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# ENABLING PD-1 AXIS INHIBITORS AGAINST SOLID TUMOURS WITH ENGINEERED ADENOVIRUSES ARMED WITH TNFA AND IL-2

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ACADEMIC DISSERTATION

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**Cover image**: the illustration in the cover is a hexagon-based pictogram that summarizes the content of this book. Art by Víctor E. Cervera-Carrascón (Helsinki, January 2021).

“Doubt is the origin of wisdom”

— René Descartes

# ABSTRACT

Over the last ten years, the use of immunotherapeutic treatments for cancer has exponentially increased due to the promising responses across different tumour types. One of the particularities of the approach is that patients can develop long lasting curative responses. Unfortunately, those encouraging complete responses happen only in a minority of patients (< 10%). Immune checkpoint inhibitors, like anti-PD-1 or anti-PD-L1, are embodiments of that kind of therapy, whose concept revolves around the concept of stopping inhibitory signals that limit the development of antitumour responses. Results obtained from clinical experiences showed that patients responding better to immune checkpoint inhibitors tend to have "hot tumours", meaning that there are effector components (such as T cells) and the microenvironment supports their function. To make immune checkpoint inhibitors work in those patients with cold tumours, the use of oncolytic adenoviruses could provide the spark to warm them. Viruses are able to trigger the immune alarm and thus, make tumours hot when their replication is restricted to the tumour.

To study the potential of oncolytic viruses to increase the efficacy of immune checkpoint inhibitors, an array of preclinical models that allowed to study the matter from different angles were used. While *in vivo* murine and hamster models provided the basis for direct antitumour effect and survival assessments, other studies including human tumour sample histocultures and other in vitro experiments were performed to understand the subjacent cellular and molecular mechanisms involved.

Due to the critical role of the T-cell compartment surrounding the activity of immune checkpoint inhibitors, different virus families were studied in terms of their ability to trigger T-cell responses. The best T-cell engagement was provided by the adenovirus candidate. An adenovirus engineered to support further antitumour immune responses was used to enable anti-PD-1 and anti-PD-L1 therapies. In both cases, the proposed strategy was able to cure all the animals. Additionally, tumours that were already refractory to anti-PD-1 still respond to virotherapy and the virus resets the tumour microenvironment sensitizing those tumours respond to anti-PD-1.

Overall, this work revealed the potential of using engineered adenoviruses to repolarize the tumour microenvironment in general and the T-cell compartment in particular to a point that complement the mechanism of action of PD-1 and PD-L1 inhibitory antibodies. That synergistic combination rendered an increased percentage of responses even in tumours previously resistant or refractory to immune checkpoint inhibitors.

# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **V. Cervera-Carrascon**, D.C.A. Quixabeira, R. Havunen, J.M. Santos, E. Kutvonen, J.H.A. Clubb, M. Siurala, C. Heiniö, S. Zafar, T. Koivula, D. Lumen, M. Vaha, A. Garcia-Horsman, A.J. Airaksinen, S. Sorsa, M. Anttila, V. Hukkanen, A. Kanerva & A. Hemminki (2020) **Comparison of clinically relevant oncolytic virus platforms for enhancing T-cell therapy of solid tumors.** Molecular Therapy Oncolytics, 17:47-60,

II **V. Cervera-Carrascon**, M. Siurala, J. M. Santos, R. Havunen, S. Tähtinen, P. Karell, S. Sorsa, A. Kanerva & A. Hemminki (2018) **TNFa and IL-2 armed adenoviruses enable complete responses by anti-PD-1 checkpoint blockade.** OncoImmunology, 7(5):e1412902,

III **V. Cervera-Carrascon**, D.C.A. Quixabeira, J. M. Santos, R. Havunen, S. Zafar, O. Hemminki, C. Heiniö, E. Munaro, M. Siurala, S. Sorsa, T. Mirtti, P. Järvinen, M. Mildh, H. Nisen, A. Rannikko, M. Anttila, A. Kanerva & A. Hemminki (2020) **Tumor microenvironment remodeling by an engineered oncolytic adenovirus results in improved outcome from PD-L1 inhibition.** OncoImmunology, 9(1):1761229,

IV **V. Cervera-Carrascon**, D.C.A. Quixabeira, J.M. Santos, R. Havunen, I. Milenova, J. Verhoeff, C. Heiniö, S. Zafar, J.J. Garcia-Vallejo, V.W. van Beusechem, T. de Gruijl, A. Kalervo, S. Sorsa, A. Kanerva & A. Hemminki (2020) **Anti-tumour efficacy in tumours refractory to anti-PD-1 mediated by adenovirus armed with TNFa and IL-2.** Science Immunology, (submitted manuscript),

The publications are referred to in the text by their roman numerals.

# ABBREVIATIONS

| | |
|---|---|
| ACK | Ammonium Chloride Potassium |
| ACT | Antigen Presenting Cell |
| APC | Antigen Presenting Cell |
| ATAP | Advanced Therapy Access Program |
| bp | Base Pairs |
| CAR T | Chimeric Antigen Receptor T cell |
| CRR | Complete Response Rate |
| CTLA-4 | Cytotoxic T-Lymphocyte Antigen 4 |
| DAMP | Damage Associated Molecular Pattern |
| DMSO | Di-Methyl-Sulfoxide. |
| DNA | Deoxyribonucleic acid Acid |
| EMA | European Medicines Agency |
| FBS | Foetal Bovine Serum |
| FDA | Food and Drug Administration (U.S.A) |
| GM-CSF | Granulocyte Macrophage-Colony Stimulating Factor |
| HIV | Human Immunodeficiency Virus |
| HSV | Herpes Simplex Virus |
| IDO | Indoleamine Deoxygenase |
| IFN | Interferon |
| IL | Interleukin |
| MDSC | Myeloid-Derived Suppressor Cell |
| MHC | Major Histocompatibility Complex |
| NDV | Newcastle Disease Virus |
| NK | Natural Killer |
| ORR | Objective Response Rate |
| PAMP | Pathogen Associated Molecular Pattern |
| PD-1 | Programmed-Death Receptor 1 |
| PD-L1 | Programmed-Death Ligand 1 |
| PD-L2 | Programmed-Death Ligand 2 |
| pRb | Retinoblastoma Protein |
| RPMI | Roswell Park Memorial Institute |
| ScFv | Single Chain Variable Fragment |
| TAP | Transporter associated with Antigen Processing |
| TCR | T-Cell Receptor |
| TGFb | Tumour Growth Factor Beta |
| TIL | Tumour Infiltrating Lymphocyte |
| TNFa | Tumour Necrosis Factor Alpha |
| vp | Viral Particle |

# 1 INTRODUCTION

At present, non-communicable diseases are responsible for the majority of human deaths worldwide (GLOBOCAN 2018). This situation is relatively new as for the majority of human history infectious diseases, especially relative to birth, were accountable for a large amount of deceases and shorter life expectancy [1]. It is noticeable when retrospectively analysing the past century, how a number of measures implemented to prevent, manage or treat those infectious diseases result in the reduction of infectious disease mortality to a tenth of the original numbers [2].

The actions taken to cut down infectious disease mortality involve better hygiene standards at multiple levels to cut down the transmission of the disease to other individuals, better disease management to reduce the negative impact on the patient's health (and also reduce the communicability period) and, remarkably, the implementation of vaccination programs.
Even if immunization techniques, such as variolation, are previously registered in history, Edward Jenner's work to fight smallpox is probably the single publication with most impact on the health of the human species. That technique described by Jenner at the end of 18$^{th}$ century is called vaccination. Although it was unknown by the time, the vaccination procedure manages to provide immunity against a certain pathogen without suffering the infection itself, a process mediated by the immune system. Harnessing the power of our own immune system led the way to, in 1980, the eradication of smallpox [3].

Even if human development resulted in wonders (such as vaccines) able to eradicate diseases, death is certain and other causes of fatality will take the lead. In that sense, the mortality landscape could change again bringing a gloomy scenario where there is a rise in infectious disease mortality associated for example to antibiotic resistance (*e.g*. methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Pseudomonas aeruginosa*) or to the appearance of new infectious agents for which we are underprepared (*e.g*. 2002-2004 SARS outbreak, 2014-2016 Ebola virus outbreak, 2019-2020 Coronavirus outbreak).

As mentioned before, the "theory of epidemiologic transition" [1] explains how in the last century, humanity moved from being a species which mortality rate is dominated by infectious diseases to an "age of mortality associated to degenerative and man-made diseases". More specifically, the current leading cause of premature death (occurred before 70 years of age) across the world is cancer (GLOBOCAN 2018). However, cancer is not a new disease but because of an increased life expectancy, there is higher incidence of it. The term cancer is a basket that includes diseases where mutated cells continue to grow and

proliferate creating new abnormal cells and vasculature to support them instead of dying [4]. To be more precise on the portrait of a cancer cell, more hallmarks can be included such as their genomic instability, the altered cellular energy administration, the self-promoting inflammation or their ability to avoid immune destruction [5].

Cancer in humans (and other species) has been recorded almost from the beginning of the history and different explanations for the disease have been proposed [6] but currently its treatment is based in five pillars: surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy. Surgery is the longest-standing approach but it has many limitations as the disease spreads and affect tissues difficult to access. In the early XX century, radiation was discovered to both cause and cure cancer, but it has strong applicability limitations, as the energy used to destroy tumours does not differentiate between healthy and cancerous cells. Chemotherapy was developed around 1950s after the observations with nitrogen mustards. Those agents exploit the fast metabolic rate of tumour cells, and harming them more but they cannot be considered purely selective for cancer cells. It is with targeted therapies, after 1980, when truly selective drugs were introduced in the therapeutic armamentarium against cancer. Those drugs are targeting specific features of cancer cells such as genes or proteins that contribute to their growth and survival. Unfortunately, the difficulty to find efficient targets together with the ability of tumours to downregulate the selected targets make challenging to provide durable response with these therapies. Immunotherapies have gained substantial interest as therapeutic agents due to the unprecedented of long-lasting responses experienced by a number of patients with advanced disease. The idea of using the immune system against cancer is not novel and it was well documented by William Coley in the 1890s [7], six years before the discovery of X-rays.

The possibility of enabling the patient's own immune system against their tumours is a utopic approach to treat cancer as it can be highly specific but also due to the immune system's ability to learn, it can compete with tumour adaptability. Obviously, the immune system on its own is not able to destroy all the tumours (otherwise, patients would not require therapies). Immunotherapies are aiming to provide the right support for the immune system to be able to clear the tumours. Currently, those immunotherapies are a promising approach but many limitations keep them away from effectively cure the majority of patients.

As other immune-related therapies (like vaccines) helped humanity to overcome previous leading causes of death, it seems that the same immune system could be a strong ally in the fight against cancer. For that, it is required to study (and ultimately understand) how to unleash the immune system and overcome all the tumour's defence mechanisms.

# REVIEW OF THE LITERATURE

## 1.1 IMMUNOEDITING: INTERACTIONS BETWEEN CANCER AND THE IMMUNE SYSTEM

Cancer immunoediting refers to the phenomena in which the immune system can both restrict and promote tumour development. This process includes three phases: elimination, equilibrium and escape [8].

### 1.1.1 ELIMINATION

Tumours undergoing the elimination phase display innate and adaptive immune mechanisms cooperating to detect and eliminate transformed cells that were able to escape cell-intrinsic mechanisms of tumour suppression [9].

The antitumour immune response starts with the detection of the tumour by the innate immune system after tissue abnormalities such as invasive growth [10] and angiogenesis [11, 12]. Those signals, together with cytokines and chemokines associated with the abnormal tissue development trigger the recruitment of more innate immune cells, NK cells and macrophages being the critical populations [13, 14] but also γδ-T cells [15]. Those immune cells can recognize developing tumours leading to the production of IFNg and other inflammation signals. At that point, the IFNg reinforces the recognition of tumour cells by innate immunity cells creating a positive feedback loop. Furthermore, IFNg has proapoptotic, antiproliferative and angiostatic ability among other antitumour effects [16]. At that stage, macrophages and NKs are able to kill tumour cells leading to the release of tumour antigens that will be collected by dendritic cells to, subsequently, migrate to the draining lymph node where they will activate tumour-specific T cells [17]. Active CD4+ and CD8+ T cells will leave the lymph node and potentially exit the systemic compartment and home in the tumour niche. Those T cells can recognize and induce cell-death in tumour cells which again perpetuate the immune cycle after the release of proinflammatory cytokines and tumour antigens [18]. As a result of the process, innate and adaptive immune cells, and other effectors such as IFNg or TNFa can be coexisting in the tumour killing cells via different mechanisms and potentially eradicating the tumour.

During this phase, the net tumour growth is negative.

