Harnessing the immunostimulatory properties of oncolytic reovirus for anticancer immunotherapy

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CHAPTER 8

Summarizing Discussion and Future Perspectives

The exploitation of the immune system to battle tumors has revolutionized the field of anticancer therapy. However, improved clinical responses to immunotherapy occur in only a subgroup of patients with solid tumors. These tumors often present with an ongoing immune response, which includes the baseline presence of immune cells, particularly T cells. Tumors with this phenotype are known as immune-infiltrated tumors. Tumors with an immune-silent phenotype, for example a large proportion of pancreatic cancers, lack this basal influx of T cells and as such barely respond to T-cell-based immunotherapy. The central theme of this thesis was to investigate and exploit the immunostimulatory properties of oncolytic reovirus as a strategy to enhance the response of pancreatic cancers to T-cell-based immunotherapy.

The use of oncolytic viruses (OVs) as anticancer agents was kickstarted by occasional observations where tumor regressions coincided with natural virus infections (1). A very well-known example, already published in 1904, is that of a 42-year-old woman with leukemia that went into remission after an infection with influenza (2). In the years following the 1950s, many human pathogens were investigated for oncolytic activity, including measles, vaccinia, adenovirus, and reovirus. More recent investigations into the therapeutic benefit of OVs have led to several clinical candidates and one FDA/EMA-approved oncolytic virus. These investigations also resulted in the culmination of various topics of debate and outstanding questions concerning the optimal application of OVs in the clinic. Here, I have used these outstanding questions, accompanied by illustrative figures comprising published and unpublished data, to summarize and discuss how the accumulated data in this thesis provides new insights and may ultimately contribute to more effective viro-immunotherapy.

REOVIRUS: ONCOLYSIS OR IMMUNE STIMULATION?

OVs, including oncolytic reovirus, comprise an emerging and highly promising class of anticancer immunotherapeutics that exploit the natural ability of certain viruses to infect and preferentially lyse tumor cells while leaving healthy cells intact. However, newer studies demonstrated that OVs might also, or even better be utilized as agents that can induce local potent immune responses.

In our studies, we observed that intratumoral administration of 10^7 plaque-forming units (pfu) of oncolytic reovirus, a dose that is comparable to the amount of reovirus pfu/kg bodyweight used for patients in clinical trials, did not result in an increased presence of apoptosis marker cleaved caspase 3 in reovirus-treated, immune-silent murine pancreatic KPC3 tumors (**Chapter 5**). Although more recent insights demonstrated that reovirus can also cause necrosis (3), a caspase-independent pathway of cell death, we also did not observe a significant reovirus-induced inhibition on the outgrowth of murine pancreatic KPC3 tumors or other preclinical tumor models (**Figure 1**). These

observations suggest that in the models that we tested, the capacity of reovirus to act directly as an oncolytic agent is limited.

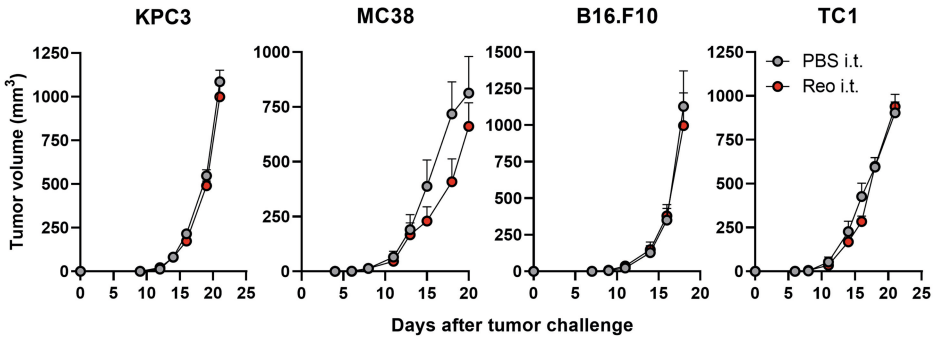

Figure 1. Reovirus administration does not affect tumor outgrowth in different preclinical tumor models. Average tumor growth curves of murine pancreatic KPC3, colon MC38, melanoma B16.F10, and lung TC1 tumors after intratumoral administration of reovirus (10^7 plaque-forming units). Data represent mean±SEM.

However, the administration of oncolytic reovirus did induce a fast interferon response (**Chapters 2, 3, 5, and 7**), including the expression of various T-cell-attracting chemokines and other interferon-stimulated genes (ISGs), which was followed by the influx of immune cells into these otherwise immune-silent tumors. Interestingly, the reovirus-induced influx of immune cells seems to be very specific for 'killer cells', since not $CD4^+$ T cells, but mostly $CD8^+$ T cells and to a lesser extent NK cells infiltrate into KPC3 tumors, as well as MC38, B16.F10, and TC1 tumors (**Figure 2**). The frequency of other immune cells, such as macrophages, dendritic cells, or neutrophils did not increase upon intratumoral reovirus administration. In contrast, whilst other OVs might also induce the influx of $CD8^+$ T cells or NK cells, often the influx of other immune cells is more prominent. For instance, after the injection of adenovirus Δ24-RGD in a syngeneic mouse model for glioblastoma (4), macrophages were the immune-cell population that was mostly enriched in the tumor. Alternatively, vesicular stomatitis virus (VSV) administration greatly enhanced the frequency of neutrophils in B16 murine melanoma tumors compared to other immune-cell populations (5). Although all these immune cells can be employed for anticancer therapy using various strategies, the observation that reovirus administration predominantly induces a potent influx of $CD8^+$ T cells in various preclinical tumor models makes reovirus especially attractive to combine with T-cell-based immunotherapy.

