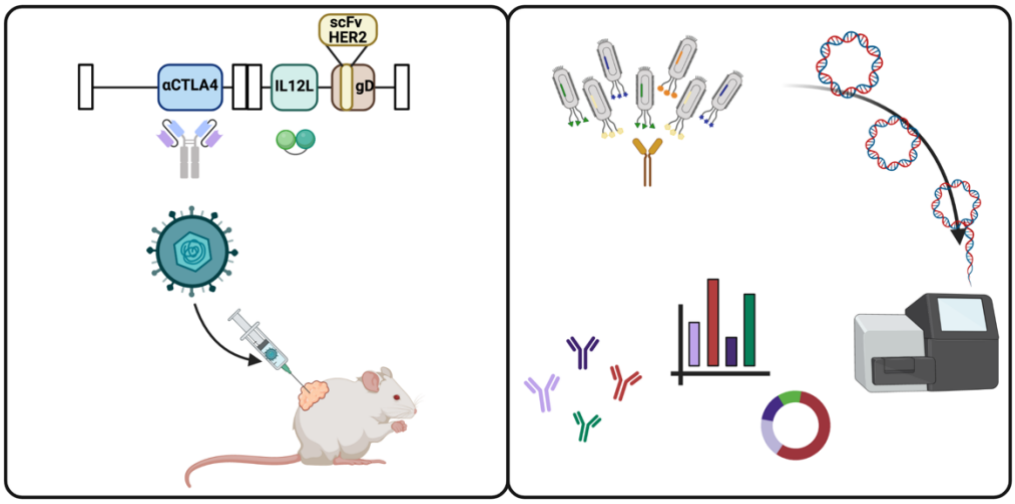
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIII CYCLE

Chiara Gentile

Development and characterization of an immunotherapeutic platform based on oncolytic Herpes simplex viruses and αCTLA-4 antibodies



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Development and characterization of an immunotherapeutic platform based on oncolytic Herpes simplex viruses and αCTLA-4 antibodies

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ABBREVIATIONS

ADCC: Antibody-dependent cell-dependent cytotoxicity
APC: Antigen-presenting cells
CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4
DAMPs: Danger-Associated Molecular Patterns
DC: Dendritic cells
EMA: European medicine agency
FDA: Food and drug administration
gD: Glycoprotein D
GM-CSF: Granulocyte-macrophage colony-stimulating factor
hPBMC: human peripheral blood mononuclear cells
HSV-1: Herpes simplex virus 1
ICB: Immune checkpoint blockade
ICD: Immunogenic cell death
ICI: Immune checkpoint inhibitors
IL-12: Interleukin-12
irAEs: Immune-related adverse events
MHC: Major Histocompatibility Complex
PAMPs: Pathogen-Associated Molecular Patterns
PD-1: Programmed death-1
PDL-1: Programmed death ligand-1
oHSV: Oncolytic HSV
OV: Oncolytic virus
TAA: Tumor-associated antigen
TSA: Tumor-specific antigen
TCR: T cell Receptor
THV: Targeted Herpes virus
TME: Tumor microenvironment

ABSTRACT

In the last decade, immunotherapy has emerged as a promising approach to treat cancer. Although encouraging results have been obtained with monotherapy treatments, a large percentage of patients still do not respond, opening therapeutic options to combination of immunotherapeutics (i.e., oncolytic viruses and mAbs targeting immune checkpoints). Unfortunately, despite combination therapies emerged as a valid option thanks to synergistic efficacy, the occurrence of systemic immune-related adverse events (irAEs) often leads to treatment interruption.

The purpose of my thesis was to implement a platform of immunotherapeutics based on oncolytic HSV (oHSV) encoding vectored immunomodulators for local cancer treatment. Interleukin-12 and αCTLA-4 antibody were identified as promising payloads, to be encoded within oHSV genome. The efficacy of these oHSVs was evaluated in *in vivo* mouse model showing significant improvement in antitumor efficacy compared to oncolytics devoid of the selected immunotherapeutic cargoes.

As viral vectored αCTLA-4 antibody resulted in significant improvement of antitumor efficacy, to facilitate preclinical to clinical translation, I isolated human/murine cross-reactive αCTLA-4 antibodies through a high throughput screening of a phage display library of $scFv_s$ by Next Generation Sequencing.

Additional work will explore the potential ability of the THV_CTLA4 to reduce irAEs occurrence thanks to the antibody confined expression within the TME.

1. INTRODUCTION

1. Cancer immunotherapy

1.1a Immune system and cancer immunoediting

Innate and adaptive immune system compartments are closely related and have intriguing overlapping functions in mediating antitumor immunity. Physiologically, these two compartments guarantee protection from microbes and non-self molecules, mounting non-specific as well as specific immune response (Olszanski 2015; Gasparri 2017).
Somatic mutations make tumor cells detectable by immunesurveillance that activates T lymphocytes to eliminate neoplastic cells (Blair 2008; Abbott 2019). However, interaction between tumor and immune cells can be controversial. On one hand, immune system effectively controls tumor growth, as demonstrated by the higher frequency in tumor development exhibited by both preclinical immune-compromised mouse model and human conditions of congenital (i.e., severe combined immunodeficiency, SCID) or acquired immune-deficiency (i.e., acquired immunodeficiency syndrome, AIDS) (Terme 2016). On the other hand, several studies have highlighted an unexpected role of immune system in promoting tumor progression (Zamarron 2011).
The role of immune system in tumor control has been explained by the three E theory, by which interaction between cancer cells and immune system includes Elimination, Equilibrium and Escape phases (fig. 1).
During the elimination phase, an active immunosurveillance guarantees the elimination of cells that accumulate mutation and become or have the potential to become malignant (Olszanski 2015; Gasparri 2017; Abbott 2019). Innate and adaptive immune response cooperates to ensure tumor cell lysis and subsequent release of tumor-specific and tumor-associated antigens (TSA and TAA, respectively). APCs (as dendritic cells, DC) uptake and expose TSA and TAA on their surface by MHCI and MHCII complexes, which together with costimulatory signals (CD80/CD86-CD28) and cytokines activate CD8 and CD4 T cells. Whereas the crucial role of CD8 T cells against tumors has been confirmed by the better prognosis associated with a higher grade of infiltrated CD8 T cells in different tumor types (i.e., melanoma, breast, ovarian and colorectal cancers), CD4 T cell role remains controversial (Muenst 2016; Mahmoud 2011). Indeed, activated CD4 T cells are crucial for CD8 T cell differentiation, especially for the generation of CD8 memory T cells (Bourgeois 2002). However, it has been demonstrated that CD4 T cells, characterized by the expression of CD25 and Foxp3 transcription factor, known as Tregs, have a crucial role in sustain tumor growth.
As long as NK and CD8 T cells prevail over Tregs and other immune-suppressive cells (including cancer cells themselves), tumor remains clinically undetectable (Oiseth 2017) and is kept under control until the Equilibrium phase. In this phase, immune system acts as a selective driving force, allowing adapted

tumor cells to survive and acquire a “tumor dormancy” phenotype. It has been demonstrated that this phase can last for years, as showed by cases of transmission of tumor from organ donors to immune-suppressed organ recipient (Swann 2007).

During this period of time, tumor cells keep accumulating mutations (immunoediting), allowing them to finally escape immune control and indefinitely growth (Escape phase).

Multiple mechanisms are involved in tumor escape. Firstly, tumor cells impair expression of different class of membrane proteins, including class I and II MHC, TAA molecules and FasL, resulting in a reduced antigen recognition by CD8 T cells and an apoptosis resistance respectively (Terme 2016). Secondly, tumor cells create an immunosuppressive tumor microenvironment (TME) through: i) secretion of immune-suppressive cytokines (i.e., IL-10); ii) recruitment of Tregs and myeloid-derived suppressor cells (MDSCs); iii) induction of phenotypic and functional switch of macrophage (to M2 phenotype) and dendritic cells; iv) upregulation of co-inhibitory lymphocyte signals (i.e., PDL-1) (Velcheti 2016; Liu 2015).

Finally, the resulted immune-suppressive TME leads to T cell anergy and/or exhaustion. Anergy is due to absence of costimulatory signals that causes CD8 T cell inability to proliferate. Chronic exposition to tumor antigen induces the acquisition of exhausted phenotype by CD8 T cells, characterized by loss of cytotoxic function and inability to secrete cytokines. Moreover, the presence of immunosuppressive cytokines (IL10, TGFβ, VEGF), together with hypoxia, induces expression of immune checkpoint molecules (PD1, CTLA-4, TIM3, LAG3) on CD8 surface (Granier 2016).

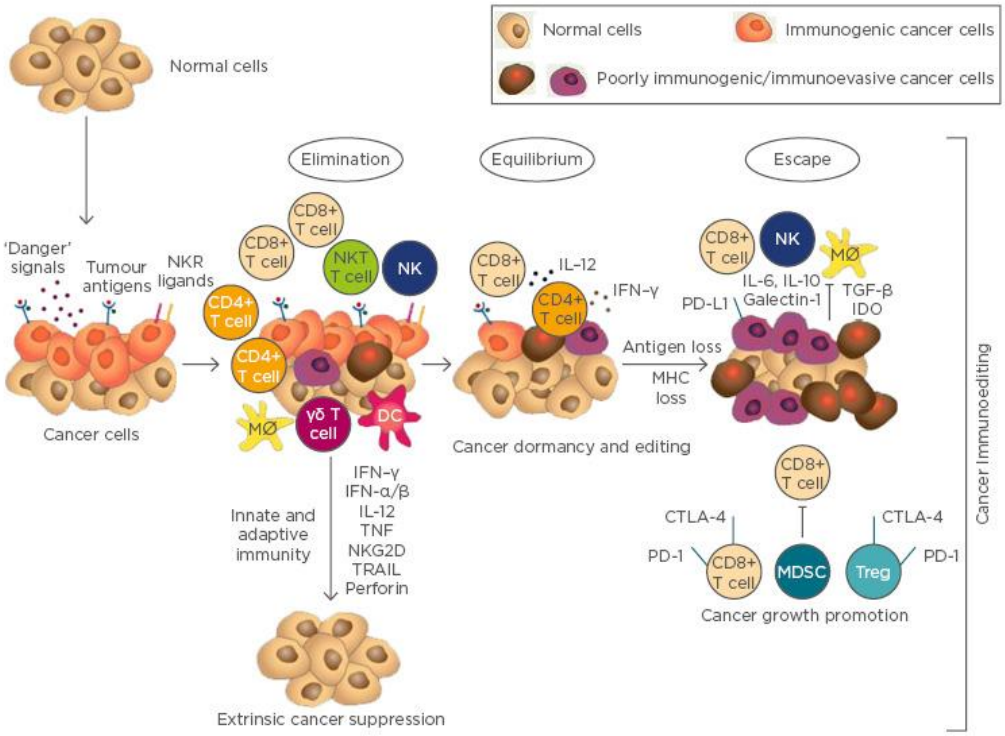

Figure 1. The three phases of cancer immunoediting. Newly transformed cells are recognized and eliminated by immune cells during the elimination phase. Tumor cells keep accumulating mutation and immune system acts as a driving force, allowing some tumor cells to survive. During this phase (equilibrium) neither immune system neither tumor cells prevails. Further adaption of tumor cells allows them to finally escape from immune system control and indefinitely grow (escape phase). (Niccolai 2017)

1.1b Cancer immunotherapy

The elucidation of the interaction between immune system and cancer, has laid the ground for the new branch of cancer treatment known as immunotherapy. Based on the knowledge that immune system is able to counteract tumor cells, the aim of immunotherapy is to activate effector immune cells against cancer. Several approaches can be used to reach this goal:

- Cancer vaccine. Cancer vaccine are mostly therapeutic treatment implemented to augment adaptive response through expression of tumor antigens. They can be directed against TSA or TAA respectively resembling personalized and off-the-self cancer vaccine (Velcheti 2016). An example of cancer vaccine is Sipuleucel-T, approved for metastatic prostate cancer by Food and Drug Administration (FDA) in 2010 (Kantoff 2010).
- Cytokines. Cytokines are molecules used by immune cells to communicate and organize a synchronous response, modulating the magnitude and the duration of the response.
- Adoptive T cell Therapy and CAR-T cells. These approaches are based on *ex vivo* amplification of natural occurring or manipulated T cells against TSA or TAA. In particular, tumor patient's TILs can be harvested and *ex vivo* amplified by IL-2 to be successively re-infused to the tumor bearing patient (Rosenberg 2015). To overcome limitations related to TILs collection (accessibility of tumor and cell yield), circulating T cell (CTLs) can be easily harvested and transduced with a chimeric antigen receptor (CAR), allowing specific recognition of tumor by MHC-I independent mechanism.
- Oncolytic viruses. Cancer cells, due to their metabolic signature are highly susceptible to viral infections. Different viruses can be modified to further improve tumor-selectivity. The cell lysis due to infection leads to tumor-antigen release and subsequent activation of antitumor immune response.
- Immune checkpoint inhibitor blockade. Physiologically, effector immune cells express different regulatory receptors on their membrane surface, allowing a fine-grain control of the immune response. Blocking or activation of these receptors through antibodies can restore functionality in anergic and/or exhausted T cells.

Oncolytic viruses and immune checkpoint blockade will be discussed deeper in the next paragraphs.

1.2 Oncolytic viruses

1.2a General features of oncolytic viruses

Oncolytic viruses (OVs) represent a promising platform for cancer immunotherapeutic treatments. Despite first evidences of the effectiveness of a viral infection in the control of tumors date back to the early 1900s, only in the last decades a more extensive knowledge of viral biology and tumor immunology has shed light on the potential of this class of therapeutics, leading to an increasing number of OVs entering in clinical trials (Martinez-Quintavilla 2019).
OVs are replication competent viruses characterized by a selective infection and replication within cancer cells, which spare healthy cells (Chiocca 2014). They include both single- or double-stranded DNA or RNA viruses and can be divided in two classes:

- viruses which naturally replicate within cancer cells. To this class belong New Castle disease virus (NDV), reovirus, parvoviruses.
- viruses which are genetically manipulated/engineered to acquire tumor selective tropism. Among others, stand out Herpes simplex virus (HSV), Measles virus (MV), Adenovirus (Ad), Vesicular stomatitis virus (VSV).

Despite this classification, all OVs, and viruses in general, have evolved mechanisms to keep infected cells alive as long as replication cycle is completed and viral progeny assembled (Finlay 2006). Accordingly, tumor cells provide permissive conditions for viral replication as they are characterized by active replication, cell death resistance, DNA damage stress, immune evasion, damped anti-viral machinery (Chiocca 2014).
In addition to their tumor-selective replication, OVs offer many other advantageous features including self-increasing dose, due to *in situ* viral amplification, and low probability of resistance generation, thanks to the involvement of multiple cytotoxicity pathways.
Thanks to these characteristics, about a hundred clinical trials, principally Phase I/II, have been started (Macedo 2020). The encouraging results obtained in last years have led, in 2015 and 2016, FDA and European Medicine Agency (EMA) to approve T-VEC, an attenuated HSV-1 expressing GM-CSF, for intra-tumoral treatment of unresectable stage 3 and 4 melanoma.
Despite promising results, several hurdles remain to be overcome. The most investigated aspect concerns OVs delivery. To date, intra-tumoral injection is mostly preferred instead of systemic intravenous administration, due to its efficiency and safety. However, this route of administration is limited to palpable and accessible tumors and could have a reduce efficacy in targeting distant and undiscovered metastasis. For these reasons, in last years, many efforts have been done to improve systemic delivery, taking into account the presence of pre-existing immunity for human viruses or induced immunity following repeated

administration and sequestration in the liver that could reduce the effective dose reached at the tumor bulk (Chiocca 2014).
Strategies under investigation include: i) use of alternative serotypes; ii) use of cell carriers; iii) PEGylation (covalent conjugation with polyethylene glycol) of the viral coat (Tesfay 2013; Kaufman 2015).