### 1.1.2 EQUILIBRIUM

The pressure exerted by antitumour immune forces, on a constantly proliferating and genetically unstable entity such as a tumour, can select for tumour subclones that are able to evade being recognized and eliminated by immune cells, reaching an equilibrium of forces. Alternatively, equilibrium phase can be reached not because of clonal selection, but after the tumour has undergone certain modifications (epigenetic or transient transcriptional regulation) that allow them to counter immune activity or reduce their sensitivity to immune effectors.

The equilibrium phase is the longest of the immune editing process; it can take decades for tumours to move to the next phase [19].

During this phase, the net tumour growth is close to zero.

### 1.1.3 ESCAPE

When a tumour develops enough mechanisms of immune resistance, or in other words is profoundly immunoedited, its growth is unrestrained by the immune system and it can be clinically evidenced [20-22].

The cumulative process of immune resistance can include tumour cells acquiring features such as the expression of immunosuppressive effectors or recruitment and enhancement of immunosuppressive immune cells. Immunosuppressive effectors secreted by tumour cells or immunosuppressive immune cells that can hamper antitumour responses are, for example, the tumour growth factor beta (TGFb), interleukin 10 (IL-10), indoleamine deoxygenase (IDO) or the enzyme arginase [23]. Direct cell to cell interaction can also mediate inhibition of immune activity in key populations such as effector T cells. Again, not only tumour cells but also immunosuppressive immune cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), or tumour-associated macrophages, can express signals that prevent antitumour immune responses such as immune checkpoint inhibitory signals (*e.g.* programmed-death ligands 1 and 2 [PD-L1, PD-L2]) [24]. Besides the prevention of antitumour immune actions, tumour cells are also able to develop resistance mechanisms that allow them to endure deleterious immune activity. IFNg insensitivity, often mediated by defective receptor signalling pathways [25], or apoptosis avoidance in cases such as the constitutively active STAT3 [26] are widely known examples of immune resistance-enabling modifications.

Another mechanism frequently developed by tumours is not strictly related with neither inhibition nor resistance of immune activities, but with the prevention of immune recognition by loss of antigen expression [27], major

histocompatibility complex (MHC) defects [28], or NKG2D shedding [29]. This line of protection against immune clearance from the tumour, seems to be particularly prevalent (40-90%)[28, 30, 31] in human tumours, which could indicate that it is also an effective way to escape from the immune elimination and promote tumour escape.

During this phase, the net tumour growth is positive.

### 1.1.4 TUMOUR REGRESSION: IMMUNOTHERAPY TO SUPPORT ELIMINATION

The mechanisms of antitumour immune activity are already described. For an escaping tumour able to resist the patient's endogenous immune system, some factor of the equation needs to be changed to return to an elimination phase. As evolutionary pressure would not support re-gaining of sensitivity against immune activities and the immune system is unlikely to gain new abilities, the only way to alter the *status quo* is to artificially enhance immune activity and dim immune suppression. In that sense, the study of each immunoediting phase gave the opportunity of fighting cancer from different angles: studying elimination helps understanding ways of supporting the immune system to fight or prevent advanced disease, studying equilibrium it has been possible to identify molecular targets of the tumour sculpting process in order to stabilize tumour cell genomes, and finally, the study of the escape has provided further knowledge on the specific mechanisms behind cloaking mechanisms and suppression mechanisms by the tumour that allows developing of adequate counter measures.

When translating the basic knowledge gained on tumour immunoediting, it was concluded that, as for many other therapeutic approaches (*e.g.* the anti-retroviral treatment for HIV or even the use of targeted therapies against cancer), an effective immunotherapy approach should aim to tackle multiple non-redundant immune pathways in order to generate a long lasting tumour immunity. In addition, an effective immunotherapy approach must be able to overcome possible iterations of the cancer immunity cycle as the tumour will attempt to escape every time [32]. In this regard, based on preclinical and clinical studies, three essential targets should be targeted when designing effective immunotherapies [33]:

- Support of T-cell activation and priming: boosting the interaction between antigen presenting cells loaded with tumour antigens and naïve and inactive tumour specific T cells, can ultimately improve the ability of the adaptive immune effectors to eradicate the tumour.

*Examples: oncolytic viruses[34, 35], STING agonists [36], TLR agonists [37], oncogene inhibitors [38], proteasome inhibitors [39], epigenetic drugs [40], or cancer vaccines [41]. Additionally, immunogenic cell-death triggered by conventional radiotherapy and chemotherapy can also favour T-cell activation and priming [42, 43].*

- Disruption of the protumourogenic microenvironment: tackling the mechanisms that support an escaping tumour can turn the balance in favour of immune elimination of the tumour. For that purpose, multiple targets have been identified as potential therapeutic targets.

  *Examples: targeting metabolites [44], promoting vessel normalization [45], targeting suppressive immune cells [46], or targeting tumour stroma [47].*

- Promotion of antitumour effector cells at the tumour niche: enhancing endogenous mechanisms of antitumour activities can help to overcome the barrier established by tumour suppression. Furthermore, artificial manipulation of T-cells can provide means generate alternative sources of antitumour effector entities.

  *Examples: Adoptive-cell therapy of T cells [48], cytokine therapies [49], T-cell engagers [50], antagonistic antibodies targeting T-cell suppressive mechanisms [51], or agonistic antibodies targeting T-cell stimulatory mechanisms [52].*

Another layer of refinement when designing effective immunotherapies comes after studying each patient's individual features. This means, focusing on the specific mechanisms is the particular tumour using to escape, but also taking into account other features of the patient such as their microbiome [53-55].

### 1.1.5 TUMOUR PROGRESSION: ESCAPING AFTER IMMUNOTHERAPY

The natural cancer immunoediting process flows from elimination to escape in those tumours that eventually require treatment. Subsequent repetitions of this immune editing process happen with every line of treatment the patient receives [21], as the tumours tend to adapt to them. The only way to halt the perpetuation of the immune editing process is to eradicate the tumour completely.

In terms of post-immunotherapy tumour progression, two different scenarios can be proposed to explain the tumour reaching the escape phase: innate or acquired immunotherapeutic resistance [56, 57]. While escaping tumours are treated with an immunotherapy and there is no return to equilibrium or elimination phase, it is classified as "innate resistant". Innate or inherent resistance can occur when some of the mechanisms developed by the tumour to prevent immune activity (both endogenous and mediated by previous immunotherapies)[58] also grants immunity against the new therapy. Situations where the tumour is poorly immunogenic (*e.g.* low mutational burden, non-UV and non-virally induced tumours)[33, 59] and the immune system fails to detect the presence of malignant cells can also explain innate resistance against immunotherapies. It is also possible that a patient's immune system is unable to mount immune responses (*e.g.* defects in key mechanisms of the immune system, chemotherapy induced severe immunodeficiency, HIV infection) in a way that prevents any potential benefit from the therapy [33].

On the other hand, tumours that initially respond to the therapy and go from escape to equilibrium or even elimination but ultimately are able to return to an "escape" status are subject of the term "acquired resistance". This scenario can be explained with the same mechanisms previously mentioned that support the transition from elimination towards escape.

## 1.2 VIROTHERAPY

Throughout history, tumour regression has been recorded in cancer patients who contracted infectious diseases. Case reports from the over a century ago were documented, where contraction of influenza was correlated to regression of patients suffering from leukaemia [60, 61]. To be noted, the benefit for the cancer patient after virus infections was assessed even before the discovery and characterization of viruses as such. Even if Edward Jenner established the vaccination method against smallpox in the late 18$^{th}$ century [62], it was almost a hundred years later, in 1898 when Martinus Beijerinck (based on previous work [63, 64]) pointed out that the agent causing the tobacco mosaic disease was able to infect plants even if the source of infection was able to pass through Chamberland candle filtration. He provided a prototypical definition of viruses calling them "*contagium vivium fluidum*" [65]. In the subsequent years to come the further diseases, including human ones, were attributed to viruses and virus-related assays were developed supporting the embryonic stages of the field of virology [66]. Only in 1939, with the fist electron microscopy pictures revealing the true form of viruses,

the so called “Dark Age” of virology came to an end allowing a rapid increase in knowledge regarding virology [67] (Figure 1).

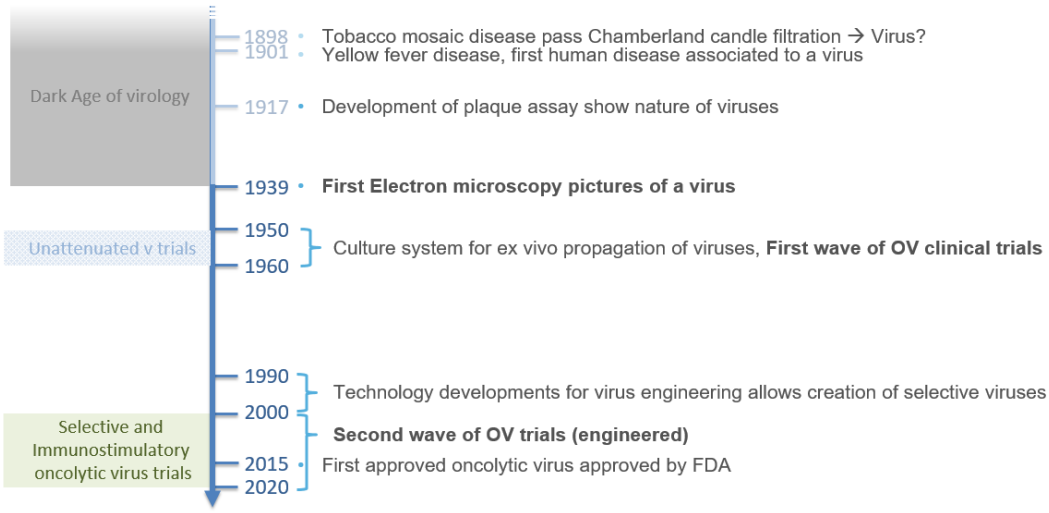


*Figure 1. Major milestones in the history of oncolytic viruses. OV; oncolytic virus*

When light was shed on the nature of viruses, they were mostly regarded as agents potentially causing different types of cancer but soon multiple clinical experiences were started to address their possibilities as antitumour agents. In that regard, the first stages of oncolytic viruses against cancer are filled with equal amounts of fascination and disappointment [66]. Some of the causes for the disappointment part can be linked to unrealistic expectations of complete responses, lack of reliable clinical trial frameworks and the use of poorly selective candidate viruses. In the 90s of the last century, the field of oncolytic viruses gained some interest albeit the opportunities granted by technological development regarding virus genome engineering. The first rationally designed viruses were developed by the early 2000’s and a wild type virus (Rigvir) was the first oncolytic virus ever approved for its use in Latvia 2004 [68]. Soon after, China approved the first genetically modified oncolytic virus for use in squamous cell carcinoma of the head and neck [69-71]. The virus, called Oncorine (H101) is an adenovirus. After that, talimogene laherparepvec has been approved for use in unresectable melanoma in 2015 [72]. On the top of those oncolytic viruses being used in the clinics of different regions of the world, a myriad of other candidates are currently being developed clinically and preclinically.

The concept of an oncolytic virus existed at the theoretical level much before it was technically possible to develop them and disregarding subtle differences, the term oncolytic virus refers to the virus-based tools that would replicate only in tumour cells leaving healthy cells unharmed [73]. If complete

selectivity for tumour cells can be difficult to find, certain degree of natural tumour selectivity is present in multiple viruses; NDV, parvoviruses, myxoma viruses, reovirus, Seneca Valley virus and Coxsackie virus [73, 74]. Further selectivity for tumour replication can be granted to those viruses as well for viruses that originally were not tumour selective, such as adenoviruses, measles viruses, herpes simplex viruses, polioviruses and VSV among others [73].

The antitumour effect triggered by oncolytic viruses is not solely dependent on the host cell lysis that occurs during the replication cycle of the virus, but also includes a plethora of immune-related phenomena that support the development of antitumour immune responses [75-77]. When an oncolytic virus lyses a tumour cell pathogen and danger associated molecular patterns (PAMPs and DAMPs) are released into the extracellular compartment of the tumour milieu which is a clear signal for attraction the attention of the immune system towards the tumour [78]. Concurrent to the release of the immune alarm in the tumour, tumour associated antigens and tumour neoantigens become available for the development of immune responses [73, 79] (Figure 2).

