Virotherapy, gene transfer and immunostimulatory monoclonal antibodies

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Malignant cells are susceptible to viral infection and consequent cell death. Virus-induced cell death is endowed with features that are known to stimulate innate and adaptive immune responses. Thus danger signals emitted by cells succumbing to viral infection as well as viral nucleic acids are detected by specific receptors, and tumor cell antigens can be routed to professional antigen-presenting cells. The anticancer immune response triggered by viral infection is frequently insufficient to eradicate malignancy but may be further amplified. For this purpose, transgenes encoding cytokines as co-stimulatory molecules can be genetically engineered into viral vectors. Alternatively, or in addition, it is possible to use monoclonal antibodies that either block inhibitory receptors of immune effector cells, or act as agonists for co-stimulatory receptors. Combined strategies are based on the ignition of a local immune response at the malignant site plus systemic immune boosting. We have recently reported examples of this approach involving the Vaccinia virus or Semliki Forest virus, interleukin-12 and anti-CD137 monoclonal antibodies.

Viruses Destroying Cancer

Vaccinia virus (Vv) is an oncolytic poxvirus with widespread historical use in humans, in particular as an efficient vaccine for the eradication of smallpox.[1] Vv therapy has also shown encouraging antitumor activity, bearing the potential to target both localized tumors and more advanced metastatic lesions.[2–4] Vv is capable of selective replication in cells with a malignant phenotype and is characterized by an enveloped double stranded DNA genome. Vv can infect a broad host range and its replication occurs rapidly within the cytoplasm, limiting the possibility of chromosomal integration.[5,6] Vv displays broad tissue tropism and is known to take advantage of several membrane fusion pathways

rather than cell surface receptors for entry into target cells.[6] Vv is highly immunogenic and efficient at spreading through the blood to distal lesions upon the activation of signaling pathways such as that transduced by the epidermal growth factor receptor (EGFR)-RAS axis.[6,7] It is thought that the antitumor effects mediated by Vv are based on three different mechanisms of action that include: (1) direct infection of tumor cells and subsequent replication leading to tumor cell lysis, with features of both necrosis and apoptosis; (2) immune-mediated cell death initiated by the release of cellular danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), as well as tumor-associated antigens (TAAs) at the site of infection, and (3) tumor vasculature collapse.[5,8]

Alphaviruses, like the Semliki Forest virus (SFV) and Sindbis virus (SIN), have also been successfully used as oncolytic agents in several preclinical models of cancer.[9–12] Alphaviruses are enveloped viruses containing a single positive strand RNA genome which, after infection, can replicate in the cytoplasm. This process induces a strong cytopathic effect that leads to cell death by apoptosis in most mammalian cells. Interestingly, propagation-deficient alphaviral vectors, in which structural genes have been replaced by a gene of interest, are also able to induce apoptosis in infected cells, although with a more delayed time-course. Apoptosis as induced by SFV vectors is dependent on the non-structural region of the genome, requires viral RNA synthesis and has been shown to occur independently of p53.[13] The fact that many tumors have lost p53 functions makes the use of alphaviral vectors very attractive for cancer therapy, as these vectors are able to overcome the anti-apoptotic state conferred by defects in the p53 signaling pathway. Apart from the aforementioned studies in which natural alphaviral strains were tested as oncolytic agents, some groups have evaluated if the induction of apoptosis by propagation-deficient alphaviral vectors might lead to tumor regression. In this context, repetitive doses of SFV or SIN vectors expressing reporter genes were able to induce the regression of tumors implanted in immunodeficient mice.[14,15] However, the antitumor efficacy of both alphaviral vectors and Vv is greatly enhanced when they express immunostimulatory cytokines, or when they are used in combination with other therapies (see below).
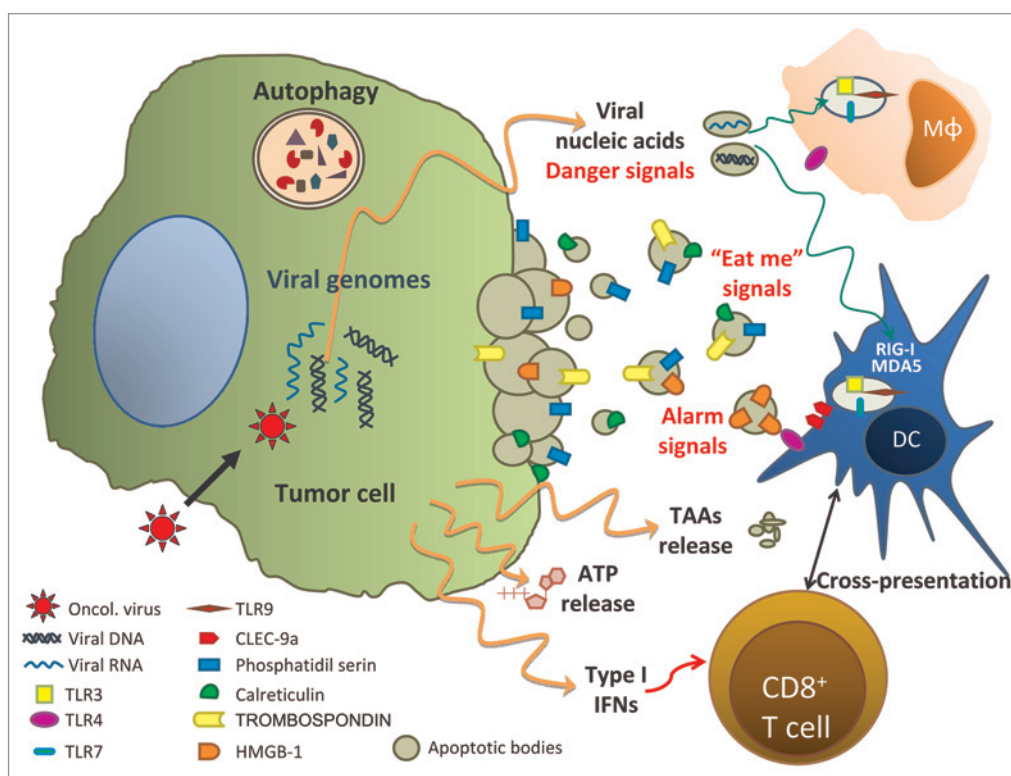
Figure 1. Immunogenic features of oncolytic virus-induced cell death. Infection of a tumor cell by an oncolytic virus will trigger apoptosis, leading to the release of tumor antigens that can be taken up by antigen-presented cells (APCs) and cross-presented to CD8+ T cells. Infected apoptotic cells can also get covered molecules that will be recognized as "eat-me signals" (e.g.., calreticulin, phosphatidylserine, and thrombospondin), and emit "alarm/danger signals" (e.g., viral genomes and glycoproteins, HMGB1). Other factors released by apoptotic cells that contribute to the activation of immune responses include ATP, whose secretion relies on the autophagic machinery, and Type I interferon (IFN).

