Harnessing the immunostimulatory properties of oncolytic reovirus for anticancer immunotherapy

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CHAPTER 1

General Introduction

THE IMMUNE SYSTEM AS A DEFENSE AGAINST PATHOGENS

The immune system protects the body against invading microorganisms. Invaders, such as viruses and bacteria, can enter the body from the outside after breaching mucosal layers. Two branches of the immune system can be distinguished, the innate and the adaptive arms. As the first line of defense, the innate immune response is initiated immediately after pathogen-specific structures are recognized by pattern-recognizing receptors such as Toll-like receptors (TLRs) or retinoic acid-inducible gene 1 (RIG-1)-like receptors (1). One important function of innate immunity is the rapid recruitment of phagocytic immune cells such as neutrophils and macrophages to sites of infection through the production of cytokines and chemokines, to quickly eliminate the pathogen. Additionally, infected cells produce interferon molecules that initiate signaling cascades to enhance pathogen detection and restrict pathogen replication (2). Innate immunity responds relatively similar to different pathogens, which is why it is considered a non-specific immune response. In contrast, the adaptive immune response is highly antigen-specific and relies on the presence of specific receptors on immune cells derived from the thymus (T cells) or the bone marrow (B cells). The adaptive immune response is initiated by antigen-presenting cells (APCs), especially dendritic cells, that process and present pathogen-derived antigens to T cells. $CD4^+$ T cells play a key role in coordinating immune responses, including the stimulation of $CD8^+$ T cells and B cells. Whereas $CD8^+$ T cells engage in the eradication of intracellular pathogens via interactions between the T-cell receptor (TCR) and foreign peptides presented in major histocompatibility class I molecules (MHC-I), a B-cell response involves the production of antibodies that promote phagocytosis of the pathogen and prohibit infection (**Figure 1**).

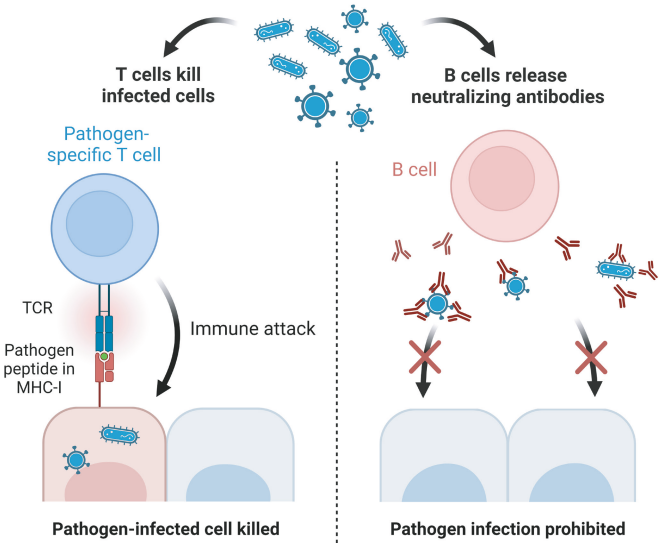

Figure 1. Adaptive immune responses upon exposure to pathogens. $CD8^+$ T cells are primed to recognize pathogen-derived peptides in MHC-I molecules on the surface of infected cells, leading to T-cell mediated killing of these infected cells. Activated B cells produce neutralizing antibodies, which bind to the pathogen and prevent the infection of cells.

EMPLOYING THE IMMUNE SYSTEM IN THE BATTLE AGAINST CANCER

The immune system not only mounts protective immune responses when it recognizes 'non-self' pathogens, it also can employ the same mechanisms to fight cancer. Although malignant cells are more similar to the host than pathogens, they still differ genetically from normal cells and, therefore, can be recognized by the immune system. Mutations in tumor cells might also give rise to the specific recognition of tumor cells by immune cells, a trait called immunogenicity. The potential of the immune system to recognize and control cancer is exploited in what is known as cancer immunotherapy. A big advantage of immunotherapy is that the tumor-specific immune response can eliminate a primary tumor, and established immune memory could also prevent cancer from recurring.