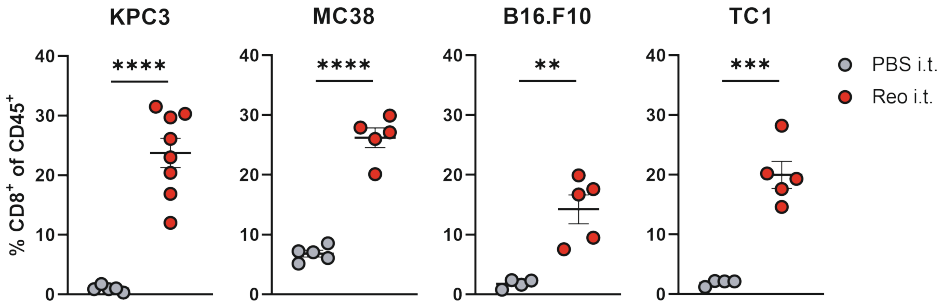

Figure 2. Reovirus administration induces an intratumoral influx of CD8⁺ T cells. Frequency of CD8⁺ T cells out of CD45⁺ immune cells in murine pancreatic KPC3, colon MC38, melanoma B16.F10, and lung TC1 tumors after intratumoral administration of reovirus (10^7 plaque-forming units). Data represent mean±SEM. Significance levels: **p<0.01, ***p<0.001, and ****p<0.0001.

Altogether, our observations demonstrate that in the case of oncolytic reovirus, the exploitation of its immunostimulatory potential should be prioritized over its use as an oncolytic agent. The oncolytic effect of reovirus might be improved by employing a much higher dosage or a modified virus that has more lytic potential, but it is unknown if this is possible without inducing adverse effects. Additionally, employing a higher dose or a more lytic virus might result in faster clearance of the virus by eliciting stronger antiviral immune responses. The fact that potent immunostimulatory effects can already be observed using a relatively low dose of reovirus further advocate that this characteristic of reovirus should be exploited for anticancer therapy.

REOVIRUS THERAPY: MONOTHERAPY OR COMBINATION THERAPY?

Applying OV therapy as monotherapy or in combination with other anticancer modalities is another important question that needs to be answered when considering clinical OV application. As was reviewed in 2020, a large proportion of clinical trials investigating the safety and efficacy of OVs were conducted with OV monotherapy (6).

However, in the case of reovirus, our data and studies by others demonstrated that the efficacy of reovirus as monotherapy is limited (7-9). As such, reovirus has often been used in combination with various other therapeutics to increase its anticancer efficacy (**Figure 3**). Given the immunostimulatory potential of reovirus (See section "Reovirus: oncolysis or immune stimulation"), especially the potential beneficial combination of reovirus with immunotherapeutic strategies warrants extensive investigation.

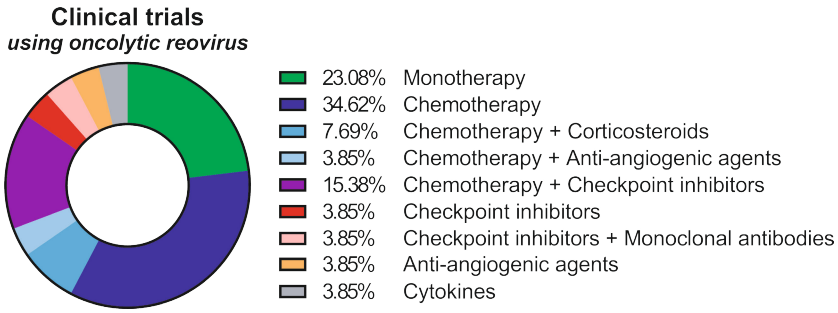

Figure 3. Combination agents used with oncolytic reovirus in clinical trials. A total of 26 clinical trials involving reovirus were assessed for their additional use of other therapeutics. Chemotherapy: reovirus with cyclophosphamide, carboplatin, paclitaxel, or gemcitabine. Chemotherapy + Corticosteroids: reovirus with bortezomib/carfilzomib and dexamethasone. Chemotherapy + Anti-angiogenic agents: reovirus with irinotecan/leucovorin/fluorouracil and bevacizumab (αVEGF). Chemotherapy + Checkpoint inhibitors: reovirus with gemcitabine/irinotecan/leucovorin/fluorouracil/bortezomib/carfilzomib/paclitaxel and pembrolizumab (αPD1)/avelumab (αPD-L1)/nivolumab (αPD-L1). Checkpoint inhibitors: reovirus with retifanlimab (αPD1). Checkpoint inhibitors + monoclonal antibodies: reovirus with atezolizumab (αPD-L1) and trastuzumab (αHER2). Anti-angiogenic agents: reovirus with lenalidomide/pomalidomide. Cytokines: reovirus with sagramostim (recombinant GM-CSF). Data was obtained from clinicaltrials.gov on 15-03-2023.