1.2b OVs mechanism of action: "heating-up" of tumor microenvironment

At the early begging of virotherapy development, scientists demonstrated that OVs mechanism of action relies on their ability to lyse tumor cells, leading to a reduction of the tumor mass. With the advent of syngeneic tumor models in immunocompetent mice, it became clear that OVs infection prompt not only cancer cell lysis, but also induction of CD8 T cell-mediated and long lasting antitumor immune response (Melcher 2011).
It is now well established that OVs act as *in situ* vaccines through two distinct but overlapping mechanisms: i) tumor cell lysis; ii) activation of antitumor immunity (fig. 2).
After infection, OVs efficiently replicate within tumor cells leading to the immunogenic lysis and release of viral Pathogen-Associated Molecular Patterns (PAMPs) and Danger-Associated Molecular Patterns (DAMPs). Immunogenic cell death (ICD), including immunogenic apoptosis, necrosis, necroptosis, autophagic cell death, is a type of cell death characterized by release of DAMPs, as ATP, High-Mobility Group Box 1 (HMGB1), and type I IFN, which enhance recruitment, activation and maturation of DC, increase MHC/peptide complex expression and stimulate production of T cell chemokines (Russel 2018).
In this *scenario*, innate immune response plays a pivotal role as, on one hand, it can counteract viral spread and, on the other hand, it contributes to the stimulation of antitumor adaptive immunity. In last years, it has become clear the key role of DNA-sensing STING pathway in inducing ICD and activating antitumor immune response. It has been demonstrated that STING-deficient mice have impaired antitumor T cell responses (Russel 2018). Recently, we have clarified that integrity of STING pathway is necessary for an adequate immunotherapeutic effect, as it is crucial for ICD and type I IFN production (Froechlich 2020).
Along with PAMPs and DAMPs, lysed cells release TAA, which, together with viral antigens, are engulfed by APCs and exposed on their surface, activating naïve or anergic T cell. Once activated, CD8 T cells expand into cytotoxic effectors and mediate antitumor immunity upon antigen recognition. Moreover, release of perforin and granzyme within the TME by immune cells may result in the killing of nearby tumor cells, even in the absence of direct antigen recognition, a process known as "immune-associated bystander effect" (Schietinger 2010).

In this way, OVs are able to convert the immunocompromised TME into an immunocompetent one, modifying the cytokine milieu and the type of immune cells recruited within the TME, also in so called “cold” tumors. This change can further promote the tumor cell recognition and lysis, leading to release of neoantigen previously hidden to immune system, promoting epitope spreading (Bridle 2010), and the antitumor response on distal uninjected tumor (abscopal effect). Results from preclinical models have revealed both systemic and long-lasting effect. Accordingly, in OPTiM trial (Phase III clinical trial using IT-delivered T-VEC) injection in one tumor induces a systemic antitumor immunity, as no evidences of virus spread from injected to uninjected lesions were proved (Andtbacka 2016). However, since the efficacy on metastasis is not potent as on primary injected tumor, combination therapies are under investigation, as will be discussed in following paragraphs.

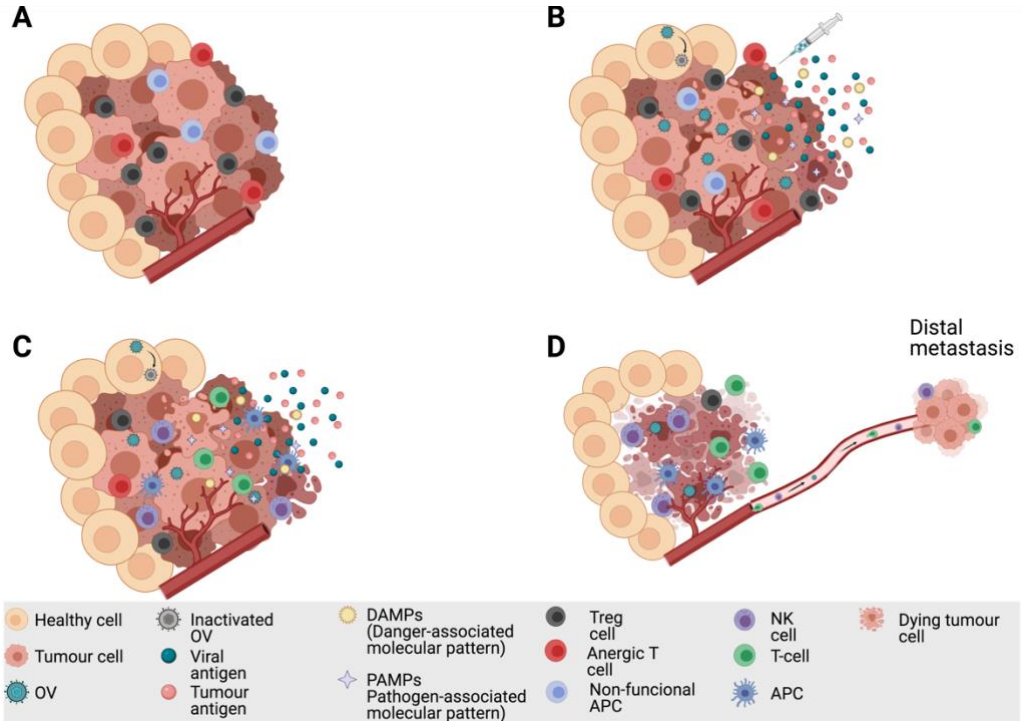

Figure 2. Mechanisms of action of oncolytic viruses. (A) In the tumor context, TME is often immunocompromised as shown by abundance of Treg cells, anergic T cells and non-functional APCs. (B) After the intra-tumoral injection, OV selectively replicates within tumor cells, whereas replication within normal cells is impaired. OV replication leads to tumor cell lysis, which event causes the release of TAAs, DAMPs, PAMPs, as well as viral antigen. (C) Those molecules are engulfed by immature APCs, prompting their maturation and subsequent activation of naïve or anergic T cells. Cytokine production leads to the conversion of the TME into an immunocompetent microenvironment, with the recruitment of activated immune cells. (D) Activated immune cells mediate the elimination of tumor cells. Moreover, the generated antitumor immunity is also able to target distal metastasis.

1.2c *Herpes simplex* virus 1: structure and replication cycle

Herpes simplex virus 1 (HSV-1) belongs to *Alphaherpesvirinae* subfamily, as it is characterized by a short replicative cycle, ability to establish a latent infection in neurons and wide range of hosts (Rechenchoski 2016).
From the external to the inner layer, HSV-1 is composed by: i) the outer envelope, a lipid bilayer containing 11 glycoproteins, crucial for interaction with host cells; ii) the tegument, an amorphous layer composed by proteins involved in the regulation of viral replicative cycle (i.e., VP16, VP22); iii) the capsid, an icosahedral structure that contains DNA.
HSV-1 genome consists of a linear dsDNA of about 150 kbp, composed by two unique units, long (UL) and short (US), flanked by inverted repeats. UL and US contain around 90 transcriptional units, each of them encodes a single protein, with minor exceptions (Kukhanova 2014).
Infection starts with recognition of the host cell, which requires more than one interaction. Initially, the glycoprotein C (gC) and glycoprotein B (gB) bind heparan sulfate (HS) on the cell membrane. This interaction is unspecific and unstable until the involvement of glycoprotein D (gD). gD specifically binds several receptors, known as herpesvirus entry mediator (HVEM), nectin-1 and nectin-2. The binding induces a conformational change in gD mediating the interaction with the heterodimer gH/gL, which mediates the membrane fusion. This event allows capsid to be released within the cytosol, where it is translocated to the nucleus via microtubules (Campadelli-Fiume 2007). Viral DNA is finally released into the nucleus and replication cycle starts (fig. 3).
Replication cycle is a tightly regulated process that includes three phases: immediate-early (IE), early (E), late (L).
IE phase starts with VP16 binding to two cellular transcription factors, HCF-1 and OCT-1. This complex associates with specific sequences and initiates transcription of the five IE genes (*ICP0, ICP4, ICP22, ICP27, ICP47*). Of these, ICP0 trigger proteasomal degradation of cellular protein involved in anti-viral defense mechanism. Right after activation of IE genes, DNA replication starts (E phase) both through cell machinery and viral newly synthetized DNA polymerase complex. Once DNA replication is completed, late genes are expressed (L phase). They encode structural proteins (tegument, capsid and glycoproteins), which are assembled with scaffolding proteins. The nucleocapsid is enveloped during fusion with the inner nuclear membrane. Moreover, those particles are enveloped again by the Golgi complex vesicles. Mature virions are finally released by exocytosis (Rechenchoski 2016; Kukhanova 2014; De Mello 2016).

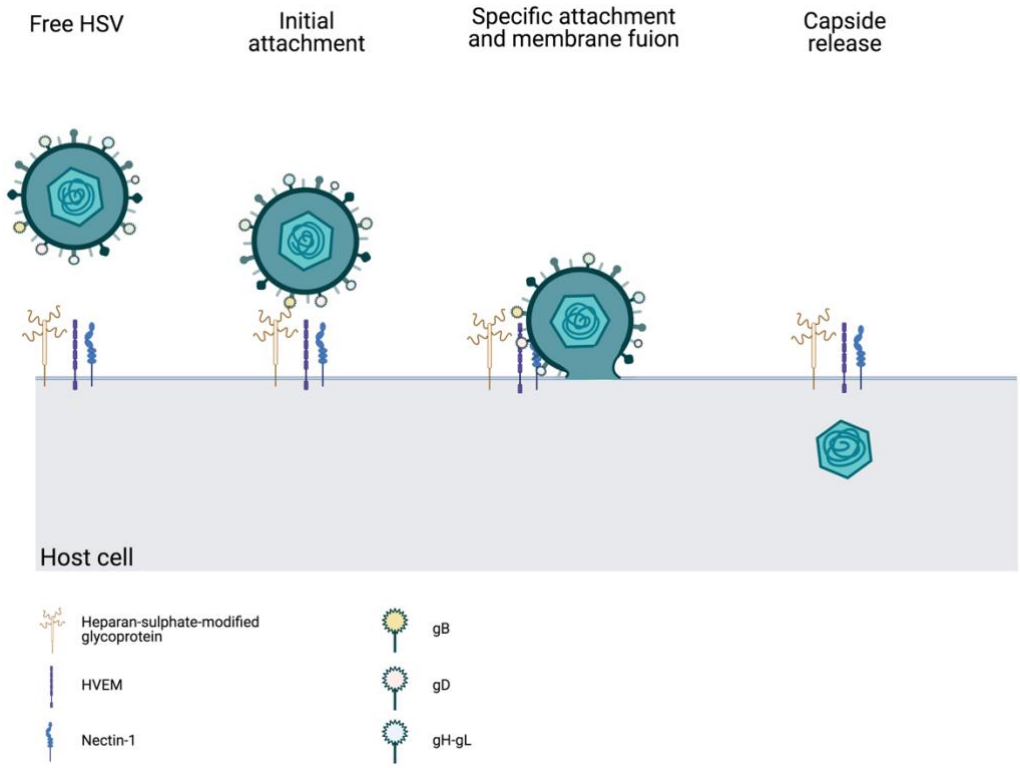

Figure 3. Wild-type (WT) HSV-1 entry mechanism. Mechanism of WT HSV-1 entry relies on the subsequent interaction of viral glycoproteins with ligands expressed on host cell membrane. First interaction between the virus and the cell is mediated by the glycoprotein B (gB) with heparan-sulphate glycoproteins. After the specific binding of glycoprotein D (gD) with HVEM and Nectin-1/2, membrane fusion occurs and viral capsid is release with the cytosol.

1.2d HSV-1 as an oncolytic virus: retargeting strategies

Among the different viral vectors that can be used as oncolytic virus, HSV-1 is one of the most promising, owing a range of advantageous characteristics including: i) natural cytolytic cycle with ability to infect majority of the malignant cell types; ii) large genome containing different non-essential genes that can be replaced with foreign genes; iii) easy manipulation and deep knowledge of its biology; iv) anti-viral drugs availability (Acyclovir and Ganciclovir).
Despite these features, the main disadvantage of HSV-1 is the high prevalence in the population (about 40-90% depending on the socioeconomic class). Prior immunity in these subjects consists of presence of neutralizing antibodies that could limit HSV-1 use as an OV especially if delivered systemically. Surprisingly, preclinical experiments in immunocompetent mice as well as results from phase I clinical trial found that anti-HSV-1 antibodies did not alter the therapeutic efficacy of intratumorally administered T-VEC (Prestwich 2009; Hu 2006).
To guarantee safety in immunotherapeutic approach, the main requirement is the selective infection of tumor cells, which spares normal ones. For HSV-1, this objective can be reached through three different approaches, schematized in figure 4.
The first investigated strategy is conditional replication. Conditional replication is obtained by mutations or deletions of genes that are usually crucial in counteracting IFN-mediated response in infected cells. As tumor cells have often impaired IFN pathway, this modification allows replication in tumor cells whereas impairs replication in normal non-dividing cells. γ34.5 gene is the best representative of these viral genes as its protein product (ICP34.5) acts suppressing the shutoff of host protein synthesis in favor of viral protein synthesis. As attenuation also affects the cytotoxic potential in tumor cells, alternative strategies to restrict viral tropism have been developed by transcriptional and tropism retargeting (Campadelli-Fiume 2011).
Transcriptional retargeting consists of placing viral genes under the control of tumor-specific promoter. All classes of viral genes (IE, E and L) have been tested for transcriptional retargeting (Lou 2018). As it has already been tested in conditional replication, γ34.5 remains one of the candidate genes for transcriptional retargeting. In rQNestin34.5, γ34.5 was placed under the control of nestin promoter. Nestin is an intermediate filament expressed during neuronal embryogenesis, but it is not expressed in normal adult brain. Its expression increased again in glial tumors. In mice preclinical model of brain tumor, treatment with rQNestin34.5 prolonged lifespan of mice (Kambara 2005).
The more recent strategy that can be used to redirect HSV-1 infection is tropism retargeting. This approach is based on manipulation of viral glycoproteins as, as previously discussed, they mediate viral entry specificity. Among the eleven glycoproteins, gD is surely the one that ensures binding specificity. For this reason, many efforts have been done to modify gD tropism. The first possible

manipulation concerns the generation of a recombinant gD, fused with a heterologous ligand able to specific bind a tumor-receptor. R5111 was the first virus generated with this approach, as it carried IL-13 fused with gD N-terminus, with the aim of targeting IL13Rα2 expressed in malignant glioma. Even if it was able to infect tumor cells expressing IL13Rα2, it retained the ability to bind gD natural ligands (Zhou 2002). In light of these findings, it was clear that retargeting needed to be accompanied by a de-targeting strategy. Thus, the second possible mechanism of retargeting requires substitution of essential amino acids, responsible for recognition of natural ligands, with single chain antibody (scFv) fragment targeting tumor proteins. Fully virulent retargeted viruses have been generated, recognizing different tumor-specific proteins including Mesothelin, HER-2 and EGRFvIII (Froechlich 2021; Menotti 2018). To ensure maximum safety also with fully virulent viruses, transcriptional and tropism retargeting can be combined, as we recently demonstrated. In SurE virus, HER-2 retargeting was combined with placement of essential immediate early α4 gene under the control of survivin promoter, highly transcribed in G2 phase of cell cycle (Sasso 2020).