The therapeutic activation of tumor-specific T cells is especially promising due to their ability to selectively recognize and kill tumor cells. Indeed, multiple T-cell-based immunotherapeutic strategies have revolutionized the treatment of cancer. An example of T-cell-based immunotherapy is the use of immune checkpoint inhibitors, where inhibitory signals on T cells or their ligand on tumor cells are blocked to reactivate dysfunctional tumor-specific T cells (3). Another example is adoptive T-cell therapy (ACT), where tumor-specific T cells are isolated and expanded *ex vivo* before re-administration to the patient (4). Additionally, cancer vaccines, including synthetic peptides, viral vectors, and DNA or RNA vectors aim to enhance the frequency of tumor-specific T cells for enhanced eradication of the tumor (5). Lastly, the use of agonistic monoclonal antibodies is emerging, which aims to improve tumor-specific T-cell responses by targeting co-stimulatory molecules (6).

Although the above-mentioned T-cell-based immunotherapeutic strategies have demonstrated remarkable clinical responses in the treatment of solid tumors, there is much room for improvement. Successful responses to T-cell-based immuno-therapeutic interventions in solid tumors mostly occur in patients where tumors have an immune-infiltrated tumor phenotype, which displays a preexisting but often dysfunctional immune response (**Figure 2**) (7,8). However, a large proportion of solid tumors does not have an immune-infiltrated phenotype, but an immune-silent phenotype. These tumors are much less susceptible to T-cell-based immunotherapeutic strategies (9). Various factors contribute to this decreased susceptibility, for instance, a lack of antigens that can be targeted due to lower immunogenicity and/or lower mutational burden, an absence of tumor-specific T-cell priming or activation, or impaired trafficking and infiltration of tumor-specific T cells into the tumor beds (10-13). An example of a tumor type with an immune-silent phenotype is pancreatic cancer, which often responds poorly to various immunotherapeutic strategies (14). The lack of effective responses to immunotherapy in this type of tumor is a huge unmet need. Therefore, strategies to transform the immune-silent phenotype of unresponsive solid tumors

to an immune-infiltrated phenotype are desperately needed to enhance the efficacy of T-cell-based immunotherapeutic strategies. In other words, we need to find ways to increase the frequency of T cells in solid tumors to enhance the efficacy of T-cell-based immunotherapy.

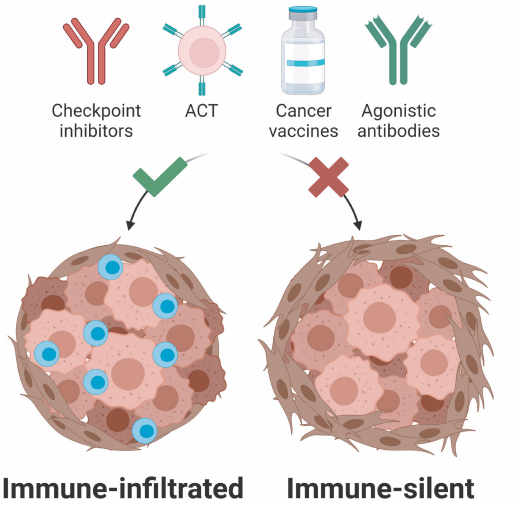

Figure 2. Immunophenotype of solid tumors determines response to T-cell-based immunotherapy. In immune-infiltrated tumors, T cells (blue) can migrate through the stromal regions (brown) into the tumor nests (red) but are often dysfunctional. Immunotherapeutic strategies such as checkpoint inhibition, adoptive cell transfer (ACT), vaccination and the use of agonistic antibodies can be effective. These therapies are less effective or non-effective in immune-silent tumors where intratumoral T cells are absent.

