺ T cell proliferation in mixed lymphocyte reactions (MLRs). However, in an immunocompetent model, no viral-specific tolerance but a generalized immunosuppression was elicited upon treatment with OAd peptide-pulsed tolDCs.

To improve the intratumoral spread of OAdS, we armed the ICOVIR15K virus with a modified version of the pore-forming toxin (PFT) Paraspurin 2 (PS2), as this toxin has been reported to display a selective antitumor activity in its active form. We presumed the toxin-mediated cell lysis would amplify the OAd's oncolytic capacity and induce an immunogenic cell death (ICD) in bystander non-infected cells. PS2-enriched supernatant treatment exhibited selective cytotoxic effects against several human and murine cancer cell lines *in vitro* and efficiently controlled tumor growth in a lung carcinoma immunocompetent model. Moreover, vaccination with PS2-treated tumor cells acted as an ICD inducer that promoted a protective antitumor immune response in a vaccination-rechallenge setting. Finally, we showed that the ICOVIR15K-PS2 OAd significantly exerted an enhanced antitumor efficacy compared with the ICOVIR15K parental virus in an immunodeficient model, expecting better outcomes when both oncolysis and toxin ICD-mediated responses could act synergistically in clinical testing, where the OAd can replicate.

Keywords: Oncolytic Adenovirus, Paraspurin, Tolerogenic Dendritic Cells, Virotherapy

RESUM

Els virus oncolítics (OVs) són capaços d'infectar preferiblement les cèl·lules tumorals i replicar-s'hi selectivament sense malmetre els teixits sans. El seu potencial en restaurar la immunitat antitumoral de l'hoste ha tornat a generar interès en oncologia. Els OVs s'han avaluat en assajos clínics com a monoteràpies o combinats amb quimioteràpia, radioteràpia i immunoteràpia. Entre aquests, els adenovirus oncolítics (OAds) han sigut àmpliament estudiats i utilitzats pel seu poder oncolític i la seva capacitat d'estimular la resposta immunitària. Malgrat això, la seva eficàcia terapèutica com a agents anticancerosos es veu limitada per la distribució restringida dins del tumor i les respostes immunitàries antivirals consegüents. Aquesta tesi doctoral aborda, en dos capítols diferenciats, diferents estratègies per superar aquests obstacles.

Sovint les respostes immunitàries antivirals esdevenen predominants després d'administrar OAds, que poden minvar o fins i tot emascarar les respostes antitumorals. A fi de contrarestar-ho, hem explorat si les cèl·lules dendrítiques tolerogèniques (tolDCs) exposades a epítops derivats d'OAds podrien induir una tolerància viral específica, afavorint així les respostes antitumorals. Els resultats van revelar que les tolDCs induïdes amb IL-10 pertanyien principalment al subtipus CD11c⁺CD11b⁺ cDC2, secretaven citocines antiinflamatòries i presentaven una capacitat reduïda d'aloresponse en reaccions limfocitàries mixtes (MLRs). No obstant, el tractament amb tolDCs pulsades amb pèptids adenovirals no va induir una tolerància viral específica, sinó més aviat una immunosupressió generalitzada.

Per a millorar la propagació intratumoral dels OAds, es va armar l'adenovirus ICOVIR15K amb la toxina formadora de porus (PFT) Parasporina 2, ja que aquesta toxina ha demostrat tenir una activitat antitumoral selectiva en la seva forma activa. Suposàvem que la lisi cel·lular mediada per la toxina ampliaria la capacitat oncolítica de l'OAd i induiria una mort cel·lular immunogènica (ICD) en les cèl·lules adjacents no infectades. El tractament amb PS2 va mostrar efectes citocides selectius contra diverses línies cel·lulars tumorals humanes i murines *in vitro*, així com controlava de manera eficient el creixement tumoral en un model de carcinoma pulmonar. A més, la vacunació amb cèl·lules tumorals tractades amb PS2 actuava com a inductor d'ICD que promovia una resposta antitumoral protectora. Finalment, vam demostrar que l'OAd ICOVIR15K-PS2 exercia una eficàcia antitumoral millorada en comparació amb el virus parental ICOVIR15K en un model immunodeficient. Anticipem resultats més prometedors quan es puguin combinar els efectes de la oncolisi i l'ICD mediada per la toxina en l'àmbit clínic, on l'OAd pot replicar-se.

Paraules clau: Adenovirus Oncolítics, Parasporina, Cèl·lules Dendrítiques Tolerogèniques, Viroteràpia

INTRODUCTION

1. CANCER IMMUNOLOGY

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2022¹. It can be defined as a complex disease characterized by uncontrolled cellular proliferation of transformed cells subject to evolution by natural selection². Tumor cells originate from normal cells undergoing accumulated mutations that circumvent cell division and death regulatory mechanisms. These outgrowths form primary tumors that, as a result of (epi)genetic changes and selective pressures within the tumor microenvironment (TME), drive the development of further cancer cells with potentially increasingly aggressive and malignant features that ultimately spread through the lymphatic system or bloodstream and invade distant parts of the body as metastases. Tumor cells that escape from intrinsic programmed cell death face external checks mediated by the immune system. Immune cells recognize abnormal proteins on tumor surfaces and become activated to destroy these cells eventually. Therefore, the effectiveness of the immune system in battling malignant cells and the tumor's ability to evade immune detection are the key factors in dictating the course of cancer.

Tumors differ from their normal tissue counterparts not only in biological behavior but also in antigenic composition. Genetic instability, a hallmark of cancer³, commonly arises from defects in DNA damage repair systems and results in the accumulation of mutated proteins that determine the tumor mutational burden (TMB)⁴. Such genomic variations could give rise to tumor antigens, presented by the major histocompatibility complex (MHC), recognized by the immune system as “non-self” and elicit antitumor immune responses. Tumor antigens are typically classified into tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). While TAAs are generally expressed by healthy cells but overexpressed or aberrantly expressed in cancer cells, TSAs are newly formed by tumor cells due to either point mutations (known as neoantigens) or oncogenic viral infections. Another source of TSAs are antigens derived from cancer-germline genes (cancer/testis antigens, CTAs) that are selectively

expressed in germline and tumor cells because of DNA demethylation⁵ (their antigens are not present on germline cells because of the lack of HLA molecules).

1.1 ANTITUMOR IMMUNE RESPONSE

The idea that the immune system plays a role in identifying and eliminating cancer cells was first proposed by Paul Ehrlich as the “cancer immunosurveillance” theory and later expanded by M.F. Burnet and L. Thomas during the twentieth century^{6,7}.

The term antitumor immunity refers to an interplay between innate and adaptive immune systems in a series of steps known as the Cancer-Immunity Cycle⁸ (**Figure 1**). The antitumor response begins with detecting damaged-associated molecular patterns (DAMPs) on stressed cells by cytotoxic cells of the innate system. Natural killer (NK), NKT, and gamma-delta ($\gamma\delta$) T cells bridge innate and adaptive responses; although being part of the T-cell lineage, they recognize antigens less specifically than conventional T cells, giving a rapid response by directly killing tumor cells via perforins and granzymes. Additionally, they initiate a pro-inflammatory environment by releasing chemokines and cytokines such as interferon-gamma (IFN γ) to stimulate further the immune response against the tumor⁹. Under these inflammatory conditions, with high levels of IFN γ , incoming macrophages are skewed toward an M1 tumoricidal phenotype. M1 macrophages are associated with a high level of phagocytosis and secretion of pro-inflammatory cytokines that contribute to tumor cell elimination. Death cancer cells release TAA and TSAs (**Figure 1, step 1**) that are captured and processed by dendritic cells (DCs) or other antigen-presenting cells (APCs) like macrophages and B cells (**Figure 1, step 2**). Mature DCs then migrate to draining lymph nodes to present these antigens on MHC class I and II to naïve T cells (signal 1), priming and activating them to target cancer-specific antigens (**Figure 1, step 3**).

To yield a robust T-cell expansion, signal 1 must be accompanied by additional costimulatory signaling through CD80 and CD86 molecules on DCs (signal 2). Moreover, depending on the antigen nature, maturing DCs orchestrate the immune response

by producing specific T cell-polarizing molecules, which influence the development of T helper (Th) cell subpopulations (signal 3)¹⁰. In general, Th1 cells are associated with a protective role against tumors¹¹. Th1 cells support antitumor immunity by secreting cytokines such as tumor necrosis factor (TNF), interleukin (IL) 2, and IFN γ , which boost CD8⁺ T cells and NK cell cytotoxicity and promote antibody production by plasma cells (activated antibody-producing B cells), enhancing both cellular and humoral responses against tumors¹².

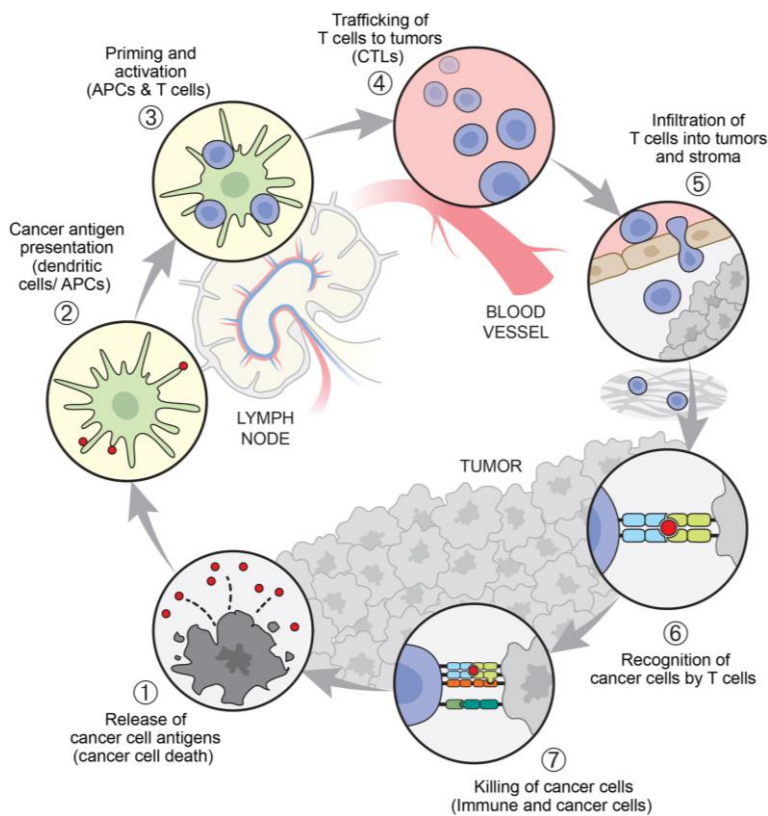


Figure 1. The cancer-immunity cycle. 1) Tumor antigens are released from dead cancer cells by cell stress or the innate immune system. 2) APCs uptake and present tumor antigens to naïve T cells in the draining lymph node. 3) Tumor-specific cells are primed and differentiated into effector cells. 4) Activated CD4⁺ (Th) and CD8⁺ (CTL) are chemoattracted to the tumor site. 5) Effector T cells extravasate and infiltrate the tumor tissue. 6) T cells recognize cancer cells. 7) Such Ag-specific recognition induces cancer cell killing. Image from reference 8.

Subsequently, cytotoxic CD8⁺ T cells (CTLs) migrate from the lymph node to the tumor site chemoattracted via a gradient of chemokines (i.e., CCL2, CCL5/RANTES, CXCL9, and CXCL10)¹³ (**Figure 1, steps 4 and 5**). Finally, infiltrating CTLs recognize and kill cancer cells specifically by interacting with their T-cell receptor (TCR) and their cognate antigen on MHC-I (**Figure 1, steps 6 and 7**). This killing releases additional tumor antigens, perpetuating the cycle and strengthening the immune response.

However, in cancer patients, this cycle is often disrupted. The continued deletion of cancer cells expressing T-cell targets may allow tumors to evolve and avoid attack. There has been growing evidence that immunosurveillance represents only one component of the complex tumor-immune system relationship, leading to the novel concept of “the three E’s of cancer immunoediting,” coined by Schreiber and colleagues¹⁴.

During cancer immunoediting, the host’s immune system shapes tumor fate in three phases, leveraging innate and adaptive immune responses¹⁵ (**Figure 2**). Elimination’s first phase involves destroying transformed cells by a competent immune system. It aligns with the classic immunosurveillance constantly exerting selective pressure on tumors. Sporadic tumor cells that manage to overcome this phase may enter an Equilibrium phase between dying and surviving tumor cells where immunoediting occurs: only the immunosuppressive or less immunogenic tumor cell clones survive. The final phase, Escape, occurs when these immunologically sculpted tumors begin to proliferate, becoming clinically detectable, and create a tumor microenvironment that evades immune activity.

Despite advancements in early cancer detection, tumors in the elimination and equilibrium phases typically remain undetectable. Consequently, most cancers are in the escape phase at diagnosis, where a complex TME limits treatment effectiveness, particularly in aggressive malignancies like pancreatic and small-cell lung cancers^{16,17}. Even though recently introduced immunotherapies such as CAR-T cells and immune checkpoint inhibitors (ICIs) (detailed in Section 2 of this chapter) resulted in a significant breakthrough, tumor-escaped patients respond differently to them^{18,19}.

Moreover, these varied responses define a second cycle of immunoediting²⁰. Thus, an in-depth understanding of immunoediting throughout tumor growth and progression and tailored new approaches for escape-phase tumors are essential to counter or reverse immune evasion mechanisms.

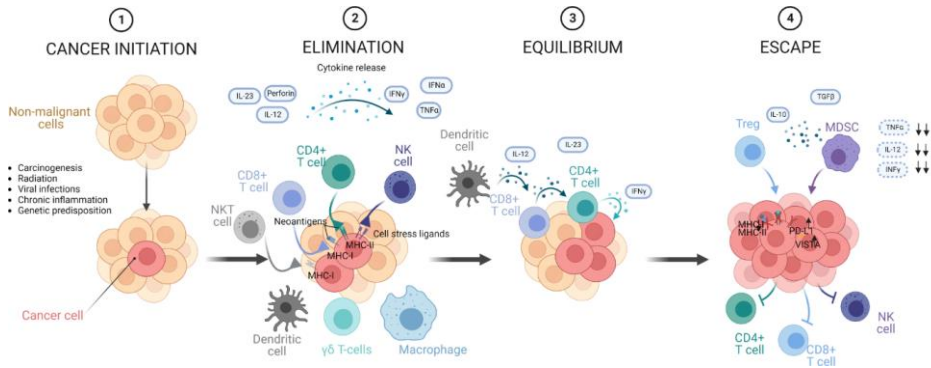


Figure 2. Cancer immunoediting phases. **1)** Aberrant cells that fail intrinsic tumor suppression mechanisms are exposed to an external control operated by innate and adaptive systems. **2)** Elimination is a phase of cancer immunoediting where both the innate and adaptive immune systems detect and destroy early tumors before they become clinically visible. In this phase, the balance is towards antitumor immunity due to an increase in expression of tumor antigens, MHC class I, Fas and TRAIL receptors on tumor cells and perforin, granzymes, IFN- $\alpha/\beta/\gamma$, IL-1, IL-12, TNF- α in the TME. **3)** In the equilibrium phase, the immune system holds the tumor in a state of functional dormancy and involves a balance between antitumor (IL-12, IFN- γ) and tumor-promoting cytokines (IL-10, IL-23). **4)** During the escape phase of cancer immunoediting, the immune system fails to restrict tumor outgrowth, and tumor cells emerge, causing clinically apparent disease. The balance is skewed towards tumor progression due to immunosuppressive cytokines and molecules such as IL-10, TGF- β , or PD-L1. Image adapted from reference 15.

1.2 TUMOR-INDUCED IMMUNE EVASION

In cancer, the immune system is not ignorant of tumor cells but actively suppressed by them²¹. During the transition between equilibrium and escape phase, immunoedited tumor cells develop multiple mechanisms to impose an immune-tolerant state essential for survival. As depicted in **Figure 3**, these mechanisms mainly focus on impairing effector T cell tissue localization, antigen encounter, and acquisition of effector function^{22,23}. A common immune evasion strategy involves altering antigen expression or the antigen processing and presentation pathways, thereby reducing tumor cells' visibility from Ag-specific CD8⁺ T cells^{24,25}. Similarly, tumor cells can cease

to express ligands for NK cell effector molecule NKG2D²⁶. Tumors may also increase the presence of inhibitory molecules such as immune checkpoint proteins (e.g., PD-L1 or CTLA-4) that, even recognized by immune cells, restrict their activation and weaken the antitumor response.

Furthermore, tumors establish an immunosuppressive TME in which cancer cells, epithelial and stromal cells, such as cancer-associated fibroblasts (CAFs), produce factors like TGF- β , prostaglandin E2, and IL-10 that disables the functionality of APCs and cytotoxicity of NK and T cells. In addition, TME also acts as a physical barrier to avoid T cell infiltration. Moreover, tumors can recruit immunosuppressive cells — including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC), and regulatory T cells (Tregs)— to their microenvironment, which contribute

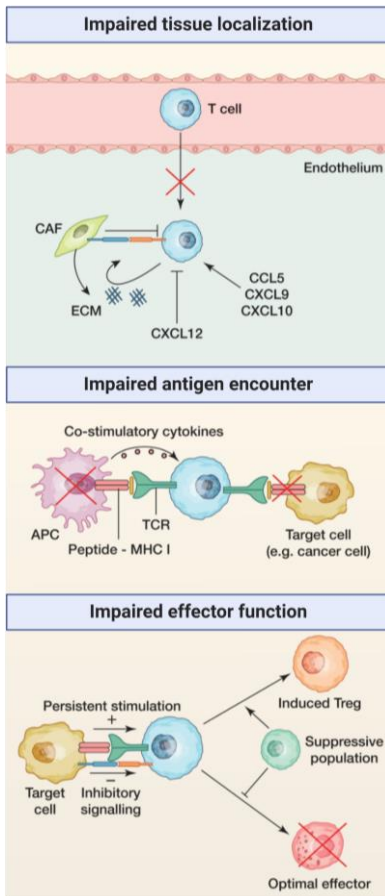


Figure 3. Mechanisms of tumor immune evasion.

Tumors employ various mechanisms to circumvent immune effector cells by: 1) Controlling T cell localization. Cancer cells reduce the expression of endothelial adhesion molecules (e.g., ICAM-1) required for circulating T cells to migrate. Activated CAFs promote the expression of CXCL12 chemokine, producing a chemorepulsive effect on CXCR4-bearing T cells; downregulation of pro-inflammatory cytokines (e.g., CXCL9 and CXCL10) contributes as well to decrease T cell recruitment in the TME. CAFs are also implicated in generating a dense ECM to generate stromal barriers that limit T-cell migration. Additionally, the production of large amounts of vascular endothelial growth factor (VEGF) by tumor cells can increase tumor endothelial FasL expression and promote the elimination of T cells. 2) Impairing antigen recognition through defects in the antigen presentation machinery (APM), such as the suppression of MHC class I expression, neoantigen loss, and alteration of cDC1 recruitment. 3) Promoting T-cell dysfunction. Chronic antigen exposure can ultimately skew effector T cells from early to later differentiated, exhausted states. Cancer cells can inhibit T cell effector functions by expressing co-inhibitory ligands (e.g., PD-L1, CTLA-4) or inducing cell death (e.g., Fas/FasL signaling). Moreover, they can enhance the abundance of Tregs and suppressive myeloid populations through their conversion from other cell types. Image adapted from reference 23.

to T cell exhaustion²⁷. Overall, interactions between cancer cells, the extracellular matrix (ECM), and stromal cells configure the TME, influencing the effectiveness of immunotherapy or other conventional treatments. Transformed cells must evade or suppress the immune response to establish a clinically detectable disease. Consequently, markers of immune activity against cancer cells, such as the infiltration of CTLs and Th1 lymphocytes in the TME and genetic signatures of T-cell activation, predict positive patient outcomes and response to immunotherapy across cancers^{28,29}.

In line with these findings and taking into account the different cells affecting the TME and the 14 hallmarks of cancer³, Liu et al. proposed a complete reclassification of four tumor immunophenotypes based on their driving factors³⁰: oncogene-driven type, stromal cell-driven type, immunosuppressive cell-driven type, and exhausted T cell-driven type (**Table 1**). Evaluating multiple biomarkers together could provide more precise prognostic and predictive information than assessing each individually.

Table 1. The four immunophenotypes of TME based on their driving factors.

Immunophenotype	Cancer	TIME features	Response to ICIs	Tumor features
Oncogene-driven	NSCLC and other KRAS/EGFR/MYC-driven tumors	<u>Cold</u> Poorly infiltrated; ↓TMB, MHC, and chemokine expression; ↑immunosuppressive ligands	Resistant	↑proliferation and angiogenesis; apoptosis resistance
Stromal cell-driven	HCC, Panc, ccRCC	<u>Cold</u> Abnormal vascular network hinders T cell infiltration	Resistant	↑stromal cells (VECs, fibroblasts, pericytes, MSCs); ↑growth factors (e.g., VEGF, PDGF, FGF); ↑collagen and ECM deposition; immature neovascularization
Immunosuppressive cell-driven	Panc, PCa, BC	<u>Hot</u> ↑immunosuppressive cells (TAMs, MDSCs, Bregs, Tregs); ↑dysfunctional T cells; ↑pro-inflammatory factors (e.g., IL-6, IL-1β, IL-17); presence of TLS	Responsive	Mature vasculature

Exhausted T cell-driven	GB	Hot ↑ immunosuppressive ligands and cells; ↑ terminally-exhausted effector cells	Resistant	Chronic inflammation
Cancer: BC, breast cancer; ccRCC, clear cell renal carcinoma; EGFR, epidermal growth factor receptor; GB, glioblastoma; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma viral oncogene homolog; MYC, myelocytomatosis oncogene; NSCLC, non-small cell lung cancer; PCa, prostate cancer; Panc, pancreatic. TIME features: Breg, regulatory B cell; IL, interleukin; MHC, major histocompatibility complex; MDCS, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; TIME, tumor immune microenvironment; TLS, tertiary lymphoid structures; TMB, tumor mutational burden; Treg, regulatory T cell. Response to ICIs: ICI, immune checkpoint inhibitor. Tumor features: ECM, extracellular matrix; FGF, fibroblast growth factor; MSC, mesenchymal stem cell; PDGF, platelet-derived growth factor; VEC, vascular endothelial cell; VEGF, vascular endothelial growth factor. Table adapted from reference 30.				

2. CANCER IMMUNOTHERAPY

Cancer immunotherapy has revolutionized the oncology field during the last decade. Unlike traditional treatments (radiotherapy or chemotherapy), which target tumors directly, immunotherapy refers to any treatment that boosts or restores the immune system’s natural defenses by either reactivating immune effector cells or blocking inhibitory pathways that cancer cells use to evade detection.

One of the most successful approaches has been using monoclonal antibodies against the inhibitory immune checkpoints (ICIs) (**Figure 4-1**). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) are the most potent examples of T cell immune checkpoint molecules that tumors use to suppress immune function by expressing CD80/CD86 and PD-L1/2 ligands, respectively. Accordingly, the blockade of CTLA-4 and PD-1/PD-L1 pathways showed long-lasting immune-mediated survival benefits in multiple cancers; they are currently indicated to treat melanoma, head and neck cancers, hepatocellular carcinoma, Merkel cell carcinoma, and some subtypes of renal and lung cancers, among others³¹. Ever since, more T-cell immune checkpoints, such as TIM-3, LAG-3, and TIGIT, have been investigated as novel ICI targets³². Researchers are also evaluating ICIs that can release the brake on other immune cells^{33,34}.

Another approach is the use of bispecific antibodies (BsAbs) (**Figure 4-2**), which are formed by two single-chain variable fragment (scFv) regions from antibodies joined by a linker; one binding domain recognizes TAAs on target tumor cells, and the other binds to CD3 on T cells. This union forces an artificial immunological synapse between the T and tumor cells without involving typical costimulatory signals, specific TCR, and MHC recognition. The FDA has approved only one BsAb to treat leukemia, though additional BsAbs are being developed against other hematologic malignancies and solid tumors³⁵. Related molecules called immune-mobilizing monoclonal T-cell receptors against cancer (ImmTACs) have shown potential; unlike BsAbs, ImmTACs use a TCR instead of an antibody fragment to recognize proteins on cancer cells, thereby allowing these molecules to bind also intracellular proteins³⁶.

However, ICI and BsAb therapies have significant effects only in hot or immunosuppressed tumors, as they rely on a certain degree of T-cell infiltration. Despite their remarkable efficacy, they also can lead to immune-related toxicities^{37,35}.

For cold tumors, adoptive cell transfer therapies (ACT) have been implemented to overcome the lack of pre-existing immunity. One form of ACT is tumor-infiltrating lymphocyte (TIL) therapy (**Figure 4-3A**), which harvests naturally occurring T cells that have already infiltrated patients' tumors. These TILs are activated and expanded *ex-vivo* and then reinfused into patients, where they can seek out and destroy tumors. Additionally, these TILs can be equipped with an engineered TCR to target specific cancer antigens (**Figure 4-3B**). In this regard, some TCRs against the so-called "public neoantigens" are currently being evaluated in clinical trials³⁸.

Besides, T cells have also been genetically modified with chimeric antigen receptors (CARs) (**Figure 4-4**). A key advantage of CAR T cells is their ability to recognize surface antigens even if they are not presented via MHC, which can render more cancer cells targeted. In contrast, TCRs can recognize surface and intracellular processed antigens, so the range of potential antigen targets is more extensive than in CARs³⁹. CAR-T therapy has achieved exciting therapeutic effects in controlling some hematological cancers, leading to their FDA approval for acute lymphoblastic leukemia and large B-

cell lymphoma⁴⁰. However, it faces more challenges in treating solid tumors⁴¹. A new class of therapy focused on CAR-engineered NK cells (CAR-NK therapy) emerges as a tumor immunotherapy alternative, offering reduced side effects and the advantage of their “off-the-shelf” nature⁴². Similarly, CAR- $\gamma\delta$ T cells are a possible source for allogeneic ACT therapy⁴³.

Despite the spotlight on the strategies described above, cancer vaccines (**Figure 4-5**) remain unique, as they can potentially prime tumor-reactive T cells while targeting a broader set of intracellular antigens. Significant efforts have been made to develop neoantigen-based therapeutic cancer vaccines^{44–46}. Unlike TAAs, neoantigens are recognized as non-self entities not subjected to central and peripheral tolerance mechanisms, making them more immunogenic and exempt from tumor-off toxicities. A new class of TSA known as non-canonical antigens (unconventional protein products that are not part of the known protein-coding genome repertoire) may be suitable for cancer vaccines because they broaden the TSA targetability and can be shared over tumors, which represents an advantage over personalized neoantigen vaccines⁴⁷. Furthermore, the success of mRNA-based vaccines against SARS-CoV-2 and the findings of a recent study in the so-aggressive pancreatic ductal adenocarcinoma (PDAC)⁴⁸ opened a new era in cancer vaccine therapy.

Precision medicine has transformed the oncology landscape, particularly immunotherapy and molecularly targeted therapies. Still, challenges persist due to limited patient responses and the development of acquired tumor resistance to current treatments⁴⁹. To address these issues, researchers are exploring therapeutic combinations integrating various treatment modalities, such as immunotherapy, targeted therapies that inhibit oncogenic signaling pathways, chemotherapy, and radiotherapy. Evidence from clinical trials suggests such combinations can increase efficacy and delay therapeutic resistance, particularly when designed rationally based on mechanistic insights^{50,51}. ICIs have been incredibly impactful in combination

strategies, with approaches like neoadjuvant⁵², adjuvant⁵³, or microbiome-modulated⁵⁴ regimens showing promise in certain tumors.

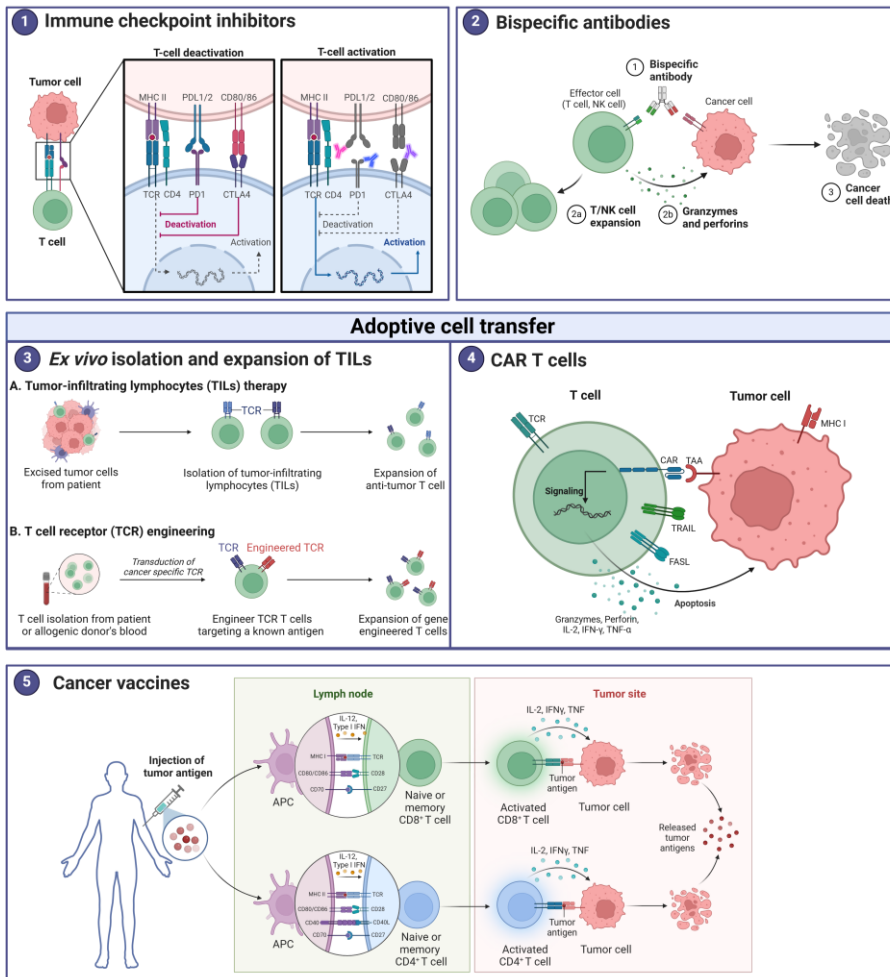


Figure 4. Highlighted immunotherapies for cancer treatment. 1) ICIs are therapeutics that block immune system checkpoints, reactivating antitumor responses. These inhibitors target regulatory mechanisms that restrain immune activity, allowing cancer cells to evade detection. By blocking these checkpoints, the immune system can recognize and eliminate tumor cells more effectively. **2)** BsAbs activate CD3⁺ T cells by bringing immune cells near cancer cells, which leads to tumor cell death. **3 and 4)** ACT boosts the natural ability of the patient's immune cells to fight cancer; in TIL therapy (3A), part of the patient's tumor is surgically removed, and the TILs from within it are isolated, expanded in the lab, and delivered back to the patient. Additionally, TCRs from these TILs can be genetically modified to recognize specific antigens, enhancing the effectiveness of the T cells (3B); T cells can also be engineered to express CAR molecules that bind to superficial tumor antigens TCR -independently (4). **5)** Therapeutic cancer vaccines expose the patient's immune system to tumor antigens carried to the lymph nodes, where they activate Ag-specific CD4⁺ or CD8⁺ T cells. Image created with BioRender.com.

3. CANCER VIROTHERAPY

As mentioned before, the tumorigenesis process is not related only to tumor cell characteristics but is deeply influenced by the TME. Thus, immunologically cold tumors, characterized by a high immunosuppressive TME that makes them immune-resistant (see Table 1), are ineffective to T cell-directed immunotherapies. Efforts to overcome the resistance of cold tumors with therapies like ICIs are focusing on turning them into hot tumors. This implies first “conditioning” tumors with an immunosensitive state before immunotherapy⁵⁵. Conditioning may be achieved through multiple mechanisms, such as immunogenic cell death (ICD)²⁸, which will be further explained in the following lines. Some chemotherapeutic agents and focal radiation regimens can inflame the TME through ICD processes⁵⁶. However, they produce highly toxic effects, and tumors at advanced stages become insensitive to these treatments. In this context, oncolytic viruses (OVs) have emerged as promising alternatives to convert immune-silent tumors into immune-active tumors.

3.1 ONCOLYTIC VIRUSES AS MULTIMODAL ANTITUMOR AGENTS

OVs are naturally occurring or engineered viruses that preferentially infect and can selectively replicate in and lyse tumor cells while leaving normal cells unharmed. The viral progeny generated during the initial infection is released into the extracellular environment, where it spreads to nearby cells. This propagation amplifies the therapeutic effects of the initially administered dose, ideally until the tumor mass is entirely eradicated.

OVs have multimodal mechanisms to eliminate cancer cells⁵⁷. Initially, OVs gained interest for their direct tumor cell lysis (oncolysis) capabilities. The dominant types of OVs include RNA viruses such as measles virus (MV), vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), Maraba virus, picornavirus, and reovirus; and DNA viruses such as adenovirus, herpes simplex virus (HSV), and vaccinia virus⁵⁸. Some OVs are inherently more infectious towards transformed cells, such as reovirus⁵⁹. Healthy tissues naturally clear these viruses, but features of tumors, such as specific cell

receptors, intracellular enzymes, or defective antiviral-response pathways, favor the replication of these viruses in tumor cells. Other viruses like HSV, vaccinia, or adenovirus have been genetically modified to target tumor cells^{60–62}.

Evidence suggests that OV_s can induce antitumor T cell responses beyond their oncolytic activity by acting as *in situ* vaccines (**Figure 5**). During oncolysis, tumor antigens, DAMPs, and PAMPs are released from infected cells, provoking a pro-inflammatory response that culminates in tumor-reactive CD4⁺ and CD8⁺ T-cell priming^{63–65}.

Additionally, OV_s can be used as delivery platforms for tumor-specific expression of transgenes that contribute to the immunomodulation of the TIME. Many transgenes have been used to arm OV_s, from cytokines, chemokines, tumor antigens, suicide genes, and ECM-degrading enzymes to ICI_s or BsAbs^{66,67}.

Several OV_s have progressed to human trials; to date, four have gained regulatory approval as commercial cancer therapies. The first OV, a picornavirus called Rigvir®, was approved in Latvia to treat melanoma in 2004⁶⁸. One year later, the oncolytic adenovirus (OAd) Oncorine (formerly H101) was approved for head and neck cancer in China⁶⁹. In 2015, the FDA approved T-VEC (also termed IMLYGIC® or talimogene laherparepvec), an HSV expressing the granulocyte-macrophage colony-stimulating factor (GM-CSF) for the treatment of advanced or non-resectable melanoma⁷⁰. Finally, in 2021, a modified HSV, named Teserpaturev/G47Δ or DELYTACT®, was approved in Japan for malignant glioma⁷¹.

3.1.1 Oncolytic virus-mediated ICD

OV_s destroy cancer cells by inducing multiple forms of ICD, potentially eliciting robust antitumor immune responses^{72,73}. ICD represents a form of regulated cell death (RCD) that differs from other RCD types because it activates adaptive immune responses against antigens expressed by the dying cell⁷⁴. The ability of RCD to drive adaptive immunity depends on two major factors: 1) the antigenicity of tumor cells and 2) the release of adjuvants from dying cells⁷⁵. Infected cells display sufficient antigenicity to

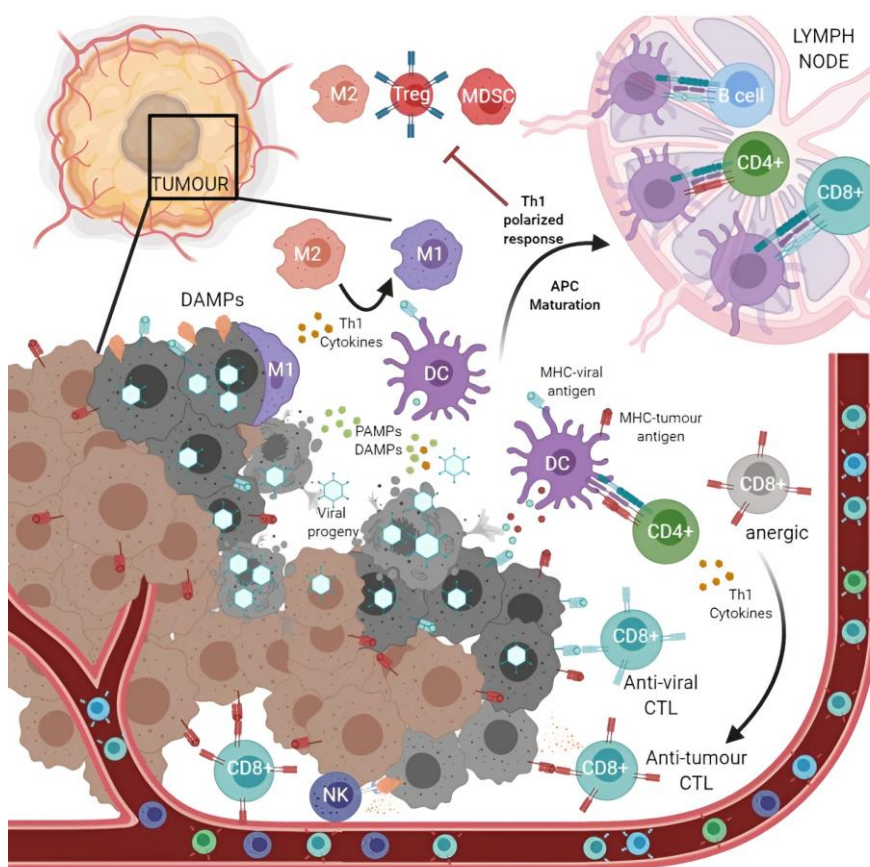


Figure 5. Overview of immunovirotherapy and *in situ* vaccination hypothesis. Tumor-infected cells express DAMPs that could recruit M1 macrophages (purple), among other innate cells, and phagocyte the tumor cell (dark grey), inducing pro-inflammatory cytokines (Th1 cytokines). In parallel, the virus induces oncolysis (light grey), releasing the viral progeny, PAMPs (green), DAMPs (orange), tumor (red), and viral (light blue) antigens. These are captured by DCs that mature and transit to the lymph node, cross-presenting and activating antitumoral and antiviral lymphocytes (CD8+, CD4+, and B cells). They circulate towards the tumor thanks to a chemokine gradient induced during the pro-inflammatory response. The Th1 polarization can revert immunosuppressed phenotypes such as M2 macrophages to M1 or anergic T cells to their activation. Moreover, the Th1 polarization in the lymph node avoids the maturation and differentiation of regulatory Tregs and MDSCs. Image from reference 110.

drive immune responses, as they express a panel of viral antigenic epitopes that escape from immune tolerance for being exogenous; malignant cells as well, mainly due to neoantigens. Besides, adjuvanticity is governed by the spatiotemporal release or surface expression of DAMPs needed to recruit and mature APCs.

Endoplasmic reticulum (ER) and reactive oxygen species (ROS) stress responses play a pivotal role in initiating ICD by generating and releasing DAMPs. Key DAMPs for cell death to be perceived as immunogenic include calreticulin (CRT), high-mobility group box 1 protein (HMGB1), adenosine triphosphate (ATP), and type I IFN⁷⁶.

CRT is an ER chaperone that translocates to the plasma membrane during ICD, providing an “eat me” signal to APCs; upon binding to CD91 on APCs, it promotes phagocytosis and antigen presentation⁷⁷. HMGB1 is passively released from the stressed cell and binds to toll-like receptor 4 (TLR4) on DCs, enhancing the cross-presentation of antigens from dying tumor cells⁷⁸. ER stress also contributes to the active release of ATP via exocytosis. ATP acts as a “find me” signal for APCs upon binding to purinergic receptor P2Y2 (P2RY2), thus facilitating the recruitment of more myeloid cells to sites of active ICD⁷⁹.

At the molecular level, changes in the composition and abundance of cytosolic double-stranded DNA (dsDNA) and dsRNA induced during tumorigenesis or by cellular stress following virotherapy are detected by pattern recognition receptors (PRRs) such as cGAS and RIG-I, respectively, resulting in activation of stimulator of interferon genes (STING) and the Myd88-cascade. Subsequently, complex downstream signaling, including IRF3 and NFκB-dependent pathways, ultimately leads to the expression of type I IFN and other pro-inflammatory cytokines. Type I IFN enhances the cytotoxic activity of CD8⁺ T cells and NKs and supports DCs in cross-priming T cells. They also stimulate macrophages to secrete pro-inflammatory mediators and suppress the immunosuppressive activity of CD4⁺CD25⁺FOXP3⁺ Tregs. Additionally, type I IFNs induce the production of chemokines CXCL9 and CXCL10 in cancer cells undergoing ICD. This is mediated through an autocrine signaling loop, further amplifying the recruitment of immune cells to the tumor site⁸⁰.

4. ONCOLYTIC ADENOVIRUSES

Among OV, adenoviruses (Ads) have been predominant as virotherapy agents due to their safety, genomic stability, and well-understood biology. They are considered safe

for not being associated with severe diseases in immunocompetent individuals. Moreover, the episomal Ad genome prevents integration into the host DNA and encodes a proofreading-DNA polymerase, ensuring genomic stability. Their ability to infect proliferating and quiescent cells and their replicative capacity (lytic cycle) allow high viral yields (10^{12} – 10^{13} viral particles/mL). High genome stability and production titers make Ads feasible for clinical applications⁸¹. Owing to these attributes, OAds are indeed the most frequently used vectors in clinical settings for cancer therapy⁸².