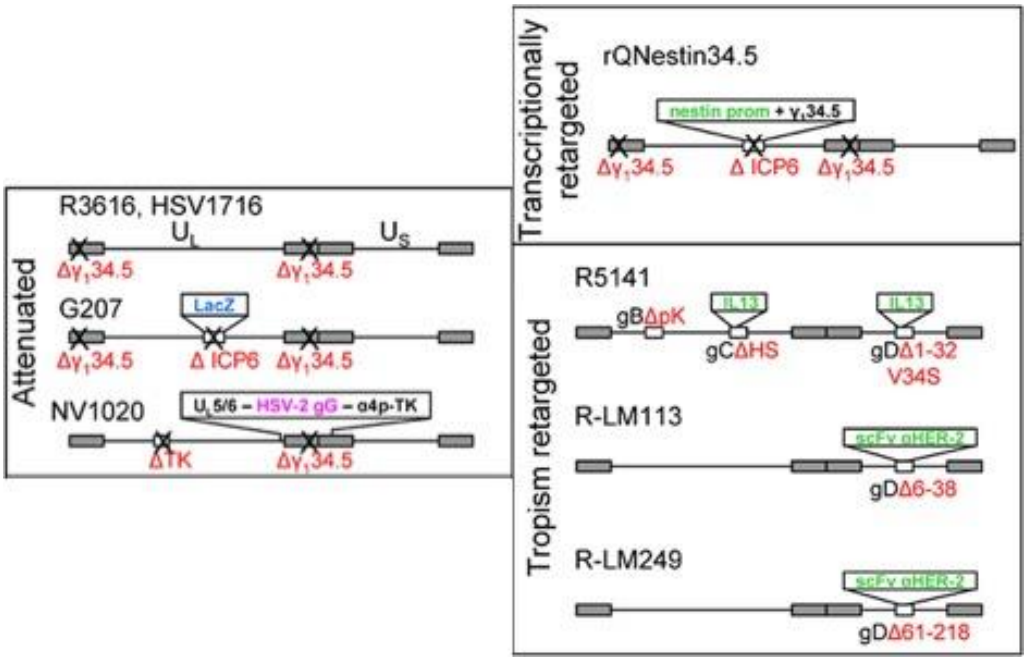

Figure 4. Schematic representation of engineered HSV-1-based oncolytic viruses. Genomes of different oncolytic HSV-1 are grouped according to the strategy adopted for tumor-restricted replication. In attenuated viruses, genes involved in the neurovirulence or in the replication (i.e., γ34.5, TK) are deleted, ensuring replication in active-dividing cells (i.e., tumor cells). In transcriptionally retargeted viruses, genes crucial for viral replication are placed under the control of tumor-selective promoter. In tropism retargeted viruses, portion of glycoproteins, crucial for the recognition of natural ligands, are deleted and replaced with $scFv_s$ targeting tumor antigens (modified from Campadelli-Fiume 2011).

1.2e HSV-1 as an oncolytic virus: arming strategies

Starting from the solely tumor selective oncolytic HSV-1 (oHSV), a second-generation of oHSV has been generated by addition of immunostimulatory transgenes within the viral genome. Thus, "armed" oHSVs have the potential to improve the antitumor efficacy by delivering directly within the TME immunostimulatory molecules.

The most loaded class are cytokines. Indeed, starting from the 1980s, cytokines have been tested as potential anti-cancer molecules, revealing a mild efficacy as monotherapy in preclinical models. However, the modest response rate, accompanied by a high systemic toxicity, has downgraded their clinical use (Berraondo 2018). Believing that a tumor-confined expression of these molecules could lead to an increased efficacy and a reduced toxicity, scientists loaded them into oHSV backbone. Among the different cytokines, IL-12 has been one of the most investigated due to its role in inducing IFNγ production, in increasing NK cell cytotoxicity and T cell proliferation as well as in suppressing angiogenesis (Del Vecchio 2007). Both attenuated and fully virulent forms of HSV-1 (i.e., M002 and R-115, respectively) have been armed with IL-12, showing an increased antitumor efficacy compared to not-armed constructs and a no significant toxicity in immunocompetent mouse model (Markert 2012; Leoni 2018; De Lucia 2020).

Despite cytokines, other classes of genes have been investigated including angiogenesis inhibitors, prodrug converting enzymes, suicide genes (i.e., TK, Toda 2001), fusogen membrane glycoproteins (i.e., GALV, Thomas 2019) and monoclonal antibodies targeting immune checkpoints (fig. 5).

Although promising results have already been achieved, more efforts have done to identify novel cargoes that can be loaded into oHSVs and further improve the antitumor immunity.

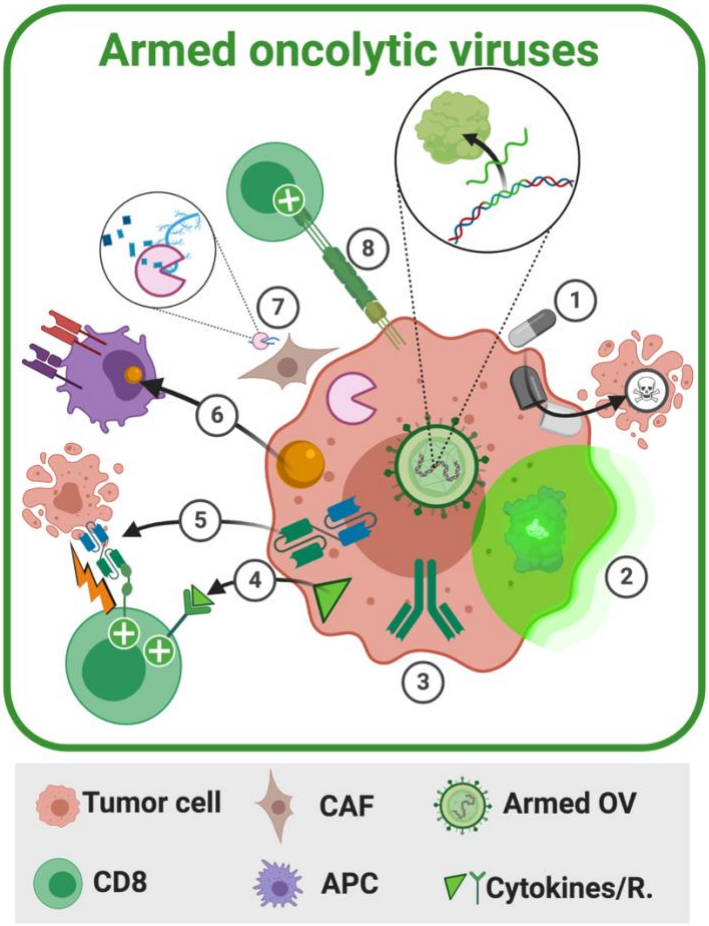

Figure 5. Classes of molecules suitable for oHSV arming. OHSV antitumor activity can be improved through insertion of different molecule genes. These payloads can be: (1) pro-drugs activators; (2) molecules for monitoring *in vivo* biodistribution; (3) monoclonal antibodies targeting immune checkpoints; (4) cytokines; (5) BiTEs targeting CAFs or tumor antigens; (6) tumor antigens; (7) extracellular matrix proteolytic cleavage; (8) ligands for co-stimulatory molecules (adapted from Sasso 2020).

1.3 Immune checkpoint

1.3a Immune checkpoint and their role in physiology and in tumor conditions

As previously described, T cell-mediated adaptive immune response requires two signals to be activated: i) binding of TCR to an antigen exposed on APC surface through MHC complex; ii) costimulatory signal (i.e., CD28, CD80, CD86, ICOS, OX40).
In physiologic condition, once T cells have been activated and have elicited their function, mechanisms of negative control occur, switching off the response and, thus, preventing damage on healthy tissues. Negative control is obtained through T cell expression of cell membrane molecules, known as immune checkpoints (Buchbinder 2016).
Among them, the first identified molecule has been cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). CTLA-4 knockout mice, characterized by the release of self-reactive T cells and resulted lethality, gave the evidence of its negative role in the regulation of T cell response (Tivol 1995). Normally, in resting T cells, CTLA-4 is mainly localized into intracellular vesicles due to a rapid internalization from the plasma membrane. Straight after engagement of stimulatory signals resulting from both TCR/antigen-MHC complex and CD28/CD80-CD86 binding, CTLA-4 is translocated to cell membrane by exocytosis of CTLA-4-containg vesicles. Once located on cell surface, CTLA-4 competes with CD28 for binding to CD80-CD86, for which it has higher affinity. Interaction between CTLA-4 and CD80/CD86 leads to shutdown of effector T cells. Moreover, CTLA-4 is able to impair T cell function through physical capture of CD80/CD86 via a mechanism known as trans-endocytosis. Removal of CD80/CD86 from APC directly reduces T cell stimulation via CD28 (Rowshanravan 2018; Qureshi 2011) (fig. 6). Beyond CTLA-4, other immune checkpoints have been identified, including PD-1. Programmed Death-1 (PD-1) is a checkpoint molecule expressed on several immunological cells, including T and B cells and NK cells. PD-1 binding with its ligands (PDL-1 and PDL-2) leads to inhibition of T cell proliferation, impairment of TNFα and IL-2 production and reduction of T cell survival (Buchbinder 2016) (fig. 6).
As described above, cells bring into play several mechanisms that allow them to evade immune recognition. Primarily, they modify TME into an immunosuppressive state, hijacking immune cell functions (i.e., induction of M2 phenotype of macrophages, regulatory state of DCs and Tregs proliferation). Tregs recruitment and proliferation play an essential role in tumor escape, as demonstrated by aberrant T cell activation and autoimmunity in Treg-specific CTLA-4 depletion model (Jain 2010). Thus, Tregs constitutively express CTLA-4 on their surface, acting as a decoy for CD80/CD86 molecules and, therefore, impairing effector T cell activation (Wei 2018).
Moreover, as a consequence of the inflammatory state, several tumors, including melanoma, breast cancer and prostate cancer, have evolved the ability to

overexpress on their cell surface inhibitory molecules as PD-L1, promoting effector T cell anergy (Ju 2020).

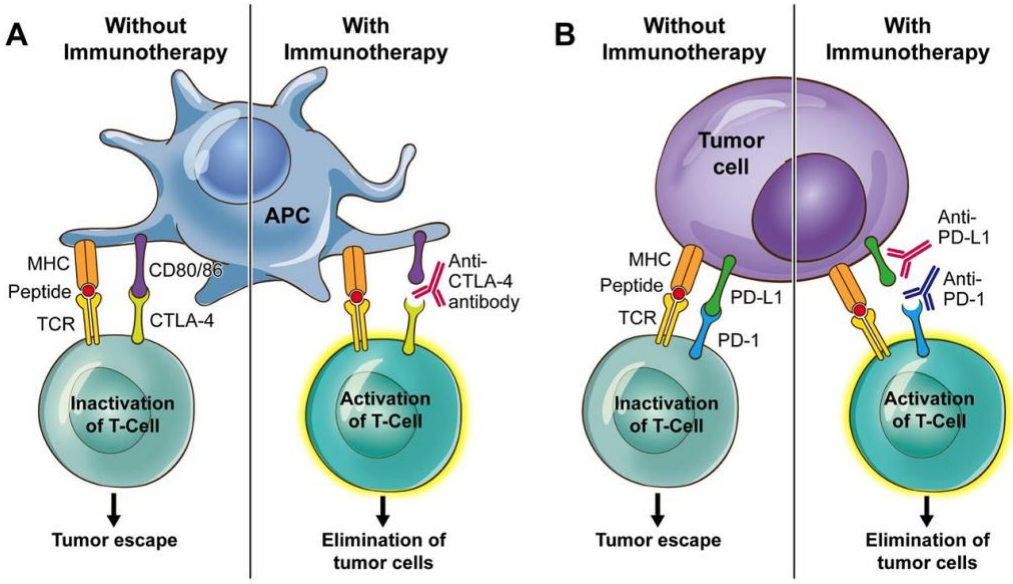

Figure 6. Role of CTLA-4 and PD-1/PDL-1 axis in tumor immunoregulation and mechanism of action of immune checkpoint inhibitors. (A) Upon activation, T cells express CTLA-4 on their surface, which competes with CD28 for CD80/CD86 binding. Binding of CTLA-4 and CD80/CD86 leads to T cell inactivation. In the tumor context, tumor cells induce Treg proliferation and the constitutive expression of CTLA-4 acts as a decoy for CD80/CD86 co-stimulatory molecules, impairing effector T cell activation. Anti-CTLA-4 monoclonal antibodies block the CTLA-4-CD80/CD86 interaction and favouring CD28-CD80/CD86 interaction, abolishing the inhibitory brake and stimulating T cell effector functions. (B) Unlike CTLA-4, PD-1 is expressed on different immune cells including exhausted T cells, B cells and NK cells. Interaction of its ligands, PDL-1 and PDL-2, leads to effector T cell inhibition. Tumor cells are able to overexpress PD-1 on their surface keeping effector T cells inactivated. Monoclonal antibodies targeting PD-1 or PDL-1 prevent the inhibitory interaction, reactivating antitumor immunity (Soularue 2018).

1.3b Immune checkpoint blockade

Based on the elucidation of the mechanisms of the immune response regulation, Allison first theorized that antibody-blockade of CTLA-4, and of an immune checkpoint molecule in general, could remove the brake from the immune response, reactivating anergic T cell.
Starting from his hypothesis, the effectiveness of monoclonal antibodies (mAbs) targeting CTLA-4, PD-1 and PD-L1 has been proved in preclinical models and clinical trials allowing to the approval for their use in several tumor indications also as first-line treatment.
Although a significant proportion of patient experienced a durable response, a large percentage of them still do not respond, prompting for combination therapy. The first combination approach has been based on CTLA-4 and PD-1 blockade. In fact, these two immune checkpoints act at different levels in T cell activation, respectively at site of priming and at inflamed peripheral tissue/tumor (Buchbinder 2016). In advanced melanoma patients, combination of ipilimumAb and nivolumAb increased not only the percentage of complete responders (57% compared to 19% and 43% of ipilimumAb and nivolumAb monotherapies respectively) but also the overall survival (Wolchok 2017). Moreover, ipilimumAb plus nivolumAb combination has also been tested in other tumor indications, including metastatic renal cell carcinoma (mRCC) and small cell lung cancer (SCLC), confirming its improved efficacy (Hammers 2017; Taylor 2015).
Despite these excellent results, several hurdles remain to be overcome. First of all, monotherapy, as well as combination treatments, are often associated with occurrence of immune-related adverse events (irAEs), due to the non-specific activation of the immune system (Boutros 2016). As expected, combined administration of different immune checkpoint antibodies (i.e., ipilimumAb plus nivolumAb) results in a higher incidence of adverse effects (Wolchok 2017).
Secondly, single agent treatment can lead to resistance development, as tumor cells can induce a compensatory upregulation of additional immune checkpoint molecules. This aspect can be partially overcome with the combination treatment (Kalbasi 2020).
Last but not least, to increase the number of patients who will benefit from the blockade of immune checkpoints, there is still a need in the identification of biomarkers useful to predict patient outcome. Both in CTLA-4 and PD-1 blockade, scientists have observed that a higher mutational tumor burden (TMB) is often associated with a better response, probably due to the release of large number of neoantigen recognizable by reactivated T cells (Wei 2018; Snyder 2014). However, response has also been observed in tumor with low TMB, opening to intriguing future investigation. Particularly, these tumors, also known as cold tumors, could find a better option in combination of immune checkpoint blockade with other immunotherapeutic strategies, as OVs.

1.3c Combination therapy with oncolytic viruses

A deeper understanding of molecular mechanisms of immune checkpoint blockade (ICB) involved in the generation of antitumor response and the awareness of needed of improved therapies for unresponsive tumors have paved the way for combination approaches.
Among different tumor types, cold tumors are continuously under investigation as they are almost completely refractory to ICB. Thus, these tumors are characterized by an almost total absence of immune cell infiltration within TME, which could explain ICB resistance.
As mentioned before, cold tumors could find a better treatment option combining OV treatment with ICB. Indeed, it is known that OV administration prompts TAA release and cytokines production and attracts immune cells, converting the "cold" TME into a "hot" one. In the sense of antitumor response, OV acts as prime/boost and combination with ICB ensures improvement of the efficacy of this response by removing the inhibitory brakes (Bastin 2016). To identify the best agent combination and the right timing of administration, several preclinical and clinical studies have been performed in different tumors.
Focusing the attention on oHSVs, first evidences of the potency of combination treatment came from Chen and colleagues. Using an immunocompetent rhabdomyosarcoma mice model, they demonstrated that combined administration of oncolytic HSV1716 and α-PD1 antibody significantly prolonged the survival of treated mice compared to placebo and monotherapy treatments. As expected, the beneficial effect was lost in immunocompromised mice, highlighting the crucial role of the immune system in the antitumor response. In agreement with their findings, characterization of the immune signature within the tumor revealed "hot" immune changes, as shown by the effector CD4 and CD8 T cell increased infiltration (Chen 2017).
Similar results were obtained by Saha and colleagues in a murine model of glioblastoma (GBM), combining oHSV G47Δ (encoding IL-12) and PD1/PDL1 blockade. Since they obtained only a modest percentage of treated mice, for the first time they set up a triple combination, adding an α-CTLA4 antibody to the combination. This treatment approach led to 77% of treated mice, which were also protected from tumor rechallenge 6 months after the initial treatment. (Saha 2017).
Supported by the promising results obtained in preclinical models, combination of oHSV and immune checkpoint inhibitors were also tested in clinical trial. Based on the promising results obtained in monotherapy, T-VEC was one of the first viruses combined both with α-PD1 or α-CTLA-4 antibodies. In both phase Ib/II trials conducted on advanced stage melanoma patients, combination increased the rate of overall response, without any increase in toxicity (Sun 2018; Chesney 2018).