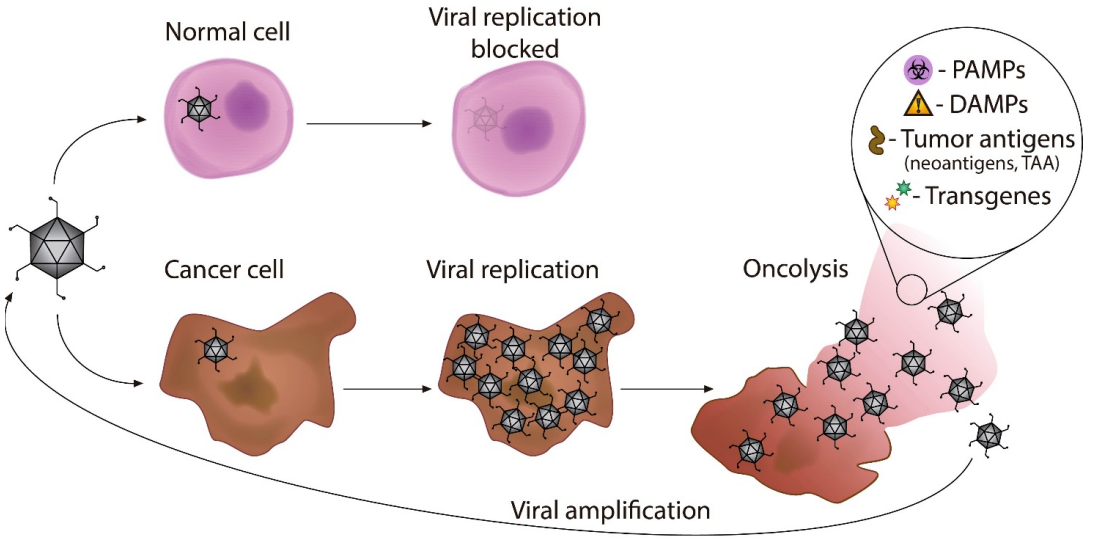


*Figure 2. General concept behind oncolytic viruses. Oncolytic viruses are therapeutic tools able to replicate and lyse in tumour cells but unable to do so in healthy cells, leaving them unharmed. Oncolytic virus mediated oncolysis releases new viral particles able to potentially transduce other tumour cells but also releasing pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs), tumour antigens (such as neoantigens or tumour associated antigens) and other products that can be armed into the adenoviral genome. (Reproduced with permission from Cervera-Carrascon V. et al. 2019. Expert opinion in biological therapy).*

In practice, the use of oncolytic viruses is hampered by different events happening in the tumour microenvironment (physical and chemical barriers preventing the spreading of the virus), in other non-tumour tissues (adverse events triggered after the presence and/or replication of the virus) and by antiviral defences against viruses (innate and adaptive immunity) [73].

Just a decade ago, it was thought that neutralising antibodies and complement mechanisms can render oncolytic viruses useless [80, 81]. But the dogma is shifting towards a direction where the virus can coexist with neutralising antibodies [82] and where antiviral responses can mean a beneficial approach to repolarize the suppressive tumour microenvironment [83-85].

### 1.2.1 ADENOVIRUS

The 7 human adenovirus species (classified alphabetically from A to G) are included in the *Adenoviridae* family and the *Mastadenovirus* genus [86]. They are sorted into 50 different serotypes [86, 87]. Their linear double-stranded DNA genome has an approximate length of 36 000 base pairs (bp) and it is encapsulated in a naked pseudo icosahedral capsid with a diameter of 70-90 nm. Such capsid is composed of trimeric hexon proteins and includes 12 penton proteins at the vertices, which also hold knobbed fibers [87].

Adenoviral infections in the human population are present worldwide during the whole year and are especially endemic in the pediatric population. Providing they are non-enveloped viruses, they are highly resistant to physical and chemical agents, including gastric acid and biliary secretions, which allow the virus to replicate in the gastrointestinal tract [88]. At room temperature, the virus can remain infectious outside the host for up to 3 weeks. The transmission of the disease occurs person to person and through water and fomites. There is no animal reservoir and only few animal models can reproduce the human disease [89], which is usually asymptomatic but sometimes includes mild respiratory distress and other symptoms like fever, eye inflammation, or diarrhea [88]. The serotypes 1, 2, and 5 of adenoviruses can remain latent for long periods in the tonsils [90].

The majority of the adenoviruses use their fiber knobs to bind the target cell receptors, such as coxsackie adenovirus receptor, desmoglein-2, or CD46 [91-94]. After the virus attaches to the cell membrane, viral pentons interact with host-cell integrins ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) and the viral particle is internalized into a clathrin-coated vesicle [95]. After internalization, the virus leaves the endosome and approaches the nucleus, where the viral genome is delivered to continue its replicative cycle [96-98]. Upon nuclear entry, early gene products (E1A, E1B, E2, E3 and E4) work towards viral DNA replication. After

replication is completed, transcription of late genes (*L1*, *L2*, *L3*, *L4* and *L5*) produces structural proteins needed for the virion assembly [98].

The assembly starts with hexons and pentons grouping around a set of scaffolding proteins (L4 22-33K) to subsequently insert the viral DNA inside the structure. Once the genome enters the capsid, scaffolding proteins are released and precursor proteins (L1 52-55K) cleaved, allowing the final maturation of the virus [99]. The replicative cycle of adenoviruses takes 24 to 36 hours to be completed, and it yields above 10 000 virions that are released to the extracellular space after cell lysis [98].

Other important virus-host cell interactions critical to be understood when understanding the adenoviral replicative cycle include the mechanisms modulating the cell cycle and apoptosis machinery. Adenovirus' E1A protein plays a crucial role in the replicative cycle of the virus as it is the main responsible for directing host cell cycle into S-phase so the virus can replicate its genome [100]. As a response mechanism to the arrest into S-phase, the host cell tends to accumulate p53 protein which would drive the cell into apoptosis. Adenoviral proteins such as E1B 55K, E4 orf6 or E1B 19K are able to avoid apoptosis by direct or indirect interaction with p53 [101]. Before the replicative cycle of the virus is completed at the host cell, apoptosis is avoided but once the new generation of viral particles are ready to be released to the extracellular milieu, E1B 19K promotes autophagy and subsequently cell lysis for the release of the viral particles formed in that cell [102]. Another important virus-host interactions are those regarding the immune signalling produced in the host cell after infection. Even if adenoviruses do not inhibit directly the cascade leading to production of IFN beta, E1A protein is able to block the mono-ubiquitination of cellular histones that take part in the expression of interferon-stimulated genes [103]. Besides the innate response inhibition, adenoviruses also hamper adaptive immune responses as E19 binds TAP (transporter associated with antigen processing) retaining it in the endoplasmic reticulum avoiding the cell-surface expression of this protein and ultimately avoiding the peptide transportation into MHC-I [104].

Adenoviruses do not have natural selectivity for replication in cancer cells, but it is a feature that can be granted after a process of genetic engineering [105]. In that sense, deletions in the early region 1A gene produces defects in the E1A activity that is widely used as a mechanism to generate tumour selectivity [106]. Alternatively to deletions in the E1A gene, tumour selectivity can also be acquired by making E1A to be conditionally expressed under a promoter that is selectively or over-expressed in cancer cells. This transcriptional targeting of cancer cells has been used in oncolytic adenoviruses that were used safely in phase I clinical trials [107, 108]. As mentioned before, E1A is able to make the host cell to transition to S-phase of the cell cycle. That phenomenon starts with E1A binding the retinoblastoma

protein (pRb). [109](Figure 3A). The role of pRb is to control the cell's replication cycle which is effectively done by creating complexes with the DNA-binding transcription factor E2F. When they are in a complexed form they are unable to bind DNA and promote cell replication [110]. When E1A binds pRb, the complex with E2F is dissolved, and E2F is free to bind specific DNA regions and promote transitioning to S-phase [111, 112]. Virtually all cancer cells are defective in mechanisms controlling cell replication (p16/pRb) [113], that is a requirement for their defining over replicative status [5]. Providing that cancer cells have deficient pRb proteins, E2F is constitutively free and the activity of E1A becomes non-essential for virus replication. That circumstance opens the way for creating replication selectivity through transcomplementation: a virus with defective E1A protein will be able to replicate in cells with constitutively free E2F (cancer cells) but not in cells with functional pRb proteins (healthy cells)[113]. A 24 bp deletion in the E1A gene renders a defective E1A product (E1A-d24), unable to bind pRb (Figure 3 B). While this deletion has been the basis for selectivity of various oncolytic adenoviruses used safely in clinical trials [83, 114-116], it has shown how transiently free E2F can lead to viral replication of these viruses, compromising their selectivity [117]. To create higher selectivity, another selectivity feature can be added (additionally to the 24 bp deletion) when the replication of the virus is controlled by a promoter dependent on high levels of free E2F [118, 119](Figure 3 C). In that sense, lysis of cancer cells is unaffected but selectivity is substantially increased [117].

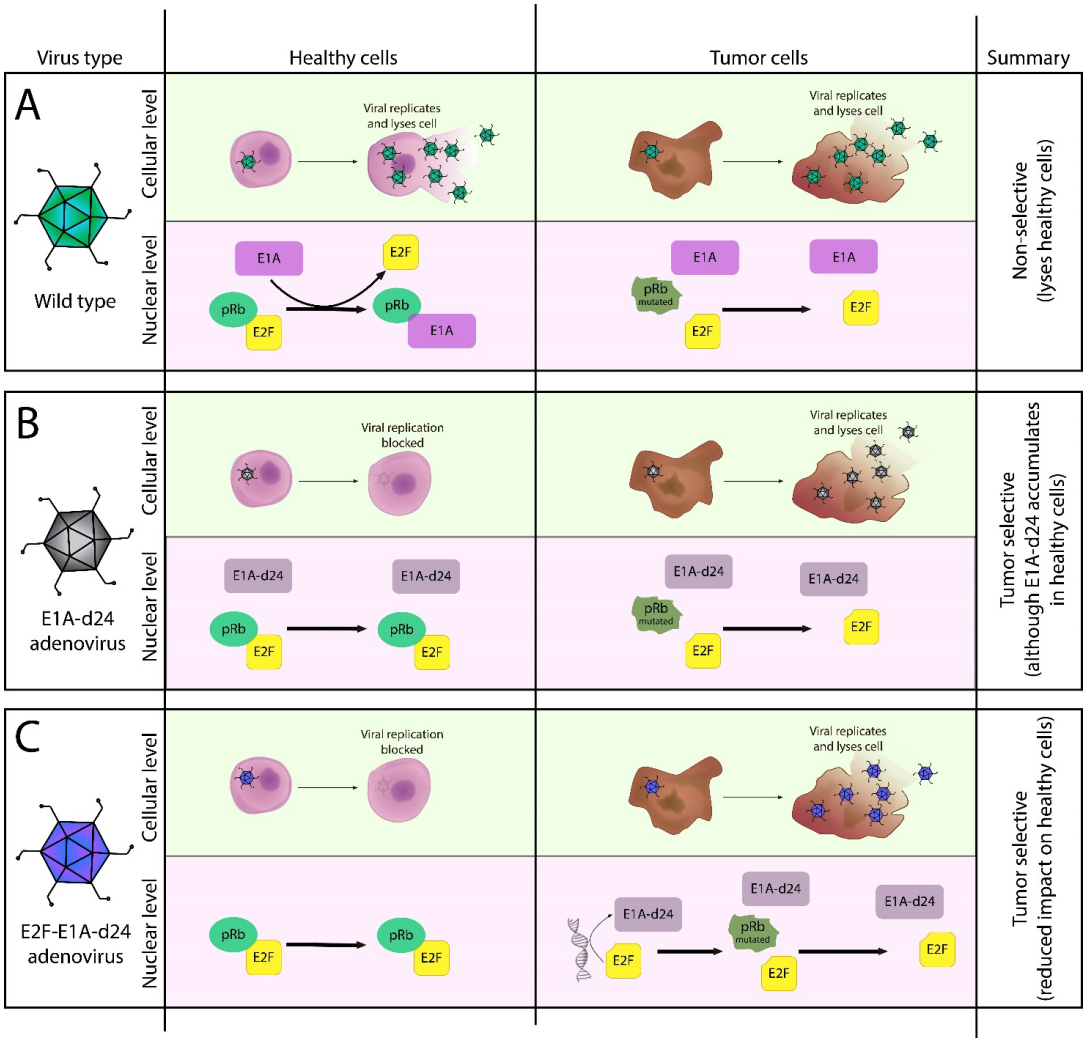


*Figure 3. E1A-mediated strategies of tumour selectivity. A) Wild type adenovirus produces E1A, a protein that binds pRb and subsequently releasing E2F and promoting entry into S-phase for viral replication and eventual cell lysis. This is the case for both healthy and tumour cells. B) Upon cell infection with a virus deficient in functional E1A protein, the virus is unable to force the cells into S-phase, but the defective E1A-d24 protein is still produced in healthy cells. In tumour cells, due to their inherently free E2F, functional E1A is not required and cell lysis occurs. C) The E2F-E1A-d24 adenovirus will not produce E1A-d24 in healthy cells as they do not have high enough levels of E2F. In cancer cells free E2F will trigger replication on the virus and it will be possible due to the defective pRb. (Reproduced with permission from Cervera-Carrascon V. et al. 2019. Expert opinion in biological therapy).*

A different selectivity strategy for adenoviruses can be established without involving E1A, but E1B, another critical tool for the virus-host interaction. Similarly as with defects in p16/pRb defects, cancer cells are required to be deficient in p53/p14ARF to sustain their uncontrolled proliferation [120, 121]. As explained before, wild type adenovirus produces E1B to inactivate p53 and avoid premature apoptosis. Deletion of E1B gene creates a similar circumstance where the virus will replicate only in cells deficient in the p53

pathway [69]. Differently than it occurs with E1A deletions, crippling the E1B 55k activity reduces replication of the virus in both healthy and tumour cells [122]. The E1B deletion approach has been embodied in oncolytic adenoviruses, such as ONYX-015 and H101 [69, 123], which were taken into clinical trials and approved in certain territories.

