UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE E BIOTECNOLOGIE MEDICHE XXX CICLO

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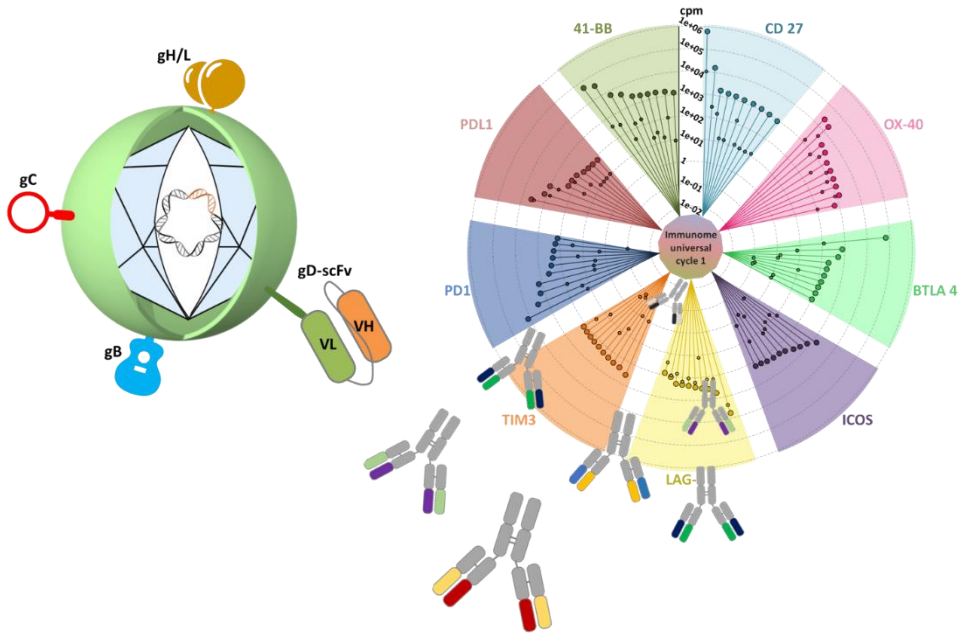
Generation and *in vitro* characterization of cancer immunotherapeutics based on oncolytic viruses and immune checkpoint inhibitors



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Abstract

In the last decade, advances in cancer immunotherapy, in all its facets, have revolutionized the way to treat cancer, becoming by now a pillar in the field of oncology. Immune checkpoint antibodies anti PD-1, PD-L1 and CTLA4 are successfully used in multiple types of cancer also as first-line therapy. Nevertheless, many patients do not respond to treatment or fall in continual relapse, which implies the need to boost anti-cancer immune response. Oncolytic viruses are a promising class of drug that counteract cancer both directly through cell lysis, and indirectly through recruitment of immune cells into the immunosuppressed tumour microenvironment. The clinical outcomes of recently approved Imlygic (Talimogene laherparepvec T-Vec) demonstrated, in a limited percentage of patients, an immune mediated anti-tumour effect. Thus, as confirmed by preclinical and clinical evidences, the combination of immune checkpoint modulators and oncolytic viruses could represent a breakthrough in cancer immunotherapy field. The purpose of this study was to generate cancer immunotherapeutics based on next-generation oncolytic viruses, and a large repertoire of monoclonal antibodies targeting the main immune checkpoints. I generated a HSV-1 based double retargeted oncolytic virus, with enhanced safety in normal cells and remarkable virulence in tumour cell lines, by exploiting the combination of replication conditioning and entry glycoprotein engineering. The *in vitro* characterization of this oHSV suggests the possibility to implement this double retargeting strategy also to exploit systemic route of administration of oncolytic viruses. In a complementary manner, I isolated a large repertoire of hundreds of monoclonal antibodies through an *ex vivo/in silico* High Throughput Screening a of phage display library of human $scFv_s$ based on Next Generation Sequencing. This strategy allowed me to rapidly identify biological active mAbs targeting immune checkpoint modulators. Additional work will explore, *in vivo*, the most suitable combinations of engineered oncolytic viruses with immunomodulatory mAbs from our repertoire, in preclinical settings of investigation.

Introduction

Oncolytic viruses

General features of OV

Oncolytic viruses (OV) are an emerging, large class of drugs for cancer treatments. The interest in viruses as anti-cancer drugs goes back to nearly a century, when important tumour regressions were observed as a consequence of naturally acquired viral infections. Thus, in 1912, an attenuated rabies virus was used in treatment of cervical carcinoma. Despite the interest, the first well established attempts to engineer viruses was reported in the '90s, thanks to the advances in technologies for genome manipulation and to the knowledge in viral biology. The first engineered OV reported by Martuza and colleagues was based on a thymidine kinase-negative mutant of Herpes simplex virus, which was demonstrated to prolong survival in a glioma nude mouse model [1]. After few years, Bischoff JR published a second vector, based on an adenoviral mutant, reporting a complete regression in over 60% of injected tumours in nude mice [2]. Despite these encouraging "historical" results, for years, the fragmented information about viral biology and tumour immunology have not allowed to get advantages from oncolytic virotherapy. Only in recent years OVs have entered clinical trials [3].

An oncolytic virus is a viral particle able to infect and kill cancer cells without damaging healthy tissues. The viral progeny released from infected cells could spread and kill bystander tumour cells, but also endothelial cells, thus reducing tumour bulk, and acting as anti-vascular agent. However, the recent advances in oncoimmunology have shifted the way of seeing the virotherapy as an immunological drug, thanks to its ability to induce adaptive tumour-specific immune responses.

An optimal OV should represent a good compromise between power and tumour selectivity, achievable in different ways, as it will be described below. To date, a plenty of naturally occurring or engineered viruses have been studied as oncolytics, including both enveloped (herpesviruses) and naked DNA (adenoviruses) and RNA viruses (i.e. Newcastle disease virus, measles). Many of these have entered early Phase I or II clinical trials as single drugs, or in combination therapies. Currently, there are about 80 completed or recruiting clinical trials, most of which with adenoviruses or herpesviruses, because of a deep knowledge in their biology [4,5]. Finally, in 2015 the Food and Drug Administration (FDA) and the European medicines agency (EMA) approved as first drug of this class the HSV-1 derived T-VEC (Imlygic, Amgen, Thousand Oaks, CA, USA) for the treatment of advanced melanoma lesions in the skin and lymph nodes. Much information is coming out from the clinical usage of T-VEC, shedding light on immunological relevance of the treatment.

OV as cancer vaccine: heating-up "cold" tumours

Vaccines targeting cancer cells are in development from years. The goal of a cancer vaccine is to induce an effective adaptive immune response against tumour-associated antigens (TAAs). It has been reported that only a small percentage of tumours share common TAAs, suggesting the need for personalized, precision medicine. Classically, these cancer vaccines consist of *ex vivo* manipulated immune cells, tumour associated antigens (TAAs) (administered as recombinant proteins, coding vectors or cancer cell lysates). All these drugs have demonstrated efficacy both in pre-clinical and clinical contexts. In this *scenario,* OVs could represent a breakthrough. As previously hinted, it is well-established that the oncolytic virotherapy can induce both cellular and humoral anti-tumour immunity, working as a cancer immunotherapeutic. Indeed, tumour cells infected with an OV activate an inflammatory cascade, attracting immune cells for innate and adaptive immune responses against cancer. This feature is principally due to the immunogenic cell death (ICD) mechanisms induced by OVs, including immunogenic apoptosis, necrosis, necroptosis, pyroptosis, and autophagic cell death. The ICD is characterized by increased exposure of calreticulin on cell membrane, and release of well-known immune-related molecules such as uric acid, high-mobility group box 1 and ATP. Moreover, viral infection induces the release of stimulating cytokines, such as IL-1, IL-6, IL-12, IL-18, IFN-γ. Along with these molecules, lysed cancer cells release TAAs and cancer related proteins (CRPs) arisen during cancer immunoediting. Then, antigen presenting cells (APC) capture both viral and tumour antigens and present them to naïve or anergic T cells. In this way, the immunocompromised tumour microenvironment (TME), characterized by overexpression of immunosuppressive and vascularization promoting cytokines like IL-10, TGF-β, TNF-α and VEGFs, turns into an "immunocompetent habitat". This effect is potentiated in "armed" OVs, in which immunostimulatory cytokines or chemokines like B7-1, IL-12, IL-18, IL-2, GM-CSF are encoded from engineered viral genomes [5-7] (Fig.1).

According to their inflammatory status, tumours can be classified into three cancer-immune phenotypes:

- Inflamed. This phenotype is characterized by the presence in tumour bulk of macrophages and tumour infiltrating lymphocytes (TILs) both $CD4^+$ and $CD8^+$, often specific for cancer cells, but anergic, because of the immune-suppressive microenvironment. This phenotype is suitable for immunomodulatory therapy.
- Immune-excluded. The main feature of immune-excluded tumours are non-penetrating TILs, which are accumulated in the surroundings of tumour parenchyma. The clinical outcome of these patients is unclear.

- Immune-desert. In this class of tumours, TILs are totally absent or present in a very limited number. This phenotype is likely referred to those tumours with no pre-existing anti-tumour immunity. Immune checkpoint blockade is almost always useless [8].

Interestingly, OVs result both in strengthening of TILs in inflamed tumours and in induction of inflammation in those tumours with poor or completely absent immune cells.

To date, the most reliable and used administration route of OVs is intra-tumour injection (IT). Indeed, even if the intravenous (IV) or intraperitoneal (IP) delivery may be preferred to get a systemic effect, IT administration avoids problems related to side effects and to eventual presence of neutralizing antibodies from pre-existing immunity against the virus. Despite many companies are dedicating efforts in advanced ways of systemic delivery (carrier cells, chelating molecules), emerging preclinical data are revealing a systemic effect of OVs also in IT injected patients. This feature of OVs has been confirmed by results from OPTIM trial (IT delivery of T-VEC) showing an important immune-mediated anti-cancer systemic effect. This phenomenon is known as *abscopal*, that is, the anti-tumour activity on distal uninjected lesions. In the beginning, this effect was thought to result from viral replication and spread from injected to uninjected tumours, but to date it has been demonstrated that in distal tumours there is no detection of virus. Recent data from T-VEC demonstrated the systemic immune response as a result of local IT activation of cancer specific T effector cells able to migrate towards distal lesions [9-11]. Unfortunately, the systemic "vaccine" effect on metastasis was not as potent as the OV injection in primary tumour, suggesting the need of combination therapies, as will be detailed in the next sections.

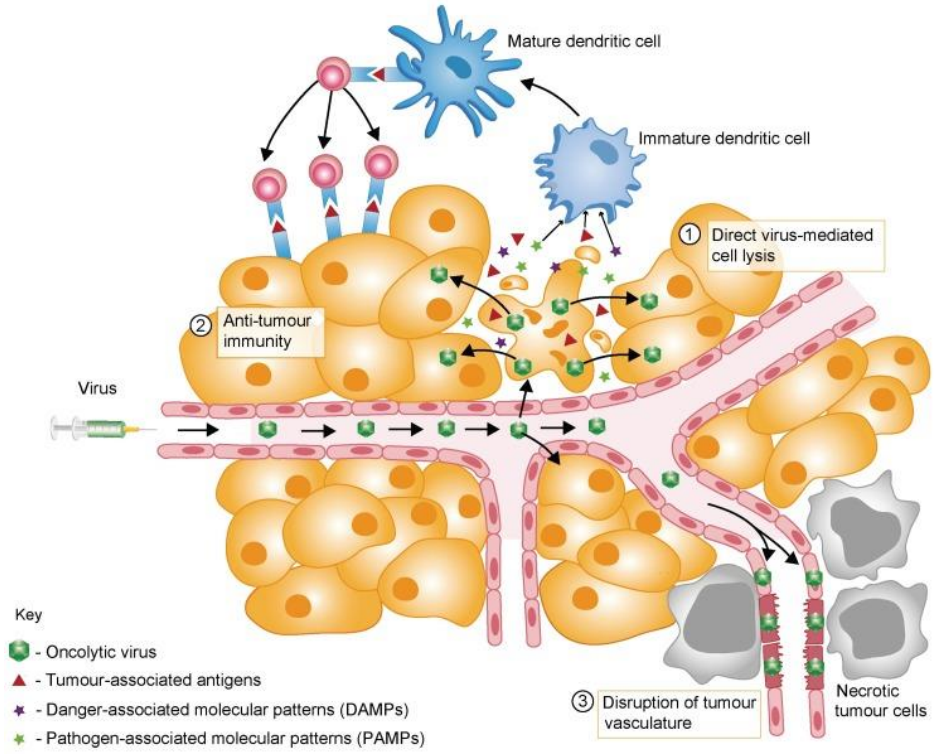


Fig. 1 Mechanisms of tumour cell death trough Oncolytic Viruses. The oncolytic virotherapy acts through several mechanisms. The box 1 shows the direct action of Oncolytic Viruses (OVs) through virus-mediated cell lysis. OVs infect and replicate in tumour cells, leading to direct cell death. The release of the progeny virus particles implies the infection of neighbour tumour cells, which results in the amplification of the initial viral input. The virus-mediated cell lysis causes the release of Tumour-associated antigens (TAAs), Danger-associated molecular patterns (DAMPs) and Pathogen-associated molecular patterns (PAMPs) that meet Antigen Presenting Cells (APCs), such as immature Dendritic Cells (DCs), leading to their maturation. This involves a local inflammation with the migration of mature DCs to lymph nodes, where they present TAAs and viral antigens to naïve T cells, leading to their maturation. The mature CD4+ and CD8+ T cells can thus induce an anti-cancer response acting on infected and uninfected tumour cells. This mechanism is represented in box 2, described as Anti-tumour immunity. In addition, as shown in box 3, the oncolytic virotherapy is able to induce the disruption of tumour vasculature by necrotic cell death, eliminating the fundamental structure for nutritional support of tumour cells [12].

Herpes simplex viruses

Herpesviruses are a large family of dsDNA, enveloped viruses, with a genome size ranging from 150 to about 250 kbp. According to the International Committee on Taxonomy of Viruses (ICTV), herpesviruses can be clustered in three main subclasses:

- Alpha-herpesviruses, including herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are characterized by a fast replicative cycle and prolonged latency in neurons. These viruses are able to infect most of vertebrates.
- Beta-herpesviruses are characterized by slow replication targeting principally dendritic cells, macrophages, epithelial cells, endothelial cells, fibroblasts. The main members of this class are cytomegaloviruses (CMVs), human herpesvirus-6A and 6B (HHV-6), and human herpesvirus-7 (HHV-7) [13].
- Gamma-herpesviruses replicate slowly, similarly to beta-herpesviruses; they become latent in lymphocytes, and can induce cellular transformation. Epstein-Barr virus (EBV) is the best characterized member of this class [14].

HSV-1 and HSV-2 are for sure the most prominent engineered herpesviruses used as oncolytics.

HSV-1 structure and replication cycle

HSV-1 has been the first isolated alpha-herpesvirus. It is widespread all over the world with a prevalence range from 40 to 90% in developed and developing countries. Usually, it infects hosts through oral or genital mucosa. Rarely it is completely eradicated after contagion and primary infection; more often, through retrograde transport, it enters in a latent state of infection in sensory neurons. Occasionally, due to stress conditions or "spontaneously", latent HSV-1 reactivates its replication, giving rise to new infective particles. HSV-1 consists of an enveloped capsid with a size of about 150-200nm. From the inner to the external layers, we can distinguish: i) the icosahedral capsid containing the viral genome, ii) the tegument, consisting of viral proteins useful for viral entry, immediate early phase of HSV-1 infection and packaging (i.e. VP1/2, VP11/12, VP13/14, VP16, VP22), iii) the envelope, consisting of the more external coating of HSV-1, which contains all the glycoproteins required for viral entry in host cells (gB, gC gD, gH/L).

HSV-1 genome is a linear dsDNA of about 152 kbp in length. Its genome consists of two unique sequences named unique long (Ul) and unique short (Us) flanked by inverted repeats. About 80 genes have been identified into the

HSV-1 genome by direct detection of transcripts and proteins or by open reading frame (ORF) predictions (Fig.2).

HSV-1 entry is a multi-step process involving envelope glycoproteins and target host receptors. The first interaction is established between cell membrane proteoglycans like heparan sulfate (HS) and viral glycoprotein gC. This first unstable interaction is reinforced by gD which interacts with HSV-1 preferential targets "herpes virus entry mediator" (HVEM) and nectin-1. Finally, host and viral membrane fusion is mediated by gH/gL complex. [15-20] (Fig.3). Once membranes have been fused, the capsid crosses the cytosol through microtubules to the nucleus, where the viral genome is released. Recently, a novel alternative entry mechanism by endocytosis has been described [21].

Viral replication is a complex, tightly regulated mechanism. HSV-1 genes can be divided into three groups according to the post infection, temporal expression: immediate-early, early and late. The expression of immediate-early (IE or α) genes is dependent on host transcriptional apparatuses and on the tegument protein VP16. The regulation of IE genes is the most complex among the transcriptional cascades involved in viral replication, due to composite *consensus* sequences upstream the core promoters recognized by viral trans activator VP16 and by the host cell proteins "coactivator host cell factor-1" (HCF-1) and Oct1. To date, five genes belonging to IE class have been identified. Of these, ICP4 and ICP27 are essential for complete viral replication. ICP4 is the major transcription regulator of HSV-1 for early and late viral genes. It acts both as an activating factor, inducing RNA Polymerase II transcription by recruiting the TFIID complex, as well as a repressor on its own promoter, according to a negative feedback [22]. ICP27 is required for maturation and cytosolic translocation of viral transcripts. Once IE genes have been activated, early genes can be transcribed and viral DNA replication starts, too. DNA replication occurs into the host nucleus from three origins of replication thanks to both host and viral apparatuses for DNA synthesis. Indeed, HSV-1 encodes its own apparatus for DNA replication including a helicase/primase complex (UL5, UL8, UL52), DNA polymerase and accessory proteins (UL30, UL42, ICP8 and UL29), and enzymes for nucleotide metabolism, including the well-characterized thymidine kinase (UL23) and others (UL39, UL40, UL50, UL2). After DNA replication, late genes are activated. One of the most characterized is ICP34.5, which is involved in reactivation of protein synthesis in infected host cells after PKR/eIF-2a axis activation. Eventually, HSV-1 particles are assembled starting from nucleus up to cell membrane passing through endoplasmic reticulum and Golgi apparatus (Fig.4) [23-25].

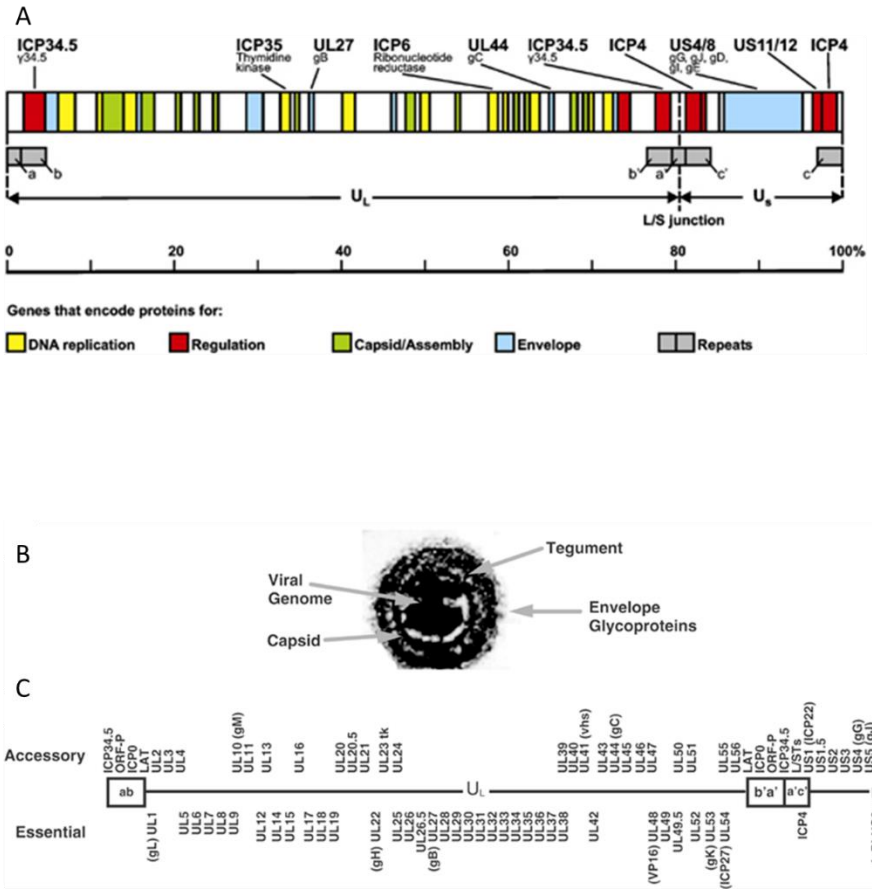


Fig. 2 Schematic representation of HSV-1 genome. The panel A shows in a colour-code the genes involved in DNA replication (yellow), regulation (red), viral assembly (green for capsid and light-blue for envelope proteins) and repeats regions (grey) [26]. The panel B shows the physical structure of a HSV-1 viral particle. Starting from the outer, the arrows indicate the envelope glycoproteins, capsid, tegument and the viral genome. The panel C shows the HSV genome, in details the distribution of Accessory (on top) and Essential (on bottom) genes. The essential genes are necessary for the replication in vitro, on contrary, the accessory genes can be deleted without influence the replication in vitro. The genes encoding glycoproteins and involved in pathogenesis are shown in parenthesis [27].

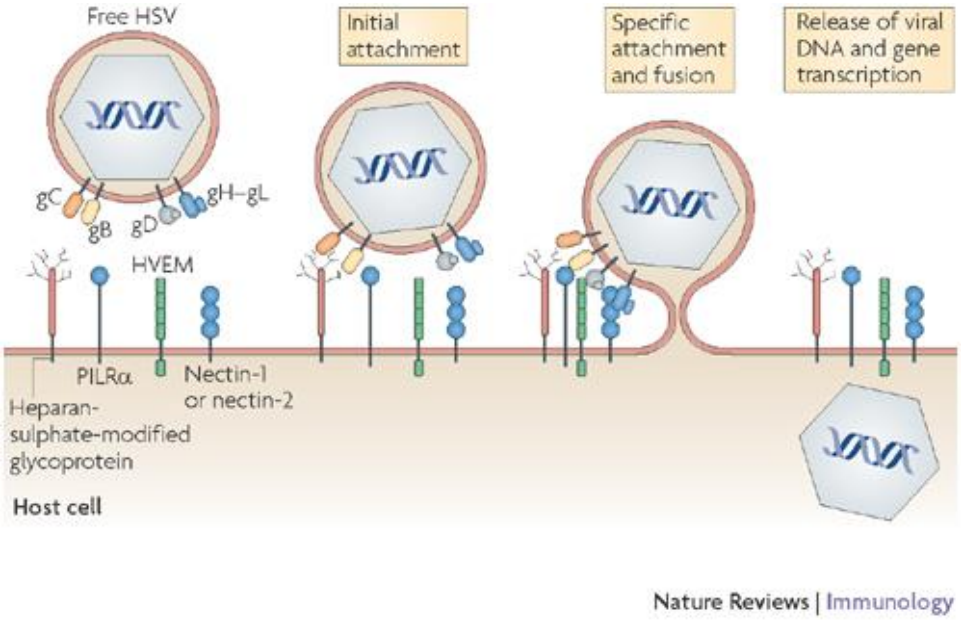


Fig. 3 Interaction of Herpes Simplex Virus entry receptors and their ligands. HSV displays on its surface five glycoproteins, gB, gC, gD, gH and gL responsible for its entry into host cells. gC and gB are involved in the initial attachment binding Heparan-sulphate glycoproteins. Moreover, gB binds Immunoglobulin-like type 2 receptor- α (PILRα), as shown in the first step of the picture. The gD glycoproteins binds herpesvirus entry mediator (HVEM), Nectin-1, Nectin-2 leading to a specific attachment and membrane fusion with the involvement of gH-gL heterodimer (second step). The viral-gene transcription occurs after the release of viral DNA into the host cell nucleus (third step) [28].