2. AIM

Cancer represents the second cause of worldwide mortality. Despite many efforts have been done to find suitable cures to eradicate cancer, conventional therapeutic approaches (i.e., surgery, radiotherapy, chemotherapy) still result unsatisfactory.
In this *scenario*, a deeper understanding of the immune system as well as of the interactions between immune and tumor cells has paved the way for immunotherapy to become a viable treatment option, especially for those tumors highly refractory to conventional therapies.
Among the different immunotherapeutic approaches, monoclonal antibodies (mAbs) targeting immune checkpoint, and oncolytic viruses (OVs), represent a turning point in anti-cancer strategies. Indeed, in recent years, an increasing number of mAbs and OVs have been tested in preclinical and clinical trials, leading to regulatory agencies approval for some of them, as the first line treatment in selected tumor indications.
Moreover, to broaden the percentage of patients that could take advantage from this approach, combination of various classes of immunotherapy (i.e., mAbs and OVs) has been exploited. This approach has shown increased efficacy, even if unspecific activation of immune cells mediated by mAbs has often been correlated with the occurrence of immune-related adverse events (irAEs).
Although improvement reached by combination approaches, advanced stages and some types of cancers still remain unassailable (i.e., pancreas, brain, breast). Studies performed on those tumors have highlighted the crucial role of the TME inflammatory status in the immunotherapy-mediated antitumor response. Against this background, modulation of the TME has become the key point to successful immunotherapy, focusing on the conversion from a "cold" to a "hot" signature. In this case, OVs treatment has the ability to recruit immune cells within the "cold" TME and the combination with immune checkpoint inhibitors may actually boost the immune response.
According to this scientific background, the purpose of my PhD project was focused on the improvement of a platform of immunotherapeutics, based on both oHSV and αCTLA-4 antibody, able to modulate the TME. To do so, I:

- Evaluated the efficacy of combination approach, identifying in the loading of an edited version of αCTLA-4 antibody gene within the viral genome the best option to potentially reduce the irAEs occurrence.
- Boosted the oHSV efficacy by addition of IL-12 gene within the viral genome. The improved efficacy allowed the lowering of the delivered dose, with optimal translational power.
- Identified a human/murine cross-reactive αCTLA-4 antibody by high throughput phage display screening, which could be not only useful for identification of novel mechanism of action of this immune checkpoint inhibitor, but also suitable for both preclinical and clinical use.

3. MATERIALS AND METHODS

3.1 Cell cultures

SKOV3 cells were cultured in RPMI Medium 1640-GlutaMAXTM-I supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin 10,000 U/mL (all from Gibco, Life Technologies, Inc.). HEK293 and HEK293-EBNA-SINEUP cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin 10,000 U/mL, 2 mM L-glutamine (all from Gibco, Life Technologies, Inc). For propagation of HEK293-EBNA-SINEUP, DMEM was also supplemented with 250 μg/mL Geneticin (G418, Gibco, Life Technologies, Inc.) and 100 μg/mL Hygromycin B (Invitrogen).
Cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

3.2 Viruses

LM-113 derives from wild-type human herpesvirus 1 strain F and contains a bacterial artificial chromosome (BAC) between UL3-UL4 viral region (Szpara 2010), flanked by LoxP sites. It also contains Cre gene, whose restricted expression within eucaryotic cells is ensured by the presence of an intron in Cre coding sequence. LM-113 carries an scFv to HER2 in gD Δ6-38 region.
To rescue the generated viruses, SKOV3 cells were plated in 12well plates and transfected with 500 ng of BAC-HSV DNAs with Lipofectamine Transfection Reagent (Life Technologies, Inc, 11668019) and grown in low serum RPMI medium at 37°C until full cytopathic effect (cpe) was reached. Produced viruses were amplified by infecting fresh plated SKOV3 cells at multiplicity of infection (MOI) 0.1 and incubated in low serum RPMI medium at 33°C until full cpe was reached. Viral particles were purified by a self-generated iodixanol gradient and titrated by plaque assay.

3.3 Modification of BAC-HSV-1

Modification of BAC-HSV-1 genomes was performed by recombineering strategy. It consists of two subsequential selection steps. Briefly, during the first step, a DNA fragment containing SacB/Amp^R/LacZ markers was amplified by PCR from a donor plasmid using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, #F-540L), following manufacturer's instructions. Oligonucleotides used for the amplification contained at the 5' end 60 base-pairs of perfect homology to the region that has to be engineered. The PCR product was purified from 1% agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, REF A9282) and electroporated into competent heat-induced SW102 cells, containing BAC-HSV-1 of interest. Following 1 hour of recovery, cells were plated on LB agar with 12.5 μg/ml Chloramphenicol, 20

μg/ml Ampicillin, 80 μg/ml X-gal, 200 μM IPTG. By replica plating, colonies were plated on LB agar containing 12.5 μg/ml Chloramphenicol and sucrose. Blue colonies unable to grow on sucrose were cultured in LB medium with 12.5 μg/ml Chloramphenicol for at least 16 hours and DNA was extracted using NucleoBond PC20 (Macherey-Nagel, 740571.100), following manufacturer's instruction. Correct insertion of the DNA cassette was verified by PCR using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, #F-540L) and Sanger sequencing.
As done for the first step, the DNA of interest was amplified by PCR from a donor plasmid using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, #F-540L) with oligonucleotides containing the same 60 base-pairs of homology used for the first step. The PCR product was purified from 1% agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, REF A9282) and electroporated into competent heat-induced SW102 cells, identified as positive after the first step. After 4 hours and half of recovery, cells were plated on LB agar with 12.5 μg/ml Chloramphenicol and containing sucrose.

3.4 *In vivo* studies

C57BL/6 mice tolerant to human HER2 (C57-HER2) were used for *in vivo* studies. Mice were challenged with 5x10^5 Lewis Lung Carcinoma cells stably expressing human HER2 (LLC-HER2) by subcutaneous injection on the right flank (day -10). At day 0 treatments started in animals previously randomized according to tumor size. THVs were intra-tumor injected five times at 1x10^8 PFU/injection or 1x10^7 PFU/injection every 2-3 days, α-m-PD-1 (BioXcell, clone RMP1-14) was administered intraperitoneally at 200 μg twice a week until day 10, α-m-CTLA-4 was administered intraperitoneally at 100 μg at day 0 and day 3. Tumor growth was monitored by measurement with a digital caliper every 3-4 days. Mice were sacrificed as soon as signs of distress or a tumor volume above 1500 mm^3 occurred.

3.5 DNA fragment preparation and libraries generation for high-throughput sequencing

For each cycle, the scFv-containing double strand DNAs were purified from superinfected E. coli TG1 cell cultures using Endo free Plasmid Maxi Kit (Qiagen, 12362). Heavy chain fragments were isolated by two-step restriction process (Sasso 2018). Briefly, full-length scFvs (VH-Linker-VL) were extracted using BamHI (R3136) and HindIII (R3104) and purified from 1.5% agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, REF A9282). To generate fragments suitable for Next Generation Sequencing (NGS), variable light (VL) chain was removed from full-length scFvs by digestion with NcoI (R3193) and XhoI (R0146). All restriction enzymes were from New England

Biolabs. Obtained fragment was purified from 1.5% agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, REF A9282).
Library preparation for sequencing and preliminary bioinformatic analysis of the data were performed at the Center for Translational Genomics and Bioinformatics, Hospital San Raffaele, Milano, Italy, as previously reported (Sasso 2015; Cembrola 2019).
Briefly, isolated VH fragments from each cycle were bar-coded by TruSeq ChIP sample prep kit (Illumina, 15023092) and sequenced to a final concentration of 10 pM with 2 × 300 nt SBS kit v3 on an Illumina MiSeq apparatus. For each sub-library, raw counts were normalized to the total number of counts, obtaining count per million (cpm) values. Obtained nucleotide sequences were translated, taking into account the correct open reading frame.

3.6 Recovery of scFv$_s$ from the enriched sub-library and conversion into full antibodies

The clones of interest were isolated from competitive elution cycle_3 and acidic elution cycle_2 by overlapping PCR (Sasso 2015). Briefly, to obtain separate VH and VL fragments, two different PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, F530S) and clone-specific primers designed within each HCDR3 and in the constant region of plasmid upstream and downstream of VH and VL. To obtain full-length scFvs, VH and VL fragments were mixed and extended by HCDR3 overlapping PCR. Recovered scFvs were converted into whole human IgG4 antibodies by cloning the corresponding VH and VL cDNAs in the SINEUP-competent 8.2VH and 4.2VL pEU vectors, expressing the constant heavy and light chains respectively (Sasso 2018). Briefly, VHs and VLs were amplified by CloneAmp HiFi PCR Premix using specific oligos. In-Fusion HD cloning kit (Clontech Laboratories, 639692) was used to clone VH in BamHI (R3136S) and BssHII (R0119L) linearized pEU8.2VH vector, and VL in ApaLI (R0507L) and AvrII (R0174) linearized pEU4.2VL vector. All restriction enzymes were from New England Biolabs.
The obtained vectors were transformed into Stellar Competent Cells (Clontech Laboratories, 636763) and the resulting colonies were screened by sequence analysis. The vectors containing the correct DNA sequences were prepared with an endotoxin-free system (EndoFree Plasmid Maxi Kit, Qiagen, 12362).

3.7 Antibody production and purification

ScFv-Fc and full αCTLA-4 antibodies were produced transfecting the expression vectors by using Lipofectamine Transfection Reagent (Life Technologies, Inc. 11668019) into HEK293 cells.
Antibodies isolated from the screening were produced co-transfecting VH and VL expressing vectors by using Lipofectamine Transfection Reagent (Life Technologies, Inc. 11668019) into the production enhanced cell line HEK293-

(40-90%)EBNA-SINEUP (HEK293_ES1), expressing the long non-coding SINEUP targeting heavy and light chain signal peptide on mRNAs (Sasso 2018). Transfected cells were grown for 10 days at 37 °C in CHO medium supplemented with 1% L-glutamine 200 mM and 1% Penicillin-Streptomycin 10,000 U/mL (all from Gibco, Life Technologies, Inc). The antibodies were purified from the conditioned media by using Protein A HP SpinTrap30 (GE Healthcare Life Science, 28-9031-32), following manufacturer's instruction.

3.8 ELISA assay

Sandwich ELISA assays were performed. Murine CTLA-4-Fc, human CTLA-4-Fc or Fc portion (negative control) were coated on NuncTM flat-bottom 96well plates at a concentration of 5 μg/mL and blocked with PS/milk 5% at 37°C for 1 hour. Samples were loaded into immobilized chimeric protein wells with PBS/milk 2.5% buffer solution and incubated for 2 hours at RT. Washes were performed with PBS1x to remove unbind antibodies. HRP-conjugated anti-IgG goat monoclonal antibody diluted in PBS/milk 2.5% buffer solution was added and incubated for 1 hour at RT. After washes with PBS 1x, plates were incubated with TMB (Sigma-Aldrich, St. Louise, USA) reagent for 10 min, before quenching with an equal volume of 1 N HCl. Absorbance at 450 nm was measured by the Envision plate reader (Perkin Elmer, 2102, San Diego, CA, USA).

		Retargeting		*Arming*	
Recombinant virus	*Parental virus*	*scFv to*	*Site of insertion*	*Cargo*	*Site of insertion*
LM-113		HER2	gD Δ6-38		
THV_αCTLA4	LM-113	HER2	gD Δ6-38	αCTLA4	UL26-UL27
THV_IL12	LM-113	HER2	gD Δ6-38	IL-12	US1-US2
THV_αCTLA4/IL12	THV_αCTLA4	HER2	gD Δ6-38	αCTLA4 and IL-12	UL26-UL27 and US1-US2

Table 1. Summary of the generated viruses. To facilitate reader comprehension, the table summarize the genomic modification carried by each virus that will be explained within this thesis.

4. RESULTS

4.1 *In vivo* combination of oncolytic virus and immune checkpoint inhibitors

As previously discussed, preclinical and clinical evidences show a significant synergy in cancer eradication between oncolytic viruses and immune checkpoint inhibitors. Our research group, in collaboration with Nouscom, has previously demonstrated that combination of human HER2-retargeted oncolytic HSV-1 (LM-113) and PD-1 blockade is effective in almost 50% of immunocompetent tumor bearing mice (Sasso 2020) leaving space to improve treatment efficacy. To test this hypothesis, we tested the triple combination of LM-113 with PD-1 and CTLA-4 blockade. To explore the efficacy of this triple combination, we exploited murine LLC (Lewis Lung Carcinoma) cells stably expressing human HER2 (LLC-HER2), to make them susceptible to LM-113 infection.
LLC-HER2 cells were subcutaneously injected in C57BL/6 mice tolerant to human HER2 (expressing human HER2 under the control of WAP promoter).
As schematized in figure 7A, ten days after implant, mice bearing well-established tumors (mean volume 115 mm^3) were randomized according to tumor size and subdivided into six experimental groups:

- Untreated
- αPD-1, receiving 6 intraperitoneal injection of a commercial anti-mouse PD-1
- αCTLA-4 (9D9), receiving 2 intraperitoneal injection of a commercial anti-mouse CTLA-4
- LM113, receiving 5 intra-tumor injection of LM113 (1x10^8 pfu)
- LM113+ αPD-1
- LM113+ αPD-1+ αCTLA4 (9D9)

Treatment efficacy was monitored through tumor size measurement (mice were sacrificed once tumors were >1500 mm^3). In accordance with previous results, single agent treatments showed no efficacy in this model and double combination of LM-113 and αPD-1 confirmed a 50% of complete response (4 animals out of 8). The triple combination exerted an additive efficacy compared to OV+PD1 as five out of 8 animals (63%) resulted tumor-free at the endpoint of the experiment (fig. 7B), with the occurrence of an additional partial responder.