Another important feature of adenoviruses when using them against cancer is their ability to enter tumour cells. Multiple differences can be found between protein expression patterns of healthy cells and tumour cells, and some of those differences can have an impact on how the virus is able to infect the target cell [79]. Serotype 5 adenoviruses, probably the best known adenovirus serotype, use the coxsackie-adenovirus receptor surface protein widely expressed in healthy cells. Many cancers downregulate the expression of the coxsackie-adenovirus receptor protein [118], which can avoid the transduction of tumour cells with the virotherapy rendering suboptimal results. Another interesting way to make adenoviruses a better tool to treat cancer is to engineer them for entering tumour cells via proteins that are widely expressed in tumour cells. That idea is exemplified by multiple serotype 5 adenoviruses that were engineered to express of different serotypes' knob proteins, like Ad5/3 or Ad5/35 adenoviruses. In that way, instead of entering the cells via Ad5 route, they will use other proteins to anchor to the cells, like desmoglein-2 (for Ad5/3 viruses), a widely expressed surface protein on tumour cells [118, 124, 125].

Multiple (18) oncolytic adenovirus candidates are included in the clinical trial registry where a total of 33 clinical trials using oncolytic adenoviruses is described (Search criteria: Database= clinicaltrials.gov, intervention treatments= oncolytic adenovirus, Recruitment status=/Suspended OR Terminated OR Withdrawn, Search date June 17$^{th}$ 2020)(Table 1).

*Table 1. Oncolytic adenovirus trials. IL-12; Interleukin-12. HSV-tk; herpes simplex virus-1 thymidine kinase. GMCSF; granulocyte and macrophage colony stimulating factor. TMZ-CD40L; trimerized membrane-bound CD40 ligand. HPV; human papilloma virus. NSCLC; non-small-cell lung cancer. TNBC; triple negative breast cancer. RCC; renal-cell carcinoma. MG1-E6E7; maraba oncolytic virus expressing E6E7. SBRT; stereotactic body radiotherapy. Nab; neutralizing antibody. BiTE; Bispecific T-cell engager. Of note, not all clinical trials using oncolytic viruses included the specific term 'oncolytic virus' under the description of the trial and thus, those studies will not appear in the previously described systematic search*

| Virus name | Serotype | Transgene(s) | Indication | Phase | Date | Other treatments | Clinical trial identifier |
|---|---|---|---|---|---|---|---|
| Ad5-yCD/mutTKSR39rep-hIL12 | 5 | IL-12 | Prostate cancer | I | Sep-15 | - | NCT02555397 |
| Ad-E6E7 | 5 | E6E7 | NSCLC | I/II | Aug-16 | MG1-E6E7, anti-PD1 | NCT02879760 |
| Ad-E6E7 | 5 | E6E7 | HPV associated cancers | I | Aug-18 | MG1-E6E7 | NCT03618953 |
| Ad-MAGE-A3 | 5 | MAGE-A3 | Melanoma, Cutaneous squamous cell carcinoma | I | Dec-18 | MG1-MAGE-A3, anti-PD1, Cyclophosphamide | NCT03773744 |
| ADV/HSV-tk | 5 | HSV-tk | NSCLC, TNBC | II | Dec-16 | Valacyclovir, SBRT, anti-PD1 | NCT03004183 |
| CAdVEC | 5/3 | anti-PDL1 | Solid tumors | I | Nov-18 | - | NCT03740256 |
| CG0070 | 5 | GMCSF | Bladder Cancer | II/III | Sep-11 | - | NCT01438112 |
| CG0070 | 5 | GMCSF | Bladder Cancer | II | May-14 | - | NCT02143804 |
| CG0070 | 5 | GMCSF | Bladder Cancer | II | Feb-15 | - | NCT02365818 |
| CG0070 | 5 | GMCSF | Bladder Cancer | II | May-20 | aPD-1, n-dodecyl-B-D-maltoside | NCT04387461 |
| CGTG-102 | 5/3 | GMCSF | Solid tumors | I | Sep-11 | - | NCT01437280 |
| CGTG-102 | 5/3 | GMCSF | Solid tumors | I | Oct-14 | - | NCT01598129 |
| CRAd-Survivin-pk7 | 5 | - | Glioma | I | Mar-17 | Neural stem cells | NCT03072134 |
| DNX-2401 | 5 | - | Glioblastoma | I | Oct-13 | Temozolomide | NCT01956734 |
| DNX-2401 | 5 | - | Glioblastoma, Gliosarcoma | I | Jul-14 | Interferon-gamma | NCT02197169 |
| DNX-2401 | 5 | - | Brain tumors | II | Jun-16 | aPD-1 | NCT02798406 |
| DNX-2401 | 5 | - | Brainstem glioma | I | Jun-17 | - | NCT03178032 |
| DNX-2401 | 5 | - | Glioblastoma | I | Oct-18 | - | NCT03714334 |
| DNX-2401 | 5 | - | Glioma | I | Apr-19 | Surgery | NCT03896568 |
| Enadenotucirev | 11/3 | - | Colon cancer, NSCLC, Bladder cancer, RCC | I | Feb-14 | - | NCT02053220 |
| Enadenotucirev | 11/3 | - | Rectal cancer | I | Apr-19 | Capecitabine, Radiotherapy | NCT03916510 |
| ICOVIR-5 | 5 | - | Melanoma | I | May-13 | - | NCT01864759 |
| LOAd703 | 5/35 | TMZ-CD40L, 41BBL | Pancreatic cancer | I/II | Nov-16 | Gemcitabine, Nab paclitaxel | NCT02705196 |
| LOAd703 | 5/35 | TMZ-CD40L, 41BBL | Multiple solid tumours | I/II | Jul-17 | - | NCT03225989 |
| LOAd703 | 5/35 | TMZ-CD40L, 41BBL | Melanoma | I/II | Oct-19 | aPD-L1 | NCT04123470 |
| NG-350A | 11/3 | anti-CD40 | Epithelial tumours | I | Feb-19 | - | NCT03852511 |
| NG-641 | 11/3 | BiTE, CXCL9, CXCL10, IFNa | Epithelial tumours | I | Aug-19 | - | NCT04053283 |
| OBP-301 | 5 | - | Melanoma | II | Jun-17 | - | NCT03190824 |
| ORCA-010 | 5 | - | Adenocarcinoma of the prostate | I/II | Sep-19 | - | NCT04097002 |
| TILT-213 | 5/3 | TNFa, IL-2 | Melanoma | I | Jan-20 | Adoptive-cell therapy (TILs) | NCT04217473 |
| VCN-01 | 5 | PH20 hyaluronidase | Solid tumors | I | Jan-14 | Gemcitabine, NAb paclitaxel | NCT02045602 |
| VCN-01 | 5 | PH20 hyaluronidase | Pancreatic adenocarcinoma | I | Jan-14 | Gemcitabine, NAb paclitaxel | NCT02045589 |
| VCN-01 | 5 | PH20 hyaluronidase | Head and neck squamous cell carcinoma | I | Jan-19 | aPD-L1 | NCT03799744 |

#### 1.2.1.1 TILT-123

In 2007, the Advanced Therapy Access Program (ATAP) started at the Cancer Gene Therapy Group (University of Helsinki). In this experimental initiative, 290 terminally ill cancer patients were treated with different oncolytic adenoviruses in a patient-by-patient. From the real patient data obtained after the use of 10 different virus constructs, many theoretical concepts regarding oncolytic adenoviruses were confirmed. The ability of these viruses to achieve transduction of uninjected metastases [126], but also their ability to cause inflammation of the tumours and achieve T-cell trafficking into those lesions [83, 127] are among the most critical highlights. In the ATAP experience, 10 different oncolytic adenoviruses were used, each of them with particular design features. The analysis of the data produced with those viruses allowed the selection of the features that rendered better results in different aspects of the drug such as biodistribution or antitumor efficacy. That information was taken into consideration to create a new oncolytic adenovirus embodying the lessons learnt during ATAP, that virus is TILT-123.

The backbone of TILT-123 is based on a serotype 5 background but it was engineered to express a serotype 3 fiber knob, to increase transduction of cancer cells. Additional engineering of the virus was performed to grant tumour replication selectivity using the previously described E2F promoter coupled to the expression of E1A-d24 for enhanced selectivity [128]. The virus backbone was armed with two cytokines: human TNFa and human IL-2. Those transgenes were inserted into the E3 region of the virus replacing non-essential open reading frames and linking the expression of these cytokines to the viral replication.

The selection of the arming device of TILT-123 was the result of multiple studies performed at Cancer Gene Therapy Group (University of Helsinki) with the aim of finding a way to enable T-cell responses against cancer [128-131]. Those studies, revealed that TNF and IL-2 were efficacious tools to increase trafficking of T cells, increasing immune activation in the tumour and ultimately delivering better antitumour efficacy than unarmed or single transgene armed versions of the same oncolytic adenovirus. In essence, the TNF component can trigger activation of immune cells [132], production of other cytokines and chemokines [133, 134] as well as causing direct necrosis and apoptosis in tumour cells [134]. IL-2 has been used as a treatment for malignant melanoma and renal cell carcinoma patients [135], as it triggers T-cell stimulation and differentiation [136-138]. TILT-123 is a virus designed around the concept of enabling T-cell activity against solid tumours. Preclinical proof-of-concept data was generated coupling the virus with T-cell therapies such as adoptively cell transferred tumour infiltrating lymphocytes (ACT-TILs) [128, 130] and chimeric antigen receptor T cells (CAR Ts)[139]. Another critical aspect of TILT-123 studied preclinically is its ability to replace

pre-conditioning chemotherapy [140] and post-conditioning recombinant IL-2 [141]. The result of the investigations of TILT-123 together with T-cell based immunotherapies led to the initiation of a clinical trial (NCT04217473) using TILT-123 to enable TILs in advanced melanoma patients.

As new immunotherapies were developed into the clinics, the use of a virus able to enable T cells was an attractive approach for multiple of those therapies. That is the case for immune checkpoint inhibitors, a therapeutic approach that changed the landscape of clinical oncology in less than a decade, whose mechanisms of action involve directly or indirectly T-cell activity. As it was seen before, the use of multiple therapies needs to be assessed for benefit and in most cases optimised for optimal results. In that sense, the work included in this thesis revolves around the use of TILT-123 to enable checkpoint inhibitors for enhanced antitumour effects against solid tumours.

### 1.2.2 OTHER VIRUSES

The main focus of this work relates to the use of adenoviruses. Nevertheless, a brief description of other clinically relevant oncolytic virus platforms is included here.

#### *1.2.2.1 Herpes simplex virus*

The *Herpesviridae* family includes Herpes simplex viruses (HSV, also known as human alphaherpesvirus) 1 and 2. HSV infections affect approximately 60-95% of adults worldwide [142]. Among the two members of the *Herpesviridae* family, HSV-1 is the most widely used species for development of therapeutic tools. Wild type HSV-1 typically causes cold sores and it usually resides in neuron's terminal ganglia [143]. It is an enveloped, double stranded DNA virus with a genome of 152 kbp, and a diameter around 155-240 nm [144].

HSV have multiple non-essential genes that can be deleted or replaced when using them as therapeutic tools. Some of those non-essential genes that are deleted, are genes related to pathogenicity of the wild type virus [145]. Most frequently, oncolytic HSV-1 viruses implement mutations or deletions in ICP34.5 (one of the main responsible genes for the neuropathogenicity of the virus), ICP6 (a subunit of a ribonucleotide reductase), and ICP47 (a gene involved on blocking antigen presentation) [146].

Multiple oncolytic HSV candidates are included in the clinical trial registry where a total of 16 clinical trials using oncolytic HSV is described (Search criteria: Database= clinicaltrials.gov, intervention treatments= oncolytic herpes, Recruitment status=/Suspended OR Terminated OR Withdrawn, Search date June 17th 2020). Those candidates can be unarmed (C134), others are armed with therapeutic transgenes. GM-CSF has been armed as therapeutic cytokine in multiple candidates (Talimogene laherparepvec [T-Vec], HF-10, OH-2). Talimogene laherparepvec, an HSV-1 deleted for ICP34.5 and ICP47 and armed with GM-CSF under a cytomegalovirus promoter [147] is the only oncolytic virus approved so far by EMA and FDA [148]. In 2015, FDA approved the use of this drug for the treatment of unresectable melanoma. Since then, there has been moderate use of it in standard clinical practice and currently is being tested for its use combined with immune checkpoint inhibitors, where there has been promising results as the objective response rate increases without major impact on the safety aspects of the combination [149, 150]. The treatment with the virus is able to induce tumour regression in uninjected lesions in an immune mediated fashion, but providing less benefit than the one seen in injected tumours [151]. While antitumour immune cells are found in non-injected metastases after the treatment with talimogene laherparepvec, the virus itself seems to lack the ability to travel between lesions.