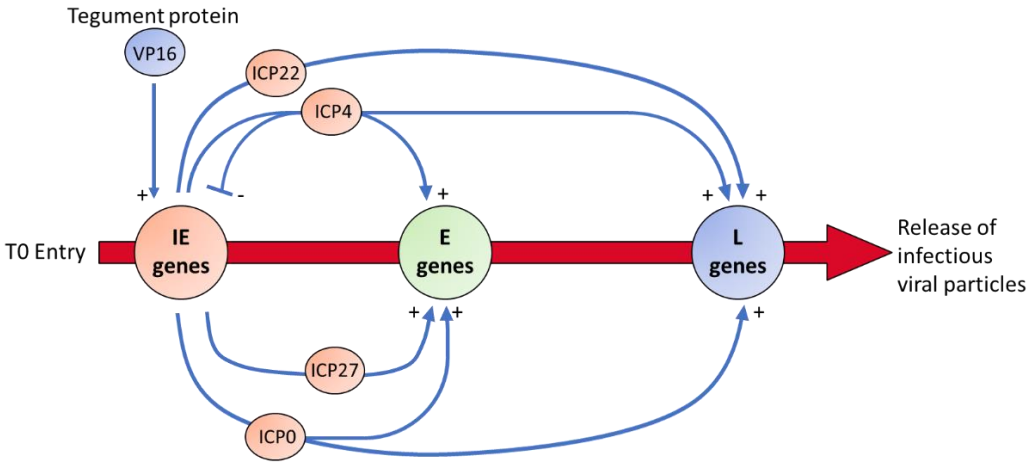


Fig. 4 Cascade of immediate early, early and late genes transcription during the HSV-1 infection. Starting from T0, the tegument protein VP16 induces the transcription of immediate early genes of HSV-1. These latter, namely ICP0, ICP4, ICP22, ICP27, induce the expression of early and late genes required for viral DNA replication and packaging. ICP4 regulation comprises a negative feedback on its own promoter.

HSV-1 as oncolytic virus

HSV-1 is one of the most exploited viruses for oncolytic therapy, both preclinically and clinically [29]. It has a number of advantages, compared to other vectors: i) easy manipulation and large genome capacity for transgene expression, ii) good replication and power to kill majority of cancer cell types, iii) the entry and/or replication in normal cell can be limited by genetic engineering, iv) anti-viral drugs are available in case of "graft versus host" (Aciclovir & Ganciclovir). The main disadvantage of HSV-1 as an oncolytic virus is its high prevalence in population that could limit viral efficacy due to prior immunity and presence of neutralizing antibodies. However, during the phase I study of oncoVEX, it has been highlighted that pre-existing immunity (assessed as neutralizing antibodies in serum) seems not to affect clinical responses and outcomes [30,31].

The most common manipulations of HSV-1 to get selective tumour clearance, saving normal cells, are attenuation and transcriptional or tropism retargeting.

1) Attenuation of virus by mutation or deletions in one or more genes responsible for virulence. To this category belong viruses deleted or in UL39 gene, encoding ribonucleotide reductase ICP6, or in $\gamma_1$34.5. The main limitation of these OVs is amenable to attenuation of virulence both in normal and in tumour cells, limiting oncolysis.
    - ICP6 is required for dNTPs production and then DNA synthesis in neural cells, where deoxynucleotide availability is limited. HSV-1 Δ ICP6 can replicate only in those cells, like tumour ones, with high proliferative rate.
    - $\gamma_1$34.5 belongs to late timing genes of HSV-1 and it is present in double copy. As a consequence of viral infection, healthy cells activate protein kinase R (PKR) in response to IFNs. PKR inactivates, by phosphorylation, the translation initiation factor eIF2α, arresting total protein synthesis. ICP34.5 recruits phosphatase 1, reactivating eIF2α and protein synthesis. Since IFN pathway is often impaired in cancer, a HSV-1vector deleted in both copies of $\gamma_1$34.5 should replicate in tumour cells, sparing normal ones. Most of HSV-1 OVs in development and in clinical trial, including the approved T-VEC, are based on this deletion. Over the attenuated phenotype, this strategy suffers of a second limitation. The PKR inactivation in tumour cells is caused by MAPK/MEK pathway [32]. Despite MEK pathway is one of the main drivers of tumour growth, it is not active universally in cancer diseases. Moreover, tumour cells could acquire resistance to Δ ICP34.5 virotherapy by MEK silencing [33]

2) Transcriptionally retargeted (TR) viruses have been developed to overcome the problems related to attenuation of deleted OVs. In TR OVs, one or more viral genes are encoded under the control of a tumour related promoter, in order to get selectivity against cancer cells. To date, both accessory and essential viral genes have been exploited to achieve transcriptional retargeting. Two of the most preclinical relevant examples of TR HSV-1 OV are: i) rQNestinHSV-1 expressing ICP34.5 under control of Nestin promoter, which has been shown to be useful in preclinical models of Glioblastoma (GBM) and brain tumours [34], ii) oHSV1-hTERT expressing the essential gene ICP4 under the control of human telomerase reverse transcriptase (hTERT) gene promoter [35].

3) The tropism retargeted viruses exploit the viral entry to achieve tumour selective viral infection. As previously described, herpesviruses entry in host cells is mediated by membrane glycoproteins. OVs of this class, combine the detargeting of glycoproteins (i.e. gD or gH) from natural receptors (i.e. HVEM or nectin-1) to retargeting to tumour membrane antigens. The retargeting can be obtained in different ways:
   - Peptide ligands fused to viral glycoproteins able to interact with tumour receptors.
   - Soluble adapters (i.e. HveC-scFv) as a bridge between gD and a target tumour protein.
   - Substitution of essential amino acids of glycoproteins gD or gH with a single chain antibody (scFv) targeting a tumour specific receptor or protein. With this approach Campadelli-Fiume and colleagues isolated non-attenuated, fully retargeted OVs targeting human HER2, demonstrating an important preclinical efficacy [36]. One potential limit of this approach, not well assessed by authors, could be the limited safety due to target receptor expression in healthy tissues (i.e. potential cardiac toxicity of a HER2 retargeted OV) (Fig.5).

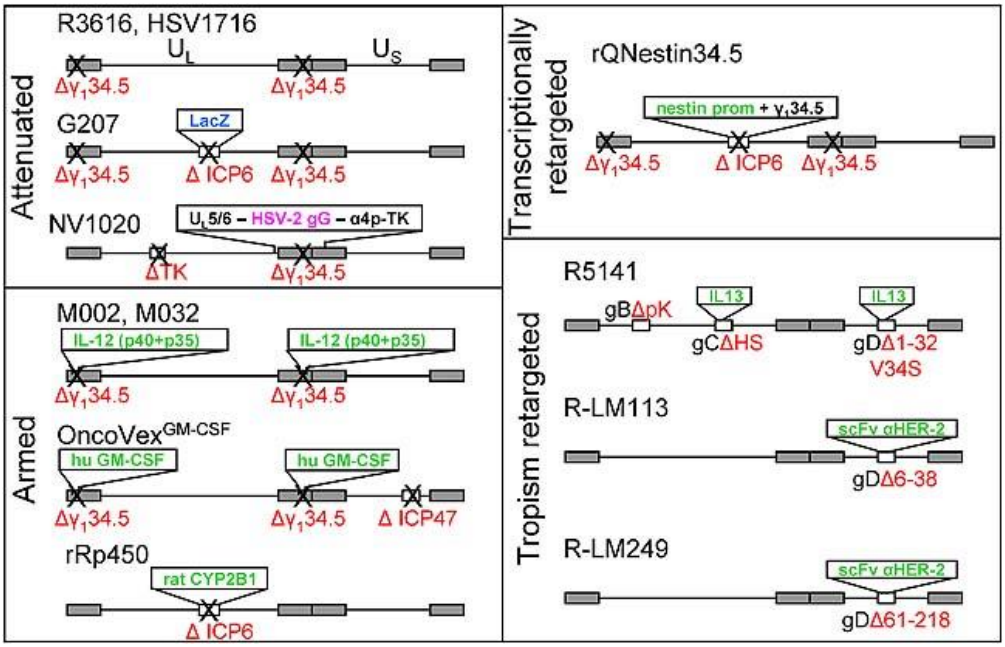


Fig. 5 Schematic representation of engineered oncolytic viruses based on HSV-1. The boxes enclose the four main groups of oHSV-1 subdivided according to different strategies for tumour restricted replication. Attenuated viruses are characterized by deletion in Neurovirulent factor ICP34.5. In armed viruses, one or more viral genes are replaced with cytokines or Cyp2b1 cytochrome. Transcriptionally retargeted oHSVs are obtained by replacing viral promoter of essential genes with a tumour specific one. The tropism retargeted oHSV-1 comprise variuos deletions of viral glycoproteins required for entry of HSV-1. The moieties deleted are usually replaced by scFv targeting a tumour antigen. Gray boxes symbolise the inverted repeats regions of HSV-1 genome. Deleted viral genes, in red, are marked as X. In green or blue are shown the transgenes encoded in selected location. HS: heparan sulfate binding site. pK: polylysine tract. TK: thymidine kinase. GM-CSF: granulocyte-macrophage colony- stimulating factor [33].

Talimogene laherparepvec (T-VEC), from lab bench to bedside

Talimogene laherparepvec, (T-Vec, tradenamed Imlygic™, formerly called OncoVexGM-CSF) has been the first OV approved by FDA and EMA for clinical uses. Its genome is deleted from both copies of *γ34.5* (ICP34.5) and from *α47* (ICP47) genes. In addition, T-VEC is armed with an expression cassette encoding the human granulocyte macrophage colony stimulating factor (hGM-CSF) inserted into the deleted γ34.5 *loci* (Fig.6). ICP47 inhibits host TAP protein required for presentation of antigens in major histocompatibility complex class I (MHC I) [37]. This protein is used by the virus to "hide" its epitopes, to escape innate and adaptive immune system responses. The deletion of this gene in T-VEC allows to improve the cancer vaccine effect by increasing neoepitopes display on cell membrane in the context of MHC I. γ34.5 deletion, as previously described, is responsible for cancer-selective replication of attenuated herpesviruses. *In situ* GM-CSF production is aimed to enhance the activation of APCs (dendritic cells and macrophages) and, thus, of effector T cells. To compare the efficacy T-VEC (expressing GM-CSF) to a non-armed version, Hawkins and colleagues used a bilateral subcutaneous tumour mouse model. They demonstrated that despite both viruses could reduce the size of injected tumours, only GM-CSF expressing T-VEC induced an *abscopal* systemic effect on the contralateral lesion [38].

In an "exploratory" phase I clinical trial, T-VEC safety was demonstrated in various metastatic tumours including malignant melanoma, breast, head/neck and colorectal cancer with injectable metastasis in cutaneous, subcutaneous or lymph nodes. Notwithstanding neither complete nor partial responses were observed, a stable disease was reported in several patients. Moreover, a local inflammation was observed in injected tumours especially in seronegative patients. Therefore, T-VEC entered in phase II study for the treatment of 50 patients with non-resectable stage III and IV melanoma. According to Response Evaluation Criteria in Solid Tumors (RECIST) the overall response rate was 26% (16% complete and 10% partial response). Interestingly, responses were observed both in injected and in uninjected lesions. In addition, it was reported an increased number of local and systemic CD8+ effector T cells combined to decrease in CD4+FoxP3+ Treg cells [39]. Finally, in a phase III clinical trial recruiting 436 patients with unresectable stage IIIB-IV melanoma, T-VEC efficacy was compared to subcutaneous injection of recombinant GM-CSF. The endpoints of this study were: i) the objective response to treatment according to World Health Organization (WHO) criteria defined durable response rate (DRR), ii) the secondary endpoints were progression-free, overall survival, objective response rate (ORR) and duration of response. The main points derived from this study were: i) the regression in

both injected (64% of which 47% complete response) and uninjected tumours (34% of non-visceral and 15% of visceral lesions) ii) the ORR of T-VEC was significantly higher (26%) than GM-CSF (5.7%). In spite of the encouraging results, no significant differences in median overall survival were observed in T-VEC treated patients compared to GM-CSF (23.3 T-VEC vs 18.9 GM-CSF months) suggesting the need for further combinational studies. The mainly reported adverse effects of T-VEC treatment were fatigue and flu-like symptoms. Thanks to these results, in October 2015 FDA approved T-VEC for local treatment of unresectable melanoma, soon followed by EMA [40-42].

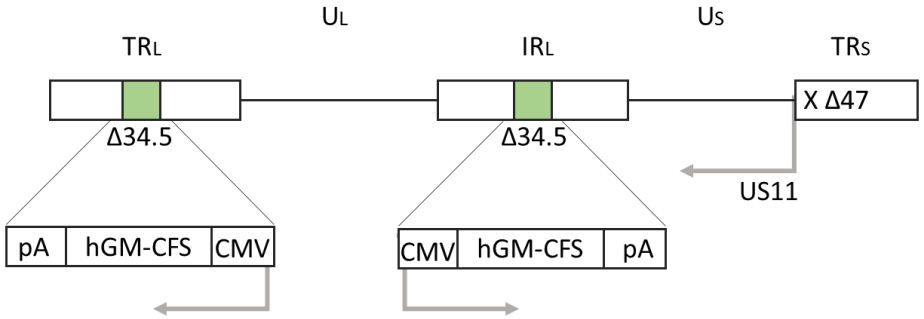


Fig. 6 Schematic representation of Talimogene laherparepvec (T-VEC) genome. T-VEC is a genetically modified herpes simplex virus (HSV) type-1 encoding GM-CSF. The production of GM-CSF by infected tumour cells leads to a localized immune response strengthening anti-tumour effect. Both 34.5 regions were deleted and replaced with two expression cassettes constituted by Cytomegalovirus (CMV) promoter, human GM-CSF (hGM-CSF) and polyA (pA). Moreover, it was deleted also in ICP7 region, required for MHCI-display of intracellular antigens.

Cancer immunoediting

Various lines of evidence have established that tumour cells and immune system establish a tug of war known as "Three E" (Elimination-Equilibrium-Escape) of cancer immunoediting (Fig.7) [43,44]. According to this model, once a normal cell turns into a cancer one, immune system is able to recognise and eliminate it. Elimination is due to innate, but especially adaptive immune responses. Innate immune cells can directly or indirectly kill tumour cells. Natural killer (NK) cells are probably the main players of innate mechanisms for cancer cell recognition and elimination. NK can identify and kill tumour cells by various TNF family ligand-receptor interactions between NK and cancer cells (i.e. CD27, OX40, CD137), as well as NK can recognize and kill by perforin and granzyme B MHC I non-expressing cancer cells [45]. Dendritic cells (DC) as well as macrophages, are antigen presenting cells (APC) able to recognize "eat me" molecules expressed on apoptotic tumour cell surface, eliminating debris from apoptosis. In addition, APCs can be stimulated by cancer-related, damage-associated molecular patterns (DAMPs), among which DNA sensing by Toll-Like Receptors (TLRs) and STING pathways seem to be among the most effective. Activated APCs express T cells costimulatory molecules CD80/CD86 and migrate into lymphoid organs, where they act as a bridge between innate and adaptive immune responses, by presenting cancer related proteins and/or TAAs to naïve CD4 or CD8 T cells, respectively, by MHC II or MHC I complex [46]. Within lymph nodes, epitope landscape is probed by T cells through T cell receptor (TCR), inducing the priming and activation of reactive T cells. Activated effector T cells infiltrate the tumour bulk recognizing by specific TCR the cognate antigen displayed in MHC I context on tumour cells surface. It has been well established that cytotoxic cells play an essential role in anti-cancer immunity, whereby CD8 cytotoxic T lymphocytes (CTLs) depletion (by αCD8 Ab) in tumour-bearing mice results in facilitated tumour growth. On the contrary, although the scientific community is dedicating great efforts to characterize immune cell subpopulations, conflicting reports abound about the CD4 T cells. For sure, CD4 T cells play an important role in the first activation and expansion of CTLs as well as they are crucial for maintenance of anti-tumour CD8 T cell memory. These features are principally attributable to the formation of the trio composed by CD4 and CD8 T cells bound to the same APC, respectively through MHC II and MHC I. In this complex, CD4 helper cells activate, by IL-2, the neighbouring CD8 T cells physically associated to the same APC [47]. More recently, Bourgeois reported a non-canonical direct interaction between CD4 and CD8 T cells via CD40–CD154 (CD40L) in the generation of CD8 memory cells [48]. In contrast, many reports point out that depletion of CD4 T cells (by αCD4 Ab) in tumour-bearing mice has strong anticancer effects. For sure, CD4 Treg subpopulation plays an essential physiological role in

inhibition of tumour-specific CTLs, but Ueha and colleagues demonstrated a stronger anti-tumour effect of total CD4 depletion compared to selective Treg (CD4, CD25, Foxp3+) abrogation [49]. Most likely, CD4 role in anti-tumour response is strongly time dependent. In the early immunoediting, CD4 cells are probably required for the full activation and expansion of CTLs, as well as they are required for development of memory T CD8 cells. On the contrary, at later stages, CD4 could limit tumour cell clearance by direct or indirect CTLs inhibition [50]. More recently, systematic studies from preclinical and clinical outcomes shed light on the importance of humoral immune response against cancer by TAA autoantibodies [51]. This process keeps cancer in check until Equilibrium phase. In this phase, sporadic transformed cells are spared by immune system due to adaptation, so that tumour cells acquire a "tumour dormancy" phenotype. In this condition cancer cells undergo genetic and epigenetic modifications driven by immune system pressure. A key role is probably assumed by pro- and anti-tumour cytokines balance. One of the main characterized pathways of equilibrium phase is the balance between the two dimeric cytokines IL-12 (anti-tumour) and IL-23 (pro-tumour) that share one of the dimer subunit, called p40. Despite the efforts, characterization of the Equilibrium phase is challenging and not fully understood. The continuous cancer immunoediting leads tumour cells to escape and indefinitely grow through several mechanisms: i) hiding TAAs by silencing mutated genes or MHC I down regulation, ii) acquiring resistance to apoptotic stimuli, iii) inducing T-cell anergizing microenvironment (see next sections) [52].

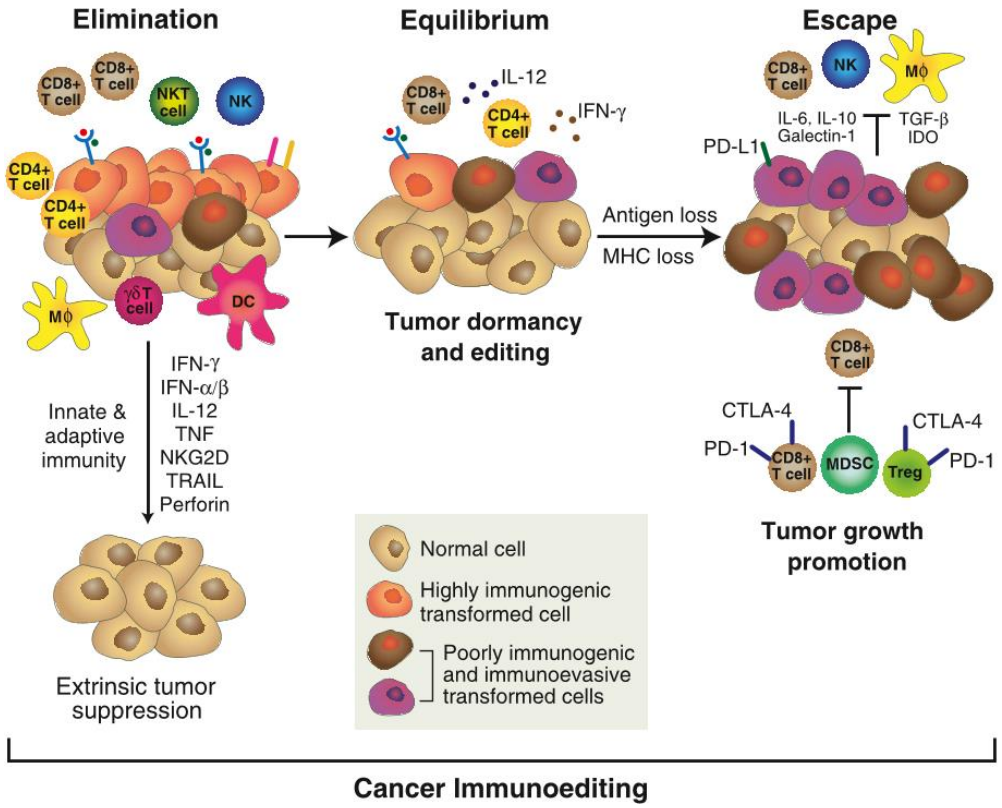


Fig. 7 The cancer immunoediting theory. Cancer immunoediting is a complex process that regards the balance between immunosurveillance and cancer establishment. It consists of three sequential phases: elimination, equilibrium, and escape. During the elimination phase, innate and adaptive immunity destroy transformed cells. Despite the effectiveness of elimination, some tumour cells can escape this process and may then enter the equilibrium phase, in which the elimination of tumour cells is prevented by immunologic mechanisms. During this phase tumour cells undergo a selection process called immunoediting and may persist in this stage for years. The persisting tumour cells may then start to grow entering the escape phase. In this phase the tumour microenvironment is well-known to be immune compromised [44].

Cancer immunotherapy

The knowledge on the tight linkage between cancer and immune system, acquired during the last decades, has generated a new branch of cancer therapy known as immunotherapy. Based on the idea that immune system itself can counteract tumour progression, the aim of immunotherapy is to reactivate CTLs against cancer. The main approaches of immunotherapy are:

- Adoptive cell therapy using autologous TILs. This approach consists of isolation and *in vitro* amplification of lymphocytes extracted from resected tumours by IL-2 supplemented media. Expanded T cells are *in vitro* tested for tumour cytotoxic activity and then reinfused into patients. To date, many clinical trials have demonstrated the effectiveness of this approach to induce complete and durable regressions of cancer disease [53].
- CAR-T cells. CAR-T cells are patients-derived engineered T lymphocytes able to recognize target cancer cells by MHC I-independent mechanism. The first attempt to generate genetically engineered T lymphocytes goes back to 1989, when Gross generated a functional T cell expressing a chimeric receptor by fusing an antibody fragment to TCR constant domain [54]. Further improvements in chimeric antigen receptors (CAR) have been achieved in 2^{nd} and 3^{rd} generation CAR-T cells by fusing antibody fragments to intracellular CD3-zeta (ζ) and additional costimulatory domains like CD28, OX40 or 4-1BB. As for TILs, CAR-T therapy requires lymphocytes isolation from each patient. *Ex vivo* rescued T cells are engineered to express the CAR by viral vectors (retroviral or lentiviral), and reinfused into patients. In August 2017, FDA approved the first CAR-T cell treatment marked as Kymriah$^{(TM)}$(tisagenlecleucel) for B cell acute lymphoblastic leukaemia.
- Immune checkpoint inhibitors. Moving beyond the more complex technologies of T cell engineering, immune modulation is based on reactivation of anergic T-cells by antibodies that block or activate regulatory receptors (see next section) (Fig.8).

Immune checkpoint landscape; blockade and activation

The regulation on T cells is the result of a balance between activating and repressing stimuli, also called immune checkpoints. As explained above, physiologically, T cell activation occurs by interaction with APC through the formation of so-called immunological synapse. The latter consists of a tripartite interaction among TCR-MHC I/II, adhesion, and costimulatory/checkpoints. The main determinant for costimulatory interaction is mediated by CD28-CD80 (B7-1)/CD86 (B7-2), respectively, on T cells and APC. A full activation of APC by TLRs pathway is required for CD80/CD86 expression. Additional late costimulatory signals are afforded by CD27, ICOS (CD278), 4-1BB (CD137) and OX40 (CD134) receptors on T cells and their ligands on dendritic or stromal cells. On the other side, inhibitory receptors are needed to inactivate T cells once the insult is eradicated, and to avoid destructive action on healthy tissue of autoreactive CTLs. The molecular players of the inhibitory pathways are more heterogeneous and involve DCs, stroma cells and Treg [55]. CTLA-4 has been the first characterized inhibitory receptor on effector T cells. It is expressed by activated effector T cells and binds to CD80/86. Thus, CTLA-4 competes with CD28, acting as decoy for CD80/86. The CTLA4-CD80/CD86 interaction induces effector T cell shutdown. Treg cells also express CTLA-4, contributing to CD80/86 decoy. In addition, as opposite to effector T cells, CTLA-4 signal transduction activates Treg inducing their maximal immune-suppressive function. Programmed death 1 (PD-1) is an additional inhibitory receptor of T cells. Its ligands, PD-L1 and PD-L2, are expressed by APCs. Additional inhibitory molecules are BTLA, TIM-3, LAG-3 and TIGIT [56]. Considering the equilibrium and escape phases of immunoediting in cancer, inhibitory axis overcomes the stimulatory ones, inducing T cell anergy.
The cellular components responsible for inhibitory TME are:

- Cancer cells. Cancer cell themselves can develop the ability to express inhibitory ligands (i.e. PD-L1, PD-L2) and produce soluble pro-tumour factors (i.e. IL-10, VEGF, TGF-β, PGE-2).
- DCs. Many literature reports highlight that DCs into tumour microenvironment have an immature or tolerogenic phenotype. These DCs contribute to T cell anergy by expressing low MHC and CD80/CD86 with high inhibitory ligands (i.e. PD-L1 PD-L2).
- Tumour associated macrophages (TAMs). As DCs, TAMs can hijack their anti-tumour function to pro-tumour according to M1-M2 paradigm. The term M1 refers to anti-tumour macrophages expressing TNFα and IL-12; whereas M2 macrophages are pro-tumour producing IL-10, TGF-β and VEGF. As expected, M2 are the most abundant macrophages into TME [57].