Immunogenic Cell Death Caused by Viral Mechanisms

Apoptosis and virus. Infection by most viruses triggers the programmed death of infected cells. Apoptosis can be induced by viral factors as a mechanism of escape and propagation or, alternatively, can be induced by cellular factors as a response to viral infection, aimed at limiting viral production and spreading. To counteract this latter mechanism, some viruses encode or co-opt factors that inhibit or delay apoptosis, resulting in more robust virus production. In these cases, a delicate balance between the inhibition and induction of apoptosis is achieved by a combinations of multiple viral products.

Viruses that are able to induce apoptosis in infected cells include adenoviruses, lentiviruses, like HIV, papillomaviruses and alphaviruses.[16] For this last group, it has been shown that the overexpression of BCL-2 in infected cells is able to block apoptosis and viral replication, hence promoting the formation of chronically infected cell lines.[17] This suggests that apoptosis might be required for completion of the alphaviral cycle.

On the other hand, many viruses, like poxviruses, have developed mechanisms to inhibit or delay apoptosis in infected cells. In the case of Vv, this is achieved by the expression of the serine protease inhibitors SPI-1 and SPI-2,[18,19] which directly inhibit the activation of caspases. Accordingly, Vv variants bearing

deletions in these genes are highly cytopathic for many cell types. Interestingly, these types of mutants replicate preferentially in tumor cells, and have been tested as oncolytic agents in preclinical models of cancer.[20]

Endogenous alarm signals from dying tumor cells. The apoptotic demise of cancer cells as induced by viral vectors has been shown to be a very efficient way to induce antitumor immune responses.[21,22] This effect is mediated by a number of mechanisms that include the release of TAAs from dying cells as well as the emission of danger signals by infected cells, including viral genomes and glycoproteins, which can provide antigen stimulation for helper T cells and ignite innate immune responses, respectively (**Fig. 1**). In most cases, programmed cell death proceeds at a steady-state without eliciting any kind of response from the immune system. Apoptotic bodies are generally engulfed and degraded by macrophages in the absence of an inflammatory response. On the contrary, certain types of cell death are more stressful and certainly much more immunogenic. By examining the death of tumor cells as elicited by chemotherapeutic agents and ionizing radiation, the laboratories of Kroemer and Zitvogel[23] discovered interesting features that indicate when the death of tumor cells results in a T-cell response against its antigens. In summary, such immunogenic features encompass:

"Eat me signals," marking apoptotic material for phagocytosis, among which the exposure of calreticulin at the cell surface.

Other molecules such as phosphatidyl serine and thrombospondin provide additional eat-me signals. An interesting mechanism of this kind seems to involve DNGR-1 (CLEC9A), a lectin expressed on dendritic cells (DCs, the major antigen-presenting cells, APCs, involved in antitumor immunity). Such a receptor is specialized in the internalization of necrotic cells[24] and has been recently found to bind exposed F-actin filaments.[25]

"Alarm signals," inducing the maturation/activation of DCs or favoring other pro-inflammatory changes. These include proteins and other factors that are normally sequestered within cells and are capable of activating pro-inflammatory receptors when released into the extracellular space. One example of this process is provided by the activation of Toll-like receptor 4 (TLR4) when the nuclear protein HMGB1 is released from a dead cell.[26] Molecules of this kind fit with the danger model proposed by Polly Matzinger, proposing that immune responses are initiated when the immune system detects tissue destruction via endogenous alarm signals.[27]

Other features: in the absence of microbial components, dying cells can provide other factors that can activate inflammation and induce immune responses. These include extracellular ATP, which can be detected by purinergic receptors[28] following its release upon the activation of autophagy (a stress response mechanism) in dying cells.[29] The importance of the local release of Type I interferon (IFN) has also been reported by several groups.[30]

However, although the immune system has probably evolved to mount responses in the presence of unscheduled cell death, these responses are overtly stronger if PAMPs are present in the milieu.