#### *1.2.2.2 Vaccinia virus*

Vaccinia virus is a species belonging to the *Chordopoxvirinae* subfamily, included in the *Poxviridae* family. Vaccinia virus has a double stranded DNA genome of approximately 190 kbp packed into an enveloped capsid, giving the viral particles the shape of a brick and a size around 270-350 nm [152]. The vaccinia virus replication cycle is unique among DNA viruses as it occurs entirely in the cytoplasm of the host cell instead of including nuclear phases as it is the case for the rest of DNA viruses [153]. Vaccinia virus creates a fast cytopathic effect on the host cell and as soon as 4-6 hours after infection, the host protein synthesis is almost ablated to give room to the production of around 10 000 viral copies in the 12-hour post infection period [154].

Some promising data has been shown in clinical trials that showed that intravenous administration of oncolytic vaccinia virus is able to transduce tumours without local administration [155]. In that sense, other studies have shown that vaccinia virus has natural tropism for tumours, mainly due to their leaky vasculature [156, 157]. Besides its affinity for tumours, further selectivity features need to be engineered into the viruses. Deleting a thymidine kinase gene from its genome limits its replication in healthy cells but it remains viable in nucleotide rich cancer cells [158, 159]. In spite of their natural tropism for tumours and the added selectivity by the thymidine kinase deletion, oncolytic

vaccinia viruses need to be further engineered to produce more selective drugs [160, 161]. Additionally, the large size of the vaccinia genome offers the possibility of arming oncolytic vaccinia viruses with one or multiple transgenes. Probably the biggest advantage of these viruses is the fact that they have been extensively used as vaccines for above two centuries [162].

Multiple oncolytic vaccinia virus candidates are included in the clinical trial registry where a total of 15 clinical trials using oncolytic vaccinia viruses is described (Search criteria: Database= clinicaltrials.gov, intervention treatments= oncolytic vaccinia, Recruitment status=/Suspended OR Terminated OR Withdrawn, Search date June 17th 2020). Among the candidates under clinical development, Pexastimogene devacirepvec is the candidate further ahead in the development line with a phase III clinical trial soon to be finished (NCT02562755) for the treatment of hepatocellular carcinoma in combination with sorafenib (a kinase inhibitor).

#### *1.2.2.3 Reovirus*

The *Reoviridae* is a family of viruses containing the genus *Orthoreoviridae*, of which the oncolytic reovirus Pelareorep is comprised. Orthoreoviruses are non-enveloped icosahedral viruses with a double capsid structure and a remarkably small genome 23.5 kbp and size 80 nm [146]. Reovirus is an acronym of respiratory and enteric virus, as initially they were not associated to any disease [163]. The majority of the human population has, at some point, been infected with reovirus, primarily during childhood [164]. The typically subclinical and self-limited infections with reovirus make them good candidates as cancer therapeutic tools especially for its use in immunocompromised patients, where it has proved to be a safe approach [165].

Pelareorep, is an isolate of type 3 Dearing reovirus that has natural preference to replicate and lyse cells with Ras pathway activated status, such as cancer cells [166]. In cells with activated Ras pathway, autophosphorilation of double stranded RNA-activated protein kinase is inhibited, disabling the innate protective mechanisms against viruses [146]. Reovirus mediated cell lysis is immunogenic and stimulates both innate and adaptive responses [167-169].

14 clinical trials are using the oncolytic reovirus, Pelareorep (Search criteria: Database= clinicaltrials.gov, intervention treatments= oncolytic vaccinia, Recruitment status=/Suspended OR Terminated OR Withdrawn, Search date June 17th 2020). And one phase III clinical trial (NCT01166542) has been completed where the virus was used in combination with

chemotherapies (carboplatin and paclitaxel) as a treatment in Platinum-refractory head and neck cancers. That trial finalised in 2014, provided positive results but not definitive for its approval by regulatory agencies. Although the use of oncolytic reovirus as monotherapy has proven largely ineffective, it seems to sensitize cancer cells to existing chemotherapeutic agents and radiation [165, 170].

## 1.3 THE USE OF ADENOVIRUSES TO ENABLE THE IMMUNE SYSTEM AND IMMUNOTHERAPIES

As discussed previously, the high variability between tumours, which additionally develop resistance to immunotherapies, suggest that antitumour treatments must include multiple non-overlapping therapies. In that way, there is an increased likelihood of efficiently activate endogenous long-lasting immunity against the treated tumour [171]. The broad impact on the tumour microenvironment of complex and flexible therapeutic agents, such as oncolytic viruses, offers interesting possibilities in that regard. In one hand, there is the fact of viruses being able to trigger multiple immune mechanisms that cover a wide range of immunostimulatory pathways [172, 173]. On the oher hand, because (wild type) viruses and humans have evolved together, different viruses generate different immune-responses [174]. Having a diverse armamentarium of viruses enables multiple possibilities to mix and match them with other immunotherapies in a way that they will synergize with each other.

Current notions of cancer and the therapeutic potential of the immune system, allowed a shift on how oncolytic viruses are used. Nowadays, they are used taking into consideration that among other antitumour mechanisms, they are able to stimulate immune responses against the tumour [118, 175, 176]. What is nowadays recognised as a virtue of the therapy, it was previously actively prevented or at least ignored, and their clinical application often included depletion of immune cells to increase the persistence of the virus [66, 177, 178]. Intrinsic features of oncolytic viruses and their replication in tumour cells provide immunostimulatory signals in the tumour microenvironment. Those features include the release of pathogen associated molecular patterns (PAMPs) as the molecular signature of the virus is recognized by the innate immune system [179], as well as damage associated molecular patters (DAMPs) providing tumour cells are lysed in an immunostimulatory fashion [179]. The presence of PAMPs and DAMPs and subsequent recognition by endogenous mechanisms increases the trafficking towards, and the activity of the immune system in the tumour [78, 180]. Additionally, when tumour cells

are lysed, tumour-associated antigens and neoantigens are released for antigen presenting cells to start the development of antitumour adaptive responses [73, 79].

As different viruses have different intrinsic properties including genome type, genome size, presence of envelope, particle size, structure, and life cycle, it is unsurprising that every virus interacts with the host, the tumour and the immune system in a different way. Taking the genome type as an example, DNA viruses (except poxviruses [181]) require to enter the nucleus to replicate. RNA viruses on the other hand can skip all the processes required to enter the nucleus as they replicate in the cytoplasm [182]. This difference, impacts in many aspects the pharmacokinetics and pharmacodynamics of the virus used as a drug as it changes the time for the virus to kill the cell but also determines the mechanisms that can be exploited to turn a wild type virus into a selective, oncolytic virus. In the same vein RNA viruses selectivity mechanisms are usually bound to defects on IFN pathways [183]. A cell suitable for virus replication with such selectivity mechanism will not trigger physiologic IFN levels and that will render poorer immune responses in the tumour milieu [184]. On the other hand, selectivity mechanisms for DNA viruses, like adenoviruses, can be tailored to target molecular features specific from cancer cells such as Retinoblastoma or P53 pathway deficiencies [184]. With this approach, it is possible to design a tumour selective virus while keeping the possibility to trigger immunogenic responses. That is one example on why adenoviruses are an attractive tool to enhance other immunotherapies. Below, more specific uses of adenoviruses with different immunotherapies will be reviewed.

### 1.3.1 VECTORED THERAPIES

In addition to the already mentioned tumour selectivity that can be achieved by genetic engineering of the adenovirus genome, it is also possible to include transgenes that will be expressed during the replication cycle in the host tumour cell. The length of transgenes that can be included in the virus without harming the replication efficiency is mostly determined by the freed space after endogenous genome is removed [79, 185]. Conceptually, the interaction between the virus and the protein encoded is double; the virus selectively vectors the transgene into the tumour, and at the same time, it is possible to find interactions between the triggered effects from both components that enhance antitumour efficacy [186].

The most remarkable features of delivering proteins with oncolytic adenoviruses when comparing to standard systemic administration are related to space and time. Space, as the expression of the cytokines is bound to the virus replication, and thus will only be produced locally at the tumour. Time,

as the viruses are replicating (at least theoretically) constantly at the tumour and so will be the release of the vectored agent. Head to head comparisons between local production of cytokines versus systemic administration of the same compound show how the former approach can significantly improve the outcome of the therapy [187]. IL-2 has been used as an immunotherapy delivered systemically in different cancer treatments [180, 188, 189]. In that approach, the cytokine is "diluted" in the patient, and the concentration of it in the tumour is the same or lower than in blood or other tissues that are subject of IL-2 effects. In contrast, when the production of IL-2 is at the tumour the ratio of IL-2 concentration between tumour and blood can be around one thousand [187]. The restriction of IL-2 effects to the tumour niche has not only double advantage as higher efficacy is achieved at the tumour but also IL-2 toxicities are diminished in the extra-tumour compartments [79, 187]. In the same example of IL-2 delivered by adenoviruses, the vectoring of the cytokine also helps to compensate weaknesses of the approach such as the short half-life of the molecule (6-8 minutes in blood [190]). Furthermore, the constant production and release of the transgene helps to avoid concentration peaks which are usually an advantage in these kinds of compounds [191, 192].

Similarly as described for IL-2, multiple other immune compounds have been armed on adenoviruses and used preclinically and clinically. A number of examples can be found in table 2.

*Table 2. Examples of immune compounds vectored by different adenoviruses.*

| Type | Transgene | References |
|---|---|---|
| Cytokines | TNFa | [132, 190] |
| | GM-CSF | [83, 116, 193, 194] |
| | IL-12 | [195-197] |
| | IL-15 | [198] |
| | IL-18 | [196] |
| | | |
| Chemokines | CCL20 | [198] |
| | IP-10 | [199] |
| | CCL3 | [200] |
| | CCL16 | [201] |
| | | |
| Immune ligands | CD40L | [115, 202] |
| | CD80 | [197] |
| | 4-1BBL | [195] |
| | OX-40L | [203] |
| | GITRL | [204] |
| | | |
| Antibodies | anti-CTLA-4 | [193, 205] |
| | anti-PD-L1 | [206] |

| | | |
|---|---|---|
| | anti-EpCAM/anti-CD3 | [207] |

Besides the possibilities of using genetic engineering to include transgenes in the genome of the virus, some novel approaches allow the delivery of therapeutic agents, like neoantigens. By using electrostatic bonds, those peptides can be attached to the viral particle in an almost instantaneous procedure [208].

### 1.3.2 ADOPTIVE-CELL THERAPIES

Some of the most promising advances in the field of immunotherapy of cancer are included under the "adoptive-cell therapy" type. This concept includes procedures where a number of cells are collected from a patient and expanded artificially outside of their body (with or without further modifications of those cells) to ultimately return those cells back to the patient as a mean of treatment.

Currently, the most successful adoptive-cell therapies are oriented to the T-cell compartment [209]. That fact, goes in line with the previously described importance of the T-cell compartment in the immunoediting process. Natural infection with (wild type) adenovirus in humans is strongly controlled by T-cell immunity [88, 210, 211]. That intrinsic interaction between T cells and adenoviruses provide a firm rationale to use oncolytic adenoviruses to enhance T-cell therapies.

#### *1.3.2.1 Adoptive-TIL therapy*

In the 1980's the efforts by S.A. Rosenberg and colleagues allowed the development and implementation in clinical trials of a form of adoptive cell therapy that used tumour infiltrating lymphocytes (TILs) [212, 213]. In this approach, a biopsy of the patient's tumour is collected and taken to the lab where the resident T cells (tumour infiltrating lymphocytes) are activated and expanded to ultimately transfer them back to the patient in order to increase the amount of T cells with antitumour effect [214]. To make the treatment effective, adoptive TIL therapy requires both a pre-conditioning and a post-conditioning treatments. The former includes the administration of lymphodepleting cyclophosphamide, and the latter includes high-dose IL-2 to keep the transferred T-cell graft active [212, 213]. Nowadays, an almost identical protocol is applied in metastatic melanoma patients rendering higher response rates (beyond 50%) than other commonly used treatments such as anti-CTLA4 or IL-2 [214]. Unfortunately, the application of the approach is

mainly restricted to melanoma, although there have been several attempts of applying the concept to other indications [215].