Moreover, Ads have also been instrumental in advancing molecular biology, particularly in understanding DNA replication, cell cycle control, RNA splicing, and oncogenic transformation^{83,84}. This extensive knowledge and their small DNA genome ease the genetic modification of Ads for therapeutic purposes.

4.1 ADENOVIRUS BIOLOGY

4.1.1 General features and classification

Ads are non-enveloped DNA viruses belonging to the *Adenoviridae* family. In humans, 57 serotypes have been identified and categorized into seven species (A–G) based on cross-reactivity patterns of neutralizing antibodies⁸⁵. Among species C, the human adenovirus serotype 5 (Ad5) primarily infects epithelial cells in the respiratory tract, resulting in mild cold-like symptoms. Ad5 has been the most widely used Ad subtype in gene therapy, vaccination, and cancer virotherapy. The viruses presented in this thesis are based on the Ad5 genome.

4.1.2 Virion structure

Ad virions comprise 13 structural proteins; 7 form the icosahedral capsid, and 6 are packaged with the linear double-stranded DNA in the particle's core. The icosahedral capsid measures 60–90 nm in diameter and constitutes 20 triangular faces. The capsid is primarily constructed from three major proteins: hexon, penton base, and fiber (**Figure 6**). The hexon protein forms homotrimers that constitute the faces and edges of the capsid, whereas the penton base forms pentameric structures at the 12 vertices, from which the fiber proteins radiate. The fiber is a homotrimer in which each protein

has three domains: an N-terminal tail that anchors it to the penton base, a central globular *shaft* that extends the fiber outside of the capsid, and a C-terminal *knob* that interacts with cellular receptors. The penton base and the fiber play a key role in viral binding and determine the adenovirus tropism⁸⁶. The hexon is the dominant immunogen for hosts' neutralizing antibodies⁸⁷.

In addition to these primary capsid proteins, minor structural proteins such as pIIIa, pVI, pVIII, and pIX are interspersed between hexons, providing structural stability and acting as cement to maintain capsid integrity⁸⁸. The viral genome, a linear double-stranded DNA, is enclosed within the capsid and is tightly associated with core proteins pV, pVII, Mu (pX), and the terminal protein (TP). These core proteins contribute to the organization and packaging of the viral genome, ensuring its stability within the virion.

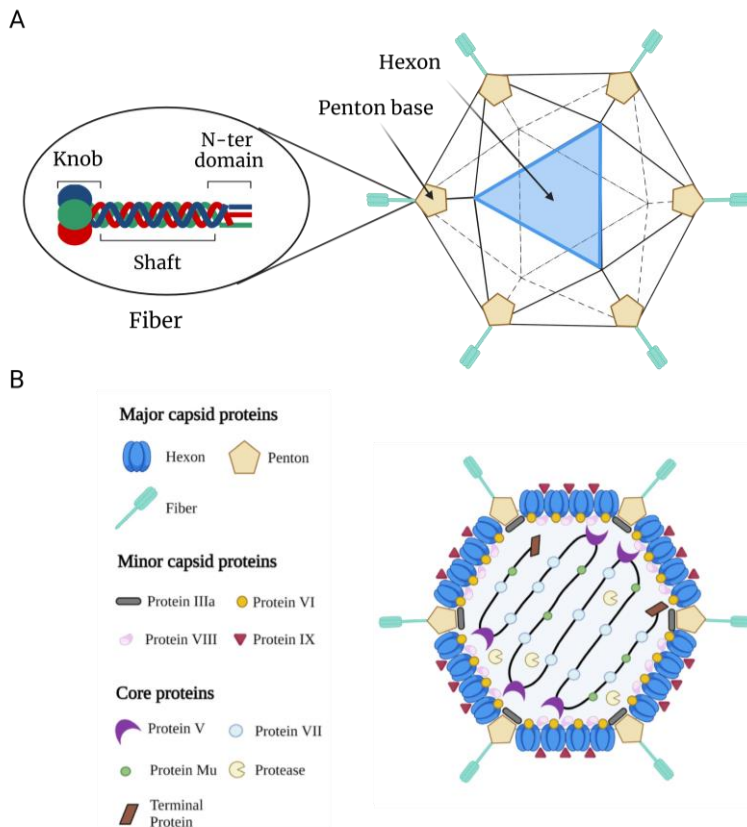


Figure 6. Structure of adenovirus virions. **A)** The outer coat of the capsid consists of three main proteins: hexon, penton base, and fiber. The hexon protein covers the faces and edges of the capsid shell. The penton base is positioned at the 12 vertices of the capsid, from which fiber protrudes. **B)** While the locations of the capsid and minor proteins are well-defined, the arrangement of core proteins and viral DNA remains controversial. Image adapted from reference 87 using BioRender.com.

4.1.3 Genome organization

The Ad5 genome is a 36 kb linear molecule of double-stranded DNA flanked by two inverted terminal repeats (ITRs) at each end containing the viral DNA replication origins. Adjacent to the left ITR lies the packaging signal (Ψ), which is involved in viral genome encapsidation. The rest of the genetic information is organized into overlapping transcription units on both DNA strands, facilitating the production of over 50 viral proteins through extensive alternative splicing.

These genes are expressed temporally regulated during the viral replication cycle. They are classified into six early transcription units (*E1A*, *E1B*, *E2*, *E3*, and *E4*), three delayed-early units (*IX*, *IVa2*, and *E2 late*), and one late transcription unit (*L*) (**Figure 7**). The *E2* and *E3* genes are divided into regions A and B using distinct polyadenylation sites. The major late promoter (MLP) controls the late transcription unit and produces a single primary transcript spliced into five families of late mRNAs (*L1* to *L5*). The late mRNAs encode the structural proteins required for assembling the viral capsid. Additionally,

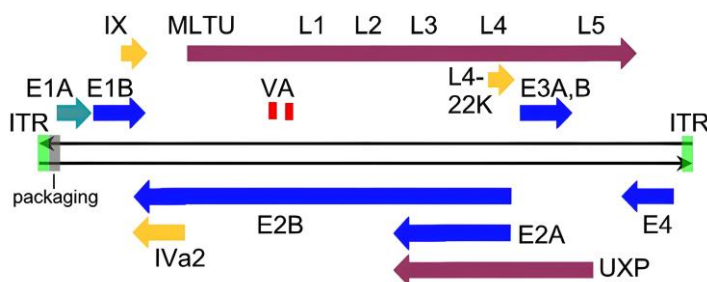


Figure 7. Genome organization of human adenoviruses. The Ad5 genome comprises 36 kb linear double-stranded DNA (represented by black lines with arrowheads indicating the 3' end). The Ad5 genes (colored arrows) are temporally transcribed as early units (*E1* to *E4*), delayed-early units (*IX*, *IVa2*, and *E2 late*), or late units (*L1* to *L5*) in both directions. Inverted terminal repeats (ITR) and the packaging sequence Ψ are indicated as green and grey boxes, respectively. The VA-RNA transcripts are depicted as red boxes. Image from reference 89.

the genome includes viral-associated (VA) genes, which encode two non-coding RNAs involved in translational regulation during infection⁸⁹.

4.1.4 Adenovirus infection cycle

Human Ads are lytic viruses that have a vital cycle that lasts about 30-36 hours and that can be divided into three phases⁹⁰: the immediate-early phase, which includes virus entry and internalization into the host cell, endosome escape, and transport to the nucleus where the expression of early genes allows viral replication; the early phase, in which early proteins are synthesized; and the late phase that involves the transcription and translation of late genes, leading to the synthesis of structural proteins required for virion assembly. The cycle ends with the lysis of cells and the release of the viral progeny to the extracellular media.

4.1.4.1 Adsorption and entry

The Ads' replication cycle initiates with the attachment to the host cell. For Ad5 it occurs through a primary high-affinity interaction between the fiber knob protein and the coxsackie adenovirus receptor (CAR), a 46 kDa transmembrane receptor and member of the immunoglobulin superfamily⁹¹ (**Figure 8, step 1**). This initial attachment is transient and insufficient for entry but serves to tether the virus to the cell surface. Subsequently, a secondary interaction occurs between the Arg-Gly-Asp (RGD) motif on the penton base protein and integrins $\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha 5\beta 1$, and $\alpha V\beta 1$. This binding triggers endocytosis through clathrin-coated vesicles⁹² (**Figure 8, steps 2 and 3**). Inside the endosome, the capsid undergoes partial disassembly due to acidification. Protein VI plays a crucial role in mediating escape from the endosome by exerting lytic activity⁹³, enabling the release of the viral nucleocapsid into the cytoplasm (**Figure 8, step 4**). Once in the cytoplasm, residual virions are rapidly transported along microtubules toward the nucleus, where they are disassembled for subsequent transcription and replication (**Figure 8, step 5**).

4.1.4.2 Early gene expression and DNA replication

Once the viral genome reaches the nucleus, the *E1A* transcription unit is expressed within 6 hours post-infection. The *E1A* promoter only requires cellular proteins to initiate transcription (**Figure 8, step 6**). One of the *E1A*-encoded proteins, the 13S RNA product or the 289 residue (289R) protein, acts as a potent transactivator. This activity, combined with the activation of host transcription factors such as E2F, indirectly activated by E1A proteins (289R and the related 243R forms), drives the expression of the other early transcription units (6-12 hours post-infection, **Figure 8, steps 10a/10b**), which are required to induce the S-phase of the cell and initiate Ad replication. The induction of S-phase entry is achieved by sequestering members of the retinoblastoma (Rb) protein family, tumor suppressors that typically inhibit the cell cycle by binding to E2F transcription factors^{83,84}. This sequestration frees E2F, enabling the expression of S-phase genes required for viral replication. However, this deregulation also triggers the accumulation of p53, a tumor suppressor that induces apoptosis as a defense mechanism. To counteract this, the *E1B* gene products 19K and 55K proteins inhibit apoptosis⁹⁴. E1B-55K inactivates p53, while E1B-19K disrupts downstream apoptotic pathways independent of p53.

The *E2* transcription unit encodes proteins critical for viral genome replication (**Figure 8, steps 11-14**), including the viral DNA polymerase (Ad pol), preterminal protein (pTP), and single-stranded DNA binding protein (ssDBP). These proteins work together to ensure efficient viral DNA replication within the nucleus.

The *E3* gene products focus on immune evasion, ensuring the infected cell survives long enough to complete the viral life cycle. For example, the E3-19K protein sequesters MHC class I molecules in the ER, preventing their transport to the cell surface and thereby reducing antigen presentation. E3-19K also interferes with antigen loading by binding to the transporter associated with antigen processing (TAP).

Finally, the *E4* transcription unit supports viral replication by stabilizing and transporting viral mRNAs and facilitating the expression of late genes.

4.1.4.3 Late gene expression and virion assembly

After accumulating early-phase proteins and initiating viral genome replication (**Figure 8, steps 14-15**), the MLP produces a single pre-mRNA that, through differential splicing and polyadenylation, generates 15 to 20 distinct mRNAs that encode late-phase proteins (**Figure 8, steps 16-19**). Most of these proteins, expressed from the *L1* to *L5* transcription units, are structural components required for virion assembly and genome packaging in the nucleus (**Figure 8, steps 20-21**). During this process, the adenoviral protease cleaves a subset of structural proteins into their mature forms, and the viral particles are assembled to generate infectious Ad virions. Finally, cell lysis and progeny release occur, mediated by the *E3*-encoded adenovirus death protein (ADP) (**Figure 8, step 22**). This is the only early gene expressed during the late phase.

4.2 DESIGN OF TUMOR-SELECTIVE ONCOLYTIC ADENOVIRUSES

A fundamental requirement in developing OAdS is to restrict their replication to tumor cells through genetic engineering; only a few viruses (reoviruses and parvoviruses) present natural tropism for tumor cells. Moreover, many Ad5-based OV's have faced challenges related to variable expression levels of the primary cell entry CAR in cancer cells⁹⁵. Two main strategies have been extensively used to achieve this selectivity. The first is transductional targeting, which aims to impair Ad entry into tumor cells at the level of virus receptor binding. The second is based on restricting Ad replication to tumor cells by manipulating different Ad transcription units.

4.2.1 Transductional targeting

Transductional targeting aims to improve Ad's ability to infect specifically target cells. The goal is to reduce the natural broad tropism of Ad5 for normal epithelial cells while enhancing its infectivity in CAR-deficient tumor cells through two main approaches: ablating the native Ad tropism (detargeting) and directing the Ad to cancer-specific receptors (retargeting).

Among employed detargeting strategies, three should be stressed: (i) genetic ablation of the native tropism determinants, such as the CAR, integrins, and heparan sulfate

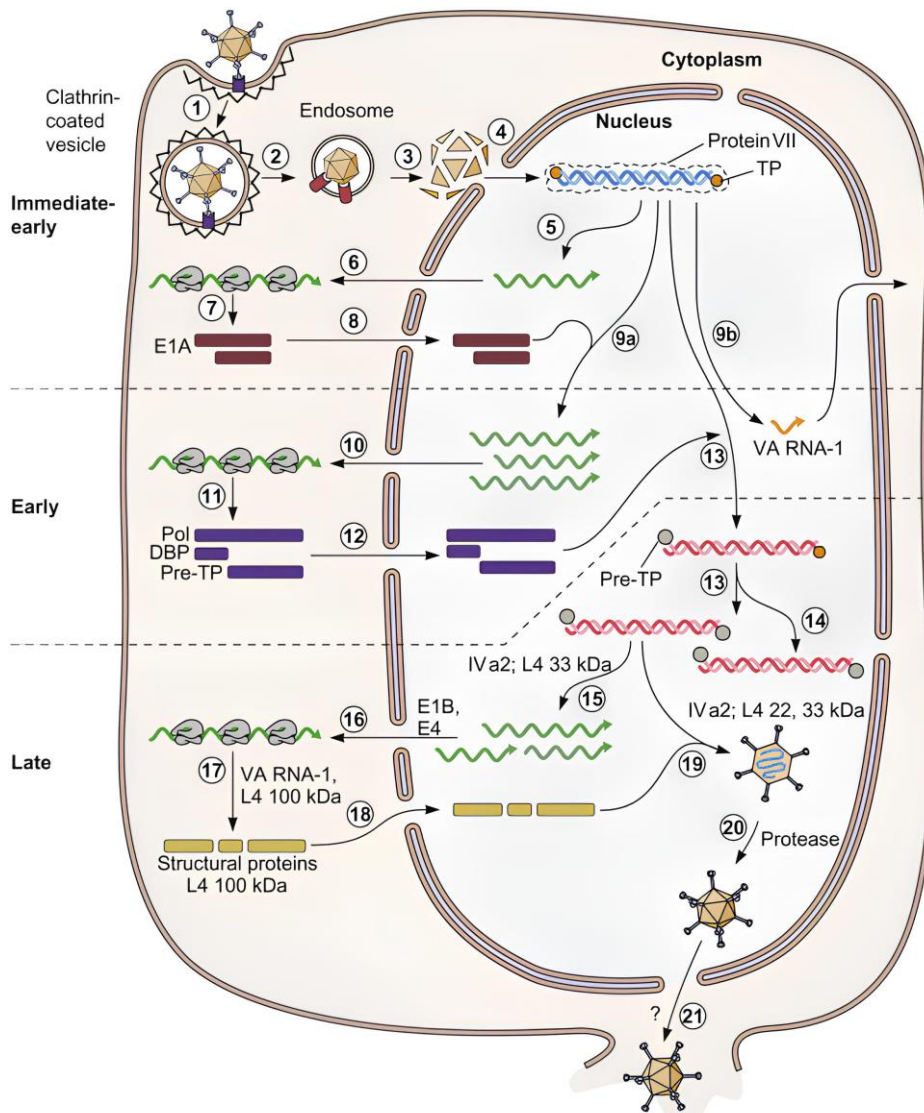


Figure 8. The infectious cycle of human adenoviruses. The infection cycle of adenoviruses can be divided into time-specific events: adenovirus cell entry (steps 1-3), capsid disassembly and genome import into the nucleus (steps 4-5), immediate-early *E1A* gene expression (steps 6-9), *E1A*-mediated transcription of viral early genes (step 10a), translation of viral early proteins (steps 11-13), viral DNA replication (steps 14-15), transcription and translation of viral late genes (steps 16-19), capsid assembly and virion maturation (steps 20-21), and virus progeny release (step 22). Image from reference 90.

proteoglycans (HSPGs); (ii) chemical modifications of the capsid (e.g., polyethylene glycol, PEG) to shield the Ad⁹⁶; (iii) “genetic pseudotyping” between different Ad serotypes⁹⁷.

Regarding retargeting, systems to modify Ad tropism are based either on adding an adapter molecule to crosslink the virus to a target cellular receptor or the genetic modifications of the viral capsid⁹⁸. One of the most successful retargeting to enhance tumor tropism is the RGD-4C peptide (CDCRGDCFC), which targets integrins, commonly overexpressed in different cancer types. The RGD in the fiber HI-loop allows using integrins as a primary receptor instead of the CAR without ablating it⁹⁹.

4.2.2 Post-entry targeting

While transductional targeting is critical to developing OAdS, retargeted viruses may still infect normal cells that express the targeted receptor. Therefore, the most crucial step in OAd development is ensuring that viral replication is strictly limited on cancer cells to minimize off-target effects on healthy tissues.

Two broad types of replication restrictions have been developed. The first is based on mutations or deletions of essential Ad replication genes in normal cells but that can be compensated only in tumor cells¹⁰⁰. One of the best examples is the Ad Δ 24, which carries a 24-base pair deletion in the *E1A* region^{101,102}. This deletion disrupts the interaction between the E1A protein and the Rb protein (pRb), a critical cell cycle regulator. As mentioned before, E1A-mediated sequestration of pRb releases the E2F transcription factor, inducing the S-phase and viral replication in normal cells. However, the Δ 24-E1A protein cannot bind to pRb in these cells, halting viral replication (**Figure 9**). Conversely, in cancer cells where the pRb is non-functional (a defect in approximately 90% of tumors), the E2F-promoting S-phase is constitutively active, allowing viral transcription and replication.

The second strategy involves inserting tissue/tumor-specific promoters or other regulatory sequences in the Ad genome to drive viral gene expression only in cancer

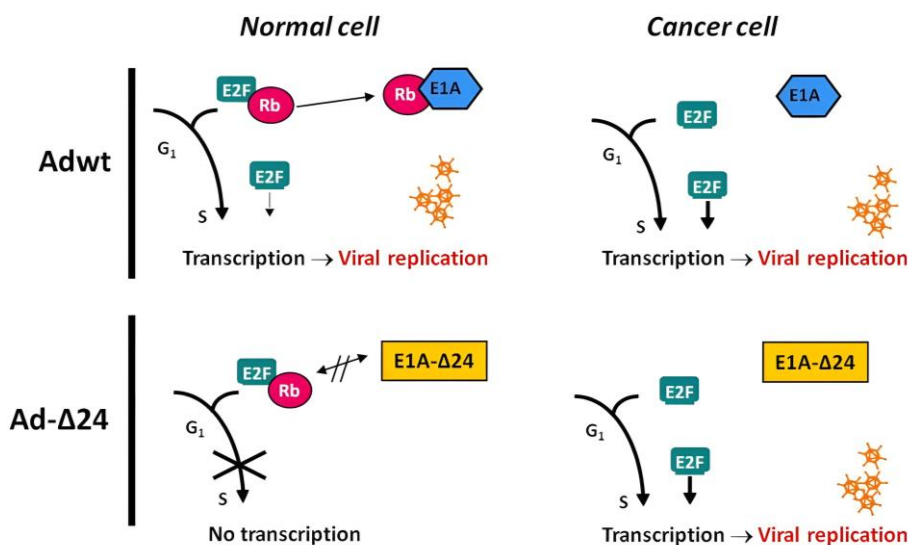


Figure 9. Δ24 selectivity mechanism. E1A protein binds and inactivates pRb to induce the S-phase of the cell cycle and viral replication. This function is redundant in tumor cells since the Rb pathway is truncated, and E2F is already free. In normal cells, Δ24 deletion avoids the dissociation of Rb and E2F, and no viral replication occurs. Image from reference 101.

cells. This has been proved successfully in OAdS using promoters such as the prostate-specific antigen (PSA), human telomerase reverse transcriptase (hTERT), MUC1, AFP, and tyrosinase promoters. A more recent approach involves taking advantage of the differential expression of specific microRNAs (miRNAs) in tumor and normal tissues¹⁰³. Cells use miRNAs to regulate gene expression of many cellular processes. miRNAs binding to target mRNAs induces gene silencing through translational repression. In this context, the insertion of liver-specific miRNAs (that are ubiquitously expressed in normal cells but downregulated in cancer cells) binding sites in the 3' untranslated region (UTR) of *E1A* and *L5* genes abrogates OAd hepatotoxicity without perturbing its antitumoral activity^{104,105}.

4.3 ICOVIR15K

ICOVIR15K (or ICO15K) is a conditionally replicative OAd designed by our group that incorporates several of the targeting strategies described above for a higher tumor specificity.

First, the endogenous promoter E1A has been modified by incorporating eight extra E2F-responsive sites arranged in four palindromes and one additional binding site for the Sp-1 transcription factor¹⁰⁶ (**Figure 10-A**). These modifications enhance E1A expression selectively in tumor cells while minimally increasing the adenoviral genome by only 151 base pairs. This compact modification allows space for the insertion of therapeutic transgenes, adding versatility to the platform. Additionally, the E1A region contains the previously described $\Delta 24$ deletion, which impairs cell replication without alterations in the Rb pathway (**Figure 10-B**).

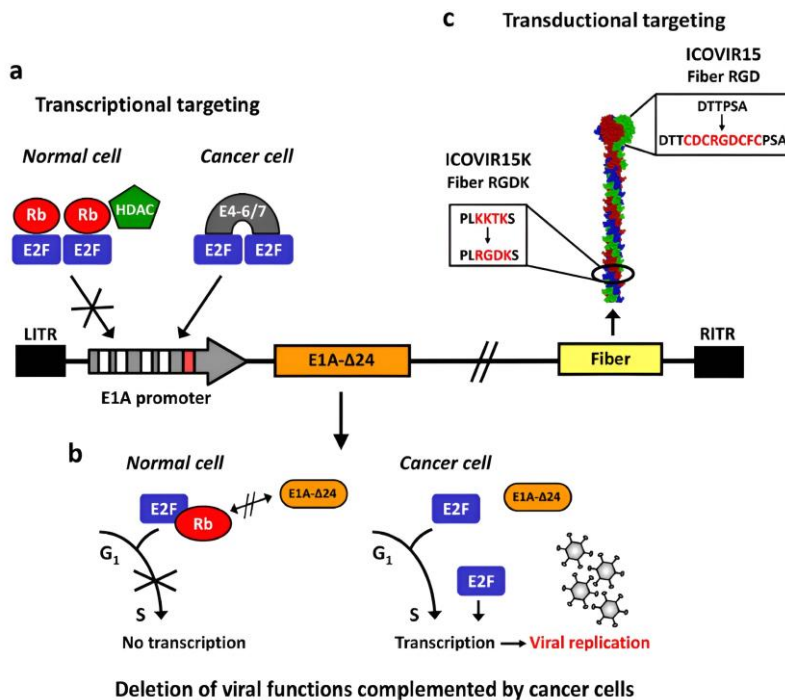


Figure 10. Schematic representation of the ICO15K genome modifications. **A)** ICO15K contains the modified E1A promoter with four E2F boxes (white squares) and one Sp1 box (red square), and **B)** the truncated E1A $\Delta 24$ protein to confer selectivity for tumor cells. **C)** The RGD motif is inserted in the fiber shaft, replacing the KKTK domain. Image adapted from reference 106.

As for transductional targeting, ICO15K combines liver detargeting and a tumor retargeting approach. The Ad fiber shaft contains a KKTK domain that binds to heparan sulfate glycosaminoglycan (HSG) in the hepatocytes, conferring a hepatic tropism¹⁰⁷. To counteract this, our group demonstrated that replacing this KKTK domain with the

RGD motif (RGDK modification) improves the tumor-to-liver ratio, resulting in a reduction of Ad-mediated hepatotoxicity and increased antitumor efficacy *in vivo* after systemic administration¹⁰⁸ (**Figure 10-C**).

This virus has shown potent antitumor efficacy and favorable toxicity profiles after systemic or intratumoral administration, and a modified version expressing the hyaluronidase is currently under clinical investigation for treating pancreatic cancer^{109,110}. ICOVIR15K is used as a platform to keep testing new modifications in the group, as the one presented in this thesis.

4.4 ARMED ONCOLYTIC ADENOVIRUSES

Although refined oncolytic viruses (OVs) with tumor-specific targeting and restricted replication (as described in Section 4.2) have demonstrated safety and selective cancer cell killing, they have proven insufficient clinical benefits in patients. Recognizing these limitations, researchers have advanced OV platforms by integrating transgenes and combining OV with other therapeutic regimens (see Section 4.5) to enhance their potency. At first, efforts centered on amplifying oncolysis. However, the increased recognition of the immune system as an essential component in the efficacy of OVs shifted strategies toward engineering viral vectors with transgenes to boost immune activation within tumors.

Transgenes can be incorporated into OAds using two main strategies. One involves inserting the transgene as an independent cassette with its own promoter and polyadenylation (polyA) signal. This approach allows for constitutive expression using general promoters (e.g., CMV promoter) or tissue-specific promoters to enhance specificity. The second strategy integrates transgenes as additional adenoviral transcription units controlled by the virus's gene expression machinery¹¹¹. For example, transgenes can be linked to viral proteins like E1A through sequences that preserve protein functionality, such as internal ribosome entry sites (IRES), or by attaching a splice acceptor and polyA signal to promote expression via the MLP¹¹². Controlling transgene expression from viral promoters is particularly appealing for

generating armed OAds since restricting its expression to virus replication adds another level of selectivity. Moreover, MLP-driven transgene expression would be limited to the late phase of the replication cycle, which is critical when therapeutic proteins might impair infected cell viability, hindering the completion of the infection cycle.

Transgene cassettes have been successfully inserted into various adenoviral genomic regions, including *E1*, *E3*, and *E4*. Notably, regions like *E3* are often entirely replaceable without altering viral replication¹¹¹. Additionally, transgenes coupled to splice acceptors and polyA signals can be placed in various regions downstream of the MLP¹¹³. The insertion location of the transgene used to arm OAds can significantly influence the expression of the transgene and the overall replication vector efficiency¹¹⁴.

One drawback of OAds compared to larger viruses is the size of the gene to be expressed; the adenoviral genome has a maximum packaging size of approximately 38 kb, which is only 2 kb bigger than the wild-type genome. Since some OAds are modified with tumor-specific promoters, this further restricts the free space for transgene insertion. For instance, in ICOVIR15K, which has a genome size of 36.1 kb, the maximum allowable transgene size is approximately 1.9 kb. As mentioned, most OAds use deletions in the immune modulatory *E3* region for large transgenes to overcome this problem.

Ads offer several key advantages over other OV for transgene incorporation. Unlike most RNA and some DNA viruses, which replicate in the cytoplasm (e.g., picornavirus, reovirus, VSV, and vaccinia virus), Ads replicate in the nucleus, allowing the use of tumor-selective promoters and other nuclear transcriptional control elements that are non-functional in cytoplasmic replication. While DNA viruses like HSV and parvovirus also replicate in the nucleus, Ads stand out due to their highly regulated and temporally ordered gene expression. The *E1A* gene, expressed early during infection, is a critical regulator, enabling precise tuning of transgene expression. Furthermore, the greater

flexibility for incorporating transgenes makes them particularly suitable for complex therapeutic strategies.

4.4.1 Types of transgenes

The insertion of transgenes into the OAds genome, also known as “arming OAds,” aims to destroy neighboring and distant uninfected cells by transgene-encoded therapeutic proteins or RNAs¹¹⁵ (siRNA, miRNA, shRNA).

4.4.1.1 Virocentric transgenes

Before oncolytic adenoviruses were seen as immunotherapeutic agents, a common genetic modification involved adding the genes for cytosine deaminase and HSV-derived thymidine kinase, the so-called “suicide genes” encoding for prodrug activating enzymes. The expression of these genes leads to the conversion of pro-drugs, such as ganciclovir (GC) or 5-fluorocytosine (5-FC), into toxic metabolites within transduced tumor cells, significantly enhancing the antitumor activity compared to unarmed oncolytic virus.^{116–118} Furthermore, OAds encoding for apoptosis inducers (e.g., p53¹¹⁹, TNF α ¹²⁰, or TRAIL¹²¹) have been published based on apoptosis stimulates Ad spread at a late stage of the virus cycle. However, these approaches may also decrease viral replication to induce apoptosis in virus-producing cells. Thus, arming OAds has evolved to amplify viral-mediated immune responses in the last decades.

4.4.1.2 Immunocentric genes

Among the most widely used modifications is the insertion of the GM-CSF transgene. This approach couples viral replication with GM-CSF production, which recruits and matures DCs. This enables T cells to be primed with TAAs released during oncolysis, as seen in clinical studies with ONCOS-102 or CG0070^{122,123}. However, GM-CSF's pleiotropic effects, such as stimulating MDSCs and TAMs, pose potential risks to pro-tumorigenic responses that must be considered. Beyond GM-CSF, other cytokines have been used preclinically: IL-12¹²⁴, IL-15¹²⁵, IL-21¹²⁶, or IL-24¹²⁷. Furthermore, OAds coexpressing combinations of cytokines and immunostimulatory ligands are brought

to clinical trials: TILT-23¹²⁸ (expressing IL-2 and TNF α) or LOAd703¹²⁹ (expressing 4-1BB and CD40L).

Armed OAdS are evolving alongside advances in immunotherapy by expressing ICIs such as anti-CTLA-4¹³⁰, soluble ligands like OX40L¹³¹, GITRL¹³², and the fusion protein SPD1-CD137L¹³³. Moreover, BsAbs-expressing OAdS have been designed to engage T cells and specific tumor antigens such EGFR¹³⁴ or FAP¹³⁵, generated in our group, FR- β ¹³⁶, or CD44v6¹³⁷ have further demonstrated potent preclinical activity. Interestingly, all mentioned approaches have been combined in a single OAd termed NG-641, armed with four transgenes: IFN α to drive DC priming, CXCL9, and CXCL10 to recruit T cells, and FAP-BiTE to redirect them¹³⁸.

4.4.1.3 TME-modulating transgenes

Additional arming strategies targeting the immunosuppressive TME have also been pursued. OAdS carrying ECM degradation-related genes can break through physical barriers between immune cells and tumor sites. For instance, hyaluronidase¹³⁹-, relaxin¹⁴⁰-, or DNase-I¹⁴¹-armed OAdS have enhanced antitumor efficacy in preclinical models by improving viral spread within tumors. OAdS have also been engineered to express antagonists targeting molecules critical for resistance to ICIs, such as TGF- β ¹⁴², further expanding their therapeutic potential.

4.5 CLINICAL EXPERIENCE WITH ONCOLYTIC ADENOVIRUSES

Adenoviruses are the most commonly used oncolytic viruses in clinical trials. As stated in Section 4.4, OAd therapies, including armed variants, exhibit firm safety profiles but have yet to demonstrate compelling efficacy as monotherapies. The most outstanding clinical data have been achieved by combining OAdS with other therapeutic regimens.

The first clinical trial of replication-competent adenoviruses (Ads) was conducted more than 60 years ago, involving 30 women with cervical carcinoma who received intratumoral or systemic wild-type (wt) Ad injections¹⁴³. While the treatment was well tolerated, with only mild flu-like symptoms reported, tumor biopsies revealed necrosis in over 50% of patients. However, no complete tumor responses were observed; all

cases showed disease progression. High titers of neutralizing antibodies (NAbs) were detected in cervical swabs two weeks post-treatment, suggesting the antiviral immune response could be associated with the virus clearance. The study underscored the favorable safety profile of Ads. Still, it highlighted the need for more potent OAd and identified antiviral immune responses as a potential barrier to effective virotherapy.

The first modified OAd to enter clinical trials was ONYX-015, a virus engineered with an *E1B-55K* deletion that was supposed to restrict replication to p53-cells, such as cancer cells¹⁴⁴. Phase I trials confirmed its safety¹⁴⁵, and phase II trials reported modest efficacy, with 14% of treated lesions showing complete or partial regression and 41% demonstrating stable disease¹⁴⁶. However, phase III trials were canceled due to a corporate takeover. Despite these challenges, Chinese researchers patented H101 (Oncorine®), a similar OAd with additional E3 deletions. A phase III trial combining H101 with cisplatin-based chemotherapy showed a response rate of 78.8% in nasopharyngeal carcinoma, leading to its regulatory approval in China. However, questions about patient cohort size, lack of a direct comparison with standard-of-care treatment, and incomplete patient follow-ups raised concerns¹⁴⁷. Additionally, subsequent studies revealed that the tumor selectivity of E1B-55K viruses was more complex than simply targeting p53¹⁴⁸ and that ONYX-015 and H101 genomic deletions negatively affected their oncolytic potency in some cancers¹⁴⁷.

These facts prompted the development of more potent and selective OAd, and second-generation OAd were developed. For example, E1A-Δ24 viruses targeting the pRb pathway (see Figure 9) and those with enhanced tumor targeting (via RGD modifications in the fiber HI-loop) were brought to the clinics. ICOVIR-5¹⁴⁹, generated in our group, was tested intravenously in a phase I clinical trial for cutaneous and uveal melanoma. Though no tumor responses were observed, 4 out of 12 patients were positive for viral genomes in metastasis¹⁵⁰, demonstrating the feasibility of OAd for systemic metastasis treatment.

Another notable example is DNX-2401 (or Delta24-RGD), which achieved significant outcomes in recurrent malignant gliomas, with 20% of 25 patients surviving beyond 3

years and more than 95% of tumor regression in 3 patients. The authors presumed that long-term survival resulted from the virus' direct oncolytic effect, followed by elicited immune-mediated antitumor responses¹⁵¹. These results earned DNX-2401 fast-track and orphan drug designations from the FDA and EMA to treat malignant glioma. In a phase I trial for newly diagnosed diffuse intrinsic pontine glioma (DIPG) patients, intratumoral infusion of DNX-2401 followed by radiotherapy led to changes in T-cell activity and tumor stabilization or size reduction in some patients¹⁵². Moreover, a recently completed phase 2 trial combining DNX-2401 with anti-PD1 in 49 patients with recurrent glioblastoma was safe with notable survival benefits¹⁵³.

Other second-generation OAdS in early clinical trials are CG7870 and ColoAd1 (Enadenotucirev). Intravesical CG7870 reported a promising 50% complete response after 6 months in a phase II trial for bladder cancer, with acceptable toxicities at a dose of 1×10^{12} vp¹⁵⁴. ColoAd1, a chimeric virus between 11 and 3 serotypes generated by “directed evolution” (Ad11/3¹⁵⁵), is being tested intravenously in different cancer types in phase I/II clinical trials. It has shown manageable toxicity in repeated-dose schedules (days 1, 3, and 5) and was detected in tumor lesions¹⁵⁶, though significant response data are pending when used alone¹⁵⁷ or in combination (e.g., anti-PD1¹⁵⁸). More recently, promising data from phase 1/2 clinical trial with AdAPT-001, an OAd expressing TGF- β trap, reverses TME immunosuppression and sensitizes tumors to ICIs, showing an overall objective response rate (ORR) of 29.1 % in an immunorefractory advanced cancer population¹⁵⁹.

Early clinical efforts have highlighted significant challenges the field must solve to become an effective anticancer therapy (detailed in Section 4.6). Advanced armed-OAdS are being developed to address such treatment limitations or enhance antitumor activity (see Section 4.4.1). When writing this thesis, more than 40 clinicals (19 still active) involving OAdS, either alone or with chemotherapy, radiotherapy, or immunotherapies, have been registered.

4.6 CHALLENGES FOR ONCOLYTIC ADENOVIRUSES

The limited clinical responses of OAd therapy, especially as a monotherapy, can be primarily attributed to several challenges that OV_s encounter after systemic administration. The tumor delivery and targeting (**Figure 11**, red zone), the intratumoral spreading (**Figure 11**, yellow zone), and the immune response in the tumor (**Figure 11**, blue zone) were identified as the main barriers to virotherapy efficacy¹⁶⁰.

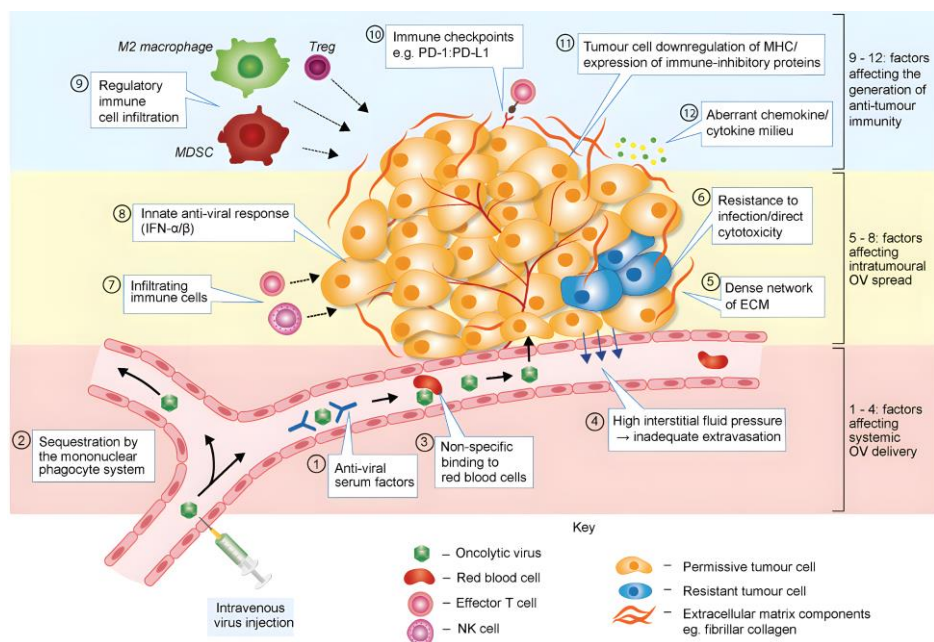


Figure 11. Challenges to effective OV_s therapy in vivo. When delivered to tumors via the bloodstream, most of the initial dose is retained by hepatic macrophages (Kupffer cells) and splenic macrophages (box 2). Additionally, OV_s can be neutralized through interactions with blood cells, complement proteins, and neutralizing antibodies (boxes 1 and 3). The high interstitial fluid pressure within the tumor hampers the extravasation (box 4). If extravasation is achieved or after intratumoral injection, several factors may limit the intratumoral viral spread and therapeutic effectiveness: the tumor stroma (e.g., ECM) (box 5), the antiviral activity, and cancer resistance to OAd-mediated cell killing (box 6-8). Finally, the immunosuppressive TME impairs the OAd-induced antitumor response (box 9-12). Image from reference 160.

4.6.1 Tumor delivery and targeting

Systemic administration of OAd_s offers better tumor coverage than intratumoral (IT) administration, but it also poses several challenges. Upon intravenous injection, the

liver and spleen sequestered a substantial portion of the viral dose due to their large vascular fenestrations, leading to hepatotoxicity¹⁶¹ (**Figure 11-2**). Liver-specific uptake is further exacerbated by the interaction of the viral capsid with coagulation factors, which increases the liver-to-tumor targeting ratio¹⁶². Kupffer cells in the liver actively phagocytose the virus¹⁶³, while serum factors such as NABs, complement proteins and antiviral cytokines promote rapid viral clearance via the reticuloendothelial system¹⁶⁴ (**Figure 11-1**). These mechanisms are particularly problematic given the high seroprevalence of wt-Ad5 in humans, with up to 90% of patients possessing pre-existing NABs.

OAdS also attach to human erythrocytes via CAR (**Figure 11-3**), though the impact on viral bioavailability is still under debate^{165,166}. The high interstitial fluid pressure within tumors impedes viral extravasation from the vasculature¹⁶⁷ (**Figure 11-4**). First-generation OAd5s relied on CAR receptors for infectivity, frequently downregulated in advanced cancers⁹⁵. While second-generation OAdS addressed this issue with RGD-targeting strategies, their intravenous activity remained suboptimal due to the abovementioned challenges.

Several strategies have been explored to overcome this first barrier. Shielding the viral capsid with synthetic polymers¹⁶⁸, albumin-binding domains¹⁶⁹, or replacing the viral capsid's hypervariable regions (HVRs) from other serotypes¹⁶⁷ helped evade pre-existing immunity. Cellular carriers, such as MSCs, have also been employed to deliver viruses to tumor sites while protecting them from immune clearance¹⁷⁰. Another interesting approach was the so-called Ab-retargeting, consisting of bispecific molecules that redirect adenovirus-specific antibodies to tumor cells, thereby labeling them for antibody-dependent cell-mediated cytotoxicity (ADCC)¹⁷¹.

4.6.2 Intratumoral spreading

Once OAdS reach the tumor, their ability to spread is often limited by the tumor stroma, which comprises various cellular components, including CAFs, immune cells (**Figure 11-7**), and an extensive ECM (composed of collagens, proteoglycans, and glycoproteins) (**Figure 11-5**). Tumor-mediated alterations in ECM remodeling

mechanisms are correlated with the development and progression of cancer¹⁷². Moreover, it acts as a physical barrier, restricting drug distribution and OAd replication within tumors¹⁷³.