Reovirus and checkpoint blockade

When aiming to exploit the immunostimulatory properties of oncolytic reovirus, as was the goal of the research described in this thesis, reovirus is often combined with other immunotherapeutic agents. Within the group of immunotherapeutic agents, reovirus is most often combined with immune checkpoint inhibitors, especially those blocking the PD1-PD-L1 axis. Here, binding of PD-L1 (expressed on tumor cells or various immune cells) to PD1 (expressed on T cells) inhibits the effector function of T cells, including tumor-specific T cells. Blocking this pathway to enhance the efficacy of T cells that are primed after OV therapy is a logical choice, since OVs can stimulate the secretion of interferons that upregulate the expression of PD-L1 on tumor cells (10-14). Indeed, we also observed an increase in PD-L1 expression on both tumor cells ($CD45^-$) and immune cells ($CD45^+$) in KPC3 tumors after intratumoral reovirus administration (**Figure 4**).

Although reovirus administration attracts a wave of T cells to the tumor, the presence of these T cells does not affect tumor growth (**Figure 1**). This suggests that these incoming T cells are not tumor-specific. Thus, even though PD-L1 is expressed, the lack of tumor-specific T-cell responses limits the efficacy of checkpoint blockade in these KPC3 tumors (**Chapters 2 and 3**). In other, more immunogenic tumors, the combination of reovirus and checkpoint blockade can be very beneficial. This was also visible in our studies, where we observed that the combination of reovirus and αPD-L1 therapy was very effective in the immunogenic murine MC38 colon tumor model (**Chapter 5**). The efficacy of this combination therapy could even be further improved by TGF-β blockade (**Addendum II**). Other studies have also demonstrated a synergistic effect when reovirus is combined with checkpoint blockade. For instance, in the subcutaneous

B16 melanoma model, intratumoral reovirus administration combined with systemic αPD1 treatment led to a significantly increased survival of mice compared to both agents alone, which was attributed to increased antitumor T-cell responses and abrogation of T_{reg} activity (15). Similar observations were made in the syngeneic EMT6 breast cancer model, where the combination of reovirus and αPD1 increased survival and tumor-specific immune responses, even leading to protection against rechallenge (14). Lastly, in the orthotopic syngeneic GL261 brain tumor model, intravenous reovirus administration significantly enhanced the efficacy of αPD1 therapy (13).

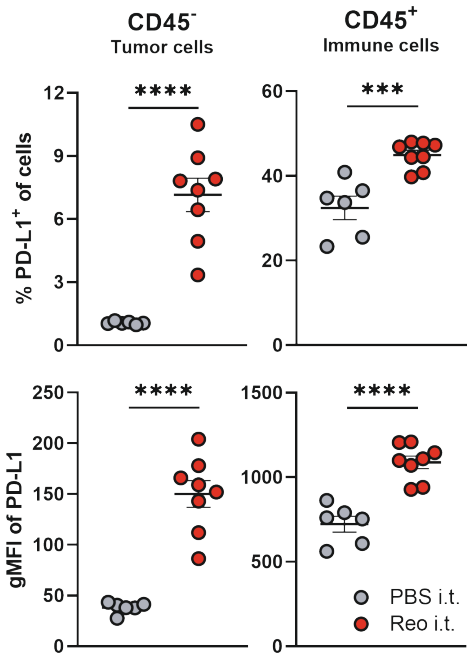

Figure 4. PD-L1 expression in KPC3 tumors after reovirus administration. Intratumoral reovirus administration increases the frequency of PD-L1$^+$ cells, as well as the intensity (gMFI) of PD-L1 expression on both the tumor cell compartment (CD45$^-$) and the immune cell compartment (CD45$^+$) in KPC3 tumors. Significance levels: ***p<0.001 and ****p<0.0001.

Since the lack of tumor-specific T-cell responses, even after reovirus administration, prevents effective combination therapy in non-immunogenic tumors, it is necessary to employ other immunotherapeutic strategies to treat these tumors.

Reovirus and SLP vaccination

In our studies described in **Chapter 2 and 3**, we observed that a significant proportion of the CD8$^+$ T cells that infiltrated into the tumor after reovirus administration was reovirus-specific, and not tumor-specific. Whereas a body of literature has shown that several OVs, including reovirus, can induce tumor-specific T-cell responses via antigenic spread, this seems to be restricted to immunogenic models with high mutational load or expression of tumor-associated or artificial antigens (16-20). Therefore, the exploitation

of virus-specific T cells may represent a solution for targeting low-immunogenic tumors to which tumor-specific responses are more difficult.