- Treg. Regulatory T cells play an essential role in tumour progression principally acting as decoy for both receptors (sequestering CD80/CD86 by CTLA-4) and soluble factors (sequestering IL-2 by IL-2r) [58].
- Several cell types from tumour microenvironment also contribute to the generation of immunosuppression. These actors differ from a tumour to another and include principally cancer-associated fibroblasts (CAFs) and cancer-associated stromal cells (CASC) but also adipocytes, endothelial cells and so on. These cells produce a plenty of immunosuppressive molecules including miRNA, cytokines, chemokines or matrix remodelling proteins [59,60].

Based on these considerations, checkpoint-based immunotherapy relies on re-activation of anergic T cells by agonist or antagonist molecules. Although many types of drugs with immunomodulatory effect have been tested, including small molecules and aptamers, the most feasible and advanced approaches exploit monoclonal antibodies (mAbs) (Fig.8) [61,62]. To date, a great deal of mAbs with immunomodulatory activity have been isolated and tested preclinically and clinically. This approach allows to rescue the cytotoxic activity of weak CTLs acting either as agonists on costimulatory receptors, or as antagonists on coinhibitory ones [63]. Until now, FDA and EMA have approved mAbs targeting the three main immunosuppressive receptors CTLA4 (Ipilimumab), PD1 (Nivolumab and Pembrolizumab) and PDL1 (Atezolizumab, Durvalumab and Avelumab). On a regular basis, regulatory agencies extend the approval of these mAbs for the treatment of several tumors including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), Head and Neck Squamous Cell Carcinoma, Hodgkin Lymphoma, Urothelial Carcinoma, microsatellite instability (MSI)-high or mismatch repair (MMR)-deficient solid tumors and Merkel-cell carcinoma (MCC). Clinical trials for additional therapeutic indications also arise, every year. Despite unprecedent response of immunotherapy, the reported anti-cancer effect is restricted to a limited percentage of patients, suggesting the need for combination therapy or boosting agents. For example, Larkin and colleagues studied the effect of Ipilinumab (αCTLA-4) and Nivolumab (αPD-1) as monotherapy or in combination in advanced melanoma. The objective response rate of combination was 57,6% compared to 19% Ipilinumab and 43.7% Nivolumab monotherapies [64,65]. Additional clinical trials of Nivolumab and Ipilinumab combination are still ongoing [66-69]. Despite the benefits arising from such combinations, about half of the patients still do not respond to therapy. To improve response rate, new antibodies targeting secondary inhibitory (TIM-3, VISTA, LAG-3, IDO, KIR) and stimulatory (CD40, GITR, OX40, CD137, ICOS) targets recently entered clinical trials

[70]. The most promising approach to improve the clinical outcome, could be the combination of the well characterized antagonist mAbs (CTLA-4 or PD-1/PD-L1) to agonist receptors (in particular OX-40) [71,72]. Meanwhile, many efforts are dedicated to identify biomarkers for response prediction and the molecular basis of resistance to cancer immunotherapy [73]. Today, it is acclaimed that a multiparametric value is needed to predict response/resistance to immunotherapy, taking into account the principal biomarkers: i) mutational load of cancer cells, ii) cancer-immune phenotypes (see previous chapters), iii) immune checkpoint molecules expression (i.e. PD-L1, PD-L2, CTLA-4), iv) microsatellite instability, v) serum markers (such as lactate dehydrogenase), vi) basic and advanced imaging (i.e. immuno-PET) [74]. Considering all this, the scientific community is unceasingly interested in isolation of newer and more powerful mAbs.

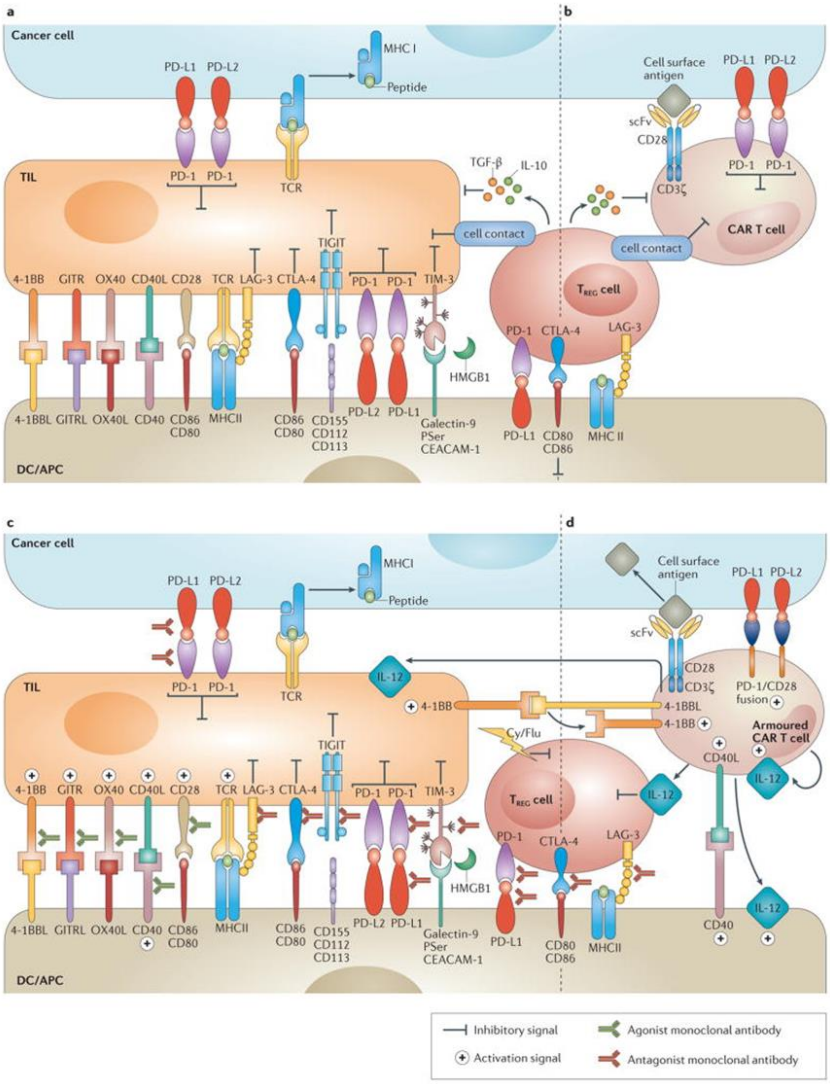


Fig. 8 Overview of immunomodulatory monoclonal antibodies and armoured chimeric antigen receptor (CAR) T cells pathways. In the panel A is shown the negative pathways that induce T cells anergy. Such factors include cell surface receptors, such as programmed cell death 1 (PD-1), Lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain 3 (TIM-3) and cytokines, such as TGF-β and IL-10. Regulatory T (TReg) cells in tumour microenvironment (TME) are involved in the inhibitory mechanisms. In the panel B is shown the inhibitory activity of TReg on CAR T cells and on endogenous T cells. The immunomodulatory monoclonal antibodies are used to overcome the immunosuppression caused by inhibitory immune checkpoints by blocking suppressive receptors, for example programmed cell-death 1 (PD-1) or cytotoxic T-lymphocyte antigen 4 (CTLA-4) and activating stimulatory receptors, such as TNFRSF9 (4-1BB) or OX40. The panel D shows the pathways for which armoured CAR T cells overcome immunosuppression associated with the TME expressing, in the example, CD40L, IL-12 or TNFSF9 (4-1BBL). Image source: Khalil, D. N. et al. Nature reviews Clinical oncology. 2016;13(5):273-290.

Structure of monoclonal antibodies and their isolation

Monoclonal antibodies (mAbs) are the primary tool in clinical use for cancer immunotherapy. The structure of Abs consists of a tetramer of two heavy and two light chains, linked each other by disulphide bounds. Both heavy and light chains contain constant and variable domains. The structure of each Ab is composed by a constant crystallisable fragment (Fc) specific to each immunoglobulin isotype (IgM, IgA, IgD, IgG, IgE), and the Fab portion containing the variable domains responsible of binding to the target (Fig.9). Although the hybridoma approach has been used for isolation of new monoclonal antibodies for years, it currently suffers from several disadvantages, including the need of humanization and no applicability for toxic or poorly immunogenic antigens (i.e. highly conserved across species) [75]. To overcome these disadvantages, one of the most used technologies to isolate mAbs exploits synthetic libraries of single-chain variable fragments (scFvs). A scFv consists of variable regions of heavy and light chains in frame-fused through a flexible linker (Fig.9). These scFvs can be displayed on the surface of yeast or phage particles, each of which physically associates its genetic information to the corresponding phenotype (i.e., a scFv clone) (Fig.9). Phage/yeast display allows to isolate a set of potential binders through several selection cycles with the target of interest (recombinant protein or a target expressed on cell surface membranes) (Fig.10) [76,77].

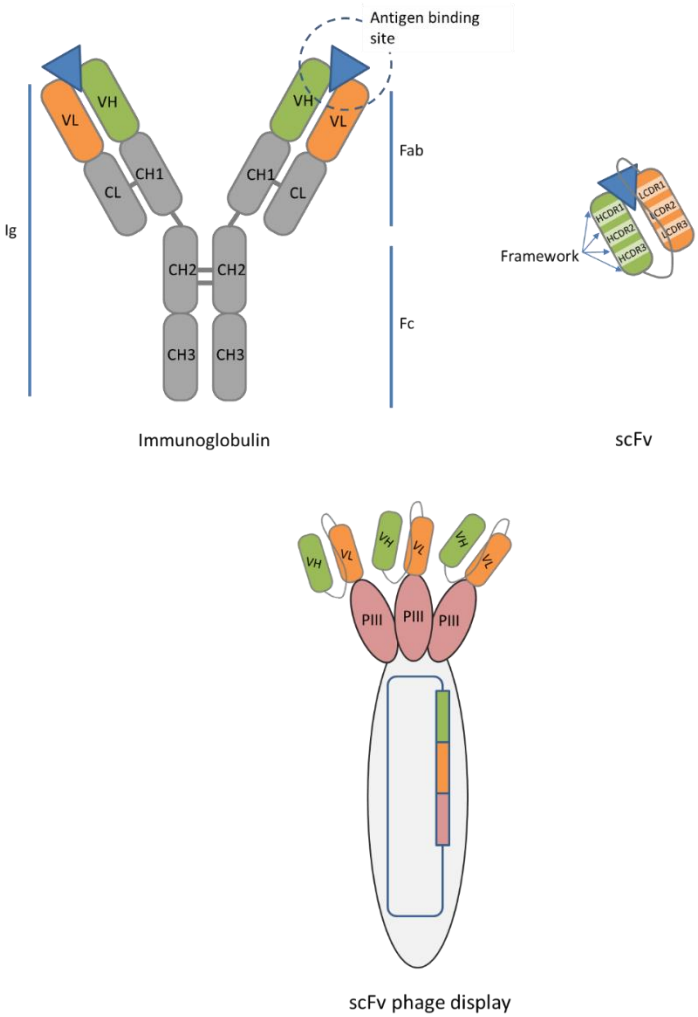


Fig. 9 Representation of mAbs, scFv and phage-scFv structure. The immunoglobulins (Ig) consist of Fab and Fc portions. Fab contains the variable domains of heavy (VH) and light chain (VL) involved in binding to the antigens (blue triangle). The single chain variable fragment is the smallest unit of an antibody able to constitute a paratope and to recognize its epitope. It consists of the variable domain of both heavy and ligth chains fused by a flexible linker peptide. In order to create a library of scFv, the mRNA from healty donor spleen is used as template to extract by PCR the variable domains, which are then randomly assembled. This repertoire of scFv is in frame fused with coat protein PIII of M13 phage. The diversity of libraries is usually arround 10^{10}.

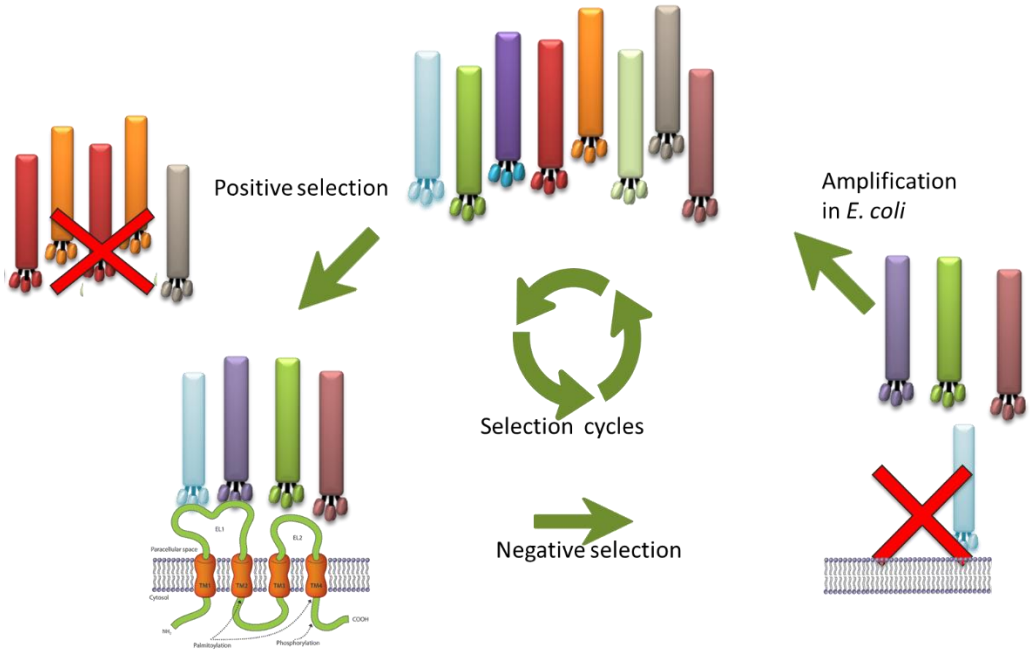


Fig. 10 Phage display platform. Potentially binder phages are selected on target protein throught panning (positive selection). Positive selection step is performed by incubating the phages with the target expressing cells or recombinant protein. The negative selection is made on target not expressing cells or recombinant protein carrier to eliminate aspecific clones. Some selection cycles are performed to enrich the potential binders. At the end of each cycle, phages are amplified by *E.Coli* infection.

Combination therapy with Oncolytic viruses

The limited efficacy of OVs and immune checkpoint modulators has opened new possibilities for combination therapies in cancer. As mentioned before, much of the effects of oncolytic virotherapy is mediated by cooperation with the immune system (cancer vaccine). Namely, one of the most interesting features of an OV is to turn immunodeficient tumours (immune-excluded and immune desert) in their inflamed counterpart. Moreover, as a consequence of OV infection and tumour cell death, the TILs display a more active immune-phenotype compared to the anergic state of the untreated condition. On the other side, cancer immunotherapy has shown its great potential on a restricted percentage of patients due to the frequent immunocompromised tumour microenvironment. Hence, according to these observations, several preclinical models and clinical trials have been developed to get full advantage from both OVs and cancer immunotherapy through their "alliance" [78-80]

By using a murine model of glioblastoma multiforme (GBM) and oncolytic HSV (oHSV G47Δ expressing mIL-12) Saha and colleagues demonstrated an additive effect of OV combination with mAbs targeting PD-1, PD-L1 and CTLA-4. Even more interestingly, they showed that the triple combination of OV+αPD-1+αCTLA-4 acted synergistically, curing most of GBM in mice and conferring complete resistance to tumour re-challenge. Depletion analysis in CD4, CD8 or macrophage cell populations suggested a complex cellular cross-talk and a fundamental role of M1 TAMs [81]. Recently, authors from Amgen published the combination of a murine version of T-VEC (OncoVEX$^{mGM\text{-}CSF}$) with αCTLA-4, giving particular emphasis to the cure of all injected tumours and to the *abscopal* effect on contralateral lesions dependent on effector CD8 T cells [82]. Eventually, the first phase 1b clinical trial of T-VEC combination with anti PD-1 pembrolizumab has been concluded. Although the endpoint of this clinical trial was the evaluation of the safety of combination, the preliminary results suggest that combination of T-VEC and pembrolizumab could actually overcome the limitations of both single therapies. To address this point, a phase III trial is currently ongoing [83].

Aims

Despite progresses in early diagnostic and care, incidence and prevalence of cancer disease is projected to increase in next decades. Indeed, if on one hand most people live longer, on the other hand the increased lifespan represents itself a "risk factor", as it rises the exposition to risk factors (lifestyle, genetics, environment pollution, etc) inducing an accumulation of mistakes in DNA and thus, neoplastic transformation. New antineoplastic drugs have been developed for most cancer types, rising up to 50% the survival chances. Nevertheless, advanced stages and some cancer types remain killer diseases (i.e. pancreas, lung, brain). Immunotherapy has revolutionized the way to treat cancer, leading to unprecedented responses in patients and filling gaps in drug repertoire for orphan cancer disease. The way to re-activate immune system against cancer are many; among these, oncolytic virotherapy and mAbs targeting immune checkpoints represent the breakthrough of last decade as cancer immunotherapeutics. Despite the preclinical and clinical success of OVs and mAbs as monotherapy, the efficacy remains restricted to a small percentage of patients, whereas their combination seems to enhance significantly each other's effect. This suggests improved performance of combinations, both in terms of safety and efficacy. In particular, most of oncolytic viruses currently in clinic or clinical trials are based on: i) attenuated vectors with a poor virulence and/or ii) non-attenuated OVs with potential not negligible side effects. Likewise, new therapeutic mAbs more powerful of those in clinic, and/or against newly discovered immunomodulatory targets are required.

The purpose of my PhD project was to generate a cancer immunotherapeutics repertoire to:

- Overcome the limitations of oncolytic virotherapy, engineering a non-attenuated HVS-1 with enhanced safety compared to those currently developed, by combining tropism and replication strategies of retargeting.
- Study the efficacy and selectivity of these vectors in tumour and normal cells.
- Generate a large repertoire of agonist and antagonist mAbs targeting the most relevant immune checkpoint modulators by high throughput "immunomic" screening of phage antibody library.
- Analyse *in vitro* the potential therapeutic effect of isolated mAbs
- Generate a proof of principle of advantages in using non-attenuated OV in combination to checkpoint inhibitors.

Materials and methods

Cell cultures

SKOV3 and SAN cells were cultured in RPMI Medium 1640-GlutaMAX™-I; HEK293 and A375 cells were cultured in Dulbecco's Modified Eagle's Medium; MRC5 cells were cultured in Minimum Essential Medium Eagle; G361 were cultured in Mc Coy's 5A Medium. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 UI ml-1 penicillin, 50 µg ml-1 streptomycin, 2mM L-glutamine. All the reagents for cell culturing were from GibcoTM, Thermo Fisher Scientific. Cell lines were purchased from the American Type Culture Collection (ATCC) or kindly donated from collaborators and cultured in a humidified atmosphere containing 5% CO2 at 37 °C.

SEAP reporter assay

The putative promoter sequences -260 to -1 for S_Survivin; -830 to -70 for L_Survivin; -400 to -24 for hTERT; -300 to -1 for CXCR4 were purchased by The Invitrogen GeneArt Gene Synthesis service and were cloned into pSEAP2-Basic (GenBank Accession#: U89937, Clontech Laboratories, Mountain View, CA, USA) upstream SEAP with restriction enzymes XhoI and HindIII. HEK293, SKOV3, A375, G361, SAN and MRC5 were transfected with the specific promoter-SEAP vectors by using Lipofectamine Transfection Reagent (Life Technologies, Inc.) and grown up for 24h or 48h. SEAP activity was dosed from conditioned media by Phospha-Light SEAP Reporter Gene Assay System (Thermo Fisher). Assays were performed according to the manufacturer's instructions in 96-well plates. For measurement of S_Survivin promoter-SEAP in response to Nocodazole, SKOV3 cells were transfected with S_Survivin promoter-SEAP vector and 8h after, Nocodazole was added to the media at final concentration of 0.1µg/ml. 12h post Nocodazole treatment, SEAP activity was dosed from conditioned media.

Viral rescue and titration by plaque assays or RealTime PCR

SKOV3 cells cultured in 24well plates were transfected with 250 ng of BAC-HSVs DNA with Lipofectamine Transfection Reagent (Life Technologies, Inc.) and grown up until full cytopathic effect (cpe) was reached. Starting from this step, viral particles were used to infect SKOV3 in a scale-up process to get

a considerable quantity of viruses. To titrate infectious viral particles, a plaque assay was performed. Briefly, at day -1, 250,000 SKOV3 cells were plated in a 12-well plates; at day 0, the media to titrate were diluted, from 1:10 to 1:10E+09, in low serum RPMI medium in a final volume of 350 µL, and incubated with SKOV3 by gently shaking 1h at 37 °C. Thus, the medium was replaced with 1ml of low serum RPMI medium, and cells were cultured in a humidified atmosphere containing 5% CO2 at 37 °C. 120h later, cells were fixed with 100% ethanol for 10' and stained with 10% GIEMSA for 15', and plaques were counted. To analyse the viral replication, the viral genome copies were titrated by TaqMan RealTime PCR (Taqman universal PCR mastermix, Applied Biosystems) from infected cells and conditioned media. Briefly, conditioned media containing both intra- and extra-cellular viral particles were diluted in liquid formulation A195 and treated with RNase-free, DNase I recombinant enzyme (Roche) for 30' at 37 °C to eliminate free viral DNA. The DNase I was inactivated with 25mM (final concentration) EDTA for 20' at 80 °C. Thus, to extract viral DNA, the enveloped HSV-1 particles were treated with SDS 0.1% (w/v, final concentration) and 100 µg proteinase K (Roche) for 1 hour at 56 °C. The extracted viral particles were diluted 1:10, 1:100 and 1:1000 and analysed by TaqMan RealTime PCR according to the manufacturer's recommendations.

Modification of BAC-HSV-1

To modify BAC-HSV-1 vectors the sacB/Ampr/lacZ recombineering was used. It consists of a two sub sequential selection steps. The first Positive selection and the second negative one. Briefly, for the first step, a DNA fragment containing sacB/Ampr/lacZ selection cassette was amplified by PCR, from a donor plasmid with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific 2 U/µl): 1X Phusion HF Buffer; dNTPs 200 µM; DMSO 3%; Primer Forward 0.5 µM; Primer Reverse 0.5 µM; Phusion Hot Start II DNA Polymerase (2 U/µL) 0.02 U/µL; template 10 ng/µl; H2O up to 50 µl. The oligonucleotides used for this amplification contained in their 5'-end at least 40 base-pairs of perfect homology to the region to be engineered. The PCR products were purified from 1% agarose gel with Wizard® SV Gel and PCR Clean-Up System (Promega). The cassettes were thus electroporated (25 mF, 2.5 kV, 200 Ohm) into electrocompetent SW102 heat-induced bacteria containing the BAC-HSV-1 of interest. After 1h recovery, SW102 cells were plated on LB agar plus 12.5 µg/ml Chloramphenicol; 20 µg/ml Ampicillin; 80 µg/ml X-gal; 200 µM IPTG. The Blue colonies were cultured in LB medium for 16 hours, and DNA was extracted by NucleoBond PC100 (MACHEREY-

NAGEL GmbH & Co. KG). The second step of recombineering was performed by transformation by electroporation of SW102 cells, derived from the first selection step, with the DNA fragment containing the promoter or scFv of interest amplified with the same 40 base-pairs of perfect homology as the first step. The negative selection was performed on plates containing sucrose.