An additional issue that compounds the tumor stromal anatomical barriers is the relatively slow replication rate of OAd, which also negatively impacts both their ability to induce bystander lysis and their efficiency in spreading throughout the TME.

4.6.3 Immune responses

The role of the immune system in cancer virotherapy remains a subject of considerable debate. Initially, the robust innate and adaptive immune responses elicited by OAd were viewed as a barrier to efficacy, primarily because these responses were thought to limit viral replication, a process traditionally considered essential for tumor regression. Clinical experience shows that viral replication in patients is restricted due to immune clearance, meaning a robust antiviral immune response is elicited despite the immunosuppressive TME (**Figure 11-9 to 12**). Furthermore, the immunogenicity of viral epitopes often results in biased immune responses against them over other epitopes, a phenomenon known as immunodominance¹⁷⁴.

Nonetheless, tumor regression has been reported in cases where OAd replication was minimal or undetectable. Preclinical studies have demonstrated antitumor efficacy using non-replicative viruses in murine models, suggesting that mechanisms other than direct viral oncolysis contribute to therapeutic outcomes. Even more provocative is the increasing preclinical evidence suggesting that preexisting antiviral effector responses might also be engaged to directly contribute to tumor eradication^{175,176}. On this matter, the delayed complete regressions observed in glioblastoma patients treated with DNX-2401, occurring months post-administration after pseudoprogression without detectable viral replication^{151,177}, have prompted a reassessment of the role of the immune system in oncolytic virotherapy.

This evolving perspective positions the immune system as an adversary that impedes viral oncolysis and, simultaneously, as a potential ally that drives antitumor responses

by reversing TME immunosuppression. Achieving a balance between these opposing effects may be critical for the success of virotherapy. The interplay between antitumor and antiviral immune responses has been a central focus of this thesis and is further described in Section 6 of this chapter.

The work described in this thesis mainly addresses the last two challenges of OAd-based therapies (limited viral spread within the tumor and the immunodominant antiviral immune response), which will be discussed in detail in the coming sections.

5. STRATEGIES TO ENHANCE BYSTANDER LYSIS AND TUMOR SPREAD

As mentioned before, improving the efficiency of virus penetration and spread in solid tumors is essential for advancing OV therapy.

Targeting ECM components or CAFs are the main strategies to improve OAd spread within the tumor. ECM-degrading enzymes have been employed to enhance viral penetration in solid tumors¹⁶⁷, with hyaluronic acid (HA) being a dominant target due to its abundance in the ECM. In our group, delivery of an HA-expressing OAd (VCN-01) showed improved dissemination and therapeutic activity in a human melanoma xenograft model¹⁷⁸ and is under clinical investigation for the treatment of advanced pancreatic adenocarcinoma¹⁰⁹. Other ECM-modulating proteins were targeted. For instance, OAd-expressing small molecules like relaxin¹⁷⁹ and decorin¹⁸⁰ affect ECM remodeling by inhibiting collagen synthesis, upregulating matrix metalloproteases (MMPs), which contribute to collagen degradation, or downregulating their inhibitors (TIMPs).

Regarding CAFs, bioselection experiments of Ads in CAFs conducted by our group demonstrated that truncation of the i-leader protein enhances viral release and propagation within these cells¹⁸¹. Another promising approach involves using the SPARC promoter to drive virus replication selectively in CAFs and other stromal cells. This strategy induces fibroblast oncolysis and has shown potent *in vitro* cytotoxicity in stroma-rich ovarian cancer xenograft models¹⁸².

Alternatively, other studies demonstrated that ultrasound cavitation-mediated delivery of OAdS can also increase the tumor penetrance of systemically delivered viruses¹⁸³.

Furthermore, viral replication in the tumor and subsequent spread from infected to uninfected cells is critical for tumor eradication. OAdS are nuclear viruses. This means that, unlike cytoplasmic viruses, the replication cycle is typically longer because it includes steps such as nuclear entry and the use of host enzymes for transcription and splicing. The time required for viral replication delays the accumulation of infectious virions, limiting the rate at which uninfected tumor cells can be lysed through subsequent cycles of infection. Clinical trials revealed that it is partly due to the antiviral immune response that is subsequently mounted, which rapidly clears the virus¹⁸⁴ (discussed in Section 6). For this reason, viral infections with rapid replication and dissemination are often believed to inflict more significant damage on tumors before being contained by the immune system^{185,186}. Multiple approaches have been described to improve OAd local release: ADP overexpression¹⁸⁷, inducing apoptosis through the deletion of antiapoptotic genes like *E1B19K*¹⁸⁸ or incorporating proapoptotic transgenes to facilitate cell death and viral release (see Section 4.4.1.1). Viral yields can also be enhanced by introducing specific miRNA-binding sites within the OAd to trap mRNAs that hamper viral oncolysis¹⁸⁹. Additionally, bioselection of mutagenized Ads has identified variants with improved release kinetics and enhanced therapeutic efficacy¹⁹⁰.

Regardless of how effectively an oncolytic virus (OV) spreads within a tumor, many cancer cells often evade infection¹⁹¹. To transform oncolytic virotherapy from a tumor-reducing approach into a potentially curative treatment, it is essential to target these uninfected (bystander) tumor cells. Several methods can achieve this. For instance, OAdS encoding for the aforementioned “suicide genes” can diffuse the toxic metabolic products to peripheral uninfected tumor cells through intercellular contact¹⁹². OAdS can be equipped with antiangiogenic genes (e.g., VEGF inhibitor¹⁹³) to enhance viral permeability and inhibit endothelial cell proliferation. Alternatively, OVs are also

armed with fusogenic membrane glycoproteins to promote bystander killing through the induction of cell-cell fusion and massive syncytia formation¹⁹⁴.

We investigated the potential of using secreted toxins as candidate transgenes to enhance OAd's tumor-debulking. Previous studies have explored the delivery of immunotoxins using adenoviral vectors. Immunotoxins consist of a tumor cell-binding component, such as an antibody or ligand, linked to a cell-killing agent, typically a toxin derived from bacteria or plants. This combination enables the toxin to specifically target and eliminate tumor cells. In this dissertation, we examined the therapeutic potential of a different toxin-based strategy, which involves combining bacterial pore-forming toxins (PFTs) with oncolytic adenoviruses (OAdS).

5.1 PORE-FORMING TOXINS

Bacterial pathogens exhibit a highly opportunistic nature, which can be attributed to the evolutionary development of effective virulence factors that can cause significant damage to eukaryotic cells. Among them, pore-forming toxins (PFTs) are a critical component of the virulence arsenal of many pathogenic bacteria. These proteins belong to the broader class of pore-forming proteins (PFPs), an ancient family found in bacteria and across all kingdoms of life. Historically, the term "hemolysin" was applied to PFTs due to their initial discovery as agents capable of lysing erythrocytes *in vitro*¹⁹⁵. Subsequent research has shown that many of these proteins are initially released as water-soluble monomers, which can bind to cellular membranes. Once they attach, they oligomerize and form pores in the targeted eukaryotic cells as integral transmembrane proteins. PFTs account for roughly 30% of all identified bacterial proteins.

PFTs are divided into two structural categories based on the mechanisms they use to penetrate cellular membranes: α -PFTs, which utilize helical structures, and β -PFTs, which form barrel-shaped pores¹⁹⁶. A unique feature of certain PFTs, which makes them particularly appealing for targeted therapeutic applications, is their requirement for activation by host proteases to oligomerize and unleash their lytic potential¹⁹⁷. One

of these toxins meeting this latter point was chosen in this work to be cloned into the genome of an OAd.

5.1.1 Parasporins from *Bacillus thuringiensis*

Parasporin (PS) is a subtype of PFTs produced from the gram-positive entomopathogen *Bacillus thuringiensis* (*Bt*). These bacteria are commonly used as insecticides, especially in the transgenic crop¹⁹⁸. As a general mode of action, these toxins are produced during the bacterium's sporulation as inclusion crystals. When ingested by insect larvae, the alkaline conditions of the midgut dissolve these crystals, releasing the monomeric protoxins into the environment. After binding to the specific receptors on the brush border membrane of the midgut epithelium, the activated toxin would lead to insect death and allow spores to germinate¹⁹⁹. During the sporulation phase, *B. thuringiensis* produces two types of parasporal toxins: Crystal (Cry) and Cytolytic (Cyt) proteins. Some *Bt* strains additionally produce insecticidal non-crystal forming proteins during vegetative growth (VIPs)²⁰⁰. Although parasporins initially were categorized as a subfamily of Cry proteins, their unexpected remarkable cytotoxicity and increasing members made them unique and were later considered a separate entity²⁰¹. Parasporins are currently defined as “*B. thuringiensis* and related bacterial parasporal proteins that are non-hemolytic but capable of preferentially killing cancer cells”²⁰². To date, 19 parasporins have been identified and classified into six groups (PS1 to PS6) with different sizes and modes of action²⁰³. Like the Cry toxins, parasporins require proteolytic processing to activate their toxicity²⁰⁴.

Based on their structure, parasporins can be classified into the three-domain and β -PFT type²⁰⁵. The three-domain parasporins resemble the structure of three-domain Cry toxins, characterized by a highly conserved five-block sequence and a distinct three-domain architecture. These toxins are initially produced as nascent proteins of approximately 80 kDa, which are processed into their active forms, typically 60–70 kDa. Examples of this type include PS1, PS3, and PS6. The β -PFT-type parasporins, in contrast, share structural similarities with MTX-like Cry toxins and exhibit limited sequence similarity to β -PFTs such as aerolysin from *Aeromonas hydrophila*. These

toxins are synthesized as inactive precursors ranging from 31 to 37 kDa, then cleaved into their active forms of 27–30 kDa. Notable members of this category include PS2, PS4, and PS5.

One of the most essential features of parasporins is their ability to discriminate cancer from non-cancer cells, which is related to specific receptor recognition. **Table 2** summarizes parasporins' cytotoxic activity across various cancer cell lines, with reported lethal concentrations (LC₅₀, exposure concentration of a toxic substance lethal to half of the tested population) values ranging from as low as 0.0017 µg/mL for PS2 against Sawano cells to 3.0 µg/mL for PS1 against HepG2 cells. Notably, all parasporins demonstrate cytotoxicity against multiple cancer cell lines, with PS2 and PS5 exhibiting the broadest range of target strains and the most potent LC₅₀ values²⁰⁶. This is one of the reasons we chose PS2 for the purposes of this thesis.

5.1.1.1 Parasporin 2

The PS2 family are pore-forming proteins included in the β-PFT subgroup. They are small polypeptides of approximately 37 kDa, and the cleavage of both N- and C-terminal inhibitory domains by serine proteases, such as proteinase K and trypsin, generates the active form of 30 kDa (**Figure 12-A**).

Upon this cleavage-mediated activation, the active toxin binds an unknown principal receptor in the cell membrane, oligomerizes, and crosses the cell membrane to form a pore that perforates the membrane and causes cell death. Insertion into the membrane requires a large conformational change from the protoxin to the toxin structures in which a protein domain extends and penetrates the membrane. Though its intrinsic selectivity remains uncertain, it has been described that binding to GPI-anchored proteins is required in the cytotoxic action of PS2 as a kind of coreceptor²⁰⁷ (**Figure 12-B**). As mentioned, PS2 has been found to have strong cytotoxic activities against various human cancer cell lines with markedly divergent target specificities. For example, it was highly cytotoxic toward human hepatocyte cancer cells (HepG2 cells) but not toward normal hepatocyte cells (HC cells)²⁰⁸ (**Table 2**).

Table 2. Cytotoxic activity of the different Parasporins shown as lethal concentration (LC₅₀) in µg/mL. Best values are bolded. Note that PS5 has also shown toxicity to normal uterus and lung cells. Table adapted from reference 206.

Cell lines	PS1	PS2	PS3	PS4	PS5	PS6
MOLT-4 (Leukemia)	2.2	0.022	>10	0.472	0.075	ND
Jurkat (Leukemia)	>10	0.018	>10	>2	0.124	ND
HL-60 (Leukemia)	0.32	0.019	1.32	0.725	1.079	ND
U937 DE-4 (Leukemia)	ND	ND	ND	0.980	>10	ND
K562 (Leukemia)	ND	ND	ND	>2	4.249	ND
HepG2 (Liver Cancer)	3.0	0.019	2.8	1.90	0.049	2.3
HeLa (Cervical Cancer)	0.12	2.5	>10	>2	0.08	7.2
Sawano (Endometrial Cancer)	>10	0.0017	>10	0.245	0.065	ND
TCS (Cervical Cancer)	ND	>2	>10	0.719	0.046	ND
Caco-2 (Colon Cancer)	>10	0.013	>10	0.124	0.30	>10
A549 (Lung Cancer)	>10	>10	>10	>2	>2	ND
T (normal cells)	>10	0.148	>10	>2	ND	ND
HC (normal hepatocyte cells)	>10	1.1	>10	>2	>10	>10
UtSMC (normal uterus cells)	>10	>2	>10	>2	0.223	ND
MRC-5 (normal lung cells)	>10	>2	>10	>2	0.273	ND
ND: not determined						

5.1.1.2 Oncolytic adenoviruses expressing Parasporin 2

The advantages brought by the generation of OAdS armed with PFTs comprise the already mentioned oncolytic effects at the tumor site: tumor cell lysis and localized transgene expression, which occur in iterative and exponentially increasing cycles. However, the key innovation of this approach lies in the potent, tumor-specific tropism of PS2. Thus, arming an OAd with PS2 presents a dual advantage: (i) PS2 enables the elimination of uninfected tumor cells via pore formation, thereby enhancing viral spread within the TME, and (ii) given the mechanism of action of PS2, we postulate that the induced cell death is of the immunogenic type (ICD), thereby contributing to tumor inflammation. Although PS2 has demonstrated minimal cytotoxicity toward normal hepatocytes, ICOVIR15K was selected as the adenoviral platform for this approach to further mitigate the risk of hepatotoxicity.

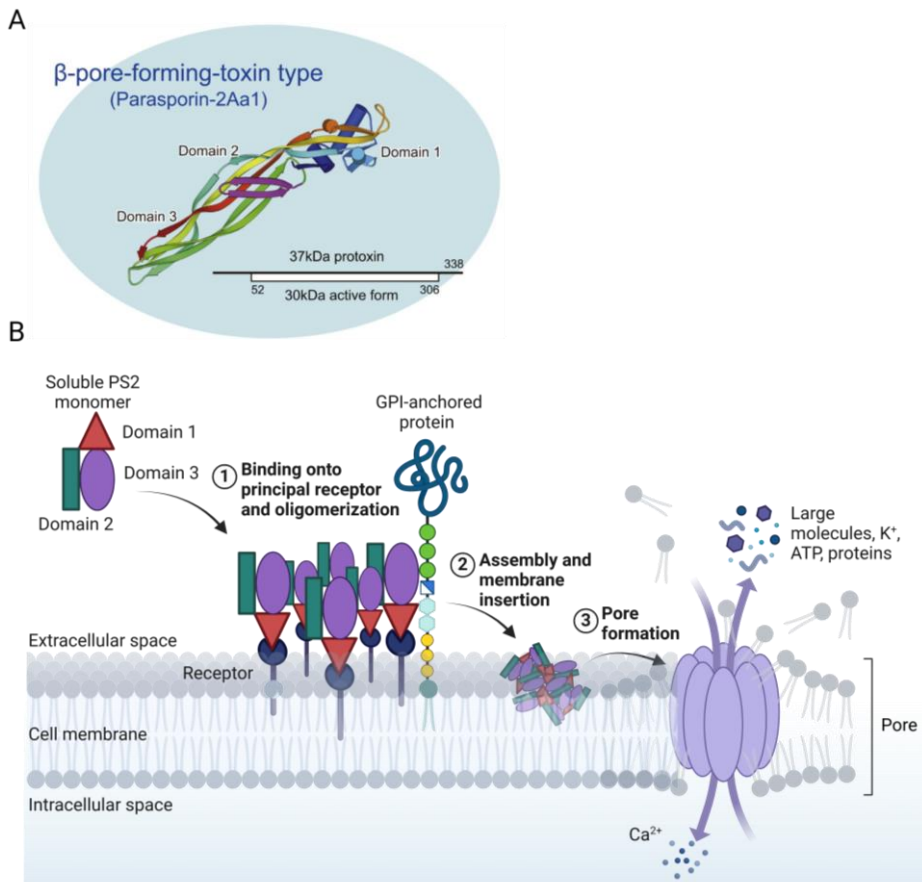


Figure 12. Parasporin 2 from *Bacillus thuringiensis*. **A)** PS2 is a β -PFT whose protein structure can be divided into three domains along its long axis. Domain 1 shows extensive structural diversity and is rich in aromatic residues, probably responsible for receptor binding. In contrast, domains 2 and 3 are both β -sandwiches with remarkably conserved folds. A prominent amphipathic β -hairpin in domain 2 is characteristic of this group of toxins and is supposed to be inserted in the membrane upon pore formation, while domain 3 plays a vital role in the interaction between the individual monomers in the oligomer. **B)** PS2 monomers produced in a water-soluble conformation bind to their primary receptor in lipid rafts of the cell membrane. Additional binding to the glycan core of GPI-anchored proteins was required for the cytotoxic action of PS2. Upon binding, the toxins were cleaved to become active and start the oligomerization process, which, in β -PFTs, occurs at the membrane surface, producing an intermediate structure known as a pre-pore. Rearrangements of this pre-pore lead to concerted membrane insertion into the hydrophobic lipid bilayer to form a disruptive pore in a cellular membrane, which promotes the influx or efflux of ions, small molecules, and proteins through the host membrane. Image A from reference 205. Image B created with BioRender.com.

6. VIRAL EPITOPE IMMUNODOMINANCE

OVs face unique challenges compared to other forms of immunotherapy due to their high immunogenicity. Antiviral immunity develops rapidly, with viruses typically cleared within 1–2 weeks post-administration, whereas tumors often persist in most cases. This vast contrast arises because, unlike antitumor CTLs, antiviral immunity did not suffer tolerogenic conditioning during the escape phase. Moreover, despite tumor neoepitope attracting interest for not being subjected to central tolerance mechanisms, they still might differ in one amino acid from their wild-type “self” counterparts. Conversely, viral epitopes are highly immunogenic for their exogenous nature (no central tolerance education).

6.1 PRECLINICAL EVIDENCE OF ADENOVIRUS IMMUNODOMINANCE

T cells compete with each other during their genesis and maturation after an antigenic challenge, leading to epitope dominance and epitope interference^{209,210}, known as immunodominance.

The immunodominance of adenoviral epitopes has been extensively studied as masking immune responses against therapeutic transgenes in adenovirus-based vaccines^{211,212}, which could be particularly relevant for OVs engineered to express tumor antigens. For example, studies of intramuscular administration of an Ad5-based vector encoding Hepatitis B surface antigen (HBsAg) and ovalbumin (OVA) in BALB/c mice demonstrated that preexisting immunity to Ad5 significantly diminished CD8⁺ T cell responses against HBsAg and OVA²¹³. Instead, the isolated CD8⁺ T cells predominantly targeted Ad5-derived epitopes. This skewing toward antiviral responses was also observed in antibody-deficient IgH^{-/-} mice, suggesting that Ad5-specific cellular immunity, rather than humoral responses, impairs the priming and expansion of CD8⁺ T cells specific to the transgene-encoded antigens. This effect becomes more intense if patients have been pre-exposed to the vector, as occurs with Ad5 in humans.

Since similar viral strains are often used in the OV field, the immunodominance observed for vector vaccines highlights current gaps in OV research and might indicate

shared mechanisms. Accordingly, data from a preclinical model of hepatocellular carcinoma using an IFN- β -armed OV also showed that OVs can antagonize anti-PD-L1 therapy by stimulating dominant antiviral CD8⁺ T cell responses that suppress antitumor T cell populations, which are the target of anti-PD-L1²¹⁴.

6.2 ADENOVIRUS IMMUNODOMINANCE IN CLINICS

Clinical evidence supports the preclinical findings of immunodominance in humans. Several trials of Ad-vectored vaccines demonstrate that preexisting Ad5-specific immune responses, such as CD8⁺ T cells or antibodies, are associated with significantly reduced induction of immune responses to the delivered antigens. This has been observed in trials involving vaccines for Ebola virus²¹⁵ and HIV^{216,217}, which reported diminished CD4⁺ T cell, CD8⁺ T cell, and antibody responses in Ad5-seropositive individuals. Additionally, the immunodominance of Ad epitopes may impact prime-boosting strategies since tumor antigens are released concurrently with viral antigens after prime-oncolysis.

Viral immunodominance is also plausible in OAdS. Since the initial steps of ONYX-015²¹⁸, nearly 50 clinical trials involving OAdS have been conducted as of the time this manuscript was written. **Table 3** highlights 16 trials focusing on applying OAdS as monotherapy across various cancer types. Only 7 of them reported complete responses (CR) in some patients. The best results reported have been 47% 6-month CRs in bladder cancer¹⁵⁴ and one long-term CR in glioblastoma (> 8 years after treatment)¹⁵¹. The fact that there is always an antiviral response, with total virus early clearance consistently occurring, but that complete tumor remissions are more challenging to achieve suggests that antiviral responses are much more efficient and may, in some way, interfere with or mask antitumoral responses. The best responses are achieved in combinatorial assays. For instance, the same OAd used in the bladder cancer NCT02365818 trial showed CR 6-month rates of 82% when combined with the anti-PD1 Pembrolizumab²¹⁹ (NCT04387461).

Table 3. Summary of active or completed clinical trials with OAdS as monotherapies registered in ClinicalTrials.gov.

OAd	Clinical trial	Phase	Cancer	Admin	CR / Related data
DNX-2401 (Delta24-RGD)	NCT00805376 ¹⁵¹	I (C)	MG	IT	3/25 (12%) CR with progression-free periods of ≥ 3 years PT; Anti-Ad5 titers in 2/3 CR
DNX-2440	NCT00562003	I (C)	OC	IP	NR
Delta24-RGD	NCT01582516 ²²⁰	I/II (C)	GBM	IT	1/19 CR >8 yrs PT, with the highest anti-Ad5 titers
ICOVIR-5	NCT01864759 ¹⁵⁰	I (C)	Melanoma	IV	None
OBP-301	NCT02293850 ²²¹	I (Unk)	HCC	IT	None
	NCT03190824	II (Unk)	Melanoma	IT	NR
TILT-123	NCT04695327 ¹²⁸	I (R)	Adv solid	IT/IV	None. SD in 9/13 uninj. lesions by CT and 11/14 lesions by PET (IT admin.)
ORCA-010	NCT04097002 ²²²	I/II (NR)	PCa	IT	None
CG7870	NCT00116155 ²²³	I/II (C)	PCa	IV	None
CG0070	NCT00109655 ²²⁴	I (Unk)	BC	IVE	48,6% CR with a median duration of the CR of 10,4 mos; 14/22 (63,6%) CR in the multidose cohorts
	NCT02365818 ¹⁵⁴	II (C)	BC	IVE	47% overall 6-mos CR
	NCT04452591 ²²⁵	III (R)	BC	IVE	79/105 (75,2%) CR at any time, of which 86% is an ongoing response at 12 mos. 14/26 (53,8%) converted to CR after repeated admin.
AdVince	NCT02749331	I/II (Unk)	NETs	IA	NR
ColoAd1	NCT02053220 ¹⁵⁶	I (C)	CRC, NCLC, BC, RCC	IT/IV	NR
	NCT02028442 ¹⁵⁷	I/II (C)	Epithelial, mCRC, BC	IV	None
NG-350A	NCT03852511 ²²⁶	I (C)	Epithelial	IT/IV	None

Phase: C, completed; NR, not recruiting; unk, unknown status. **Cancer:** Adv solid, advanced solid tumors; BC, bladder cancer; GBM, glioblastoma multiple; HCC, hepatocellular carcinoma; mCRC, metastatic colorectal; MG, malignant gliomas; NETs, neuroendocrine tumors; NCLC, non-small cell lung cancer; OC, ovarian cancer; Panc, pancreatic; PCa, prostate; RCC, renal carcinoma. **Administration:** IA, intraarterial; IP, intraperitoneal; IT, intratumoral; IV, intravenous; IVE, intravesical. **Response:** CR, complete response (≥ 95% reduction in tumor size); mos, months; CT, computed tomography; NR, not reported; PET, Positron emission tomography; PT, post-treatment; SD, stable disease; uninj, uninjected; yrs, years.

Moreover, the therapeutic effects observed in glioblastoma patients treated with DNX-2401, where tumor regressions occurred several months post-administration and without detectable viral replication, strongly suggest an immune-mediated mechanism of action. Interestingly, the best-responding patient had the highest pre- and post-treatment anti-Ad5 antibody titers.

Despite the success of OV therapy, which may depend on achieving an optimal balance between antiviral and antitumor immune responses, the latter remains underexplored²²⁷. Most clinical trials primarily report tumor infiltration and inflammation markers without clarifying adaptive immune responses targeting unique tumor antigens. Specific T-cell responses against TAAs like MAGE-A3, MAGE-A1, NY-ESO-1, and mesothelin have been observed after repeated intratumoral injections of ONCOS-102²²⁸, and yet these are antigens broadly expressed in a wide array of malignancies. More efforts should be made to integrate biomarkers that appropriately discern between virus and tumor-specific adaptive immune responses of the particular tumor under investigation.

6.3 STRATEGIES TO OVERCOME ADENOVIRUS IMMUNODOMINANCE

Different strategies have already been proposed to redirect the immune system from the virus to the tumor. Heterologous prime-boost regimens, based on administering two distinct vectors encoding the same antigen, proved effective for some OV therapies. Bridle et al. demonstrated that priming with an adenoviral vaccine expressing a TAA and boosting with an oncolytic VSV armed with the same antigen significantly enhanced antitumor activity. Notably, this regimen redirected the immune response from predominantly targeting viral antigens to targeting tumor antigens²²⁹. Consequently, clinical trials of such strategy are currently ongoing²³⁰ (NCT02285816, NCT02879760). Coating the adenoviral capsid with MHC-I-restricted tumor-specific peptides²³¹ or expressing tumor antigens^{214,232} and BsAbs^{233,134} has favored the antitumor response *in vivo*. We have also demonstrated that OAds encoding tumor-associated epitopes fused to the viral protein E3-19K promote MHC-I epitope presentation independently from the TAP protein. This leads to antitumor

immune responses that compensate for the adenoviral epitope immunodominance²³⁴. Another interesting approach is the so-called oncolysis-assisted DC vaccination, in which combining OAds with tumor epitope-loaded DCs shifted the immune response toward effective tumor-specific antitumor immunity²³⁵.

Although these studies support the need for fine-tuning between antiviral and antitumor responses, they failed to fully solve the hurdle of viral epitope immunodominance. Even when immune responses toward cancer epitopes are favored in these settings, viral epitopes can still be presented and induce antiviral immune responses; most of the approaches described above do not address virus-specific CTL-mediated clearance from the TME. Moreover, many of these strategies rely on MHC-I expression, frequently downregulated in cancer cells. This highlights the need for innovative approaches to manage antiviral immunity while enhancing antitumoral immune activity and maintaining the balance between them. In this thesis, we explore the use of tolerogenic dendritic cells (tolDCs) as a novel strategy to achieve this.

6.3.1 Tolerogenic dendritic cells (tolDCs)

DCs are central players in regulating immune responses by inducing immunity or establishing tolerance through interaction with other immune cells^{236,237}.

Tolerogenic DCs are considered semimature or mature-resistant DCs, meaning they can present antigens to T cells similarly to mature DCs, but in a way that promotes T cell dysfunction rather than activation²³⁸. DCs play an important role in the generation of central immune tolerance in the thymus²³⁹ and also exert peripheral tolerance via several mechanisms: induction of clonal anergy, metabolic modulation of T cells, secretion of anti-inflammatory cytokines, and clonal deletion²⁴⁰ (**Figure 13**). The advantage of tolDCs is that they can be loaded with an antigen to specifically target their T cells without affecting other immune responses.

Several protocols have been reported for generating tolDCs *ex vivo* from hematopoietic precursors or peripheral blood monocytes: treatment with pharmaco-

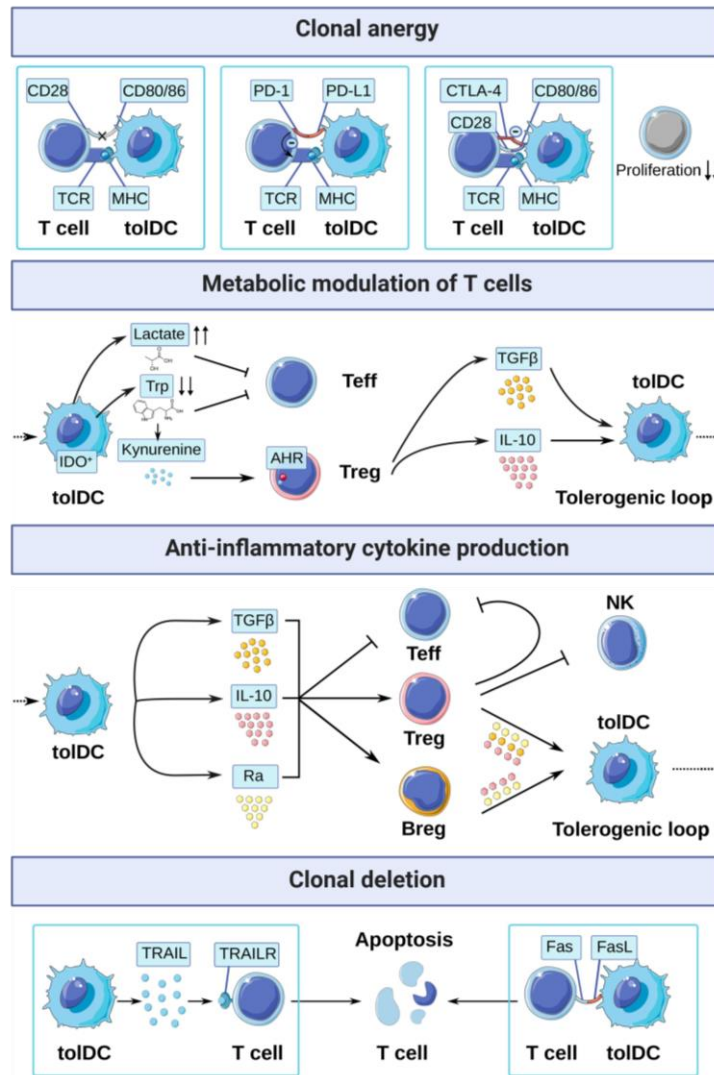


Figure 13. Mechanisms of toIDCs tolerance induction. 1) Clonal anergy in T cells can result from interactions with toIDCs via three main pathways: (i) insufficient co-stimulatory signaling from toIDCs, (ii) interaction of PD-L1 on toIDCs with PD-1 on T cells, and (iii) binding of CTLA-4 on T cells to CD80/CD86 molecules on toIDCs and their subsequent transendocytosis and degradation, which leads to reduced CD28 co-stimulatory signaling. As a result, T cells are unable to produce IL-2 and proliferate. 2) IDO⁺ toIDCs create immunosuppressive environments by generating metabolites like lactate or kynurenine (through the aryl hydrocarbon receptor, AHR) that suppress effector T cells (Teff) and promote Treg differentiation, respectively. 3) ToIDCs also secrete tolerogenic cytokines and metabolites, such as IL-10, TGF- β , and retinoic acid, which further suppress Teff activity and encourage differentiation of Tregs and Bregs, creating a feedback loop that sustains tolerance. 4) toIDCs induce apoptosis in T cells through the Fas/FasL signaling and by producing the TRAIL cytokine. Image adapted from reference 240.

logical and immunosuppressive agents such as vitamin D3 (VitD3), dexamethasone, rapamycin, NF- κ B inhibitor; culturing in the presence of anti-inflammatory cytokines like IL-10 and TGF- β ; or genetic modifications to selectively inhibit or activate critical molecules and pathways, such as the suppression of costimulatory molecules CD40, CD80, and CD86²⁴¹.

TolDC therapy has demonstrated safety and efficacy in different experimental models of autoimmune diseases, such as multiple sclerosis (MS), type 1 diabetes (T1D), rheumatoid arthritis (RA), or Crohn's disease. Moreover, data from phase I clinical trials have shown that therapy with tolDCs is safe and well tolerated by patients suffering from these disorders²⁴². In the gene therapy field, Kushwah et al. showed treatment with IL-10 tolDCs induced tolerance towards adenoviral vectors, diminishing antiviral immune responses after vector readministration and enhancing transgene expression in a lung disease model²⁴³. However, this approach has never been investigated in the context of OAd, which is addressed in this thesis.

HYPOTHESES AND OBJECTIVES

The main objective of this thesis was to counteract the low intratumoral viral spread and the viral epitope immunodominance by arming OAd with parasporin 2 PFT and using virus-specific tolerogenic dendritic cells, respectively.

Hypothesis 1: The administration of IL-10-induced tolDCs pulsed with OAd epitopes before OAd treatment mitigates antiviral immune responses, thereby favoring the antitumoral immune responses.

Hypothesis 2: The expression of a secreted-PS2 from an OAd improves the virus's oncolysis and tumor debulking while promoting ICD in bystander non-infected cells, which contributes to redirecting immune responses toward tumor antigens.

Specific objectives were set for two independent projects:

Induction of immunological tolerance to OAd with tolDCs-based conditioning

- To develop a murine immunocompetent model in which the response against tumor and viral epitopes can be measured upon OAd administration.
- To generate and phenotypically characterize tolDCs, and validate *in vitro* their tolerogenic function.
- To assess *in vivo* whether adenovirus-specific tolerization using tolDCs can bias the immune response from viral to tumor antigens.

Oncolytic adenoviruses expressing the pore-forming toxin PS2

- To test the specificity and functionality of the modified PS2 in suitable *in vitro* assays.
- To evaluate the PS2 ICD-inducing capacity *in vitro* and in tumor-bearing immunocompetent mice models.
- To generate and characterize ICO15K-based OAd expressing PS2 toxin
- To evaluate the antitumor efficacy of PS2-expressing OAd in tumor-bearing immunodeficient mice models.

MATERIALS AND METHODS

1. HANDLING OF BACTERIA

In order to obtain enough plasmid DNA, its amplification in bacteria was required. For this reason, the plasmid should have a replication origin that allows its replication on the desired strain and a gene that confers antibiotic resistance to select bacteria and avoid contamination. In this work, the *Escherichia coli* strain DH5 α has been used for this purpose. The SW102 strain has been used to perform homologous recombination.

1.1 PREPARATION OF ELECTROCOMPETENT AND CHEMOCOMPETENT BACTERIA

1.1.1 Electrocompetence

To induce electrocompetence, 10mL of LB (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl) were inoculated with an *E.coli* stock (conserved at -80°C with 15% glycerol) and was grown overnight at 37°C in agitation. The next day, the 10mL preculture was used to inoculate 1L of LB at 37°C in agitation until an OD600 of 0.5-0.6. Then, the culture was distributed in 250mL SORVALL bottles and kept 40min on ice to stop bacterial growth. Further manipulation of bacteria was carried on ice. Bacteria were centrifuged 15min at 4000g and 4°C in a SORVALL centrifuge. The supernatant was discarded, and the pellet was gently washed with 4°C cold bi-distilled water (ddH₂O). This centrifugation/washing process was repeated 3 times. In the last wash, the pellet was resuspended in 45mL of water with 15% glycerol. Bacteria were centrifuged one more time and resuspended in 3mL of ddH₂O containing 15% glycerol. The OD600 of a 1:100 dilution of the suspension should be close to 1 (equivalent to 2.5x10⁸ bacteria/mL). Finally, the bacterial suspension was aliquoted and stored at -80°C.

1.1.2 Chemocompetence

To induce chemocompetence, 5mL LB were inoculated with the *E.coli* glycerine stock, and it was grown overnight at 37°C in agitation. The next day, 3mL of the overnight culture was used to inoculate 15mL of LB at 37°C in agitation until it reached an OD600 of 0.6-0.7. Then bacteria were incubated 20min on ice and centrifuged for 10min at 2000g and 4°C. The supernatant was discarded, and the pellet was gently resuspended

in 9mL of CaCl_2 0.1M, and incubated on ice for 30min. Then a centrifugation of 10min at 2000g and 4°C was performed. The resulting pellet was resuspended in 4.8mL CaCl_2 0.1M and 15% glycerol. Appropriate aliquots were prepared and stored at -80°C.

1.2 TRANSFORMATION OF COMPETENT BACTERIA

1.2.1 Electroporation

Frozen electrocompetent DH5 α aliquots (50 μ L) were thawed on ice and mixed with a maximum of 10 μ L pre-cooled DNA (10-1000pg for transformations or >400ng for recombinations). The mixture was gently mixed, incubated 5min on ice, and transferred into an ice-cold 0.2 cm electroporation cuvette. The electroporation was performed at 50 μ F, 1500V, and 125 Ω with the Electro Cell ManipulatorTM ECM 630. Immediately, bacteria were recovered in 1mL LB and incubated for 60min in agitation at 37°C. The recovered culture or dilution was plated on LB plates supplemented with the corresponding selection antibiotic and incubated at 37°C (or 32°C for virus recombinations) overnight. The next day, desired clones were inoculated for mini or midi-preps, as described in Section 1.3.

1.2.2 Chemical transformation

Frozen chemocompetent DH5 α aliquots (50 μ L) were thawed on ice and mixed with 2 to 10 μ L of DNA and incubated for 1min on ice. Then a heat-shock was induced at 42°C for 30-45sec flicking the tube. Once the shock finished, bacteria were put on ice for 2min. After that, bacteria were recovered in 1mL of LB for 1h at 37°C in agitation. Bacteria were centrifuged at 2000g for 5min, most of the supernatant was discarded, and the pellet resuspended with the remaining volume. The resuspended bacteria were plated on LB plates supplemented with the corresponding selection antibiotic and incubated at 37°C overnight. The next day, desired clones were inoculated for mini or midi-preps, as described in Section 1.3.

1.3 PLASMIDIC DNA EXTRACTION FROM BACTERIAL CULTURES

Plasmid DNA was obtained from saturated *E. coli* cultures according to protocols based on alkaline lysis with SDS. DNA was prepared at a small and large scale.

Small-scale plasmid DNA preparations (mini-preps) were performed following an adapted protocol²⁴⁴ using Qiagen midi-prep kit reagents. A colony grown in an LB-antibiotic dish was inoculated in 5mL of LB-antibiotic and incubated overnight at 32°C (for SW102) or 37°C (for DH5α). 500μL of the overnight culture were saved to prepare the glycerol stock, and the rest was centrifuged at 2000g. The pellet was resuspended in 200μL of pre-cooled solution R3 with previously added RNase indicated by the kit and incubated 5 minutes on ice. After that, 200μL of solution L7 were added, and the mixture was blended by inversion and incubated 5min at RT. Finally, 200μL of precooled solution N3 were added, and the mixture was mixed by inversion and let it on ice for 20min until a white precipitate appeared. Then, it was centrifuged 10min at 15000g, and the clear supernatant was collected without taking the white pellet that corresponds to cellular DNA, proteins, and SDS. Next, 0.75 volumes (high copy) or 1.3 volumes (low copy) of isopropanol were added and mixed with a vortex. The plasmid DNA was precipitated by centrifugation for 10min at 15000g, and the DNA pellet was washed with 70% ethanol. It was centrifuged 5min at 15000g, discarding the supernatant, and the pellet was dried and resuspended in 50μL of TE or ddH₂O.

Large-scale DNA preparations (midi-preps) were prepared from 100mL (Low-copy plasmids) or 50mL (High-copy plasmids) of saturated bacteria culture using the NZY Midiprep kit (Nzytech), following manufacturer's instructions.

1.4 HOMOLOGOUS RECOMBINATION IN BACTERIA

Genetic modification of the adenovirus genome was carried out with the bacterial artificial chromosome (BAC) recombination-mediated genetic engineering (recombineering) method described by Warming et al.²⁴⁵. The homologous recombination of the BACs is performed in SW102, which contains the integrated temperature-sensitive defective λ-prophage. The prophage recombination machinery

is controlled by a temperature-inducible promoter (activation at 42°C). Electroporation of the heat-shocked bacteria with a donor DNA fragment flanked by homology arms (>40bp) to the targeted site in the BAC results in the insertion of the DNA by homologous recombination. This method has been adapted by Stanton et al.²⁴⁶, with a BAC carrying the whole adenovirus genome, which is called AdZ (Ad with zero cloning steps). *SacB* and *Ampicillin* (Amp) resistance genes from Stanton et al. protocol were replaced by *rpsL-neo* cassette, a faster and more cost-effective method²⁴⁷.

Genome modification was performed in two steps. In the first step (positive selection), the *rpsL-neo* cassette is inserted in the adenovirus genome region to modify. The *neo* gene in the *rpsL-neo* cassette confers resistance to kanamycin. Thus, positive selection can be achieved by plating the heat-shocked bacteria after electroporation in LB agar plates supplemented with kanamycin (kana) and chloramphenicol (Cm, the pAdZ BAC backbone confers resistance to this antibiotic). In the second step (negative selection), the heat-shocked bacteria containing the pAdZ-*rpsL-neo* were electroporated with the DNA of interest flanked by homology arms targeting the *rpsL-neo*. The *E. coli* strain SW102 is intrinsically resistant to streptomycin (Strep) due to mutations in the *rpsL* gene, but introducing the wild-type *rpsL* gene into the bacteria exerts dominant phenotypic effects over mutated *rpsL*²⁴⁸. After the second transformation, recombinant clones were those that had incorporated the inserted DNA and had lost *rpsL-neo* cassette, being consequently sensitive to Kana and resistant to Strep.