Tumor antigen cross-presentation. Cross-presentation is a function mediated by a specialized population of DCs, consisting in the ability of these cells to uptake exogenous antigens and introduce them into the MHC Class I antigen-presenting pathway. Under normal conditions, endogenous polypeptides degraded by the proteasome are the main source of peptides presented in the context of MHC Class I molecules. However, specific DCs subsets can divert antigens from endosomes to the cytosol and present exogenous peptides on MHC Class I molecules.[31] This phenomenon is instrumental to mount a specific cytolytic T-cell response against viral antigens and is clearly upregulated by Type I IFN.[32] In mice, there are specific subsets of CD8α+ DCs that excel at cross-presentation and cross-priming,[33] whose equivalents in humans have been identified in CLEC9A+CD141+CD11c cells.[34]

In the case of tumor cells, an important question is how to route TAAs to cross-presentation. Spontaneous cross-presentation of tumor antigens in the steady-state may prove counterproductive, leading to peripheral tolerance and inactivating or deleting responsive T cells. In order to be efficient, cross-presentation needs DCs to be matured by alarm signals or by microbial molecules.

A possibililty in this sense is provided by the induction of immune responses via cytopathic alphaviral vectors and Vv, which has been shown to proceed via antigen cross-presentation.[35,36] In these studies, it has been demonstrated that the apoptotic demise induced by an alphaviral vaccine vector could facilitate the uptake of apoptotic bodies by DCs and other APCs, resulting in an enhanced immune response against the alphavirus encoded antigen by a cross-presentation mechanism. Similarly, tumor lysis caused by Vv infection results in TAA uptake by DCs, leading to the induction of potent CD8+ cytotoxic T lymphocyte (CTL) responses that target both infected and non-infected tumor cells as well as to an enhancement of the immunostimulatory capacity of DCs.[37] Interestingly, such an immune response developed irrespective of the route of virus administration.[36] Furthermore, a recombinant Vv expressing CD40 ligand (CD40L) also enhanced antigen presentation, stimulating the generation of antigen-specific T cells.[38] More recently, the DC receptor CLEC9A has been shown to mediate the cross-priming of CTLs during Vv infection in mice.[39] Hence, cross-priming may be a feature shared by the immune responses to many viruses and cytopathic viral vectors.

Viral nucleic acids as alarm signals. PAMPs are molecular structures that are widely present in microbes and that are detected by the host via so-called pattern-recognition receptors (PRRs).[40] In viruses, PAMPs can be represented by both structural proteins and, mainly, nucleic acids. Reaching the intracellular microenvironment is an important issue for the recognition of "foreign" nucleic acids, especially for DNA. Apart from the subcellular localization, viral RNA is detected by cells as non-self because of the formation of double-stranded RNA (dsRNA) structures during the replication of RNA and DNA viruses,[41] as is the case for both SFV and Vv,[42,43] or the presence of triphosphate groups at the 5' end of the molecule.[44] PRRs recognizing viral PAMPs include TLRs in endosomes and retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) (**Fig. 1**). Thus, viral dsRNAs can be recognized by TLR3 (within endosomes), ssRNA with viral features by TLR7/8 (within endosomes) as well as by RIG-I and melanoma differentiation-associated gene 5 (MDA5) in the cytoplasm, and viral DNA genomes by TLR9 (within endosomes), recognizing unmethylated CpG-rich DNA motifs in endosomes,[45,46] as well as by stimulator of interferon genes (STING in the cytosol). The hallmark of these nucleic acid-sensing pathways is that they potently promote the production of Type I IFN through TRIF-, MYD88- or IPS1-dependent pathways, as well as many other inflammatory cytokines. These are key mechanisms for initiating the innate immune response after a viral infection, which can be exploited to use viral vectors in cancer gene therapy.

In the particular case of vaccination with alphaviruses, dsRNA is probably detected as a "danger signal" providing an additional adjuvant effect to the vector-encoded antigen. Several studies suggest that this signal promotes the induction of Type I IFNs, which are the main cytokines involved in innate immune responses against viral infections. In fact, Leitner et al.[47] demonstrated that the protective effect induced by a SIN vector expressing a melanoma antigen was lost in mice lacking the Type I IFN receptor. Several studies to determine which PRRs are crucial for the induction of Type I IFN by alphaviral vectors suggest that this process is independent of TLRs, and rather is mediated by RIG-I.[48,49]