VH fragment extraction and sequencing

After three cycles of panning of phage display scFvs, the double strand DNA phagemids containing the scFvs were isolated from cultures of superinfected E. coli TG1 cells using GenElute HP Plasmid Maxiprep Kit (Sigma-Aldrich). The full length scFvs, containing both VH and VL, were excised by double digestion with restriction enzymes BamHI and HindIII (New England Biolabs) and purified with Wizard® SV Gel and PCR Clean-Up System (Promega) from 1.2% agarose gel. From the purified scFv sub-libraries, a second enzymatic excision by NcoI and XhoI (New England Biolabs) was performed to isolate VHs, that were then purified with Wizard® SV Gel and PCR Clean-Up System (Promega) from a 1.4% agarose gel. Library preparations, sequencing and preliminary analysis of the data were performed at the Center for Translational Genomics and Bioinformatics, Hospital San Raffaele, Milano, Italy. For the preparation of the barcoded libraries, TruSeq ChIP sample prep kit (Illumina) was used. A coupling scheme for bar-code was implemented, to sequence VHs as a mixture of several sub-libraries. The barcoded samples were diluted to a final concentration of 10 pM and sequenced with 2×300 SBS kit v3 on an Illumina MiSeq platform. Paired-end reads were assembled at the Center for Translational Genomics and Bioinformatics, Hospital San Raffaele (Milano, Italy) and the fraction joined reads was about 0.9 for each sample. To deeper analyse the data, the unique sequences for each sub-library were translated to a protein sequences to strengthen the information about enriched paratopes. VH sequences found to be enriched in two or more target-specific sub-libraries and stop codon bearing VHs were discarded. Sequences were thus sorted according to counts per million reads into cycle#3. Ranked VHs were defined as target specific when: i) cpm at cycle#3 were ≥ 85; ii) Δ (cpm cycle#3 - cpm cycle#2) ≥ 0.

Recovery of scFvs of interest from the enriched sub libraries

PD-1_1, PD-1_2, PD-1_3, PD-1_4, PD-1_5, PD-1_6, PD-L1_1, PD-L1_2, PD-L1_3 PD-L1_4, PD-L1_5, LAG-3_1, LAG-3_2, LAG-3_3, LAG-3_4, LAG-3_5, LAG-3_6, LAG-3_7, LAG-3_8, LAG-3_9, LAG-3_10 clones were isolated from the corresponding cycle#3 sub-library by overlapping PCR. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used to perform two extension reactions to obtain firstly single VH and VL fragments, and next the full scFv. The overlapping primers were designed within the corresponding HCDR3 regions and in constant region of plasmid upstream and downstream of VH and VL. The reactions were assembled as follow: 150 ng of template (PD-1, PD-L1 or LAG-3 cycle#3) for the first PCR amplifying separately VH and VL fragments; 10 ng of template for extension PCR to reconstitute the full scFv. Each reaction was performed with 0.5 µL Phusion DNA Polymerase (0.02 U/µL); 10 µL 5x Phusion HF Buffer; 1 µL dNTP mix; 0.5 µM forward primer; 0.5 µM reverse primer; 1.5 µL DMSO; H2O to a final volume of 50 µL. The primer sequences are not indicated for protection of intellectual property. The success of the rescue was evaluated by Sanger sequencing.

Antibody production and purification

For the conversion of the selected scFvs (PD-1_1, PD-1_2, PD-1_3, PD-1_4, PD-1_5, PD-1_6, PD-L1_1, PD-L1_2, PD-L1_3 PD-L1_4, PD-L1_5, LAG-3_1, LAG-3_2, LAG-3_3, LAG-3_4, LAG-3_5, LAG-3_6, LAG-3_7, LAG-3_8, LAG-3_9, LAG-3_10) into whole IgG4, the VH and VL was amplified with specific primers and purified with Wizard® SV Gel and PCR Clean-Up System (Promega) by 1.3% agarose gel. The PCR reactions were assembled as follows: 30-60 ng of template; 12.5 µL mix PCR; 1.5 µL of 5 µM forward primer; 1.5 µL of 5 µM reverse primer; H2O to a final volume of 25 µL. The primer sequences are not indicated for protection of intellectual property. In-Fusion HD cloning kit (Clontech Laboratories, Mountain View, CA, USA) was used to insert the variable fragments in vectors expressing the constant antibody heavy and light chains. The VHs were cloned in the Peu 8.2 vector, previously linearized with BamHI and BssHII (New England Biolabs), and the VLs were cloned Peu 4.2 vector, linearized with ApaLI and AvrII (New England Biolabs). Stellar Competent Cells (Clontech Laboratories, Inc, MountainView,CA, USA) were transformed with obtained vectors and the colonies were screened by digestion and sequence analysis.

The correct preps were co-transfected in HEK293-EBNA by using Lipofectamine Transfection Reagent (Life Technologies, Inc.) and grown up for about 10 days at 37 °C in serum-free CD CHO medium (Gibco, Life Technologies, Inc.) supplemented with 5 ml of L-glutamine 200 mM (Gibco, Life Technologies), 5 ml of Penicillin-Streptomycin 10.000 U/mL-10 mg/mL (Sigma-Aldirch) in 150mm Corning® tissue-culture treated culture dishes. The conditioned media were collected and the antibodies were purified by using Protein A HP Spin-Trap or High-trap Protein A HP (GE Healthcare Life Sciences, New York, USA).

In vivo studies

C57-HER2 mice were used for in vivo studies. Mice were implanted subcutaneously on the right flank with 5X10^5 LLC-HER2 cells (day 0). 10^8 LM113 pfu were injected intra tumourally alone or in combination with intra-peritoneally treatment with 200 μg α-mPD-1 (BioXcell, clone RMP114). Tumor growth was measured by caliper every 3-4 days using the formula LxW2/2 (L as the largest and W the smallest diameter of the tumor). Animals were sacrificed as soon as signs of distress or a tumor volume above 2000 mm^3 occurred.

Results

Generation of oncolytic viruses

Identification of tumour-selective promoters

As mentioned before, the restriction of virulence in cancer cells by replication conditioning is a prominent advantage in virotherapy. To identify potential tumour-specific promoters, I combined reports from scientific literature to gene reporter assays. First, I selected two "pan-tumour" promoters (Survivin and hTERT) and one more restricted to our main target of HER2-related malignancies (CXCR4).

Survivin, encoded by the *BIRC5* gene, is a well-known inhibitor of apoptosis (IAP), physiologically expressed in G2/M phase of cell cycle. During this phase, survivin interacts with microtubules of mitotic spindle and is required to prevent apoptosis. Its expression is rapidly turned off in G1 phase. Contrariwise, survivin is a driver of neoplastic transformation. Since survivin activation is downstream of leading oncogenic cell-signalling pathways (tyrosine kinases receptors, PI3K, AKT, HIF, ERK), it is overexpressed in a plethora of cancers and cancer cell lines, but almost undetectable in the corresponding, healthy tissues (both in human and in mouse) [84-89]. In cancer, survivin is often associated to overexpression of other oncogenes (i.e. HER2) and angiogenetic factors, and its expression correlates to poor prognosis, drug/radiation resistance and tumour relapse [90-92]. A very low expression has been reported in few normal adult tissues and cells with renewal ability like vascular endothelial cells, polymorphonuclear cells, T cells, erythroid cells and hematopoietic progenitor cells [93].

Human telomerase reverse transcriptase hTERT represents the catalytic subunit of telomerase. The latter is required for maintenance of the length of telomeres, and its expression is physiologically repressed in postnatal somatic cells, inducing the shortening of telomeres and cell senescence. hTERT is widely used in research laboratories to create cell lines, thanks to its immortalizing activity. Reactivation of telomerase in somatic cells is often involved in oncogenesis and has been reported in over 90% of cancerous cells [94]. Like Survivin, also hTERT expression is not 100% restricted to cancer cells. Increasing evidences report a basal expression in normal cells especially in lymphocytes, mesenchymal stem cells (MSCs), endometrium and testis for maintenance of senescence, or during S phase of cell cycle [95-97].

The pan-tumoural overexpression of Survivin and hTERT makes their respective promoters an attractive target for cancer restricted replication of OVs.

CXCR4 is the receptor of CXCL12 chemokine. It has been reported that CXCR4 is expressed in 23 cancer types, including ovarian and breast cancer

promoting tumour growth, progression and metastasis [98-100]. Its expression is often associated to HER2 in breast and ovarian primary tumours and in metastasis, correlating with poor survival of patients [101]. In spite of tumour correlation, CXCR4 is widely expressed in normal tissues including bone marrow, MSCs, immune system, endothelial cells, vascular smooth muscle cells, gastrointestinal tract, alveolar epithelial cells and in central nervous system (CNS) [102,103]. CXCR4 plays an essential role in homing, retention and mobilisation of haematopoietic CD34+ stem cells, migration of many immune cells (naïve lymphocytes and unprimed T cells, monocytes, B cells, NK cells, dendritic cells, mast cells) [104].

Therefore, I explored the mRNAseq database of The Cancer Genome Atlas (TCGA) (Institute TCGA Genome Data Analysis Center (2016): Firehose stddata__2017.9.24 run. Broad Institute of MIT and Harvard.) to compare systematically the expression of my three target genes in normal and tumour tissues. As expected, Survivin and hTERT showed a stronger tumour-restricted expression, compared to CXCR4 (Fig.11). Indeed, expression in normal tissues was restricted i) to thymus for survivin, ii) to gastrointestinal tract, pancreas, thymus and thyroid for hTERT, iii) fairly widespread in healthy tissues for CXCR4.

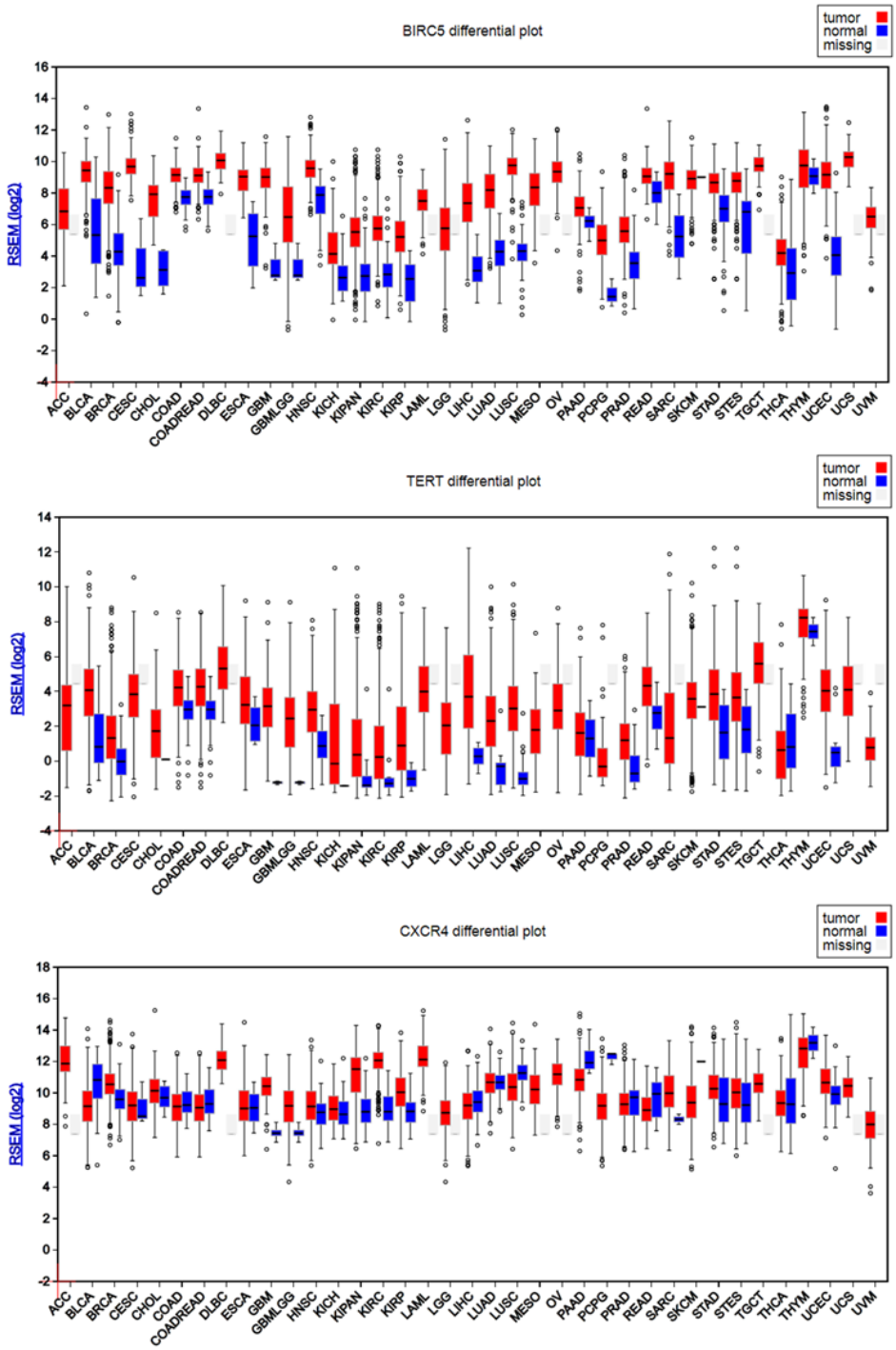


Fig. 11 *In silico* study of tumour-selective promoters by mRNASeq database analysis. The panels show the analysis of mRNA expression from The Cancer Genome Atlas of the three target genes (*BIRC5*, *hTERT*, *CXCR4*). The boxes compare the expression of mRNAs in

tumour tissues (red boxes) and into their normal counterparts (blue boxes). The missing reports are in grey. Abbreviations: ACC (Adrenocortical carcinoma), BLCA(Bladder Urothelial Carcinoma),,BRCA(Breast invasive carcinoma), CESC(Cervical squamous cell carcinoma and endocervical adenocarcinoma), CHOL(Cholangiocarcinoma), COAD(Colon adenocarcinoma), COADREAD(Colorectal adenocarcinoma), DLBC(Lymphoid Neoplasm Diffuse Large B-cell Lymphoma), ESCA(Esophageal carcinoma), FPPP(FFPE Pilot Phase II), GBM(Glioblastoma multiforme), GBMLGG(Glioma), HNSC(Head and Neck squamous cell carcinoma), KICH(Kidney Chromophobe), KIPAN(Pan-kidney cohort(KICH+KIRC+KIRP)), KIRC(Kidney renal clear cell carcinoma), KIRP(Kidney renal papillary cell carcinoma), LAML(Acute Myeloid Leukemia), LGG(Brain Lower Grade Glioma), LIHC(Liver hepatocellular carcinoma), LUAD(Lung adenocarcinoma), LUSC(Lung squamous cell carcinoma), MESO(Mesothelioma), OV(Ovarian serous cystadenocarcinoma), PAAD(Pancreatic adenocarcinoma), PCPG(Pheochromocytoma and Paraganglioma), PRAD(Prostate adenocarcinoma), READ(Rectum adenocarcinoma), SARC(Sarcoma), SKCM(Skin Cutaneous Melanoma), STAD(Stomach adenocarcinoma), STES(Stomach and Esophageal carcinoma), TGCT(Testicular Germ Cell Tumors), THCA(Thyroid carcinoma), THYM(Thymoma), UCEC(Uterine Corpus Endometrial Carcinoma), UCS(Uterine Carcinosarcoma), UVM(Uveal Melanoma)

In vitro characterization of tumour-selective promoters

Thus, by combining literature reports to bioinformatic tools of regulatory elements prediction and Encyclopedia of DNA Elements (ENCODE), I identified the putative promoter sequences for the three analysed genes in -260 to -1 for Survivin; -400 to -24 for hTERT; -300 to -1 for CXCR4 (positions are relative to the CDS) [105-114]. To assess the tumour-selective activity I generated reporter gene constructs by cloning the selected promoters upstream of the secreted alkaline phosphatase cDNA (SEAP). I transfected the reporter vectors into five human tumour cell lines of different origin, SAN, G361 and A375 (malignant melanoma), SKOV3 (ovarian adenocarcinoma), HEK293 (embryonic kidney) and in human normal MRC5 cells (normal lung fibroblasts). As expected, the Survivin, hTERT and CXCR4 promoters were active in all the selected cancer cells; contrariwise, only a low or just detectable activity was observed in MRC5 cells. As a control, the cytomegalovirus (CMV) promoter activity was also assessed in the same cell lines. The comparable potency of CMV promoter in normal and tumour cell lines confirmed the idea that selected promoters were actually tumour specific. Unsurprisingly, it was not possible to define a universal ranking of promoter strengths, due to peculiar combination of promoter activity-cell line (i.e. CXCR4 in A375, or Survivin in SKOV3). Despite this, promoters have fairly consistent strengths across different tumour and normal cell types (Fig.12). Among the three promoters, CXCR4 showed the highest variability across the analysed tumour cell lines, being also the most active in normal MRC5 cells. Telomerase and Survivin promoters showed comparable activities, but taking into account both gene reporter assays and bioinformatic analysis, I selected Survivin as the promoter of choice for designing replication-conditional OV.

One additional reason for selecting Survivin deals with the evidence that its promoter was also particularly active in SKOV3 cells, which are tipically used as a suitable line for HSV-1 production. Once chosen the region of Survivin promoter as the target sequence, which I named short Survivin (S_Survivin), I tried to extend the minimal promoter length to evaluate whether a wider extension could have conferred higher strength to the promoter activity. So, I evaluated the strength of a longer promoter covering the region -830 to -70 ATG (long Survivin, L_Survivin), which is partially overlapping with the previous one (Fig.13). As before, I tested L_Survivin promoter by SEAP reporter assays in comparison to S_Survivin form and to CMV promoter in SKOV3 and MRC5 cells. In this assay, I evaluated the SEAP production both at 48 and 72h post transfection to mimic the critical timing of HSV-1 infection.

The short length of Survivin promoter showed a higher activity than the long one, similar to that of CMV, without perturbing the tumour selectivity (Fig.13). To confirm that S_Survivin promoter was responsive to physiological cell-cycle stimuli, I used Nocodazole to arrest cells in G2/M phase since, as mentioned before, Survivin is particularly active in this phase of cell cycle. Therefore, I transfected SKOV3 with short Survivin SEAP reporter vector and treated with 0.1µg/ml Nocodazole. Twelve hours post treatment, I measured SEAP activity from conditioned media. As expected, the activity of short Survivin in Nocodazole treated cells was at least 5-fold higher compared to DMSO (control) treated cells (Fig.14). Considering the power, the "safety" and the more compact size, compared to long form, I selected the "short Survivin" as promoter for HSV-1 engineering.

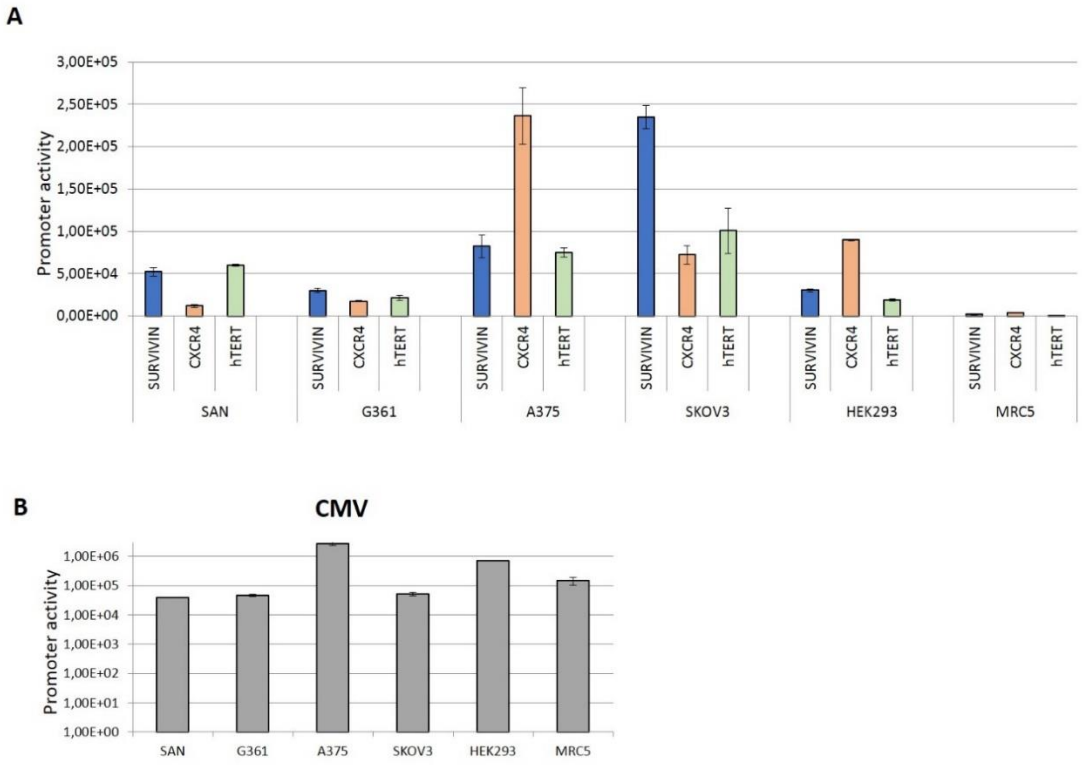


Fig. 12 Promoters expression in normal and tumour cell lines. The promoter activity has been assessed by SEAP reporter assays. In the panel A, the expression of the three selected promoters, namely Survivin (Blue bars), CXCR4 (orange bars) and hTERT (Green bars) was assessed in tumour (SAN, G361, A375, SKOV3, HEK293) and normal (MRC5) cells. The panel B shows the expression of the CMV promoter (Grey bars) in both tumour and normal cells.

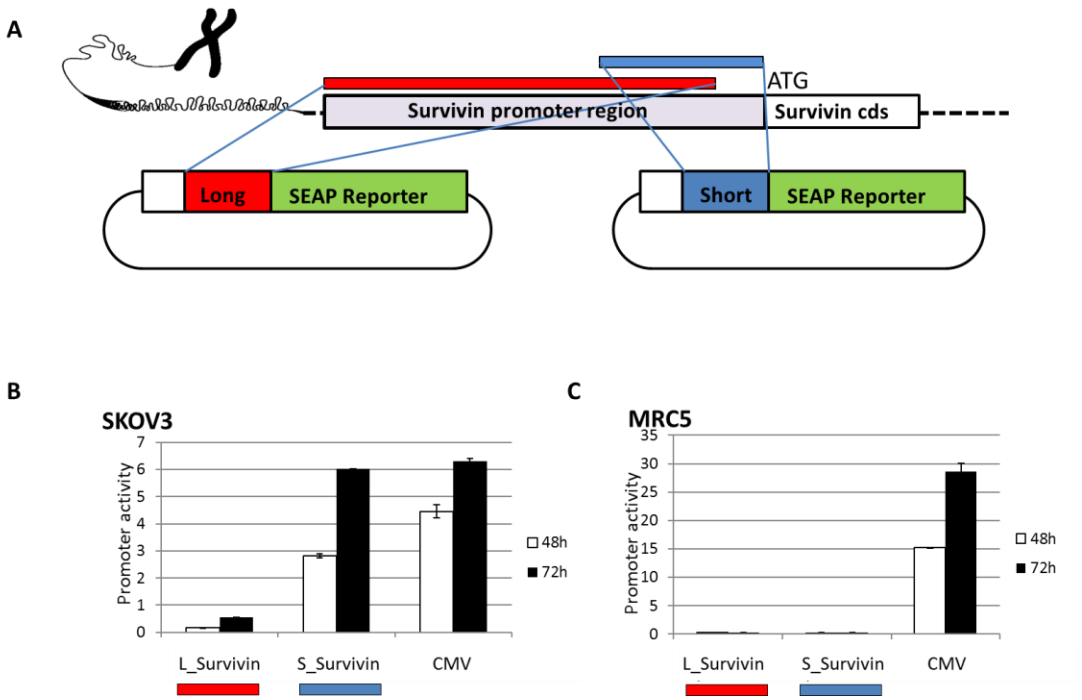


Fig. 13 Survivin promoter length optimization. (A) A schematic representation of survivin promoter region subcloned into vectors for SEAP reporter assays. The Long Survivin promoter (Red) is from -830 to -70; the Short Survivin promoter (Blue) is designed over -260 to -1 from ATG. (B and C) SEAP activity measured from conditioned media 48h (white bars) and 72h (black bars) after transfection in SKOV3 (B) and MRC5 (C).

