Armed oncolytic viruses: A kick-start for anti-tumor immunity

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ABSTRACT

Oncolytic viruses (OVs), viruses that specifically result in killing tumor cells, represent a promising class of cancer therapy. Recently, the focus in the OV therapy field has shifted from their direct oncolytic effect to their immune stimulatory effect. OV therapy can function as a "kick start" for the antitumor immune response by releasing tumor associated antigens and release of inflammatory signals. Combining OVs with immune modulators could enhance the efficacy of both immune and OV therapies. Additionally, genetic engineering of OVs allows local expression of immune therapeutics, thereby reducing related toxicities. Different options to modify the tumor microenvironment in combination with OV therapy have been explored. The possibilities and obstacles of these combinations will be discussed in this review.

1. Introduction

1.1. The anti-tumor immune response and the immune profile of tumors

The innate and the adaptive immune system work together to detect transformed cells and remove them before they form a tumor [1]. The anti-tumor response starts with the release of tumor associated antigens (TAA) from dying cancer cells and accompanying signal molecules, which attract and activate cells of the innate immune system [2,3]. Whereas NK and γd-T cells can recognize and kill tumor cells directly, antigen presenting cells (APCs), such as DCs and macrophages, take up TAAs to activate the adaptive immune system [4,5]. The maturation of APCs by the accompanying danger signal molecules determines the skewing to a preferred T helper cell (Th) 1 response. These Th1 signals constitute of pro-inflammatory cytokines, such as interleukin (IL)-12, type I interferons (IFNs) and tumor necrosis factor (TNF), and damage-associated molecular pattern molecules (DAMPs), such as nuclear protein HMGB1, heat-shock proteins and ATP, [3]. The Th1 cytokines stimulate the generation of tumor specific cytotoxic CD8+ T cells (CTLs), which are crucial effector cells in the antitumor response [6]. Subsequently, effector T cells, including T helper cells and CTLs, are attracted to the tumor site via a gradient of T cell attracting chemokines, including chemokine (C-C motif) ligand 2 (CCL2), CCL5/ RANTES, chemokine (C-X-C motif) ligand 9 (CXCL9), and CXCL10 [7]. At the site, CTLs recognize and kill tumor cells mediated by MHCI-T cell receptor interactions. If the immune system succeeds in destruction of the beginning tumor, the host remains free of cancer.

In some cases, tumor cells are reprogrammed to evade the immune system resulting in an equilibrium between dying tumor cells and tumor cells surviving the immune attack. As a consequence, a selection of immunosuppressive or less immunogenic tumor cell variants is introduced, which cannot be eliminated by the immune system [8]. These tumor cells establish a tumor microenvironment (TME), in which the function of anti-tumor immune cells is attenuated (Fig. 1) [9]. **First of all,** tumor cells and stromal cells (endothelial and epithelial cells and fibroblasts) produce factors such as transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2) and IL-10, that disrupt APC maturation in the TME [3,7,9]. As a result, DCs isolated from the TME often display a partly matured, immune suppressive phenotype and secrete cytokines that induce non-favorable Th2 responses [7]. **Secondly**, tumors inhibit infiltration of effector T cells by repressing the production of T cell attracting chemokines CXCL9/10 and modification of CCL2 [7,10].
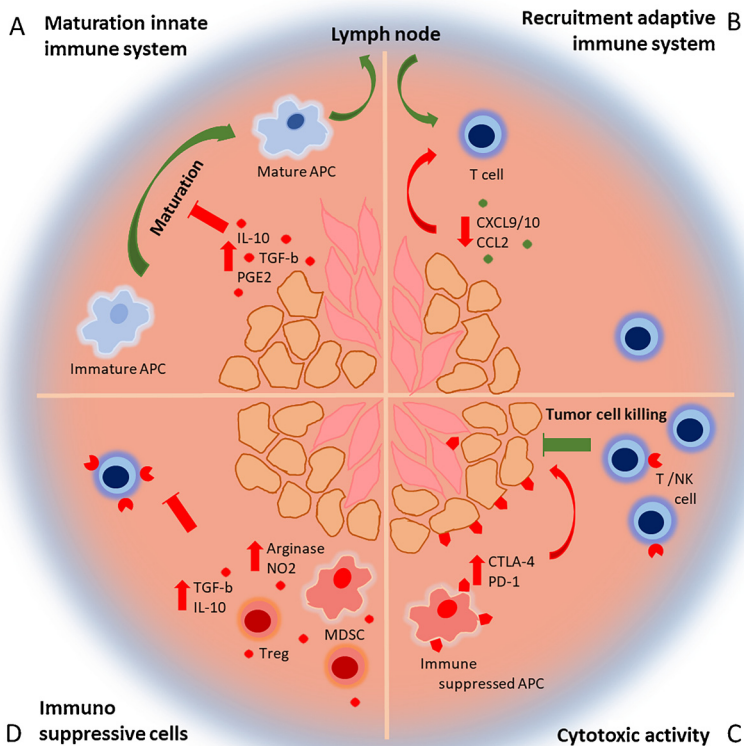
Fig. 1. The immunosuppressive tumor micro environment. (A) Tumor cells (orange) and stromal cells (pink) secrete immune suppressive molecules, which inhibit the maturation of APCs. Matured APCs migrate to the lymph node to activate the adaptive immune system. (B) As a result, activated T cells migrate to the tumor driven by a chemokine gradient. However, the secretion of chemokines is lowered in the tumor resulting in reduced T cell infiltration. (C) T cells that enter the TME to target the tumor cells are inhibited by immune suppressive receptors expressed by the tumor, stromal cell, but also immune suppressed APCs. (D) Tregs and MDSCs are recruited to the TME, which secrete more immune suppressive molecules and inhibit the T cell response even further.

Thirdly, effector T cells that can infiltrate the tumor are attenuated by expression of several immunosuppressive molecules and persistent exposure to tumor antigens. As a result, T helper cells and CTLs isolated from the TME often present an exhausted phenotype, characterized by high level expression of immune checkpoint receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [10]. Ligation of these receptors with their ligands expressed on tumor and stromal cells, but also immunosuppressed APCs, leads to inhibition of the tumor specific T cell response. **Fourthly**, regulatory immune cells such as CD4+ regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) are recruited to the tumor site. Similar to tumor cells, Tregs secrete IL-10, Indoleamine 2,3-dioxygenase (IDO) and TGF-β, leading to further attenuation of the T cell response [7,11]. Furthermore, Tregs consume IL-2, which is indispensable for T cell activation [7]. MDSCs contribute to the suppression of effector T cells through production of arginase and nitric oxide, which deprives T cells from amino acids necessary for proliferation [12].