The plasmid pAdZ5-CV5-E3⁺ provided by Richard Stanton was modified to obtain the pAdZICOVIR-15K plasmid. This backbone has been used for all the modifications described in this thesis. Briefly, for positive selection, glycerinated bacteria containing the desired backbone were inoculated in 5mL of LB with Cm + Strep antibiotics (12.5µg/mL, and 1mg/mL, respectively) and incubated overnight at 32°C in constant agitation. Then 25mL of LB Cm + Strep were inoculated with 0.5mL of the previous culture and incubated at 32°C with agitation until it reached an OD600 between 0.5-0.6. At this moment, culture was divided into two Falcon tubes with equal volumes.

One was kept on ice until further processing (non-induced). The other tube was induced by heat-shock at 42°C for 15min in a water bath with agitation, followed by chilling on ice for 15min (induced). From that moment, manipulation was performed on ice to maintain electrocompetence. Both cultures (induced and non-induced) were centrifuged 5min at 4000g and 4°C. The supernatant was discarded, and the bacteria pellet was resuspended in 12mL of cold ddH₂O water. This washing step was repeated thrice. After the last wash, the pellet was resuspended in approximately 300μL of ddH₂O. Then, 50μL-aliquots of the induced and uninduced cultures were transformed with ≥200 ng of the *rpsL-neo* DNA flanked by the desired homology arms. Bacteria were recovered in 1mL of LB and incubated for 2h at 32°C with constant agitation. From this recovery, 100μL were plated into LB plates containing Cm and Kan (12.5μg/mL, and 15μg/mL, respectively) and incubated overnight at 32°C. About 20-24 hours later, the number of colonies was counted to determine the induced non-induced ratio and the recombination efficiency. Then, colonies from induced plates were picked and inoculated in 4mL LB supplemented with Cm and Kana and incubated at 32°C and 250 rpm. The next day, mini-preps of DNA were performed (detailed in Section 1.3) to screen for recombinant clones; 17,5μL of the mini-preps were digested with the appropriate restriction enzymes (New England Biolabs and Takara/Clontech) to discern parental than recombinant clones in electrophoresis on 1% agarose gels. Positive clones were stored at -80°C in LB with 15% (v/v) glycerol.

A similar procedure was followed for the second step. The clones obtained in the previous step were inoculated in 5mL of LB Cm + Kan and cultured overnight at 32°C. The next day, the bacteria were prepared for electroporation, as described previously. Again, over 400 ng of DNA (insert) containing the desired modification were transformed. After the recovery incubation, 100μL from a 1:10 and 1:25 dilution were plated in LB agar supplemented with Cm and Strep, and plates were incubated overnight at 32°C. After 36h, colonies were picked and inoculated in 4mL of LB-Cm + Strep to perform mini-preps and check recombinant clones as above.

2. CELL CULTURE

2.1 CELL LINES

The cell lines used in this thesis for the *in vitro* experiments are summarized in the following table:

Table 4. Cell lines used in this thesis. *CT26 cells transduced with a lentiviral vector expressing the human coxsackievirus and adenovirus receptor (hCAR). FBS: Fetal Bovine Serum

Cell line	Tumor type	Origin	Medium	FBS
A549	Lung adenocarcinoma	Human	DMEM	10%
Caco2	Colorectal adenocarcinoma			
HEK-293	Embryonic kidney			
HepG2	Hepatocellular carcinoma			
HT29	Colorectal adenocarcinoma			
NP9	Pancreatic tumor			
NP18	Pancreatic tumor			
Jurkat	Acute T-cell Leukemia		RPMI	
B16-F10	Melanoma	Murine	DMEM	
CMT64.6	Lung adenocarcinoma			
MC38	Colon adenocarcinoma			
CT26	Colon adenocarcinoma		RPMI	
CT26hCAR*	Colon adenocarcinoma			

The cancer cell lines A549, Caco2, HEK-293, HepG2, HT29, B16-F10, MC38, and CT26 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). NP9 and NP18 cell lines were provided by Dr. Alberto Villanueva (Institut Català d'Oncologia, Barcelona, Spain). CMT.64 cell line was provided by Dr. Stephan Kubicka (Hannover Medical School, Hannover, Germany), and the most permissive clone to adenovirus infection was previously isolated and expanded by our group, generating the CMT64.6²⁴⁹. In studies involving immunocompetent murine models treated with OAds (see Figure 14), CT26hCAR cells were created by transducing CT26 cells with a lentiviral vector expressing the Ad5 human coxsackievirus and adenovirus receptor (hCAR). This modification was designed to enhance the virus's infectivity and facilitate the evaluation of therapeutic effects in these models.

2.2 CELL MAINTENANCE AND COUNTING

All cells were maintained in the appropriate media described in **Table 4** (Gibco, Thermo Fisher Scientific) supplemented with inactivated (56°C, 30min) fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and 1% of penicillin-streptomycin (PS, Gibco-BRL) (100U/mL and 100µg/mL, respectively). They were incubated at 37°C in a humidified atmosphere with 5% CO₂. When cultures reached 80-90% confluence, cells were washed once with PBS and detached with trypsin-EDTA. Then, cells were resuspended with media to a final volume of 10mL. Cell suspensions were used to split the cells 1:3 to 1:20, depending on the cell line. Cell counting was performed with an automatic cell counter TC20TM (Bio-Rad) according to the manufacturer's instructions.

2.3 ISOLATION OF MURINE BONE MARROW-DERIVED DENDRITIC CELLS

Euthanized C57BL/6 mice were used as a source of bone marrow (BM) cells, which were processed similarly to already published protocols^{243,250–252}. Briefly, tibias, femurs, and humeri are dissected by cutting at the joints in the RPMI medium to loosen the muscle tissue. Bones are carefully scrubbed with sterile gauze to remove the residual soft tissues and placed for 2 minutes in 70% ethanol for disinfection. Then, they are soaked back in RPMI medium. In a laminar flow hood using sterile utensils, both ends of bones were cut with scissors to expose the bone marrow, which is flushed out 2-3 times with a syringe filled with complete RPMI medium. After dissolving any clusters by pipetting, the cell suspension is centrifuged twice in RPMI medium for 500g for 5 minutes. BM cells were cultured for 7 days in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor ([rmGM-CSF] Peprotech, Rocky Hill, NJ) only to generate normal DCs or in the presence of 70 ng/ml rmIL-10 (also from Peprotech) to generate IL-10-modified tolerogenic DCs. At days 3 and 6, half of the medium is removed and replaced with fresh cytokine-complemented medium. BMDCs were ready for use on day 7, during which either they were used as is or infected with Ad5 particles (3000vp/cell) or pulsed with peptides (7µg/mL or 2µM, depending on the peptide stock solution) (GenScript) for 24 h. To obtain mature DCs, at day 7, they were treated with *E.coli* LPS (1µg/mL) for another 24h. An essential aspect of tolDC

production is ensuring their stability when exposed to pro-inflammatory conditions. Most tolDC generation protocols incorporate a maturation phase using agents like LPS to establish a state in tolDCs that resist activation. Additionally, this maturation step stimulates the expression of C-C chemokine receptor 7 (CCR7), which is required for tolDCs to migrate to the lymph nodes²⁵³.

2.4 ISOLATION OF MURINE SPLENOCYTES AND TDLN

Spleens from euthanized C57BL/6J mice were surgically resected under sterile conditions and placed in falcons containing RPMI 10% FBS until their processing. Cells strainers (70µm) were placed in a petri dish containing 5mL of RPMI 10% FBS and spleens were mechanically disrupted with the plunger of a sterile syringe. The medium in the petri dish was passed through the strainer to ensure complete disaggregation. The cell suspension was centrifuged for 5 minutes at 750g, and the supernatant was discarded. Red blood cells were lysed by resuspending and incubating the pellet with 5mL of ACK lysis buffer for 5 minutes at RT. The lysis was stopped rising the falcon with complete medium. Then cells were washed thrice with PBS using a Pasteur pipette to remove the remaining fat. Finally, splenocytes were resuspended in RPMI 10% FBS, a 1/100 dilution was counted, and cell concentration was adjusted as needed.

For ELISpots using tumor-draining lymph nodes (TDLNs), the inguinal TDLNs from both sides of the animal were extracted and preserved in 1.5 mL tubes with 1mL PBS 2% FBS until being processed. Then, TDLNs were placed in a 600mm dish with a 70µm strainer, and 750µL 2% cold PBS were added. TDLNs were smashed through the strainer with a 5mL syringe plunger and transferred in a new 1.5mL tube. Then, the dish was washed with an additional 750µL 2% cold PBS and moved to the tube. Centrifugation at 10000rpm 1min at 4°C was performed. Finally, cells were resuspended with 1mL 2% PBS or media and were counted.

2.4.1 Fluorescent labeling of mouse splenocytes with CFSE

Splenocytes ($1-3 \times 10^7$) were resuspended with 1mL of 5µM CellTrace™ CFSE dye (Thermo Fisher Scientific) in PBS (1:1000 dilution). Cells were incubated at 37°C for 20

minutes, protected from light. The reaction was then blocked by adding at least 5 volumes of PBS 5% FBS. Cells were incubated for 5 extra minutes at RT and centrifuged at 300g for 5min. The supernatant was discarded, and pellets were resuspended in 5mL PBS 5% FBS. This washing step was repeated twice. After the last washing, pellets were resuspended in RPMI 10% FBS. Supplementation with β -mercaptoethanol (Sigma, St. Louis, MO, USA) is recommended. Cells were counted, and concentration was adjusted as needed.

2.5 CRYOPRESERVATION AND CELL THAWING

For long-term storage, cells were collected by trypsinization and pelleted by centrifugation. Cells were resuspended with cold freezing medium (90% FBS, 10% DMSO) and distributed in cryotubes at 0.5mL/tube. Tubes were placed in a Mr. Frosty freezing container at -80°C for 24h and then were stored in a liquid nitrogen tank. For cell thawing, cells were rapidly thawed in a water bath at 37°C and diluted with a prewarmed medium in a 15mL tube. Centrifugation at 1000g for 5 minutes was done to pellet cells. Then cells were resuspended and seeded at the desired density.

2.6 MYCOPLASMA TEST

All cell lines were routinely tested for contamination of *Mycoplasma hyorhinis*, *M. arginine*, *M. Pneumoniae*, *M. fermentas*, *M. orale*, *M. pirum*, *Acholeplasma laidlawii*, and *Spiroplasma mirum* by qPCR using the following primers:

Table 5. Primers for testing mycoplasma.

Primer	Sequence (5'→3')
MICO-1	GGCGAATGGGTGAGTAACACG
MICO-2	CGGATAACGCTTGCGACTATG

Tested cells were cultured in overconfluence for a week without antibiotics. A 1/100 dilution of the medium from this culture was used as a template for the testing qPCR. If the result was positive, cells were discarded or treated with Plasmocin™ (Invivogen) at 25 μ g/mL for 2 weeks and tested again.

3. RECOMBINANT ADENOVIRUSES

The adenovirus used in this thesis is derived from the human adenovirus serotype 5 (Ad5), specifically from the virus ICOVIR-15K backbone, which has been previously described (see Section 4.5 of Introduction).

3.1 CONSTRUCTION OF EXPRESSION VECTORS ENCODING PARASPORIN

For all clonings, the vector was digested by the indicated restriction enzymes (New England BioLabs) and separated by gel electrophoresis; the corresponding band was sliced from the gel and purified with Monarch®DNA gel extraction kit (New England BioLabs) according to manufacturer's protocol. Transgenes were synthesized by PCR and then digested to perform the ligation. PCRs were performed according to the manufacturer's protocol (NzyTech, Ref.MB146) with the conditions described in **Table 6**. Thermocycler was programmed as indicated in **Table 7** for annealing of complementary oligonucleotides.

Table 6. PCR conditions to generate transgenes. * $\leq 5^{\circ}\text{C}$ the mean of the melting temperature of both primers (often $50\text{--}60^{\circ}\text{C}$). #1minute/Kb length of the DNA fragment. Primers' working concentration: $10\mu\text{M}$.

Stage	Description	Temperature	Time	Cycles
1	Initial denaturation	96°C	5 minutes	1
2	Denaturation	96°C	30 seconds	30
	Annealing	Depending on primers*	Depending on transgene#	
	Extension	72°C	2 minutes	
3	Final extension	72°C	7 minutes	1
4	Cooling	4°C	HOLD	1

Table 7. Thermocycler programs for annealing complementary oligonucleotides.

Stage	Description	Temperature	Time	Cycles
1	Initial denaturation	96°C	5 minutes	1
2	Annealing	96°C ($-1^{\circ}\text{C}/\text{cycle}$)	1 minute	60
3	Cooling	4°C	HOLD	1

PS2Aa1 gene sequence from *B.thuringiensis* A1547 strain was obtained from publically available resources (Protein Data Bank ID: 2ZTB). To construct a PS2 expression vector under the CMV constitutive promoter, EcoR1 and XbaI restriction sites were added to the *PS2Aa1* fragment by PCR and subsequently cut with these enzymes to clone into a pTwist-CMV vector. Removal of the N-terminal PS2 segment is a prerequisite for cytotoxicity, and additional removal of the C-terminal segment accelerates the cytotoxic action. Cleavage furin sites at N- and C-terminal ends were further inserted in the resulting PS2-encoding plasmid with the best consensus cleavage sequence of furin, according to Shiryaev et al.²⁵⁴. The first furin site at N-terminal was added by cloning annealed oligonucleotides “SalBmg-Fw” and “SalBmg-Rv” with Sall-BmgBI enzymes, generating the pCMV-PS2AaFu plasmid; the second at C-terminal, by cloning the oligonucleotides “BsrXho-Fw” and “BsrXho-Rv”, and cut with BsrGI and XhoI enzymes (giving the pCMV-PS2AaFu2 plasmid as a result) (**Table 8**). This was made to replace the proteinase K cleavage sites in the wild-type protein, allowing PS2 to be activated during secretion (since furin is endogenously expressed in most eukaryotic cells).

To increase the PS2 transgene cytotoxicity, PS2Ab1 from *B.thuringiensis* TK-E6 strain fragment (PS2Ab GenBank AB-186914) was cloned into PS2Fu2 with AgeI-XbaI enzymes to replace the original *PS2Aa1* sequence.

Of note, the pCMV-PS2Aa1-Fu2 plasmid initially has the mouse Ig heavy chain signal peptide (MGWSCIIILFLVATATGVHS, 19 amino acids). In order to maximize PS2 secretion, the first methionine of *PS2* gene was removed, and the human chymotrypsinogen B1 signal peptide (trypsinogen from now on) was cloned before the *PS2* gene, as described by Cheng et al.²⁵⁵, in the pCMV-PS2AbFu2 plasmid with Not-Sall enzymes. The resulting final plasmid was named pCMV-PS2Ab-FuT, which was the one used to generate the OAd for this thesis. Both PS2Ab and trypsinogen signal peptide sequences were ordered to GenScript Biotech (New Jersey, USA) after doing a codon humanization using The Codon Optimization Tool website (Integrated DNA Technologies, IDT). In addition, the Kozak sequence was placed right before this signal

peptide at the N-terminal to ensure translation initiation and the SV40 polyadenylation signal at the C-terminal end.

Table 8. DNA sequences used for generating PS2 expression vectors.

Description	Sequence (5'→3')
Trypsinogen signal peptide (humanized)	ATGGCTTTCCTGTGGCTCTTGCTGCTGGGCATTGCT TGGTACTACGTTTCGGA
SalBgm-Fw	TCGATTTGGATAATCAGGTGGTGGAGACTTCCAGTC GACCCAGAGACTGAGAAGGGAC
SalBgm-Rv	GTCCCTTCTCAGTCTCTGGGTCGACTGGAAAGTCTCC ACCACCTGATTATCCAAA
BsrXho-Fw	GTACAGCCTGCTAGATCTCTTCTTGATGAGCAGCAAA GACGAAAGCGCGAAACGC
BsrXho-Rv	TCGAGCGTTTCGCGCTTTCGTCTTGCTGCTCATCAAG AAGAGATCTAGCAGGCT

The linearized vector and digested PCR products (insert) were ligated with Takara's DNA Ligation Kit, Mighty Mix (Cat.#6023), following the manufacturer's instructions (the recommended vector:insert ratio is 25 fmol vector:25-250 fmol from insert). Finally, electrocompetent or chemocompetent bacteria were transformed (see 1.2) with 2 to 5μL of the ligation and incubated overnight at 37°C. The next day, mini-preps of DNA (Section 1.3) and analysis of restriction patterns were performed to screen for clones with the newly ligated plasmid.

3.1.1 Sanger sequencing

The clones of the ligation were checked by restriction pattern analysis. Moreover, they were sequenced to confirm and discard transgene mutations. Sequencing was performed with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific). For the sequencing setup, 20μL-reaction mixes were prepared, containing 100-200ng DNA, 3.2 μM of the primer, 4μL 5X Sequencing Buffer, 2μL BigDye™, and ddH₂O to reach the final volume. Sanger sequencing of reactions was

performed by STAB VIDA (Portugal). The primers used are detailed in **Table 9**, and PCR conditions in **Table 10**.

Table 9. Primer for sequencing pCMV-PS2 plasmids.

Primer	Sequence (5'→3')	Checked plasmid
SPeukF	GATGGTCCTGTATCATCCTGT	pCMV-PS2Aa1-Fu2
TwistF	GTGCTGGCCATCACTTTGGCA	pCMV-PS2Ab-Fu2 pCMV-PS2Ab-FuT

Table 10. PCR conditions for sequencing.

Stage	Description	Temperature	Time	Cycles
1	Initial denaturation	96°C	1 minute	1
2	Denaturation	96°C	30 seconds	50
	Annealing	50°C	15 seconds	
	Extension	60°C	2 minutes	
3	Cooling	4°C	HOLD	1

3.1.2 Calcium phosphate transfection

After clone confirmation, a large-scale DNA preparation was performed (explained in 1.3) and transfected into HEK-293 cells by the calcium phosphate-based method. Monolayers of HEK-293 cells at a confluence of 70% were used. For each plasmid to be transfected, the following mixture was prepared:

Table 11. Proportion of reagents for calcium phosphate transfections.

Reagent	24-well plate	6-well plate	p10 plate
CaCl ₂ 2M	3.9µL	19.5µL	62.5 µL
DNA	0.6µg	3µg	15µg
ddH ₂ O	up to 32.4µL	up to 162µL	up to 500 µL

The solution was mixed up softly for 5 minutes. Another 1.5mL tube containing 32.4µL, 162µL or 500µL of HBS 2X (NaCl 274 mM, HEPES 50 mM, and NaH₂PO₄ 1.5 mM in H₂O, pH adjusted to 7.05 with NaOH) was prepared depending on the plate-size. The solution containing the DNA was added drop by drop to the tube. The mixture was

incubated for 20 minutes at RT and added dropwise to the plate. Two hours later, calcium phosphate precipitates became visible. At 16h post-transfection, the medium was removed and exchanged for fresh medium.

3.1.3 Transfection with PEI

For generating large amounts of PS2 supernatants, p15 plates were usually transfected with PS2-encoding plasmids with polyethylenimine (PEI)-based transfection method. At 2 hours before the transfection cells' medium was changed to FBS-reduced Opti-MEM™ (Gibco, Thermo Fisher Scientific):

Table 12. Proportion of reagents to transfect a p15 plate with the PEI-based method.

Reagent	p15 plate	
	PEI (1mg/mL)	Opti-MEM™
PEI Tube	105µL	895µL
DNA Tube	42µg	up to 1mL

The solution was mixed softly for 5 minutes. The DNA tube was poured over PEI tube drop by drop while vortexing and left incubating at RT for 10-15 minutes until the appearance of small precipitates. Then, the mixture is added to cells dropwise to the plate. After 4 hours, the medium was aspirated and replaced with the normally used growth medium.

3.1.4 Parasporin supernatant generation and concentration

HEK-293 cells p15 plates were transfected with PEI with either PS2-encoding plasmid or their equivalent empty plasmid as a control. The day after transfection, the medium was carefully changed to OptiMEM™ to allow PS2 production. Supernatants were harvested every 3-4 days (2 or 3 rounds approximately, as long as transfected cells look healthy) and concentrated (if needed) 20-fold with Amicon Ultra-15 filter units with a molecular weight of 10kDa (Merck Millipore).

3.2 CONSTRUCTION OF RECOMBINANT ADENOVIRUSES

As explained before (Section 1.4), a parental backbone of pAdZ-ICOVIR15K should contain an *rpsl-neo* cassette in the desired position to replace it with a transgene. The transgene was generated by PCR according to **Table 6**. The product was purified by gel electrophoresis excision according to Monarch®DNA gel extraction kit (New England BioLabs) or Quiaquick PCR product purification kit (Qiagen) as convenient.

3.2.1 Construction of PS2AbFuT-expressing oncolytic adenovirus

The pCMV-PS2Ab-FuT plasmid was used as a template to generate the SAS-Kozak-Trypsinogen-PS2Fu2 transgene with "FiSASKPS2FuT" and "PS2AbFuTR" primers (**Table 13**). The left arm included a homology sequence to the adenovirus genome after the fiber gene, the Kozak, and the SAS splice acceptor. The right arm included the stop codon, polyA signal, and extra DNA homologous to the adenovirus genome after the fiber gene. The product was purified, and homologous recombineering (detailed in 1.4) was performed into pAdZ-ICO15K-RpsLNeo, generating the pAdZ-ICO15K-Fi-SAS-PS2FuT.

Table 13. Primers for generating the ICOVIR15K-Fi-SAS-PS2FuT OAd.

Primer	Sequence (5'→3')
FiSASKPS2FuT	CATTGCCCAAGAATAAAGAATCGTTTGTTACTTATGACT CGTACTATTGTTATTCATCCAGGCCGCCACCATGGCTT
PS2AbFuTR	TGGGGCTATACTACTGAATGAAAAATAAACACGTTGAA ACATAACTTTATTACAGACCGATAGTTTGTG

3.2.2 Construction of tumor neoepitope-expressing oncolytic adenoviruses

For immunization studies, the so-called "ICO15-d6.7/19K-B16 TMG" (or ICO15-OVA257) and "ICO15K-CMV-TMG08" were used, which encode for B16-F10 tumor neoepitopes or TAAs CD8⁺ epitopes, respectively. These viruses were generated previously in our group using ICOVIR15 or ICOVIR15K backbones that had an E3-6.7K and E3-19K deletion (d6.7/19K) and the RGD in the fiber HI-loop. Selected neoepitopes or TAAs were designed as cDNA encoding the epitope as 27mer or 9 mer amino acids,

also known as minigenes. The minigenes were concatenated one after another, generating a tandem minigene (TMG). TMGs of both viruses were cloned after Ad5's fiber under a CMV promoter, as late genes are not translated in murine cell lines^{256,257}

3.2.3 Adenovirus generation by calcium phosphate transfection

The recombinant viral DNA was transfected in a 6-well plate of HEK-293 by calcium phosphate-based method (explained in Section 3.1.2). No plasmid linearization is required for pAdZ vectors since these plasmids incorporate a self-excising system. Once the plasmid enters the cell, the endonuclease I-SceI is expressed and releases the viral genome, and the viral cycle begins. After several rounds of replication (about 7 days post-transfection), foci of cytopathic effect are detectable. Then, cells were collected together with the supernatant (cell extract, CE) and underwent 3 rounds of freeze/thaw cycles to release the viral particles from the cells. The CE was used for further amplification steps.

3.3 CLONE ISOLATION BY PLAQUE PURIFICATION ASSAY

Serial dilutions from the transfection CE, ranging from 10^{-1} to 10^{-7} , were prepared in DMEM 5% FBS. 100 μ L of each dilution was used to infect one 6-well of 80% confluent A549 cells for 4 hours at 37°C. After removing the media and washing cells with PBS, 3mL of a 1:1 solution of DMEM 5% FBS and 1% agarose pre-warmed was added to the cells. Once solidified, 2mL of fresh medium was added over the agarose matrix. The plates were incubated at 37°C until the appearance of plaques was evident (5-8 days post-infection). Then, the medium was removed, and the plaques were picked through the agarose matrix. The pipetted agarose/medium was resuspended in 500 μ L of DMEM 5% FBS and used for further amplification steps.

3.4 AMPLIFICATION AND PURIFICATION OF ADENOVIRUSES

Amplification and purification allowed us to obtain enough amounts of adenovirus in the appropriate formulation to be used for *in vitro* and *in vivo* assays.

3.4.1 Amplification of oncolytic adenoviruses

After clone selection (explained in 3.3), 250 μ L from the plaque suspension were used to infect a 6-well of confluent A549 cells. When the cytopathic effect (CPE) was observed, cells were harvested and subjected to 3 freeze/thaw cycles. From this point on, viruses were sequentially amplified by scaling up. In general, 1:20 dilutions of the viral suspension from each step were used as inoculum for the next expansion step, while the rest was stored at -80°C as backup stocks. Infections were performed in DMEM 5% FBS and dishes were incubated until cells showed evident signs of CPE. Infected cells were collected by pipetting and subjected to 3 freeze/thaw cycles. In the final harvesting step, cells were pelleted by centrifugation and resuspended in a maximum volume of 10mL before performing freeze/thaw cycles for purification. 50mL of supernatants were also collected for the first round of ultracentrifugation.

3.4.2 Purification of oncolytic adenoviruses

Oncolytic adenoviruses were purified by cesium chloride (CsCl) density gradient centrifugation. First, cell pellets were subjected to 3 freeze/thaw cycles to release the viruses from cells. Debris was cleared by centrifugation at 1000g for 10 minutes, and the supernatant was collected. Cell pellets were resuspended in 10mL of the supernatant stored in the last step of the amplification process, and tubes were centrifuged again to wash the pellet. This process was repeated until a final volume of 42mL of virus supernatant was reached. Discontinuous CsCl gradients were prepared in ultracentrifugation tubes (Beckman Coulter). For the first layer of the gradient, 2.5mL of a solution with a concentration of 1.35g/mL was added to the tubes' bottom. The second layer consisted of 2.5mL of a CsCl solution at 1.25g/mL, which was carefully added on top of the first layer. Gradients were completed by carefully adding 7.5mL of the virus on top of the second layer. Tubes were ultracentrifuged for 2 hours at 150000g (35000rpm, SW40 Ti rotor, Beckman) and 10°C. With these conditions, viral particles are separated from cell debris according to size and appear as 2 bands at the interface between 1.25 and 1.35g/mL layers. The upper band corresponds to empty viral capsids and was removed by suction. The lower band of interest was collected

and placed on ice for further purification and concentration. A second centrifugation step using a continuous CsCl gradient was performed. The solution containing the virus was brought up to 24mL with the CsCl solution at 1.35g/mL and distributed into 2 ultracentrifuge tubes. The second centrifugation was carried out overnight at the same conditions as the first one but without centrifugation brakes. After this, the upper band was discarded by suction, and the band corresponding to the full viral particles was collected in a maximum volume of 2mL. Buffer exchange was performed by three consecutive dialyses of 2 hours with desired buffer Tris-NaCl, supplemented with glycerol to a final concentration of 5% (v/v). Then aliquots were stored at -80°C for long-term storage.

3.5 TITRATION OF ADENOVIRUSES

3.5.1 Determination of physical viral particles by spectrophotometry

This method quantifies the viral particles (vp) from a purified adenovirus stock without discrimination between infective or defective particles. It is based on the determination of the absorbance of viral DNA at a wavelength of 260nm. Three different dilutions (1:5, 1:10, and 1:20) of the purified viral stock were prepared in lysis buffer (Tris 10 mM, EDTA 1 mM, 0.1% SDS, pH 8.0) and incubated for 10min at 56°C. Then, the OD₂₆₀ and OD₂₈₀ were measured using a spectrophotometer. The final concentration of the virus was calculated with the following formula, considering that the extinction coefficient of adenoviruses is 1.1×10^{12} per OD unit:

$$\text{Titer (vp/mL)} = \text{OD}_{260\text{nm}} \times \text{sample dilution} \times 1.1 \times 10^{12}$$

The ratio between the absorbance at 260nm and 280nm should be around 1.3, giving an idea of the integrity of the purified sample.

3.5.2 Determination of functional viral particles by anti-hexon staining

This technique allows the determination of functional infective viral particles (Transducing Units, TU) in purified stocks and cell extract samples. The method is based on the detection of viral protein hexon in infected cells. Serial 1:10 dilutions of the viral

stock were prepared in DMEM 5% FBS in triplicates in a final volume of 100μL in 96-well plates. A549 cells (1×10^5 cells) were added to the wells in a volume of 50μL, and plates were incubated for 36h at 37°C. The medium was aspirated, and the cells were dried for 5 minutes at RT. Then 100μL/well of cold methanol were added and incubated 10min at -20°C to fix and permeabilize the cells. Methanol was removed, and wells were washed twice with PBS²⁺ containing 1% BSA. Cells were incubated for 1-2h at 37°C with 1:5 diluted anti-hexon antibody obtained from the hybridoma 2Hx-2 (ATCC, Manassas, VA, USA). Cells were then washed thrice and incubated with an anti-mouse secondary antibody conjugated with the fluorochrome Alexa-488 (Thermo Fisher Scientific) diluted 1:500 for 1-2 hours. Finally, cells were washed and the viral titer was determined by counting stained cells using an inverted fluorescence microscope. The following formula was used to calculate the concentration of transducing units (TU) in the stock:

$$\text{Functional titer (TU/mL)} = \frac{\text{mean of positive cells}}{100\mu\text{L}} \times \text{Dilution factor} \times 1000\mu\text{L}$$

Table 14. Titers of purified viruses used in this thesis. Vp, viral particles; TU, transducing units

Virus	Physical titer (vp/mL)	Functional titer (TU/mL)	Ratio vp/TU
ICO15K-Fi-SAS-PS2FuT	1.56×10^{12}	1.4×10^{11}	11.14
ICO15K	2.67×10^{12}	2.1×10^{11}	12.71

3.6 CHARACTERIZATION OF ONCOLYTIC ADENOVIRUSES

3.6.1 Isolation of viral DNA from infected cells (Hirt's)

This method has been used to confirm the DNA genome from isolated clones. A549 cells seeded in a 100mm plate were infected with the corresponding adenovirus until complete CPE was observed. Cells were harvested and centrifuged 5min at 1000g. The supernatant was discarded, and the cell pellet was resuspended in 1mL of PBS. The cell suspension was pelleted again by centrifugation and resuspended in 350μL of ddH₂O. Then, 350μL of Hirt's solution 2X (10 mM Tris pH 8.0, 20 mM EDTA, 1.2% SDS, and

200µg/mL of proteinase K) was added. The sample was mixed and incubated for 1h at 56°C. After incubation, 200µL of NaCl 5M was added dropwise while vortexing. The mixture was incubated at 4°C for 8-16h until a white precipitate (corresponding to the cellular DNA) appeared. The suspension was centrifuged for 30 min at 15000g at 4°C, and the viral DNA's upper phase was collected. Incubation with RNase at a final 100 µg/µL concentration was carried out for 1h at 37°C. Samples were mixed with one volume of phenol-chloroform and centrifuged for 5min at 13000g. The upper phase containing the DNA was collected, mixed with two volumes of ethanol supplemented with 2% sodium oxaloacetate, and incubated for 1h at -20°C. Samples were centrifuged for 20min at 13000g, the supernatant was discarded, and pellets were washed once with 70% ethanol. Finally, the supernatant was discarded, and airdried DNA pellets were resuspended in 25µL TE pH 8.0.

3.6.2 Isolation of viral DNA from purified viral particles

This method has been used to verify the identity of each generated virus-purified stock. A 50µL-aliquot of purified virus (typically, 2×10^{10} vp results in 1 µg of viral DNA) was incubated with: 16µL EDTA pH 8.0 (0.5 M), 20µL SDS (10%), 8µL Proteinase K (10 mg/mL) and up to 400µL of TE pH 8.0. Then, samples were incubated for 2h at 56°C to dissociate viral particles. DNA was isolated by phenol-chloroform extraction and ethanol precipitation, as described above. DNA pellets were resuspended in 25µL TE pH 8.0.

3.6.3 Characterization of viral genomes

Genomes of recombinant adenoviruses were characterized by checking the restriction patterns. 500ng of viral DNA were digested with the desired restriction enzyme according to the manufacturer's instructions. Furthermore, adenovirus genomes were sequenced (as explained in Section 3.1.1) to discard potential recombinations with the wild-type E1A present in HEK293 cells (transfection) and to discard mutations in the transgenes after the amplification process in A549 cells. The primers for the sequencing are described in **Table 15**.

Table 15. Primers used for sequencing ICO15K-Fi-SAS-PS2FuT OAd.

Region	Primer	Sequence (5'→3')	Target sequence covered
E1a	Oligo11	GTGTTACTCATAGCGCGTAA	E2F cassettes and the Sp1 target sequence
	Ad670F	ATCTTCCACCTCCTAGCCAT	Δ24 mutation
Fiber	iRGDLinker1F	CATTAATGAAATATTTGCCACAT CCTCTTACAC	Transgenes inserted after fiber (upstream)
	iRGDLinker4R	GTGGTGGTGGGGCTATACTACT GAATGAAAAATGAC	Transgenes inserted after fiber (downstream)
	Fiberup4	CATCCGCACCCACTATCTTCA	KKTK modification in the ICO15K backbone

4. IN VITRO ASSAYS

4.1 *IN VITRO* TOXIN CHARACTERIZATION METHODS

4.1.1 MTS assay

To determine PS2-induced cytotoxicity, the colorimetric MTS assay was performed. The CellTiter 96® AQueous One Solution Reagent (Promega) was used. This reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. Assays are performed by adding 20μL of tempered CellTiter 96® AQueous One Solution Reagent into culture wells in 96-well plates containing cells and PS2 supernatants in 100μL of culture medium. Plates were incubated for 1–4 hours in a humidified, 5% CO₂ atmosphere. Afterward, the absorbance was immediately read at 490nm with a 96-well plate reader (Perkin Elmer Victor X2 Multimode Microplate Plate Reader). The quantity of formazan product, measured by 490nm absorbance, is directly proportional to the number of living cells in the culture.

4.1.2 ATP release assay

The extracellular ATP release upon PS2 treatment was continually monitored in real-time by using The RealTime-Glo™ Extracellular ATP bioluminescent assay (Promega).

Briefly, 96-well plates with seeded cells were set up to collect all the desired timepoints to be measured. Then, 50 μ L RealTime-Glo™ Extracellular ATP Assay Reagent was mixed and coincubated with cells in culture at the time of the treatment, and plates were read every hour for 24h using a standard plate-reading luminometer (Perkin Elmer Victor X2 Multimode Microplate Plate Reader). No supernatant sampling is required.

4.1.3 CFSE-based method to titrate PS2 supernatants

An approximate quantification of the PS2 toxin in supernatants from HEK293-transfected cells was set up using Jurkat cells, which were suspension cells susceptible to PS2. Briefly, 20000 Jurkat were stained with CFSE and left at 37°C 3h in a falcon tube to discard spontaneous CFSE release. Then, stained cells were washed twice with PBS and treated in 96-well plates with PS2 (either concentrated 20x or not) or ICO15K-Fi-PS2AbFuT supernatants at different dilutions (serial dilutions 1:2 up to 1:64 for non-concentrated supernatants; from 1:10 to 1:640 for concentrated supernatants). After 1h, the CFSE fluorescence was measured at 485/535nm in a standard plate-reading fluorometer (Perkin Elmer Victor X2 Multimode Microplate Plate Reader). As pores were formed through cells due to PS2, the intracellular CFSE can be released and detected. The average fluorescence of untreated Jurkat (background CFSE release) and Triton X-100-treated cells (maximum CFSE release) were taken as a negative and positive control, respectively. Triplicates of each condition were performed.

The absorbance detected was directly proportional to the amount of PS2, and the % of cell lysis was calculated. The function *approxfun* from R package *stats* was used to interpolate the desired cell lysis percentage in the function. Then, the Lytic Units per milliliter (LU/mL) of each supernatant were estimated using the following formula:

$$\text{Titer (LU/ml)} = [\text{n}^\circ \text{ of cells} \times (\% \text{ cell lysis}/100) \times 1000] / [(150/\text{Interpol. Dil.})],$$

in which 1000 is applied for conversion to mL, 150 is the total volume analyzed in μ L, and the interpolated dilution is the dilution of supernatant corresponding to determined % of cell lysis.

4.2 RECOMBINANT ADENOVIRUS-BASED *IN VITRO* ASSAYS

4.2.1 Adenovirus-mediated cytotoxicity assay

Different human or mouse cell lines were seeded in 96-well plates in DMEM supplemented with 5% FBS and infected with serial dilutions of the parental virus or the modified viruses (**Table 16**). On days 4-6 post-infection, plates were washed with PBS and stained for total protein content with the bicinchoninic acid (BCA) kit (Pierce Biotechnology). Absorbance was determined, and the percentage of cell survival was calculated by normalization to values of uninfected wells. The inhibitory concentration that killed 50% of the cells (IC_{50}) was calculated with GraphPad Prism v8.0 (GraphPad Software Inc.) using a dose-response non-linear regression with a variable slope.

Table 16. Detail of the initial multiplicity of infection (MOI), serial dilutions, and number of cells used for cytotoxic *in vitro* assays.

Cell line	Initial MOI (TU/cell)	Viral dilutions	Cells/well
A549	200	1/5	30000
HepG2	200	1/5	30000
MC38	2000	1/5	20000

4.2.2 Adenovirus production assay

A549 (10^5) were seeded in 24-well plates and incubated overnight. Cells were infected with the parental virus and tested viruses at MOI of 20 in 500 μ L of DMEM 5%FBS for 4 hours. Then, cells were washed once with PBS and fed with fresh media. Cell extracts and supernatants were collected over desired timepoints and were prepared by 3 freeze/thaw cycles. The functional titer was determined with the anti-hexon staining method (see Section 3.5.2). Results are expressed as the number of TU produced by a single cell, considering the functional titer and the number of cells on the day of the infection.

4.2.3 Oncolysis-mediated enhanced PS2-mediated cytotoxicity

Oncolysis-mediated enhanced PS2-mediated cytotoxicity was evaluated with the xCELLigence Real-Time Cell Analyzer System (Agilent). 100 μ L of growth media was added to each well of an Agilent E-Plate 96 to obtain background readings. MC38 and HepG2 cells (1×10^3) were resuspended in 100 μ L growth media and then seeded in the E-Plate 96. After overnight tumor cell adherence to the well bottom, the medium was carefully removed, and cells were treated with 150 μ L PS2AbFuT plasmid or ICOVIR15K-Fi-PS2FuT supernatants. Cell index (relative cell impedance) values were monitored every 3 minutes for 50-70 hours and normalized to the maximal cell index value.

4.3 IMMUNOLOGY TECHNIQUES

4.3.1 Flow cytometry

Flow cytometry analysis was performed with a Gallios cytometer (Beckman Coulter). The antibodies used in this thesis are listed in **Table 17** (except the ones used for tumor immunophenotyping, which are included in **Table 18**). Cells were washed twice with PBS (centrifugation at 750g for 5min) and stained for viability with LIVE/DEAD fixable stain (dilution 1:1000, Thermo Fisher Scientific) 30min at RT in the dark. Then, after three washes with PBS 5% FBS and 0,01% NaN_3 , direct surface staining with corresponding antibodies was performed for 20min on ice in the dark. For indirect surface staining, three more washing steps were done after primary antibodies stainings, followed by incubation for fluorochrome-coupled secondary antibodies for 20min on ice in the dark.

For intracellular staining of cytokines, 3×10^5 - 1×10^6 cells were stimulated with Brefeldin A Solution (BioLegend) at 37°C for 4h (1:1000). Cells were harvested and stained for viability and cell surface antigens. After two washing steps, cells were resuspended in 100 μ L/well for microwell plates (or 250 μ L for tubes) with Fixation/Permeabilization solution for 20min at 4°C (BD Cytofix/Cytoperm™, BD Biosciences). Then, after two washes in 1 $\times$ BD Perm/Wash™ buffer (BD Cytofix/Cytoperm™, BD Biosciences), cells

were stained for a fluorochrome-conjugated anti-cytokine antibody diluted in the BD Perm/Wash™ buffer for 30min at 4°C in the dark. Finally, two more washing steps were done, and cells were resuspended with the staining buffer for flow cytometric analysis.

All flow cytometry data were analyzed with the FlowJo software v10 (Tree Star).

Table 17. Flow cytometry antibodies. mAb, monoclonal antibody; pAb, polyclonal antibody; unconj, unconjugated. *Indirect staining with secondary anti-rabbit AF647 Ab (Thermo Fisher).