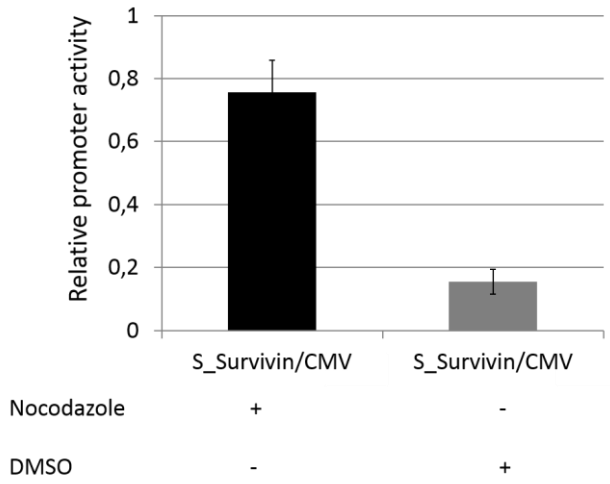


Fig. 14 Nocodazole treatment with Short Survivin SEAP assay. After SKOV3 cells were transfected with short Survivin SEAP reporter vector, they were arrested in G2/M phase with 0.1 µg/ml of nocodazole. 12 hours post treatment, the SEAP activity was analysed. As the image shows, the Survivin promoter has 5-fold higher activity (black bar) in Nocodazole treated compared to DMSO-treated cells (grey bar).

Generation and comparison of different layouts of Survivin replication conditional HSV-1

In my study, I therefore chose to drive the expression of the essential viral gene ICP4 by the S_Survivin promoter. To generate the S_Survivin replication conditional oncolytic HSV-1 virus, I used the recombineering cloning system. The starting vector was the wt Human herpesvirus 1 strain F (GenBank accession number: GU734771.1), containing a bacterial artificial chromosome (BAC) inserted between UL3-UL4 viral genes [115,116]. The BAC construct also encodes eGFP under control of early HSV α27 promoter, to facilitate the monitoring of infections. Since the regulatory elements of HSV-1 are not fully understood, I decided to compare the effectiveness of two deletions/insertion sites upstream both copies of ICP4. As starting point I used, as reference sites, an extended deletion from Zhang et al (-600 to -1 from ICP4 CDS start site) [35] and a more restricted deletion (-650 to -300 from ICP4 CDS start site) from Bloom et al. [117]. In order to keep the Kozak consensus sequence of ICP4, the reference Zhang deletion was reduced from -600/0 to -600/-20 bps from ATG (Fig.15). ICP4 is present in two copies in HSV-1 genome, as it is located in a short repeated region. To replace both endogenous promoters with S_Survivin, I performed two positive, followed by two negative, steps of *sacB/Amp*^{r}*/lacZ* recombineering (see M&M). According to the deletion, the corresponding BAC-HSV-1 constructs were named, respectively, B_RC and Z_RC (RC: replication conditional). After quality checking of BAC constructs (PCR amplicon size, digestion profile, Sanger sequencing) (data not shown), I rescued the viral particles by BAC transfections and subsequent amplifications in SKOV3 cell line, since the latter is a good cell factory for HSV-1 (see M&M). To verify preliminarily that the replication of RC_oHSVs was tumour selective, I infected SKOV3 and normal MRC5 cells with wild-type and RC viruses at same MOI and monitored infections 72h later. As can be shown in figure 16, both Z_RC and B_RC showed a limited replication and spread in MRC5 cells, compared to the wt strain, LM55. Single interspersed green cells observed upon MRC5 infection with both RC-HSVs suggested that RC viruses were able to enter normal cells and that they were not able to replicate efficiently in the non-transformed cellular background. Contrariwise, the spread of RC_HSVs in tumour SKOV3 was not limited by promoter replacement.

To select the best layout for further assessments, I evaluated in quantitative assays viral replication in terms of genome copies (gc) and infectious viral particles produced upon infection with B_RC and Z_RC OVs. Thus, I infected

SKOV3 cells at multiplicity of infection (MOI) of 0.1 with both Z_RC and B_RC. The quantitative TaqMan real-time PCR assays revealed that at all analysed time points (72h, 96h, 120h post infection) the genome copies (gc) produced per cell (gc/cell) in the B layout were at least the double, compared to those produced in the Z layout (Fig.17). To rate the virus concentration as infectious dose produced (plaque forming units, pfu), I performed plaque assays with conditioned media from Z_RC or B_RC infected SKOV3 cells. Surprisingly, despite a similar genome replication, infectious dose produced by B_RC was a hundred times greater than those rescued by Z_RC (Fig.17). From these notes, I decided to continue my investigation with B_RC layout to which from now on I will refer as Survivin_oHSV.

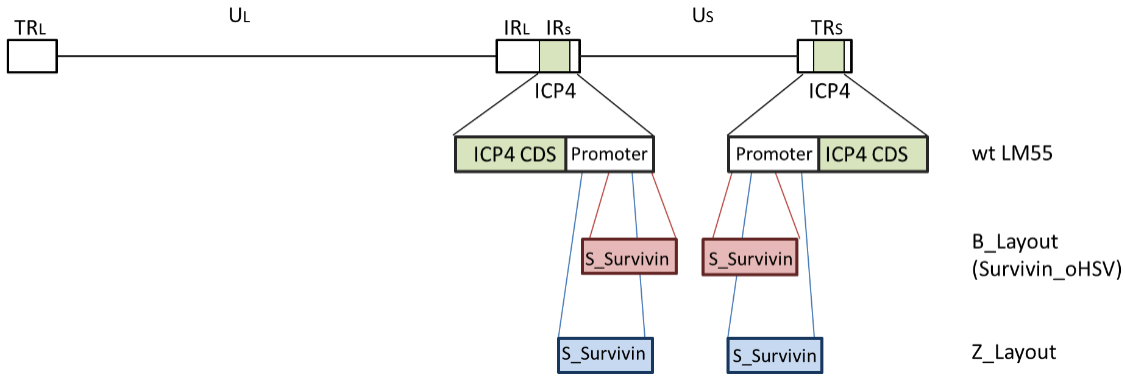


Fig. 15 Schematic representation of replication conditional HSV-1 generated. The cartoon shows the regions of endogenous ICP4 promoters replaced with Short Survivin promoter. In red is the restricted deletion of B layout from -650 to -300 from ICP4 CDS (green) start site; in blue the extended deletion -600 to -20 of the Z layout is shown.

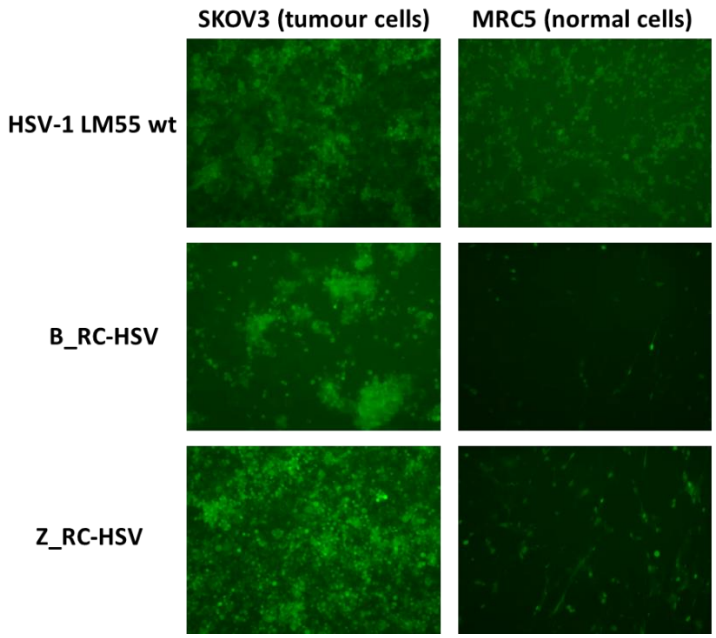


Fig. 16 Replication of RC_oHSV and wt HSV-1 in tumour SKOV3 and normal MRC5 cells assessed by eGFP spread. The fluorescence microscopy images of SKOV3 and MRC5 infected cells reveal that wt HSV-1 LM55 is able to spread both in tumour and normal cells (top panel). Contrariwise, B and Z layout of RC_oHSV infect SKOV3 tumour cells, but have a limited ability to replicate in normal MRC5 cells, as single green cells are observable (middle and bottom panels).

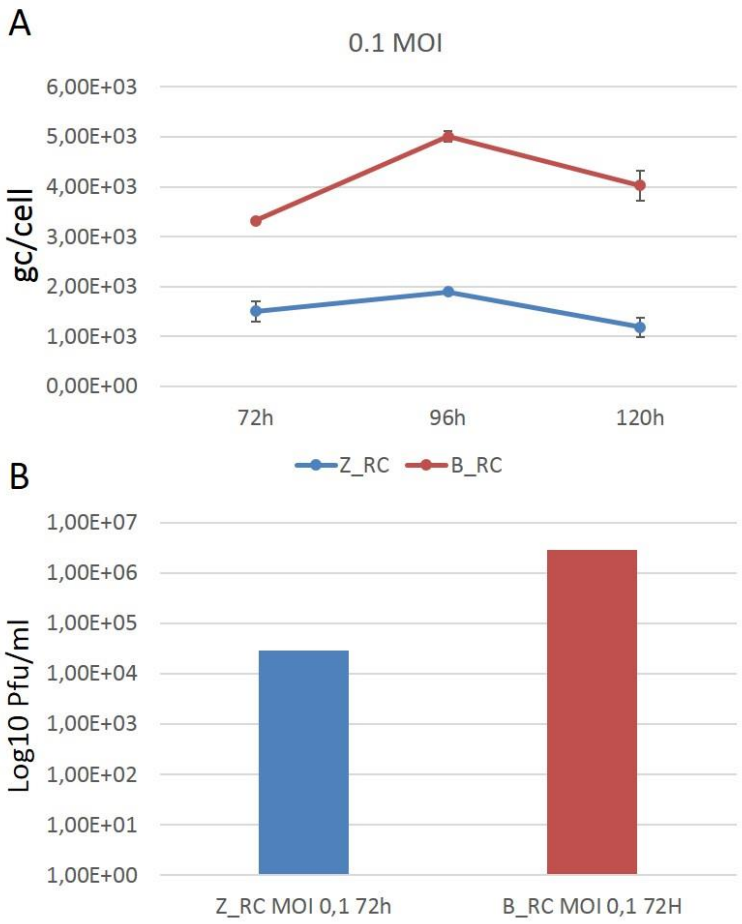


Fig. 17 Evaluation of replication and viral yield of B and Z Replicational Conditional oHSV. After infection of SKOV3 cells at 0.1 MOI the viral replication (panel A) in terms of genome copies and the infectious viral particles (panel B) of B_ and Z_ Replicational Conditional oHSVs were evaluated. The genome copies (gc) per cell (gc/cell) produced by B_RC virus are double to Z_RC (panel A). The infectious viral particles produced by B_RC is hundred times higher than that produced by Z_RC (panel B).

Survivin_oHSV specifically replicates in cancer cells

To study the selective oncolytic potential of transcriptionally retargeted Survivin_oHSV, its activity was compared to the parental wild-type counterpart, LM55. First, I infected tumour cell lines (SKOV3, G361, A375, SAN), as well as normal MRC5 cells at 0.1 MOI, monitoring infection up to 24h. The use of eGFP (encoded in BAC downstream of an immediate early promoter) as reporter was useful for monitoring infection and spread of viral particles, as shown in figure 18. The data show that spread of Survivin_oHSV is similar, or slighty lower, compared to the one observed for wt LM55 in SKOV3 and SAN tumour cell lines. Surprisingly, Survivin_oHSV spread seems even higher than the one observed for wt LM55 in A375 melanoma cells. As expected, the "single cell" green phenotype in normal MRC5 cells suggests that Survivin_oHSV enters normal cells, but does not replicate in survivin promoter non-competent cells. On the contrary, the spread of wt LM55 in normal MRC5 is not restricted, looking equivalent to what observed in tumour cell lines. The standard light-field images (phase-contrast) underline a direct correlation between viral spread in terms of eGFP expression, and induced cytopathic effect (cpe), evident as dead cells that round up and become detached (Fig.18). As aforesaid, the wt LM55 still replicated and killed, to similar extents, both normal and tumour cell lines. The G361 cells appeared resistant to wt HSV-1 infection, therefore it has been discarded from analysis of viral replication. Next, I titrated the viral genome copies 24h after infection, in order to assess the actual replicative potential of the Survivin_oHSV. Figure 19 shows that the virus replicates in all the three tumour cell lines, while it is just barely detected in normal MRC5 fibroblasts. The replication of RC Survivin_oHSV in these experimental conditions (MOI 0.1 and 24h post-infection), decreased by 4-fold in SAN cells and approximately 10-fold in SKOV3, compared to the parental wild-type virus. Notably, SKOV3 were the cells with the best performance in gc/cell, in agreement with the reporter assay data for the S_Survivin promoter. A limited impairment in replication of engineered viruses compared to wt is, however, expected, since each manipulation may result in virus attenuation, both in normal and in tumour cells. Of note, in A375 Survivin_oHSV replicated similarly or even better than wt LM55, indicating a tumour/virus combination dependent mechanism (Fig.19).

To exclude a late toxicity of RC Survivin_oHSV in normal MRC5 cells, I expanded the experimental conditions, relative to MOI and time points. Therefore, I infected SKOV3 and MRC5 at 0.01, 0.02, 0.1, 0.5 MOI and

monitored the cytopathic effect for up to 96h. In figure 20 the representative time points of 48h and 96h post infection (pi) are shown. The results showed that the cytopathic effects of Survivin_oHSV and wt LM55 in tumour cell line SKOV3 were quite similar. On the other hand, normal MRC5 cells supported very well the replication of wt LM55 at all analysed MOI, up to reaching a full cpe at 96h pi. A very different effect was evidenced in MRC5 cells infected with Survivin_oHSV at 0.01 and 0.02 MOI, that showed a non-infected like phenotype for up to 96h pi. However, a dose-dependent cytopathic effect was observed in MRC5 infected at highest MOI of 0.1 and 0.5 with Survivin_oHSV (Fig.20).

To better understand the kinetics of infection and replication, multiple cycles of infection are usually required. Thus, I planned infections at lower multiplicity of infection compared to the conditions described in figure 17 to assess the genome copies/cell values. Replicates of SKOV3, A375 and MRC5 cells were infected at MOI 0.01 and 0.03 with LM55 and Survivin_oHSV and after 24h, 48h and 72h pi, viral particles were titrated by TaqMan, to generate a complete overview of viral replication as a function of time and dose. The results of the assay in figure 21 confirmed, even under these conditions, the tumour-restricted tropism of the replication conditional Survivin_oHSV. Indeed: i) during the time course of infection, Survivin_oHSV exhibited a similar trend of replication as wt LM55 in human survivin positive tumour cell lines; ii) despite the same trend, replication of Survivin_oHSV was confirmed to be weaker than that, observed for the wild-type LM55 strain; iii) wt HSV-1 LM55 replicated equally well in normal and in tumour cells; iv) no viral replication of Survivin_oHSV was appreciable in normal MRC5 infected at low MOI of 0.01; v) Survivin_oHSV replication in MRC5 was barely detected 72h pi at 0.03 MOI (at least 1000 fold less than wt) (Fig.21). Also looking at oncolytic activity in tumour cell lines, the recombinant RC virus could reach a delayed but complete cytopathic effect compared to parental wt virus LM55 (Fig.22 representative 0.03 MOI). Moreover, while Survivin_oHSV did not form plaques in normal MRC5, the wt LM55 replicated quickly, in agreement to genome replication assessed previously (Fig.22).

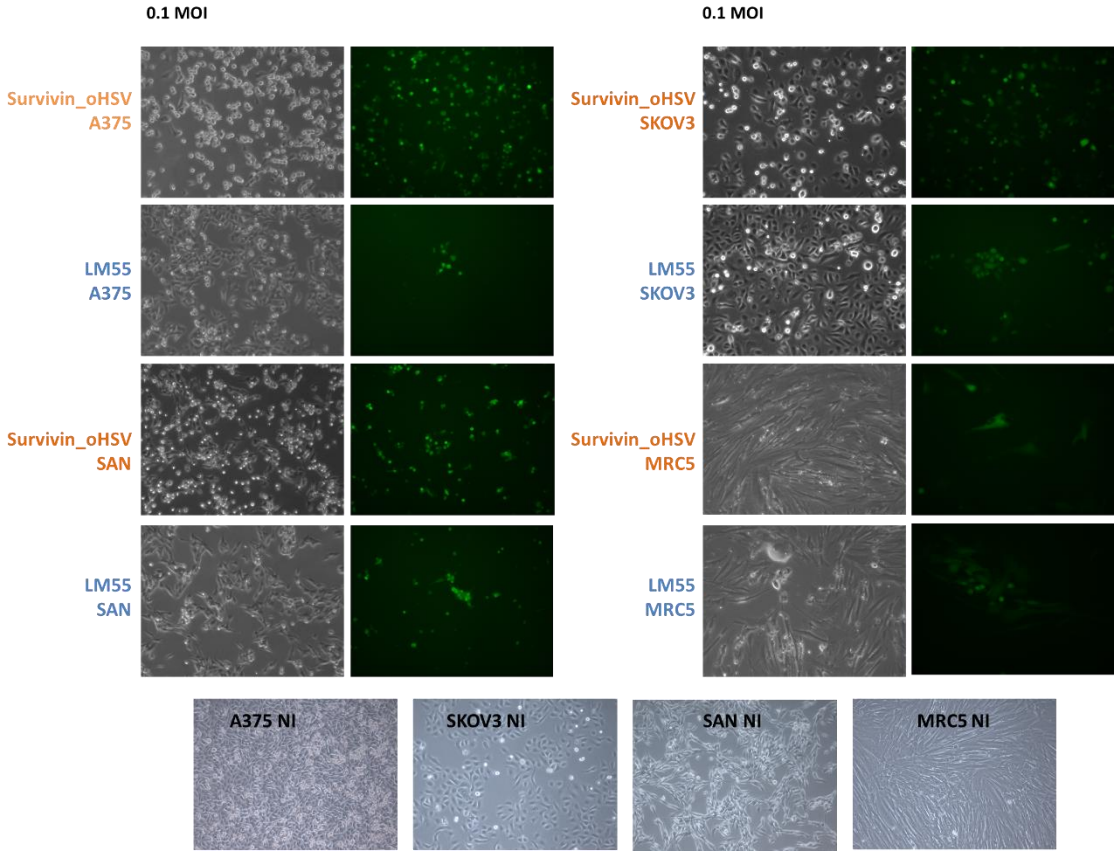


Fig. 18 Evaluation of viral spread and cytopathic effect of Survivin_oHSV and wt LM55 in tumour and normal cell lines. The panels show the viral replication 24h post infection at 0.1 multiplicity of infection with the RC Survivin_oHSV and wt LM55. The viral spread is evaluable as eGFP positive cells under fluorescence microscopy (right images); the cytopathic effect is evident as round and dethatched cells by phase-contrast microscopy (left images). On the bottom the non-infected cells are shown.

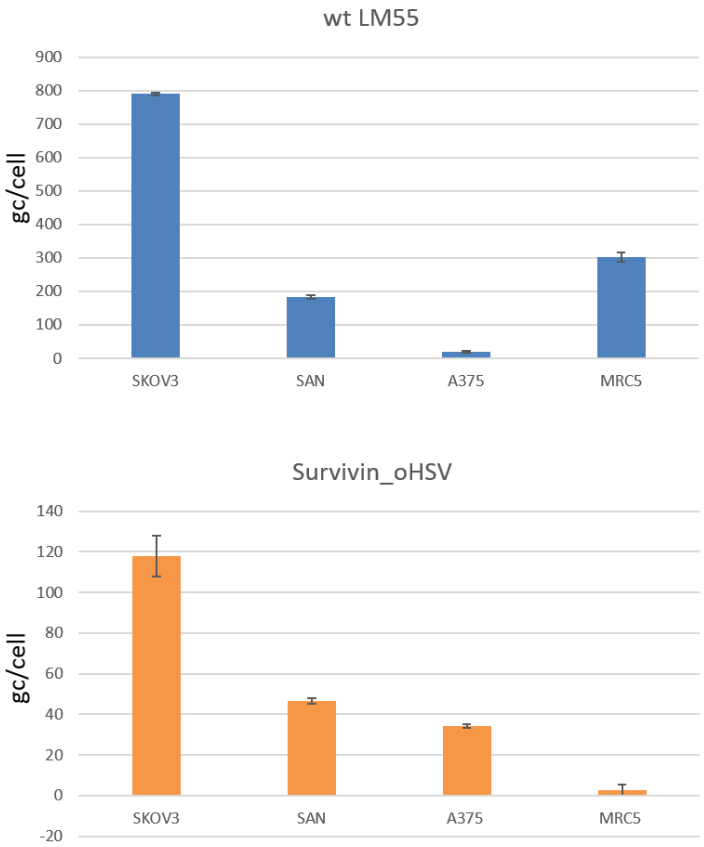


Fig. 19 Replication of Survivin_oHSV and wt LM55 in tumour and normal cell lines.

SKOV3, SAN, A375 and MRC5 cells were infected at 0.1 MOI with Survivin_oHSV and wt LM55. 24h post infection, the genome copies (gc) produced per cell were assessed by RealTime PCR. The viral yields are clustered per virus type. In both panels the wt LM55 and RC Survivin_oHSV are respectively represented as blue and orange bars.

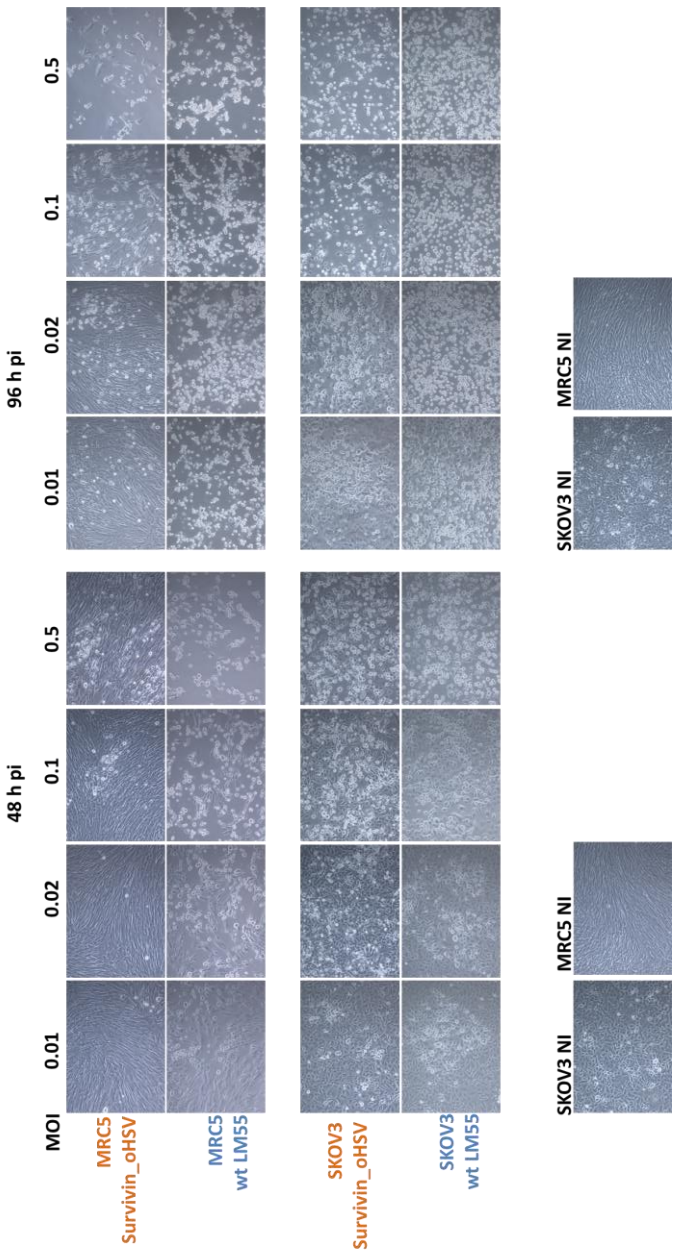


Fig. 20 *In vitro* oncolytic activity of RC Survivin HSV and LM55. To monitor the cytopathic effect in tumour (SKOV3) and normal (MRC5) cells, the two cell types were infected with Survivin_oHSV and the control wt LM55 at different MOI (0.01, 0.02, 0.1, 0.5) and for different timepoints post infection (up to 96h). The image shows two representative time points, 48h and 96h pi. In MRC5 cells, the control LM55 shows an early (48h pi) cytopathic effect at low MOI (0.01-0.02), in comparison to the Survivin_oHSV that shows a limited effect at the same MOI (0.01-0.02), also up to 96h pi. However, a dose-dependent cytopathic effect was shown at high MOI (0.1-0.5). In contrast, the cytopathic effect elicited in SKOV3 cells is similar for both viruses. On the bottom the non-infected cells are shown.