Despite all these evasion mechanisms, CTLs and Th1 T helper cells are still considered to be the most crucial effector cells in anti-tumor immunity and their infiltration into the TME is associated with good prognosis in various types of cancer [13]. Proper activation of these cells is key for an effective antitumor response and the abundance of mechanisms used by tumors to suppress these cells offers many targets for cancer immunotherapy strategies.

At the moment, multiple strategies to target the TME are being explored. Recent successes have led to the FDA approval of checkpoint inhibitors anti-CTLA-4 (clinical responses in 10–15% of treated patients) and anti-PD1 (clinical responses in 30–40% of patients) for treatment of melanoma [14,15]. Clinical trials have shown that dual, synergistic blockage improved antitumor responses against melanoma, indicating that it might take more than one approach to induce powerful and long lasting anti-tumor immunity [16,17]. However, systemic administration of these checkpoint inhibitors, as well as other immunotherapies, often coincides with severe immune-related adverse

effects similar to autoimmune diseases [16,17]. A promising treatment option to potentially overcome this obstacle is oncolytic virotherapy.

1.2. Oncolytic viral therapy

Oncolytic virotherapy is an approach that uses oncolytic viruses (OVs), either with natural tropism for neoplastic cells or genetically modified to enhance selectivity for tumor cells [18,19]. Tumor cells often lack an adequate antiviral response, making them more susceptible to OV infection than healthy cells. The viral infection leads to tumor regression through two distinct mechanisms: direct killing of tumor cells by replication dependent induced cell death and promotion of an antitumor response towards all tumor cells, including non-infected cells, by inducing immunogenic cell death. Types of immunogenic cell death, such as immunogenic apoptosis, necrosis and autophagic cell death, are characterized by the release of TAAs in combination with DAMPs and viral pathogen associated molecular patterns (PAMPs) [19]. Following the secretion of DAMPs and cytokines, more innate immune cells, such as macrophages, DCs, NK cells and neutrophils infiltrate the tumor environment. The immune stimulating cytokine secretion leads to maturation of APCs and hence presentation of TAAs and viral antigens to activate the adaptive immune system in the lymph nodes. Cytotoxic T cells will start infiltrating the tumor again and specifically eliminate cancer cells. Simultaneously, memory T cells are formed, which improves protection against new tumor challenges in mouse models [20,21]. Therefore, it is evident that OV therapy can function as a 'kick start' for the antitumor immune response by providing TAAs in an immunogenic manner and inducing infiltration of immune cells.

Recently, the focus in the oncolytic virotherapy field has shifted from their oncolytic effect to their immune stimulatory effect. Recombinant OVs armed with immune modulators further enhance the activation of the immune system and overcome the immunosuppressive TME [18]. The first armed OV approved by the FDA is oncolytic Herpes-Simplex-Virus (HSV)-1 expressing GM-CSF showing improvement of

melanoma treatment, but no cure yet (T-VEC) [22]. Therefore, many more immune modulator armed OV therapies followed and their obstacles and opportunities have come to light. This review will give an overview of the state-of-the-art therapies used in combination with immune modulators to treat cancer patients and give a hint on potential future directions.

2. Immune therapy

Immune therapies for treatment of cancer aim at overcoming the tumor immune suppressive environment and at increasing antitumor immunity. Most immune therapies target directly or indirectly the inhibitory or stimulatory receptors on immune cells and are often based on monoclonal antibodies. Other therapies intend to restore the intratumoral balance of cytokines and chemokines into a more favorable inflammatory TME to attract and activate immune cells. The most interesting noncellular therapies for combination with OV therapy for solid tumors are described below.

2.1. Cytokines

Cytokines are key players in stimulating and regulating antitumor immune responses. For this reason, one of the first immune therapeutic approaches in cancer treatment was the administration of recombinant cytokines. As described before, the most essential cytokines in the antitumor response are IL-12, GM-CSF, IL-2 and IL-2-related cytokines IL-15 and IL-21, and they all stimulate different parts of the immune system [23]: GM-CSF recruits APCs to the TME [17], IL-12 is normally expressed by APCs and stimulates polarization of T helper cells to a Th1 phenotype, IL-2 is a T cell growth factor and improves T cell expansion [9], and the IL-2-related cytokines promote survival of T cells, but also play an important role in NK cell activation [23]. Despite the their plethora of immune modulatory actions, cytokines have lost their popularity as a monotherapy, because of their low objective response rates and non-negligible side effects upon systemic administration [24,25].

2.2. Inhibitory receptors

One of the most promising immune therapeutics are checkpoint inhibitors. Inhibitory receptors such as CTLA-4 and PD-1 act as checkpoints to avoid over activation and are expressed by T cells and inhibit the T cell activation in the lymph nodes and survival in the TME [26]. Several checkpoint inhibitors have been approved by the FDA of which the first were against CTLA-4 and PD-1. Treatment with these inhibitors results in reactivation of the suppressed immune cells [14,15]. In addition, blocking of CTLA-4 can lead to depletion of Tregs [25]. Combinations of a-CTLA-4 antibody and a-PD-1 antibody have shown to increase responses to treatment of advanced melanoma. However, the frequency of severe immune-related adverse events was also enhanced in clinical studies (16.3 − 27.3% in monotherapy group vs. 55% in combination therapy group) [16]. Another disadvantage of these therapies is the fact that tumors can become resistant towards checkpoint inhibitors [27]. As a result, a variety of other co-inhibitory receptors, such as lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin and mucin receptor protein 3 (TIM-3), have recently been identified. LAG-3 is normally activated by its ligand MHCII on APCs and indirectly reduces T cell proliferation [28]. Interaction of TIM-3 with its ligand galectin-9, expressed by Tregs, induces cell death in Th1 cells [28]. Blockage of these inhibitory receptors can unleash potent antitumor CTL responses and are now in clinical or preclinical development [17,28].

2.3. Co-stimulatory receptors

Agonistic antibodies are also in development to activate co-stimulatory receptors expressed by T cells, such as 4-1BB and OX40 [17].