Antibody	Species	Clone	Company	Dilution	Application
Anti-CD80-FITC	Mouse	16-10A1; mAb	BioLegend	1:500	BMDC characterization
Anti-CD86- PE-Cy7	Mouse	GL-1; mAb	BioLegend	1:200	BMDC characterization
Anti-I-A/I-E Antibody (MHC-II)-APC	Mouse	M5/114.15; mAb	BioLegend	1:500	BMDC characterization
Anti-CD11b-PerCP/Cy5.5	Mouse	M1/70; mAb	BD Biosciences	1:100	BMDC characterization
Anti-CD11c-PE	Mouse	N418; mAb	Miltenyi Biotec	1:50	BMDC characterization
Anti-CD103-FITC	Mouse	M290; mAb	BD Biosciences	1:100	BMDC characterization
Anti-MerTK-AF700	Mouse	DS5MMER; mAb	eBioscience	1:100	BMDC characterization
Anti-CD4-BV510	Mouse	RM4-5; mAb	BioLegend	1:400	BMDC characterization
Anti-CD4-PE/Cy7	Mouse	GK1.5; mAb	BioLegend	1:200	Splenocyte ICS/MLR
Anti-CD8α-APC/Cy.5.5	Mouse	53-6.7; mAb	BD Biosciences	1:100	BMDC characterization
Anti- CD8α-PE/Cy5	Mouse	53-6.7; mAb	BioLegend	1:200	Splenocyte ICS/MLR
Anti-CD3-FITC	Mouse	17A2; mAb	BioLegend	1:200	Splenocyte ICS
Anti-CD3-APC	Mouse	17A2; mAb	BioLegend	1:400	MLR
Anti-IFNγ	Mouse	XMG1.2; mAb	BD Biosciences	1:200	Splenocyte ICS
Anti-Calreticulin (unconj.)*	Mouse	pAb	Abcam	1:100	ICD assays

4.3.1.1 Tumor immunophenotyping

The immunophenotyping of tumors was performed with the support of Lúdia Franco Luzón (PhD) and Ainhoa Muñoz Merino from Cancer Immunotherapy Group, IDIBELL). Briefly, tumors were first mechanically digested using blades and chopped into 2-4 mm pieces. Mechanical digestion was followed by enzymatic digestion using Tumor

Dissociation Kit, Mouse (130-096-730, Miltenyi), and gentleMACS™ Octo Dissociator with Heaters (130-096-427, Miltenyi) following the manufacturer's instructions. Samples were filtered by 40 µm cell strainers (352340, Falcon) to obtain single-cell suspensions, and erythrocytes were removed using Red Blood Cell Lysis Buffer Hybri-Max™ (R7757, Sigma).

We used a complete panel of antibodies for flow cytometry staining, including markers for general immune populations and their phenotypical activity (**Table 18**). To avoid unspecific labeling, we used FcR Blocking Reagent, mouse (130-092-575, Miltenyi), and to exclude dead cells, we used Zombie NIR™ Fixable Viability Kit (423105, Biolegend). Cells were acquired using a CytoFLEX SRT Benchtop Cell Sorter (Beckman Coulter), and data was analyzed using FlowJo Software (Beckman Coulter). The strategy of flow cytometry analysis is represented in **Table 19**.

For the representation of results, flow cytometry data was normalized against vehicles and changes in cell populations are shown as a percentage of viable or CD45⁺ cells.

Table 18. Antibodies used for tumor immunophenotyping. A 1:100 dilution was applied for all antibodies.

Antibody	Catalog #	Company
CD19 Antibody, anti-mouse, PE-Vio 615, REAfinity	130-111-890	Miltenyi
CD25 Antibody, anti-mouse, PE-Vio 615, REAfinity	130-123-028	Miltenyi
FITC anti-mouse CD3e	155604	Biolegend
PerCP/Cyanine5.5 anti-mouse CD279 (PD-1)	109120	Biolegend
PerCP/Cyanine5.5 anti-mouse CD11c	117328	Biolegend
PE anti-mouse CD4	116006	Biolegend
Brilliant Violet 421 anti-mouse CD8a	100738	Biolegend
Brilliant Violet 650 anti-mouse CD223 (LAG-3)	125227	Biolegend
Brilliant Violet 650 anti-mouse CD69	104541	Biolegend
APC anti-mouse TIGIT (Vstm3)	142106	Biolegend
APC anti-mouse CD137	106110	Biolegend
Alexa Fluor 700 anti-mouse NK-1.1	108730	Biolegend
Alexa Fluor 700 anti-mouse CD62L	104426	Biolegend
PE/Cyanine7 anti-mouse CD152	106314	Biolegend

PE/Cyanine7 anti-mouse/human CD44	103030	Biolegend
Brilliant Violet 510™ anti-mouse CD45	103138	Biolegend

Table 19. Panel strategy followed for tumor immunophenotyping.

FITC	PerCP/ Cy5.5	PE- TxRed	PE	PE/Cy7	BV421	AmCyan	BV650	APC	AF700	APC/Cy7	Mix, Tube
CD3	PD1	CD25	CD4	CTLA4	CD8	CD45	LAG3	TIGIT	NK1.1	Zombie	A) Lymphoid exhaustion/late activation
CD3	CD11C	CD19	CD4	CD44	CD8	CD45	CD69	4-1BB	CD62L	Zombie	B) Lymphoid effector cells

4.3.2 Mixed lymphocyte reaction (MLR)

To determine the stimulatory capacity of DCs, 2×10^4 iDCs, cDCs, or toIDCs from C57BL/6 mice pulsed with the appropriate peptide or infected with ICO15K virus were incubated with 1×10^5 allogeneic CFSE-stained splenocytes (three replicated per condition) from BALB/c mice (ratio 1:5) for 5 days. Then, cells were harvested and stained with a viability marker and then with α CD3, α CD4, and α CD8 antibodies (see Table 17). The CFSE dilution within the living CD3⁺CD8⁺ T cell subset of splenocytes was measured by flow cytometry.

For syngeneic mixed lymphocyte reactions (SMLR), either BMDCs and splenocytes from C57BL/6 were used.

4.3.3 Mouse IL-10 ELISA

Supernatants from MLR cultures were harvested and were analyzed for mouse IL-10 cytokine using an ELISA kit (ELISA MAX™ Standard Set Mouse IL-10, Cat. No. 431411), according to the manufacturer's protocol.

5. IN VIVO ASSAYS

5.1 MOUSE MODELS AND PROCEDURES

All animal studies were performed at the IDIBELL facility (AAALAC unit 1155) and approved by the Ethics Committee for Animal Experimentation from Biomedical Research Institute of Bellvitge (IDIBELL). Animals were humanely euthanized according

to pre-established endpoint criteria to minimize suffering and in compliance with institutional and international ethical guidelines for animal care and use. In all experiments, tumors were measured weekly under isoflurane anesthesia with a digital caliper and tumor volume was determined with the equation:

$$V \text{ (mm}^3\text{)} = \pi/6 \times W^2 \times L,$$

where W and L are the tumor's width and length, respectively.

For immunodominance demonstration studies, 6-8 week-old female BALB/c (Charles River) were subcutaneously implanted with CT26hCAR tumors (2×10^6 tumor cells) in one flank. Mice were randomly allocated into groups ($n=4/5$ group) when the tumor volume average reached 60-65mm³. Then, tumors were treated intratumorally at days 15 and 18 post-tumor inoculation with either 40μm STING agonist CDN (ADU-S100, InvivoGen, Cat. code tlr1-nacda2r-05) or 3×10^8 TU ICO15K OAd. Tumor volume was monitored for 21 days, and mice were sacrificed to isolate TDLNs for ELISpots (see Section 5.2).

For MLR or SMLR assays or detecting antiviral and antitumoral immune responses, 6-8 week-old female C57BL/6 mice (Charles River) were immunized by intravenously or intramuscularly injecting 2×10^{10} vp of the corresponding OAd, and after 7 days, they were sacrificed to isolate splenocytes for MLRs (see Section 4.3.2) or ELISpots.

For antitumor efficacy studies using PS2 SNs, 6-8 week-old female C57BL/6 mice were implanted subcutaneously with CMT64.6 (1×10^6 tumor cells) in one flank. Mice were randomized into two groups ($n=4/\text{group}$) when tumors reached an average tumor volume of 70mm³. Finally, PS2 or Ctrl SNs were injected intratumorally (50μL) on days 12, 16, and 19 post-tumor inoculation, and tumor volumes were measured until day 27.

To evaluate PS2 ICD-inducing capacity, 8 week-old female C57BL/6 mice were subcutaneously inoculated in the left flank with 5×10^5 MC38 cells treated previously (for 15-20min at 37°C) with either PS2-containing SN or their control counterpart. One week later (14 days later in case of prime-boosting), a rechallenge with another 5×10^5

non-treated alive MC38 cells were implanted in the right flank. Tumor volume of both vaccinated and rechallenge sides were monitored over time. Once rechallenge tumors were evident, animals were sacrificed for ELISpot or immunophenotyping studies (see Section 4.3.1.1). If required, surgical tumor resection of large primary tumors was performed under isoflurane anesthesia. Surgical incisions were closed with wound clips, which were removed 7–10 days later. Analgesia and local antisepsis was also administered.

For OAd-mediated antitumor efficacy studies, 8 week-old male and female NOD/scid/*IL2rg*^{-/-} (NSG) mice (bred in house) were implanted subcutaneously with 7×10^6 HepG 2 cells (2 tumors per animal). Animals were randomly distributed into three treatment groups ($n=9-10/\text{group}$) when tumors reached an average volume of approximately 70mm^3 . Then, animals were treated intravenously with 4×10^{10} vp in $200\mu\text{L}$ of the corresponding OAd, or treated with $200\mu\text{L}$ PBS. Tumor volumes were follow-up over time, and animals were sacrificed at day 40 post-treatment. Tumor samples were collected for further immunohistochemistry and qPCR analyses.

5.2 ENZYME-LINKED IMMUNOSPOT ASSAY (ELISPOT)

Enzyme-linked immunospot assay (ELISpot) measures the frequency of cytokine-secreting cells, IFN γ in our case, at a single-cell level in response to the stimulation with a specific antigen.

All steps until the development of the plates were performed under sterile conditions. 96-well ELISPOT plates (Multiscreen plates, Millipore, Billerica, MA) were treated with $30\mu\text{L}$ of 35% ethanol per well for 1min and washed 5 times with ddH $_2\text{O}$. Plates were incubated overnight at 4°C with a murine IFN- γ -specific capture antibody (clone AN-18, BD 551309, San Jose, CA) at $4\mu\text{g}/\text{mL}$ diluted in PBS at a final volume of $100\mu\text{L}/\text{well}$. The day after, the antibody was discarded and plates were washed 5 times with PBS and blocked with $200\mu\text{L}/\text{well}$ RPMI+10%FBS medium for at least 1 hour at RT. Then, $100\mu\text{L}$ of 2.5×10^6 cells/mL suspension (see Section 2.4) were added to each well (2.5×10^5 cells/well). Peptides used for the stimulation of splenocytes were: E1a₂₃₄₋₂₄₃

(SGPSNTPPEI) and E1b₁₉₂ (VNIRNCCYI) as Ad5 H-2^b-restricted epitopes (C57BL/6 strain); DBP7 (LPKLTPFALH) and Hex3 (KYSPSNVKI) as Ad5 H-2^d-restricted epitopes (BALB/c strain; or the correspondent according to the experiment (B16-F10 TAA epitopes 8mers/9mers). The OVA₂₅₇ (SIINFEKL) and Ndufs1 9-mer (ILAAVSNMV) peptides were also used for pulsing BMDCs. All peptides were synthesized by GenScript Biotech (New Jersey, USA) at >95% purity and resuspended with DMSO at 1mg/mL. The peptide stimulation was performed at a working concentration of 7µg/mL or 2µM (depending on the stock solution) diluted in media. PepTivator®AdV5 hexon (Milenyi Biotec) was resuspended with sterile ddH₂O according to the manufacturer's instructions.

Phorbol-myristate-acetate (PMA at 15ng/mL) plus ionomycin (250ng/mL) were used as a positive control of stimulation, and only media as the negative control. Plates were incubated with the splenocytes or TDLNs for at least 18h (using 9mer as stimuli) or 36h (for 27mers) at 37°C and 5% CO₂. Cells and medium were removed, and the plates were washed 5 times with PBS. Then, the biotinylated anti-murine IFNγ secondary antibody (clone RA-6A2, BD 55156, San Jose, CA) was added at a concentration of 1µg/mL in PBS 0.5% FBS at a final volume of 100µL/well and incubated for 1-2 hours at RT. Next, the antibody was removed, and the plates were washed 5 times with PBS. The streptavidin-ALP (ExtrAvidin Ref E2636-2ML Sigma E2636, Sant Louis, MO) diluted 1:2500 in PBS 0.5% FBS (100 µL/well) for 1h at RT. Again, 5 washes with PBS were performed before adding the substrate BCIP/NBT solution (50µL/well, Ref B1911-100ML, Sigma, St. Louis, MO). Plates were developed for 15-30min until spots emerged when the plate was washed with tap water to stop the reaction. Spots were counted after the plates were dry using the Bioreader 6000-E (Biosys, Germany).

5.3 IMMUNOHISTOCHEMISTRY (IHC)

Paraffin-embedded blocks were cut into 4-µm thick sections with a microtome and deposited into poly-L-lysine treated slides. Sections were deparaffinized by subjecting them to a battery of three xylols, two absolute ethanol, two 96% ethanol, one 70% ethanol, and one 50% ethanol (5 minutes each). Finally, the sections were rehydrated by submerging them in ddH₂O for 5min and rinsed thrice with PBS 0.1% Triton-X100.

Antigens masked during routine fixation were retrieved by immersing the slides in sodium citrate solution (pH 6.0) and heating them for 1h30min in a decloaking chamber. After rinsing with ddH₂O for 5min, endogenous peroxidase activity was blocked by incubation for 10 minutes in 0.3 % H₂O₂ dissolved in ddH₂O. Then, sections were washed once with ddH₂O (5min) and thrice with ddH₂O during (5min). Next, sections were blocked to reduce unspecific binding with blocking buffer (PBS 1% BSA + 20% Goat Serum) for 1 hour at RT. All the incubations were performed in a humidity chamber.

Primary antibody incubation was performed using an anti-E1A adenovirus-2/5 (sc-430, Santa Cruz, CA, USA) diluted 1:200 overnight at 4°C. After temperating slides for 30min, 3 washings with PBS 0.2% Triton-X100 for 5 minutes were performed. Next, sections were covered with anti-mouse EnVision™+ Dual Link (anti-mouse IgG + anti-rabbit IgG conjugated with HRP) (Dako Laboratories, Glostrup, Denmark, ref. K4063) and incubated for 1h at RT. Then, slides were washed 3 times more with PBS 0.2% Triton X-100 (5min) and developed by covering the sections with the chromogenic substrate DAB+ (EnVision™ Kit, Dako Cytomation K3468) for approximately 10min until a brown precipitate appeared (the revelation time depends on the sample). The reaction was stopped by rinsing the slides with tap water thrice for 5min. Finally, the sections were counterstained with hematoxylin, dehydrated with ethanols, cleared with xylene, and mounted in DPX (VWR International). For Hematoxylin/Eosin staining, standard methods were performed.

5.4 DETECTION OF VIRAL GENOMES IN TUMORS BY REAL-TIME qPCR

Frozen tumor samples were mechanically disrupted with micropestles for microcentrifuge tubes (VWR, ref. 211-2100). Then, the Maxwell® RSC simplyRNA Tissue Kit protocol (Promega, Cat.#AS1340) was followed. Briefly, 200µL of chilled 1-Thioglycerol/Homogenization Solution was added to the tubes to further smash tumors until a homogenate was formed. Homogenates were kept on ice while the other tumors were being processed. Shortly before processing samples on the Maxwell® Instrument, 200µL of Lysis Buffer (Part# MC501C) were added to 200µL of

homogenate and vortexed vigorously for 15sec to mix. Then, all 400µL sample was transferred to well #1 (the largest well) of the Maxwell® RSC Cartridge. Finally, 7µL of DNase I Solution were added to well #4 (yellow reagent). After adding the blue DNase I Solution, the reagent in well #4 becomes green. For Maxwell® Automated RNA purification from tumor homogenates, plungers and 50 µL of Nuclease-Free Water Elution tubes were added where they corresponded on the cartridges placed in the deck tray. Finally, the instrument's run instructions were followed. Automated RNA extraction was performed in approximately 1h.

Next, reverse-transcription of RNA samples was performed using the PrimeScript RT reagent kit (Takara Bio, San Jose, CA, USA), following the manufacturer's protocol. In short, a master mix of 4 µL 5xPrimeScript Buffer, 1µL PrimeScript Enzyme, 1µL Oligo dT, and 1µL random 6mers was prepared (volumes x 1 reaction), and 7µL of this mix were added into each reaction tube. Then, 13µL of diluted DNA containing 1µg of total RNA in RNase-free water was added. The reaction was incubated in a thermocycler at these conditions: i) 37°C for 15min; ii) 85°C for 5sec; iii) 4°C HOLD. The resulting cDNA samples were frozen at -20°C until analysis by qPCR.

The analysis of cDNA samples was performed in a LightCycler 480 Instrument II (Roche, Basel, Switzerland). To quantify the viral genomes and PS2 transcripts in the tumor, 50ng cDNA in the presence of SYBR Green I Master (Roche) were used. Standard curves for viral genomes and PS2 were prepared by serial dilutions of known copy numbers of adenovirus and PS2-encoding plasmids, respectively. The primers used to detect Ad5 (hexon) and PS2 cDNAs are listed in **Table 20**, and PCR conditions, detailed in **Table 21**.

Table 20. Primers used for the detection of viral genomes and PS2 molecules.

Primer	Sequence (5'→3')
Ad18852	CTTCGATGATGCCGCACTG
Ad19047R	ATGAACCGCAGCGTCAAACG
PS2qPCR Fw	CTTACTGCGAAGATAGCACGGG
PS2qPCR Rv	CTGGAAGTCAAAGGAGCTGACG

Table 21. qPCR conditions for the detection of viral genomes and PS2 molecules.

Stage	Description	Temperature	Time	Cycles
1	Activation	95°C	10 minutes	1
2	Amplification cycles	95°C	15 seconds	40
		60°C	1 minute	
		72°C	7 seconds	

6. STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software v8.0 and R software v3.6.3. Generally, all results were expressed as means $\pm$ SEM ($\pm$ SD if indicated). Pvalue<0.05 was taken as the level of significance. First, data were assessed for normality with the Shapiro-Wilk test; parametric or non-parametric tests were performed depending on the result. Even so, most data were analyzed using non-parametric tests, which are more appropriate for small sample sizes. For mean group comparison, the test election criteria were summarized in **Table 22**:

Table 22. Statistical test election criteria for group comparison. *Depending on whether all groups were compared with each other (Tukey) or only against a group control (Dunnett's).

	Parametric		Non-parametric	
	<i>Paired</i>	<i>Unpaired</i>	<i>Paired</i>	<i>Unpaired</i>
2 groups	PairedTwo-Tailed T-test	Two-tailed T-test	Wilcoxon signed-rank test	Mann-Whitney test
≥ 3 groups	Paired One-way ANOVA	One-way ANOVA	Kruskall-Wallis	Kruskall-Wallis
Post hoc test	Tukey	Tukey/Dunnett*	Dunn	Dunn

For *in vivo* studies followed over time, the corresponding statistical test was applied at each timepoint. For the immunodominance demonstration ELISpot, box plots were graphed showing the interquartile range (IQR). Upper and lower whiskers extend from the hinge to the largest value no further than $1.5 \times \text{IQR}$ (R v3.6.3). Correlations of parametric data were assessed by Pearson correlation and non-parametric data by Spearman correlation (GraphPad Prism v8.0 and R v3.6.3).

Figures 14-C, 20-B, 21-B, 22-B, 29-B, 31, 34-B, and 37, as well as their associated statistical analysis, were obtained using R software with the support of Marcel Costa Garcia (PhD); analyses of Figures 41 and 42 were performed using R software aided by Pau Fiol Mata (both from Cancer Immunotherapy Group, IDIBELL).

RESULTS

1. VIRAL IMMUNODOMINANCE IN THE CONTEXT OF ICO15K VIRUS

As mentioned before, the immunogenicity of viral epitopes often results in biased immune responses against them over other epitopes, including tumor epitopes, which may induce impaired antitumor T-cell responses.

To validate this assumption, we designed an *in vivo* experiment in which CT26hCAR-tumor-bearing mice were treated intratumorally with two doses of a STING agonist (c-di-AM(PS)₂ (Rp, Rp); Invivogen), ICO15K OAd, a combination of both agents or left untreated (PBS) (**Figure 14-A**). To evaluate how these treatments influenced the balance between antitumor and antiviral immune responses, an immunocompetent mouse model with murine tumors was used. However, CT26 cells exhibit low susceptibility to infection by human Ad5; for that reason they were engineered to express the human CAR receptor via lentiviral transduction, thereby enhancing their virus infectivity. Tumor monitoring revealed that only the groups treated with the STING agonist demonstrated significantly better tumor growth control compared to ICO15K-treated animals, which exhibited growth patterns similar to PBS controls (**Figure 14-B**). These results align with previous findings on the potent antitumor efficacy of STING agonists in established CT26 tumors²⁵⁸ and the fact that the virus is unable to replicate in murine cells, so no oncolysis could be induced. Moreover, the STING agonist's effectiveness was also reflected in the number of spots obtained against the tumor cell line in an ELISpot assay (**Figure 14-C**). Conversely, in the OAd-treated animals, this antitumoral immune response decreased while potent antiviral responses were detected (Adv5 mix), suggesting viral-induced immune responses were masking the antitumoral responses.

We included the “combo” group as well, expecting this putative virus interference would weaken antitumoral immune responses compared to the STING agonist alone. Although the mean value in this group was similar to the ICOVIR15K group, which pointed in that direction, no rotund conclusions can be drawn due to the considerable intragroup heterogeneity. Surprisingly, antiviral responses barely existed in this latter condition. Besides, it is worth mentioning that some saline-treated animals developed

spontaneous tumor immunity just for being inoculated with CT26hCAR cells, similarly as Aliche et al. reported²⁵⁹. What is clear is the virus alone elicited strong antiviral responses and moderate antitumoral responses, the latter not as potent as the ones generated by a non-viral agent. This encouraged us to explore the DC-based viral tolerization strategy, with its results detailed in the next section.

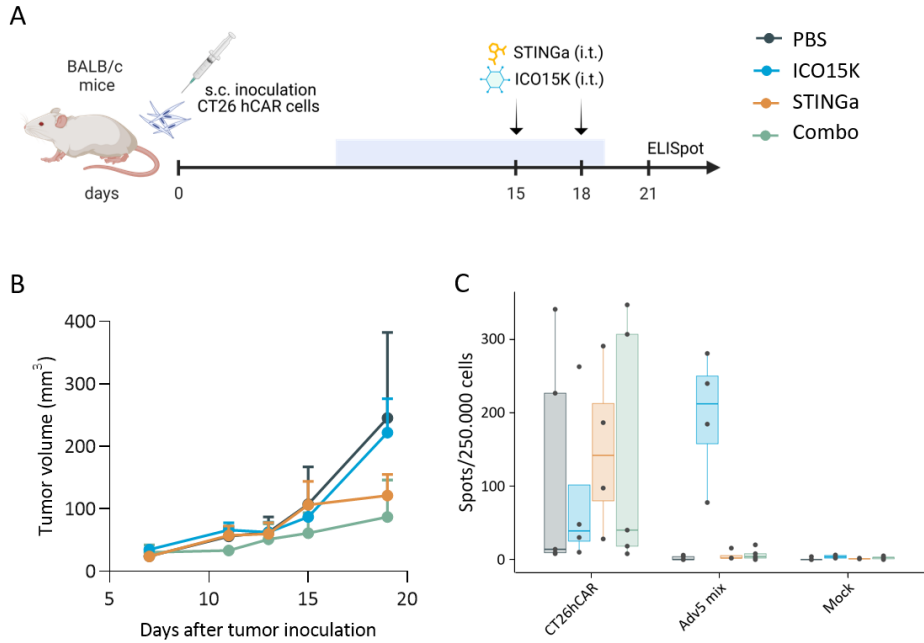


Figure 14. Antitumoral immune responses of STING agonist immunotherapy are hindered in the presence of OAdS. Mice with established subcutaneous (s.c.) CT26hCAR tumors were treated intratumorally (i.t.) with ICO15K, a STING agonist (STINGa), a combo of both, or left untreated (n=4-5/group). Arrows indicate the days of treatment. A) Tumor growth was monitored over time (colored box), and B) antitumor and antiviral immune responses were measured with an ELISpot assay using tumor-draining lymph nodes (TDLNs) from treated animals. Mean $\pm$ SEM was plotted. Data statistics were analyzed by unpaired Kruskal-Wallis test and Dunn's *post hoc* test. C) The tumor cell line or Adv5 peptide mix was used as stimuli. The number of spots was normalized in each case to spots per 250.000 TDLNs. Box plots show the interquartile range (IQR). Upper and lower whiskers extend from the hinge to the largest value no further than 1.5 * IQR. Image A created with BioRender.com. Adv5, adenovirus serotype 5.

2. INDUCTION OF IMMUNOLOGICAL TOLERANCE TO ONCOLYTIC ADENOVIRUSES USING DENDRITIC CELLS

Given that the viral epitope immunodominance phenomenon to adenoviruses has been broadly studied, we wanted to explore the feasibility of inducing immunological tolerance to OAds using tolerogenic dendritic cells. To our knowledge, this method was only reported for adenoviral vectors in gene therapy using the IL-10 cytokine as a tolerogenesis-inducing agent²⁴³. The remarkable ability of dendritic cells to direct Ag-specific immune responses, combined with their tolerogenic properties in the appropriate context, represents an advantage we could leverage to modulate antiviral responses and shift the balance toward antitumor immunity. Our group implemented a protocol for generating tolDCs from mouse bone marrow cells using low doses of GM-CSF while omitting IL-4 from the standard protocol. Originally described by Lutz et al.²⁵², this approach yields tol-BMDCs characterized by low expression of MHC-II, CD40, CD80, and CD86, as well as a resistance to maturation when stimulated with LPS. Thus, mouse bone marrow cells were cultured by adding GM-CSF $\pm$ IL-10 every 3 days for 6 days to generate BMDCs or BMtolDCs.

GM-CSF is a growth factor related with bone marrow precursor mobilization and DC differentiation. Initiation of colony formation could be seen at various sites since day 3, with cells aggregating together (**Figure 15, day 4**). Conversion of cells into adherent macrophages (elongated cells) was also seen. Immature BMDCs were obtained on day 6 of the culture in the presence of murine GM-CSF and large-sized colonies of BMDCs were formed at various sites. Treatment of immature BMDCs on day 6 of culture with maturation stimulus LPS (1 μ g/ml) for 24h resulted in the maturation of BMDCs, as evidenced by an increased population of cells with branched and extended morphology (arrows), in contrast with the rounded BMDCs at early stages of the culture. Optical microscopy images suggested this spherical morphology is maintained in IL-10-cultured BMDCs, with few cells showing discrete dendrites (**Figure 15, day 7**). However, no significant morphological changes were observed at this resolution between culture conditions.

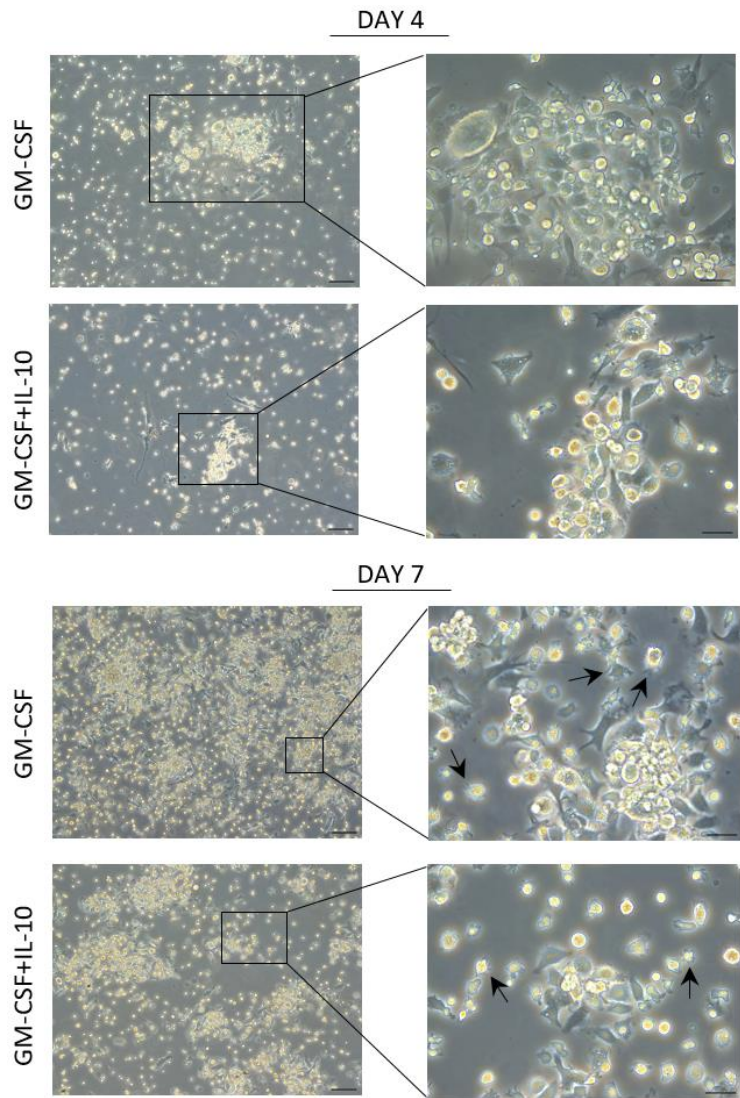


Figure 15. Morphological observation of murine BMDCs during the differentiation procedure.

Bone marrow is flushed out from the bone cavity using a 21-gauge needle attached to a syringe filled with completed growth media. Then, BMDCs are cultured in RPMI-1640 with 10% FBS containing 20 ng/ml rmGM-CSF and 70ng/ml rmIL-10 for generating tolDCs. Fresh medium is supplemented on days 3 and 6. Black arrows point at dendrites formation protruding from DCs. Pictures were captured by an inverted microscope with a digital imaging system (Leica DMI1; Leica Microsystems, Germany). Scale bars: 100µm (left panel); 30µm (right panel). rm, recombinant murine.

2.1 Tolerogenic phenotype characterization of IL-10-induced tolDCs

BMDC cultures were phenotypically characterized by flow cytometry to assess the semimature profile so distinctive of tolDCs. Surface expression of CD11c (a murine DC-specific marker) was analyzed in BMDCs harvested on days 7-8, and the CD11c⁺ population was selected to evaluate the expression of costimulatory molecules such as CD80 (not shown), CD86 (**Figure 16-A and B**), and MHC-II (**Figure 16-C and D**). The phenotype of cDCs (panels A and C) and tolDCs (panels B and D) were analyzed by comparing the variation of these markers in mature DCs (matured with either LPS or ICO15K infection) regarding immature DCs (iDCs, $\emptyset$) of both DC types. Although a basal expression of activation markers was evident in immature DCs (iDCs), as indicated by the area under the grey dotted line in the double-positive quartiles, the addition of LPS significantly increased this double-positive population across all conditions. On this matter, the net increase in the double-positive:total CD11c⁺ ratios for CD86 and MHC-II showed no significant differences between cDCs and tolDCs (CD86: 0.79 ± 0.09 for cDCs versus 0.72 ± 0.14 for tolDCs; MHC-II: 0.71 ± 0.06 for cDCs versus 0.64 ± 0.06 for tolDCs). These findings deviate from the widely accepted notion that tolDCs exhibit a maturation-resistant phenotype, where the addition of LPS would significantly enhance the expression of costimulatory molecules in mature cDCs but not in tolDCs. Instead, our results align more closely with reports suggesting that differences in CD86 and MHC-II expression between cDCs and tolDCs are relatively subtle²⁶⁰.

Contour plots revealed that a high viral dose (3000 vp/cell) only partially upregulated the CD86 marker on cDCs (area above the grey dotted line), whereas tolDCs maintained basal expression levels similar to their iDC counterparts. As for MHC-II molecules, virus-induced maturation was less pronounced, with the double-positive population resembling that of iDCs. Thus, even at a high viral dose, cDCs exhibited suboptimal maturation, as indicated by limited CD86 expression and only a slight increase in MHC-II levels, failing to achieve the robust maturation observed with LPS stimulation. This result was unexpected, as the virus, being an exogenous agent, was anticipated to act as a strong maturation stimulus.

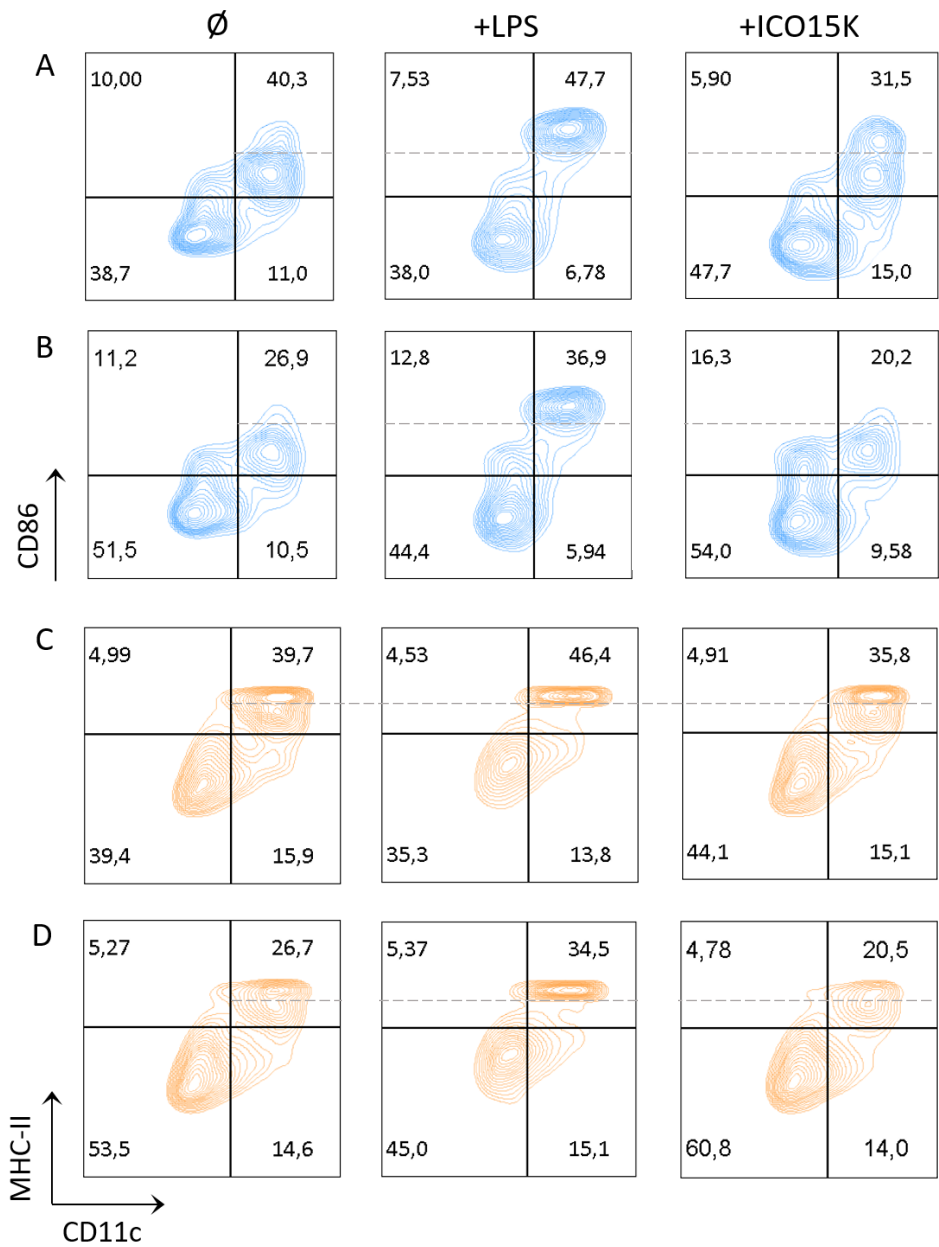


Figure 16. Expression of coestimulatory activation markers of murine cDCs and toIDCs in different treatment conditions. Both cDCs (panels A and C) and toIDCs (panels B and D) cultures were treated at day 6 with LPS (1µg/ml) or ICO15K (3000vp/cell) maturation stimuli. Frequencies of CD86 (blue) and MHC-II (orange) versus CD11c live cells are shown. vp: viral particles.

Looking for other markers that allowed us to clearly differentiate tolDCs from iDCs and cDCs, we first tested the MerTK receptor tyrosine kinase, which was highly expressed on dexamethasone-induced murine tolDCs^{261,262}. However, no remarkable differences in MerTK cell surface expression levels were observed among DC types (**Figure 17-A**). In contrast, IL-10-induced tolDCs did result in higher production of the anti-inflammatory cytokine IL-10 (statistically significant compared with iDCs), accordingly with a tolerogenic profile (**Figure 17-B**).

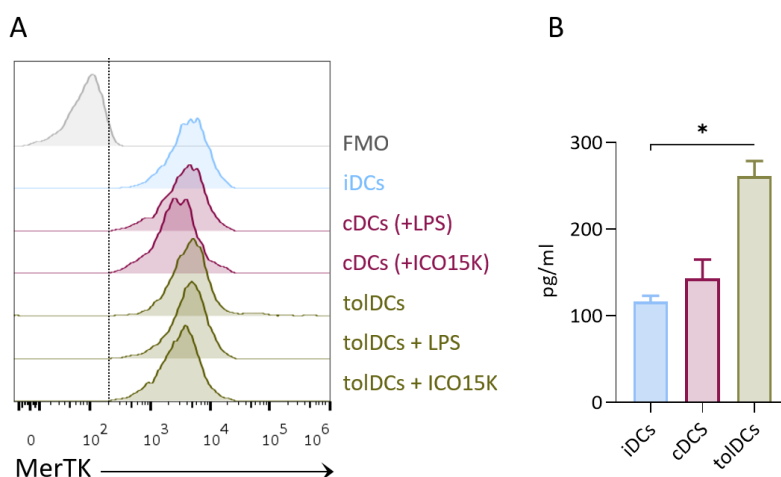


Figure 17. IL-10-induced murine tolDCs do not upregulate MerTK expression levels but secrete higher amounts of IL-10 compared with iDCs and mature cDCs. A) The indicated DC subsets were stained with MerTK-AF700 antibody and detected by flow cytometry. The histograms are gated for alive CD11c⁺ DC cells. **B)** A mouse IL-10 ELISA was performed with supernatants harvested from DC cultures. The absorbance in wells was read at 450nm using a microplate reader and the IL-10 concentration (pg/ml) was interpolated. Mean $\pm$ SEM of triplicates is plotted. * $p \leq 0.05$ Kruskal-Wallis and Dunn's *post hoc* test; $n=3$ per group. FMO, fluorescence minus one.

To better characterize our DC cultures, we also assessed key markers that determine the subset class of these cells. In general, DCs can be subdivided into three main subsets: conventional DCs (cDCs, also referred to as myeloid DCs), monocyte-derived DCs (moDCs), and plasmacytoid DCs (pDCs). cDCs can be further subdivided into cDC1, cDC2 and migratory DCs (migDCs)²⁶³. Both cDC1 and cDC2 can behave as migratory or lymphatic tissue-resident subsets. In the case of cDC1, the distinction relies on the

differential expression of either CD8 α (resident cDC1) or CD103 (migratory cDC1) molecules. On the other hand, the CD11b molecule is characteristic of cDC2 cells regardless of location, and they also express CD4²⁶⁴; the only way known to differentiate between migratory or resident is based on differential expression levels of both MHC-II and CD11c, where migratory cDCs exhibit only intermediate levels of CD11c, but high expression of MHC-II, and the other way round for resident cDC2 cells²⁶⁵.

In this context, our results indicated that almost 70% of living cells in cultures expressed the CD11c marker (**Figure 18**, histogram), though the range can vary among cultures, being around 40-50% in Figure 16. Nonetheless, what is entirely consistent is that the vast majority of them were CD11b⁺, suggesting they belong to the cDC2 subset. Whether their nature is migratory or resident would have to be deeply investigated.

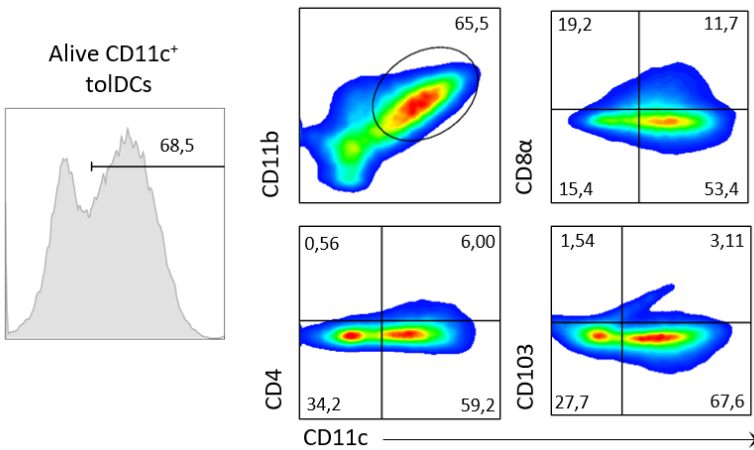


Figure 18. Murine CD11c⁺ tolDCs exhibit a cDC2 marker. CD11c⁺ gated tolDC cells were assessed by flow cytometry for the indicated markers to elucidate their DC subset nature. Results revealed most of them expressed the CD11b molecule, which is a marker of migratory DCs contained in lymph nodes. FCM plots with population frequencies (%) are shown.