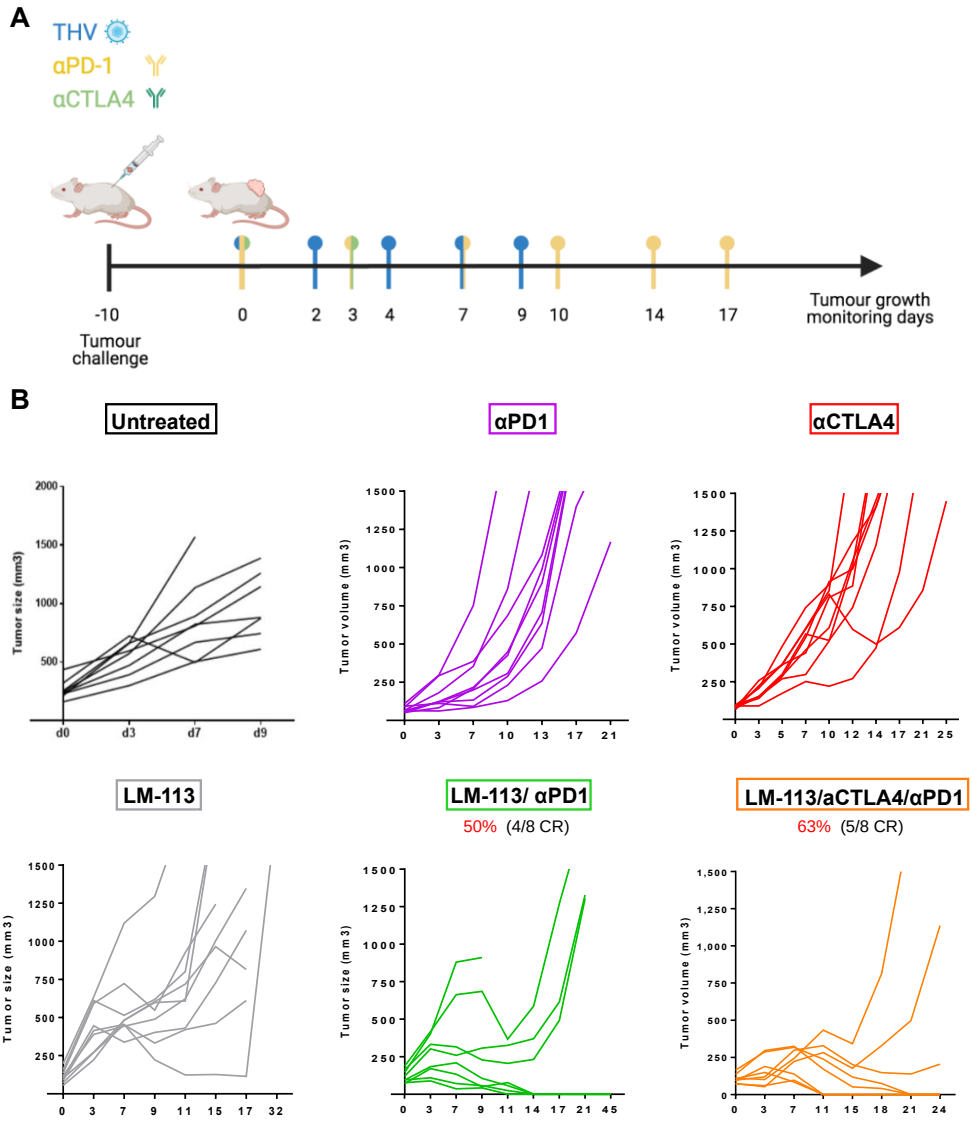

Figure 7. ***In vivo*** **activity of LM113 in combination with immune checkpoint inhibitors.** (A) Schematic representation of the experimental setting. HER2-LLC cells were used to challenge hHER2 transgenic/tolerant C57BL/6 mice by subcutaneous injection. Ten days after implantation, mice were treated with six intra-peritoneal injection of αPD-1 (200 µg/injection), two intra-peritoneal injection of αCTLA-4 (100 µg/injection), five intra-tumor injection of LM-113 (1x10^8 pfu/injection) or a combination of them depending on the experimental group. (B) Graphs show growth curve of untreated, αPD-1, αCTLA-4, LM-113 or combo-treated tumors. Each line represents an individual HER2-LLC tumor followed over time. Percentages indicate the response rate.

4.2 *In vitro* optimization of commercial αCTLA-4 antibody suitable for HSV-1 arming

Despite the combination of immune checkpoint blockade has been proved to exert remarkable synergy in antitumor efficacy, the increased incidence of immune-related adverse events (irAEs) cannot be underestimated (Boutros 2016). These events are attributed to the systemic administration of two or more antibodies that can lead to the activation and expansion of autoreactive T cells breaking self-tolerance (Postow 2018).
In this *scenario*, the confined expression of the antibody within the TME could ensure a selective activation of tumor-resident T cells.
OV has been widely exploited as shuttle for immunoadjuvants (Sasso 2020). This approach allows to encode payloads of interest within OV's genome to produce a protein of interest before cancer cells undergo lysis.
Based on this concept, we decided to encode αCTLA-4 antibody into LM-113 genome.
The size of payload expression cassette (promoter-payload coding sequence-polyA) could be a limiting factor for packaging capacity. The classic IgG antibodies consist of heavy and light chains encoded separately by two different expression cassettes (or expressed as bicistronic construct) (fig. 8A).
To optimize the minimal size of the antibody, not at expense of effector function that requires Fc portion, I designed and cloned the murine 9D9 αCTLA-4 mAb as both full antibody (two vectors encoding heavy and light chains) and as scFv-Fc. In the scFv-Fc layout, VH and VL sequences were joined by a flexible linker, mainly constituted by glycine and serine amino acids, and followed by the nucleotide sequence of IgG2A Fc.
To evaluate the productivity and binding capacity of full mAb *versus* scFv-Fc, both constructs were transfected into HEK293 cell line and recombinant proteins were purified by protein-A affinity chromatography. Purified mAbs were loaded on polyacrylamide gel in non-reducing condition to preserve disulfide bounds that respectively join tetramer and dimer for full mAb and scFv-Fc.
As shown in figure 8B, scFv-Fc antibody exhibited the expected size of ~140 kDa, confirming its ability to correctly fold and assemble.
To complete the *in vitro* characterization, I compared the binding affinities of the scFv-Fc and full mAb by ELISA assay on murine CTLA-4 recombinant protein. As shown in figure 8C, scFv-Fc was able to bind CTLA-4 in a similar fashion to the full antibody. Based on these experiments, we decided to clone scFv-Fc version of murine 9D9 antibody into LM-113 genome.

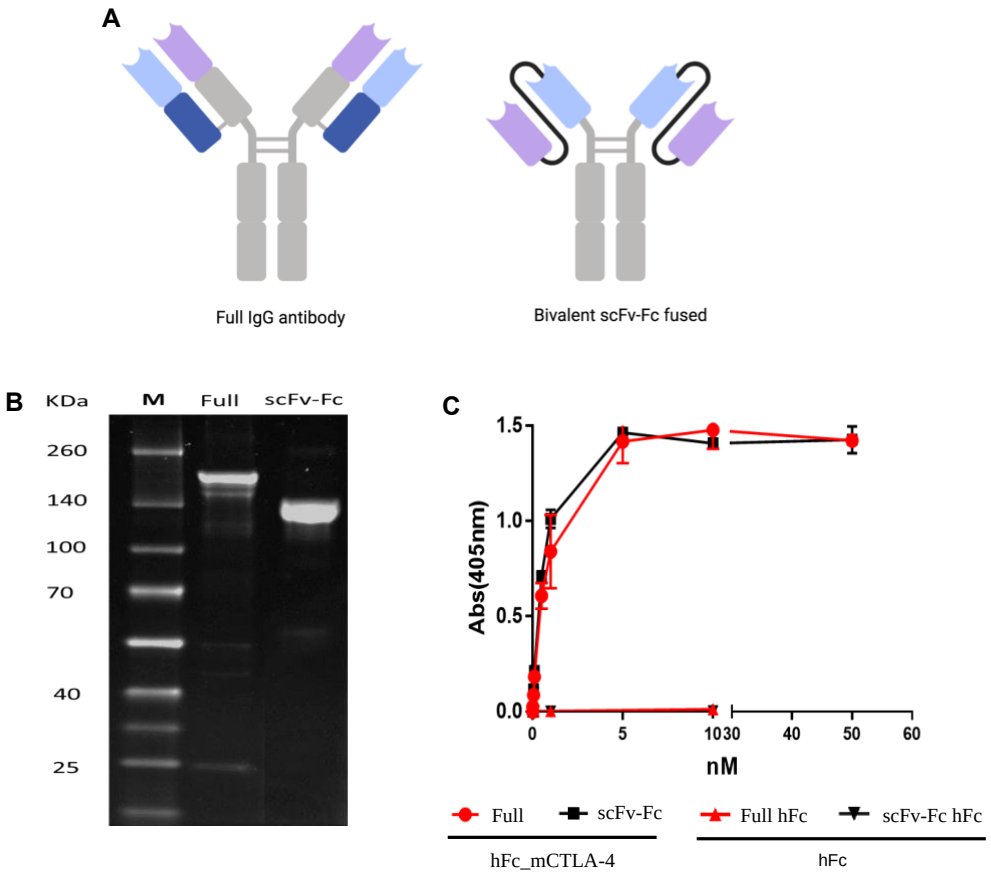

Figure 8. *In vitro* generation and characterization of bivalent scFv-Fc αCTLA4 antibody. (A) The cartoon shows the structural differences between classic IgG antibody and the generated bivalent scFv-Fc antibody. (B) Recombinant antibodies were loaded on polyacrylamide gel in non-reducing condition, showing both the expected size (150 kDa and 140 kDa respectively). (C) The graph shows the binding specificity of both full IgG and scFv-Fc antibodies in recognizing murine CTLA-4 protein.

4.3 Generation of αCTLA-4_armed HSV-1 oncolytic virus and *in vivo* efficacy evaluation

A mouse codon usage-optimized sequence of αCTLA-4 9D9 scFv-Fc was generated and cloned into an intergenic *locus* of LM-113 genome (Menotti 2008). We previously proved that insertions in UL26-UL27 intergenic *locus* are not detrimental for viral productivity and thus selected for αCTLA4 scFv-Fc insertion (De Lucia, 2018).
The LM-113 construct containing the BAC region was exploited to generate the novel vector of interest by recombineering-mediated homologous recombination (HR). Briefly, as the HR is a quite rare event, in order to facilitate the selection of positive clones, recombineering consists of two subsequent steps of positive and negative selection. In the first step, a selection cassette containing *sacB/Amp^r/lacZ* was inserted into the UL26-Ul27 *locus* by 50bp homology arms (HA). *Amp^r* and *lacZ* are positive markers and can be used to identify clones that have inserted the cassette in a desired *locus*. On the contrary, *sacB* encodes for levansucrase enzyme that converts sucrose to levan, a harmful molecule for bacteria. This gene was used as a counterselectable marker in the second step, during which the αCTLA-4 DNA construct replaced the first selection.
Following the recombineering, PCR amplicon size and Sanger sequencing were used to prove the *bona fide* insertion and the correct generation of αCTLA4-armed LM-113, hereinafter referred to as Targeted Herpes Virus_αCTLA4 (THV_αCTLA4) (fig. 9). I rescued the viral particles by transfection and subsequent amplification in HER2+ SKOV3 cell line. Infectious viral particles were purified by iodixanol gradient for *in vivo* testing.

As preliminary to efficacy studies, the *in vivo* expression of αCTLA-4 scFv-Fc encoded by the virus was assessed in a small group of LLC-HER2 tumor-bearing mice. I collected the sera at the endpoint of the experiment. As shown in figure 10, ELISA assay revealed that all mice treated with THV_αCTLA4 showed nanograms quantity of CTLA-4 antibodies, confirming that the virus was able to express the cargo.
Thus, THV_αCTLA4 was tested *in vivo* in the previously described established tumor model of LLC-HER2. Tumor size was monitored up to the endpoint of the experiment. As expected, the efficacy of double combination of THV_αCTLA4 + αPD1 was comparable to the triple combination with αCTLA4 recombinant antibody. Indeed, 4 out of 6 mice responded to the treatment (3 complete responders, 1 partial responder) (fig. 11).

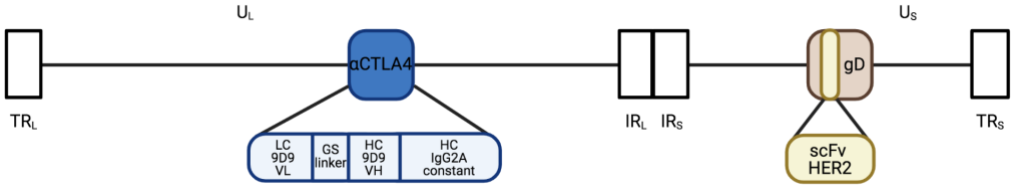

Figure 9. Schematic representation of the generated THV_αCTLA4. The cartoon shows the gD gene, carrying the replacement of aa 6-38 with HER2 scFv, and the CTLA-4 scFv-Fc gene inserted within UL26-UL27 region.

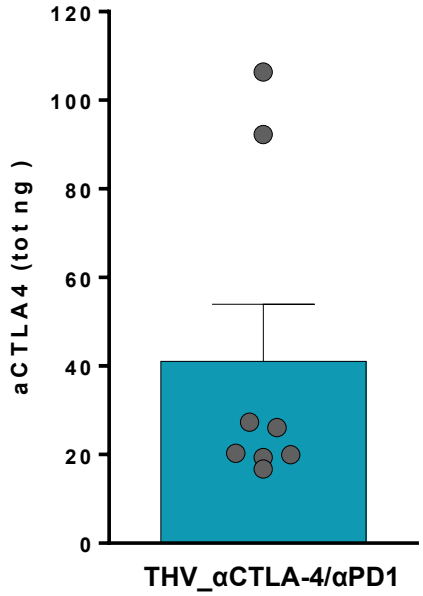

Figure 10. THV_αCTLA4 successfully mediates αCTLA-4 expression *in vivo*. Sera from mice treated with THV_αCTLA-4 were collected at the end point of the experiment and were analyzed by ELISA assay for the evaluation of the αCTLA-4 levels. The average expression is shown by the histogram. Each dot represents a single animal.

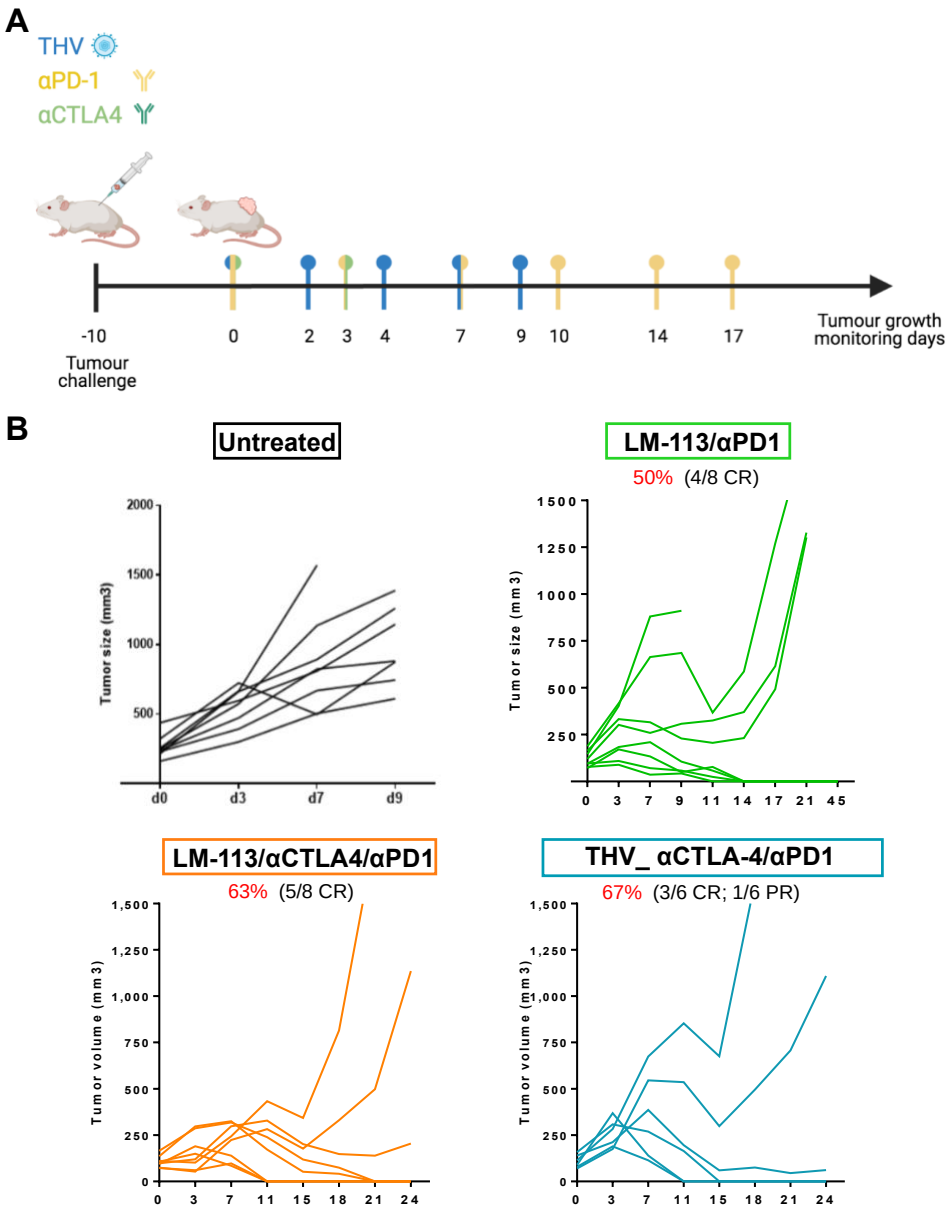

Figure 11. *In vivo* activity of THV_αCTLA4 in combination with α-PD1. (A) Schematic representation of the experimental setting. At day -10, hHER2-transgenic/tolerant mice were challenged with HER2-LLC cells by subcutaneous injection. Treatment started in randomized established tumors at day 0. Five intra-tumor injection of LM-113 or THV_αCTLA4 (1x10^8 pfu/injection), two intraperitoneal injections of αCTLA-4 (100 µg/injection) and six intra-peritoneal injection of αPD-1 (200 µg/injection) were performed. (B) The volume of the tumor over time is depicted. Each line reflects the progression of a single tumor. Percentage indicates the rate of response as sum of partial and complete responders.