Stimulation of these two receptors by their ligands or monoclonal antibodies induces activation, proliferation and survival of T cells. Another interesting target is CD40, which is expressed on DCs. CD40 ligation with CD40L or therapeutic antibody stimulates DC maturation and presentation of antigens, which leads to efficient T cell priming [28]. More stimulatory co-receptors are explored today, such as B7.1 and GITR [29–31], which might benefit the effect of current therapies.

2.4. BiTEs

Another class of therapeutics constitute of dual specificity recombinant antibodies, also called bispecific T cell engagers (BiTEs), which have shown promise as anti-tumor therapeutics. These antibodies simultaneously bind to CTLs via the T cell receptor (TCR) and a tumor antigen expressed on the tumor cell resulting in bypassing MHC dependent antigen presentation [32]. An example of such therapy is Blinatumomab, which engages the CTL to CD19+ tumor cells. Currently, it is approved by the FDA for treating acute lymphoblastic leukemia, which is an hematologic malignancy [32].

3. Combination of immune therapy and oncolytic virotherapy

Therapeutic treatment of solid tumors could be enhanced by combination treatment of both immune and OV therapy. By introducing these discussed immune stimulators, checkpoint inhibitors and cytokines as immune modulators in viral vectors, adverse events can be reduced, resistance reverted and treatment responsiveness enhanced.

3.1. Combination of OVs with cytokines and chemokines

Several viruses have been engineered to express different cytokines or chemokines. Cytokines and chemokines are attractive transgenes, because they are encoded by small genes and are in general easy to build in a viral genome. Moreover, they often have pleiotropic effects, which means they can target different immune cells simultaneously [23,32]. A complete overview of all cytokines and chemokines used in oncolytic virotherapy is given in Table 1.

3.1.1. GM-CSF
The most extensively studied transgene is the cytokine GM-CSF. GM-CSF promotes DC recruitment and maturation. GM-CSF has been successfully used to arm HSV and this armed virus has been approved by the FDA under the name of T-VEC for treatment of metastatic melanoma patients [22]. Besides HSV, other viruses have also been armed with GM-CSF [33,34]. Phase 1 clinical trials in patients with colorectal and hepatocellular carcinoma, neuroblastoma and Ewings sarcoma have proven the efficacy and safety of oncolytic vaccinia virus (VV) expressing GM-CSF (Pexa-Vec) [35–37]. In addition, two different adenovirus serotypes expressing GM-CSF induced long term survival of patients with, amongst others, ovarian, colon, pancreatic and breast cancer, with no severe side effects [34,38]. Biopsies obtained from these patients with different metastatic tumors showed increased infiltration of T-cells and macrophages, which also correlated with patient survival and hence the additive effect of GM-CSF as vectorized immune modulator [38].

3.1.2. IL-12
IL-12 both activates and promotes survival of NK cells, but also Th1 effector cells [39,40]. Several viruses have been armed with IL-12 and tested in different tumor models (Table 1) [41–46]. HSV-IL-12 induces tumor infiltration of effector T cells, NK cells and APCs in neuroblastoma and glioma mouse models [43,45]. When comparing HSV-IL-12 with HSV-GM-CSF, HSV-IL-12 was demonstrated most effective in tumor growth inhibition of injected tumors as well as metastases in a squamous cell carcinoma mouse model and a prostate cancer mouse model [47,48]. In addition, mice were better protected against re-

Table 1
Combination therapy of armed oncolytic viruses and immune modulators.

Transgene	Virus	Tumor	Additive immunologic effects	Toxicity
Cytokines				
GM-CSF	HSV [22,48,121]	Adenocarcinoma [33], Metastised (phase I)[a] [22,34] Breast cancer [121], Melanoma (phase I)[a] [121,122]	Improved peripheral blood mononuclear cell response [124]	Grade 1 and 2 [22,34],
	AdV [34,110,122]		CD3+ T cell infiltration [33]	
	MV [33]		Long-term immunity against rechallenge with tumor cells [33]	
	VSV [123]			
	NDV [124]			
IL-12	AdV [108]	Neuroblastoma [45], Glioma [43], Prostate [48], Squamous Cell Carcinoma [46,47], Melanoma [108]	Infiltration of macrophages, T helper, CTL and NK cells [43,45,47,48]	No signs [44,46,47]
	HSV [30,43–45,47,48]		Improved survival [108] and protective against rechallenge [47]	
	VSV [46]			
IL-2	NDV [52–56]	Melanoma [54,55], Hepatoma [52,53], Squamous Cell Carcinoma [51]	Infiltration of T helper and CTL [51–56]	No signs [53,54]
	HSV [51]		Immunity against rechallenge with tumor cells [51–54]	
IL-15	VSV [57]	Colon carcinoma [57], Melanoma [58,127]	Increase in tumor specific CTLs in the blood [57]	IL-15 only detectable in tumor [58]
	NDV [58]		Infiltration of T helper and CTL [58], [126]	
	IAV [125]		Immunity against rechallenge with tumor cells [58]	
	HSV [126]		Increases survival in mouse model [58,127]	
	IAV [127]			
IFN-β	MV [61]	Non-small cell lung cancer [128], [129], Mesothelioma [61], Pancreatic adenocarcinoma [59]	Improved survival mouse model [61]	Not reported
	VV [128]			
	VSV [129]			
	NDV [59]			
IFN-γ	NDV [55]	Melanoma [55], Mammary and colon carcinoma[62]	Increased cytokine expression and improved DC maturation [62]	Not Reported
	VSV [62]		Increased T cell infiltration [55]	
Others: IL-18[30], [108], IL-17[130], TNF[55], MIP1a[131], FLT3L[131]	NDV [55]		Improved T cell responses	Not reported
	VSV [130]			
	AdV [108]			
	HSV [30]			
Chemokines: CCL5[65], CCL2[67], CCL19[132], CXCL11[64], [133]	VV [65,66,122,123]	Colon carcinoma	Improved DC maturation [65]	Not reported
	HSV [67]		Improved infiltration T helper cells and CTLs [64], [65], [132], [133]	
			Induces a Th2 response, but reverts to a Th1 response in combination with DC vaccination [65]	
Co-stimulatory ligands				
B7.1/CD80	HSV [84]	Neuroblastoma[84], Melanoma (patients)[a] [86]	Immunity against rechallenge with tumor cells [84]	Low grade [86]
	VV [86]		Response in 3/11 patients [86]	
4-1BBL/CD137L	VV [89]	Melanoma	No difference in DC maturation Infiltration of CTL	Not reported
CD40L	AdV [92]	Melanoma[93],	More Th1 cytokines [92]	No signs [92]
	AdVdd [90]	Solid tumor (patients)[a][92]	Infiltration of T helper, CTL, NK, DC, MDSC [93]	
	VV [93]		Improved survival, but not with armed VSV [90]	
	VSV [90]			
OX-40L	AdV [95]	Melanoma, lung carcinoma	Infiltration of T helper and CTL	Not reported
GITRL	AdV [31]	Melanoma	Infiltration of T helper and CTL	No signs
LIGHT	AdV [99]	Prostate	Recruitment of effector T cells Reduced Treg suppression	Not reported
CD70	VV [98]	Colon adenocarcinoma	Reduced tumor growth	Not reported
Checkpoint inhibitors				
anti-CTLA4	MV [75]	Melanoma	Infiltration of T helper and CTL [75], [108]	No signs [75], [108]
	AdV [74,108]		Decreased infiltration of Tregs [75]	
anti-PD1 or PD-L1	MV [75]	Melanoma	Infiltration of T helper and CTL [75], [83]	No signs [75]
	VV [83]		Decreased infiltration of Tregs [75], [83]	
	MYXV [81]		Improved survival [81]	