ONCOLYTIC VIRUSES AS IMMUNOSTIMULATORY AGENTS

One promising strategy to increase intratumoral T-cell density is treatment with oncolytic viruses (OVs) (15). The use of OVs as anticancer agents is emerging and inspired by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval of talimogene laherparepvec (T-Vec) in 2015. T-vec is a herpes simplex virus type 1 (HSV-1) encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) that increased survival and demonstrated favorable tolerability in advanced-stage melanoma patients (16). Besides T-vec, two other OVs have been approved globally for the treatment of cancer; a picornavirus named Rigvir was approved in 2004 for the treatment of melanoma in Latvia, and in China the use of a genetically modified adenovirus named H101 was approved in 2005 for the treatment of nasopharyngeal carcinoma in combination with chemotherapy. Currently, there is an immense pipeline of over 200 registered clinical trials investigating the therapeutic application of various OVs as single agents or as part of combination therapies (17).

OVs can demonstrate anticancer activity by their preferential replication in transformed cells, either as a natural characteristic or after genetic modification. Continued replication of the OV in malignant cells can eventually induce oncolysis, which might impair tumor outgrowth. Besides their oncolytic function, accumulating (pre)clinical evidence suggests that OVs might elicit a stronger antitumor effect through their capacity to function as immunostimulatory agents. Indeed, the percentage of scientific publications containing the search terms 'oncolytic virus' (OV) and 'immune response' (IR) increases yearly (**Figure 3**).

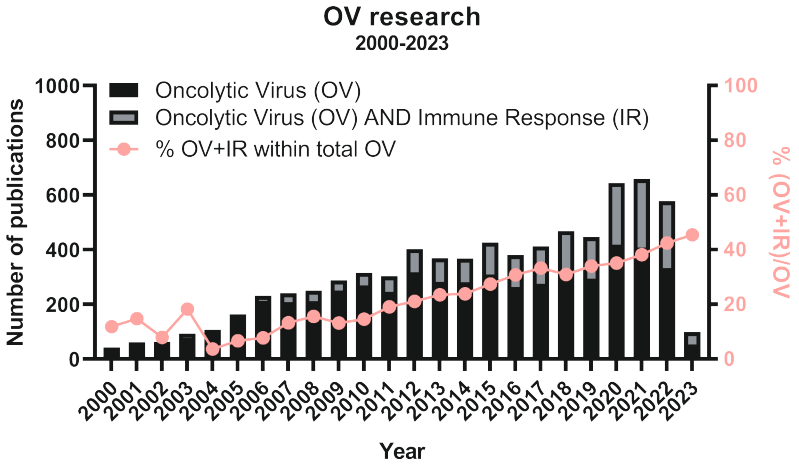

Figure 3. Number of publications focusing on immunostimulatory properties of OVs since 2000. Number of publications from the years 2000-2023 containing the search term 'Oncolytic Viruses' (OV), either alone or in combination with search term 'Immune Response' (IR). Right y-axis depicts percentage of OV+IR papers within total number of OV publications. Data obtained from PubMed Central® on 23-02-2023.

The idea to use OVs as immunostimulatory agents for anticancer therapy is supported by observations that OV treatment could induce an environment that is particularly favorable for the priming of T cells (**Figure 4**). For instance, the release of virus-derived nucleic acids, pathogen-associated molecular patterns (PAMPs), and/or damage-associated molecular patterns (DAMPs) during OV infection optimally induce the maturation of dendritic cells (18,19). Simultaneously, dying tumor cells are a source of virus and tumor antigens, leading to the priming of tumor- and virus-specific $CD4^+$ and $CD8^+$ T-cell responses (20,21). This process is further enhanced by the OV-induced interferon (IFN) response, which involves the release of T-cell-attracting chemokines, promotes antigen presentation, and recruits immune cells to the tumor (22). Thus, the OV-induced tumor-reactive immune response is believed to be not only a crucial aspect of the therapeutic efficacy of OVs themselves (23,24) but may also be utilized to sensitize tumors for other types of immunotherapy by enhancing immunogenicity or by attracting activated $CD4^+$ and $CD8^+$ T cells to nonresponsive tumors (15,25).