In **Chapter 3**, we exploited the ability of reovirus-specific T cells to recognize and kill virus-infected tumor cells. We demonstrated that a synthetic long peptide (SLP) vaccine-induced preinstalled pool of reovirus-specific $CD8^+$ T cells was recruited to the tumor upon intratumoral reovirus administration and effectively delayed tumor growth. Antiviral $CD8^+$ T cells were shown to reside in a range of both murine and human tumors, including melanomas, brain metastases, and endometrial, lung, and colorectal cancers (21-25). In contrast to most of the tumor-specific T cells present, the $CD8^+$ T cells specific for common viral pathogens, such as Cytomegalovirus (CMV), Eppstein-Barr virus (EBV), or Influenza virus exhibited phenotypes more in line with active effector cells, which could be activated upon stimulation with their cognate antigen (21,22). Furthermore, exploiting OV-specific T cells instead of other T cells specific for other viruses adds some sort of tumor-specificity to the system, due to the specific replication of the OV in malignant cells. In this way, only tumor cells are converted into target cells for the previously established OV-specific T cells.

Although this is a promising approach, a lot of steps are necessary before the combination of vaccination and OV therapy can be clinically applied. For instance, reovirus-epitopes for human HLA class I types need to be identified, to allow the specific priming of reovirus-specific T cells and not the induction of reovirus-specific NAb responses that would occur when for instance vaccines would be used that comprise complete reovirus proteins in their original conformation. Alternatively, it would be an option to provide overlapping sequences of reovirus proteins, which circumvents the need to identify reovirus-specific T-cell epitopes. Lastly, besides an SLP, other formats might be considered to deliver reovirus-specific T-cell epitopes, such as the mRNA-containing lipoplex nanoparticles that have recently been used to deliver neoantigens to prime tumor-specific T-cell responses in pancreatic cancer (26).

Reovirus and CD3-bsAbs

In addition to the exploitation of the specificity of reovirus-specific T cells, these T cells can also be employed to target cancer cells by bypassing their specificity. In **Chapter 2**, we made use of CD3-bispecific antibodies (bsAbs) to redirect the recruited reovirus-specific T cells to the tumor and induce tumor-specific killing. Since CD3-bsAbs activate T cells via binding to CD3, the interaction between MHC class I and the T-cell receptor is redundant and any T cell, including virus-specific T cells, can be employed to target tumor cells (27-29). Although T cells induced by vaccination or other strategies might also be employed by CD3-bsAbs (30), we were the first to demonstrate that the treatment of intratumoral reovirus injection followed by systemic CD3-bsAb administration (Reo&CD3-bsAbs) resulted in the fast regression of local and distant tumors. This effect was dependent on the expression of the targeted tumor antigen on tumor cells. Therefore, for the clinical application of reovirus and CD3-bsAbs, the

selection of the appropriate tumor antigen is of utmost importance. For effective bsAb therapy in humans, the ideal target antigen needs to be selectively and abundantly expressed on tumor cells but should also be essential for tumorigenesis, to avoid downregulation or immunologic selection for tumor cells without expression of the antigen. Although the ideal target for PDAC has not been identified, a plethora of tumor antigens is currently being evaluated (30). Additionally, various OV-CD3-bsAb platforms are extensively investigated in preclinical studies (31). Based on our data, we argue for a fast translation of this highly promising immunotherapeutic combination to the clinic.

In **Chapter 7**, we investigated whether the efficacy of Reo&CD3-bsAb therapy could be further improved by TGF-β blockade. As is reviewed in **Chapter 6**, TGF-β is one of the most potent and pleiotropic regulatory cytokines and is involved in almost all stages of tumor growth, including initiation, progression, and spread (32). TGF-β signaling influences virtually all innate and adaptive immune cells, which includes the stimulation of inhibitory regulatory T cells and the inhibition of cytotoxic $CD8^+$ T cells (33). Additionally, TGF-β plays a role in the exclusion of T cells from the tumor beds. Thus, in the context of cancer, these pleiotropic functions of TGF-β make it an interesting, but complex, target for therapy.

This complexity was also visible in our studies, since TGF-β blockade antagonized Reo&CD3-bsAb combination therapy in KPC3 tumors but enhanced the percentage of complete responses to this therapy from 50% to 100% in MC38 tumors. This demonstrates that intertumoral differences can determine whether TGF-β blockade improves or impairs the efficacy of (viro)-immunotherapeutic strategies, and an increased understanding of these intertumoral differences is required to predict which individuals would most likely benefit from TGF-β neutralization as an addition to Reo&CD3-bsAb therapy.

Reovirus and other forms of anticancer therapy

Besides the combination with immunotherapeutic strategies, reovirus is also often combined with chemotherapeutic agents (**Figure 3**), and synergistic effects were sometimes observed. This enhanced treatment efficacy might be mostly attributed to increased tumor cell death. For instance, the treatment of prostate cancer cell lines with reovirus combined with various chemotherapeutic agents led to increased cell death compared to both agents alone (34). Similarly, combined treatment of reovirus and docetaxel demonstrated superior antitumor efficacy in subcutaneous human prostate PC3 tumors engrafted in nude mice (34). Others have demonstrated the improved efficacy of reovirus therapy after combination with cisplatin, gemcitabine, vinblastine, and/or paclitaxel in the murine melanoma B16.F10 model (35), various non-small cell lung cancer cell lines (36) and the Cal27 tumor model for head and neck cancer (37). The combination of reovirus and chemotherapy was demonstrated to be safe in multiple clinical trials (38,39) and demonstrated antitumor responses in a Phase II study in patients with head and neck cancer (40). Combined, these data suggest that the

addition of chemotherapy might be mostly beneficial to enhance intratumoral cell death. However, since chemotherapeutic drugs have also demonstrated immunostimulatory potential (41,42), an interesting avenue for further research might be to investigate whether a combination of reovirus and chemotherapeutic drugs could not only lead to lead to enhanced oncolysis, but also to enhanced immune stimulation.