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Table 1 (*continued*)

Transgene	Virus	Tumor	Additive immunologic effects	Toxicity
Combinations				
GM-CSF + IL-12	AdV [104,105]	Melanoma	Secreted cytokine profile shifted from Th2 to Th2 response [105] Infiltration of T helper, CTL, NK and DC [104], [105] Immunity against rechallenge with tumor cells [105]	Not reported
IL-12 + IL-18	AdV [106]	Melanoma	Infiltration of T helper, CTL, NK	Not reported
IL-12 + CCL2	HSV [67]	Neuroblastoma	Reduced tumor growth	Not reported
B7.1 + IL-12	AdV [41]	Melanoma	Infiltration of T helper, CTL and DC	Not reported
B7.1 + IL-18	HSV [132]	Neuroblastoma, Prostate	Reduced tumor growth No significant difference in survival	Not reported
B7.1 + GM-CSF	AdV [85]	Melanoma	Infiltration of T helper, CTL and DC Immunity against rechallenge with tumor cell	Not reported
4-1BBL + IL-12	AdV [42]	Melanoma	Infiltration DC, T helper and CTL	No signs
Others				
HPGD	VV [100]	Several tumor models	Expression of Th1 cytokines, CXCL10/11, CCL5 Decreased infiltration MDSC More DCs secreting IL-12 in LN	Not reported
TRIF	VV [100]	Renal cell carcinoma	Increased immune stimulatory cytokine response Improved survival	Not reported
DAI	VV [103]	Melanoma	Improved CD8 + cell infiltration Reduced tumor growth	Not reported

[a] indicates patient studies, whereas other studies were performed in mouse models.

challenges with tumor cells in the HSV-IL-12 treated group, indicating the formation of a long term, anti-tumor response [47]. Similarly, adenovirus armed with IL-12 showed improved tumor reduction compared to adenovirus armed with GM-CSF in a thyroid cancer rat model [49]. In conclusion, multiple studies demonstrate that OVs armed with IL-12 yield better anti-tumor effects than vectorized GM-CSF and can even be further improved by combining different immune modulators.

3.1.3. IL-2 and IL-15

IL-2 and IL-15 both signal via cytokine receptors of the common γ chain family and are important for the stimulation, proliferation and survival of T cells and NK cells [23,50]. Systemic treatment with IL-2 is associated with major adverse side effects in humans [24]. Therefore, local delivery of IL-2 by OVs has been tested by several research groups [51–56]. In these murine studies, reduced tumor growth and increased T cell infiltration of the tumors was reported. No distress was observed in mice and IL-2 production was limited to the tumor site, which might indicate less side-effects when administrated to humans [53–55]. Also, mice were protected from re-challenge with tumor cells suggesting induction of long term tumor specific immunity and thus show promise as vectorized immune modulator [52–55].

Despite the promising effects of IL-2, IL-15 showed to have several advantages over IL-2. In contrast to IL-2, Il-15 can stimulate only NK and effector T-cells, whereas IL-2 has also the undesirable effect of stimulating Tregs [50]. Even though systemic treatment with IL-15 induces less toxic effects local expression of IL-15 mediated by armed Vescilular Stomatitus virus (VSV) enhanced anti-tumor activity compared to VSV treatment combined with systemic IL-15 in murine models [57]. Vectorization by NDV demonstrated that IL-15 induced more CTL infiltration and increased activation of tumor-specific effector cells resulting in improved survival rates compared to IL-2 in a melanoma mouse model [58]. Altogether, these studies indicate that treatment with OVs expressing IL-15 is more efficient than systemic treatment with IL-15 or treatment with OVs expressing IL-2.

3.1.4. Type I and II interferons

IFN-α/β are important in antiviral responses, but also play a role in anti-cancer immunity by inducing DC maturation and CTL and NK cell activation [23,32]. In addition, they result in upregulation of MHC I expression on tumor cells and can have direct effect on cell proliferation. Only a few studies have investigated OVs armed with type I interferons [59–61]. The direct anti-tumor effects of IFN-β expressed by the measles virus (MV) and NDV has been demonstrated in immune deficient mouse models leading to some improvement of the viral therapy. Buijs et al. showed that a virus expressing IFN-β interfered with oncolytic NDV replication, whereas Willmon et al. and Li et al. found that viral IFN-β expression did not interfere with VSV and MV replication [59–61]. However, the immune stimulating effect of virus mediated IFN-β expression still needs to be assessed in an immunocompetent model to determine the potency of IFN-β as transgene.

Type II interferon (IFN-γ) is an important Th1 effector cytokine, secreted by activated Th1 cells, CTLs and NK cells [23]. IFN-γ upregulates MHC I expression in tumor cells and promotes Th1 skewing via an autocrine loop [23]. A recent study describing VSV expressing IFN-γ suggested that this virus induces a stronger immune response by increasing MHC I antigen presentation on tumor cells, enhancing DC maturation and attracting T cells to the tumor site by inducing CCL2 expression in mice [62]. The authors did not observe any difference in viral replication, even though IFN-γ is also known for its antiviral activity. In another murine study, treatment with NDV- IFN-γ did not result in a significant beneficial effect compared to unarmed NDV [55]. In conclusion, arming OVs with IFN-γ has been shown to be effective, but efficacy may depend on the virus used.