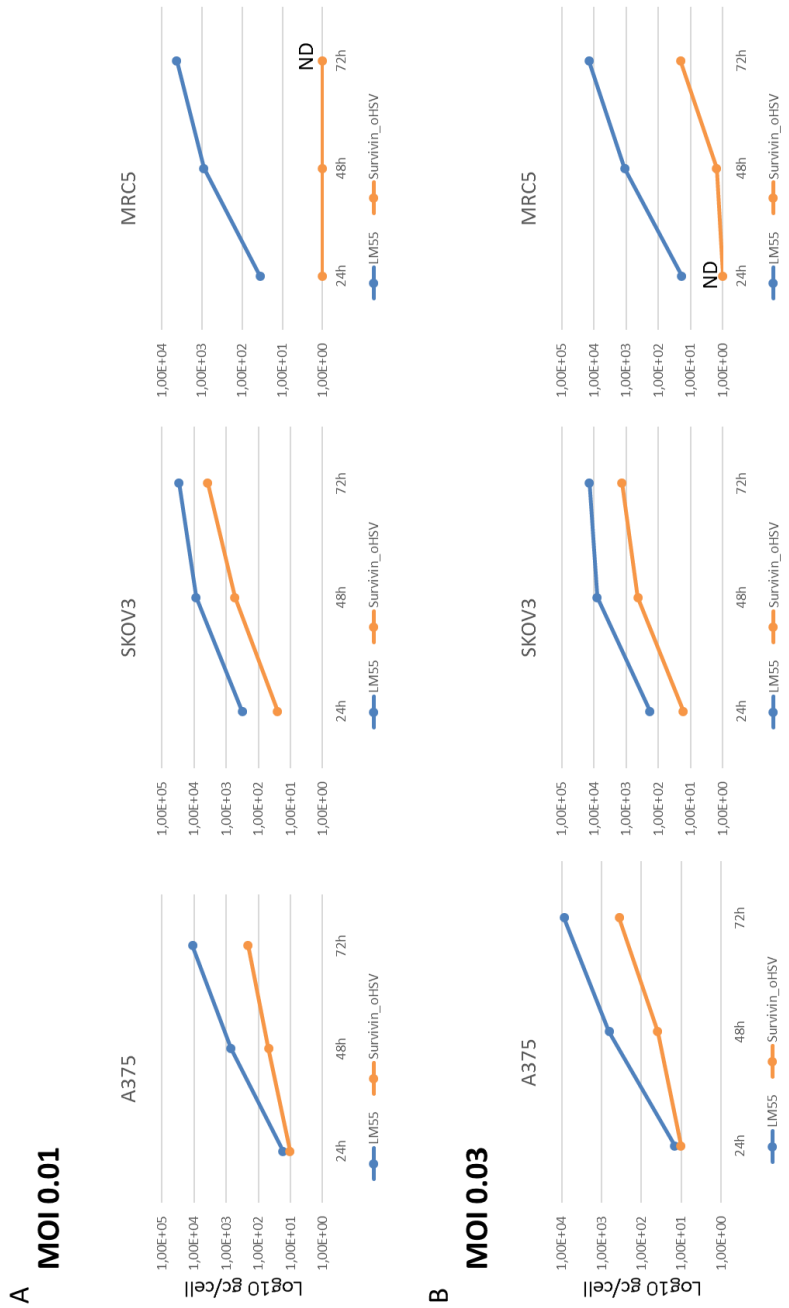


Fig. 21 Comparison of Survivin_oHSV1 and wt LM55 replication. Survivin_oHSV1 and wt LM55 replication were assessed in the survivin positive tumour cell lines (SKOV3 and A375), and in normal MRC5 cells. Cells were infected at 0.01 (panel A) and 0.03 (panel B) MOI. The growth curves were assessed from 24 to 72h post infection. The wt LM55 is reported in blue; the RC Survivin_oHSV in orange. ND: not detected

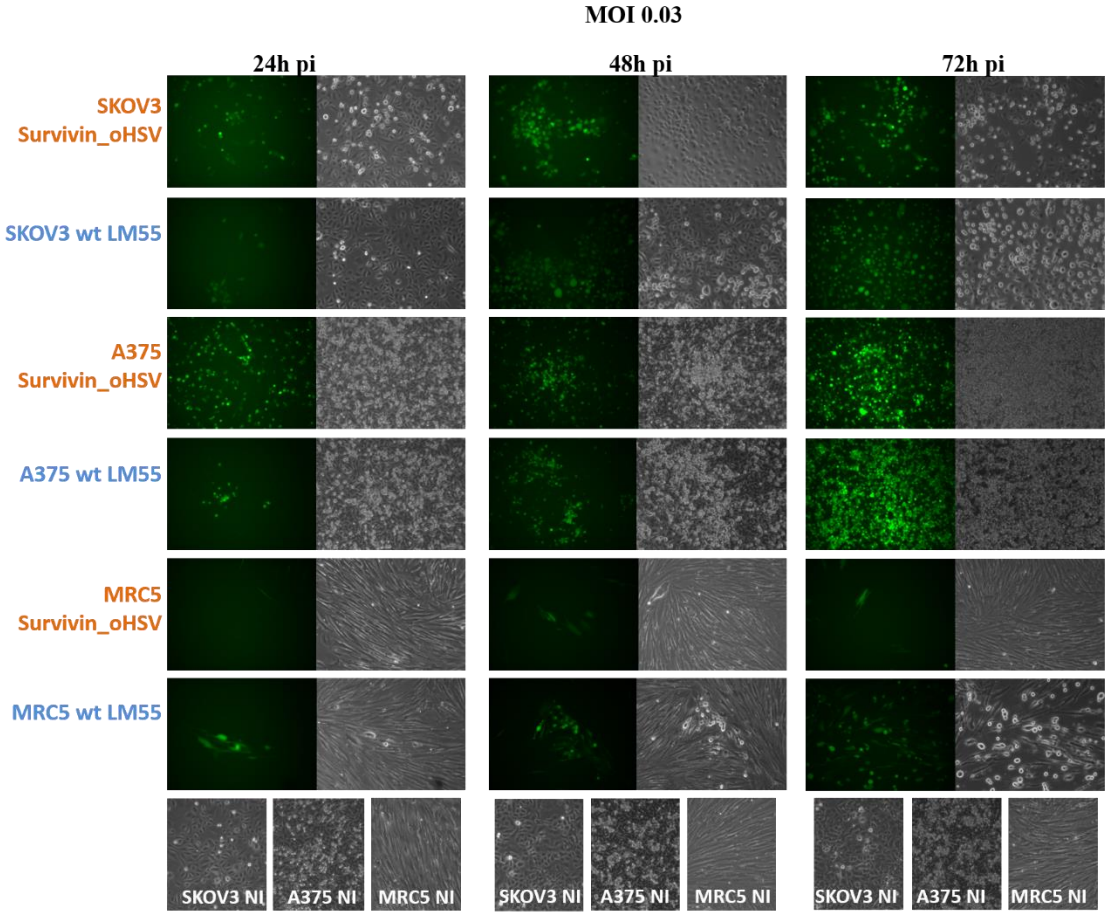


Fig. 22 Evaluation of viral spread and cytopathic effect of Survivin_oHSV and wt LM55 in tumour and normal cell lines. The images show the viral replication in SKOV3, A375 and MRC5 cells by Survivin_oHSV and wt LM55evaluated 24h, 48h and 72h post infection at 0.03 MOI. The viral spread is evaluable as eGFP positive cells under fluorescence microscopy (left panels); the cytopathic effect is evident as round and dethatched cells by phase-contrast microscopy (right panels). On bottom are shown the non-infected cells at the indicated time post infection.

Is two better than one? Double retargeting of oncolytic virus

The results of the replicative potential of the Survivin_oHSV demonstrated that replication conditioning of HSV-1 by survivin promoter mediates a selectivity for tumour cells up to 3 orders of magnitude, compared to the parental wt virus. Despite the effectiveness of this latter, a complete abrogation of replication in normal cells can't be achieved, especially at high MOI. These results are in agreement with literature data, showing a residual replication of RC OVs in normal cells, probably due to promoter leakiness [35]. To date, the potential adverse effects of this phenomenon have not been deeply assessed, since oncolytics in clinics or clinical trial are mostly attenuated and administered intratumorally. The emerging knowledge about the role of tumour-related proteins (i.e. Survivin or hTERT) in normal tissue is shedding light on the need for additional safety [118,119]. To ensure the safety of a conditionally replicative oncolytic virus, the addition of a second retargeting is, indeed, desirable. Thus, to implement the safety of our transcriptional retargeted Survivin_oHSV, I decided to combine the post entry transcriptional retargeting, to an entry retargeting. Due to the strong correlation between the two oncogenes Survivin and HER2, I decided to target the latter. Moreover, additional evidences suggest that HER2 amplification is not restricted to breast and ovarian cancers, but often occurs in other serious malignancies including those of the cervix, uterus, colon, prostate, pancreas, as well as non-small cell lung cancer and glioblastoma multiforme [120-125]. One of the most relevant approaches for retargeting of viral particles is the engineering of envelope viral glycoproteins with antibody fragments (scFvs). As mentioned before, Campadelli's group demonstrated that replacing amino acids 6 to 38 of HSV-1 gD with a Trastuzumab-derived scFv targeting HER2, abrogates the virus ability to enter through endogenous receptors HVEM/Nectin-1, thus allowing infection solely via HER2 receptor [126]. A relevant point is that the so generated OV, named LM113, is a non-attenuated virus. Therefore, once entered a cell, it should replicate indistinctly in normal and tumour cells. Beyond the detargeting from endogenous ligands, little is known about toxicity in healthy tissues expressing HER2. About that, both scientific literature [127] and protein expression database (The Human Protein Atlas) [128] indicate a considerable expression of HER2 in normal adult tissues as skeletal muscle, heart, bronchus, bladder, skin, breast, uterus (Fig.23).

To get an additive or synergistic safety of both transcriptional and receptor retargeting, I replaced by recombineering the aa 6-38 of gD glycoprotein in Survivin_oHSV, with the Trastuzumab-derived scFv (Fig.24). Trastuzumab is a monoclonal antibody to HER2, used in the treatment of breast and gastric/gastroesophageal cancer [129]. Potentially, this double retargeted OV should enter solely in HER2+ overexpressing cells and replicate through survivin promoter, sparing both HER2+ normal tissue and Survivin+

replicative normal cells. This double retargeted oHSV was named SurE_oHSV (Survivin_Erbb2_oHSV) also as a good wish for its "sure" effect. In addition, to compare the safety of single *vs* double retargeting, I also obtained from Campadelli's group the only gD retargeted virus (LM113). The bullet points below summarize the different constructs with the corresponding features:

- LM55 is the wt strain F of HSV-1 inserted into a BAC
- Survivin_oHSV is the replication conditional HSV-1, in which both copies of the endogenous promoter of ICP4 were replaced by Survivin promoter
- LM113 is a tropism-retargeted HSV-1, in which aa 6-38 of gD glycoprotein have been replaced by Trastuzumab-derived scFv
- SurE_oHSV is a double retargeted OV obtained by combining gD retargeting to Survivin_oHSV

First, to evaluate whether the predicted safety of SurE_oHSV was achieved at the expense of potency, I infected SKOV3 cells in a large range of MOI (from 0.0005 to 0.05) and monitored the infection up to 150h pi. Unpredictably, as shown in phase-contrast (Fig.25) and fluorescence (Fig.26) images, the double retargeted SurE_oHSV not only preserved the replication potency, but also exerted a very virulent phenotype, also at low MOI. The titration of viral particles from the conditioned media of the previous experiment, confirmed the reinvigoration in replication compared to Survivin_oHSV up to wt-like level (from 6E+03 to 1E+04 gc/cell) (Fig.27).

To study the effects of single *vs* double retargeting, I infected tumour and normal cells with LM55, Survivin_oHSV, SurE_oHSV, LM113 at 0.01 and 0.03 MOI. As tumour cells, I used SKOV3, since they are both Survivin+ and HER2++. Unfortunately, A375 cells do not express HER2, thus, they weren't useful for this characterization [130]. As a normal cell line, I exploited MRC5, almost negative for both HER2 and Survivin. The results regarding replication (Fig.28) and cytopathic effect (Fig.29) were as follows:

- As asserted in previous assays, LM55 replicated both in tumour and in normal cell lines.
- As expected, Survivin_oHSV replicated in SKOV3 tumour cell line even if less efficiently, compared to wt LM55. On the contrary, it entered in MRC5 (via endogenous ligands HVEM/Nectin-1), but its replication appeared limited (observable as single eGFP+ cells in Fig.30).
- LM113 replication in SKOV3 was comparable to that of wt LM55, due to high expression of HER2. In MRC5, despite LM113 entry was limited (observable as few eGFP+ cells, due to low HER2 display on cell surface), its spreading to bystander cells was somehow effective, probably because of some HER2 expression and passage through tight junctions (Fig.30).

- SurE_oHSV replication appeared more virulent than that, observed for Survivin_oHSV, LM113 and LM55 in tumour SKOV3, as appreciable by emptying in cell number 72h pi (Fig.29). An apparent decrease in replication happened at 72h (Fig.28) pi since, due to cell lysis, SurE_oHSV titer began to fall in the absence of sufficient host cells to be infected. On the contrary, an additive safety effect was achieved in MRC5 cells, appreciable as few single infected cells without any type of spread and toxicity (Fig.30).

An overview in virus performance is reported in Table 1 for all the generated vectors.

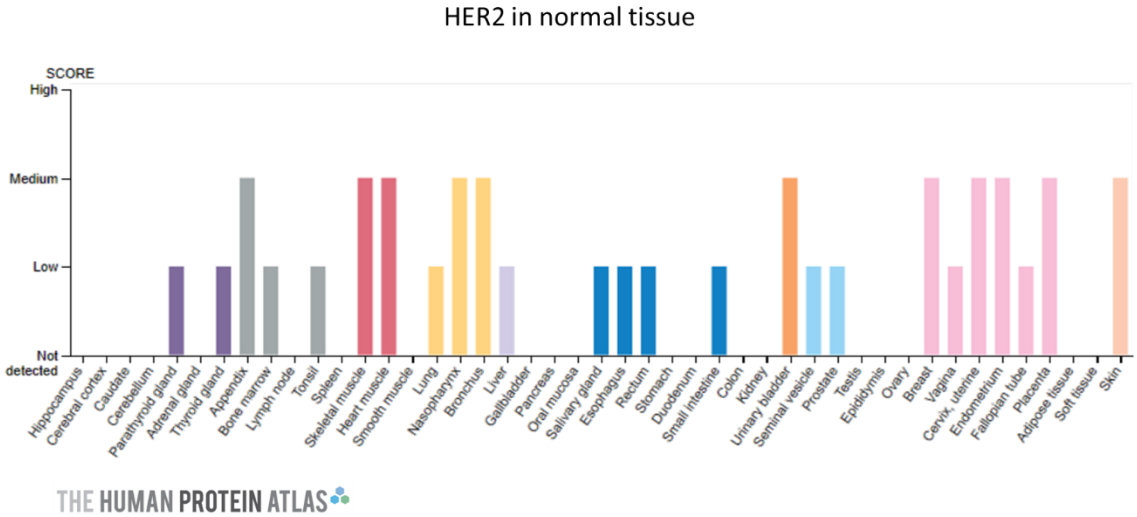


Fig. 23 Analysis of human HER2 expression in healthy tissue obtained by immunohistochemistry data from The Human Protein Atlas. The graph shows HER2 expression for each of the 44 analysed tissues. The color-coding is based on tissues with functional features in common.

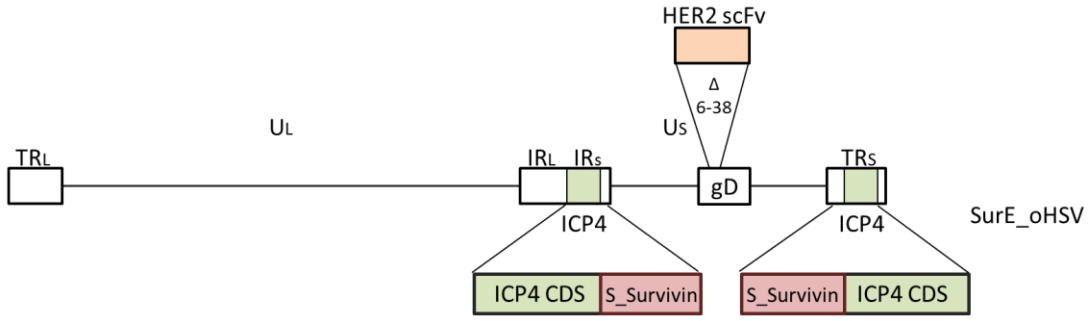


Fig. 24 Schematic representation of double retargeted oncolytic virus: SurE_oHSV genome. Starting from the previously engineered Replication Conditional HSV (Survivin_oHSV) backbone, in which both copies of wt ICP4 promoter were replaced with Short Survivin Promoter (S_Survivin), a further safety condition was added by retargeting the entry mechanism of HSV into host cells. The region of wt gD glycoprotein (6-38 bp) were substituted with HER2 scFv sequence, obtaining a selective infection only in HER2+ cells. This new oncolytic virus was named SurE_oHSV (for extended SurvivinErbb2_oncolyticHSV).

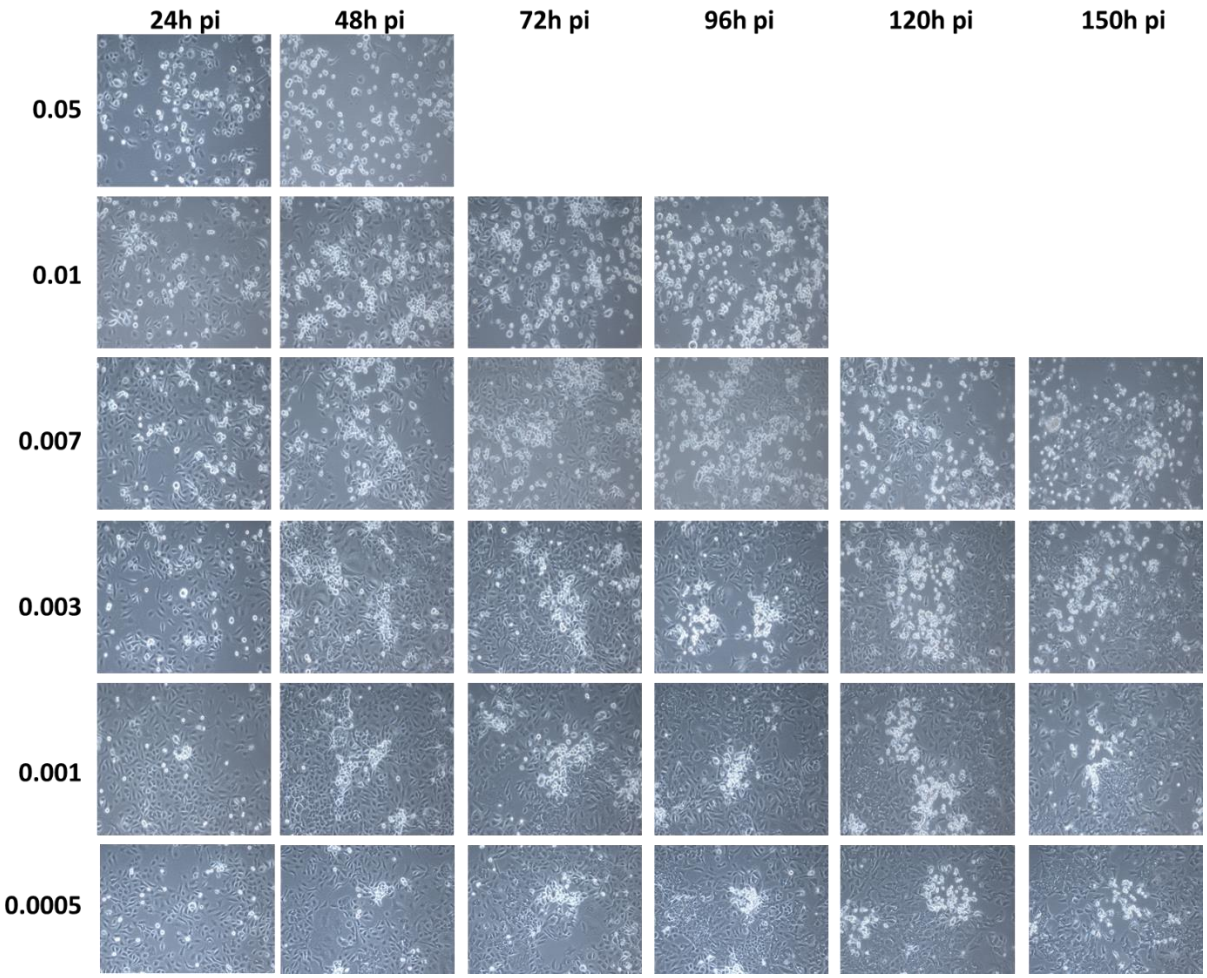


Fig. 25 Replication of double retargeted oHSV SurE_oHSV in tumour SKOV3 cell line assessed as phase-contrast microscopy. SKOV3 cells were infected with SurE_oHSV over a wide range of MOI (0.05 to 0.0005). The report shows the cytopathic effect at indicated time points of observation. The missing images were due to complete cytopathic effect.

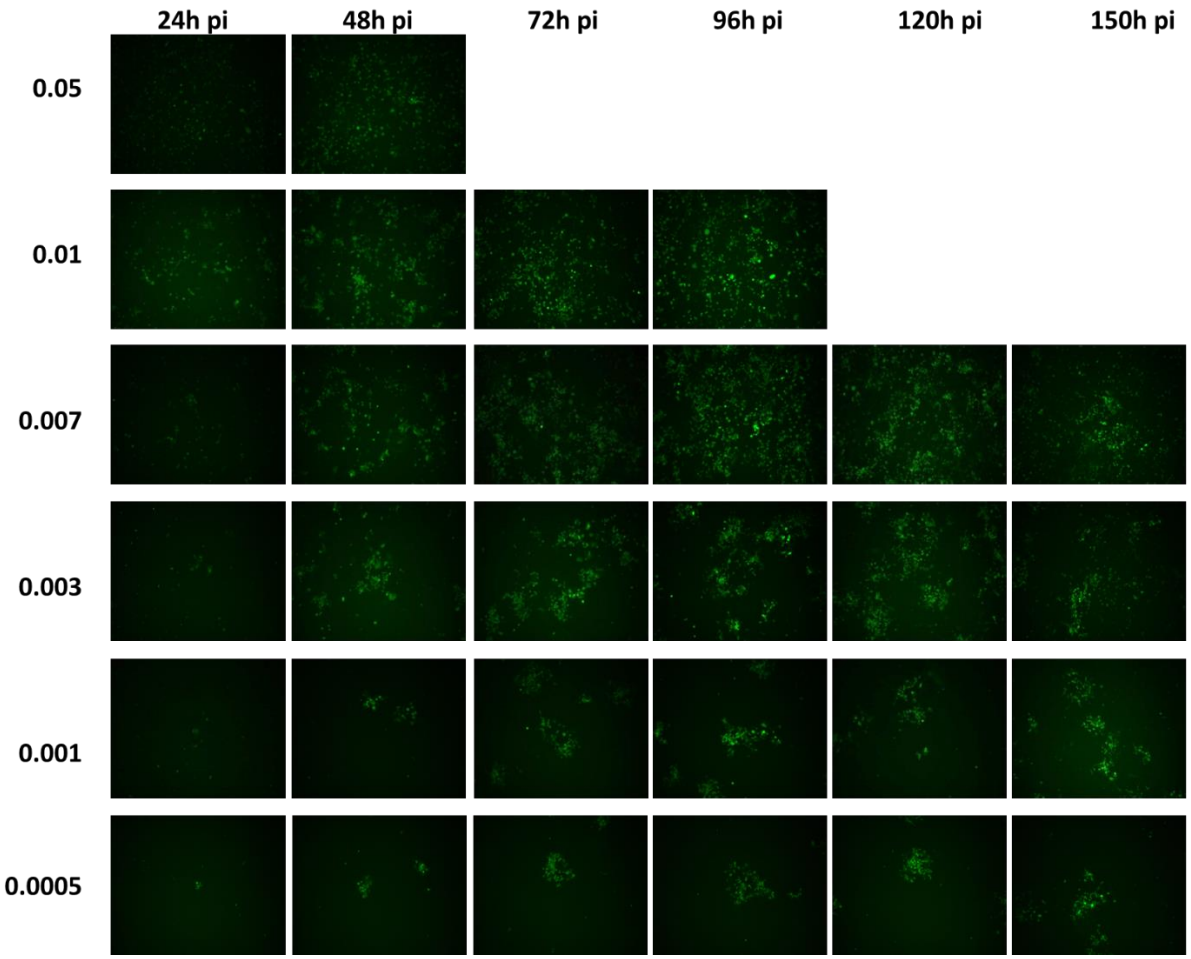


Fig. 26 Replication of double retargeted oHSV SurE_oHSV in tumour SKOV3 cell line evaluable as eGFP positive cells under fluorescence microscopy. Tumour SKOV3 cells were infected with SurE_oHSV as in figure 25. The report shows the amplification and spread of viral particles at indicated time points of observation. The missing images were due to complete cytopathic effect.