In conclusion, the research described in this thesis advocates for applying reovirus as part of a combinatorial approach, and not as monotherapy. However, exploitation of the immunostimulatory potential of reovirus requires careful evaluation of the immune phenotype of tumors to determine which immunotherapeutic strategy will induce optimal results when combined with reovirus. Although the field of cancer immunotherapy, including OV research, is predominantly focused on and might prefer the induction of potent endogenous tumor-specific T-cell responses, we demonstrated that virus-specific T cells can also be very useful to target tumor cells. Especially for low-immunogenic tumors where endogenous tumor-specific T cells are lacking, I propose that combining reovirus with CD3-bsAbs might lead to better anticancer efficacy compared to the commonly used checkpoint inhibitors. Alternatively, combining OVs with the adoptive transfer of *ex vivo* cultured tumor-specific T cells might be promising, where OV treatment can induce a local chemokine gradient to facilitate the recruitment and trafficking of these transferred T cells to the tumor and increase their antitumor efficacy (43-48). Lastly, further investigation into intertumoral differences is required to assess the factor, process, or cell type that determines whether TGF-β blockade will be beneficial for the efficacy of combined reovirus and T-cell-based immunotherapeutic strategies.

ROUTE OF ADMINISTRATION: LOCAL OR SYSTEMIC DELIVERY?

Another important outstanding question for OV therapy is the choice of administration route. Local delivery of OVs is clinical practice for the FDA/EMA-approved OV T-VEC (49,50) and is used in many preclinical studies, including the majority of studies described in this thesis, and ensures efficient delivery to the tumor site. However, in a large number of clinical studies, reovirus and other OVs are administered intravenously (6,51,52). A major and clinically-relevant advantage of intravenous delivery is that it does not rely on injectable tumor lesions, which are often lacking in the majority of cancer types. Additionally, multiple lesions can be targeted at once by systemic administration.

The effect of the administration route on OV delivery into tumors

Consideration of the route of OV administration is mostly focused on the efficient delivery of the OV itself to the tumor site (6,53). We observed that intravenous administration of reovirus resulted in very limited viral presence in tumors compared to intratumoral administration, even when a 10-fold higher dose of virus is used for

the infusion (**Chapters 2 and 3**). Similar observations were made in immunodeficient mice bearing human pancreatic BxPC3 tumors, where intratumoral administration of oncolytic Newcastle disease virus (NDV) led to the detection of viral RNA in 4 out of 6 tumors, while intravenous administration of NDV in the same dosage led to the detection of viral RNA in only 1 out of 6 tumors (54). This is most likely explained by the fact that systemic delivery results in the spread of the OV throughout the body, leaving fewer infectious particles available to infect tumor cells when compared to direct, intratumoral administration.

The low OV detection in tumors after intravenous administration is also observed in clinical studies. For instance, in a Phase I dose escalation study with an oncolytic vaccinia virus, evidence of viral infection in the tumor 8 days after intravenous administration could only be observed in 2 out of 8 tumor biopsies (55). Similarly, in a Phase I dose escalation study with an oncolytic adenovirus in patients with cutaneous and uveal melanoma, evidence of virus genomic particles in tumors could be detected in 4 out of 7 patients (56). Lastly, in a Phase I study with oncolytic reovirus in 9 patients with brain tumors, immunohistochemistry analysis revealed the presence of reovirus σ3 protein in 6 out of 9 patients, but in very low levels (13). Although these studies and others indicate that intravenous OV delivery is safe and well-tolerated, the detection of high titers at tumor sites is not yet demonstrated and might contribute to the moderate clinical responses observed after intravenous OV therapy. Future research should also reveal whether increased delivery of reovirus to tumors will result in increased antitumor responses and improved survival. These parameters might be correlated, since in a Phase II study investigating intravenous delivery of reovirus to 13 patients with metastatic melanoma, reovirus could be detected in tumor biopsies of only 2 patients, whom both displayed a longer progression-free survival (80 and 87 days) compared to the median survival of 45 days (57).

The effect of the administration route on the OV-induced immune response

Although efficient delivery of the OV itself is currently the main focus, the OV-induced immune response might be a more appropriate parameter to investigate, especially in the context of combining OV administration with T-cell-based immunotherapy (see also the section 'Reovirus therapy: monotherapy or combination therapy?'). In our studies, we observed that priming of reovirus-specific T cells does not depend on a specific route of administration (**Chapter 3**). In fact, reovirus infection of tumors was not even required to mount a potent systemic reovirus-specific T-cell response, suggesting that uptake of a virus particle by an antigen-presenting cell, without specific replication in tumors, is already sufficient for the priming of reovirus-specific $CD8^+$ T cells. But, although priming of reovirus-specific T cells was similar between injection methods, intratumoral administration induced more efficient trafficking of (reovirus-specific) $CD8^+$ T cells to the tumor, presumably due to increased expression of T-cell-attracting chemokines *Cxcl9* and *Cxcl10* and other ISGs (**Chapter 3**).