Vv dsRNA can trigger the initiation of a suicide response in virus-infected cells.[50] Furthermore, it has been shown that a "danger signal" initiated by Vv can elicit innate immune responses that are mediated by TLR2 and MyD88, leading to the production of pro-inflammatory cytokines, such as Type I IFN. This pathway plays an important role in shaping innate and adaptive immune responses to Vv in vivo.[51] The highly destructive nature of Vv is

known to cause the release not only of PAMPs but also of many DAMPs.[6] DAMPs are nuclear or cytosolic proteins with defined intracellular function that, upon release from damaged or dying cells, also activate effector cells from the innate immune system.[52] Collectively, these alarm signals initiated by virotherapy induce a "first-line" innate immune response that can subsequently lead to potent tumor-specific immune responses, possibly clearing residual disease and providing long-term immunosurveillance against tumor relapse.[6] The idea of endogenous moieties reflecting tissue stress and damage was primarily proposed as the danger model by P. Matzinger.[53] It is quite possible that infection-denoting molecules and endogenous alarm signals cooperate to elicit and regulate inflammation as an evolutionary conserved mechanism to fight dangerous infection.

Virus-Enforced Expression of Immunostimulatory Molecules

Cytokines: interleukin-12 (IL-12) and others. Although the viral induction of apoptosis in tumor cells is able to promote antitumor responses, these are often limited by the poor accessibility of tumor cells or dominated by immune responses developed against the virus itself. This last point is suspected to be important for replicating viruses that produce high amounts of viral proteins. In this situation, viral antigens might compete with tumor antigens on the same APC, probably inhibiting antitumor responses. However, tumor cells expressing viral antigens on their surfaces might also stimulate APCs to cross-prime tumor-specific T cells, favoring in this way antitumor responses.[54] Anticancer immune responses can be enhanced by combining virus-induced apoptosis with immunostimulatory proteins that are able to activate and expand immune cells or enhance the performance of APCs. These types of molecules can be administered as recombinant proteins or expressed directly from expression cassettes within viral vectors. Immunostimulatory proteins include, among others, pro-inflammatory cytokines like IL-2, IL-12, IL-15, IL-18, and granulocyte macrophage colony-stimulating factor (GM-CSF). Other molecules that can be used to potentiate immune responses include chemokines, co-stimulatory molecules (see below), and inhibitors of immunosuppressive checkpoint mechanisms.

One of the cytokines that has shown stronger antitumor activity is IL-12, a heterodimeric protein consisting of two subunits (p35 and p40), which plays a key role in the induction of Type I T-helper responses.[55] IL-12 enhances the function of cytotoxic immune cells, including CTL and natural killer (NK) cells, and has potent IFNγ-dependent therapeutic activity. In addition, the antitumor properties of IL-12 are potentiated by its antiangiogenic effects.[56] Several viral vectors, such as adenovirus, retrovirus, or alphavirus, have been used to deliver IL-12 to tumors, in vivo, resulting in localized expression of the cytokine and antitumor efficacy.[10,57,58] By using a SFV vector expressing IL-12 (SFV-IL-12), we have previously shown that it is possible to completely eradicate a high proportion of tumors derived from colon adenocarcinoma, melanoma or lung carcinoma cells implanted in immunocompetent mice.[59,60] The antitumor immune responses induced by SFV-IL-12 appear to be mediated by CD8+ T cells and

to require both a high expression of IL-12 and SFV RNA replication. The SFV-IL-12 vector has also shown efficacy in a model of spontaneous hepatocellular carcinoma, as induced in woodchucks by infection with woodchuck hepatitis virus (WHV), an hepadnavirus similar to human hepatitis B virus (HBV).[61] In this clinically relevant model, SFV-IL-12 induced partial tumor responses and a transient decrease of WHV viremia, but no complete tumor regression was observed. Some viral vectors expressing IL-12, like adenovirus- or canarypoxvirus-based vectors, have been used in cancer patients in the context of Phase I clinical trials. Albeit promising results observed in preclinical studies, these vectors only showed modest therapeutic effects in humans.[62,63]

GM-CSF is known to improve the survival, performance and recruitment of DCs. An oncolytic Vv has been shown to benefit from the expression of cytokine, which improved its therapeutic effects.[64,65] This virus has shown promising results in a Phase II clinical trial.[64] Despite these data, negative results obtained in randomized clinical trials using GM-CSF as an adjuvant have made the use of this cytokine highly controversial.[66,67] In fact, it has been shown that high doses of GM-CSF can induce the expansion and function of myeloid-derived suppressor cells (MDSC), leading to the inhibition of antitumor immune responses.[68] Separating the local effects of GM-CSF, involving the recruitment and differentiation of APCs, from its systemic effects, corresponding to the expansion of MDSCs, would be desirable for the optimal use of this cytokine.

However, the limited results generally obtained in spontaneous tumor models and clinical trials suggest that these cancer therapies need to be improved, something that could be achieved by either using more potent vectors and combining several immunostimulatory molecules.

Co-stimulatory molecules. Antigen presentation requires: (1) a cell that processes and presents the antigen on MHC molecules, and (2) a T cell that specifically recognizes the antigen via its T-cell receptor (TCR) complex. When an APC presents an antigen loaded onto MHC molecules to a T cell, additional signals are required to complete the activation of both the APC and the T cell. These additional signals are provided by co-stimulatory molecules expressed on the surface of both cell types. Viral vectors expressing these costimulatory molecules have been used in cancer gene therapy to strengthen the activation of immune cells against cancer.