2.2 Allogeneic T cell proliferation is impaired with IL-10-induced tolDCs

In addition to phenotypic characterization, the most crucial property defining a tolerogenic dendritic cell is its ability to inhibit or reduce T-cell responses. The capacity of tolDCs and of cDCs to activate T cells was investigated in mixed lymphocyte reactions (MLRs) by quantifying T cell proliferation. The principle of a mixed lymphocyte reaction is that T cells (present in splenocytes) from one donor proliferate in response to APCs, DCs, from a genetically distinct donor. This response is triggered by the recognition of mismatched MHC molecules between unrelated donors, which elicits a T-cell immune response. A key advantage of the MLR assay is its robust baseline response, which occurs without the need for additional stimulatory compounds. Appropriately generated tolDCs are supposed to suppress this inherent T cell proliferation, demonstrating their immunoregulatory capabilities. In our case MLRs were performed by coculturing 1×10^5 CFSE-labeled splenocytes from C57BL/6 mice with either 2×10^4 cDCs or tolDCs derived from BALB/c mice for 5 days (ratio splenocytes/DCs 5:1). Proliferating T cells were monitored by dye dilution by flow cytometry (**Figure 19-A**).

As expected, mature cDCs (+LPS) were the condition that favored the most T cell proliferation due to the allogeneic reaction between mouse strains. In comparison, the proportion of proliferative CD8⁺ T cells was substantially diminished when IL-10 was added to BMDCs, being statistically significant in tolDCs infected with the ICO15K virus (**Figure 19-B**). However, some inconsistencies were also observed. The first was that iDCs were found to induce allogeneic T cell proliferation as similar to cDCs in some MLRs (**Figure 19-B**). In contrast, they should have shown comparable values as tolDCs, just as the experiment illustrated in **Figure 19-A**. The other one was the significant variability observed in the condition where cDCs were infected with the ICO15K OAd. In **Figure 19-A**, these cells displayed behavior more akin to iDCs or tolDCs, inhibiting allogeneic T-cell proliferation rather than acting as mature cDCs. However, in other MLR assays, the virus did promote noticeable T-cell activation, albeit at levels lower than those achieved with LPS treatment (**Figure 19-B**).

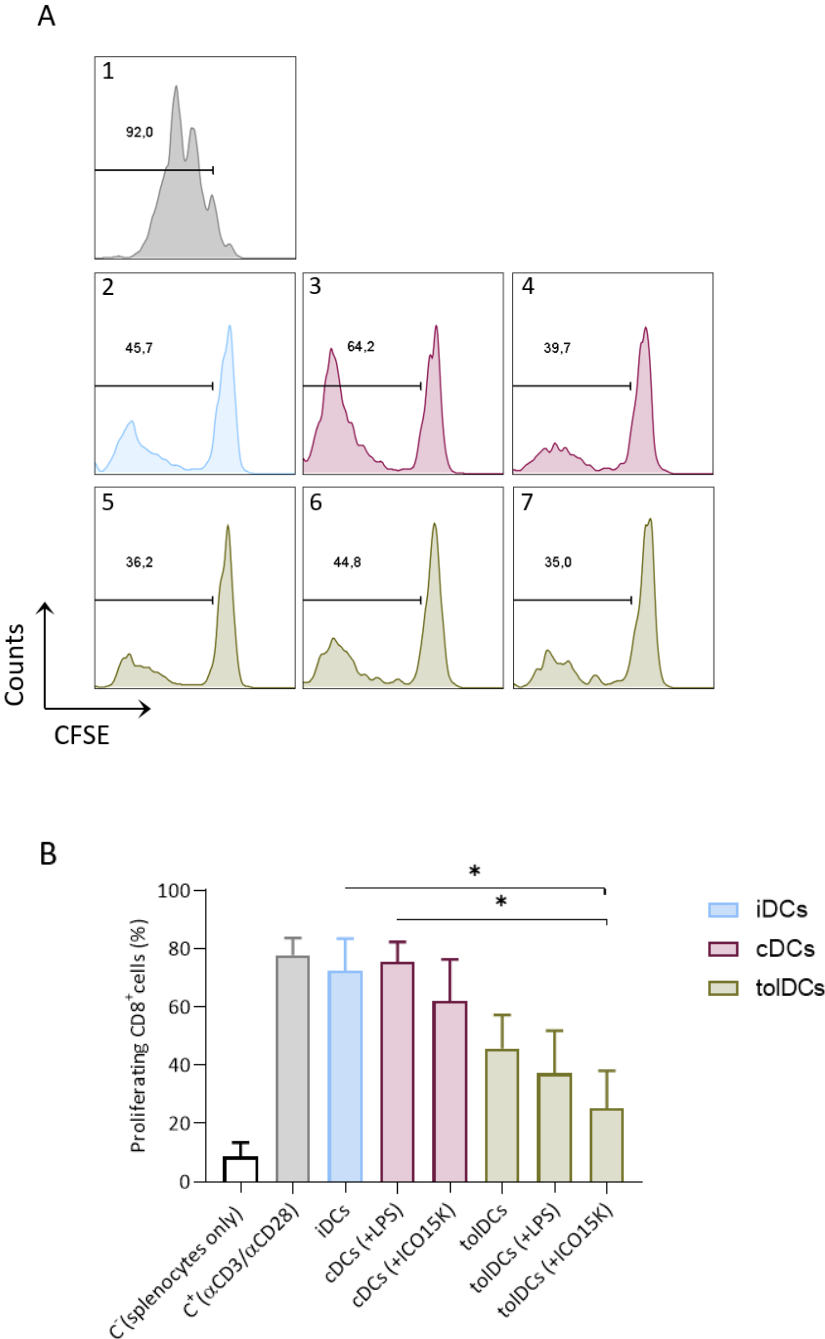


Figure 19. Suppressive activity of IL-10-stimulated BMDCs on allogeneic T-cell proliferation. CFSE-labeled splenocytes from BALB/c mice were cocultured with DCs derived from C57BL/6 (MLR), and after 5 days, T cell proliferation was measured by dye dilution **A**) Representative histogram plots of CFSE-gated CD3⁺CD8⁺ T cells, with their respective proliferation frequencies indicated (bars). **B**) Mean $\pm$ SEM of three independent MLRs. The α CD3/ α CD8 antibody-mediated stimulation was used as positive control. * $p \leq 0.05$ One-way ANOVA and Dunnett's *post hoc* test. MLR, mixed lymphocyte reaction. 1: Proliferation *Ctrl; 2: iDCs; 3: cDCs (+LPS); 4: cDCs (ICO15K); 5: tolDCs; 6: tolDCs +LPS; 7: tolDCs +ICO15K.

These findings, coupled with the observation in Figure 16 that ICO15K treatment did not upregulate CD86 and MHC-II expression to the same extent as LPS, led us to conclude that using the whole virus as a maturation stimulus was not consistently effective. As a result, subsequent experiments utilized Ad5 peptides to pulse DCs, ensuring proper antigen capture, processing, and presentation.

Despite these unexpected outcomes, the experiment demonstrated that IL-10-treated tolDCs exhibited a tolerogenic functionality, enabling them to effectively suppress alloresponses in MLRs.

2.3 Mature cDCs prime antigen-specific T-cell responses in a syngeneic setting

To confirm that cDC-triggered T-cell proliferation in the previous MLRs was antigen-specific, a syngeneic mixed lymphocyte reaction (SMLR) was performed. Unlike an MLR, in an SMLR, the DCs and T cells proceed from the same mouse strain, eliminating the possibility of alloreactive responses. Therefore, to induce immune responses, DCs must undergo maturation and be pulsed with an antigen to effectively present it and activate T cells that have previously encountered such antigen.

In order to recreate these conditions, on day 6 of DC culture, DCs (cDCs or tolDCs) were pulsed with the OVA₂₅₇ peptide, an irrelevant peptide (Ndufs1, a neoepitope from the CMT64.6 cell line, which shared the same MHC haplotype-restriction as OVA) or left unpulsed. Then, they were cocultured for 5 days with CFSE-stained splenocytes from an OVA-immunized C57BL/6 mouse. This immunization was done one week before the coculture, by injecting intravenously an OVA-encoding OAd that expresses the OVA

epitope under the constitutive CMV promoter (ICOVIR15-d6.7/19K-B16-TMG generated in our group) (**Figure 20-A**). Finally, CFSE dilution of the T CD8⁺ subset was once again used as an indicator of lymphocyte proliferation and measured by flow cytometry (**Figure 20-B**).

We verified successful immunization with the OVA-encoding virus, as approximately 4% of splenocytes stimulated by the immunization secreted IFN γ upon rechallenge with the OVA257 peptide (**Figure 20-C**), indicating their activation. Interestingly, only mature OVA-pulsed cDCs could promote the highest T-cell proliferation when cocultured with OVA-specific T cells, even being a small proportion of the total splenocyte suspension. Conversely, when these splenocytes were cocultured with an irrelevant peptide-pulsed cDCs, T-cell proliferation decreased, suggesting that the priming of T cells was triggered in an Ag-specific manner.

While this experiment did not directly confirm the antigen specificity of tolDCs, the comparable percentages of CFSE dilution observed for both DC types in the irrelevant peptide condition, along with the notable reduction of the CFSE dilution induced by OVA-pulsed tolDCs, suggested a potential retention of Ag-specific T-cell proliferation caused by the latter. Nevertheless, whether these differences between OVA-pulsed tolDCs and Ndufs1-pulsed tolDCs were due to an Ag-specific tolerization requires further validation through additional replicates or another type of experiment to confirm these trends.

2.4 tolDCs pulsed with viral peptides trigger a general reduction of both antiviral and antitumoral immune responses

Given the observed tendency of tolDCs to more effectively suppress T-cell proliferation in MLRs (see Figure 19), we sought to evaluate their potential to induce tolerance against Ad5 epitopes, thereby enhancing the efficacy of a subsequent OAd administration. To achieve this, it was first necessary to establish a robust model for quantifying immune responses against both viral and tumor epitopes. With these responses effectively measured, we could then determine whether the application of

Ad-tolerization using tolDCs impacted antitumor responses relative to antiviral responses; in other words, if the tolerization strategy could attenuate the strong antiviral responses while simultaneously unmasking the antitumoral responses as a result.

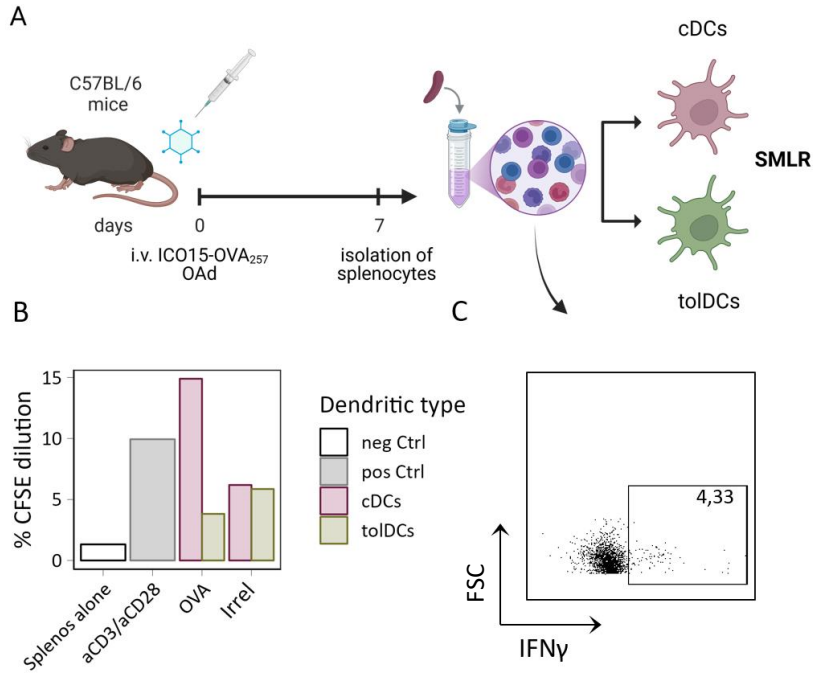


Figure 20. OVA-pulsed mature DCs prime Ag-specific T cell responses in an SMLR. **A)** A syngeneic mixed lymphocyte reaction (SMLR) was performed by coculturing for 5 days cDCs or tolDCs from C57BL/6 mice with splenocytes from another immunized C57BL/6 mouse with the virus ICO15-d6.7/19K-B16-TMG, which encodes for the OVA₂₅₇ 8-mer epitope (2×10^{10} vp i.v. injection). **B)** DCs used for SMLR cocultures were previously treated with LPS ($1 \mu\text{g}/\text{mL}$) and then pulsed with $2 \mu\text{M}$ OVA₂₅₇ or an irrelevant peptide of the same MHC haplotype (antigen presentation negative control). Splenocytes were stained with CFSE dye, and the percentage of CFSE dilution was measured as an indicator of T-cell proliferation. No statistics could be applied as this experiment was performed once. **C)** Splenocytes from the immunized mouse were challenged with the OVA 8-mer peptide. An intracellular cell staining (ICS) was performed to detect the frequency (%) of IFN γ -secreting OVA-CD8⁺ T cells. Irrel, irrelevant; i.v., intravenous; pos, positive; neg, negative; Image A created with BioRender.com.

We successfully detected antitumor immune responses when tumor epitopes were incorporated into the viral construct¹¹⁰. Following a similar setup, we induced both antiviral and antitumor immune responses by immunizing mice with an OAd encoding

CD8 TAAs from the B16F10 cell line (ICO15K-CMV-TMG08) (**Figure 21-A**). The ELISpot analysis evidenced that, apart from the response to the tumor cell line, immune responses to tumor epitopes were significantly lower compared to the strong reactions elicited by the highly immunogenic adenoviral E1a peptide (**Figure 21-B**), which supported the viral immunodominance hypothesis.

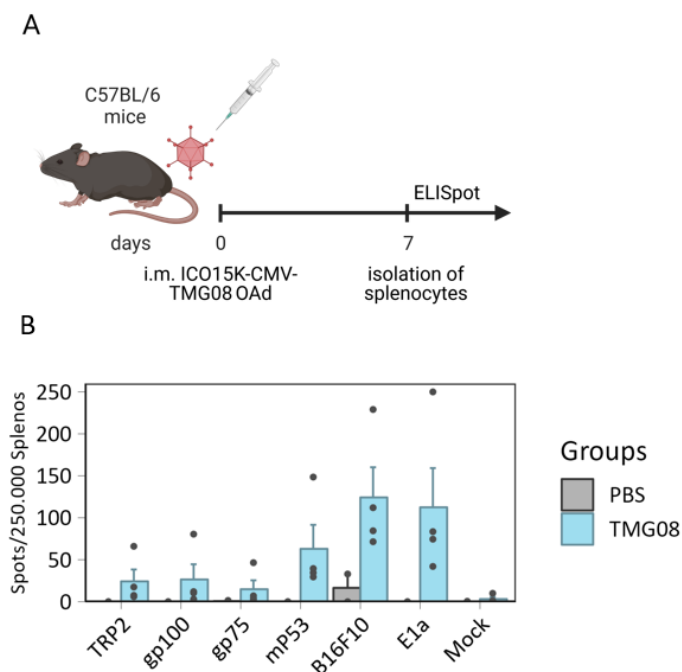


Figure 21. Immune responses against both viral and tumor epitopes are detected upon immunization with an OAd encoding B16 epitopes. A) C57BL/6 mice were immunized intramuscularly (i.m.) with 2×10^{10} vps of ICO15K-CMV-TMG08 OAd (from which CD8 epitopes of the B16F10 cell line in tandem minigenes were expressed, $n=4$) or not (PBS, $n=2$). **B)** Splenocytes were isolated one week later and stimulated with the same CD8 epitopes, the cell line, or the adenoviral epitope E1a for an ELISpot assay. The number of spots was normalized for 2.5×10^5 splenocytes. Mean $\pm$ SEM bars are plotted. vps, viral particles. Image A created with BioRender.com.

In light of these results, we decided to move to an *in vivo*, applying a similar preventive approach protocol some authors used to tolerize against autoimmune disease-related antigens^{266,251}. Briefly, we pretreated mice systemically with tolDCs previously co-pulsed with E1a, E1b, and a mix of hexon (PepTivator®AdV5 hexon) adenoviral peptides 3 days before injecting the ICO15K-CMV-TMG08 OAd, followed by another tolDCs

treatment 4 days after OAd administration, aiming to boost the tolerization. The co-pulsing strategy was chosen to allow tolDCs “to see” as many OAd antigens as possible, expecting this could reinforce the OAd tolerization. Seven days later –when immune responses were supposed to be configured– we performed an ELISpot with TDLNs against both tumor and OAd peptides (**Figure 22-A**). Disappointingly, treatment with tolDCs reduced immune responses in all conditions, suggesting they were exerting a general rather than viral-specific immunosuppression, even though these differences between groups were only statistically significant for the E1b peptide (**Figure 22-B**). Not so striking was the variation for the hexon pool mix condition, which could be attributed to what is already known: that the cellular response to the human Ad5 is dominated by the E1a and E1b epitopes²⁶⁷.

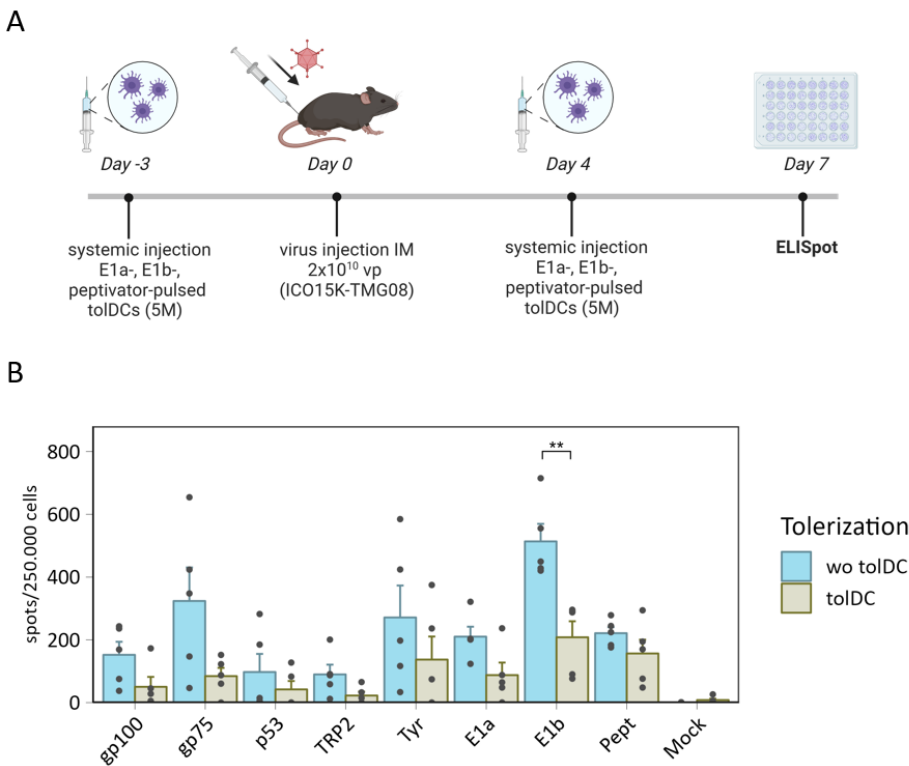


Figure 22. tolDC treatment fails in reducing viral-specific immune responses. A) Treatment timeline. Prior to and after ICO15K-CMV-TMG08 immunization (2×10^{10} vps), 5×10^6 tolDCs pulsed with E1a, E1b, and hexon adenoviral peptides were administered intravenously (tolDC) or not (without tolDC). **B)** Antitumoral and antiviral immune responses were measured with an ELISpot assay at day 7 post-virus administration, comparing both groups. The number of spots was normalized in each case to spots per 2.5×10^5 cells. Mean $\pm$ SEM bars are plotted ($n=5/\text{group}$). $^{***}p \leq 0.01$ by Mann-Whitney test and Dunn's *post hoc* test. i.m., intramuscular; vps, viral particles. M, million. Image A created with BioRender.com.

Following a critical evaluation of the results obtained, research efforts in this direction ceased after encountering experimental challenges and inconsistencies regarding the existing literature. It became clear that the project's anticipated outcomes were not fulfilled and could not be further validated within the given timeframe and resources. Consequently, efforts were shifted toward investigating more promising strategies to enhance the therapeutic potential of OAdS, such as the one detailed in the next section.

3. ONCOLYTIC ADENOVIRUSES EXPRESSING PORE-FORMING TOXINS

3.1 Study of PS2Ab antitumor cytotoxic activity

The potent antitumor properties in parasporins make them promising candidates for development as anticancer agents. Among these, parasporin 2 stands out for its broad toxicity spectrum against cancer cells, as reported in the literature (see Table 2). In particular, for this work, the initially chosen PS2Aa1 from *B. thuringiensis* A1547 strain²⁶⁸ was replaced by the PS2Ab from *B. thuringiensis* TK-E6 strain²⁶⁹ due to its higher cytotoxicity, although having 84% homology with the former PS2 subtype. As detailed in Materials and Methods, two furin cleavage sites and the human trypsinogen signal peptide sequence were inserted before *PS2Ab* to allow its activation and extracellular secretion, respectively.

The *PS2AbFuT* sequence, hereinafter referred to as PS2FuT or just PS2, was inserted into expression plasmids and transfected into HEK293 cells to produce PS2-enriched supernatants. Then, different human and murine tumor cell lines were treated with these supernatants at dilutions ranging from 1:2 to 1:8. Nuclear condensation and swelling in PS2-sensitive cells were appreciated under light microscopy at 2h after

treatment (**Figure 23**, black arrows). At this time, the most sensitive cells, like HepG2, presented a more advanced phenotype of detached cells due to toxin-mediated cell lysis (**Figure 23-C**). By contrast, A549 cells remained intact, confirming their resistance to PS2 (**Figure 23-A**).

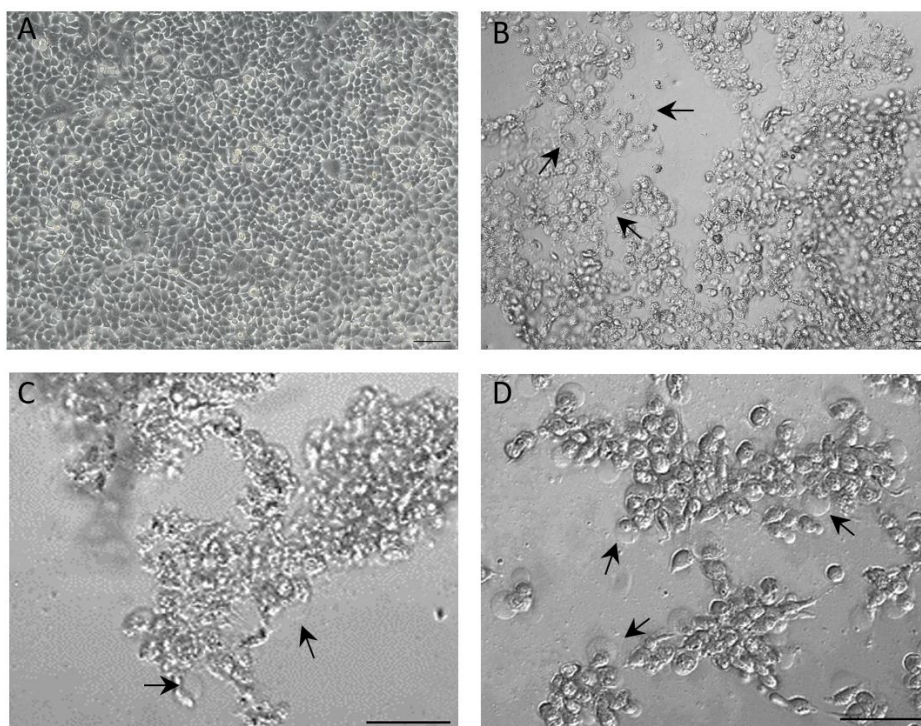


Figure 23. Morphological effects of PS2AbFuT SN on a panel of tumor cell lines. Human A549 (**A**) and HepG2 (**C**), and murine CMT64.6 (**B**) and MC38 (**D**) cells were incubated with HEK293-transfected with a PS2FuT expression plasmid supernatant. After 2h, pictures were taken under the microscope. Cytoplasm cell swelling due to pore formation can be seen (black arrows). Pictures were captured by an inverted microscope with a digital imaging system (Leica DMI1; Leica Microsystems, Germany). Scale bar: 50 μ m.

These observations were consistent with results in MTS assays, in which cell viability was lost at different PS2 SN dilutions in a more extensive panel of human and murine tumor cells (**Figure 24**). The more sensitive a cell type is to the toxin, the less metabolic activity was measured at a higher dilution of PS2 supernatant. Among the tested cell lines, HepG2, MC38, and CT26 cells exhibited the highest sensitivity, with CT26 cells

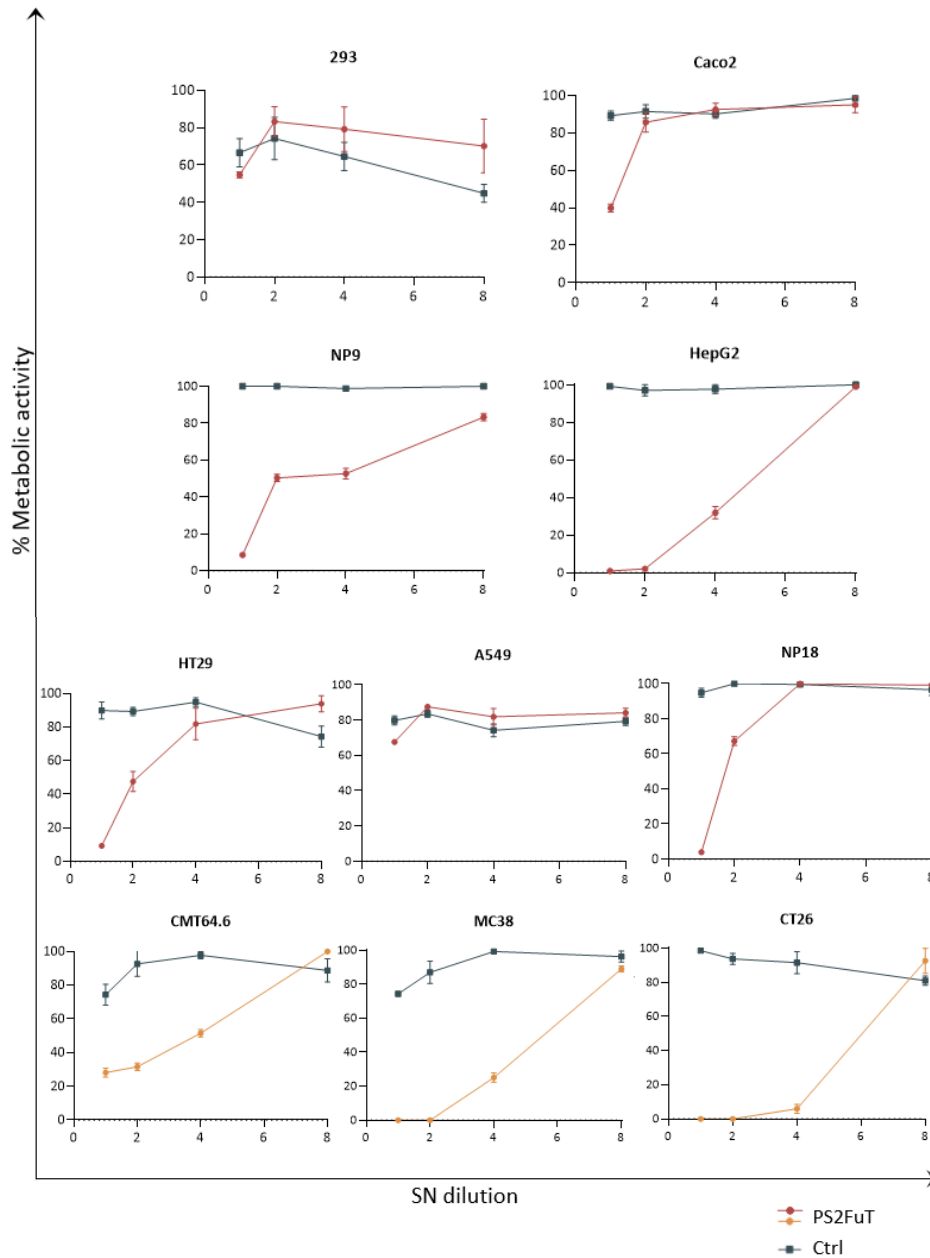


Figure 24. Cytotoxic activity of PS2FuT supernatants across tumor cell lines. MTS assays were performed by treating a panel of human (red) or murine (yellow) tumor cell lines with PS2FuT SNs for 24h at 37°C to quantify the percentage of cell viability (or metabolic activity). The mean of triplicates $\pm$ SEM is plotted. Ctrl, control.

being the most affected, showing only about 6% viability even at a 1:4 dilution. The

fact that PS2 resulted inactive against HEK293 and A549, presumably due to lack of putative unknown receptors, was one of the main reasons to exploit this toxin in the context of OAdS since these are the cell lines used for adenovirus transfection and production, respectively. Overall, PS2 exhibits potent and selective cytotoxic activity against various types of human and mouse cancer cells *in vitro*.

More importantly, when we moved to a therapeutic setting and treated thrice CMT64.6-bearing mice intratumorally with PS2FuT supernatant, an efficient tumor growth control was observed compared with the control (supernatants from empty plasmid-transfected cells) (**Figure 25**).

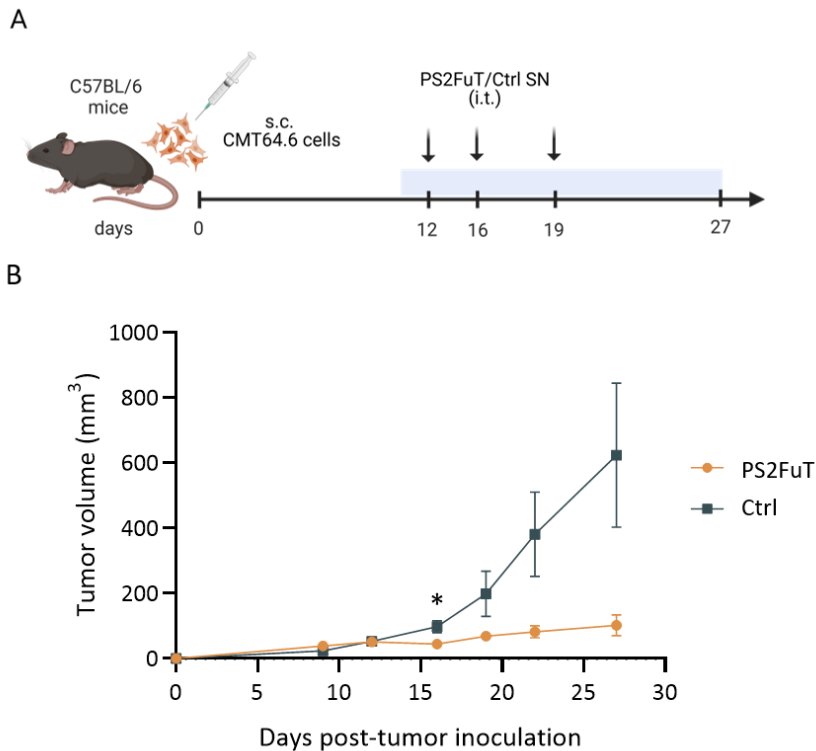


Figure 25. Intratumoral administration of PS2FuT supernatant controls tumor growth in a therapeutic setting. C57BL/6 mice with CMT64.6 established tumors ($n=4/\text{group}$) were treated intratumorally (i.t.) with 3 doses of concentrated PS2 or control supernatant, and tumor volumes were monitored over time (colored box). All data are presented as means $\pm$ SEM. * $p \leq 0.05$ Mann-Whitney test and Dunn's *post hoc* test at each timepoint. i.t., intratumoral; s.c., subcutaneous; Ctrl, control (empty plasmid supernatant). Image A created with BioRender.com.

3.2 PS2-induced immunogenic cell death

One of the primary objectives of this thesis was to use the PS2 as a transgene to arm our OAd, aiming to leverage its potent anticancer cytotoxicity to enhance the viral spread within tumors. Furthermore, if PS2 secreted from the OAd could also trigger immunogenic cell death, it could lead to the sustained release of DAMPs by a pool of uninfected cells exposed to the toxin. These danger signals would likely be identified by the immune system and explicitly associated with tumor antigens presented by these cells. Thus, this could open an opportunity to shift the immune response balance from viral to tumor epitopes.

Extracellular ATP can function as a DAMP and is a key biomarker for determining whether an agent induces immunogenic cell death or not. Thus, a bioluminescence-based assay was performed to measure the ATP release upon PS2 SN treatment of several cell lines (**Figure 26**). Accordingly, ATP was detected in the PS2-sensitive NP9, HepG2, and MC38 cells but not in the resistant A549 cell line.

It is well-known that tumor cells treated by ICD inducers emit DAMPs, including but not limited to calreticulin (CRT). CRT translocates from the ER to the cell membrane surface, where it functions as a novel uptake signal for APCs. Thus, we sought to evaluate whether CRT translocation to the cell surface could be measured using PS2-sensitive CT26 cells compared to PS2-resistant A549 cells. To detect the actively translocated CRT rather than the intracellular CRT, which passively diffused following pore formation, cells were incubated with PS2 supernatants and stained with the LIVE/DEAD™ viability dye and an α CRT antibody at very early time points. As anticipated, no CRT surface expression was observed by flow cytometry in A549 cells (**Figure 27-A**). In contrast, CRT translocation was detected in CT26 cells but only within the dead cell population starting at 2 hours post-treatment (**Figure 27-B**). Ideally, translocated CRT should have been observed in the live cell fraction. These findings suggested that PS2 pore formation might occur so rapidly that by the time cells were incubated with the α CRT antibody, they were already dead, allowing the antibody to penetrate through the pores or leading to the release of intracellular CRT. In either

case, the detected CRT likely originated intracellularly in dead cells following pore formation.

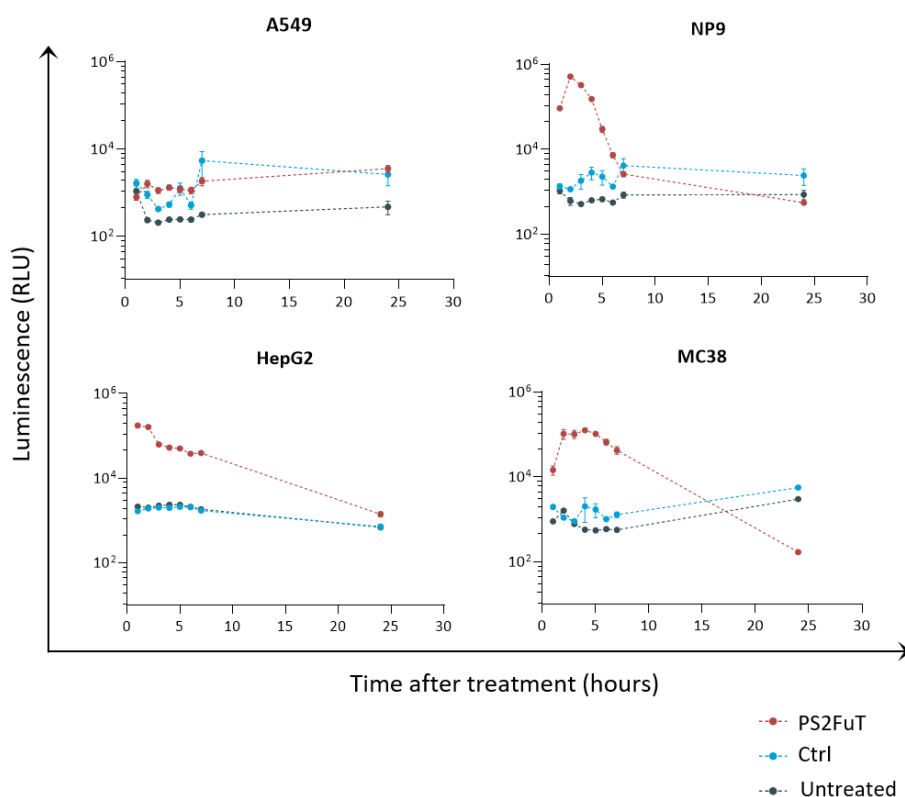


Figure 26. PS2FuT supernatant induces ATP release on PS2-sensitive cells. A panel of human and murine cell lines were treated with PS2 supernatant, the empty plasmid supernatant (Ctrl) or left untreated and the RealTime-Glo™ Extracellular ATP Assay Reagent (Promega) was added at that time. The resulting luminescent signal was read for 24h as an indirect measure of ATP release. Means $\pm$ SEM of bioluminescence in relative light units (RLU) are plotted.

Given that our proposed ICD inducer acts by forming pores, detecting calreticulin through this method proved challenging. What was evident, however, was that the toxin selectively induced cell death in CT26 cells, while A549 cells remained unaffected.

The gold standard approach to assess the ability of dying cells to trigger adaptive immunity involves vaccination assays in immunocompetent syngeneic mice²⁷⁰. Mouse

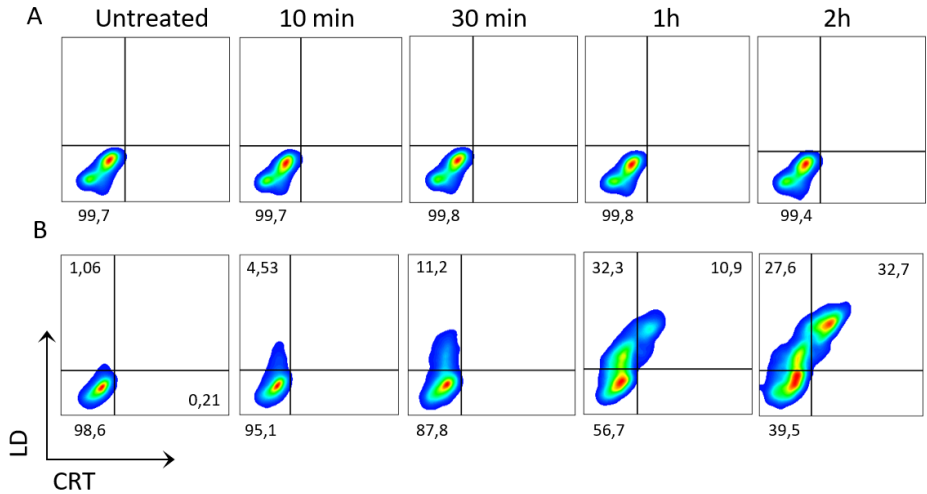


Figure 27. Calreticulin molecule detection in PS2-sensitive cells. A549 (A) or CT26 (B) cells were treated with PS2 supernatant, and a time-course of calreticulin (CRT) expression was established at early time points by flow cytometry. CRT was detected only in the PS2-sensitive cell type. FCM plots of viability versus CRT with the most relevant population frequencies are shown. LD, LIVE/DEAD™ (Thermo Fisher).

cancer cells were exposed to a potential ICD inducer *in vitro* and then administered as a vaccine subcutaneously. One week later, mice were challenged subcutaneously with living cancer cells of the same type and followed over several days for tumor incidence and growth. Not only the percentage of tumor-free mice but also the growth rate of tumors potentially developing despite a vaccine-induced adaptive immune response are commonly employed as indicators of (at least some) degree of immunogenicity.

In this case, MC38 cells were used in C57BL/6 mice to model this sort of experiment with PS2 (**Figure 28-A**). Results using this protocol are depicted in **Figure 28-B**, in which PS2-treated MC38 cells did not develop tumors (vaccinated side). Concerning the rechallenge side, mice vaccinated with PS2-incubated cells showed a delay in tumor growth compared to the control group, suggesting an adaptive antitumor immune response was induced.