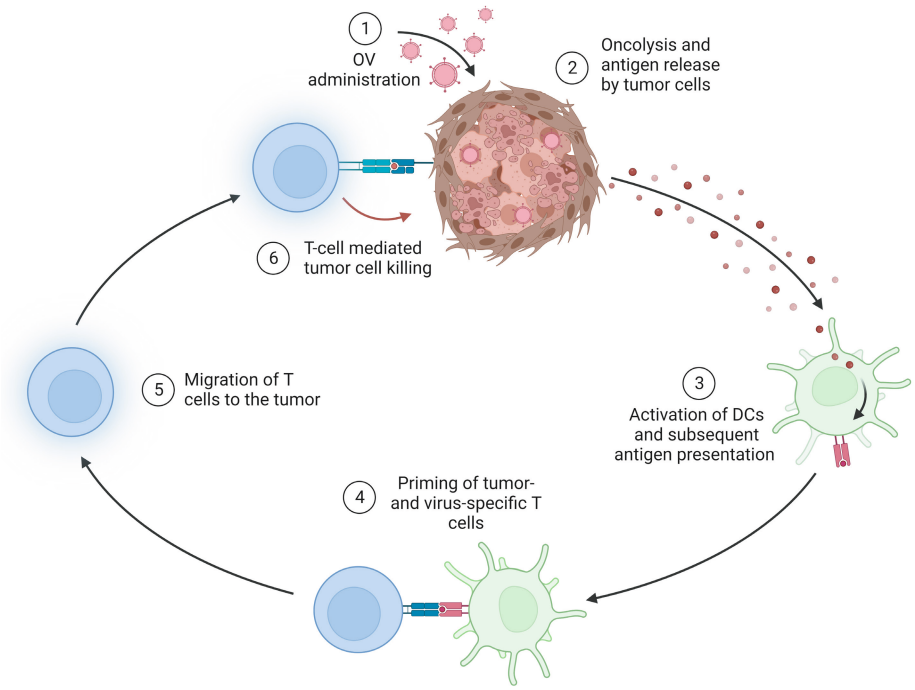

Figure 4. Model of the immunostimulatory properties of OVs. OV replication causes oncolysis, which can induce the release of tumor-specific and virus-specific antigens. The subsequent uptake and presentation of these antigens by dendritic cells (DCs) leads to the induction of tumor- and virus-specific T cells. OV infection and replication also can induce a type I interferon (IFN) response that causes the release of T cell-attracting chemokines. The tumor- and virus-specific T cells are attracted by these chemokines and migrate towards the tumor to exert their function.

ONCOLYTIC REOVIRUS

Various viruses can demonstrate oncolytic and immunostimulatory potential, including Vaccinia virus, HSV-1, and Adenovirus (26,27). In particular, the mammalian reovirus type 3 Dearing strain (T3D) is one of the leading OVs under clinical evaluation and displays an excellent safety record in clinical trials (28,29). Previously known as Reolysin®, reovirus type 3 Dearing is currently manufactured as pelareorep for therapeutic anticancer application by Canadian company Oncolytics Biotech Inc.

Reoviruses are non-enveloped, double-stranded (ds) RNA viruses. The outer and inner capsids protect its genome, which consists of 10 dsRNA segments termed large (L1-3), medium (M1-3), or small (S1-4) (30). These gene segments encode 8 structural proteins (λ1-3, μ1-2, and σ1-3) and 2 non-structural proteins, μNS and σNS (**Figure 5**). Reoviruses were first isolated in the 1950s from pediatric stool samples and were termed reovirus (respiratory enteric orphan virus), since at the time of discovery reoviruses were not associated with any known disease. In most individuals, reovirus infection occurs asymptomatically during childhood, confirming its classification as an orphan virus.

Besides T3D, two other reovirus serotypes circulate in humans, named serotype 1 Lang (T1L) and serotype 2 Jones (T2J).