One strategy has relied on reinforcing the activation of APCs through the use of CD40 receptor agonists. An example of this approach is provided by the expression of CD40L, a glycoprotein usually found on activated T cells that can bind CD40 on the surface of DCs and B cells, inducing their activation.[69] Expression of this molecule with adenoviral vectors has shown a high degree of efficacy in several tumor models, inducing a great percentage of complete tumor eradications mediated by CD8+ T-cell and/ or NK-cell activation and upregulation of MHC Class I/II molecules, among other effects. Results from a rat hepatocellular carcinoma model,[70] a mouse lymphoma model,[71] as well as from a Phase I/II clinical trial on bladder carcinoma patients[72] well represent the efficacy of this strategy. Of note, an agonist monoclonal antibody targeting CD40 has shown promising antitumor activity in pancreatic cancer patients.[73]

The expression of co-stimulatory molecules aimed at attaining optimal T-cell activation has also shown a good degree of success in numerous animal models and some clinical trials. Examples of these strategies are represented by the expression of OX40L, CD80 and 4–1BBL (CD137L) molecules, which are normally found on activated APCs and bind to OX40, CD28 and 4–1BB on activated T cells, respectively.[74–77] In general, stimulation of these receptors enhances T-cell proliferation, survival and the acquisition of effector functions.

The potential of OX40L as an antitumor agent was demonstrated by expressing it with a replication-deficient adenovirus, an approach that was able to induce a significant suppression of tumor growth and an increased survival among tumor-bearing mice.[78] Deficient CD28 stimulation during T-cell priming results in anergy,[79] a phenomenon that is thought to occur naturally in tumors due to the immunosuppressive microenvironment that reigns in malignant tissues. In order to circumvent this effect, efforts have been directed to co-express TAAs and CD80 co-stimulatory molecules in viral vectors. Kudo-Saito and coworkers have used poxvirus-derived vectors to express carcinoembryonic antigen (CEA) plus a triad of T-cell co-stimulatory molecules, namely, CD80, ICAM-1 and LFA-3, in a CEA-expressing mouse tumor model.[80] Complete tumor remissions were observed in most of the animals, with T-cell responses detected not only against CEA, but also against other tumor antigens (antigen spreading). A similar strategy was followed in a clinical trial enrolling melanoma patients.[81] I this setting, 31% of objective clinical responses were observed, with one patient achieving a complete response that lasted for more than 22 months.[81] In a randomized Phase II clinical trial, a variant of the same viral vector expressing prostate specific antigen led to a median 8–9 mo overall survival advantage in castration-resistant prostate cancer patients.[82] This approach is presently undergoing registration to Phase III trials for the same indication.[82]

Another strategy consists of expressing CD80 alone[83,84] or in combination with cytokines such as GM-CSF,[85] IL-2,[86,87] or IL-12.[63,88–90] Adenovirus-, HSV- and poxvirus-derived vectors were used for the expression of these molecules in tumors. The antitumor activity of CD80 was generally improved in all these scenarios, acting synergistically with the mentioned molecules. However, CD80 expression needs to reach bright levels, since lower levels may be suppressive because of the preferential ligation of the CTLA-4 co-inhibitory receptor.[91]

In the case of 4–1BB signaling amplification, systemic administration of agonist antibodies have shown potent antitumor effects.[76] Thus, stimulation of this T-cell receptor represents a powerful tool against cancer, which has also been explored through the expression of 4–1BBL from viral vectors. In this scenario, the group of Shu-Hsia Chen has shown remarkable antitumor efficacy in mice treated with a combination of adenovirus vectors expressing 4–1BBL and IL-12, respectively.[92] This strategy resulted in long-term remission of liver metastases in more than 50% of treated mice, an effect that was shown to be dependent on NK and CD8^{+} T cells. Similarly, Huang and colleagues have shown that the antitumor effects triggered by an oncolytic adenovirus co-expressing 4–1BBL and IL-12 could be improved by

co-administration of DCs.[93] Moreover, intratumoral injections of semi-allogenic DCs transduced with an IL-12 -expressing adenovirus have been demonstrated to synergize with the systemic administration of agonist anti-CD137 monoclonal antibodies.[94]

Despite promising expectations raised by the use of oncolytic vectors, the effect of these viruses are usually hampered by a vigorous immune reaction against the virus itself. In order to circumvent these unwarranted responses, some oncolytic vectors expressing co-stimulatory molecules have been evaluated in the context of an impaired immune system. Thus, the sublethal irradiation of tumor-bearing mice was able to increase the antitumor effect of a recombinant Vv expressing 4–1BB. This involved the reduction of antiviral antibody titers, the stimulation of MHC-I Class I expression and viral persistence, as well as the rescuing of effector-memory CD8^{+} T cells.[8] An alternative that has been successfully used to control immune responses against oncolytic viruses in mice is the use of low doses of cyclophosphamide.[95] In a clinical trial, the combination of this immunosuppressive agent with an oncolytic adenovirus in patients with advanced solid tumors resulted in higher rates of disease control than the treatment with virus only.[96]