Although T-cell influx in tumors might be lower after intravenous OV administration compared to intratumoral administration, this may not necessarily result in lower efficacy of OV and T-cell-based immunotherapy. For example, we observed that intravenous reovirus administration followed by CD3-bsAbs was able to induce potent tumor regressions and significantly improve survival in both KPC3 (**Chapters 2, 5, and 7**) and MC38 (**Chapter 7**) tumor models. Direct comparisons between therapeutic outcomes of viro-immunotherapeutic strategies after intratumoral or intravenous OV administration are lacking, but effective antitumor responses have been observed after intravenous reovirus administration combined with αPD1 therapy in a murine brain tumor model (13), with an intravenously-administered vaccinia virus in combination with αPD1 therapy in murine pancreatic neuroendocrine tumors and metastases (58) and after intravenous administration of oncolytic alphavirus M1 in combination with αPD-L1 in murine melanoma B16.F10 and murine prostate RM-1 tumors (59). Thus, effective combination therapy is feasible when OVs are administered intravenously.

Combined, we demonstrated that the infection of tumors by reovirus is related to the route of administration, with intratumoral reovirus administration resulting in greater infection of tumors compared to intravenous administration. However, intratumoral administration is not required for effective combination therapy, since intravenously administered OVs are also capable to sensitize tumors for T-cell-based immunotherapy. Interestingly, reovirus infection is not restrained to the injected tumor site. This is evidenced in our studies where reovirus was administered to only one tumor in mice with bilateral tumors, we were also able to detect virus and T cells in the non-injected, distant tumor (**Chapter 2**). Similarly, the addition of CD3-bsAbs resulted also in potent antitumor responses in these tumors, even though they were not intratumorally injected with reovirus. Possibly, reovirus itself can migrate from one tumor to the next. However, since we also observed low levels of reovirus in the tumor-draining lymph node, it is also possible that reovirus migrated to the distant tumor by associating with immune cells, as has been observed before (60,61). These observations provide interesting avenues for further research. Altogether, our data suggest that combined reovirus and T-cell-based immunotherapy can result in effective antitumor responses after both intratumoral and intravenous reovirus administration, and even in the context of metastatic disease.

PREEXISTING IMMUNITY: BARRIER OR BRIDGE FOR EFFECTIVE THERAPY?

Another outstanding question that is closely related to determining the optimal route of OV administration, is whether preexisting immunity against an OV influences the efficacy of OV therapy. This is especially relevant in the context of systemic administration, where OVs might be more susceptible to clearance by preexisting neutralizing antibodies compared to local, intratumoral administration.

In **Chapter 4**, we summarized the current literature regarding the effect of preexisting immunity on both the OV infection and replication, as well as the OV-induced immune response. Preexisting immunity, especially in the form of neutralizing antibodies (NAbs), is prevalent against several viruses that besides their application as OVs, also circulate in the human population or are used as vectors for vaccination. These include Adenovirus serotype 5 (Ad5) (62,63), Herpes simplex virus type 1 (HSV-1) (64,65), Vaccinia virus (55,66,67), and reovirus (7,9,38,39,68-70). For Ad5 and HSV-1, their capacity to infect cells in order to replicate is impaired in preexposed animals (71,72). This illustrates the importance of investigating the possible effects of preexisting immunity on the efficacy of reovirus therapy.

The influence of (preexisting) NAbs on the efficacy of reovirus as an oncolytic agent

In **Chapter 5**, we confirmed that the majority of human cancer patients also present with circulating NAbs against reovirus. Therefore, we preexposed mice to reovirus to induce high levels of circulating NAbs, and observed that viral infection was significantly impaired in preexposed mice. NAbs also counteracted reovirus-mediated control of tumor growth, since the antitumor efficacy of reovirus was much improved in mice that could not produce NAbs, and again reduced in these mice upon the transfer of NAbs. Thus, NAbs hamper the effective use of reovirus as an oncolytic agent.

NAbs ensure fast removal of infectious reovirus particles and thus likely prevent a large proportion of reovirus particles to reach the tumor and exert their oncolytic effects. Since the majority of the human population has circulating reovirus-specific NAbs, this might explain why reovirus monotherapy has not yet reached optimal efficacy in prior clinical studies (7,73). Various strategies can be employed to enhance reovirus infection and the efficacy of reovirus therapy, for instance combining reovirus with chemotherapeutic agents that can ablate the production of NAbs upon reovirus exposure (74,75), or depletion of $CD4^+$ T cells (**Addendum I**). However, chemotherapy and $CD4^+$ T-cell depletion cannot eliminate preexisting Nabs, thus this may only be successfully employed in reovirus-naive individuals. Alternatively, an option might be to load reovirus on immune cells, such as T cells, dendritic cells, or monocytes before administration, to shield the virus and prevent NAb-mediated clearance (60,76,77).