3.1.5. Effector cell attracting chemokines

Insufficient infiltration of effector lymphocytes in the tumor often correlates with low efficacy of T cell stimulating immunotherapies [7,10]. Therefore, OVs armed with chemokines, which attract effector cells, may improve antitumor efficacy in addition to the endogenous chemokine release upon viral infection. Important chemokines in the TME are CCL2, CCL5 (RANTES), CXCL9 and CXCL10, which attract Th1

cells and CTLs and CCL22, which attracts undesirable Tregs [63]. Combination of OV therapy with a chemokine modulating cocktail, which induces production of CCL5 and CXCL10 while reducing CCL22, was shown to promote trafficking of T helper cells and CTLs to the TME and resulted in improved survival in a murine colon cancer model [64]. Administration of viruses armed with CCL5 or CCL2 both resulted in increased numbers of infiltrating Th1 cells in colon cancer and neuroblastoma [65–67]. In conclusion, these studies have shown that the use of OVs armed with chemokines efficiently increased the infiltration of T cells into the tumor. However, none of the studies have assessed the infiltration of immune cells in distant tumors and tumor reduction has not been mentioned.

3.2. Combination of OVs with blocking of co-inhibitory receptors

The most popular immunotherapeutics are the checkpoint inhibitors, anti-CTLA-4 or anti-PD-1/PD-L1, yielding promising effects but coinciding with major adverse effects, as described above [68]. In addition, some tumors are resistant to these immunotherapies, dependent on the immunogenicity of the tumor and the suppression of the anti-tumor immune responses in the TME [69]. Combination of systemic immunotherapeutics with localized OV therapy may enhance their therapeutic efficacy and may overcome tumor resistance and reduce immune related adverse effects.

3.2.1. CTLA-4

CTLA-4 is a checkpoint inhibitor that inhibits early stages of T cell activation in the lymph nodes, but also stimulates undesirable Treg functions [28,68]. Blockage of CTLA-4 signaling releases a brake on T cell activation and even depletes intratumoral Tregs [28]. Intratumoral administration of NDV combined with systemic treatment with anti-CTLA-4 (Ipilimumab) showed improved antitumor effect mediated by CTLs and NK cells in a murine melanoma model [70]. Studies using combinations of systemic CTLA-4 blockade with other OVs (VV [71,72] and VSV [20]) showed prolonged survival in renal [71], lung [72] and mammary [20] tumor models, long term protection to re-challenge with tumor cells [71,72], and even cured mice [20]. Moreover, T-VEC combined with systemic CTLA-4 blockade has been evaluated for therapy in melanoma patients, which yielded a tumor growth control that was significantly greater than observed after both monotherapies [73]. The incidence of severe adverse effects was similar to Ipilimumab monotherapy [68], [73]. Local expression of anti-CTLA-4 induced by administration of adenovirus armed with anti-CTLA-4 resulted in significantly higher concentrations of the antibody in the treated tumor, while plasma levels remained at concentrations indicated as safe in murine models [74]. However, Engeland et al. demonstrated that treatment with MV armed with anti-CTLA-4 was less efficient than intratumoral treatment with MV combined with systemic administration of anti-CTLA-4, presumably because the major site of action of CTLA-4 is in the lymph node and not in the periphery [75]. Though local expression may lead to transient expression of the transgene in tumor draining lymph nodes, this study indicated that systemic anti-CTLA-4 therapy may be required for optimal responses.

3.2.2. PD-1 and PD-L1

With a completely different mechanism of action, PD-1 is also a checkpoint inhibitor expressed by effector T cells [68]. While CTLA-4 inhibits T cells in early activation stages in the lymph nodes, PD-1 signaling limits the function of activated T cells at later stages of the immune response taking place in tumors and tissues [68]. Multiple OVs such as MV [76], reovirus [77,78], and VSV [79,80], have been combined with systemic PD-1 blockade in treatment of murine glioblastoma [76], melanoma [77,78], and acute myeloid leukemia [79] tumor models, resulting in enhanced influx of CTLs in the tumors and prolonged survival in mice. Specifically CTLs and NK cells, but not T helper cells, were shown to mediate the beneficial effects [77,79,81].

Moreover, a phase 1b clinical trial with the FDA approved T-VEC combined with PD-1 resulted in good response rates in patients [82]. Arming Myxoma Virus (MYXV) with anti-PD-1 did result in improved survival in mice as well [81]. However, local expression induced by administration of MV or VV armed with anti-PD-1 or anti-PD-L1 demonstrated to be as efficient as therapy with unarmed VV combined with systemic anti-PD-1/anti-PD-L1 treatment in murine tumor models [75,83]. In conclusion, these studies suggest that OVs combined with checkpoint inhibitors improve treatment, but the benefits of vectorization differ per oncolytic virus.

3.3. Combination of OVs with activation of co-stimulatory receptors

3.3.1. B7-1

B7-1 (CD80) is expressed by APCs and a potent co-stimulatory molecule for T cells. B7-1 provides co-stimulation via interaction with CD28 on T cells, but inhibits T cells through interaction with CTLA-4 [28]. Nevertheless, several OVs armed with B7-1 have been tested in (pre)- clinical trials for the treatment of melanoma [41,84–86]. Replication defective HSV expressing soluble B7-1 induced a prolonged tumor specific immune response in mice bearing neuroblastoma tumors [84]. VV expressing B7-1 was eventually evaluated in melanoma patients, where the treatment was well tolerated, but only few patients responded [86]. Overall, OV expression of B7-1 seems to have additional benefits in combination with other transgenes, but not as a single transgene.