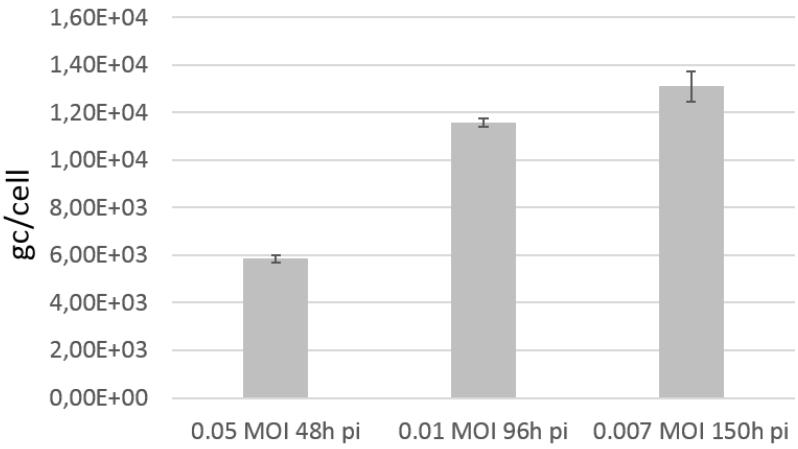


Fig. 27 Evaluation of SurE_oHSV replication in SKOV3, assessed as genome copies produced per cell. Viral particles from the previous experiment were dosed as viral genome copies by RealTime PCR. For each MOI, the viral replication was evaluated at the end point of infection (full cpe). The analysed MOI and time points are indicated on graph.

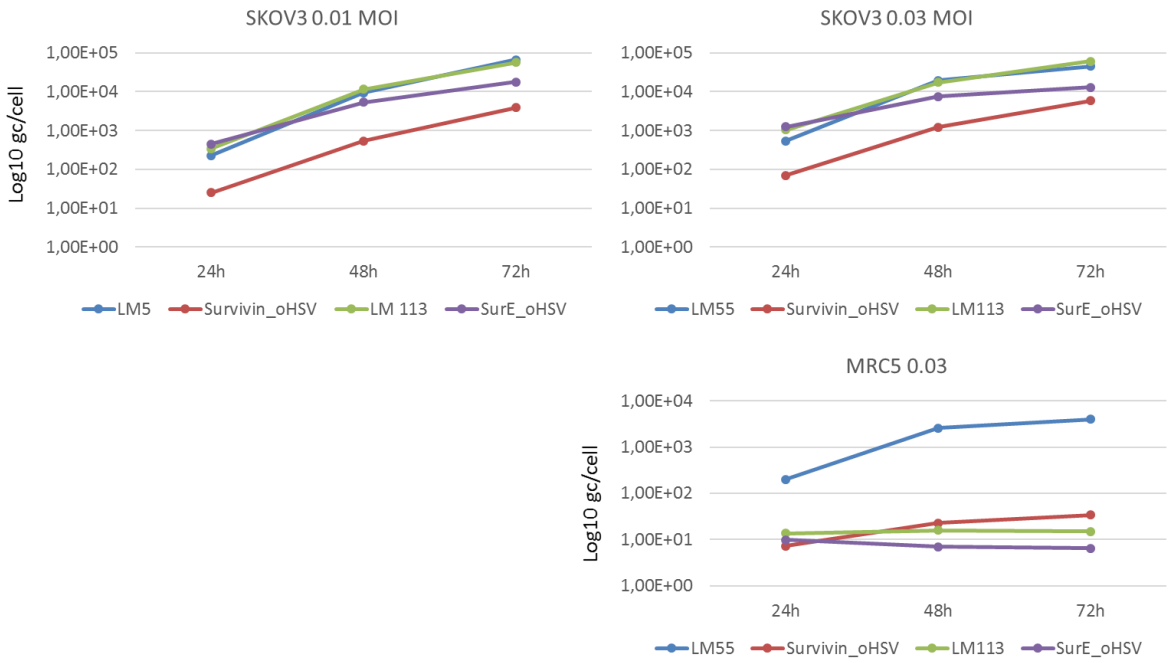


Fig. 28 Comparison of the single vs double retargeting oHSV replication. To study the effect of single (Survivin_oHSV, LM113) and double (SurE_oHSV) retargeting on viral replication, tumour and normal cells (respectively SKOV3 and MRC5) were infected with wt LM55, LM113, Survivin_oHSV and SurE_oHSV at 0.01 and 0.03 MOI. Genome copies were evaluated at different time points 24h, 48h, 72h post infection. LM55 replicated in SKOV3 and MRC5 cell lines without difference. The single retargeted, Survivin_oHSV, replicated in SKOV3 cells with less efficiency compared to the wt; in MRC5, the viral replication of RC oHSV was limited. LM113 in SKOV3 (HER2+) cells replicated efficiently, as the wt; in MRC5 due to a low expression of HER2 receptor, the viral replication was low. The double retargeted SurE_oHSV, had a high viral replication in tumour cell line, though its yield decreased at 72h pi because of exhaustion of live cell, as substrate, due to cell lysis. The combo retargeting of replication and entry mechanism synergises with the lowest viral replication in normal cell lines compared with the all other viruses.

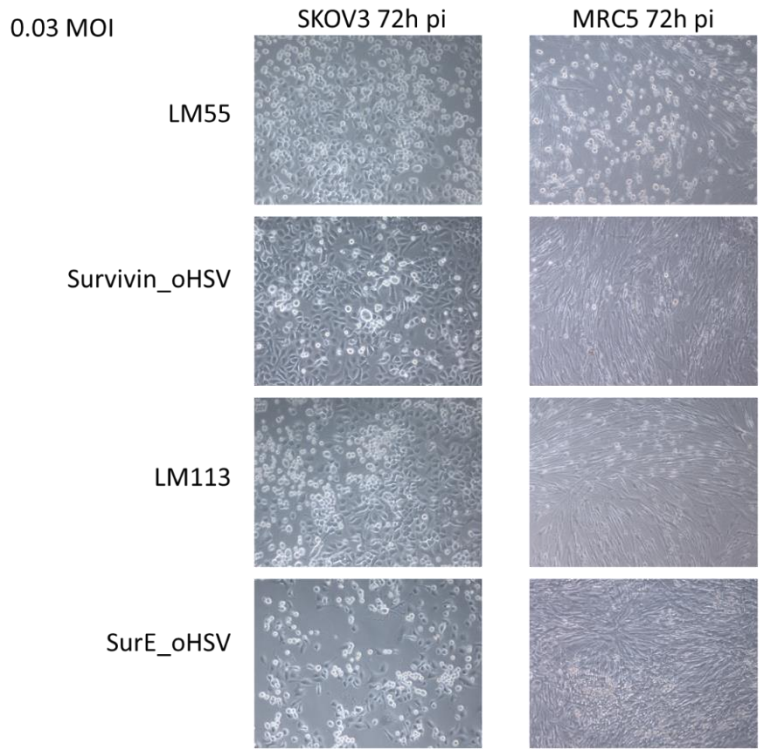


Fig. 29 *In vitro* study of the effects of the double retargeting on selective tumour killing. Single- (LM113, Survivin_oHSV), double- (SurE_oHSV) retargeted viruses and wt LM55 were used to infect tumour SKOV3 (left panels) and normal MRC5 cells (right panels) at 0.03 MOI. Images produced by phase-contrast microscopy were recorded 72h post infection and cytopathic effect is evident as detaching round cells.

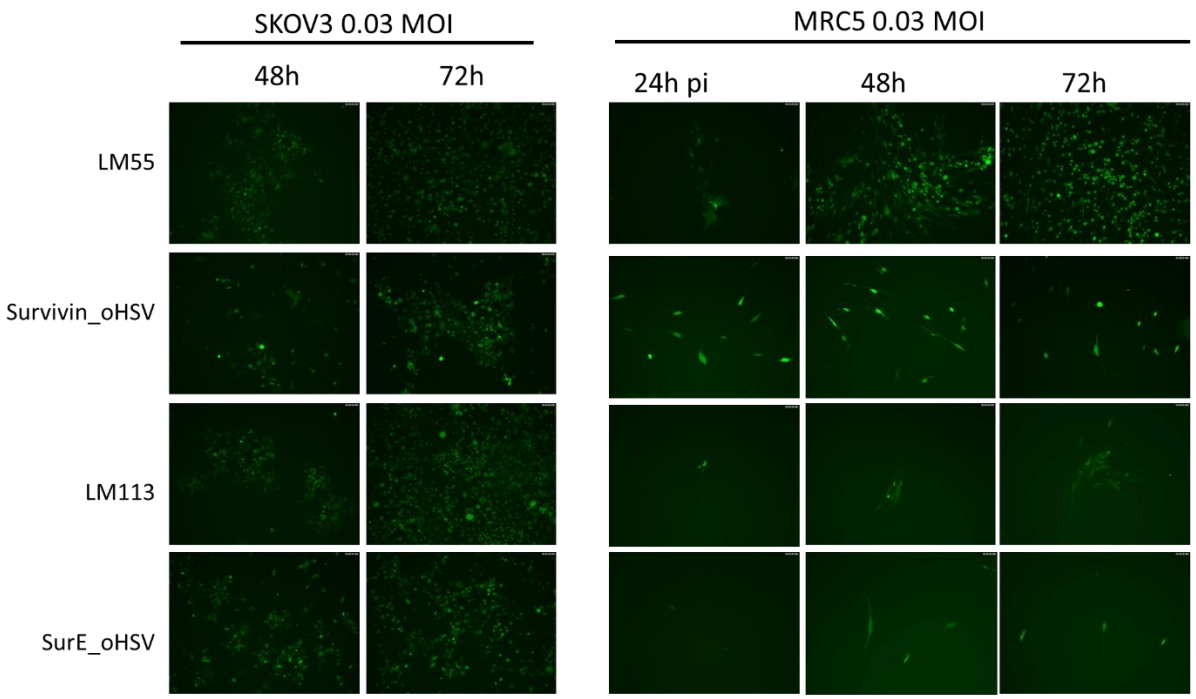


Fig. 30 Effects of single and double retargeting on viral spread. Single- (LM113, Survivin_oHSV), double- (SurE_oHSV) retargeted viruses as well as wt LM55 were used to infect tumour SKOV3 (left panels) and normal MRC5 cells (right panels) at 0.03 MOI. Images produced by fluorescence microscopy were recorded 24h, 48h and 72h post infection.

	SKOV3			MRC5		
Virus	ENTRY	REPLICATION	CPE	ENTRY	REPLICATION	CPE
LM55	++ HVEM/Nectin-1	++ by endogenous ICP4 promoter	+++	++ HVEM/Nectin-1	++ by endogenous ICP4 promoter	+++
Survivin_oHSV	++ HVEM/Nectin-1	+ by survivin promoter	+	++ HVEM/Nectin-1	+/- By survivin promoter leakiness	+
LM113	++ HER2	++ by endogenous ICP4 promoter	++	+/- HER2	++ by endogenous ICP4 promoter	+
SurE_oHSV	++ HER2	++ by survivin promoter	+++	+/- HER2	+/- By survivin promoter leakiness	-

Table 1 Summary of viral tropism in tumour SKOV3 (left side blue) and normal MRC5 (right side green) cells. For entry and replication: +: weak entry/replication; ++: medium entry/replication; +/-: limited entry/replication. For each box it is indicated the preferential way of entry or replication. For cytopathic effect (CPE): +++: massive cpe; ++: good cpe; +: limited cpe; -: no cpe.

Immunome repertoire generation

Massive parallel screening and selection of human scFv$_s$ targeting immune checkpoint modulators

Since the goal on my project was to generate a repertoire of cancer immunotherapeutics, we decided to isolate a large collection of human antibodies against major Immune Checkpoints (IC), namely, LAG-3, PD-L1, PD-1, TIM3, BTLA, TIGIT, OX40, 4-1BB, CD27 and ICOS, in collaboration with professor De Lorenzo's group. To this aim, we developed a novel strategy for high throughput sequencing-based screening (HTS) of phage display libraries. The main hurdle of this kind of screening is related to "quality" of protein target in terms of stability and preserved folding. To bypass this limitation, we took advantage of expression of target IC in their native conformation on T lymphocytes. Indeed, as explained in introduction, most of IC are expressed on T lymphocytes cell surface in response to activation and/or stimulation. To exploit this T cell feature, it was set up an activation protocol of human peripheral blood mononuclear cells (hPBMCs) to use these cells as substrate for the first cycle of selection. The phages eluted from this first cycle were potentially enriched for scFv$_s$ targeting our target immunomodulators, thus henceforth we referred to this sub-library as 'Immunome Library'. To split and enrich phages specific for each target, starting from Immunome Library, Fc-fused recombinant proteins were used to perform two subsequent parallel cycles of selection.

Identification of target specific clones by Next Generation Sequencing and mAbs production

To select individual phage clones targeting each of the ten targets, I combined Next generation sequencing technology (NGS) to phage display. This approach allows to identify potential binders, according to their enrichment profile. In particular, the sequences can be analyzed following the trend of enrichment between selection cycles, as well as the representativeness within each cycle. Therefore, once selection cycles were performed, I extracted the double strand phagemid DNAs from each sub-library. To identify the clones of interest, I sequenced the VH regions from extracted DNA by massive parallel sequencing on the MiSeq Illumina platform (see Materials & Methods Section for details). Obviously, a decrease in complexity of sub-library was expected starting from Immunome Library (cycle#1) to target specific cycle#3, due to progressive counter selection of non-specific clones and increase in preponderance of target specific ones. Considering this, to optimize costs and output (i.e. number of reads per sample) I mixed together VH from cycle #2 and #3 of each target in the same run of sequencing, using two different barcodes. On the contrary, I dedicated a whole run of MiSeq to cycle#1 Immunome Library to achieve the deepest possible coverage. For each target, 10 to 20 million of reads were obtained. After the sequencing and elimination of non-joined sequences performed at the Center for Translational Genomics and Bioinformatics, Hospital San Raffaele, I performed an in-depth analysis of data. First, I removed from analysis the VH sequences found in two or more target-specific sub-libraries, presumably due to the enrichment of Fc binders shared by the 10 recombinant proteins (still present, despite the negative panning steps). In the same way, the clones without the classical framework backbone or encoding stop codons into the scFv sequence, were taken out from the list of potential binders, due to biased unspecific biological enrichment (Fig.31). I ranked the resulting filtered sequences by representativeness at cycle#3 to identify those with the highest level of enrichment. To trap the most relevant clones, I introduced a threshold filter of 85 counts per million (cpm) at cycle#3. These stringency criteria allowed me to identify the best potential binders for 9 out of 10 targets. Indeed, TIGIT selection was not fruitful, probably due to weak expression on hPBMCs. In figure 32, the top 10 sequences for each target were shown in relation to cpm at cycle#2 and #3. In addition, by phylogenetic analysis, I evaluated the heterogeneity of the top ten binders for each target, named as target_ranking, reported in figure 33 together with detailed trend of enrichment from cycle#1 to #3.

To demonstrate the effectiveness of the screening, we decided to characterize the best scFvs for three out of the nine targets.

A limitation of HTS approach is that detailed information is obtained exclusively for VH sequences. Moreover, since the selection of potential best binders was performed *in silico*, no isolated clones were available. To identify the VL linked to VH of interest and to recover "physically" the clones from the phage display sub-libraries, I set up a molecular method [131]. I optimized a clone-specific PCR protocol exploiting the unicity of hypervariable HCDR3 sequence (Fig.34) (see also M&M). I started to rescue clones targeting PD-1, PD-L1 and LAG-3, considering the clinical relevance of these IC.

To test the binding of rescued scFvs, I converted them into fully human IgG4 by sub-cloning VH and VL into eukaryotic expression vectors encoding constant domain of heavy and light chains (Fig.35). The heavy and light chain coding vectors were co-transfected in HEK293EBNA cells and IgGs were purified by affinity chromatography from conditioned media. Starting from the top enriched target specific clone, I converted scFvs up to obtain at least five effective antibodies for each of the three targets for further characterizations. Indeed, some mAbs (i.e. LAG-3_2, LAG-3_4, LAG-3_5, LAG-3_6) were excluded from analysis because of low productivity or instability (precipitation).

For all the target proteins, good binders (nanomolar Kds) according to ELISA assays were identified. The best mAbs were also assessed for their biological activity revealing both the ability to efficiently induce T cell proliferation and cytokines production (Ref., Data not shown; from professor De Lorenzo's group). Furthermore, preliminary data suggest a relevant *in vivo* anti-tumor activity of some novel anti-PD1 and anti-PD-L1 mAbs in a mouse preclinical model.

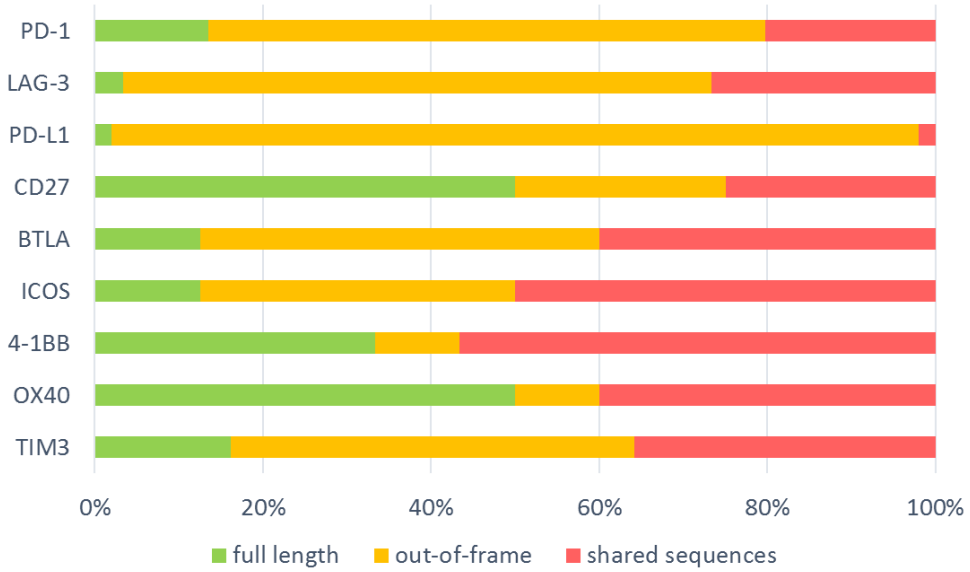


Fig. 31 Results from application of filters to the sequence frequencies. The image shows the different percentage of full length, out-of-frame and shared sequences of $scFv_s$ for all targets. The full length scFvs are in green, the out-of-frame scFvs are in orange and the scFvs shared in more than one target are in red. The targets CD27, OX40 and 4-1BB show a higher percentage of full length scFvs. PD-1, BTLA, ICOS and TIM3 show a discrete percentage of full length scFvs. LAG-3 and PD-L1 show a higher percentage of out-of-frame sequences.

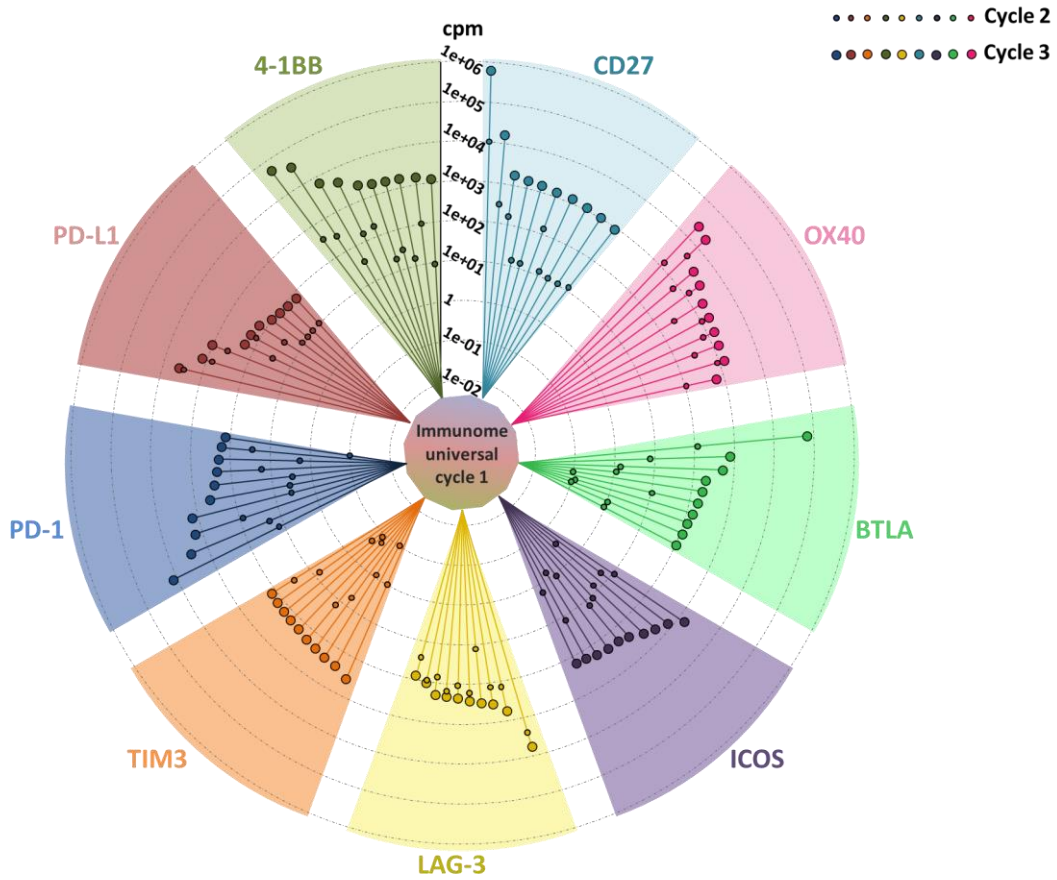
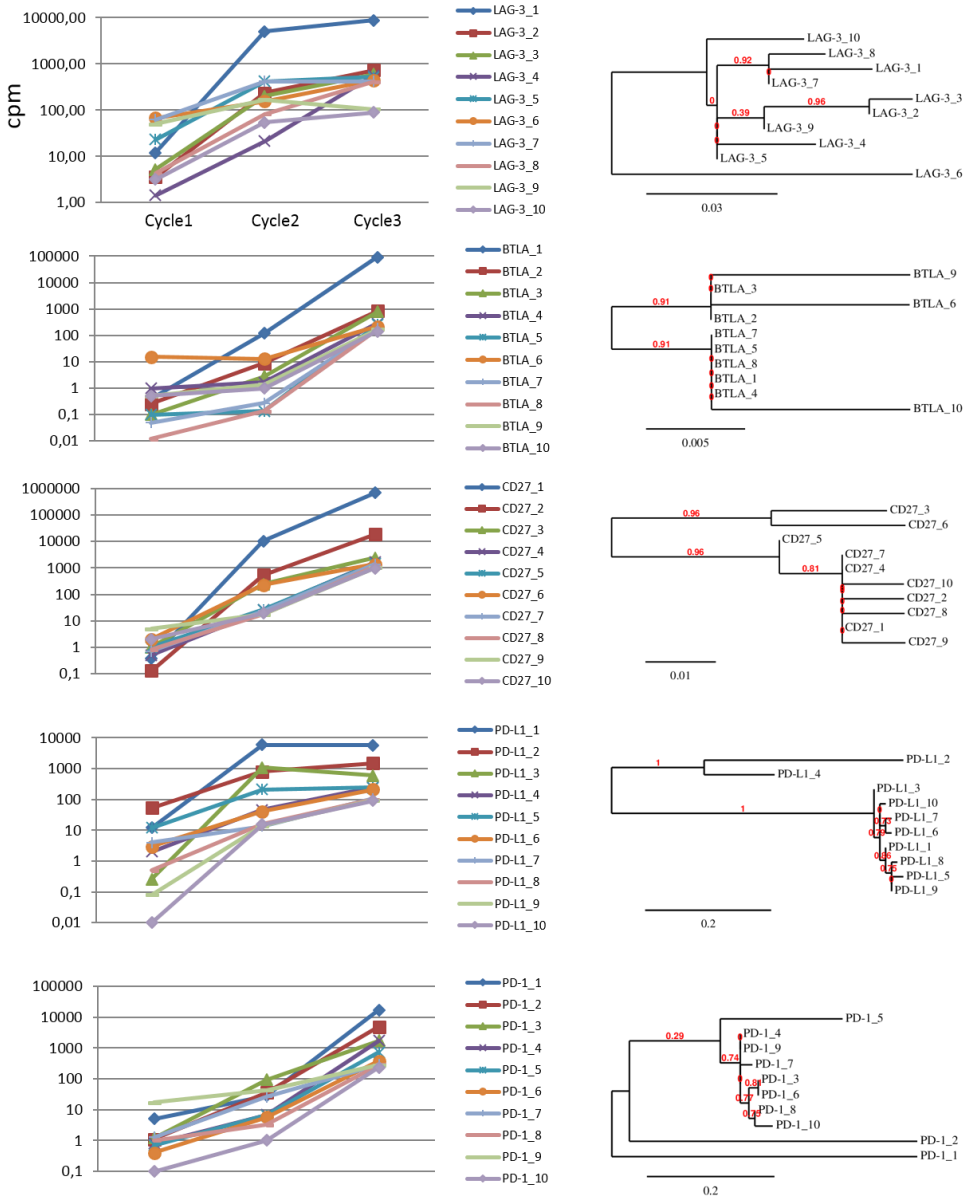