Strengthening the Patient's Immune System with Immunostimulatory Monoclonal Antibodies

Monoclonal antibodies have been invaluable molecular tools to specifically trace proteins for biotechnology applications. In therapy, they have been used to specifically target destruction mechanisms to malignant cells as well as to block growth factors such as pro-angiogenic factors. These agents, in combination with chemotherapy, have exerted a profound impact in cancer management.[97] Monoclonal antibodies have also revolutionized immunology providing knowledge on glycoproteins that act as surface receptors in immune cells and soluble cytokines that mediate cell-to-cell communication. It was soon realized that such antibodies could stimulate receptors or block them, mimicking or interfering with natural ligands.

The in vivo administration of some of these agents has shown therapeutic effects against transplanted tumors. This is the case of antagonist antibodies targeting the inhibitory receptors CTLA-4, PD-1, TIM-3 and LAG-3[98] as well as of agonistic antibodies targeting CD137, CD134, CD40, CD27 or GITR. The mechanism of action of both anti-CTLA-4[99,100] and anti-PD-1/anti-PD-L1[101,102] antibodies have been extensively characterized in both mice and humans. Administration of CTLA-4-blocking antibodies enhances the activation of T cells and decreases the immunosuppressive functions of Treg, while PD-1/PD-L1-blocking antibodies reverse immunosuppression, T-cell anergy and apoptosis, both approaches leading to enhanced antitumor responses. The role of other checkpoint inhibitors like TIM-3 and LAG-3 has started to emerge[103,104] and the use of reagents targeting these molecules, in various combinations, has shown to increase the activation of T cells in mouse models of cancer and autoimmunity.[105,106]

Currently, an extensive series of highly promising clinical trials is bringing this concept toward clinical reality. An anti-CTLA-4 monoclonal antibody is already registered for metastatic melanoma and trials with anti-PD-1 and anti-PD-L1 (B7-H1)

Table 1. Strategies for antitumor therapy based on the combination of viral vectors and monoclonal antibodies

mAb*	Viral vector†	Tumor type	Antitumoral mechanism	References
CD137	Ad-IL-12	Melanoma, colon cancer, metastatic liver tumor	CD8^{+} and NK cells	120, 121
	Vaccinia (Vvdd)	Mammary, colon adenocarcinoma	CD8^{+}, NK cells and neutrophils	113
	SFV-IL-12	Melanoma, lung cancer	CD8^{+} and CD137 upregulation	60
CD137+OX40	Ad-IL-12	Large colon carcinoma	CD8^{+} and CD4^{+} cells	122
CTLA4	VSV	Mammary	CD4^{+} (early) and CD8^{+} (late) cells	123
	Vaccinia-p53	Sarcoma	CD8^{+} and CD4^{+} cells	124
	Vaccinia-CEA/TRICOM	Colon adenocarcinoma	CTL, increased T-cell avidity	125
TNFα (Infiximab)	oAd	Ovarian cancer	Increased apoptosis	126
EGFR (Cetuximab)	oAd	Head and neck squamous cell carcinoma	Enriched CD133^{+}/CD44^{+} cells in tumors	127
CD25	VSV + IL-2	Melanoma	Multiple	128
	Ad-αCTLA4	Mammary, cervical carcinoma	CD8^{+} and NKT cells	129
VEGF (Bevacizumab)	oAd	Anaplasyc thyroid carcinoma	Enhanced viral distribution	130
	Vaccinia	Pancreas, prostate, lung tumors	Inhibition of vasculature	131
	Ad-mda-7	Lung tumor	Tumor cell apoptosis & reduced VGEF and CD31 expression	132
	HSV	Sarcoma, advanced gastric cancer	Enhanced virus spread/ anti angiogenesis	133, 134
	HSV-angiostatin	Glioma, sarcoma, gastric cancer		135

*The molecule for which the mAb is specific is indicated. †Ad, first generation adenovirus; VSV, Vesicular stomatitis-virus; oAd, oncolytic adenovirus; Vaccinia-CEA/TRICOM, recombinant vaccinia expressing murine B7-1, ICAM-1, and LFA-3 in combination with human CEA (carcinoembryonic antigen); mda-7, melanoma differentiation associated gene-7; HSV, herpes simplex virus.

agents are progressing with excellent results.[107–109] Although in some cases adverse reactions in the form of excessive inflammation and autoimmunity have been reported, these were generally controllable and benign. Antibodies targeting the stimulatory molecules CD40 and CD137, or their ligands, have also been tested in early Phase I trials and are showing some promise.[110] Combinations of these monoclonal antibodies with gene therapy has already been associated with synergic effects (**Table 1**). A recent study has shown that the concomitant targeting of both immune stimulatory and inhibitory checkpoints with antibodies can enhance the effects of radiotherapy against established breast cancer in mice.[111] Along similar lines, the efficacy of vaccines is also enhanced when some of these immunostimulatory monoclonal antibodies are co-administered. Further experiments testing both the efficacy and tolerability of these immunomodulatory antibody combinations in preclinical mouse models are necessary before the launch of clinical trials.