4.4 Enhancement of THV_αCTLA4 efficacy through addition of vectored mIL12: generation of the oncolytic viruses and *in vivo* evaluation of their efficacy

Our results demonstrated that THV_αCTLA4 is as effective as the combination of the unarmed THV with immune checkpoint inhibitors but with a potential reduction in the development of side effects.
To further improve effectiveness of THV_αCTLA4, we decided to encode a second transgene within its genome to boost the antitumor immune response. Among the different possible cargoes, we recently demonstrated that interleukin 12 (IL-12) is a powerful transgene in immunosuppressive tumors including LLC and GBM (De Lucia 2020; Cheema 2013). Moreover, the use of IL-12 encoding OVs (HSV and adenoviruses) is currently evaluated in different clinical trials (NCT04806464, NCT02555397, NCT02062827).
The natural mature form of IL-12 (p70) is a heterodimer composed by p40 and p35 linked by disulfide bond. We previously described a THV armed with IL-12 in which p40 and p35 subunits were expressed as bicistronic construct by internal ribosome entry site (IRES). Interestingly, p35 and p40 can assemble with other subunits (i.e., p40-p19) to form the heterodimeric cytokine IL-23. To avoid the production of non-desired IL-23, and to improve the production of IL-12, here, we generated a next-generation IL-12 construct by fusing p35 to p40 by a flexible linker.
Thus, I again used the recombineering cloning technique to modify the parental LM-113 or the THV_αCTLA4 to obtain both the single IL-12 armed (THV_IL12) and the double IL-12/αCTLA-4 armed (THV_αCTLA4/IL12) viruses. In both cases the selected *locus* for the insertion of IL-12 was the intergenic region between US1 and US2 (fig. 12), as successfully used for the insertion of different transgenes (De Lucia 2020; Menotti 2018).
As for THV_αCTLA4, positive clones were confirmed by PCR and Sanger sequencing to check the insertion of both transgenes in the selected *locus*.
To evaluate the antitumor activity of the latter generated viruses, I performed *in vivo* studies using the very same mouse model shown before. After ten days from the implantation, mice bearing well-established tumors were randomized according to tumor size and subdivided into three experimental groups:

- Untreated
- LM-113, receiving 5 intra-tumor injection of LM-113 (1x10^8 pfu)
- THV_IL12, receiving 5 intra-tumor injection of the virus (1x10^8 pfu)

As IL-12 is known to be a potent activator of the immune response, virus was firstly tested as monotherapy. As already demonstrated, monotherapy of LM-113 was ineffective in this model. Surprisingly, as shown by the tumor volume growth curves in figure 13B, 10 animals out of 12 (80%) receiving THV_IL12 resulted tumor-free at the endpoint of the experiment, confirming the crucial role of the immune system in the oHSV-mediated antitumor response.

Nevertheless, despite the excellent result, this high effectiveness would not allow to appreciate any synergistic effects related to the double armed virus. Thus, I decided to test the THV_IL12 in a suboptimal condition, lowering the viral load by one logarithm (from 1x10^8 to 1x10^7 pfu/injection). To verify whether this dosage was still effective and to evaluate the potential synergistic effect of the cargoes of the double armed oHSV, I run the following scheme:

- Untreated
- THV_IL12 low dose, receiving 5 intra-tumor injection of the virus (1x10^7 pfu)
- THV_αCTLA4/IL12, receiving 5 intra-tumor injection of the virus (1x10^7 pfu)

As shown in figure 14B, THV_IL12 was still effective at a lower dose, reaching 45% of efficacy (5 out of 11). Moreover, THV_αCTLA4/IL12 showed a higher percentage of complete responders (60%) in monotherapy, confirming the synergistic effect of the two cargoes. In addition, the good efficacy obtained with a reduced dose represents a strength for the translational aspect.

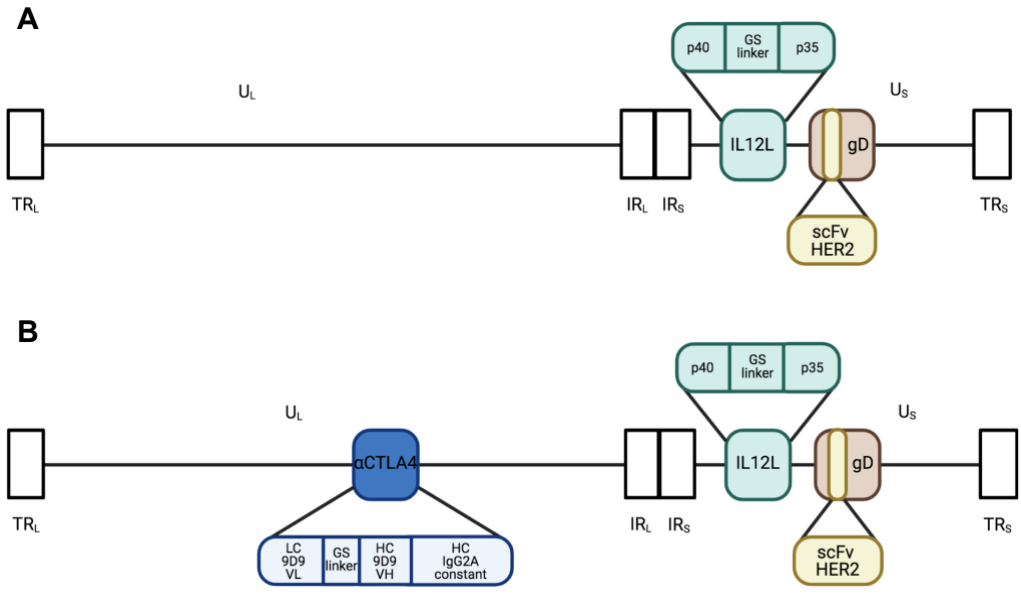

Figure 12. Schematic representation of generated THV_IL12 and THV_αCTLA4/IL12. (A) The cartoon shows the gD gene, carrying the replacement of aa 6-38 with HER2 scFv, and the IL-12 gene inserted within US1-US2 region. (B) The cartoon shows the gD gene, carrying the replacement of aa6-38 with HER2 scFv, the IL-12 gene inserted within US1-US2 region and the αCTLA-4 scFv-Fc gene inserted between UL26 and UL27.

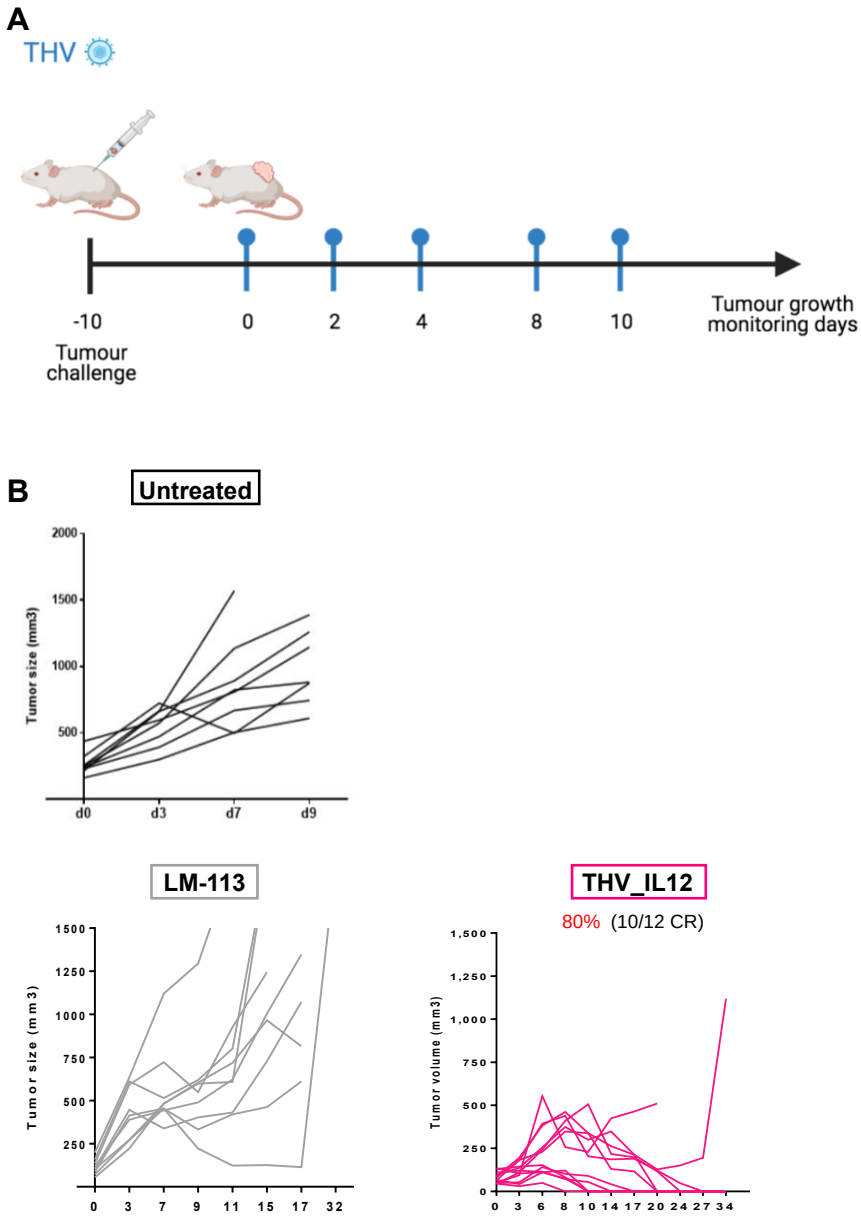

Figure 13. *In vivo* activity of THV_IL12. (A**)** Schematic representation of the experimental setting. At day -10, hHER2-transgenic/tolerant mice were challenged with HER2-LLC cells by subcutaneous injection. Treatment started in randomized established tumors at day 0. Five intra-tumor injection of LM-113 or THV_IL12 (1x10^8 pfu/injection) were performed. (B) Tumor volumes over time are shown. Each line reflects the progression of a single tumor. Percentage indicates the rate of response.

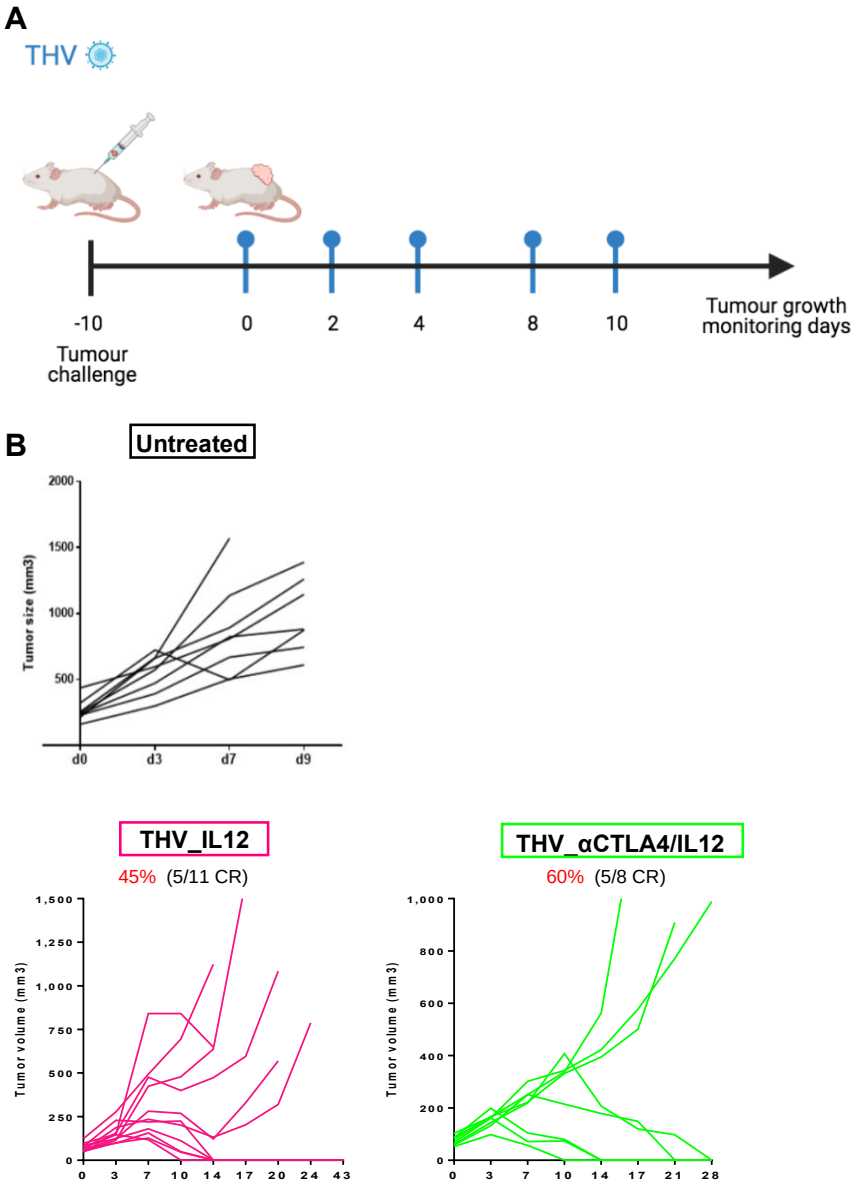

Figure 14. *In vivo* activity THV_IL12 and THV_αCTLA4/IL12 at a lower dosage. (A**)** Schematic representation of the experimental setting. At day -10, hHER2-transgenic/tolerant mice were challenged with HER2-LLC cells by subcutaneous injection. Treatment started in randomized established tumors at day 0. Five intra-tumor injection of THV_IL12 or THV_αCTLA4/IL12 (1x10^7 pfu/injection) were performed. (B) Tumor volumes over time are shown. Each line reflects the progression of a single tumor. Percentage indicates the rate of response.

4.5 Parallel screening and selection of human/murine cross-reactive CTLA-4 scFvs

In light of the promising results obtained from *in vivo* treatment with CTLA-4_armed viruses, we evidenced the need for the isolation of novel, proprietary αCTLA-4 antibodies. As the mechanism of action of αCTLA4 antibodies relies on Fc effector function through its interaction with Fc receptors (FcRs), the *in vivo* experimentations are essential to study the biological activity of novel mAbs (Vargas 2018). Many αCTLA-4 mAbs investigated in clinical trials or even approved (i.e., ipilumumAb) do not recognize mouse or rodent CTLA-4, and thus, have required challenging preclinical validation exploiting humanized mouse models or non-human primates (Du 2018; Levisetti 1997). To overcome this hurdle, with the aim to make preclinical to clinical transition easier, our efforts were dedicated to isolate human/murine cross-reactive αCTLA-4 antibodies.