The effect of (preexisting) NAbs on reovirus efficacy as a T-cell-attracting agent

Although the presence of preexisting NAbs hampers the use of reovirus as an oncolytic agent, the reovirus-induced influx of T cells was surprisingly not affected in preexposed mice (**Chapter 5**). A similar observation was made in a study where immunocompetent naive or NDV-exposed B16.F10-bearing C57BL/6J mice were intratumorally injected with NDV (78). While viral replication was decreased in preexposed mice, the intratumoral influx of $CD8^+$ T cells was comparable between naive and preexposed animals. These observations illustrate that the presence of reovirus in a tumor might not directly correlate with the presence of T cells.

It is commonly accepted that the reovirus-induced expression of ISGs is responsible for the attraction of T cells to the tumor. However, in the presence of NAbs, the reovirus-induced expression of ISGs was impaired, but a remaining moderate expression of some ISGs, including T-cell-attracting chemokine *Cxcl9*, was still observed. We hypothesize that this moderate expression might already have been sufficient to attract T cells to the tumor. Alternatively, it is possible that the administration of reovirus to preexposed mice did not completely preclude effective viral infection and ISG expression, but that this response was already quenched by NAbs at the moment of analysis. Additionally, the presence of the virus itself, the expression of ISGs, and the influx of T cells may differ kinetically in preexposed mice compared to naive mice.

Lastly, various studies comparing the proteome and the immunopeptidome note that there is a limited correlation between the presence of viral antigens and the presentation of epitopes (79,80). Thus, even though the presence of NAbs significantly decreases the number of genomic copies of reovirus, this might not preclude the presentation of reovirus-epitopes in MHC-I and the presence of reovirus-specific T cells in the tumor. Further studies to directly compare the kinetics of reovirus presence in the tumor, the expression of ISGs, as well as the intratumoral influx of T cells in naive versus reovirus-preexposed mice could hopefully answer these remaining questions.

Additionally, it would be interesting to investigate why the presence of NAbs shifts the specificity of reovirus-specific T cells from being specific for the $\mu1_{133\text{-}140}$ epitope to being specific for the $\mu1_{422\text{-}430}$ epitope. Since the reovirus-specific NAbs are also directed towards the reovirus μ1 protein, it is possible that reovirus particles bound to NAbs are processed differently, leading to the presentation of other epitopes on the surface of infected cells (81). Although we demonstrated in **Chapter 5** that these $\mu1_{422\text{-}430}$-specific T cells could still be employed by CD3-bsAbs, it would be interesting to investigate if inducing a preexisting pool of T cells with this specificity by SLP vaccination would also lead to impaired tumor growth upon intratumoral reovirus administration.

Do preexisting T cells influence the anticancer efficacy of reovirus therapy?

In parallel, the question was raised whether the presence of reovirus-specific T cells impairs the efficacy of reovirus therapy. Interestingly, the work presented in **Chapter 3** demonstrated that the preexisting presence of a large pool of reovirus-specific T cells enhanced the antitumor efficacy of reovirus monotherapy, without impairing reovirus infection in the tumor. Since we also demonstrated that depletion of $CD8^+$ T cells did not improve reovirus infection (**Chapter 5**), our data suggest that $CD8^+$ T cells are not involved in the clearance of reovirus.

Since mice were vaccinated with a reovirus-specific $CD8^+$ T-cell epitope-containing SLP before intratumoral reovirus administration, we did not induce preexisting reovirus-specific NAbs that could counteract viral infection and ISG expression upon subsequent therapy. Installing this pool of virus-specific T cells prior to intratumoral reovirus

administration resulted in a faster and bigger influx of T cells that were mostly reovirus-specific and caused a delay in tumor growth (**Chapter 3**). Thus, we demonstrated for the first time that the presence of preexisting reovirus-specific T cells does not impair, but instead improves the efficacy of reovirus monotherapy. For future experiments, it would be interesting to investigate whether having this preinduced pool of reovirus-specific T cells could also lead to enhanced efficacy of Reo&CD3-bsAb therapy.

Altogether, we concluded that the presence of NAbs prevents the use of reovirus as an oncolytic agent, but not its T-cell-attracting capacities. Therefore, reovirus can still be employed for effective combination therapy with T-cell-based immunotherapy. This is very promising for the clinical application of reovirus, where patients presenting with high levels of preexisting NAbs might not be eligible for effective reovirus as monotherapy but could still be susceptible to a combinatorial strategy comprising reovirus and T-cell-based immunotherapy. Additionally, we delivered conceptual evidence that taking advantage of a (preexisting) virus-specific immune cell population provides an exciting new approach in the cancer immunotherapy field.

CONCLUDING REMARKS

In this thesis, we unraveled the immunostimulatory potential of oncolytic reovirus and investigated how these immunostimulatory characteristics could be exploited for effective anticancer immunotherapy.

In summary (**Figure 5**), we demonstrated that administration of oncolytic reovirus does not lead to strong oncolytic effects in tumors (**1**), but instead unleashes a very potent immune response, including the priming of reovirus-specific $CD8^+$ T cells (**2**). For the first time, we showed that these reovirus-specific $CD8^+$ T cells can be employed for anticancer immunotherapy (**3**), by either bypassing their specificity (with CD3-bsAbs) or by exploiting their specificity (via installing a preinduced pool using SLP vaccination). Besides the induction of reovirus-specific $CD8^+$ T cells, reovirus administration also leads to very fast B-cell responses. We are the first to demonstrate that the presence of neutralizing antibodies (NAbs) restricts the use of reovirus as an oncolytic agent (**4**), but that the reovirus-induced influx of $CD8^+$ T cells is retained and the use of reovirus in combination with T-cell-based immunotherapy can still result in potent antitumor responses. Lastly, we showed that blockade of TGF-β does not impair reovirus infection and reovirus-induced expression ISG expression (**5**) or the reovirus-induced attraction and activation of T cells (**6**), but that intrinsic differences between preclinical tumor models can determine whether TGF-β blockade is a beneficial addition to combined reovirus and T-cell-based immunotherapy.