Fig. 32 Snapshot of best ten scFv$_s$ per target from immunome screening. The screening procedure started from the universal cycle#1 (inner multicolour circle) performed by incubation of naive library Delta on activated PBMCs expressing all the target proteins. Each section of the pie chart describes the enrichment profiles for the best ten scFvs targeting the indicated targets, and scored according to their counts per million values within the second and third selection cycles. The lines within each sector connect the individual enrichments, obtained after cycle#2 (small circles) and cycle#3 (large circles). Cycles#2 and #3 were both performed on the recombinant proteins.


cpm
10000,00
1000,00
100,00
10,00
1,00
Cycle1
Cycle2
Cycle3
LAG-3_1
LAG-3_2
LAG-3_3
LAG-3_4
LAG-3_5
LAG-3_6
LAG-3_7
LAG-3_8
LAG-3_9
LAG-3_10
LAG-3_10
LAG-3_8
LAG-3_1
LAG-3_7
LAG-3_3
LAG-3_2
LAG-3_9
LAG-3_4
LAG-3_5
LAG-3_6
0.92
0.96
0.39
0.03
100000
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100
10
1
0,1
0,01
BTLA_1
BTLA_2
BTLA_3
BTLA_4
BTLA_5
BTLA_6
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BTLA_9
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CD27_1
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CD27_1
CD27_9
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PD-L1_1
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PD-1_1
0.29
0.74
0.2

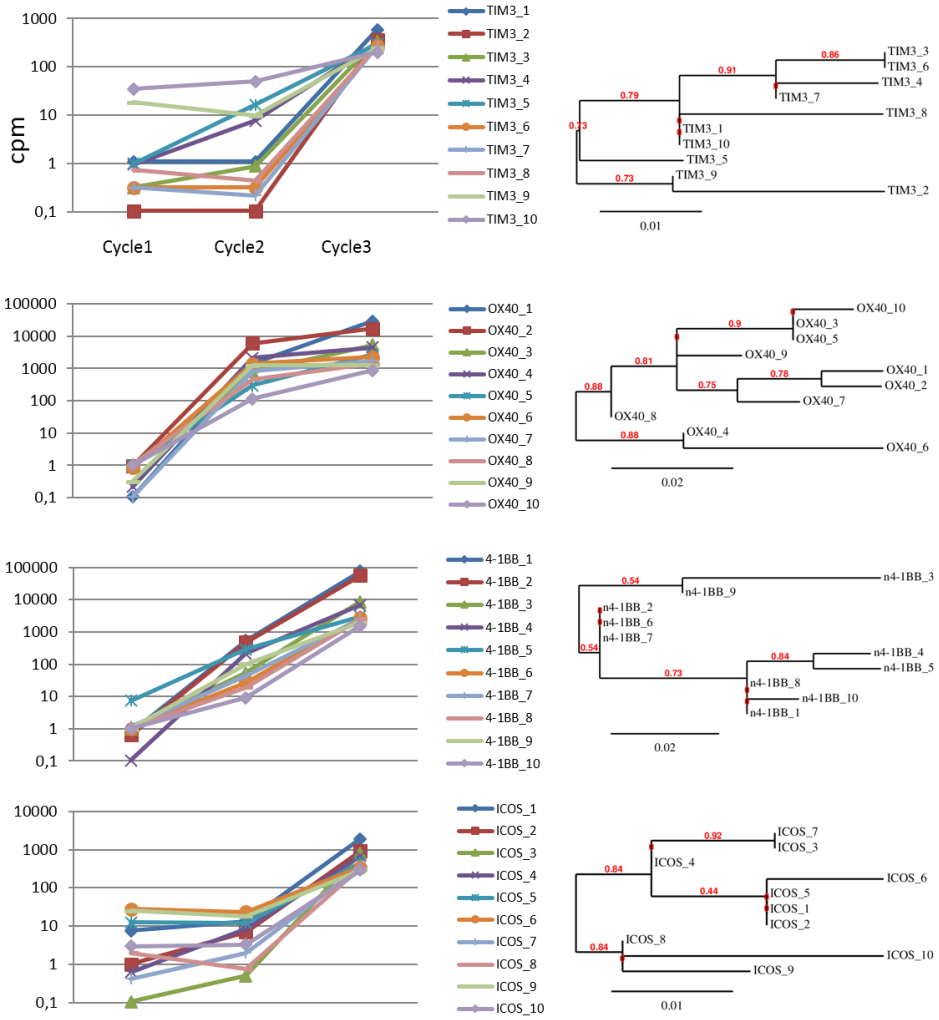

Fig. 33 Detailed trends of enrichments and phylogenetic correlations between the top ten enriched scFvs for each target protein (see also previous page). For each of the indicated targets, the left panel shows the representation of relative enrichments across the three selection cycles, assessed as counts per million. On the right side, the dendrograms report the phylogenetic clustering of the ten most enriched clones assessed by translated scFv sequences (Phylogeny.fr.).

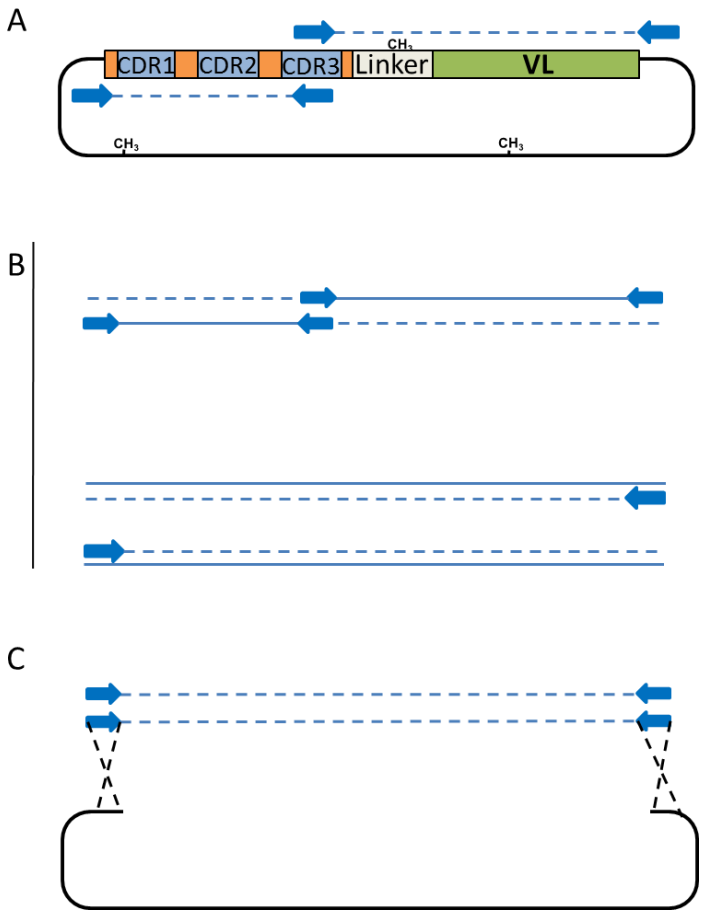

Fig. 34 Scheme of molecular rescue of clones of interest from enriched sub-libraries. The picture shows the rescue strategy based on overlapping PCR technology. Starting from the top, in A, the first step is based on two independent PCR reactions, that amplify separately the upstream and the downstream regions of the whole scFv. The fragments obtained from this PCRs share an overlapping region within the HCDR3 region. In B, the second step consists annealing, elongation and amplification of the overlapping fragments, to re-construct the full scFv. The full length scFv is sub-cloned into an expression vector (C).

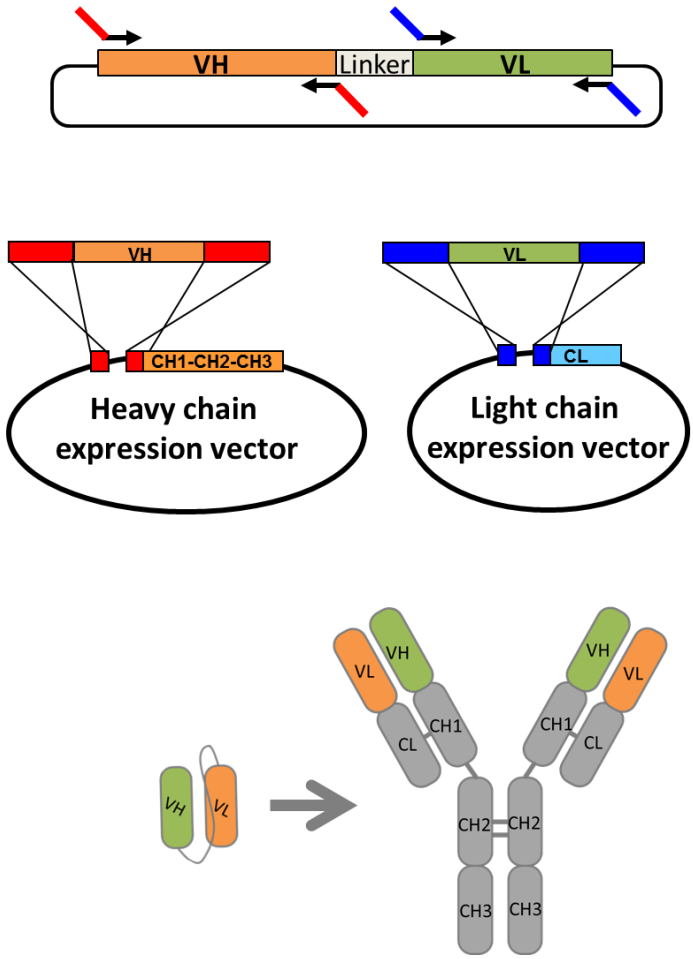

Fig. 35 Conversion of scFv into a full human IgG. The selected scFvs are converted into whole human IgGs inserting the variable fragments (orange and green, respectively, for variable heavy and light chains) in vectors expressing the constant antibody heavy and light chains. The obtained plasmids were co-transfected in HEK293-EBNA and grown up in serum-free CD CHO medium. Immunoglobulins were purified from conditioned media 10 days after transfection by Protein A.

In vivo combination of oncolytic virus and immune checkpoint inhibitors

Having successfully generated SurE_oHSV and a collection of antibodies, to be characterized for their suitability in preclinical settings, the final efforts of my experimental activity within the PhD timeframe were dedicated to establish the best conditions for large scale production of the oncolytic virus. In the meantime, I decided to generate a proof of concept set-up to evaluate, *in vivo*, the combination of virotherapy and immune checkpoint blockade. To this aim, the gD-retargeted LM113 oHSV was combined to immune checkpoint blockade with a suitable PD-1 monoclonal antibody. The mouse model selected for this preclinical evaluation was that of LLC-HER2. Briefly, human HER2 tolerant mice (immunocompetent C57-HER2 mice) were implanted subcutaneously with murine LLC-HER2 cells (a murine cell line stably expressing human HER2). Ten days after implant, the tumours were well established (mean volumes 115 mm^3); mice were then subdivided into 4 experimental groups for treatments, as summarized in Fig. 36:

- Untreated
- LM113: receiving 5 intra-tumour injection of LM113 (10^8 pfu)
- αPD-1: receiving 6 intraperitoneal injections of a commercial anti mouse PD-1
- LM113+αPD-1: receiving the combination of previously described treatments

Just modest delays in tumour growth were observed in single treated animals; contrariwise, a strong anti-tumour effect was evidenced in animals receiving the combination of LM113 with anti PD-1. Five out of 8 animals receiving the combination of immune checkpoint inhibitor and LM113 oHSV resulted tumour free (62.5%); one additional animal relapsed after an initial tumour regression. To evaluate the anti-tumour immune response, the tumour-free animals were rechallenged on the opposite flank with the same cells of the first challenge. All the mice were totally protected from rechallenge and rejected the tumour, suggesting that the combination of oHSV and immune checkpoint blockade induced an effective and long-lasting anti tumour immunity.

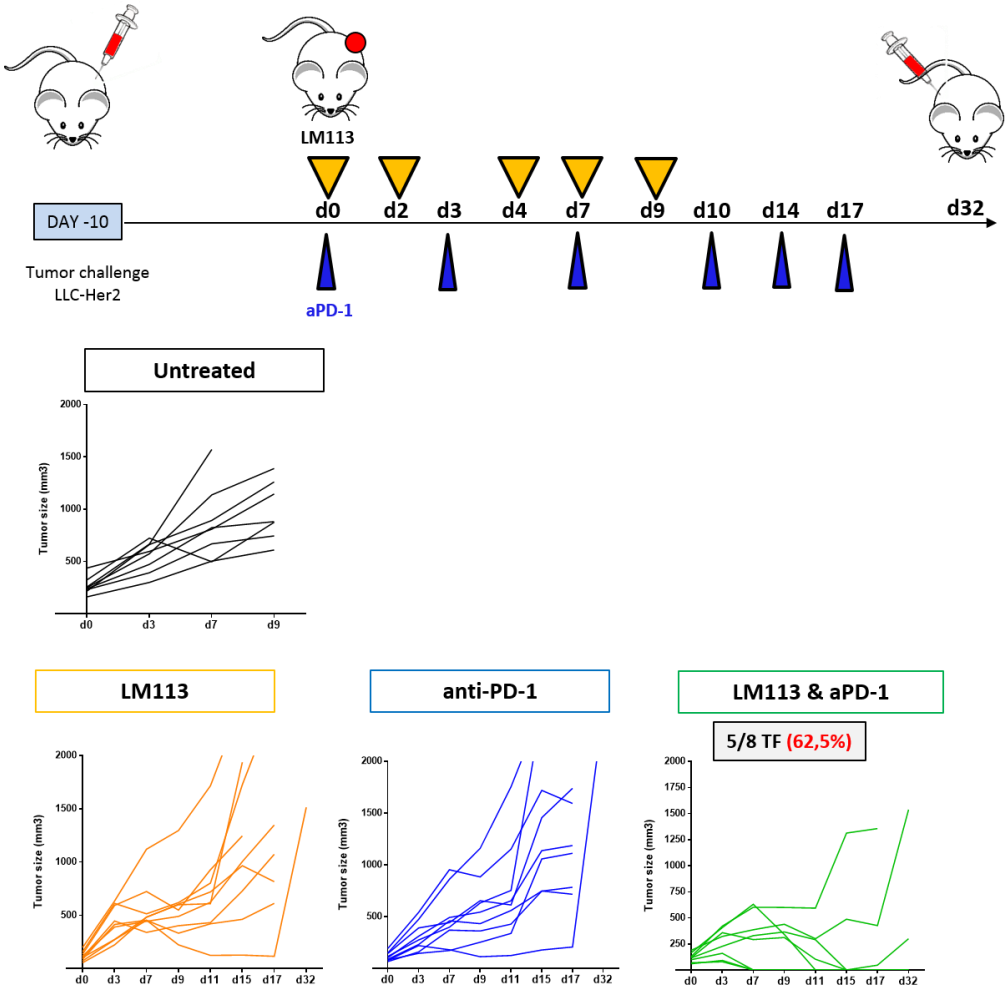

Fig. 36 In vivo activity of LM113 in monotherapy or in combination with immune checkpoint blockade. C57-HER2 mice were injected with LLC-HER2 cells. Ten days later, mice were treated as indicated in the scheme. The tumour size was evaluated up to day 32.

Discussion

The basic research conducted on the relationships between immune system and cancer have led, in the past few years, to the rapidly progressing field of cancer immunotherapy, revolutionizing the way to treat cancer patients. For sure, immune checkpoint modulators have been the main breakthrough of last decade in cancer therapy, driving to an incessant rate of approval of monoclonal antibodies by regulatory agencies. Despite this, many efforts are still dedicated to understand why these immunomodulatory mAbs exhibit only a limited efficacy, working in some patients, but not in others. Recent clinical outcomes suggest the need to combine IC inhibitors with drugs able to boost anticancer immune responses. One of the most promising approach is to induce an improved display of cancer-related proteins and tumour-associated antigens.

Meanwhile, in an apparently distinct field, oncolytic viruses have acquired rising clinical relevance thanks to the knowhow in engineering tumour-specific viral vectors. The skill of oncolytic viruses to induce tumour cell death is well established since decades, but the newly characterized immunogenic cell death is changing the way to define OVs. Indeed, OVs bring to an "immunologically noisy" tumour cell death that induces the display of TAAs, viral proteins and cytokines able to recruit immune cells and to revert the immunologically desert cancers into inflamed tumours. Thanks to this feature, it is by now conventional to define OVs as cancer vaccines. Nevertheless, clinical outcomes from T-VEC (Talimogene laherparepvec, Imlygic or OncoVexGM-CSF) treated patients show a good efficacy on injected tumours, but still limited *abscopal* effect on metastasis, as cancer relapse often happens. Taking together the limitations of IC inhibitors and OVs, their combination looks to be a foregone approach. In recent preclinical evidences and clinical trials, the combination of T-VEC with anti PD-1, PD-L1 or CTLA-4 resulted in an amazing drug synergism.

In this context, I decided to generate a repertoire of cancer immunotherapeutics exploiting both OVs and IC modulators. During my PhD I generated a novel non-attenuated oncolytic HSV-1, with potentially improved safety compared to those in clinic and clinical trials. In our strategy, to generate a non-attenuated OV, we decided not to remove genes associated to virulence. To spare normal cells and provide the tumour selective killing, I generated a double retargeted oncolytic virus by combining, for the first time, the entry to the transcriptional retargeting. First, I focused on ICP4 viral gene, as it encodes a key

transcriptional regulator of HSV-1. I generated a replication conditional OV by replacing each of the two endogenous ICP4 promoters with a pan-tumour one. Among a collection of tested promoters, I selected Survivin as the best one, due to both tumour stringency and potency. Previously reported HSV-1 OV driven by Survivin promoter were hard to translate to clinics, since they were based on amplicon virus [134]. To generate a clinical feasible OV, starting from the full length wt strain-F of HSV-1, I modified both endogenous *loci* of ICP4 without additional deletions. This OV, named Survivin_oHSV, showed a good oncolytic effect *in vitro* in various cancer cell lines and a limited toxicity in normal cells.

One of the main hurdles of oncolytic virotherapy is attributable to the route of administration. To date, in most clinical trials, OVs are intratumourally injected to avoid dilution and toxicity in normal non-target tissues. To bypass these limitations, I decided to couple the entry retargeting to the replication constraint, by replacing an essential region of the glycoprotein D (gD) with an scFv targeting the oncoprotein HER2. The toxicity in normal cells of this II generation OV, which I named SurE_oHSV, was barely detectable. Within the cellular models I tested, SurE_oHSV achieved a three Log10 gain in selectivity for cancer cells, compared to wt HSV-1. Interestingly, the safety of the novel OV was achieved without affecting the potency of the parental vector, and the addition of gD retargeting to Survivin_oHSV, reinvigorated its virulence in cancer cells. While the reasons for this occurrence have not been demonstrated, we can speculate an effect by ICP4 transactivator on the expression of the scFv against HER2. This double retargeting approach represents a proof of principle useful for the generation of further OVs targeting tumour overexpressed membrane antigens not tightly restricted to tumour cells. Additional work will provide further evaluation of the *in vivo* efficacy of this OV in mouse preclinical model.

To complete our cancer immunotherapeutic repertoire, I set up a high throughput screening (HTS) of a human scFv phage library to isolate monoclonal antibodies targeting immune checkpoint molecules LAG-3, PD-L1, PD-1, TIGIT, TIM3, BTLA, OX40, 4-1BB, CD27 and ICOS. I combined an *ex vivo* screening performed on hPBMCs (expressing ICs) to NGS. This approach has allowed us to identify the enriched scFvs targeting immune checkpoints in their native conformation. Starting from the selection performed on hPBMCs and referred as Immunome Library, to facilitate the identification of target specific scFvs, two additional selection cycles were performed, in parallel, with the recombinant proteins LAG-3, PD-L1, PD-1,

TIGIT, TIM3, BTLA, OX40, 4-1BB, CD27 and ICOS. All selections were fruitful, with the exception of TIGIT, probably due to its limited expression on hPBMCs. A global overview of the screening revealed that, despite for most of the targets, the clone enrichments already occurred at cycle#2, their representativeness was significantly improved after the third cycle, resulting in an easier identification and isolation. This technology allowed us to isolate a repertoire of hundreds of scFvs targeting the main immune checkpoint pathways LAG-3, PD-L1, PD-1, TIM3, BTLA, OX40, 4-1BB, CD27 and ICOS. As proof of principle, scFvs anti LAG-3, PD-1 and PD-L1 were converted into fully human IgG4 revealing nanomolar to sub-nanomolar affinities for their targets. The validation of biological activity of selected mAbs was assessed in comparison to the clinical gold standard Nivolumab, by evaluating T-cell proliferation and cytokines secretion. Interestingly, several mAbs from our repertoire showed an enhanced activity compared to Nivolumab. These results support the conclusion that *ex vivo/in silico* HTS could be a fruitful way for developing clinically relevant mAbs targeting immune checkpoints for cancer therapy.

In conclusion, my work was aimed to obtain molecular repertoires of improved vectors for virotherapy, and a wide collection of antibodies for immune checkpoint modulation in cancer. Both the endpoints were reached, as shown by the *in vitro* characterizations of the viral constructs, leading to a novel, safe and effective OV, and by the proved efficacy of representative mAbs from the wide collection, in increasing T-cell proliferation and cytokine secretion. The most recent literature, together with preliminary data obtained in our laboratories, lends strong support to the initial hypothesis, according to which combination of virotherapy with immune checkpoint modulation confers undoubted improvements, compared to monotherapy, in innovative cancer treatments [78-83]. Thus, the current work represents a solid start point for the identification of the most suitable combinations of the double retargeted SurE_oHSV oncolytic virus with immunomodulatory mAbs from our repertoire, in preclinical settings of investigation.

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