Our group has developed a strategy for high throughput screening (HTS) of phage display libraries of scFvs based on Next Generation Sequencing (Sasso 2015; Sasso 2018).

As depicted in figure 15, the selection of CTLA-4 specific scFvs was based on three rounds of selection performed in collaboration with the research group of Prof. C. De Lorenzo. According to the previous screening optimization, aiming to identify clones with specificity for the target protein in its native conformation, the first selection cycle was performed on activated human peripheral blood mononuclear cells (hPBMCs). Indeed, as explained in the introduction, T lymphocytes naturally express on their surface CTLA-4 after activation. To enrich the potential binders, isolated phages were further selected by two additional selection rounds performed on recombinant CTLA-4 protein. The latter consisted of two positive panning on recombinant chimeric CTLA-4-Fc protein followed by two negative rounds on Fc portion, to remove unspecific binders recognizing the Fc domain.

To ensure the identification of human/murine cross-reactive CTLA-4 scFvs, two parallel third cycles were performed using human or murine recombinant CTLA4-Fc proteins.

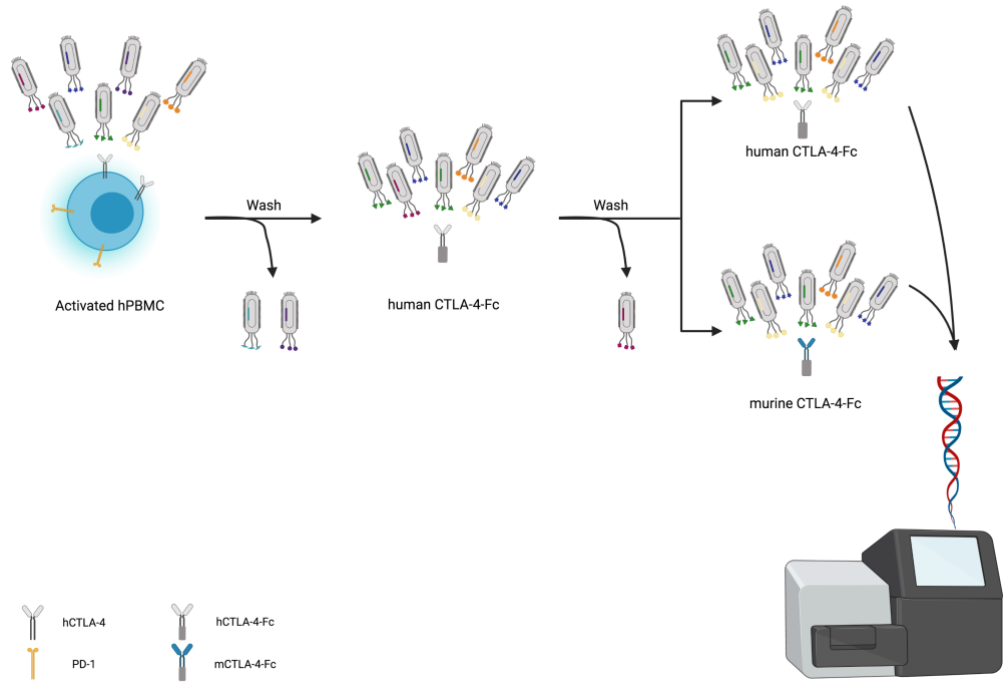

Figure 15. Phage display screening workflow. The cartoon shows the workflow of the selection cycles. Phage library was firstly incubated with activated hPBMC. After the binding, unbound clones were washed away and specific binders were eluted, amplified and incubated with recombinant CTLA-4-Fc fused (second cycle). After a negative panning on Fc, enriched clones were amplified and incubated with recombinant human or murine CTLA-4-Fc protein in two parallel cycle_3. After a second negative panning on Fc, clones were eluted and amply. Phagemid DNAs was isolated and sequenced on MiSeq Illumina platform.

4.6 Identification of CTLA-4 specific binder through Next Generation Sequencing

To widen the range of possible binders and identify even the least represented clones, I coupled the phage screening to Next Generation Sequencing (NGS). This approach allows to identify target-specific scFvs based on enrichment trend alongside the cycles as well as on the representativeness within each cycle.
Hence, at the end of the selection cycles, I extracted the dsDNA from superinfected *E. coli* cultures. Firstly, I extracted valid insert of scFv fragments (full length VH-linker-VL) by enzymatic restriction, which I further digested to obtain VH portions to avoid the identification of truncated clones (linker-VL, VH-linker).
VH fragments deriving from each sub-library (human cycle_2, human cycle_3 and murine cycle_3) were sequenced at the Center for Translational Genomics and Bioinformatics, Hospital San Raffaele in Milan. Briefly, each sub-library was labelled with a different barcode and samples were pooled into a single run of sequencing performed on MiSeq Illumina platform.
For each sequence identified by NGS, I obtained the number of reads, expressed as counts per million (cpm), and the corresponding protein sequence. The first parameter allowed me to evaluate the abundance of a specific clone within a cycle and the trend of enrichment between the different selection cycles, whereas protein sequence enabled me to identify clones with silent nucleotide changes. Therefore, I performed a detailed analysis of NGS data. Firstly, starting from human cycle_3, within the top 100 ranking sequences, I discarded those bearing stop codons or indels causing frameshift.
Since the selection was performed using Fc-fused proteins, I expected that a small number of clones could bind Fc portion despite negative selection rounds have been performed. Thus, I compared the in-frame sequences with data obtained from screenings previously done on different targets. Sequences that resulted commonly enriched also in other screenings were discarded, being potential Fc reactive clones.
The remaining in-frame sequences, representing the 15% of the sub-library (fig. 16), were further analyzed. To determine the most enriched clones, I ranked the remaining sequences in human cycle_3 by representativeness (cpm values). Four clones, named ID1, ID4, ID5 and ID8 were highly represented within this cycle. Among them, ID1 was the most enriched showing the highest number of reads (cpm) (fig. 16). Moreover, as shown in diagram in figure 17, comparison of the aforementioned clone abundancies between cycle_2 and human cycle_3 revealed that all clones resulted more enriched after the selection on the human protein, suggesting a high specificity for it.
Finally, I compared the ranking position of the identified clones also in murine cycle_3 to highlight potential cross-reactive clones. As represented in figure 17, ID1 and ID8 were highly represented after selection on the murine CTLA-4 protein and thus were predicted as cross-reactive. On the contrary, although ID4

and ID5 were also found in the murine cycle_3, their enrichment could not be considered significant.

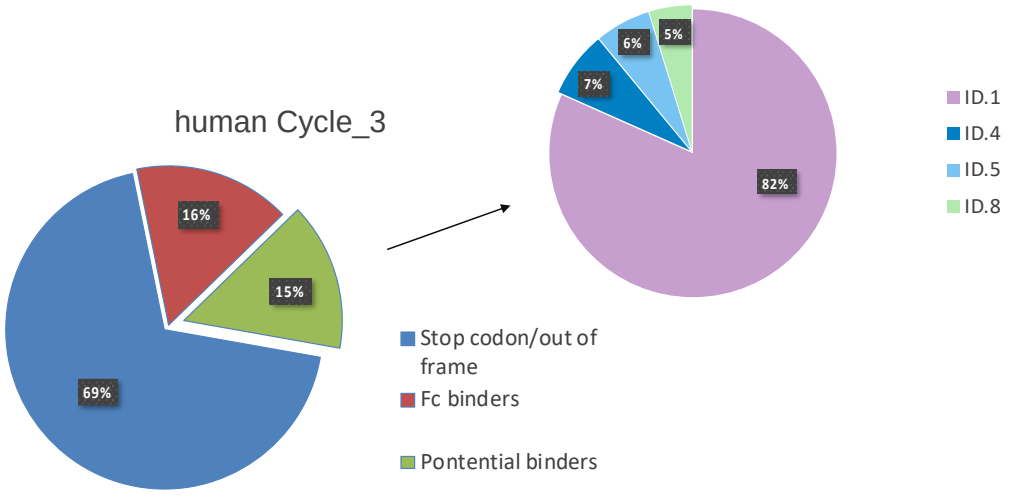

Figure 16. Sequence distribution within cycle_3 sub-library performed on human CTLA-4 protein. The pie chart shows the distribution in terms of percentage of sequences bearing stop codon or out of frame mutations (blue slice), sequences shared with other screenings (red slice) and potential CTLA-4 binders (green slice). A deeper analysis of the latter slice is represented in the smaller pie chart, showing the representativeness (as cpm) of the identified clones.

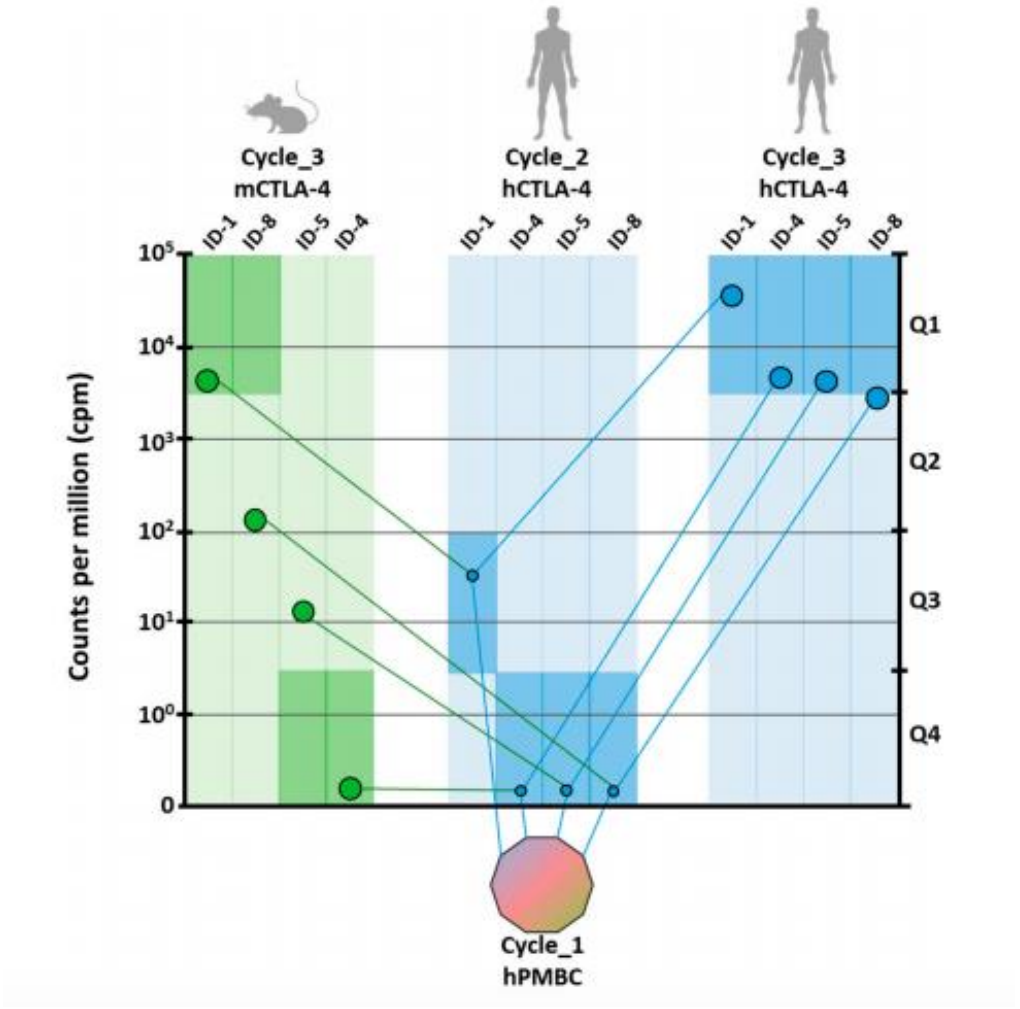

Figure 17. Clone enrichments throughout selection cycles and comparison between selection on human *versus* murine CTLA-4. The chart illustrates the enrichment trend of the four identified clones. Starting from the first selection on activated hPBMC, identified by colored decagonal, the four clones started to be select after a first cycle of selection on recombinant protein. Parallel selection with human (blue panel; left side) or murine (green panel; right side) CTLA-4 determined a comparable enrichment of ID1 and ID8 in both cycle_3, analyzed as increment of cpm values. Specific enrichment of ID4 and ID5 was observed in panning on human CTLA-4.

4.7 Recovery of the scFvs from the library and conversion into antibodies

To study biological properties of the isolated scFvs, I converted them into fully human antibodies. As clones of interest were not physically isolated from the entire library, a rescue strategy was applied to fish out ID1, ID4, ID5 and ID8. Indeed, it is well known that HCDR3 region is the most hypervariable within variable domain, and it is almost unique for each clone (Schroeder 1998). Thus, to ensure the specific-sequence recovery from the library, I designed two pairs of overlapping primers annealing within the hypervariable HCDR3 of the given clone. These clone-specific primers were coupled to universal ones annealing into constant framework region of variable heavy and light chains. The two PCR fragments were thus purified and mixed in equimolar ratio to reconstitute the entire scFv. A schematic representation of the molecular approach is depicted in figure 18. Reconstituted scFvs were analyzed by Sanger sequencing, confirming the identity of clones.

As previously mentioned, the mechanism of action of αCTLA4 antibodies is taught to be dependent on both inhibition of CTLA4 binding to its ligands (CD80 and CD86) and on Treg depletion dependent of engagement of FcRs by Fc portion. For this reason, we strategically decided to convert scFvs into both human IgG4 (low/null Fc activity) and IgG1 (high ADCC, CDC) to split the contribution of both mechanisms of action.

Variable heavy and light domains were separately sub-cloned into mammalian expression vectors encoding constant region to produce human IgGs mAb (VH in hIgG4 and hIgG1 Fc expressing vectors; VL in kappa light constant expressing vector) (fig. 19).

The generated vectors were co-transfected in HEK293-EBNA-SINEUP cells. This cell line represents an optimized version of the HEK293-EBNA, as it expresses the long non-coding SINEUP RNA, which is able to enhance translation of selected proteins, in addition to Epstein-Barr virus protein EBNA-1, which stabilizes plasmids maintaining them in an episomal form (Sasso 2018). Antibodies were purified from conditioned media by affinity chromatography with Protein A, and they were evaluated by electrophoresis on polyacrylamide gel under non-reducing conditions (fig. 19C). ID4 mAb resulted poorly soluble and was thus discarded for next characterizations. In collaboration with Prof. C. De Lorenzo's group, we evaluated the binding affinity and the cross-reactivity of the rescued clones. As predicted by the bioinformatic analysis, ID1 and ID8 were able to bind both human and murine CTLA-4, whereas ID5 only bound the human protein (fig. 20). Moreover, a more detailed investigation revealed that ID1 and ID8 had a wide range of biological effects including activation of T and NK cells by cytokine production induction, Tregs depletion by induction of ADCC and promoting tumor cell death (Passariello 2020).

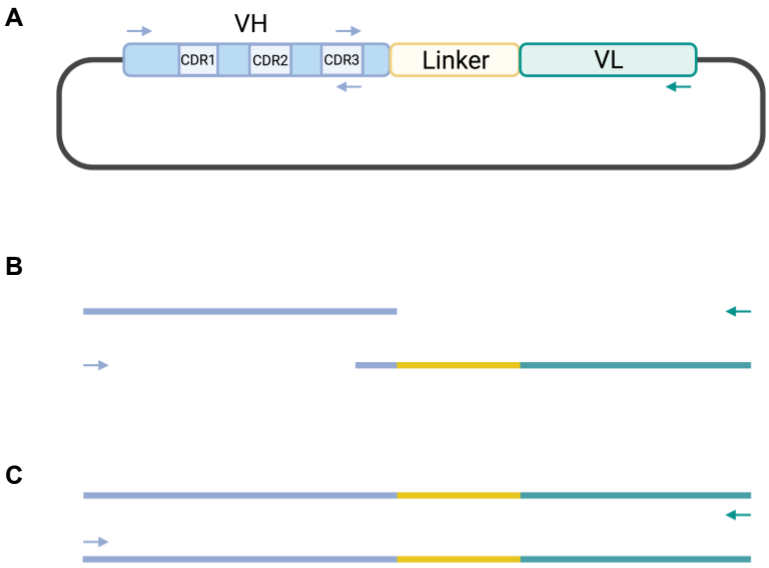

Figure 18. Molecular mechanism for the isolation of clones of interest from the sub-library. The cartoon shows the mechanisms through which the overlapping PCR allows the recovery of the clones of interest. (A) VH and linker-VL fragments are separately amplified using two pairs of oligonucleotides. (B) The obtained fragments, sharing the CDR3 region, are annealed and extended to generate the full-length fragment. (C) Full-length scFv is amplified using the external oligos.