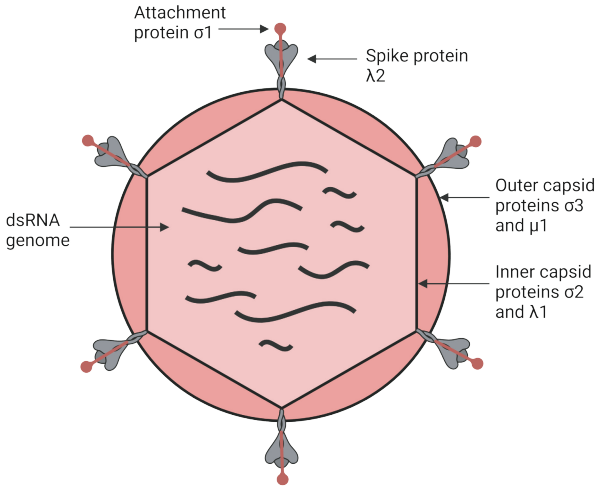

Figure 5. Schematic representation of mammalian reovirus Type 3 Dearing. Depicted are the locations of reovirus proteins and its double stranded RNA (dsRNA) genome.

Reovirus T3D (hereafter named reovirus) shows an inherent preference for replication in malignant but not healthy cells (31,32). Early, pivotal studies demonstrated that high activity of the intracellular Ras signaling pathway allowed for efficient reovirus replication (33). The Ras signaling pathway is upregulated in many cancer types, often due to an activating oncogenic mutation, where it regulates various cellular processes such as cell proliferation and survival (34). Upon infection with reovirus, healthy cells activate their natural defenses upon recognition of the double-stranded RNA structures, which includes the phosphorylation of double-stranded-RNA-dependent protein kinase (PKR). Phosphorylation of PKR leads to phosphorylation of eukaryotic initiation factor 2α, which can inhibit the translation of viral genes (35). However, in cancer cells with a highly active RAS pathway, the phosphorylation of PKR is inhibited, and viral translation is not prohibited. Besides an activated Ras pathway, other factors might also contribute to the preferential replication of reovirus in tumor cells, such as the increased expression of the reovirus entry-receptor junctional adhesion molecule A (36-38) or cellular proteases such as cathepsin B and L (39) which allow efficient viral uncoating and thus replication in tumor cells. Ultimately, sensitivity to reovirus-induced oncolysis is likely to be dependent on multiple cellular and molecular determinants, many of which still need to be uncovered.

The preferential replication of reovirus in tumor cells, combined with its non-pathogenic nature in humans, makes reovirus attractive to use as an oncolytic agent for anticancer therapy. Already in 1998, Coffey et al demonstrated that a single intratumoral injection of reovirus could result in the regression of established NIH 3T3 tumors or human U87 glioblastoma tumors in 80% of severe combined immunodeficient mice (40).

Other preclinical studies demonstrated that reovirus could induce regressions of subcutaneous and orthotopic gliomas (41), as well as colorectal C26 liver metastases (42) or prostrate xenograft tumors (43). But, although reovirus has demonstrated some tumor regressions as a monotherapy in certain cancer types, results from clinical trials all point toward a growing consensus that reovirus is unlikely to have sufficient clinical efficacy as a single agent. Various aspects might contribute to the limited efficacy of oncolytic reovirus as monotherapy, such as a limited oncolytic potential of the virus itself or the large heterogeneity of cells within tumors, including virus-resistant cell populations. In addition, early investigations suggested that the presence of neutralizing antibodies (NAbs) in patients, either preexisting or induced upon therapy, might limit the therapeutic efficacy of reovirus, especially when reovirus is administered intravenously (44). However, knowledge regarding the effect of (preexisting) NAbs on the efficacy of reovirus therapy remains underexplored.