3.3.2. 4-1BB

4-1BB is a surface protein primarily present on activated T cells and NK cells. 4-1BB signaling promotes Th1 skewing over Th2, protects T cells from activation induced cell death and enhances cytotoxic activity of T cells and NK cells [28]. 4-1BB signaling has been shown to be more potent in T cell activation compared to the CD28 co-stimulation by B7-1 [87]. A combination of oncolytic VV with systemic anti-4-1BB administration in a breast cancer model resulted in increased survival and tumor infiltration by CTLs compared to both monotherapies in patients (40% survival vs. 0% survival) [88]. Local expression of 4-1BB ligand (4-1BBL) by oncolytic VV in mice has demonstrated to enhance tumor regression and this effect was enhanced even more in combination with lymph node depletion in order to slow down viral clearance [89]. Local injection of the armed virus resulted in an improved CTL/Treg ratio in the TME. In another study, vectorization of both IL-12 and 4-1BBL by adenovirus increased T helper, CTL and DC infiltration, which resulted in improved survival of the mice [42]. In other murine models, DC vaccination combined with the armed adenovirus combined treatment yielded even better results and DCs showed enhanced migration to the tumor draining lymph node, where they activated T cells [42]. These studies have shown that vectorization of this immune modulator in OVs enhances its therapeutic efficacy.

3.3.3. CD40

The maturation and activation status of DCs is often a limiting factor in the induction of antitumor immune responses. Ligation of the CD40 receptor provides a strong activating signal to DCs, resulting in upregulation of MHC II and co-stimulatory molecules and production of IL-12, which is important for skewing of T cells towards a Th1 phenotype [28]. As DC activation takes place in the TME, treatment with OVs armed with a stimulatory CD40 antibody or CD40 ligand (CD40L) may lead to enhanced therapeutic efficacy.

Adenoviruses armed with CD40L have been tested in melanoma mouse models and patients with different types of cancer [90–92]. Treatment with a CD40L expressing VV resulted in tumor growth inhibition and increased infiltration of effector T cells, NK cells, DCs in a melanoma mouse model. However, also numbers of Myeloid derived suppressor cells increased of which its effects are unknown [93]. When evaluated in a clinical trial, patients showed disease control for 3–6

months and a systemic tumor specific immune response was induced [92].

3.3.4. OX40

Similar to checkpoint inhibitors, more and more co-stimulatory targets, such as OX40 and GITR, are being discovered as anti-tumor targets. The OX40 receptor is expressed by activated T cells and induces the production of Th1 and Th2 cytokines upon interaction with OX40 ligand (OX40L) [28,94]. OX40 ligation also directly blunts the suppressive effects of Tregs [28,94]. Arming of OV with OX40L has been described once: adenovirus expressing OX40L led to suppression of melanoma, lung and colon tumor growth in mice. This effect was mediated by Th1 cells and CTLs and an increase in Th1 rather than Th2 cytokine expression [95]. A study combining systemic OX40L therapy with systemic 4-1BBL therapy and IL-12 expressing adenovirus, revealed increased expression of Th1 cytokines. thereby the antitumor CTL response were enhanced leading to tumor rejection in a colorectal cancer model involving liver metastases compared to OX40L monotherapy and combined 4-1BBL and IL-12 armed OV treatment [96]. This makes OX40 an interesting transgene, but more research needs to be performed to elucidate its beneficial effects in other tumor models and OVs.

3.3.5. GITR

GITR is a stimulatory receptor expressed on activated T cells, but it is also constitutively expressed on Tregs [28,94]. Activation of the receptor promotes proliferation and cytokine production in T effector cells, whereas it inhibits Tregs [28,94]. Expression of GITR ligand itself by an adenoviral vector resulted in increased infiltration of T helper cells and CTLs and suppression of tumor growth, leading to prolonged survival [31]. Systemic administration of a stimulating GITR antibody together with intratumoral injection of an adenoviral vector armed with IFN-α resulted in enhanced tumor growth inhibition in injected and distant tumors compared to single treatment in colon and pancreatic murine cancer models [97]. This makes GITR ligand attractive as a transgene, but similar to OX40L, more research needs to be performed. More research needs to be done towards these targets, just like for the newly discovered LIGHT and CD27 anti-tumor targets [98,99], (Table 1).

3.4. Combination of OVs with other immune stimulatory approaches

3.4.1. Prostaglandin E2 blockade

Prostaglandin E2 (PGE2) is a principal mediator of inflammation expressed by tumor cells and immune cells and stimulates accumulation of MDSCs in the TME. Moreover, PGE2 induces IDO and IL-10 expression in DCs, whereas it reduces IL-12 expression, which is necessary to induce a Th1 response [7,10]. A recent study, revealed PGE2 as an important mediator of resistance to OV therapy and other immunotherapies [100]. Treatment of mice with VV expressing a PGE2 inactivating enzyme named hydroxyprostaglandin dehydrogenase (HPGD), resulted in a decreased number of MDSCs in the tumor and yielded a better response than other PGE2 blocking agents [100]. The virus was also shown to sensitize an otherwise resistant renal tumor to anti-PD-1 treatment [100]. Thus, blockade of PGE2 showed promising results but this approach needs further validation in other tumor models and with other OVs.

3.4.2. Pathogen receptors

Toll like receptors (TLRs) are part of the innate immune system and recognize pathogenic molecules such as bacterial lipids and proteins or viral DNA and RNA [101]. The type of TLR that is activated upon infection is an important determinant for the skewing of the subsequent adaptive immune response [101]. Manipulation of TLR signaling can therefore switch the induced immune response from Th2 to Th1.

TRIF mediates TLR3 signaling, whereas other TLRs signal via MyD88 [101]. Moreover, this pathway induces the production of Th1 cytokines instead of Th2 cytokines. To manipulate TLR3 signaling, a VV was engineered to express TRIF to increase TLR3 signaling [102]. In addition, the viral particle was deglycosylated, resulting in reduced Th2 responses. Treatment of mice with deglycosylated VV-TRIF resulted in increased production of Th1 but not Th2 cytokines in the TME. Also, the virus induced infiltration of T helper cells and CTLs. These CTLs were both virus and tumor cell specific. VV-TRIF showed improved antitumor efficacy in colon and renal tumor models compared to VV-GM-CSF (Pexa-Vec). Similar results were obtained using intracellular pattern recognition receptor DNA-dependent activator of IFN-regulatory factors (DAI) as an vectorized immune modulator [103]. These studies show that the antitumor immune response can be switched from Th2 to Th1 by manipulating intracellular pathogen receptor signaling pathways in tumor cells.

3.4.3. BiTEs

BiTEs (bispecific T cell engagers) are a class of bispecific monoclonal antibodies that have shown promising anti-tumor effects [104]. However, they have a very short half-life and therefore require continuous infusion. Vaccinia virus (VV) armed with a BiTE with specificity for the TCR and a TAA was tested in a xenograft lung cancer mouse model. The study showed increased tumor cell killing through T cell activation and skewing towards Th1 responses [105]. A disadvantage of BiTEs is that they target only one TAA and therefore stimulate the immune response directed to only that TAA, while other immune-modulatory transgenes induce immunity to multiple TAAs that are released by dying tumor cells.