Strategies of Combined Therapy

Combinatorial immunotherapy of cancer is at its infancy. It is premature to say that combinations of multiple agents will become a landmark in cancer therapy but many of us tend to believe in this concept. For instance, combinations of multiple immunostimulatory monoclonal antibodies and immunogenic cell death inducers have shown promising effects (**Fig. 2**).

Until recently, oncolytic viruses had not been tested in combination with immunostimulatory monoclonal antibodies. Our groups have recently provided two interesting examples using anti-CD137 monoclonal antibodies and two different viral vectors that cause tumor cell death: Vv and a SFV vector encoding IL-12. Although the latter virus cannot propagate, it replicates genomic RNA in infected cells, causing apoptosis.

In the first combination study, we utilized a genetically engineered strain of oncolytic Vv (Vvdd). The Vvdd mutant strain contains a double deletion of the viral thymidine kinase (TK) and viral growth factor (VGF) genes that reduces pathogenicity but enables the potent oncolytic activity of wild-type Vv to be retained.[112] As previous studies had reported that the antitumor effects of oncolytic Vv correlate with increased host immune responses, we rationalized that improved therapeutic effects may be achieved by using oncolytic viruses in combination with a potent immunostimulatory reagent.

In immunocompetent mice bearing established subcutaneous breast and colon carcinomas, we demonstrated that the intratumoral injection of Vvdd combined with the systemic administration of anti-CD137 monoclonal antibodies significantly reduced tumor growth relative to either treatment alone.[113] Furthermore,
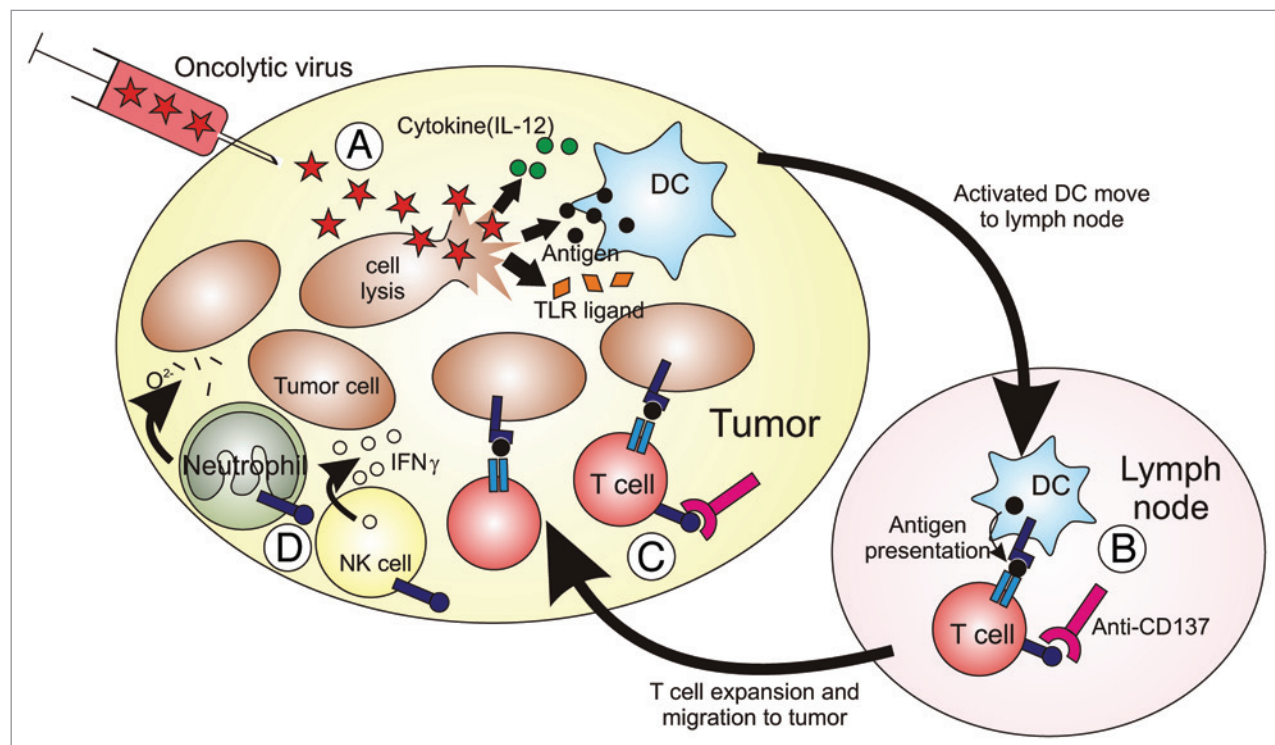
Figure 2. Oncolytic viruses can synergize with immune agonists against cancer. (**A**) Following delivery of an oncolytic virus (OV), a proportion of tumor cells are lysed releasing antigens. OV products are released and can act as Toll-like receptor (TLR) ligands, which together with cytokines encoded in the OV can activate immune cells, including dendritic cell (DCs). (**B**) Activated DCs move to lymph nodes and present antigens to T cells. Agonist antibodies such as anti-CD137 antibodies can enhance T-cell expansion. (**C**) T cells move to tumor and react against cancer cells through interactions between their T-cell receptor (TCR) and tumor-associated antigen presented in the context of MHC molecules. Anti-CD137 antibodies can also help maintain T-cell activity at the tumor site. (**D**) Other immune effector cells, recruited by the inflammatory microenvironment established by OV, can deliver additional antitumor agents, such as reactive oxygen species (O_2^-) and interferon γ (IFNγ).

the combined therapy reduces the development of pulmonary metastatic lesions.