Instead of applying reovirus as monotherapy, its potential might be better manifested in rationally-designed combination strategies (45,46). For instance, the administration of reovirus in combination with radiotherapy (47,48) or chemotherapeutic agents (49-51) has resulted in an enhanced therapeutic outcome compared to reovirus alone, which could often be attributed to increased direct cytotoxicity. But, not much is known about the immunostimulatory properties of oncolytic reovirus, and consequently, research into the possible benefit of combining oncolytic reovirus with immunotherapy is lacking. Only a few studies have explored the immunostimulatory properties of reovirus, for example by combining reovirus administration with immune checkpoint blockade. Indeed, intratumorally or systemically administered reovirus sensitized tumors to subsequent blockade of the PD-1/PD-L1 axis in preclinical models of multiple myeloma (52), glioma (22), and breast cancer (53). However, since the efficacy of immune checkpoint blockade mostly relies on the presence of tumor-specific T cells, the combination of reovirus and immune checkpoint blockade might be predominantly successful in immunogenic tumors where tumor-specific T cells can be elicited. This highlights the need for new viro-immunotherapeutic strategies that can benefit patients with less immunogenic, immune-silent tumors where T cells are mostly absent.

TGF-β SIGNALING AS ANOTHER BARRIER TO EFFECTIVE IMMUNOTHERAPY

The exploitation of OVs, and in particular oncolytic reovirus, to transform the tumor microenvironment (TME) of solid, immune-silent tumors to enhance the efficacy of T-cell-based immunotherapy is a new and exciting avenue. But, the lack of intratumoral T cells in immune-silent tumors might not be the only problem that limits effective T-cell-based immunotherapy in solid tumors. The pleiotropic cytokine transforming growth factor β (TGF-β) is considered one of the key factors responsible for the exclusion and suppression of immune cells from the tumor. In pre-malignant cells, TGF-β acts as a

tumor-suppressing cytokine that induces apoptosis and regulates proliferation (54). However, in certain types of cancer, such as pancreatic cancer, non-small cell lung cancer, and colon cancer (55-57), tumor cells can become insensitive to TGF-β-induced cytostatic effects, and TGF-β functionally switches into a tumor-promoting cytokine by stimulating cancer cell migration and invasion, extracellular matrix (ECM) remodeling, epithelial-to-mesenchymal transition (EMT) and the formation of an immunosuppressive TME (58). Amongst others, its immunosuppressive functions include inhibiting the generation, intratumoral influx, and function of $CD4^+$ and $CD8^+$ T cells, as well as dendritic cells (59). These characteristics hint towards a potential beneficial effect of TGF-β blockade on the efficacy of T-cell-based immunotherapy. Preclinical evidence already demonstrated that TGF-β blockade could enhance the efficacy of checkpoint blockade (60), but whether TGF-β blockade could work synergistically with OV therapy to optimally improve the therapeutic efficacy of T-cell-based immunotherapy is not yet investigated.

AIM OF THIS THESIS

The studies described in this thesis aimed to elucidate the immunostimulatory potential of oncolytic reovirus and to investigate how these immunostimulatory characteristics could be exploited for effective anticancer immunotherapy (**Figure 6**). After reovirus administration to the tumor (**1**), we hypothesize that a potent immune response will be elicited (**2**). We aim to identify how this immune response can be employed for antitumor immunotherapy (**3**), but also whether the emergence of reovirus-specific immune responses (**4**) might prevent or contribute to the antitumor effect of oncolytic reovirus. Lastly, we will assess whether blocking immuno-inhibitory signaling pathways in the tumor influences the function of reovirus (**5**) or the reovirus-induced immune response (**6**) and can thus be employed to improve the efficacy of viro-immunotherapy.

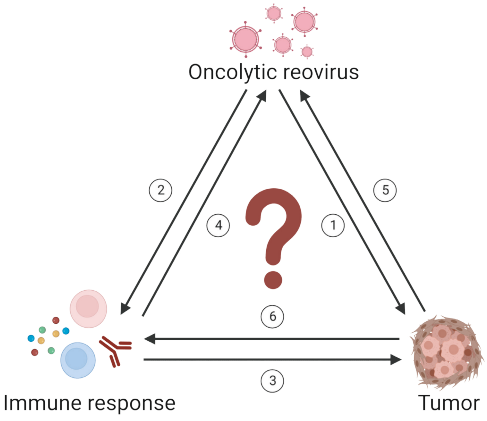

Figure 6. Harnessing the immunostimulatory potential of oncolytic reovirus for anticancer immunotherapy. Arrows and numbers indicate various processes that will be investigated in this thesis, to ultimately understand how the immunostimulatory properties of oncolytic reovirus can be employed for effective anticancer therapy.