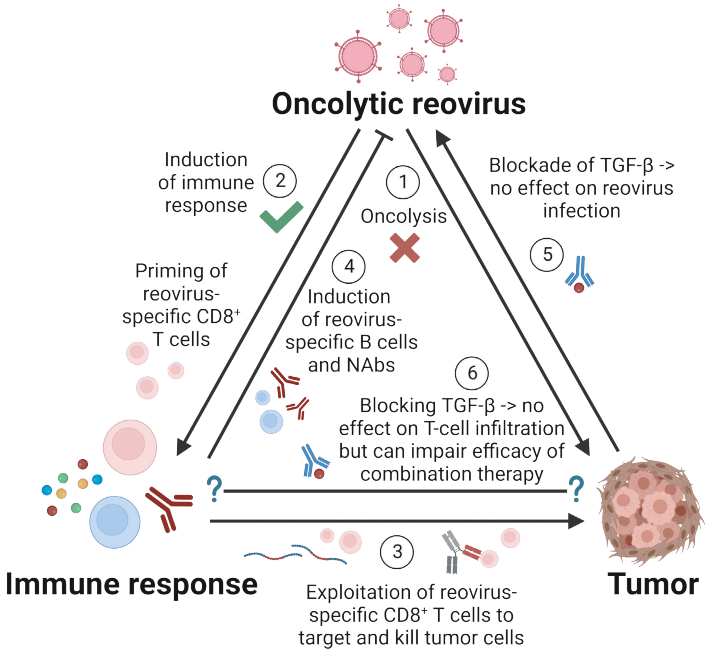

Figure 5. Harnessing the immunostimulatory potential of oncolytic reovirus for anticancer immunotherapy. Reovirus administration does not induce strong oncolysis (**1**) but unleashes a potent immune response (**2**). The reovirus-induced influx of T cells CD8$^+$ T cells can be exploited for anticancer immunotherapy, even if they are reovirus-specific (**3**). Reovirus-specific B-cell responses hamper the use of reovirus as an oncolytic agent, but not its T-cell-attracting ability (**4**). Blockade of TGF-β does not affect reovirus infection (**5**) or the reovirus-induced immune response (**6**), but intertumoral differences dictate whether TGF-β improves or impairs the efficacy of reovirus and T-cell-based immunotherapy.

Although we extensively investigated the use of oncolytic reovirus as an immunostimulatory agent to increase the efficacy of T-cell-based anticancer immunotherapy, there still are some fundamental questions that remained unanswered. For instance, we observed that CD4$^+$ T-cell depletion completely abrogates NAb production and improves the antitumor efficacy of reovirus. However, it is highly puzzling why these mice don't present with viremia and weight loss, in contrast to B-cell deficient mice that also don't have NAbs but do succumb to reovirus-induced pathology. Future studies should investigate the immunological processes underlying these observations. Additionally, we do not know why and how the presence of NAbs induces a shift in the specificity of reovirus-specific CD8$^+$ T cells that are present in the tumor, as well as the implications of this shift in specificity. We did observe that these T cells can still be employed by CD3-bsAbs, but it would be interesting to investigate whether preinstalling a pool of these 'other' T cells by SLP vaccination also leads to delayed tumor outgrowth upon intratumoral reovirus administration. Lastly, it is of utmost importance to identify the factor(s), mechanism(s), or cell type(s) that determine whether TGF-β blockade provides a benefit to the efficacy of reovirus and T-cell-based combination therapy.

Besides answering fundamental questions, a few topics need to be further investigated regarding the clinical translation of our observations. For example, to be able to exploit our novel concept of installing a preexisting T-cell pool that enhances reovirus efficacy in the clinic, prior identification of the human reovirus epitopes is needed. Subsequently, we need to determine which vaccination strategy would be most effective in inducing reovirus-specific $CD8^+$ T cells. Future research should also hunt for an appropriate tumor antigen that can be used for targeting by CD3-bsAbs. The question arises whether there is an appropriate tumor antigen that is expressed by multiple tumor types, or if tumor-specific (or even patient-specific) identification is necessary. Furthermore, it would be highly beneficial to identify or design a safe and non-invasive way to remove NAbs in seropositive patients and/or prevent NAb responses in seronegative patients, to further increase the efficacy of reovirus and T-cell-based combination therapy.

Altogether, the data accumulated in this thesis provides an increased understanding and new insights regarding the use of oncolytic reovirus for anticancer therapy. The collected data described here should prove instructive for future decisions regarding both fundamental investigations as well as the therapeutic application of oncolytic reovirus, and may ultimately contribute to more effective viro-immunotherapy for patients.

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