3.5. Combining different immune modulators

To further improve the efficacy of OV treatment, different combinations of therapies are being exploited. OVs armed with several cytokine combinations have been tested with promising results. Adenovirus expressing both GM-CSF and IL-12 induced infiltration of effector T cells, NK cells and activated APCs combined with DC vaccination in a melanoma mouse model, as well as long-term protection to re-challenge with tumor cells [106]. Moreover, viral expression of GM-CSF and IL-12 in the TME shifted the intratumoral cytokine profile from a Th2 to a preferred Th1 response and improved DC migration to the tumor site [106,107]. Similarly, viral co-expression of IL-12 and IL-18 showed enhanced therapeutic efficacy and increased infiltration of DCs and effector T cells compared to mono-expression of IL-12 in murine models [41,42,108]. The combination of IL-12 and IL-18 showed to have synergistic effects on IFN-γ production, an important Th1 effector cytokine, by T cells and NK cells and polarization towards a Th1 response [28,109]. Combining IL-12 expression with CCL2, an important T-cell chemokine, resulted in enhanced neuroblastoma growth inhibition compared to administration of armed HSV alone demonstrating the potential of combining different cytokines and chemokines in mice [67].

In addition, cytokines have also been combined with immune stimulatory receptor ligands. Treatment with adenovirus armed with GM-CSF and B7-1 in a melanoma mouse model resulted in prolonged survival and resistance to tumor re-challenge compared to treatment with unarmed adenovirus [85]. Similarly, treatment with oncolytic adenovirus armed with IL-12 as well as B7-1 resulted more often in complete regression in a melanoma mouse model compared to adenovirus armed with IL-12 alone [41]. Combination of three armed OVs with IL-12, IL-18, and B7-1 yielded significant better results in inhibition of local and distant tumor growth in a neuroblastoma mouse model, compared to single vector treatment demonstrating the possible synergistic effects between cytokines and co-stimulatory receptors [30].

The combination of checkpoint inhibitors and cytokines work equally well. Co-administration of oncolytic adenovirus expressing GM-CSF and oncolytic adenovirus expressing anti-CTLA-4 has been shown

to yield additive antitumor activity in a lung cancer mouse model [110]. Furthermore, T-VEC in combination with systemic treatment with anti-CTLA-4 resulted in significantly improved tumor growth control rates in patients with advanced melanoma compared to both monotherapies alone [73]. Currently T-VEC in combination with anti-PD-1 is being evaluated in clinical trials [111], but the results are still unknown. In addition, Sorensen et al. engineered a replication deficient adenovirus expressing a TAA. By combining administration of this virus with systemic administration of CD40 stimulatory antibodies and CTLA-4 blockage, tumor growth of melanoma was reduced and long term survival was observed in 30–40% of mice in contrast to the mono-therapies [112]. Thus, stimulation of both DCs and T cells is necessary for long term anti-tumor immune response. Therefore, these (pre-) clinical trials with combination-therapies demonstrate the potential to improve current therapies.

Taken together, these studies provide a strong rationale for further evaluation of OVs as a local delivery vector for immunotherapies targeting co-stimulatory and co-inhibitory receptors on anti-tumor effector cells or other parts of the TME.

4. Discussion and future directions

OVs represent a class of promising agents to treat cancer. Besides their direct oncolytic effects, they can function as a 'kick-start' for anti-tumor immunity. Combining OV therapy with existing immune thera-pies enhances the potential of both therapies by synergizing their ef-fects. Many different combinations have been tested and are summar-ized in this review (Table 1). These studies have broadened our understanding of the strengths of OV immune therapies, but also of its limitations. As a result, these learning lessons enable us to discuss the potential future directions and further considerations in deciding on effective immune modulators and viral combinations and reducing risks.

4.1. Effective immune modulators

One of the greatest advantages of OVs armed with immune mod-ulators is that this therapy often induces safer systemic and/or more effective localized concentrations of the modulator than systemic monotherapy [58,86,92]. Nevertheless, vectorization of checkpoint inhibitors can also limit the potency of the immune therapies by in-correct localization and timing and no resilience in case of tumor re-sistance. With regard to localization, a checkpoint receptor, such as CTLA-4, is mainly functional in the lymph node and requires systemic delivery of the blocking antibodies [72]. For instance, the anti-CTLA-4 armed MV proved to be less effective than monotherapy with CTLA-4 inhibitors [75]. Similar results were found for PD-L1 inhibitors deliv-ered by MV and VV [75,83], illustrating the second disadvantage of OVs armed with checkpoint inhibitors: the timing of administration of combined therapies. Rojas et al. and Gao et al. demonstrated that sys-temic administration of immune therapeutics given shortly (1–3 days) after OV therapy resulted in additive efficacy of the combination therapy in contrast to immune therapeutics given prior to OV admin-istration [71,113]. However, timing strategies may differ per virus as was seen with the armed MYXV where vectorization of anti-PD-1 did result in additive effects [81]. In addition to localization and timing, multiple studies have shown the development of resistance towards these checkpoint inhibitors over time in which new inhibitory mole-cules were upregulated on the cancer cells rendering the therapy in-effective [27]. Systemic administration of checkpoint inhibitors would allow for an easier transition between checkpoint therapies if necessary. Therefore, we think that arming viruses with checkpoint inhibitors might not be the most effective combination strategy.

An alternative approach to consider would be the targeting of APCs, NK cells and CTLs by arming viruses with immune stimulating agonists and cytokines. These immune cells express a known and stable subset of cytokine and co-stimulatory receptors and hence circumvent the treatment resistance as is seen with checkpoint inhibitors. Several stu-dies have shown the additive effects of OVs armed with these agonistic agents compared to OV therapy alone (Table 1). In addition, the loca-lized expression of agonistic agents reduces adverse effects in contrast to systemic administration and still allow combination therapy with checkpoint inhibitors [58,114,115]. Based on the reviewed studies, we reason that both cytokines and agonists targeting co-stimulatory re-ceptors are very promising as immune modulators in the OV treatment against solid tumors.