Together with the previously mentioned natural interaction between adenoviruses and the T-cell arm of the immune system, the use of oncolytic adenoviruses together with adoptive TIL therapy offers interesting possibilities based on the local delivery of a specific cargo oriented to boost the graft antitumour activity. A study performed by Tähtinen S et al [216], had the goal of providing empirical results on which cytokines are able to enhance further the antitumour efficacy of adoptive TIL therapy. TNFa and IL-2 were the cytokines with improved results. In that sense, including IL-2 is coherent with the post-conditioning high-dose IL-2 given in the standard adoptive TIL therapy protocols used in the clinics. The implications of TNFa seem to origin in its ability to increase T-cell trafficking and other beneficial interactions with various immune cells [129, 217]. These results led to the construction of an oncolytic adenovirus armed with those cytokines; Ad5/3-E2F-D24-TNFa-IL2 also named TILT-123 [128]. The virus has been studied in several preclinical studies [128, 130, 218] showing how the administration of the virus is not only more efficacious than the adoptive TIL therapy approach on its own [128] but also that the use of the virus allows skipping both pre and post-conditioning [140, 187]. As the virus is able to produce IL-2 constantly at the tumour site, the transferred TILs that end-up (re)entering the tumour will not have such a fierce competition for the cytokine with other immune-cells, for example regulatory T cells present at the bone marrow [219, 220]. Skipping pre and post-conditioning treatments allows the avoidance of toxicities associated to those, lethal in many cases [221]. In a broader sense, it can be claimed that the beneficial interaction between the virus and the transgenes expressed in the tumour is not only enhancing the TIL graft but also other endogenous immune populations rendering antitumour memory [128] and abscopal effect [222]. Because of this extensive work with TILT-123 and the great interest of the results those provided, a clinical trial was set up in which the mentioned virus is used together with adoptive TIL therapy (NCT04217473).

In a similar approach an adenovirus (TG1042, non-replicative) coding for IFNg, was designed to boost immune activity at the tumour site and help overcoming suppression to eventually support TIL activity. That virus was studied preclinically and subsequently in a Phase I/II clinical trial [223]. In that trial the overall response rate (ORR) was 38.5% (n=13) with a complete response rate (CRR) of 23.1%. An important observation from that trial is also the fact that the adenoviral treatment was well tolerated among all patients.

With further understanding on the key features on the mechanisms that limit the efficacy of adoptive TIL therapy, it would be possible to develop new therapies, including oncolytic viruses, aiming to overcome its limitations and provide more and better responses.

#### *1.3.2.2 Adoptive-TCR therapy*

T-cell receptor (TCR) therapy is an approach that merges principles of adoptive cell therapy and gene therapy. T cells from a patient are isolated, usually from blood, then genetically engineered with TCR alpha and beta chains targeted against a tumour epitope, and ultimately returned to the patient. To artificially express exogenous TCRs on T cells, viral vectors such as retroviruses or lentiviruses are used. In that sense, T-cell receptor engineering includes several challenges such as the requirement to select a correct TCR for each patient or the technical difficulty of pairing the exogenous alpha and beta chain while minimizing mispairing with endogenous chains [224]. Similarly as for other adoptive-cell therapies, therapeutic limitations appear when the cell transfer is not able to traffic towards tumours, when the suppressive tumour microenvironment prevents their activity, but also when off-target toxicities appear [225].

As for other immunotherapies, TCR therapy was initially developed on melanoma [226]. Studies showing the presence of T cell clones with antitumour efficacy were targeting tumour associated antigens MART-1 and gp100, allowed starting transducing autologous T cells to create antitumour T cells out of T cells that originally were not able to recognise tumour cells [227].

A total of 115 clinical trials using TCR therapy (source: clinicaltrials.gov; search criteria: "T-cell receptor–engineered T-cell therapy"; date of search: May 1st 2020) have been conducted. The number of trials initiated per year is steadily increasing from the beginning of the century. The majority (78%) of those studies are Phase I trials, and currently there is no Phase III trial ever conducted on the field. Multiple TCR therapies are being developed, mainly for solid tumours, but the above mentioned limitations are a pressing issue preventing optimal application of the therapy.

An additional benefit of TCR therapy is the multiple possibilities that it offers to gain understanding of how antitumour T cells behave in different conditions. In that sense, models such as the B16.OVA cell line (a metastatic melanoma cell line derived from B16-F0, engineered to express chicken ovalbumin) paired with the OT-I transgenic T cells (engineered to recognise ovalbumin) provide a framework that many researchers use in their studies to broaden the knowledge of cancer immunotherapy [228]. Work with this model has provided critical information on how adenoviruses can enhance adoptive T-cell therapies against cancer even in the presence of antiviral immunity [216]. Additionally, the use of adenoviruses can complement single-target therapies such as TCR-therapy as it helps to generate antitumour

responses oriented towards different epitopes, and increase the trafficking towards the tumour [129].

#### 1.3.2.3 Adoptive-CAR T therapy

Conceptually, TCR therapy and chimeric antigen-receptor T cell (CAR T) therapy are alike in many aspects. The biggest difference is that instead of transducing T cells with exogenous T-cell receptor alpha and beta chains, T cells receive a new receptor with an antibody-like receptor. Those antibody components include a single chain variable fragment (ScFv) that is coupled to intracellular signalling domains [229]. Since the development of the first CAR Ts in the late XX century, modifications mainly restricted to the intracellular signalling domain allowed the establishment of more CAR T therapy generations [230]. The second generation CAR Ts resulted after the inclusion of a co-stimulatory signalling domain (CD28 intracellular domain) after the CD3 zeta domain, to enhance proliferation. Third generation CAR Ts included a second intracellular stimulatory domain (*e.g.* 4-1BB, OX-40, ICOS), aimed at increasing the survival of the cell. In the past years, a new generation of CAR Ts has been proposed after the implementation of cytokine-releasing features into their design [231].

Tisagenlecleucel and axicabtagene ciloleucel have been approved by EMA and FDA as advanced therapies for blood cancers. Despite the remarkable clinical outcome of CAR T therapy in multiple haematological tumour indications, the same cannot be said about its use on solid tumours, where only scarce responses are assessed [184, 232]. The majority of limitations of other adoptive T-cell therapies are also applicable to CAR T therapy: lack of T-cell penetration into the solid tumour, reduction in activity and persistence as a result of the tumour immune suppressive mechanisms, antigen loss or heterogeneity in antigen expression [233-236]. Also similarly as with previously described therapies, oncolytic viruses could help to overcome those limitations as they promote trafficking of T-cells to the tumour, provide immunostimulatory signals and also promote de novo antitumor adaptive responses after oncolysis (although there is also evidence of epitope spreading after CAR T therapy [237, 238]).

The attractive combination of these therapies have been the subject of various groups aiming to get the best from each approach [139, 184, 230]. Regarding oncolytic adenoviruses, recent studies where cytokine and/or chemokine armed oncolytic adenoviruses used in combination with CAR T therapy rendered better antitumour efficacy. Also importantly, proof of concept data was generated on how the addition of these viruses was able to overcome CAR Ts limitations such as tumour trafficking or long term antitumor responses. Other studies aimed to understand the interaction of the therapies provided different angles like reduction of T-cell anergy [206, 239],

or prevention of tumour escape [240]. A study on the combination of therapies addresses it from an interesting angle where CAR Ts are used as carriers of oncolytic viruses to deliver them in the tumour with the aim to reduce neutralisation by antiviral antibodies [241].

#### *1.3.2.4 Other adoptive-cell therapies*

The most frequently studied cell type used as adoptive-cell therapy are T cells. Nevertheless, other cell types are being studied in similar approaches trying to obtain therapeutic benefit from the particular abilities of those cell types

Dendritic-cell adoptive cell therapy is another interesting approach focused on their abilities as antigen-presenting cells and thus as key initiators of adaptive responses. In 2013, Sipuleucel-T was approved for the treatment of asymptomatic or minimally symptomatic metastatic (non-visceral) castrate resistant prostate cancer in male adults in whom chemotherapy is not yet clinically indicated becoming the first dendritic-cell therapy approved by EMA (approved in 2010 by FDA for a similar prostate cancer indication). Besides Sipuleucel-T, many other clinical trials have been designed to study the feasibility of using dendritic cells as cancer therapy [242]. In parallel with the idea of using oncolytic viruses to enable T-cell therapies, multiple approaches including both therapies are being developed [195, 202, 243-245].

Other promising cell subset under scrutiny for usage as cancer therapy are natural killer cells. Providing the ability of this population to detect and lyse cells with low expression of major histocompatibility complexes (MHC), it can be a useful approach to apply on cancer as many tumour cells hide from adaptive responses by downregulating MHC expression at their surface [246, 247]. Many viruses also trigger MHC downregulation to “hide” from the immune system but a number of studies show a positive outcome on the natural killer cell subset after the use of oncolytic viruses [246-249]. Besides the direct effect on lysing tumour cells, having effector natural killer cells in the tumour microenvironment can also help to revert immune suppression by production of immunostimulatory cytokines such as TNFa and interferons [250, 251].

### 1.3.3 IMMUNECHECKPOINT THERAPIES

The immune system is an entity highly regulated to prevent deviations in homeostasis triggered by excess or defect of immune responses against their multiple targets. Immune checkpoints are one embodiment for regulation of

self-tolerance and immune responses. In that sense, immune checkpoints are divided according to their nature: inhibitory ones (those decreasing immune responses) and stimulatory ones (those increasing immune responses).

In the context of cancer, the evolutionary pressure exerted by the immune system together with the high plasticity of cancer cells, might result in the expression of inhibitory immune checkpoints [252]. At the tumour, the upregulation of those pathways translates in signals inducing self-tolerance resulting in the avoidance of tumour clearance by the immune system. The existence and understanding of those regulatory pathways was achieved in the last decade of the XX century mainly by the work conducted by the teams lead by T. Honjo [253] and J. Allison [254] which granted them the Nobel Prize of Medicine in the year 2018. A direct application of this knowledge was pursued by the administration of monoclonal antibodies targeting those inhibitory pathways. Clinical data obtained after the use of those drugs have changed the landscape in terms of therapeutic options for cancer patients in a constantly growing number of indications [241]. Probably the most impactful aspect from the approach is that unlike many of the previously standard therapies, those immunotherapies are able to provide long term responses, in many cases categorized as complete responses. Nevertheless, only a minority of patients display complete responses, and only 10-40% of them respond at all [255]. Probably, the technical simplicity of the approach (monoclonal antibodies administered intravenously), is another important factor contributing to the success of inhibitory checkpoint blockade. Other immunotherapies like the previously described adoptive-cell therapies also rendered cancer free patients, but the practical limitations of cell-based approaches is exponentially higher than these antibodies.

The relative novelty of the approach also means that the practical experience with these drugs is limited. Tackling some of those uncertainties could help increasing the number of patients that obtain complete responses from these therapies which would make a major impact on society. Already now, after almost ten years after the approval of the first drug of this class [256], there are some hints pointing to the features that correlate with response to monoclonal antibodies targeting immune checkpoints. Tumour infiltrating lymphocytes present in the tumour niche [257, 258], upregulation of inflammatory signals [259-261], and a high mutational burden [262], rank among the most predictive factors for response to immune checkpoint inhibitors [77]. The expression of certain proteins associated to these pathways is also under investigation for its potential prognostic value [263].

In the pursue of a higher response rate in cancer patients, the use of oncolytic viruses provide a rational approach to study, as they have provided signals of induction of T-cell presence at the tumour [129], as well as increasing the proinflammatory signature in the environment either by

mechanisms relative to their nature (PAMPs) or by the expression of immunostimulatory transgenes [78, 128]. The role of tumour mutational burden could be impacted if the virus mediated oncolysis exposes tumour associated antigens to mount adaptive responses. Providing that both immunotherapies have non-overlapping mechanisms of action, it is paramount to understand and optimize the co-administration strategies as it could influence the overall result of the therapy [264].

#### *1.3.3.1 Immune checkpoint inhibitors*

Immune checkpoint inhibitors (ICIs) are used in cancer to block the immunosuppressive ability of certain checkpoint inhibitors. Currently, approved ICIs target CTLA-4, PD-1 or PD-L1. Additionally, there is a longer list of immune checkpoints (LAG-3, TIM-3, TIGIT, CD96, BTLA, B7-H3, VISTA…) that are being targeted in earlier phases of development with slightly different effects on immune activities [252, 265]. Even if the particular fine tuning of the immune system can vary among ICIs, the principle of rational combination with oncolytic adenoviruses is widely applicable among them. For that reason, and due to the higher availability of clinical data from blockade of CTLA-4 and from PD-1 pathway, a special attention on those ICIs will be drawn in the current work.

##### 1.3.3.1.1 CTLA-4 inhibitors

In 2011, FDA and EMA approved the use of Ipilimumab, a monoclonal antibody against CTLA-4, for the treatment of patients with unresectable melanoma [266]. It was the first ICI ever approved. CTLA-4 (CD152) belongs to the B7/CD28 protein family and its activation triggers suppression of effector T-cell activities. Even if ipilimumab was originally approved as monotherapeutic agent against melanoma, currently it is mostly used in combination with nivolumab (an anti-PD-1 ICI). Additionally EMA has approved the use of ipilimumab for renal cell carcinoma patients who have not been treated before and are at moderate or high risk of the disease getting worse.