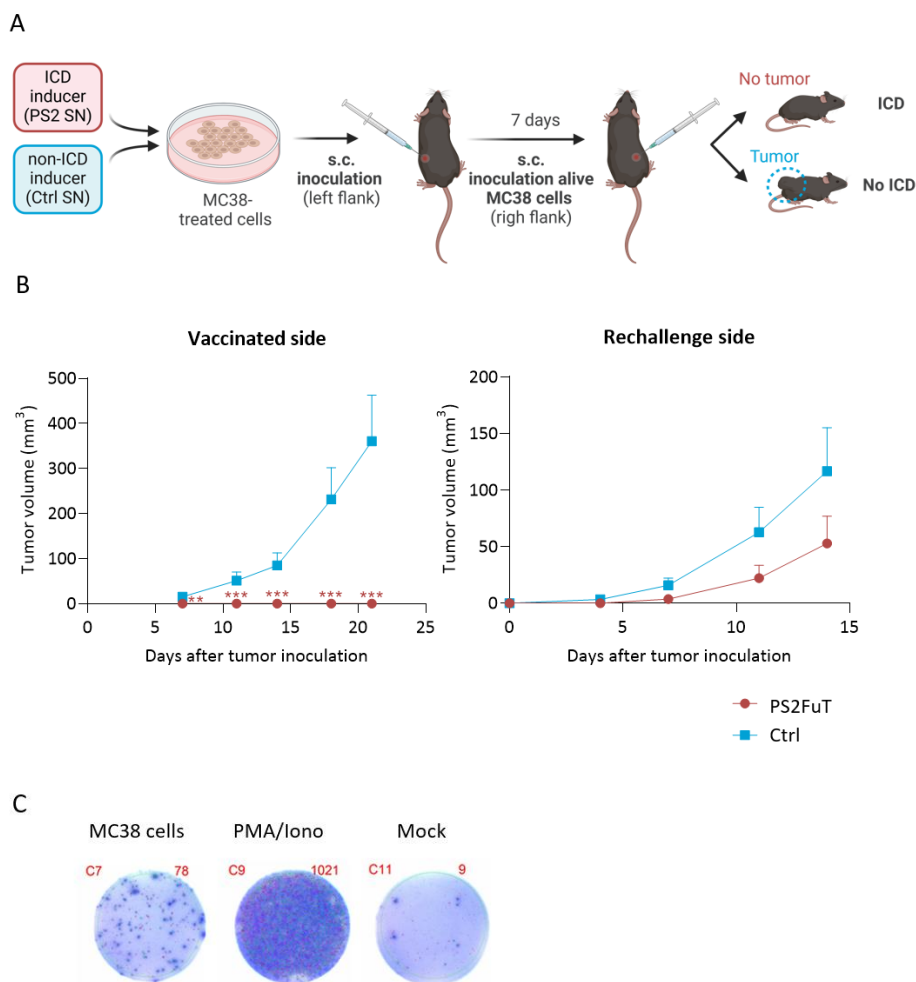


Figure 28. Prophylactic gold-standard model to assess ICD in vivo. **A)** Mouse cancer cells succumbing in vitro to a potential inducer of ICD are used as a vaccine. The ability of mice to reject (tumor incidence) or control (tumor growth) a rechallenge with living cancer cells of the same type inoculated 1 week later is considered a sign of protective anticancer immunity. **B)** Immunocompetent C57BL/6 mice were vaccinated subcutaneously with control- or PS2-treated MC38 cells ($n=7-8$ /group, respectively). Seven days later, a rechallenge with alive MC38 cells was made in the contralateral flank, and tumor volumes were followed up. **C)** ELISpot wells from rechallenge TDLN of a responder mouse. All data are presented as means $\pm$ SEM. Statistical significance was analyzed using Mann-Whitney and Dunn's post hoc tests at each timepoint (** $p \leq 0.01$; *** $p \leq 0.001$). Image A created with BioRender.com. s.c., subcutaneous; PMA/Iono, phorbol myristate acetate/ionomycin; ICD, immunogenic cell death.

Moreover, the antitumor efficacy monitoring was stopped once tumor growth tendencies were enlightened to perform an ELISpot assay using either vaccinated or rechallenge side TDLNs from these animals. A specific antitumor immune response against the MC38 cell line was detected in one out of eight PS2-treated animals (**Figure 28-C**).

Next, we performed a similar experiment by vaccinating animals twice with PS2-treated MC38 cells before applying the rechallenge with alive MC38 in the contralateral flank, pretending this prime-boost would increase the vaccine's immune protection. However, this time, we observed that most animals with the PS2FuT condition formed tumors, though they presented a significant tumor growth delay compared to the control group. Moreover, the differences in the rechallenge side were not enhanced regarding the standard vaccination-rechallenge setting (**Figure 29-A**). Even though, when establishing a threshold that discerned between stabilized tumors from the exponential-growing tumors in rechallenges (500mm^3), we found many more responders in the PS2FuT (75%, 6/8 mice) than in controls (42.8%, 3/7 mice) (**Figure 29-B**).

Since a considerable antitumor efficacy was triggered, we decided to complement this *in vivo* by immunophenotyping both vaccinated and rechallenge side tumor-cell suspensions. A panel of antibodies was applied to identify lymphoid effector cells and lymphoid exhaustion/late activation markers (see details in Table 19). Myeloid cells were also selected by discard.

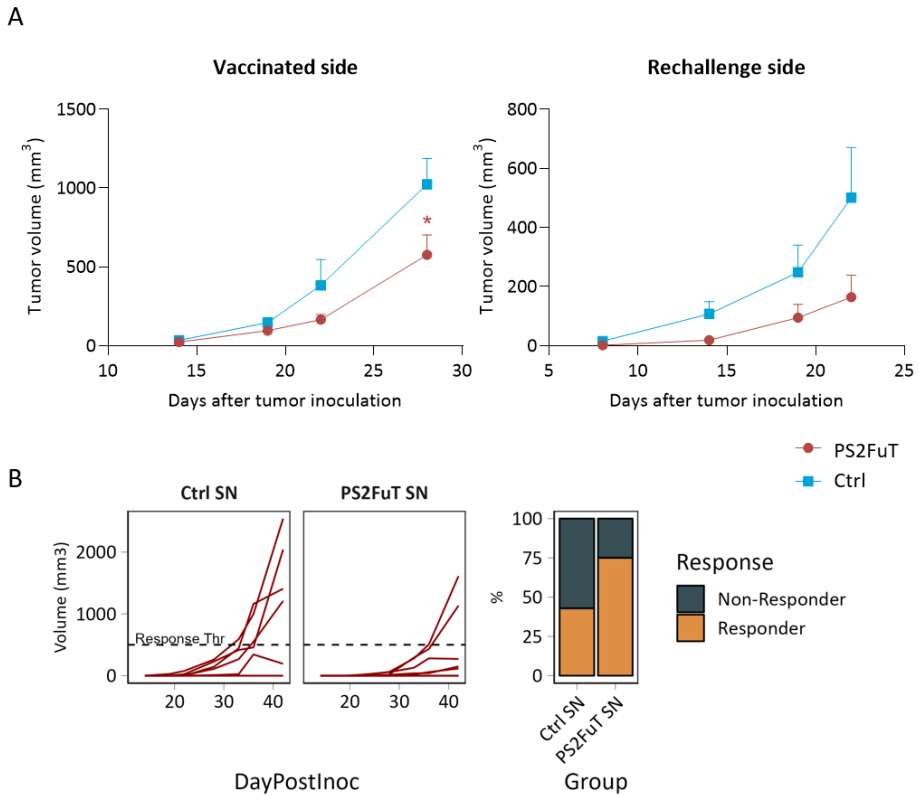


Figure 29. PS2FuT supernatant prime-boost confers similar antitumor protection than the normal *in vivo* vaccination-rechallenge setting. **A)** C57BL/6 mice were subcutaneously injected twice with MC38 cells treated with PS2 or control supernatants in the left flank in a lapse of 7 days. Then, they were rechallenged with MC38 alive cells in the contralateral flank and tumor growth on both sides. **B)** Proportion of responders and non-responders among PS2FuT (n=8) and Control (n=7) groups, considering as responders those individuals whose rechallenge tumors were beneath 500mm³. Data are presented as means $\pm$ SEM. *p $\leq$ 0.05 Mann-Whitney test and Dunn's *post hoc* test at each timepoint. DayPostInoc, days post-inoculation. Thr, threshold.

A broad analysis of immune cells revealed a noticeable, though not statistically significant, increase in T, NK, and B cells in tumors from the rechallenge group compared to their vaccinated counterparts. The only exception was observed in B cells from PS2-treated animals, where the high variability in the vaccinated tumor samples obscured any clear trend. Overall, it suggested that some immune activation might have occurred upon vaccination. If so, however, these putative immune responses were present in both control and PS2-treated groups, indicating that the effect was not attributed to the PS2-treated MC38 vaccine (**Figure 30**).

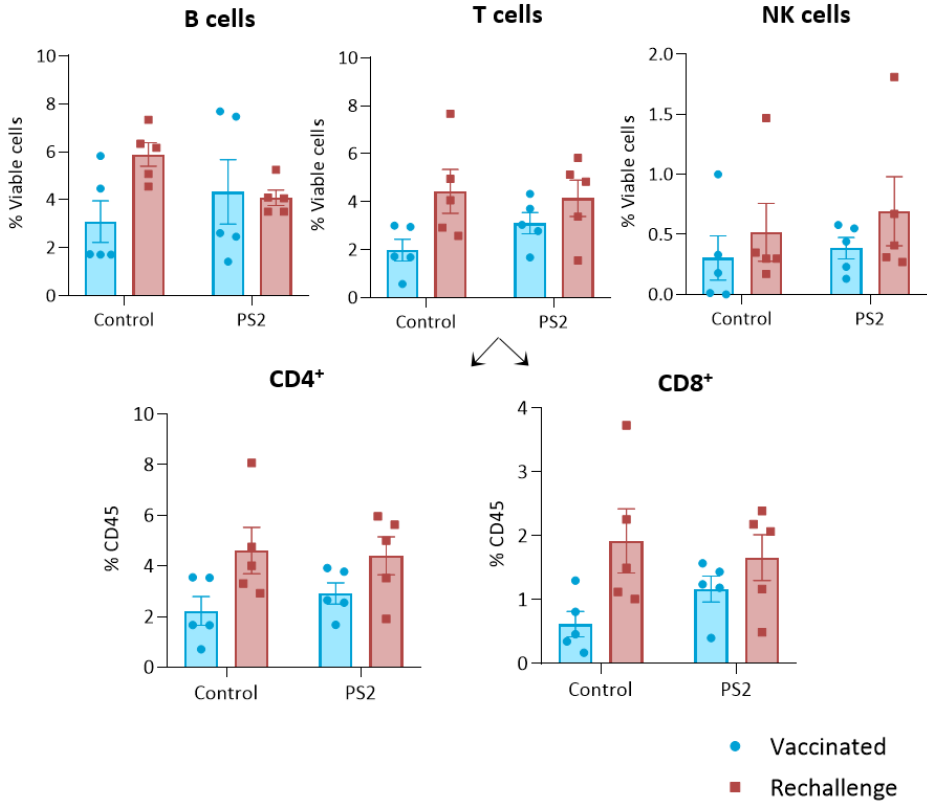


Figure 30. Proportion of effector cells from vaccinated and rechallenge side tumors. Tumors from the in vivo depicted in Figure 29 were processed and stained with a panel of antibodies for immunophenotyping characterization ($n=5/\text{group}$). Population frequencies on the indicated alive cell type are separated by treatment and tumor side. CD4⁺ and CD8⁺ cell populations were gated over CD45⁺CD3⁺ markers. Means $\pm$ SEM are plotted. Wilcoxon matched-pairs signed rank test was applied to assess the statistical significance between vaccinated vs. rechallenge tumors within a treatment on each immune cell population. In contrast, the Mann-Whitney (unpaired) test was used on each tumor side between treatments. In both cases, the *post hoc* test was Dunn's.

Nonetheless, upon reanalyzing these populations and distinguishing responders from non-responders based on a predefined tumor volume threshold of 500 mm³ (Figure 29-B), a significant increase in T and NK cells—the key antitumor cytotoxic effector cells— was observed in responder animals on the rechallenge side, regardless of the treatment applied. Interestingly, animal #13 from the control group exhibited a spontaneous response, behaving as a responder (Figure 31-A). These findings suggested, despite this outlier, a correlation between responsiveness and heightened

cytotoxic immune activity. Furthermore, PS2-treated cell vaccination notably promoted a greater number of responder animals, as depicted in **Figure 31-B**. It is important to note that some responder animals either did not develop tumors on the vaccinated and/or rechallenge side, or their tumors were too small to be analyzed, so they were not represented in these graphs.

In summary, the fact that in the presence of PS2 many more responders were observed (Figures 29-B and 31-B) and that T cell and NK cell frequencies were markedly higher in the non-treated tumors (rechallenges) of responder animals underscored the potential of PS2 to enhance both innate and adaptive immune responses. Furthermore, this also pointed to the fact that PS2-mediated ICD processes were actively occurring in MC38 cells.

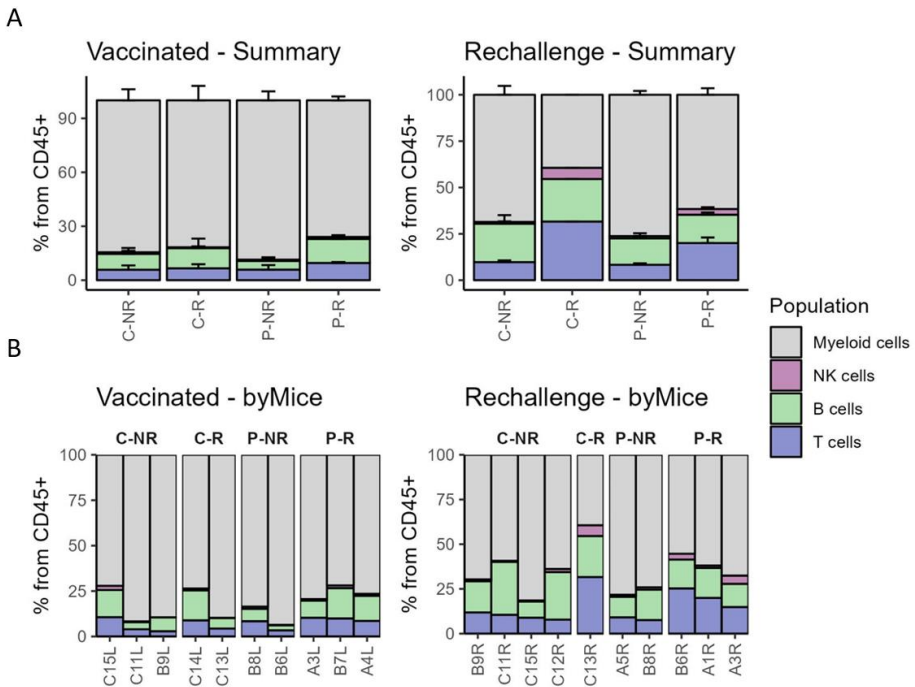


Figure 31. Summary of the immunophenotyping characterization by treatment and response. Stacked graphs showing effector cell frequencies among the CD45⁺ lymphocyte component integrating the group and response (**A**) or itemized by animal (**B**). Means $\pm$ SEM are plotted in A, and Mann-Whitney and Dunn's *post hoc* tests were applied for each immune cell subset between responders vs. non-responders, independently of the treatment administered. Significant p-values were obtained for T cells ($p=0.0095$) and NK cells ($p=0.0381$). C, control; NR, non-responder; P, PS2; R, responder.

Similarly, when examining T cell subsets in detail, both CD4⁺ and CD8⁺ T cells were generally more abundant in responder animals, either in vaccinated (**Figure 32-A**) or rechallenge (**Figure 32-B**) tumor sides. However, these findings should be interpreted cautiously rather than definitively, as dividing the data by treatment response resulted in some groups containing only one or two animals, making statistical analysis infeasible. Still, these results highlighted promising trends that warrant further investigation in larger animal cohorts to validate them.

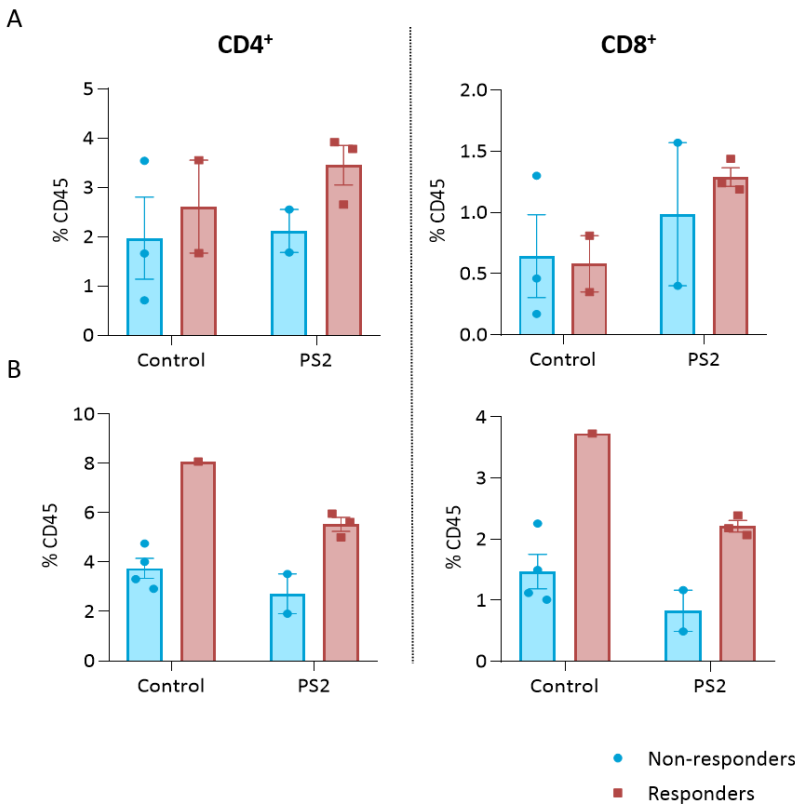


Figure 32. Estimated frequencies of CD4⁺ and CD8⁺ T cells based on treatment response. Deconvolution of the T cell population to distinguish the CD4⁺ and CD8⁺ subsets in vaccinated side (**A**) and rechallenge side (**B**) tumors. Means $\pm$ SEM are plotted over CD45⁺ cells.

Furthermore, the staining of CD8⁺ T cells showed that responder individuals exhibited a notably higher number of PD1⁺TIGIT⁺ exhausted cells, suggesting a prior adaptive

immune activation probably played a role in driving the favorable responsiveness. In contrast, differences in the expression of the 4-1BB activation marker were less pronounced (**Figure 33**, left and middle panels). This pattern of immune activation/late exhaustion aligns with the presence of CD4⁺ Tregs in responder animals (**Figure 33**, right panel), indicating that while an immune response had been triggered, it might have been suppressed or exhausted by the TME at the time of analysis.

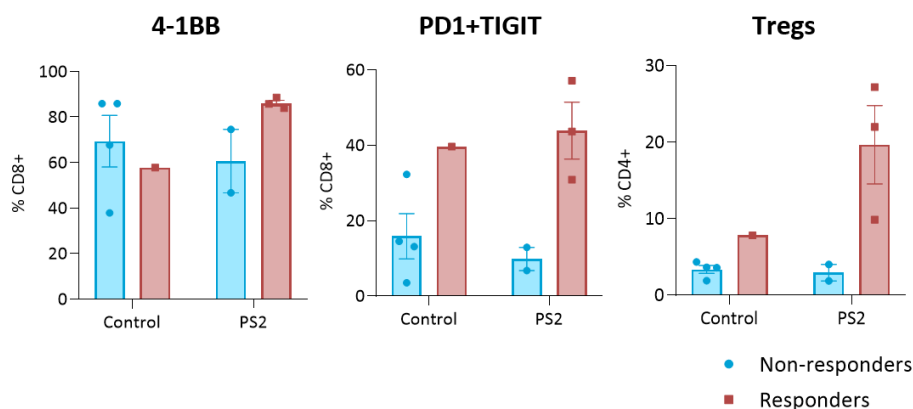
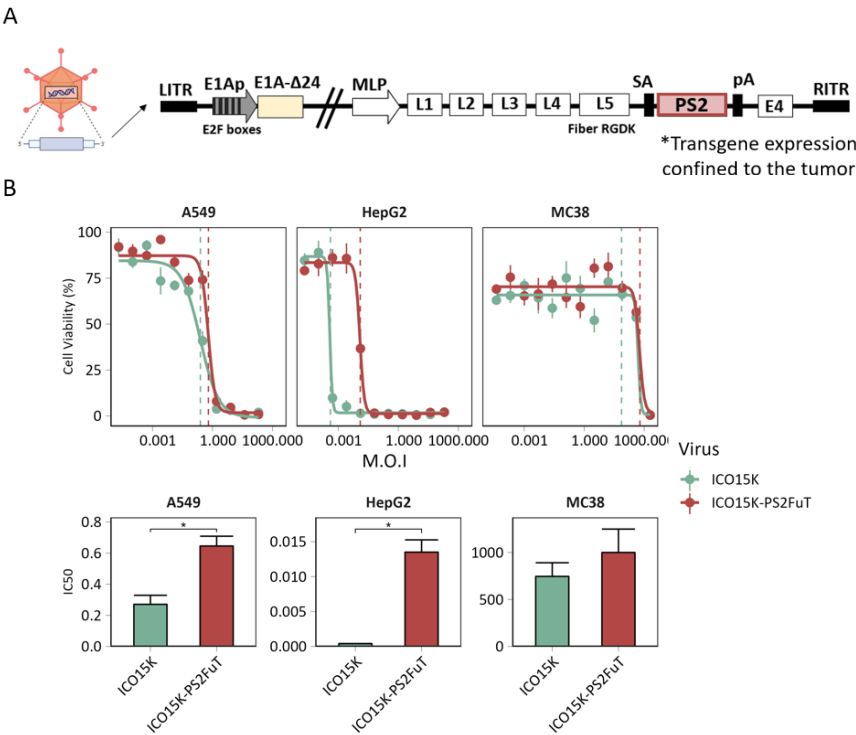


Figure 33. Activation and exhaustion markers analyzed on T cells from rechallenge side tumors based on responsiveness. Frequencies of CD8⁺ T cells expressing the activation markers 4-1BB and late exhaustion markers PD1 and TIGIT (left and middle panels). Percentages of CD4⁺ Tregs were also assessed (right panel). Means $\pm$ SEM are plotted.

3.3 *In vitro* cytotoxicity and production yields of OAd expressing PS2

Having validated the antitumoral and ICD-inducing capabilities of PS2, we introduced the *PS2FuT* construct within the ICO15K backbone by homologous recombination. The transgene was strategically placed downstream of the fiber gene using a splicing acceptor (**Figure 34-A**) to ensure its expression is tightly regulated by viral replication. Therefore, this design guarantees that the toxin's activity is confined to the tumor. The oncolytic properties of the modified virus were assessed by dose-response cytotoxicity assays in human A549 and HepG2 cell lines and the murine MC38 cell lines after 5 days of infection. A significant loss of cytotoxicity of the PS2-virus was observed in human cells compared to the parental virus (**Figure 34-B**), which was reflected by a higher IC₅₀. ICO15K-PS2FuT had an IC₅₀ of 2.5 and 31 times higher in A549 and HepG2 cells,

respectively. Moreover, the differences between cell lines were already notable in the parental virus, with the IC₅₀ being 617 times higher in HepG2. Altogether, it was confirmed that HepG2 cells are less permissive to adenovirus replication or oncolytic effect than A549, and when the PS2 transgene was added, this difference was much more evident. However, we anticipated that this limitation could be offset by the high sensitivity of HepG2 cells to the toxin. In an *in vivo* context, both the virus and PS2 cytotoxicities would likely work synergistically to kill HepG2 cells (detailed in Figure 38). As expected, none of the OAdS efficiently replicated in MC38 cells; though some murine cell lines showed some semipermissivity to OAdS (e.g., CMT64.6 cells), human adenoviruses cannot replicate in murine cells.



Virus	IC ₅₀	Cell line
ICO15K	2.48×10^{-1}	A549
	4.02×10^{-4}	HepG2
	7.40×10^1	MC38
ICO15K-PS2FuT	6.23×10^{-1}	A549
	1.25×10^{-2}	HepG2
	6.15×10^2	MC38

Figure 34. Cytotoxicity profile of ICO15K-PS2FuT virus. **A)** Schematic representation of the oncolytic adenovirus used in this project. **B)** A549, HepG2 and MC38 cell lines were infected with serial dilutions, ranging from 200 (A549 and HepG2) or 2000 (MC38) to 0 MOIs, with ICO15K and ICO15K-PS2FuT viruses. After 5 days post-infection, cell viability was determined by BCA staining. Means $\pm$ SEM of quadruplicates are plotted. Mann-Whitney and Dunn's *post hoc* tests were applied for the IC₅₀ statistics. * $p \leq 0.05$. SA, splicing acceptor; pA, polyadenylation signal, MOI, multiplicity of infection; BCA, bicinchoninic acid assay. IC₅₀, half-maximal inhibitory concentration.

Once the oncolytic properties were confirmed, we compared the viral production of our ICO15K-PS2FuT with the parental ICO15K within the virus-producing A549 cell line. We infected A549 cells at MOI 20 and monitored the amount of transfecting units per cell (TU/cell) produced in cell extracts (CE) (**Figure 35**, solid lines) and released in supernatants (SN) (**Figure 35**, dotted lines). No statistically significant differences in total virus production in CEs were observed after infection despite the tendencies of higher PS2-virus production compared to ICO15K. At the same time, the functional viral particles released to the supernatant were analyzed, and the PS2-virus also showed a similar number of infection units released as ICO15K, which was slightly higher in the last timepoint (72h). Overall, these results indicate that, despite the observed loss of cytotoxicity in the armed OAd, the transgene insertion did not compromise the viral fitness in terms of virus production yields in A549 cells.

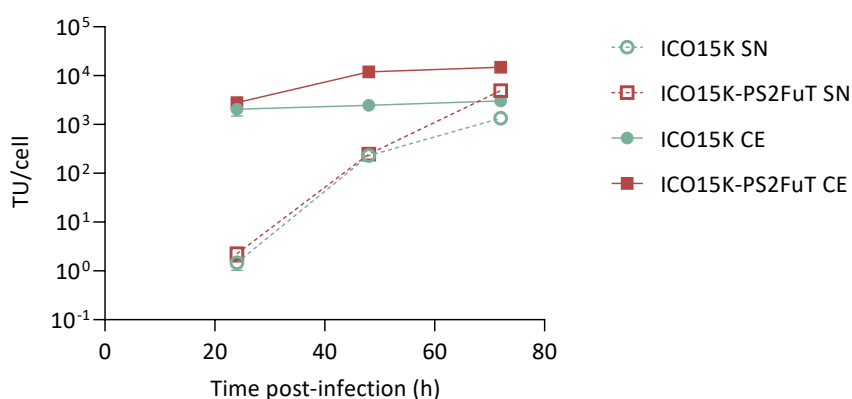


Figure 35. Kinetics and efficiency of transgene expression by the PS2-armed OAd compared with ICO15K parental virus. A549 cell line was infected with ICO15K or ICO15K-PS2FuT viruses. Cell extracts (CE) and supernatants (SN) were harvested and titrated at indicated timepoints using an anti-hexon staining-based method. Mean values $\pm$ SEM are plotted ($n=3$). TU, transducing units. Mann-Whitney test and Dunn's *post hoc* test were applied to these data.

3.4 PS2 is expressed and secreted from ICO15K-PS2 OAd, enhancing the killing in virus-resistant cells

After evaluating both the cytotoxic effects and virus production capabilities of the OAd candidate, we assessed the PS2 activity within the oncolytic virus. Since no Tag sequence was appended to the transgene—fearing potential alterations in protein folding or interference with furin-cleavage site exposure necessary for toxin activation—we opted for an indirect detection strategy, taking advantage of the replicative-deficient condition of human OAd in murine cell lines.

A time-course cell death assay was conducted using the xCELLigence system, which employs impedance-based technology to continuously monitor the killing of adherent cells. Impedance increases when cells attach and proliferate but drops when they detach due to cytotoxic effects, such as those induced by PS2. MC38 cells, previously shown to be PS2-sensitive yet resistant to OAd infection, were used as targets. Upon treatment with supernatants from the PS2-armed virus, the killing kinetics mirrored the one induced by PS2FuT SN derived from plasmid expression. Conversely, treatment with ICO15K SN neither caused significant killing nor supported cell growth, likely because the virus-containing medium was unsuitable for proliferation, although cells were not permissive to infection. Therefore, the observed differences in cytotoxicity between the two OAd could be exclusively attributed to the addition of PS2-mediated effects (**Figure 36-A**).

By contrast, when we moved to a human cell line such as HepG2, which is susceptible to OAd infection, the killing differences were no longer evident since the virus oncolysis was so potent that it masked the detection of PS2-mediated cytotoxicity (**Figure 36-B**).

Therefore, this experiment suggested that the toxin was effectively expressed and secreted by the virus, remained active, and had the potential to kill sensitive tumor cells.

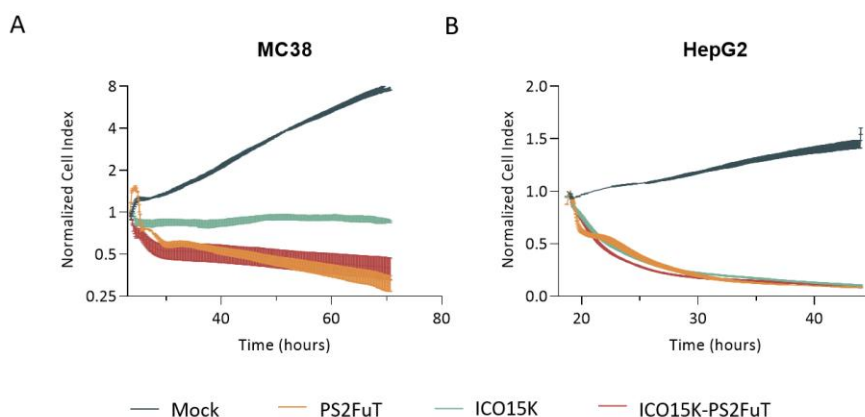
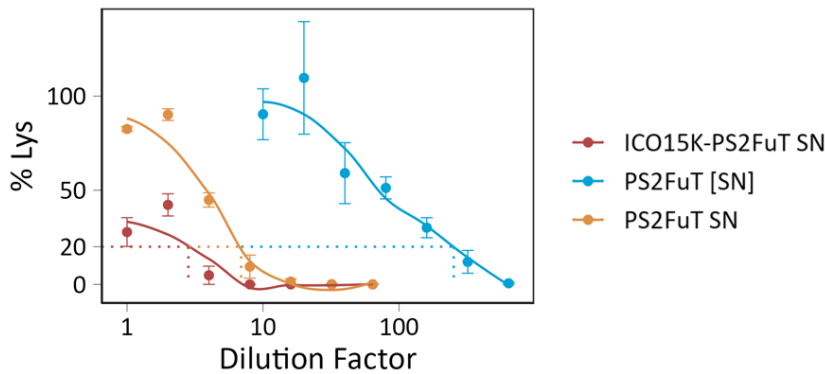


Figure 36. PS2FuT toxin is expressed and secreted from OAd-infected cells, improving the cytotoxic potential of ICO15K in virus-resistant cells. MC38 and HepG2 cells were seeded and treated with concentrated ICO15K and ICO15K-PS2FuT viruses' supernatants (green and red curves, respectively), or with supernatant from a PS2-encoding plasmid (yellow curve). The impedance at well bottoms was measured every 3 minutes for 50-70h and normalized to baseline impedance values with medium only (mock). The mean $\pm$ SEM of triplicates is shown.

3.5 Implementation of a CFSE-based method to titrate PS2 from OAd supernatants

Since all PS2 characterization experiments relied on toxin-containing supernatants, establishing a method to quantify toxin titers within each supernatant batch became essential to ensure consistency and facilitate the normalization of experimental conditions. To address this, an indirect titration approach was tested using CFSE release from PS2-affected cells. Jurkat cells, selected for their high sensitivity to PS2 and resistance to the virus, were ideal for this method as they are suspension cells, which simplified the protocol. These cells were stained with CFSE and treated with serial dilutions of toxin supernatants (either produced from expression plasmid or PS2-OAd-transfected HEK293 cells). Then, CFSE fluorescence was measured after 1 hour, where higher PS2 concentrations were assumed to correspond to increased pore formation in cells, causing greater CFSE release and higher fluorescence intensity.

With this premise in mind, this method allowed us to estimate killing curves produced by different supernatants and the calculation of lytic units per milliliter (LU/mL) based on a set percentage of cell lysis. For instance, the 20x concentrated PS2 SN has to be diluted 37 times more than the non-concentrated SN to kill 20% of cells; otherwise stated, the LU/mL of the concentrated PS2 SN was 37 times higher. On the other hand, PS2 levels in supernatants from OAd-infected cells were 2.4 times lower compared to non-concentrated plasmid-derived PS2 supernatants (**Figure 37**).



Condition	Dilution (for 20%)	Titer (LU/mL)
PS2FuT SN	6.9	1.8×10^5
PS2FuT [SN]	252.7	6.7×10^6
ICO15K-PS2FuT	2.82	7.5×10^4

Figure 37. PS2FuT secretion from expression vectors is more efficient than from OAdS. Jurkat cells were stained with CFSE and treated with several dilutions with concentrated or not concentrated supernatants from PS2FuT-encoding plasmid-transfected HEK293 cells, or the supernatant of ICO15K-PS2FuT-infected A549 cells. CFSE release fluorescence was measured upon PS2-mediated cell disruption. PS2 supernatants can be indirectly titrated with this approach as LU/mL. Data are plotted as means $\pm$ SEM of triplicates. Lys, lysis; LU, lytic units.

Taken together, these findings suggested that PS2 production was more efficient using expression vectors than from an OAd, a factor to be considered for further antitumor efficacy *in vivo* studies.

3.6 ICO15K-Fi-PS2FuT-mediated oncolysis enhances antitumor efficacy *in vivo*

Next, we assessed whether PS2 secretion from the OAd observed *in vitro* could improve the antitumor efficacy of the HepG2 tumor model. It has previously been shown and demonstrated in our hands that these cells were very susceptible to PS2 (see Table 2 and Figures 23-24) and had an acceptable OAd-mediated cytotoxicity profile (Figure 34). First, HepG2 tumor-bearing NSG mice were randomized into treatment groups and treated with a single intravenous administration of PBS, ICO15K, and ICO15K-PS2FuT (4×10^{10} vp/mouse) when the tumor volume reached a mean of over 70 mm^3 . Tumor growth curves revealed an enhanced antitumor efficacy of the PS2-armed OAd, being significant at earlier timepoints regarding PBS compared to the parental virus ICO15K (**Figure 38-A**). Although the tendency of a more delayed tumor growth remained until the end of the efficacy experiment (day 40 post-virus administration), no significant differences but a trend in a slightly improved survival of mice were observed in ICO15K-PS2FuT compared to ICO15K. (**Figure 38-B**). Despite this, it should be noted that the insertion of the PS2 transgene did notably increase the oncolytic cytotoxic potency of the OAd over the parental virus, even though having the ICO15K-PS2FuT had a 31-fold loss of cytotoxicity compared to ICO15K (Figure 34).

Body weight monitoring was performed to assess the toxicity induced by the viruses. As depicted in **Figure 39**, mice treated with both OAds showed a rapid and significant decrease in body weight during the initial days following virus administration, being more severe in the ICO15K parental virus. Unexpectedly, PBS-treated mice also experienced a gradual weight decline, possibly due to stress from handling or other external factors influencing them while the experiment was conducted.

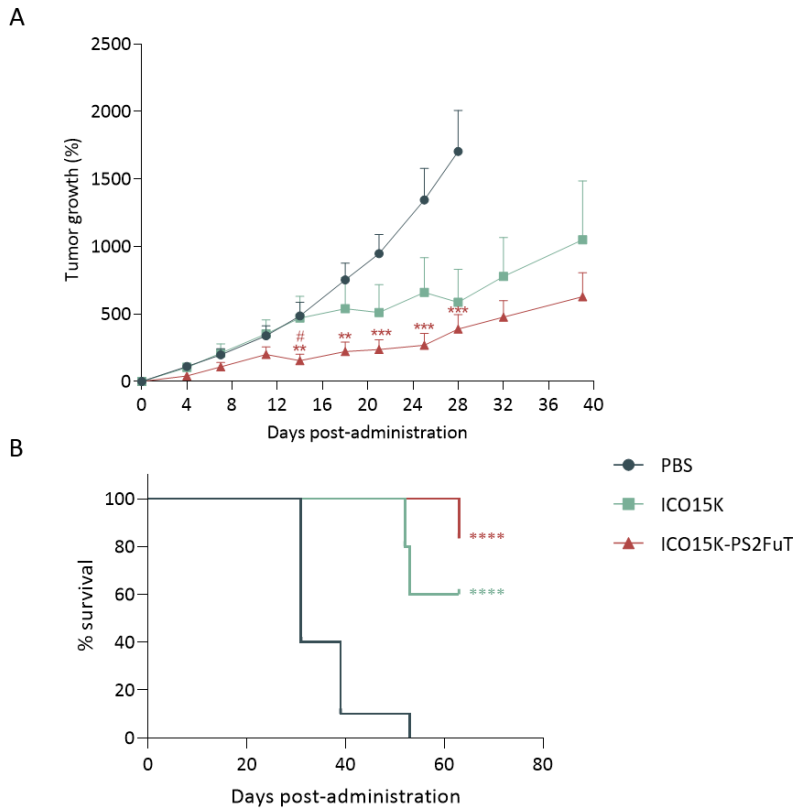


Figure 38. Antitumor efficacy of ICO15K-PS2FuT OAd in an immunodeficient mouse model. A) HepG2-bearing NSG mice were treated intravenously with 4×10^{10} viral particles (vp) of ICO15K-PS2 ($n=10$), the parental virus ICO15K ($n=9$), or with PBS ($n=10$) once tumors reached $\sim 70 \text{ mm}^3$. Tumor measures and animal weight were determined twice a week. The percentage of tumor growth was plotted. All data are presented as means $\pm$ SEM. Statistical significance was analyzed using the Kruskal-Wallis test and Dunn's post hoc test for each time point (*/ $\#p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). *vs PBS; #vs ICO15K. **B)** Kaplan-Meier survival curves of the experiment, applying the log rank test for statistical significance. **** $p \leq 0.0001$ vs PBS.

The maximum weight loss among OAd-treated groups occurred at day 7, with body weight variation exceeding 15%. This later toxicity (mainly hepatotoxicity) was reflected in the survival rates, which were comparable between the two OAds: 70% survival (7/10) for the ICO15K-PS2FuT-treated group versus 77.7% survival (7/9) for the ICO15K parental virus group. The remaining animals from both OAd treatments recovered in the following days until they reached weights comparable to those of the

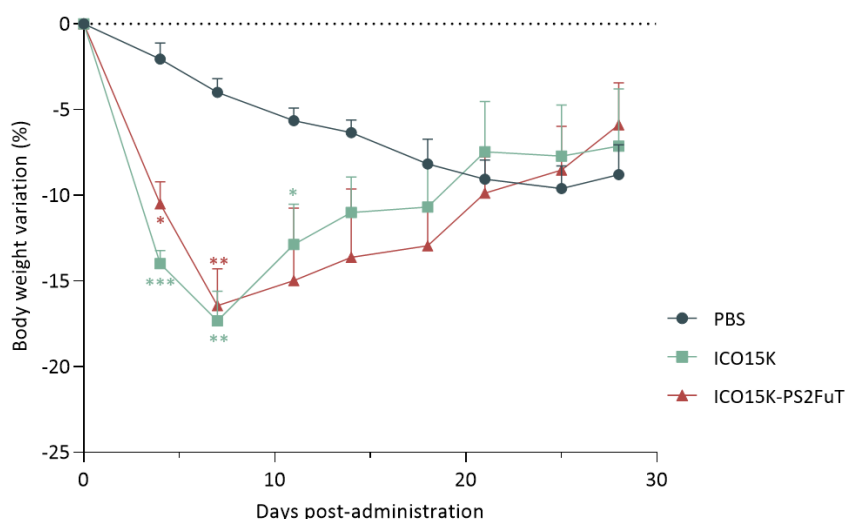


Figure 39. Body weight variation in the HepG2 antitumoral efficacy assay. The body weight of animals was monitored closely after virus administration (from day 5) to detect early late toxicities. Mean $\pm$ SEM by Krukall-Wallis and Dunn's post hoc tests were applied at each timepoint. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ vs. PBS.

PBS group. By the end of the experiment, weight variations across all groups stabilized to under 5%, showing a trend toward further reduction.

The key conclusion from these results is that the insertion of the PS2 transgene did not exacerbate a certain anticipated degree of OAd-associated hepatotoxicity compared to the ICO15K virus. This is particularly significant given that the ICO15K backbone has been used to develop VCN-01¹⁷⁸, a derivative OAd currently undergoing clinical trials. These findings support the safety profile of the PS2-armed virus and its potential for clinical application without introducing additional toxicity concerns.

3.7 OAd5 detection in HepG2 tumors

Immunohistochemical (IHC) stainings of paraffin sections from HepG2 tumors were performed for Ad5 E1a protein and hematoxylin as a contrast to analyze the localization of ICO15K and ICO15K-PS2FuT viruses. As **Figure 40** shows, the virus could only be detected in those tumors from animals that received the intravenous injection of either OAd. The distribution of anti-E1a staining (dark brown) across the tumor was

similar in both groups, with areas in which clustered cells showed an intense positive signal. Positive cells were close to necrotic areas (marked as N), suggesting an adenovirus-mediated progressive tumor oncolysis inside the tumor. Note that these images perfectly illustrate the stromal barriers encapsulating tumor sections (dashed lines or marked as S), impeding the OAd from distributing homogeneously. In 200x images, a group of elongated cells arranged in thin rows disposed more in the longitudinal than the transversal plane can be identified. This is the typical appearance of fibroblasts that, in most cases, were confining virus-positive cells in small areas.