An interesting finding arising from this study is the critical role for both adaptive and innate immune cells in the therapeutic effect. Following combined Vvdd and anti-CD137 treatment, we found increased numbers of CD11b⁺ and CD11c⁺ myeloid cells within tumor-draining lymph nodes, greater infiltration of CD8⁺ effector T cells and NK cells, as well as a sustained presence of neutrophils at the tumor site. An important therapeutic role for these immune cells was confirmed by depletion studies. In addition, the use of IFNγ-deficient mice revealed a critical role for this cytokine. Our findings demonstrate that immunostimulatory antibodies can significantly enhance the efficacy of oncolytic viruses in a potent combination approach against cancer.

In a different approach, we took advantage of the immunostimulatory properties of CD137 agonist antibodies to potentiate the antitumor efficacy of SFV-IL-12.[60] Although SFV-IL-12 had shown potent antitumor effects by itself in different tumor models, its efficacy appears to depend on both tumor type and viral dose. Since IL-12 may have toxic effects when expressed at high levels, a combinatorial regimen would have the advantage to allow for the use of lower doses of the viral vector, increasing the overall safety of the therapy.

A strong antitumor synergy was observed in mice bearing melanomas or lung carcinomas treated locally (intratumoral administration) with SFV-IL-12 and systemically (intravenous administration) with anti-CD137 monoclonal antibodies.[60] In addition, a therapeutic effect was observed in non-treated nodules within the same animals, suggesting that this type of therapy may also be effective for treating metastases. As observed when SFV-IL-12 was used as single agent, the antitumor effects of the combination regimen seemed to be exclusively mediated by CD8⁺ T cells. In fact, a striking consequence of the combined therapy was a massive increase in the total number of circulating CD8⁺ T cells, with a high proportion of them being specific for tumor antigens. A unique finding of this study that may explain the synergy observed in this model is the fact that, upon SFV-IL-12 injection, CD137 was upregulated in tumor-infiltrating CD8⁺ T cells. Having more CD137 on their surface, CD8⁺ T cells could bind anti-CD137 monoclonal antibodies more efficiently, hence becoming protected from apoptosis and stimulated in their function. This effect has not been described in other studies using anti-CD137 monoclonal antibodies in combination with IL-12, suggesting that SFV replication may also play a role in this effect. In fact, we showed that synergy was lost when systemic anti-CD137 monoclonal antibodies were combined with recombinant IL-12 and an UV-inactivated SFV vector (which is

unable to replicate) given intratumorally.[60] An additional interesting finding of this study is that humoral responses against SFV were significantly reduced when SFV-IL-12 was co-administered with the anti-CD137 monoclonal antibodies.[60] The capacity of anti-CD137 agonist antibodies to suppress ongoing humoral responses acting on T helper cells has been previously described[114] and may be used as an alternative approach to dampen antiviral antibody responses.

Clinical/Translational Perspectives

Translational research involving viruses as oncolytic agents and monoclonal antibodies poses a number of hurdles. Experience in clinical development teaches us to wait until at least one of the agents has received approval. However, if a combination treatment shows evidence for synergistic effects, combinations should be considered early in development.[115,116]

Additional obstacles come from the cost to produce some of the immunogenic vectors, including those used in our studies, at good manufacturing practice (GMP) level.[117] However, in our opinion the cost-benefit and risk balance favors combined interventions of this kind in late-stage cancer patients. Such trials could certainly be a nightmare for regulatory agencies since virotherapy, gene therapy and monoclonal antibodies converge in a carefully devised strategy. However, regulatory barriers and industrial barriers are currently under reconsideration. Society needs affordable development of therapies for lethal diseases and exploration of therapeutic combinations early in development.

The rationale of making a tumor lesion, or many tumor lesions, immunogenic while immunostimulatory monoclonal antibodies are used to enhance cellular immunity is a very appealing and conceptually attractive strategy. In fact, the first experimental evidences in mice are very promising. The spectacular effects are probably not going to be limited to Vv, alphaviruses and antibodies directed to CD137. On the contrary, we expect other immunostimulatory monoclonal antibodies to be beneficial as well, considering even triple combinations or other combinations as pioneered by the group of Mark Smyth.[118,119]

From a practical point of view, a liaison with industry for manufacturing and developing immunostimulatory monoclonal antibodies is a must. Then, Biotech companies should provide means to produce these viral agents under GMP and arrangements can be done in liaison with the pharmaceutic industry for these combinatorial approaches. Funding for clinical development is a serious obstacle at this stage, but provided that the two agents are available, Phase I/II trials become logistically feasible and hold clear promise. We must get ready to take this step forward.

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