CTLA-4 is constitutively expressed in regulatory T cells and it can be expressed in other T-cell subsets after activation [267]. Transient CTLA-4 expression on the cell membrane is triggered after T-cell activation after which the protein in retrieved and stored in intracellular vesicles [268]. Effector T-cells that express CTLA-4 and other checkpoint inhibitors are often characterized as exhausted T cells [269]. CTLA-4 related immunosuppression is mediated by the binding of the CTLA-4 protein to B7 co-stimulatory signals

such as CD80 and CD86. In that process, CTLA-4 outcompetes (due to the higher affinity for CD80 and CD86) CD28 for the binding of those molecules, ablating the stimulatory signal required for efficient T-cell activation [270]. Regulatory T cells can play a role in immunosuppression when they are present in the tumour microenvironment by reducing the availability of co-stimulatory signals, by direct out competition for the stimulatory signals [271] but also by stripping out CD80 and CD86 from APCs in a process known as trans-endocytosis [272]. The prognostic value of CTLA-4 expression in tumours remain unclear, it has been associated with both increased [273] and decreased survival [274].

Using oncolytic viruses and CTLA-4 inhibitors it is an approach currently studied in 6 clinical trials (Search criteria: Database= clinicaltrials.gov, intervention treatments=[anti-CTLA-4] AND [oncolytic virus], Study status=/withdrawn, Search date May 31st 2020)(Table 3). Rationale in combining those therapies is the fact that direct oncolysis from the virus will release tumour associated antigens in a context with higher T-cell activation (as CTLA-4 is blocked and the threshold for T-cell activation is lower). In that sense, more antitumour T-cells will be activated and migrate to the tumour to exert antitumour functions.

*Table 3. EMA approved indications for PD-1 inhibitory antibodies*

| Anti-CTLA-4 + Oncolytic virus trials | | | | | | |
|---|---|---|---|---|---|---|
| aCTLA-4 used | Oncolytic virus used | Other intervention/treatments | Indication treated | Phase | Entry date | Trial identifier |
| Ipilimumab | Pexa-Vec (Vaccinia virus) | - | Advanced solid tumours | I | Nov-16 | NCT02977156 |
| Ipilimumab | HF10 (HSV) | - | Melanoma | II | May-17 | NCT03153085 |
| Tremelimumab | Pexa-Vec (Vaccinia virus) | Durvalumab (aPD-L1) | Colorectal cancer | I/II | Jul-17 | NCT03206073 |
| Ipilimumab | Cavatak (Coxsakievirus) | - | Uveal melanoma (Liver metastases) | I | Jan-18 | NCT03408587 |
| Ipilimumab | Talimogene lagerparepvec (HSV) | Nivolumab (aPD-1) | Breast cancer | I | Dec-19 | NCT04185311 |

Legend: HSV=Herpes simplex virus

None of the listed clinical trials included adenovirus-based candidates and in general the direct combination of anti-CTLA-4 and oncolytic adenoviruses has not been studied preclinically as such. Instead of administering both drugs separately, multiple preclinical studies have used the flexibility of adenoviral engineering to create anti.CTLA-4 coding adenoviruses [193, 205, 275]. The results from the use of adenoviruses for local delivery of anti-CTLA-4 show the advantages of having the ICI directly produced at the tumour: increased concentration at the tumour and its draining lymph nodes while the concentration of the antibody in the rest of the body is lower. In this way, anti-CTLA-4 associated adverse events after off-tumour targeting could be reduced. At the same time, this approach has the risk of the difficulty to stop ICI production in case of severe adverse immune events.

Overall, CTLA-4 inhibitors have minimal use at present, few indications approved for its use, and they are consistently outperformed (mainly in terms of safety) by other ICIs (*i.e.* PD-1 and PD-L1 inhibitors). Nevertheless,

potentially interesting strategies regarding the use of anti-CTLA-4 and oncolytic adenoviruses are yet unexplored, they might be more attractive if the antibody itself gains some clinical use.

#### 1.3.3.1.2 PD-1 and PD-L1 inhibitors

A second wave of ICIs was developed to block the PD-1 inhibitory pathway. In 2014 FDA approved pembrolizumab and nivolumab two monoclonal antibodies targeting PD-1. The following year (2015) EMA also approved both drugs for their use in melanoma. Since then 23 new approvals have been granted by EMA (pembrolizumab: 12, nivolumab: 10, cemiplimab: 1) (Table 4) and even a larger number of indications granted by FDA.

*Table 4. EMA approved indications for PD-1 inhibitory antibodies*

Time of approval granted by EMA (for any line of treatment or combination)

| PD-1 inhibitors | 2015 | | | | 2016 | | | | 2017 | | | | 2018 | | | | 2019 | | | | 2020 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Indication | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 |
| Melanoma | N | P, P | | | | N | | | | | N | P | | | | | | | | | | |
| Non-small-cell lung cancer | | | N | | N | | P | P | | | | | P | | P | | | | | | | |
| Renal cell carcinoma | | | | | N | | | | | | | | | | | N | | | P | | | |
| Heand and neck cancer | | | | | | | | | N | | | | | | P | | | | | P | | |
| Classical Hodgkin lymphoma | | | | | | | | N | P | | | | | | | | | | | | | |
| Cutaneous squamous-cell cancer | | | | | | | | | | | | | | | | | | C | | | | |
| Urothelial carcinoma | | | | | | | | | | N | P | | | | | | | | | | | |

Legend: N=Nivolumab, P=Pembrolizumab, C=Cemiplimab

Soon after the approval of PD-1 inhibitors, another set of monoclonal antibodies targeting PD-L1 were developed and approved for non-small-cell lung cancer, Merkel cell carcinoma and urothelial carcinoma (Table 5). With 3 approved uses by EMA, atezolizumab is the most successful candidate, followed by avelumab and durvalumab with a single indication approved for each drug.

*Table 5. EMA approved indications for PD-L1 inhibitory antibodies*

Time of approval granted by EMA (for any line of treatment or combination)

| PD-L1 inhibitors | 2015 | | | | 2016 | | | | 2017 | | | | 2018 | | | | 2019 | | | | 2020 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Indication | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 | Q3 | Q4 | Q1 | Q2 |
| Non-small-cell lung cancer | | | | | | | | | | | At | | | | D | | At | | | | | |
| Merkel cell carcinoma | | | | | | | | | | | Av | | | | | | | | | | | |
| Urothelial carcinoma | | | | | | | | | | | At | | | | | | | | | | | |

Legend: At=Atezolizumab, Av=Avelumab, D=Durvalumab

CD279 is a cell surface receptor that can be present on T cells and other immune cells. It was thought to be involved in programmed cell death

functions for T cells [253], and thus it received the name "Programmed cell death protein 1", also known as PD-1. Further understanding on the protein functions, allowed to reassess its function as inhibitory immune signal for adaptive responses [276]. Follicular helper T cells constitutively express PD-1 [277], but beside the majority of circulating T cells do not express PD-1 until TCR engagement or exposure to cytokines (*e.g.* IL-2, IL-7, IL-15, IL-21, TGFb) induce it [278, 279]. Additionally, B cells, myeloid dendritic cells, mast cells, and Langerhans cells have been discovered expressing PD-1 [280-284] with different regulation mechanisms and certainly less known relevance in terms of antitumour effect. PD-1, a homologous protein for CD28 [276], has two known ligands: PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC). Both Pd-1 ligands can be found in a wide variety of cell types including immune cells (such as APCs or suppressor cells of myeloid origin) but also non-immune cells (such as epithelial cells or tumour cells)[285, 286]. The major extrinsic source of PD-L1 and PD-L2 upregulation is IFNg [287], but they can be intrinsically upregulated by different phenomena such as chromosomal alterations, epigenetic modifications, aberrant oncogenic and tumour suppressor signals [288]. Downstream effects triggered after PD-1 interaction with its ligands mainly affect the TCR/CD28 and IL-2 associated positive feedback resulting in a reduction of cytotoxic activity, cytokine production (IL-2, IFNg, TNFa), cell proliferation, and cell survival [289-292]. In that sense, the most critical activity of PD-1 signalling occurs when there is a simultaneous TCR dependent stimuli for the T cell [285, 287, 293]. PD-1 is transiently upregulated soon after T-cell activation as well as in chronic immune activation [294]. In cancer, PD-1 expression has been found in both tumour infiltrating and circulating antitumour T cells, being associated with a lower ability to exert effector functions [295-299].

Although PD-1 and PD-L1 inhibition was initially thought to be equivalent, the empirical use of these molecules has revealed subtle and complicated differences between them. The first difference might be understood from the fact that PD-L1 blockade will not avoid T cells expressing PD-1 from being suppressed by PD-L2 signals expressed in a variety of solid and liquid malignancies with the potential of impacting the fate of antitumour T cells [300-302]. Mirroring the two ligands for PD-1, it has been studied that besides PD-1, PD-L1 can bind CD80 (B7.1)[303] which can reduce the availability of this co-stimulatory signal for T cells impacting in their ability to proliferate and driving active T cells into apoptosis [303, 304]. Meta analyses comparing the efficacy of PD-1 and PD-L1 blockade in similar indications fail to reach a homogeneous conclusion [305, 306], but in any case it seems that efficacy profiles are not drastically different.

The use of PD-1 inhibitory antibodies together with oncolytic viruses is a strategy with evident theoretical rationale, as each therapy has the potential to enhance the other. ICIs have limited possibilities to deliver antitumour effects

if there are no TILs in a tumour, and that is one of the clearest effects of different oncolytic viruses. On the other hand, T cells that home the tumour after the immunostimulatory signals fired by an oncolytic virus are potentially susceptible to be inactivated by inhibitory signals such as those in the PD-1 pathway, which can be prevented with the use of anti-PD-1. Currently, 28 clinical trials are listed in trial records with an embodiment of this approach (Search criteria: Database= clinicaltrials.gov, intervention treatments=[anti-PD-1] AND [oncolytic virus], Study status=/withdrawn, Search date May 31st 2020)(Table 6).

*Table 6. Listed clinical trials involving the use of PD-1 inhibitors and oncolytic viruses*

| Anti-PD-1 + Oncolytic virus trials | | | | | | |
|---|---|---|---|---|---|---|
| aPD-1 used | Oncolytic virus used | Other intervention/treatments | Indication treated | Phase | Entry date | Trial identifier |
| Pembrolizumab | Cavatak (Coxsakievirus) | - | NSCLC, bladder cancer | I | Jan-14 | NCT02043665 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | Placebo | Melanoma | III | Oct-14 | NCT02263508 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | - | Hepatocellular carcinoma | I | Jul-15 | NCT02509507 |
| Pembrolizumab | Cavatak (Coxsakievirus) | - | Melanoma | I | Oct-15 | NCT02565992 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | - | Head and neck | I | Dec-15 | NCT02626000 |
| Pembrolizumab | DNX-2401 (Adenovirus) | - | Glioblastoma, gliosarcoma | II | Jun-16 | NCT02798406 |
| Pembrolizumab | Cavatak (Coxsakievirus) | - | NSCLC | I | Jul-16 | NCT02824965 |
| Pembrolizumab | MG1-MAGEA3 (Maraba virus) | Ad-MAGEA3 (adenoviral vector) | NSCLC | I/II | Aug-16 | NCT02879760 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | - | Melanoma | II | Nov-16 | NCT02965716 |
| Nivolumab | Talimogene lagerparepvec (HSV) | Biomarker | Lymphoma, non-melanoma skin cancer | II | Dec-16 | NCT02978625 |
| Pembrolizumab | ONCOS-102 (Adenovirus) | CyPh | Melanoma | I | Dec-16 | NCT03003676 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | - | Sarcoma | II | Mar-17 | NCT03069378 |
| REGN2810 | Pexa-Vec (Vaccinia virus) | - | Renal cell carcinoma | I | Sep-17 | NCT03294083 |
| Nivolumab | Pelareorep (Reovirus) | Carfilzomib, Dexamethasone | Plasma cell myeloma | I | Jul-18 | NCT03605719 |
| Nivolumab | Talimogene lagerparepvec (HSV) | - | Malignant pleural effusion | I/II | Jul-18 | NCT03597009 |
| Pembrolizumab | Pelareorep (Reovirus) | - | Pancreatic cancer | II | Oct-18 | NCT03723915 |
| Pembrolizumab | ADV/TSK-tk (Adenovirus) | Valacyclovir, SBRT | NSCLC, breast cancer | II | Dec-18 | NCT03004183 |
| Pembrolizumab | MG1-MAGEA3 (Maraba virus) | Ad-MAGEA3 (adenoviral vector), CyPh | Melanoma, squamous cell skin carcinoma | I | Dec-18 | NCT03773744 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | - | Melanoma | II | Feb-19 | NCT03842943 |
| HX 008 | OH2 (HSV) | - | Solid tumors | I | Mar-19 | NCT03866525 |
| Nivolumab | Talimogene lagerparepvec (HSV) | Trabectedin | Sarcoma | II | Mar-19 | NCT03886311 |
| Pembrolizumab | Talimogene lagerparepvec (HSV) | - | Melanoma | II | Aug-19 | NCT04068181 |
| Pembrolizumab | Cavatak (Coxsakievirus) | - | Melanoma | II | Nov-19 | NCT04152863 |
| Nivolumab | Talimogene lagerparepvec (HSV) | Ipilimumab (aCTLA-4) | Breast cancer | I | Dec-19 | NCT04185311 |
| Pembrolizumab | TMV-018 (Measles virus) | 5-Fluorocytosine | Gastrointestinal cancer | I/II | Dec-19 | NCT04195373 |
| Pembrolizumab | Cavatak (Coxsakievirus) | MK-7684 | Melanoma | I/II | Mar-20 | NCT04303169 |
| Pembrolizumab | ONCR-177 (HSV) | - | Solid tumors | I | Apr-20 | NCT04348916 |
| Pembrolizumab | OH2 (HSV) | - | Solid tumors | I/II | May-20 | NCT04386967 |