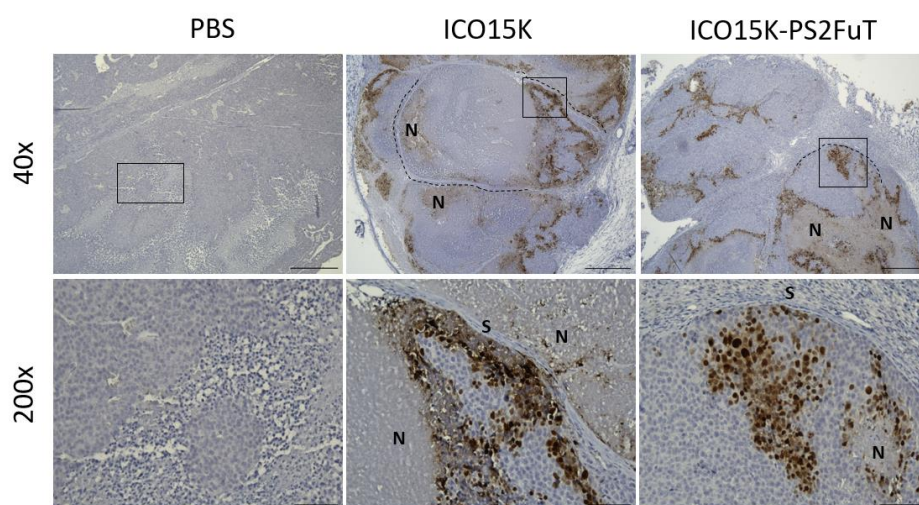
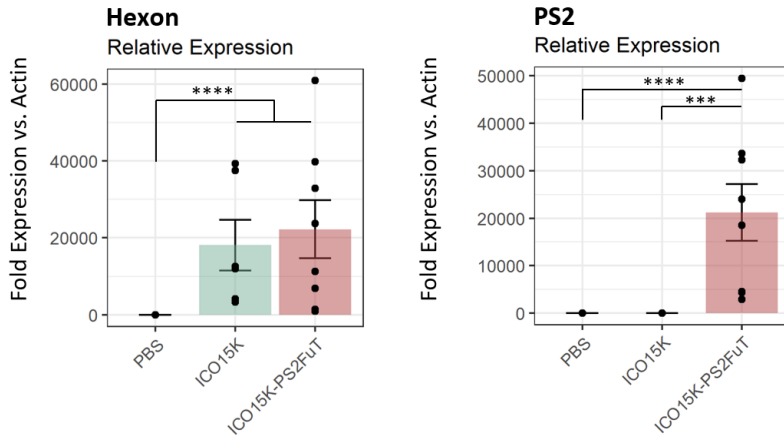


Figure 40. Immunohistochemical staining of E1a of HepG2 tumors. Representative immunohistochemical hematoxylin stains of HepG2 tumors from the antitumor efficacy in vivo (Fig. 38) were performed to assess the presence of the virus by staining the early adenoviral protein E1a (marked in dark brown). Necrotic zones were unspecifically stained, so they should not be considered positive in the IHC evaluation (marked with an N in the images). Stromal barriers encapsulating tumors were indicated as S or dashed lines. Pictures were captured by an inverted microscope equipped with a digital imaging system (Leica Mateo; Leica Microsystems, Germany). Scale bar: 500µm (upper panel) and 100µm (lower panel).

As a reinforcing quantitative study of the presence of OAd particles within the tumors, qPCRs spanning the adenoviral *hexon* and *PS2* genes were carried out in cDNA samples obtained from tumor lysates. No significant differences in hexon gene relative-to-actin expression levels were detected between ICO15K and ICO15K-PS2FuT viruses despite the latter being less cytotoxic for HepG2 cells (Figure 34). According to what was

observed in IHCs, PBS-treated tumors showed negligible amounts of Ad particles (Figure 41-A, left panel). Regarding the PS2 gene, its expression could only be detected in ICO15K-PS2FuT-treated tumors, as expected (Figure 41-A, right panel).

A



B

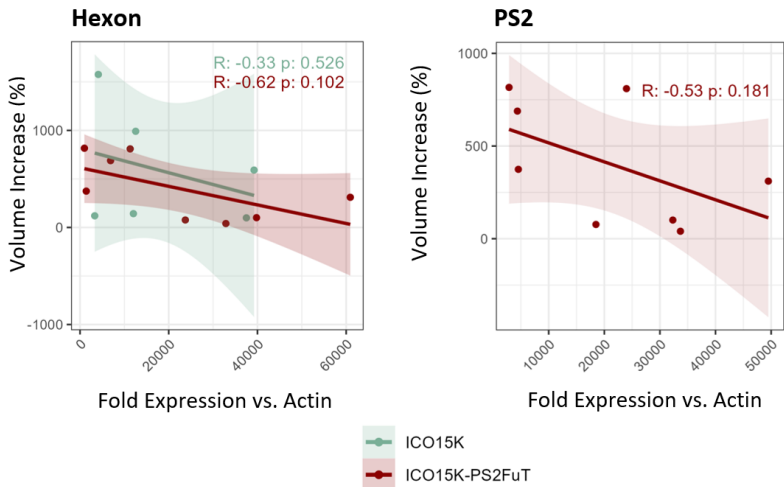


Figure 41. OAd and transgene detection by qPCR in HepG2 tumors. A) cDNA from tumor lysates of the antitumoral efficacy study (Fig. 38) was obtained and analyzed in duplicates for OAd and PS2 expression rate by a SYBR Green approach with primers spanning the hexon and PS2 genes within the adenoviral genome in 384 well-plates. Murine actin expression rate was used as a housekeeping reference to calculate the final relative expression of Hexon and PS2. Negative control with water and tumors from the PBS group did not amplify at all. Positive controls of OAd-infected cells were also included and amplified accordingly. Data are presented as means $\pm$ SEM. *** $p \leq 0.001$; **** $p \leq 0.0001$ by Kruskal-Wallis test and Dunn's *post hoc* test **B)** Pearson correlations between volume increase and actin-relative expression of hexon and PS2 genes, with their associated Pearson coefficient R and p-values.

Pearson correlations between volume increase from the antitumor efficacy experiment (Figure 38) and the fold expression (relative to actin) for each gene were also analyzed. Though not statistically significant, a negative correlation trend was observed between these two variables, meaning they might be inversely proportional (**Figure 41-B**). Nonetheless, further experiments with more samples/groups should be performed to confirm these assumptions.

In summary, this second project explored the integration of PS2 as an anticancer agent in oncolytic OAd therapy to enhance tumor cell killing and potentially trigger ICD. PS2 gene from *B. thuringiensis* TK-E6 strain, selected for its potent cytotoxicity, was engineered to be incorporated into the ICO15K OAd backbone. In vitro experiments showed selective cytotoxicity against PS2-sensitive cancer cells, such as HepG2 and MC38, among others, while resistant lines like A549 remained unaffected. In vivo, PS2-treated MC38 cells acted as a tumor vaccine, delaying tumor growth upon rechallenge and increasing cytotoxic T and NK cell populations in responder animals. The PS2-armed OAd demonstrated enhanced antitumor efficacy compared to the parental virus in a HepG2-bearing immunodeficient model without increasing hepatotoxicity. Immunohistochemical and qPCR analyses confirmed viral replication and PS2 expression in those tumors. These findings underscore PS2's therapeutic potential as an ICD-inducing agent and open the possibility to study the role of PS2-armed OAd in immunocompetent models for synergistic tumor clearance.

DISCUSSION

Cancer cells generate a complex immunosuppressive TME in which the immune system is silenced to avoid being detected and eliminated. Since the TME plays a key role in reprogramming tumors and affects the antitumor efficacy of checkpoint blockade²⁷¹, new strategies combining TME inhibitors and ICIs are being pursued²⁷². Over the last decade, growing evidence has indicated that inducing cancer cell death to stimulate the immune system is a promising approach in cancer immunotherapy²⁷⁰. In this context, the appeal of OV's relies on their ability to induce an ICD while taking benefit from their inherent tumor tropism. Another advantage of OV's compared to small-drug ICD inducers is that their mediated ICD is amplified by the process of viral replication and spread, simultaneously causing cell lysis that releases tumor antigens. Adenovirus is the most frequently used virus in virotherapy and the first to be approved for clinical use. Several features make Ads suitable as OV's, such as high transgene insertion capacity, efficient quiescent and dividing cell transduction, non-integrative episomal genome, and low pathogenicity. Moreover, OAd's cause a multimodal cell death process involving necroptosis, pyroptosis, and autophagy²⁷³.

1. Circumventing the viral epitope immunodominance

Despite the host's immune system rapidly clears the virus, preclinical evidence shows the immune-mediated response is a central mechanism of OAd's therapeutic activity independently of viral replication^{274,275}; paradoxically, the antiviral signaling can prime the immune system for a collateral antitumor response²⁷⁶. Such was the case that OAd's are currently designed as immune adjuvants encoding for immunostimulatory molecules and combined with different immunotherapies²⁷⁷.

A major problem when dealing with oncolytic viruses is that they "dominate" the immune response, causing the immune system to primarily focus on foreign viral antigens, with the less immunogenic tumor antigens –derived from normal proteins that are overexpressed or mutated– passing unnoticed. Adenoviral immunodominance could bias the immune response towards the virus instead of the tumor²⁷⁸. Thus, addressing this imbalance between antiviral and antitumor immunity is crucial for improving OAd efficacy.

Several studies combined OAds with chemotherapeutic agents such as cyclophosphamide –which at low doses is shown to have immunomodulatory properties– to prolong their persistence²⁷⁹. However, such a strategy indiscriminately suppresses T cells targeting tumor and viral antigens. Others generated OAds encoding for multiple murine neoepitopes to boost antitumor immunity since tumor antigens also come from the OAd itself²³². The problem with this approach is that it requires prior knowledge of tumor antigens for each patient or cancer type. Coating the viral capsid with MHC-I epitopes or patient-derived tumor epitopes enhanced tumor-specific immune responses and efficacy against melanoma. Still, this design allows one-round immunization because the viral progeny will not be coated. Previously in our group, we used the OAd ICOVIR15 to express tumor epitopes fused to the viral protein E3-19K, facilitating their TAP-independent presentation and increasing their immunogenicity *in vivo*²³⁴. However, this method does not allow large transgene insertions.

With these premises in mind, we envisioned using tolDCs to induce an OAd-specific tolerization in a way that preserves and potentially enhances the antitumor immune response. DCs are highly specialized immune cells with a natural capacity to finely distinguish between self- and nonself antigens and orchestrate Ag-specific immune responses. This is due to their unique functional ability to induce adaptive immune responses (it is the only APC able to activate naïve T cells) or promote immune tolerance, depending on the cytokine and growth factor surrounding signals they receive from their environment. This plasticity is essential for maintaining immune homeostasis and defending against pathogens while preventing inappropriate immune reactions. Precisely for this reason, *ex vivo*-generated tolDCs have demonstrated favorable outcomes in restoring therapeutic Ag-specific tolerance in patients suffering from autoimmune diseases²⁵³ and have also been tested in organ transplantation²⁸⁰. Thus, unlike traditional immunosuppressants, DCs can be directed to generate specific tolerance towards the antigens of interest while reducing off-target immune suppression and preserving the overall immune function.

Notably, the work by Kushwah et al. demonstrated that tolDCs pulsed with adenoviral vectors effectively suppressed Ad5-specific T cell responses, enabling vector readministration and sustained transgene expression²⁴³. These findings encouraged us to explore a similar strategy in OAd. However, it must be noted that our objective was far more challenging. Viral-vectored gene therapy primarily aims to suppress antiviral immune responses, optimize transgene delivery, and allow repeated vector administration. In this kind of therapy, antiviral responses are often considered obstacles to be suppressed or bypassed. In contrast, OAd-based therapies face the task of simultaneously addressing antiviral and antitumoral immune responses. While it is critical to modulate antiviral immune responses to prevent premature viral clearance and support tumor lysis, these responses cannot be entirely eliminated. A controlled antiviral response is imperative to unleash the ICD processes needed to overcome the immunosuppressive TME and promote the subsequent tumor antigen recognition. This balance underscores the dual role of antiviral responses in OAd therapy as both a potential hurdle and a critical component for inducing robust antitumoral immunity. Successfully navigating this delicate duality, by selectively tolerizing toward viral antigens, is essential for maximizing the therapeutic potential of OAd-based strategies.

For the above-discussed purposes, we generated our tolDCs inspired by Kushwah's and other protocols²⁵¹ and previously compared the effects of supplementing BMDC cultures with either vitD3 or the cytokine IL-10. As a result, adding IL-10 yielded better results regarding tolDC generation and reduced allostimulatory capacity, in line with Boks et al. reported²⁸¹. They performed a comparative analysis of five *ex vivo* protocols for generating human tolDCs—utilizing VitD3, dexamethasone, TGF- β , rapamycin, or IL-10— and evaluated their suitability for clinical applications. Key criteria included migratory capacity, ability to induce Tregs, and the stability of the tolerogenic phenotype under inflammatory conditions, ensuring therapeutic safety. Among the protocols, IL-10-based tolDC generation emerged as the most effective, demonstrating superior stability of the tolerogenic phenotype and stronger Treg suppressive functions. Boks et al. also revealed that co-maturation was indispensable for the stability of the phenotype and the migratory capability in all protocols tested.

However, in their study, IL-10 DCs displayed a limited migratory capability due to a reduced CCR7 expression. This limitation was further corroborated by a comparative study conducted by Adnan et al., which assessed various tolerogenic DC protocols²⁸². Thus, according to these studies, our CD11c⁺CD11b⁺ cDC2 tolDCs had more resident than a migratory profile. Despite this drawback, IL-10 DCs were shown to induce higher levels of IL-10⁺CD4⁺ Tregs compared to tolerogenic DCs generated using alternative protocols, such as those involving protein kinase C inhibitors (PKCI), vitamin D3, dexamethasone, TGF- β , rapamycin, or peroxisome proliferator-activated receptor γ combined with all-trans retinoic acid. Furthermore, Tregs induced by IL-10 DCs and PKCI-treated DCs exhibited superior suppressive capacity relative to Tregs induced by other protocols²⁸³.

We validated several markers to characterize tolDC cultures phenotypically. There were no significant differences between our tolDC and cDCs regarding activation markers, such as MHC-II or CD86, expression levels. Moreover, these differences varied between BMDC cultures, which made it difficult to assert the common notion that tolDCs present an intermediate phenotype between iDCs and cDCs of costimulatory molecules^{260,284}. DC-mediated tolerance induction is not confined to a specific subset or limited to “classical” immature DCs. Neither does it depend merely on “maturation yes or no” but on the extent of maturation combined with newly identified quantitative and qualitative molecular changes²⁸⁵. Due to the lack of expression of costimulatory molecules and MHC-II, tolDCs can induce tolerance in T cells through different mechanisms (see Figure 13).

The fact that no specific-tolDC distinctive markers have been identified or they are dependent on the tolerogenic agent used further complicates its characterization. For instance, MerTK was found to be a specific marker for dexamethasone-treated tolDCs^{261,286}, with increasing surface levels only in tolDCs compared with iDCs and cDCs. However, IL-10-induced tolDCs did not show such an increase (see Figure 17-A). Interestingly, MerTK expression was selectively upregulated by various corticosteroids, while other immunosuppressive agents such as IL-10, VitD3, and retinoic acid did not

induce its expression²⁸⁷. This selective induction highlights a specific molecular signature linked to the tolerogenic agent employed for generating tolDCs. Moreover, it underscores the functional diversity among tolerogenic agents, as each triggers distinct molecular pathways and outcomes contributing to immune tolerance induction.

Characterizing DCs is a complex, arduous task, with controversial literature as a reference. Altogether, the common phenotypical observation after tolerogenic DC differentiation showed that it is impossible to describe a unique phenotype for these cells due to the heterogeneity of differentiation protocols. The key seems to be in simultaneously evaluating multiple molecules like the ones mentioned above and many others. What is clear is that our tolDCs presented a pronounced shift toward the anti-inflammatory IL-10 cytokine production (Figure 17-B), another differentiating feature of tolDCs²⁶⁰.

In any case, the most crucial finding lies in the functional assay results, in which we demonstrated that our tolDCs exhibit a markedly reduced capacity to induce alloresponses (see Figure 19). As ICO15K-pulsed DCs were the condition with more considerable variability in inhibiting T-cell proliferation, we concluded that pulsing with the OAd was too inconsistent to be used for *in vivo* experiments. Besides, it would also be interesting to demonstrate *in vitro* that an Ag-specific tolerization is achieved; for instance, by coculturing tolDC-induced regulatory T cells with the Ag to be tolerized, naïve T cells and mature cDCs, and see whether these Ag-specific regulatory T cells can inhibit and effector response that otherwise would be mounted. Another possibility is what Mansilla et al. proposed: treating mice with Ag-specific tolDCs or PBS (sham) and, after several days, challenging their splenocytes with either the Ag in question, PMA/Iono or culture medium (negative control) for 48h. Then, they added 1 μ Ci per well of [3H]thymidine for the last 18h, and the radioactivity incorporated into the dividing cells was determined using a scintillation counter²⁸⁴. Alternatively, splenocytes can be CFSE-stained before the culture, as we did.

Concerning the *in vivo* to demonstrate whether a pre-treatment of tolDCs previously pulsed with Ad5 peptides could reduce antiviral and thereby enhance antitumoral immune responses, we performed the experiment illustrated in Figure 22. After OAd treatment, another dose of Ad5-pulsed tolDCs was administered to reinforce the tolerogenic effect. We decided to pulse them with a mix of the most immunogenic OAd5 peptides, which is a reliable way for them to be captured, processed, and presented by tolDCs. Our results revealed an unexpected general reduction of both antiviral and antitumor epitopes upon priming-boosting animals with OAd peptides-pulsed tolDCs. Some authors claimed that the major effect of the tolerization-inducing agent on BMDCs occurs during DC maturation over the last 2 days of the culture²⁸⁸. Moreover, the timepoint and duration of IL-10 supplementation are crucial in the resulting tolerizing mechanism. As stated by Raker et al., when IL-10 is present during the whole culture, tolDCs favor the induction of Tr-1-like cells. In cultures of iDCs exposed to IL-10 without undergoing maturation, tolDCs are generated to induce T cell anergy or deletion. Adding IL-10 only during the maturation step differentiates DCs to a stable tolerogenic subpopulation that induces suppressive CD4⁺ and CD8⁺ Tregs²⁸⁹. Therefore, the suppressive function of Tr1 cells, primarily driven by their secretion of IL-10 and TGF- β ^{236,290}, may account for the overall T cell hypoactivity observed. This cytokine-mediated suppression may influence antiviral and antitumor T-cell responses in an antigen-independent fashion. Thus, the induction of more cell-to-cell regulatory immune response that implies antigen presentation should be carried out. Additionally, the ability to transfer tolerance from tolerized to naïve mice is a hallmark of antigen-specific tolerance that also must be addressed.

The success of tolDC therapy depends on their effective migration to the lymph nodes or inflamed tissues to induce long-lasting immunoregulatory responses. Despite showing promising results in clinical trials, the broader application of *ex vivo* tolDC remains difficult. The process of isolating, purifying, and culturing autologous primary DCs (or their progenitor cells) involves complex handling and can be cost-prohibitive. Moreover, how many cells must be administered and how to sustain the semimature state of tolDCs, especially after *in vivo* delivery, must be standardized^{253,291}.

Additionally, tolDCs produced through this method are typically tailored for individual patients to address histocompatibility constraints, further complicating scalability and accessibility. An emerging alternative that overcomes both limitations is the *in situ* induction of tolDCs, where a tolerogenic payload is delivered to DCs *in vivo*²³⁶. In this regard, nanoparticles can simultaneously deliver controlled amounts of self-antigen and tolerogenic agents to individual DCs directed by DC-specific targeting ligands. In the context of virus-specific immune tolerance, synthetic particles loaded with rapamycin (SVP-Rapa) have demonstrated suppressing anticapsid humoral and cell-mediated immune responses when co-administered with adeno-associated virus vectors. This approach fosters immune tolerance, enhances transgene expression, and enables repeated vector administration^{292,293}. These nanoparticles likely emulate apoptotic bodies, which DCs engulf in a manner that favors anti-inflammatory signaling and tolerogenic antigen presentation²⁹⁴.

Demonstrating the OAd epitope immunodominance was not easy on our hands. The approach shown in this thesis suggested that the OAd reduced antitumoral immune responses while favoring potent antiviral ones when compared with the STING agonist agent alone (see Figure 14). However, nothing can be concluded concerning whether the OAd interferes with the STING agonist when coadministered due to the high variability in spot numbers within the “combo” group. Indeed, this is a problem we recurrently had in most of the experiments of this work, preventing us from obtaining statistically significant differences and making categorical conclusions, just supportive or encouraging tendencies.

We also tried similar settings and quantified the immune responses using tumor neoepitope-derived peptides rather than the tumor cell line. In all cases, we used already known reagents capable of eliciting antitumor efficacy, assuming that it might be translated into detectable antitumor responses in an ELIspot assay. Finding an *in vivo* setting where antitumoral immune responses could be measured was a main bottleneck we had to cope with to answer whether the antiviral:antitumoral immune response ratio changed after an OAd-tolerizing treatment. However, as stated by P.K.

Srivastava in his comprehensive review: “immunogenicity and efficacy are not the same and should not be conflated.” In other words, neoepitopes that drive tumor control *in vivo* did not trigger robust CD8⁺T cell responses detectable by flow cytometry or ELIspot. Moreover, the former binds MHC-I poorly, contributing to a lower T cell avidity that is physiologically required to avoid autoreactive T cells²⁹⁵. Furthermore, due to the negative selection processes in the thymus, peripheral neoepitope-reactive T cell repertoire must be substantially restricted compared with the corresponding repertoire against foreign antigens, favoring the viral epitope immunodominance theory. Besides, given that one of the criteria used to select peptides for *in vitro* assays was their high MHC-I affinity, CD8 responses measured *in vitro* do not predict the antitumor activity *in vivo*. This could explain why we failed to illustrate the viral immunodominance phenomenon via tumor-predicted neoepitopes. We only achieved it by using the tumor cell line instead and immunizing mice with OAd-expressing tumor epitopes (see Figure 21).

To add more complexity, some saline-treated animals also developed spontaneous antitumoral responses. This is supported by the work of Alicke et al., which observed elevated rates of rechallenge rejection following surgical resection of primary tumors in four out of five commonly used syngeneic tumor models²⁵⁹. This evidence, therefore, highlights the importance of expanding the sample size for antitumor efficacy *in vivo* settings to discern whether an antitumoral treatment is more or less efficacious.

Despite the evidence provided throughout this thesis supporting the dominant OV-specific T-cell responses over tumor-specific ones, it would be unfair not to mention that this issue is still subjected to debate, as other studies claimed that preexisting OV or other pathogen-specific immunity can be leveraged to promote the induction of a tumor-specific responses²⁹⁶. For example, Tähtinen et al. preimmunized mice with a tetanus vaccine, challenged them with B16-OVA tumors, and finally treated them with an OAd coated with MHC-I-restricted tumor-specific and tetanus-specific-MHC-II-restricted peptides. As a result, a tetanus-specific CD4⁺ T-cell memory response was generated, reinforcing the antitumor immune response mediated by the OAd²⁹⁷.

Another way to achieve this is by delivering viral epitopes into the tumor so antiviral T cells in the TME can be activated. In this regard, Chen et al. proved that an OV-mediated delivery of the so-called “virus-specific bystander T cell” epitopes (OV-BYTE) could redirect the cytotoxicity of these bystander virus-T cells to improve tumor control²⁹⁸. Similarly, intratumoral administration of a recombinant adeno-associated virus expressing the Vaccinia virus-derived B8R protein reactivated previously primed Vaccinia-specific CD4⁺ and CD8⁺ T cells, resulting in delayed growth of murine DT6606 pancreatic tumors²⁹⁹. It could be that the OV intrinsic immunogenicity varies among the OV platforms, making them more or less dominant over tumor-specific immune responses. Studies on viral gene therapy vectors pointed in this direction³⁰⁰

2. Enhancing the virus intratumoral spread and ICD by generating a PS2-armed OAd

Once an OAd reaches the tumor mass, the extent of its subsequent propagation is key in determining its potency. This is, in fact, what made OVs different from other anticancer drugs. Mathematical models of systemic oncolytic virotherapy suggest that successful tumor eradication hinges on two critical factors: i) the initial density and spatial distribution of infectious foci within the tumor and ii) the final size of the viral infectious zones arising from each infected cell (virus spread)³⁰¹. In the case of OAdS, the intratumoral spread is achieved by the release and local migration of the virion progeny. However, other viruses directly transfer from the infected to neighboring cells. Regardless of how thoroughly an OV infection disseminates within a tumor, many cancer cells will evade direct infection¹⁹¹. Effectively targeting these uninfected bystander cancer cells is crucial if oncolytic virotherapy aims to become curative and not just a tumor debulking approach³⁰².

Motivated by this, we armed the ICO15K OAd with a modified version of the PFT Parasporin 2. Not only did this toxin elicit cytocidal effects in a broad spectrum of cancer cell lines (see Figures 23 and 24), but it also had ICD-inducing properties, even the most decisive one, that is, the ability to confer a significant degree of protective

antitumor immunity in the gold standard vaccination-rechallenge setting (see Figures 28-B and 29-A). Moreover, the PS2-based vaccination generated more responder animals than the control group, which presented higher T and NK effector cell populations (see Figure 31). Many pathogens express toxins that form pores in cell membranes, so it is not surprising that the immune system has evolved to detect this type of cell lysis. Thus, PFTs seem ideal candidates for increasing the ICD induced by OAdS. Nonetheless, mechanistic insights confirming such ICD processes should be performed. Other authors proposed that a PS2 subvariant killed via apoptosis was associated with an increased expression of the tumor suppressor gene PAR-4 and the inhibition of the PI3K/AKT pathway³⁰³. It must be denoted that we got all these results, either statistically significant or trends, by working with PS2 supernatants as a toxin validation proof-of-concept. Next steps include moving to PS2 purified preparations, expecting even more encouraging outcomes since they will be more concentrated and could be quantified.

Besides favoring intratumoral virus spread, we hypothesized that PS2-mediated immunogenic cytotoxicity in non-infected bystander tumor cells would collaterally enhance immune responses against tumor antigens: cells only killed by the secreted toxin would be emitting danger signals without presenting any viral antigens. Cells lysed by the toxin cannot present toxin-derived epitopes because the toxin is not an endogenous protein. Still, it is a protein attached to their membrane, and only professional APCs can process and present exogenous proteins by cross-presentation. Therefore, we presumed the immune system would associate the danger with tumor epitopes. This way, we might redirect the immune responses toward the antitumoral side, diminishing the viral immunodominance.

Bacterial-derived toxins have increasingly experimented high interest in tumor treatment due to their potent and unspecific toxicity. Such unspecificity, however, can constitute a disadvantage for inaccessible tumors that require systemic delivery. PFTs offer a solution to this concern since they have inhibitory domains that prevent their premature action. Moreover, systemic delivery would hinder its oligomerization since

the toxin would be diluted in the bloodstream. Thus, placing a PFT into an OAd will allow this systemic delivery while confining the toxin action within the TME due to OAd oncotargeting properties. This will add an extra level of selectivity, besides the intrinsic tumor selectivity of the PS2 *per se* and the fact that for its active form, it must be cleaved in both ends by furin proteases.

Previously in our group, the aerolysin toxin was modified to become active upon cleavage with tumor stroma-specific proteases such as MMP-9, improving the *in vivo* antitumor efficacy of ICO15K in MMP-9⁺ cells without causing irreversible toxicity³⁰⁴. However, tumor cells increase the extracellular matrix (ECM) stiffness to form protective physical barriers where tissue inhibitors of metalloproteinases (TIMPs) are also expressed; some tumors indeed are described to perpetuate fibrosis by inhibiting MMPs that would otherwise degrade them³⁰⁵. We were interested in optimizing this approach and modified PS2 with a furin-sensitive linker, allowing it to be activated once expressed by the OAd within the tumor cell. Unlike MMPs –present and variably expressed in the TME– furin is a cellular endoprotease that activates numerous proprotein substrates within intracellular secretory pathway compartments. Additionally, furin has been found overexpressed in several cancer types³⁰⁶. Our preliminary results evidenced that a modified PS2 with such linkers to remove the inhibitory domain was active against tumor cells. PS2 demonstrated greater *in vitro* cytotoxicity compared to aerolysin supernatants, and it also did not affect A549, the cell line used for virus production. By contrast, aerolysin did so.

Parasporins exhibit notable anticancer activity *in vitro*, highlighting their potential as therapeutic agents for oncology. However, their antitumor efficacy *in vivo*, particularly in animal models like mice and rats, remains poorly understood. The scarcity of data on their acute and chronic toxicity in these models represents a significant limitation, as many compounds that demonstrate promising effects in cultured cancer cells fail to exhibit similar activity in living organisms. It is imperative to thoroughly investigate their toxicity profiles for clinical applications to ensure human safety. A few studies have suggested the anticancer potential of purified crystal proteins from

B. thuringiensis in animal models^{307–310}. However, the absence of sequencing data for these proteins leaves their exact involvement unverified. Among the registered parasporins, only PS4 has undergone *in vivo* toxicity testing, with a reported LD₅₀ of 160 µg/kg in male ICR mice via subcutaneous administration³¹¹. While this toxicity is lower than many bacterial toxins, PS4 still caused significant kidney function impairment and damage to proximal renal tubules. Interestingly, oral administration of PS4 did not result in observable toxicity, underscoring the importance of the delivery route in mitigating adverse effects.

Despite extensive research on isolating parasporins and analyzing their *in vitro* anticancer activity, cellular toxicity, and mechanisms of action, these findings require validation in animal models before human trials can proceed. Although over 19 parasporins have been registered, each showing specific anticancer activity against various human cancer cell lines, only PS4 has been studied *in vivo*. In this work, we provided an expanded *in vivo* knowledge concerning PS2. As mentioned before, PS2 demonstrated potent tumor growth inhibition in tumor-bearing mice and conferred protective immunity by inducing ICD in immunocompetent models, suggesting not only its direct antitumor efficacy but also its capacity to stimulate long-term immune memory against the tumor.

ICO15K-PS2FuT was successfully generated and purified, and its *in vitro* characterization indicated no significant loss of cytotoxicity and replication capacity occurred due to transgene insertion in the A549-producing cells (see Figure 34-B). Moreover, production yields were neither altered (see Figure 35), indicating viral fitness was not compromised. However, though being likewise infective, HepG2 cells posed more virus resistance that was intensified in the PS2-armed OAd. A virus production assay should be performed, too, using these cells to better characterize how the OAd behaves within them. We also incorporated the best eukaryotic signal peptide in our hands to enable PS2 secretion from OAd-infected cells. To contextualize, during the synthesis of secreted proteins, the signal peptide is recognized as it emerges from the ribosome and is bound by the signal recognition particle (SRP), temporarily

pausing translation. The ribosome-SRP complex is then directed to the cytoplasmic surface of the endoplasmic reticulum (ER), which interacts with the SRP receptor. The signal sequence is subsequently transferred to a protein-conducting channel called the translocon. Once the ribosome is engaged with the translocon, translation resumes, and the nascent protein is threaded through the ER membrane into the lumen. As the protein translocates, the signal peptide is cleaved by signal peptidase, resulting in the mature protein. This protein is then processed through the Golgi apparatus and ultimately secreted from the cell via the classical secretory pathway³¹².

In our case, the final candidates were the secrecon and trypsinogen signal peptides, with the latter working much better regarding PS2 cytotoxic effects. According to Güler-Gane et al., the signal peptidase could not recognize the signal sequence and cut the signal peptide when the first position of the translated protein began with the amino acids tyrosine (Y), cysteine (C), glutamine (Q), and proline (P). No transgene expression or activity could be observed as a consequence³¹³. With these considerations and for our PS2 gene that began with the amino acid Y, we further optimized the construct by adding two extra alanines (defined as favorable for signal protease) in between the trypsinogen and the *PS2Fu* gene.

Moreover, both trypsinogen and *PS2Fu* DNA sequences were codon-optimized toward human-biased codon usage. Codon optimization should be tailored for both high expression and efficient replication. In their study, Núñez-Manchón et al. concluded an optimized codon usage enhanced transgene expression but reduced adenoviral replication, limiting the therapeutic effect. In contrast, using non-optimized codons, the OAd replication was maintained, which boosted the oncolytic impact³¹⁴. By doing the same analysis using their ONATRY tool, we found that our *PS2FuT* gene was quite balanced (**Figure 42**), even humanized.

As shown in Figure 38-A, the ICO15K-PS2FuT OAd improved the antitumoral efficacy compared to the parental unarmed ICO15K OAd in a PS2-sensitive immunodeficient tumor model (HepG2-bearing NSG mice), even replicating worse than ICO15K. Nonetheless, optimal conditions for the configuration of the virus are uncertain. For

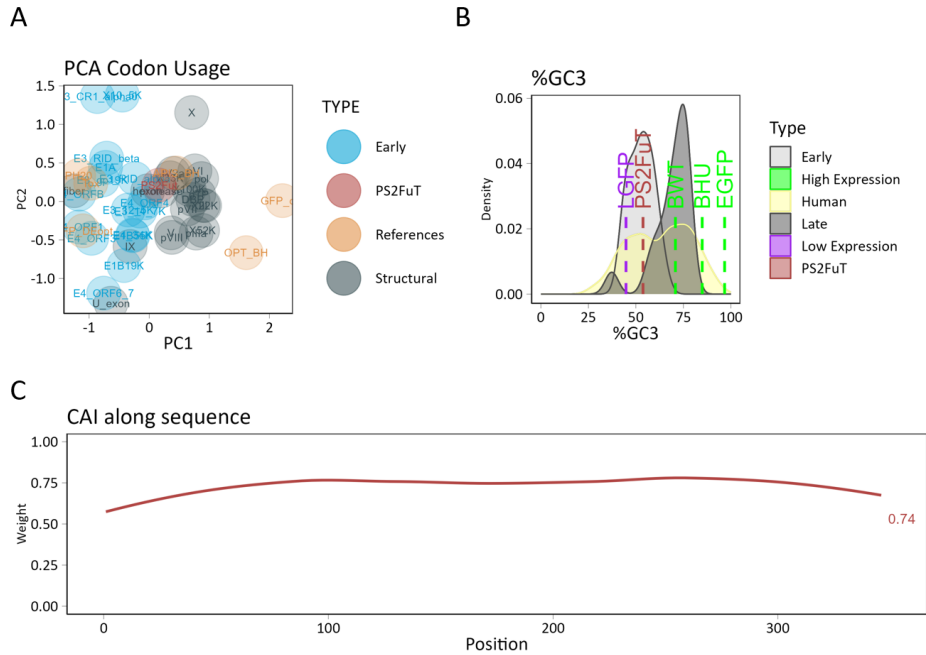


Figure 42. The humanized PS2FuT transgene shows an optimal codon usage that allows a balanced virus and transgene expression. Two key parameters should be considered when arming therapeutic transgenes under the adenoviral major late promoter. **A)** Codon usage PCA in relation to adenoviral genes. Positive PC1 values correlate with low GC3 content (and high AT3). Human optimal codon-usage transgenes (e.g., EGFP) generally cluster near late structural and replication-related genes in the PC1 component (X-axis), except for the Ad5 fiber. In contrast, low-codon optimized genes (e.g., LGFP) were close to early regulatory viral genes. The PS2FuT sequence, even humanized, overlapped with the wild-type bee hyaluronidase (BWT), which is used as a reference for balanced codon usage. **B)** Percentage of GC3 content. GC3 codons are associated with human mRNA stability and with higher translation efficiency. For optimal viral fitness, it should be around 50-70%. **C)** The codon adaptation index (CAI) along the PS2FuT sequence. CAI is a measure of the synonymous codon usage bias for a DNA sequence in relation to the codon usage of a particular species. It ranges from 0 to 1, being 1 if a gene always uses, for each encoded amino acid, the most frequently used synonymous codon in the reference set. PS2FuT CAI value was 0.74, very similar to the 0.73 CAI value of the BWT.

cloning the PS2 transgene, the traditional insert region after virus fiber —linked to the major late promoter that is only active upon replication in tumor cells— was chosen with the presence of the SAS splicing acceptor, which promoted high levels of transgene expression³¹⁵, and the 5' Kozak sequence GCCGCCACC upstream of the start codon to maximize transgene yields. Still, the ICO15K-PS2FuT supernatant presented fewer PS2 titers than those coming from plasmid-transfected cells (see Figure 37).

Other splicing acceptors may be considered to enhance transgene expression –as reported with the 40SA³¹⁶ – as long as it does not compromise viral fitness.

Another aspect to take into account is the presence of rare codons, which is independent of the codon usage optimization. Transgenes enriched in rare codons result in decreased protein synthesis rates as their cognate tRNA are less abundant^{317,318}, which varies among species. When analyzing our *PS2FuT* gene using the *bioinformatics.org* codon usage online tool (in collaboration with Dr. C. Fillat), we found three rare codons, considering as rare the codons present in the human genome in a frequency <1% (or <10‰): GCG (Alanine), ATA (Isoleucine) and ACG (Threonine). The humanized PS2FuT sequence used these codons at a frequency of approximately 30‰. However, their frequency in the human codon usage is between 6-8‰, which can delay transgene translation. Thus, removing these rare codons in the *PS2* sequence could enhance its expression levels within the OAd.

Regarding OAd toxicity concerns, we know from previous results in the group that our OAds injected systemically exit from the bloodstream in the liver, spleen, and tumors due to the endothelial fenestration size. The epithelial normal cells susceptible to infection are only hepatocytes. Thus, ICO15K was chosen as the adenoviral platform for this virus to minimize hepatotoxicity³¹⁹. No additional hepatocyte harm was expected for *PS2* insertion since this cell type has been reported to have resistance to the toxicity of *PS2*²⁰⁵. Indeed, there were no differences in the toxicity profile between our ICO15K-*PS2FuT* OAd regarding the ICO15K parental virus (as depicted in Figure 39). However, a cytotoxicity study in quiescent or cell cycle-arrested hepatocytes and other normal cells should be conducted to confirm the lack of toxicity of this virus.

Systemic OAd administration is also modeled for being clinically relevant, enabling the OAd to reach not only superficial but also deeper tumors and distant metastases. However, several challenges of intravenous injection, such as antiviral immune response and physical stroma barriers, result in limited penetration of OV in solid tumors. To overcome this, one possibility is to use MSC carrying OAd to deliver ours. MSCs are considered the most promising OV carriers based on their inherent tumor-

homing and immune-silencing properties. Several preclinical and clinical studies have highlighted the transportation capability of OV-loaded MSCs. For example, an autologous MSC-carrying OAd called Celyvir has recently completed its phase I/II clinical trial³²⁰.

Another major concern related to OAd is that human adenoviruses do not replicate efficiently in mouse tumor models, showing up to a 6-log viral replication deficiency compared with human cancer cells³²¹. This lack of permissivity represents the main challenge to studying transgene and OAd immune-mediated mechanisms of action with the virus replication and oncolysis. Despite this limitation of the preclinical immunocompetent models, OAd have shown T-cell responses against tumor antigens and T-cell-mediated antitumor activity in OAd semi-permissive hamster models²⁷⁵. In this regard, we collaborated with Dr. Cristina Fillat's lab (IDIBAPS) in evaluating the response of murine pancreatic cancer KPC cells to adenoviral replication, demonstrating that KPC cells display enhanced permissivity for Ad5 replication than any of the other murine cells tested³²². These were encouraging results, as we could test whether both PS2-mediated ICD and ICO15K-PS2FuT oncolytic potency synergize and enhance the overall antitumor efficacy in immunocompetent models. Finally, studies aiming at identifying the receptors that dictate PS2 tumor selectivity would be of great interest to screen which patients would benefit from a putative PS2-based OAd therapeutic product.

CONCLUSIONS

1. Treatment with ICO15K OAd elicits strong antiviral immune responses but decreases the antitumoral immune responses when compared with a STING agonist drug, suggesting the viral epitope immunodominance may account for these differences.
2. tolDCs generated from BMDCs and cultured in the presence of GM-CSF and IL-10 exhibited similar expression of the costimulatory markers MHC-II and CD86 compared to mature DCs (cDCs), as well as the MerTK molecule. However, they were superior regarding the anti-inflammatory IL-10 cytokine secretion levels.
3. IL-10-induced tolDCs are able to suppress T-cell proliferation when cocultured with allogeneic splenocytes in MLR assays, denoting their tolerogenic condition in impairing alloresponses.
4. Immune responses against both viral and tumor epitopes can be detected upon immunization with OAd-encoding epitopes from the B16-F10 melanoma tumor cell line.
5. Treatment with tolDCs pulsed with Ad5 peptides before and after OAd administration fails to modulate viral-specific immune responses. Still, it promotes generalized immunosuppression of both tumor and viral epitope-recognizing activated T cells.
6. Supernatants from cultures transfected with PS2FuT-expressing plasmids exert selective cytotoxic activity against certain human and murine cancer cell lines.
7. Intratumoral treatment with PS2FuT-concentrated supernatants enhances tumor growth control in CMT64.6 tumor-bearing immunocompetent mice.
8. Vaccination with PS2FuT-treated tumor cells acts as an ICD inducer that confers a protective antitumor immune response in a vaccination-rechallenge immunocompetent model.
9. *PS2FuT* transgene is expressed and secreted from OAd-infected cells, enhancing the cytotoxic potential of the ICO15K virus *in vitro* in PS2-sensitive cells.
10. The ICO15K-PS2FuT armed OAd can improve *in vivo* antitumor efficacy of ICO15K in subcutaneous PS2-sensitive tumors without causing added toxicities in immunodeficient mice, expecting major benefits when both virus oncolysis and toxin ICD-mediated responses could act synergistically in an adenoviral replication-permissive immunocompetent setting.

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