4.2. Viral combinations

Another point of consideration is the combination of the virus and the immune modulator. The virus determines both the 'kick-start' ef-fect, which initiates the inflammation, and the concentration and duration of expression of the vectorized immune modulators. However, viruses differ in the effectiveness in killing the target cell or in ex-pressing the immune modulator. For example, in a study comparing VSV with a replication defective adeno virus, both expressing CD40L, it was shown that the replication defective virus was superior in in-creasing survival rates over the replicative virus [116]. This is probably because of the low immunogenicity of the replication defective virus resulting in reduced viral clearance by the immune system and hence more immune modulator secretion. However, whether the benefits of a weaker immune response hold true for all therapies remains unknown and probably depends on the virus.

In addition to oncolytic activity, the efficacy of the OV immune therapy is influenced by the efficiency of a virus to express the immune modulators. For instance, for NDV it has been shown that the insertion site in the virus determines the expression levels of the transgene [56]. The effect of the insertion site may also hold true for other OVs, but is often not discussed. In addition, the size of the immune modulators is also of importance. Studies using a complete monoclonal antibody, only the light chain or a single-chain variable fragment showed that smaller proteins were more effectively produced than the larger proteins by cells infected with VV. However, the single chain fragment without an IgG domain was degraded faster in the TME, resulting in similar anti-tumor activity as the monoclonal antibody [83]. As not all viruses tolerate large insertions, this information should be considered when deciding which modulator to incorporate. However, predicting the ef-ficiency of the modulator expression remains difficult, because differ-ences in expression levels can occur between tumors independent of the virus [110].

4.3. Reducing risks

While moving forward with OV immune therapies to the clinic, safety ought to be considered once more. So far, no adverse effects have been reported on the therapies combining OV with immune therapies in murine models or clinical trials. Nevertheless, adverse effects of the viral infection, such as excessive viral replication or expression of the immune modulators leading to an overreaction of the immune system cannot be excluded yet. A possibility to reduce the risks on adverse effects would be the incorporation of a fail-safe mechanism into the OV therapy to abort viral replication if necessary. Only for HSV, drugs are available to inhibit viral replication [117]. Alternatively, virus with attenuated virulence or viruses with a different host range may be used. In addition, incorporation of suicide genes, such as thymidine kinase [117], rat cytochrome P450 [118] or cytosine deaminase [119], would allow inhibition of viral replication by using drugs as well. Moreover, it could add to the tumor lytic efficacy of the therapy [120]. During OV therapy, the immune modulator is not incorporated into the host genome and therefore expression levels are dependent on viral re-plication which is usually transient. Thus, controlling the viral re-plication would reduce the risks on severe adverse effects of the
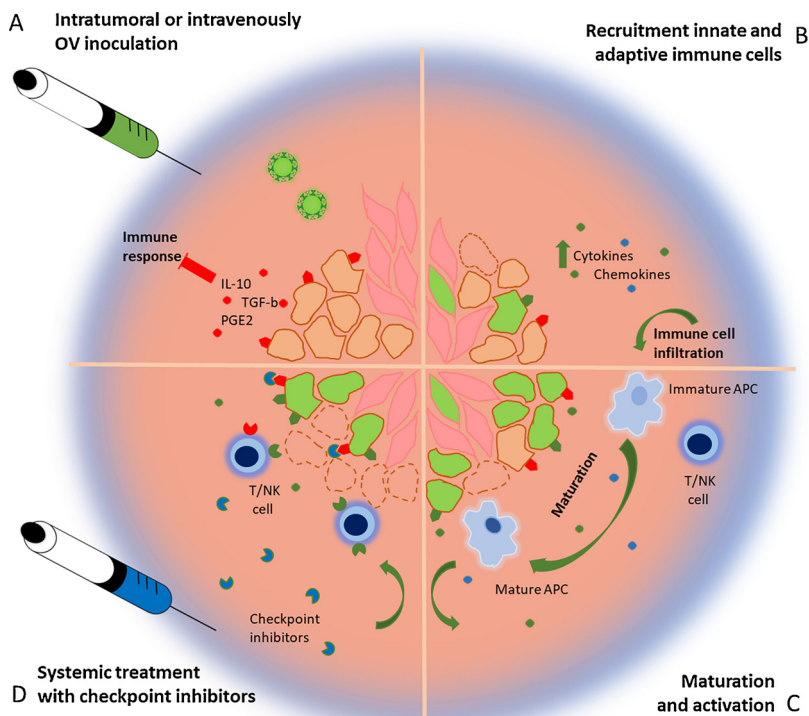
Fig. 2. Combining oncolytic viral therapy with immune modulators. (A) Weak immunogenic tumor cells in an immunosuppressive TME are infected by oncolytic viruses (green) armed with immune modulators. (B) Tumor cells start secreting viral induced cytokines and chemokines, but also the immune modulators, which improves the immune activation. As a result, immune cells start to infiltrate the TME. (C) Occasionally, the virus will induce an immunogenic cell death. The TAAs and immune stimulating environment will result in the maturation of APCs. (D) The APCs will activate the adaptive immune system upon which T cells will start infiltrating the tumor attracted by the secreted chemokines. Possible activity of immune suppressive ligands, such as PD-L1, will be prevented by the circulating immune checkpoint inhibitors resulting in clearance of the tumor.

immune modulators.

In conclusion, the overall effect of the oncolytic immune therapy depends on the interplay between virus, immune modulator and tumor. Not only tumors develop over time, but the immune system as well. This means that every stage of immune activation should be considered while deciding on the incorporation of an immune modulator (Fig. 2). Weak immunogenic tumors in an immunosuppressive TME will likely benefit from potent oncolytic viruses, cytokines and innate stimulating agonists to activate the initial innate immune response. If eventually the tumor is inflamed, effector T cells and NK cells responses can be improved by immune activating agonists, such as 4-1BBL, and checkpoint inhibitors. The vectorization of both cytokines and immune activating agonists could reduce possible adverse effects compared to systemic administration, whereas systemic delivery of checkpoint inhibitors improves the timing and localization of the treatment. Future directions will have to explore multiple combinations of dually armed OVs allowing to overcome tumor heterogeneity or at least to use both OVs and immune modulators to their full potential.

Conflicts of interest

The authors declare no conflict of interest.

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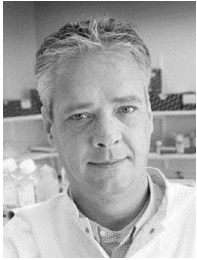
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