OUTLINE OF THIS THESIS

Here, we first studied the reovirus-induced immune response in detail in immunocompetent mice bearing murine pancreatic KPC3 tumors in **Chapter 2**. We observed that intratumoral administration of reovirus leads to a fast interferon response, which is followed by an influx of immune cells, especially $CD8^+$ T cells. A proportion of these $CD8^+$ T cells recognized reovirus itself, but not the tumor. Therefore, we employed CD3-bispecific antibodies (CD3-bsAbs) that could activate these $CD8^+$ T cells, irrespective of their virus-specificity, to kill tumor cells. The combination of reovirus and CD3-bsAbs proved to be highly effective in inducing tumor regression and survival. The reovirus-specific $CD8^+$ T-cell response is further dissected in **Chapter 3**. Here, we identified the immunodominant $CD8^+$ T-cell epitope of reovirus in the applied mouse strain, which allowed us to study the kinetics, distribution, and phenotype of reovirus-specific T cells. We show that reovirus-specific T cells are potent effector cells that are enriched in the tumor after intratumoral reovirus administration, which suggested that they may recognize and kill reovirus-infected tumor cells. A synthetic long peptide (SLP) vaccine containing this reovirus-derived $CD8^+$ T-cell epitope was designed to induce high levels of reovirus-specific T cells before virotherapy. Upon intratumoral reovirus administration, these T cells were reactivated and migrated toward the tumor, which lead to significantly delayed tumor growth. Together, the research in **Chapters 2 and 3** demonstrates two different manners in which reovirus-specific T cells can be exploited for effective anticancer therapy.

Since a substantial percentage of the human population has supposedly encountered reovirus during their lifetime, we wondered whether preexisting immunity would have implications on the efficacy of reovirus (combination) therapy. **Chapter 4** reviews the current literature on the effect of preexisting immunity against various OVs on their efficacy when used as anticancer therapeutic agents. In **Chapter 5**, the effect of preexisting immunity on reovirus replication and its oncolytic potential, as well as the efficacy of reovirus-based immunotherapeutic strategies is experimentally addressed. We observed that the presence of preexisting neutralizing antibodies impairs reovirus replication and the reovirus-induced interferon response and hampers the use of reovirus as oncolytic agent. We demonstrated in **Addendum I** that depletion of $CD4^+$ T cells can abrogate NAb production and enhance the anticancer efficacy of reovirus as monotherapy. Furthermore, we demonstrated in **Chapter 5** that the reovirus-induced intratumoral T-cell influx was not impaired by preexposure, and that potent antitumor responses can still be observed in the context of preexisting immunity.

Lastly, we investigated whether TGF-β blockade might further improve the efficacy of viro-immunotherapy. **Chapter 6** provides an extensive summary of preclinical and clinical evidence that illustrates how the combined inhibition of TGF-β signaling and the use of OVs might increase the efficacy of immunotherapy. We then investigated whether TGF-β blockade could improve the efficacy of reovirus-based immunotherapeutic

strategies in **Chapter 7** and **Addendum II**. We demonstrated that TGF-β blockade significantly improved the efficacy of reovirus and CD3-bsAbs or reovirus and αPD-L1 in the preclinical MC38 colon carcinoma model, but surprisingly impaired the efficacy of reovirus and CD3-bsAbs in the pancreatic KPC3 tumor model. We elaborated on various intertumoral differences that might be contributing to this differential effect, such as baseline T-cell density, stromal composition, or the different effects of TGF-β blockade on the reovirus-induced T-cell influx into the tumor. Finally, **Chapter 8** provides a summary and discussion of the findings of this thesis in the context of recent literature.

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