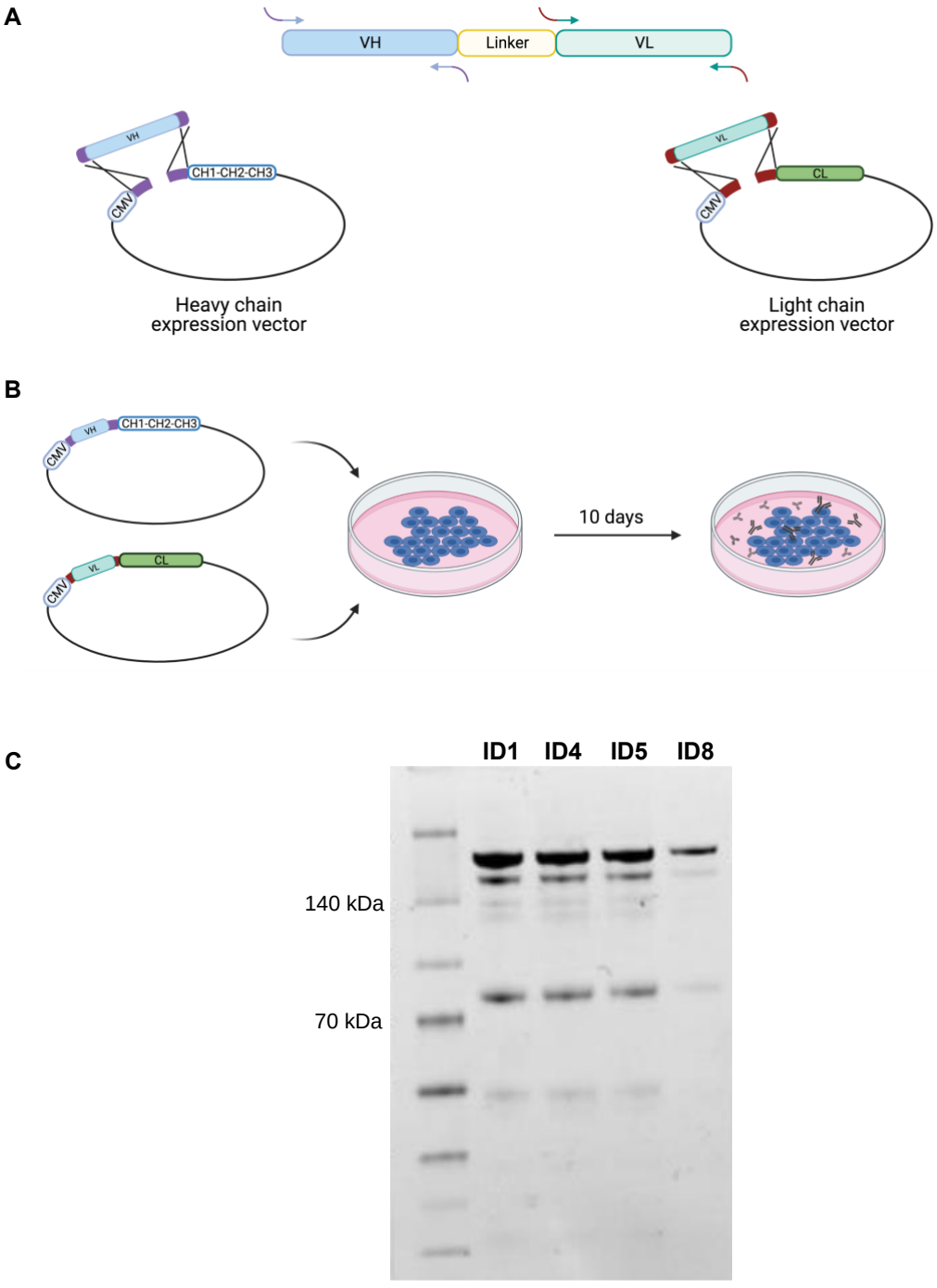

Figure 19. Production of the rescued antibodies. (A) To convert scFvs into full IgG4 antibodies, VH and VL fragments are amplified using a specific pair of oligonucleotides, which simultaneously add a homology region required for the sub-cloning into expression vectors, encoding the constant portion of heavy or light chain. (B) VH and VL expression vectors were co-transfected into HEK293-EBNA-SINEUP cells and the conditioned medium was collected 10

days after transfection. Antibodies were purified by affinity chromatography. (C) Antibody correct folding was assessed loading purified antibodies on polyacrylamide gel by Coomassie staining.

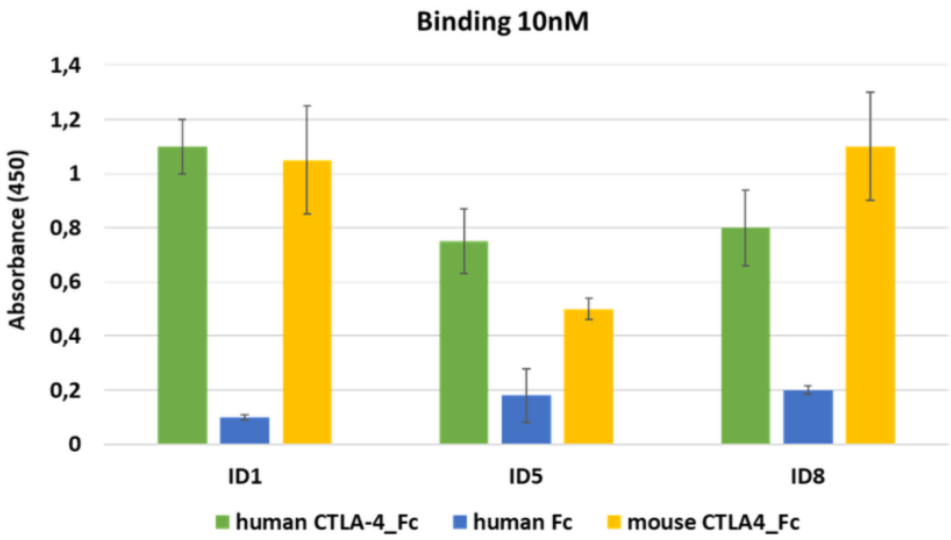

Figure 20. Rescued antibodies bind both human and murine CTLA-4. Differences in cross-reactivity and binding affinity between ID1, ID5 and ID8 were assessed by ELISA assay performed recombinant protein. The histogram shows the differences in the binding affinity between ID1, ID5 and ID8 to human CTLA-4-Fc (green bars) and murine CTLA-4-Fc (yellow bars). Fc portion was used as negative control (blue bars).

5. DISCUSSION

Over the past decade, increased knowledge of the interaction between immune system and tumor cells has led to breakthrough of immunotherapy as a valid treatment option for tumors that do not respond to conventional therapies. Accordingly, OVs have emerged as a promising class of immunotherapeutics, thanks to an increased capability in manipulating viral vectors, and to a better characterization of their mechanisms of action. Alongside the reduction of the tumor mass by direct tumor cell lysis, OVs are able to boost the antitumor immune response. Indeed, tumor cell death occurs in an immunological way, leading to release of viral and tumor-associated antigens and cytokines production, which recruit and activate immune cells within the TME. In this way, OVs possess the potential to modify even immunocompromised TMEs, converting them into immunocompetent ones. This feature could be further improved by arming OV with genes encoding for immunostimulatory molecules.

The power of OVs has been tested in several clinical trials, showing promising results. However, several hurdles remain to be overcome to improve the efficacy of this approach. Among them, the intra-tumor administration, which is often preferred, limits the treatment to primary and palpable tumors. Nevertheless, as demonstrated in OPTiM trial (Phase III clinical trial using IT-delivered T-VEC), OV treatment is able to induce a systemic antitumor response, even though the efficacy on metastasis is not as potent as on primary injected tumors (Andtbacka 2016).

Monoclonal antibodies (mAbs) targeting immune checkpoints have represented the very first effective immunotherapeutic, as demonstrated by the increased number of mAbs approved by regulatory agencies over the last years. However, although a significant number of patients experienced a durable response, a large percentage of them still do not respond, spurring for investigation of combination therapies. Indeed, if on one hand, administration of different mAbs showed increased response rate, on the other hand, it also augmented the occurrence of immune-related adverse effects (irAEs).

Taking into account the limitations of both classes of immunotherapeutics, combination of OVs and mAbs targeting immune checkpoints (ICIs, immune checkpoint inhibitors) could improve not only the efficacy of the respective monotherapies, but also their safety. Indeed, OV treatment prompts the recruitment of immune cells within the TME as well as the production of immunostimulatory molecules, and ICI administration augments the antitumor immune response through re-activation of effector T cells (Bastin 2016). Based on this concept, we and others have demonstrated in several tumor models that combination of OVs with ICIs leads to increased efficacy compared to the relative monotherapies (Sasso 2020; Saha 2017).

According to this background, the aim of my PhD project was an effort to improve the efficacy of oHSV platform by exploiting both the combination with ICIs and the arming strategy.

Starting from the combination of LM-113 (fully virulent HSV-1 based oncolytic virus, tropism retargeted by replacement of a moiety of gD with scFv recognizing HER2 antigen) with αPD-1 (Sasso 2020), I exploited the triple combination, adding αCTLA-4 to the previous one, in an established tumor setting *in vivo* based on LLC cells. According to scientific literature (Saha 2017), triple combination increased the overall response rate, showing an additive effect.
As described above, significant improvement in efficacy following ICIs treatment is often accompanied by occurrence of immune-related adverse events (irAEs). To bypass this hurdle, I decided to encode αCTLA-4 antibody into LM-113 genome, confining the expression of the antibody within the TME and selectively re-activating tumor-resident T cells. However, because of the size of cargo represents a limitation, I firstly decided to optimize the minimal effective size of the antibody without limiting the effector functions. scFv-Fc represents interesting antibody derivative, whose effectiveness has been proven in different tumors (Sokolowska-Wedzina 2017; Robinson 2018). Thus, starting from murine 9D9 αCTLA-4, I generated the full antibody and the scFv-Fc format, which showed a comparable binding affinity for the target. Optimized version of αCTLA-4 was used to generate an armed LM-113, named THV_αCTLA4.
Prior to the effectiveness evaluation, I assessed the *in vivo* cargo expression, demonstrating that THV_αCTLA4 successfully mediates the production of αCTLA-4 in nanograms/volume concentrations in serum. Unfortunately, there are no references in literature about the minimal amount of antibodies required for efficacy. Nevertheless, it has been recently demonstrated that synthetic DNA encoding αCTLA-4 antibody mediates serum expression levels between 58μg/mL and 85 μg/mL, which resulted in tumor regression in Sa1N and CT26 tumor models (Duperret 2018).
After confirmation of the payload expression *in vivo*, I tested the efficacy of THV_αCTLA4 in the same *in vivo* model used before. The efficacy resulted unaffected by the confined expression of αCTLA-4, also demonstrating that the provided amount of αCTLA-4 antibody is already sufficient to stimulate the antitumor immunity. Due to the confined expression of αCTLA-4 antibodies within TME, THV_αCTLA4 could reduce the occurrence of irAEs and so, additional work will be focused on the characterization of this aspect.

As we have already demonstrated that IL-12 is a powerful transgene (De Lucia 2020), I decided to encode a second-generation IL-12 as a second payload into THV_αCTLA4 genome to further improve the efficacy of the oHSV platform. The new version of IL-12 was optimized by physically joining p40 and p35 subunits through a flexible linker, avoiding the assembly of p40 with p19 to form undesired IL-23.
To evaluate the efficacy improvement, I tested single IL-12 armed (THV_IL12) and double IL-12/αCTLA-4 armed (THV_αCTLA4/IL12) generated viruses in the usual *in vivo* tumor setting. Thanks to the effectiveness of IL-12, I obtained interesting response rate after monotherapy treatment with THV_IL12, also with

dose lowered of one Log_{10} (from 1x10^8 pfu/injection to 1x10^7 pfu/injection). As expected, administration of the solely THV_αCTLA4/IL12 induced an increased response rate compared to THV_IL12. Results obtained with the lowered dose make the generated viruses a promising starting point for the translation into clinical settings.

Based on the promising results obtained *in vivo* with αCTLA-4, we evidenced the need to isolate proprietary antibodies, which would be human/murine cross-reactive. This feature would allow not only *in vivo* experimentation for identification of new biological activities, but also the overcoming of expensive humanized mouse or non-human primates' models.
Our group has already set up a strategy to select and isolate target-specific $scFv_s$ by combining high-throughput screening of phage display libraries with Next Generation Sequencing (Sasso 2015; Sasso 2018). Isolation of CTLA-4 specific $scFv_s$ was performed through subsequent selection cycles, first on activated hPBMCs, then on recombinant CTLA-4-Fc protein. Selection on activated hPBMCs ensures the recognition of the target in its native conformation, whereas additional cycles performed on recombinant CTLA-4-Fc protein ensure enrichment of specific clones. To ensure the identification of human/murine cross-reactive $scFv_s$, third cycle was parallelly performed on human or murine recombinant CTLA-4-Fc proteins.
To prove the *bona fide* of the methodology and evaluate the cross-reactivity, as well as the biological activity of identified clones, I converted them into full IgG4 (low/null Fc activity) and IgG1 (ADCC, CDC) antibodies. Two antibodies, named ID1 and ID8, were identified as highly cross-reactive by NGS and showed nanomolar and sub-nanomolar K_d values, confirming high affinity for both human and murine proteins. A more detailed analysis of the biological activities mediated by ID1 and ID8 antibodies revealed that they were both able to induce T cell and NK activation by induction of cytokine production in a similar or even more efficient fashion to IpilimumAb, used as a positive control. Additionally, both ID1 and ID8 were able to mediate Treg depletion by ADCC mechanism in a similar manner as IpilimumAb.
These observations make the newly discovered antibodies a significant tool for further studies in mouse models. Moreover, since they lack antagonistic activity, ID1 and ID8 may provide reduced frequency of irAEs, which will be investigated in the next future.

6. CONCLUSION

In conclusion, my work resulted in the enhancement of an immunotherapeutic platform that considered both efficacy and safety.
Firstly, I developed an oHSV armed with an optimized αCTLA-4 antibody, whose efficacy was verified in an *in vivo* mouse model in combination with PD-1 blockade. The potential ability of THV_αCTLA4 to restrict the frequency of irAEs represents, however, its greatest power. As a result, additional *in vivo* research will be performed to evaluate this trait.
Furthermore, I generated a second generation of THV_ αCTLA4 by adding an immunostimulatory gene encoding for an optimized IL-12. As shown by *in vivo* characterization, viruses armed with IL-12 resulted even more successful, to the point of reducing the necessary treatment dose. These results constitute an optimal starting point for clinical translation, reducing the amount of virus that must be produced.
Secondly, as shown by the *in vitro* characterization, I isolated novel αCTLA-4 antibodies endowed with stimulatory effects on NK and T cell activation as well as inhibitory effect on tumor cell growth. Since these antibodies lack antagonistic activity, they may be able to provide effective antitumor activity without the occurrence of irAEs, which are linked to antagonistic activity in IpilimumAb (Yang 2018). As a result of the demonstrated human/murine cross-reactivity, *in vivo* characterization will be based on assessing the frequency of irAEs in a mouse model.
Finally, the technology used for the isolation of αCTLA-4 antibodies may be considered as a valid approach to isolate other immune checkpoint mAbs, possibly resulting in variants that may avoid the side effects related to the approved antibodies.

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