Legend: CyPh=Cyclophosphamide, HSV=Herpes simplex virus, NSCLC=non-small-cell lung cancer, SBRT=Stereotactic Body Radiation Therapy

Of those trials, above 50% (15/28) are focused on studying HSV viruses, including the only approved oncolytic virus in the western market (talimogene laherparepvec) in combination with pembrolizumab or nivolumab. The remaining trials include coxsackievirus (5 trials), adenovirus (3 trials), reovirus (2 trials), vaccinia (1 trial), maraba (1 trial) and measles (1 trial)

oncolytic viruses together with a PD-1 inhibitor candidate. None of the trials involving adenoviruses are completed yet for conclusions to be made. At the preclinical level, there are multiple studies showing how when the therapies are combined it results in an increase in tumour growth control and enhances the development and sustaining of immune responses against the tumour [307, 308]. Other preclinical studies using oncolytic adenoviruses [309] showed that they are able to increase the presence of CD8 T cells in the tumour as well as increasing the PD-L1 expression in the tumour, creating a good foundation to study the combination deeper.

The same rationale described for the combination of PD-1 inhibitors and oncolytic viruses, is behind multiple trials using oncolytic viruses with PD-L1 inhibitors (Search criteria: Database= clinicaltrials.gov, intervention treatments=[anti-PD-L1] AND [oncolytic virus], Study status=/withdrawn, Search date May 31st 2020)(Table 7)

*Table 7. Listed clinical trials involving the use of PD-L1 inhibitors and oncolytic viruses*

| Anti-PD-L1 + Oncolytic virus trials | | | | | | |
|---|---|---|---|---|---|---|
| aPD-L1 used | Oncolytic virus used | Other intervention/treatments | Indication treated | Phase | Entry date | Trial identifier |
| Atezolizumab | LOAd703 (Adenovirus) | Gemcitabine, Nab-paclitaxel | Pancreatic cancer | I/II | Mar-16 | NCT02705196 |
| Durvalumab | ONCOS-102 (Adenovirus) | - | Colorectal cancer, Ovarian cancer | I/II | Nov-16 | NCT02963831 |
| Durvalumab | Pexa-Vec (Vaccinia virus) | Tremelimumab (aCTLA-4) | Colorectal cancer | I/II | Jul-17 | NCT03206073 |
| Atezolizumab | Talimogene lagerparepvec (HSV) | - | Colorectal cancer, breast cancer | I | Aug-17 | NCT03256344 |
| Atezolizumab | Talimogene lagerparepvec (HSV) | - | Breast cancer | I | Jan-19 | NCT03802604 |
| Durvalumab | MEDI5395 (NDV) | - | Solid tumors | I | Mar-19 | NCT03889275 |
| Atezolizumab | Pelareorep (Reovirus) | Trastuzumab, letrozole | Breast cancer | I | Sep-19 | NCT04102618 |
| Atezolizumab | LOAd703 (Adenovirus) | - | Melanoma | I/II | Oct-19 | NCT04123470 |
| Avelumab | Pelareorep (Reovirus) | Paclitaxel | Breast cancer | II | Jan-20 | NCT04215146 |

Legend: HSV=Herpes simplex virus, NDV=Newcastle disease virus

Of the 9 clinical trials involving oncolytic viruses and PD-L1 inhibitors, adenovirus candidates include 33% (3 trials) of the total, while HSV and reovirus have 2 trials each, and vaccinia virus and NDV have 1 ongoing trial each. None of the trials including adenoviruses have finalised yet but, again, the already mentioned preclinical data supporting the combination of oncolytic viruses with PD-1 inhibitors also support their use together with PD-L1 inhibitors. Some studies aimed at study the possible differences arising from the selection of the immune checkpoint target when combined with oncolytic viruses. Capuccini F. et al [113] produced data showing that while PD-1 inhibition enhances tumour growth control when used together with their Adenovirus and Vaccinia platforms, PD-L1 inhibition is unable to provide similar results. Those results might indicate that PD-1/PD-L2 interactions can be a critical source of immune inhibition in their model.

Despite the theoretical rationale for the use of oncolytic adenoviruses together with PD-1 and PD-L1 inhibitors, there are major uncertainties

regarding the practical aspects of the idea. Some of those include the sequence of administration, the length of the treatment, the selection of the immune checkpoint inhibitor, as well as the possibility of using an arming device for the virus that would be able to increase even further the efficacy of the therapeutic approach.

#### *1.3.3.2 Immune checkpoint stimulators*

In parallel to immune checkpoint inhibitors that aim to block the activity of suppressive pathways, an opposite approach is to stimulate the activity of several immunostimulatory pathways with immune checkpoint stimulators (ICS). The typical embodiment of this approach is with monoclonal antibodies or soluble protein factors with the ability to activate the receptor they are designed to bind. OX40, 4-1BB, GITR and ICOS are the most representative targets [252]. The clinical development of these molecules is not closely as advanced as for immune checkpoint inhibitors, and there are no approved ICS for any indications by EMA or FDA. It is likely that the slow progress of this is linked to the life-threatening cytokine-release syndrome suffered by six volunteers at a Phase I clinical trial studying the safety of an immune super agonist [310]. Currently there are oncolytic adenoviruses embodying the immune checkpoint stimulation approach under clinical development like LOAd703 (NCT03225989, NCT04123470, NCT02705196). LOAd703 is a chimeric serotype (5/35) oncolytic adenovirus, engineered to express two immunostimulatory agents: a membrane bound trimer of CD40L and 4-1BBL. Other viruses in earlier stages of drug development are coding for ICS molecules like OX40L or 4-1BBL [311, 312]. While some preclinical studies with ICS coding virus managed to induce tumour growth control and a clear shift towards antitumour activity [313], other studies showed a strong PD-L1 and PD-L2 upregulation that crippled the therapeutic result of the approach [311, 312]. The upregulation of immunosuppressive factors can be understood as a counter response to the intense stimulation produced intrinsically by the virus and the ICS concurrently.

While the concept of immune checkpoint stimulators is relatively new, previously characterized targets such as CD40, CD40L, CD28, CD80, CD86, CD27 and CD70 could be included in the same category [252]. When increasing the list of ICS, the list of oncolytic adenoviruses engineered to activate those pathways is longer [115, 197, 202, 243].

### 1.3.4 OTHER IMMUNOTHERAPIES

The field of immunotherapy for cancer is a blooming area for development of new approaches. Besides the already mentioned modalities of immunotherapy, there is an ever-growing number of them that will not be deeply discussed here.

In many cases, when a new immunotherapy is conceived, it can be somehow implemented into the oncolytic virus idea. Examples of other promising immunotherapies based on an oncolytic adenovirus are those ones coding for antibody-based cell engagers [207, 314], or the oncolytic adenoviruses used as vectors for non-cell based cancer vaccines [208, 315].

# 2 AIMS OF THE STUDY

The aims of this study are

1. to evaluate the impact of different oncolytic viruses on the tumour immune microenvironment in the context of T-cell therapy (I);

2. to assess the potential antitumour benefits derived from the use of engineered adenoviruses together with immune checkpoint inhibitors (II, III); and

3. to study if tumours refractory to immune checkpoint inhibitors respond to virotherapy (IV).

# 3 MATERIALS AND METHODS

## 3.1 TUMOUR MODELS

### 3.1.1 MOUSE MODELS [II, III, IV]

Animal experiments involving mice were performed on 4-6 week old animals purchased from Envigo (Indianapolis, IN, USA). Those animals were housed in biosafety level 2 conditions. Animal work started one week after arrival of the animals to respect quarantine guidelines. All the cell lines used in animals were tested to be pathogen free prior to engraftment (Surrey Diagnostics Ltd, Granleigh, UK). Tumours were originated after subcutaneous injection of cells in a volume of 100 µL of serum free media. When tumours were above 4 mm as maximum diameter, animals were randomized into different treatment models.

#### *3.1.1.1 B16.OVA (melanoma)*

B16.OVA is a murine melanoma cell line engineered to express chicken egg albumin (ovalbumin). It has been a widely used cell line in immunology due to the possibilities that it offers in terms of studying the generation of immunity against a xenogeneic protein. It was designed by Richard Vile's research group to whom we are thankful for such a useful gift. It was cultured in RPMI 1640 supplemented with 10% FBS, 5 mg/mL G-418 (Roche, Basel, Switzerland), 2 mMol L-Glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA, except if otherwise indicated). 37ºC and 5 % $CO_2$ were the culturing conditions used to culture the cells. In vivo studies with B16-OVA started with subcutaneous delivery of 2.5 x $10^5$ cells in the lower flank of C57BL/6-OlaHsd mice. 11 days after engraftment, majority of animals presented palpable tumours (above 4 mm in maximum diameter) and they were included in treatment phase of the studies.

#### *3.1.1.2 4T1 (triple negative breast cancer)*

4T1 (ATCC, CRL-2539) is a murine breast cancer cell line cultured in RPMI-1640 medium supplemented with 10% FBS, L-Glutamine (2 mMol), penicillin (100U/mL), streptomycin (100 mg/mL)(all from Sigma-Aldrich, St. Louis, MO, USA). 37ºC and 5 % $CO_2$ were the culturing conditions used to culture the cells. 1 x $10^5$ 4T1 cells were implanted in the lower left flank of BALB/cf3h animals. The majority of animals presented tumours above 4 mm by day 8 post-engraftment.

### 3.1.2 HAMSTER MODELS [I]

Hamster-based animal experiments were performed on 4-6 week old male Syrian hamsters (French colony) purchased from Charles River Laboratories (Wilmington, MA, USA). Those animals were housed in biosafety level 2 facilities and the animal work started only after one week quarantine was completed. All the cell lines used in animals were tested to be pathogen free prior to engraftment (Surrey Diagnostics Ltd, Granleigh, UK). Tumours were originated after subcutaneous injection of cells in a volume of 100 µL of serum free media. When tumours were above 4 mm as maximum diameter, animals were randomized into different treatment models. The advantage of using Syrian hamster models allow to study human adenoviruses in an environment of semi-permisivity which allows self-amplification of the virus. That feature is not present in other murine models and it is what makes Syrian hamsters a precious model for us.

#### *3.1.2.1 HapT1 (pancreatic carcinoma)*

A Syrian hamster syngeneic pancreatic cell line has been used for *in vitro* and *in vivo* studies. The cancer cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, L-Glutamine (2 mMol), penicillin (100U/mL), streptomycin (100 mg/mL)(all from Sigma-Aldrich, St. Louis, MO, USA). 37ºC and 5 % $CO_2$ were the culturing conditions used to culture the cells. *In vivo* studies included the subcutaneous administration of 2 x $10^6$ cells.

#### *3.1.2.1 DDT1-MF2 (leiomyosarcoma)*

DDT1-MF2 is a syngeneic cancer cell line for Syrian hamster models. This cell line has been used in an *in vivo* study. The cell line was cultured *in vitro* with DMEM medium supplemented with 10% FBS, L-Glutamine (2 mMol), penicillin (100U/mL), streptomycin (100 mg/mL)(all from Sigma-Aldrich, St. Louis, MO, USA). 37ºC and 5 % $CO_2$ were the culturing conditions used to culture the cells. For the engraftment of tumours, *2.5* x $10^5$ cells were subcutaneously injected in the upper right flank of the animals.

### 3.1.3 HUMAN MODELS [III]

#### *3.1.3.1 Patient derived histocultures (urological malignancies)*

Surgically removed malignant tissue was sampled and processed into a single cell suspension first by mechanical disruption of the tissue onto small fragments that were digested overnight with a set of enzymes (collagenase type I (170 mg/L), collagenase type IV (170 mg/L), DNase I (25 mg/mL) and elastase (25 mg/mL) (all enzymes from Worthington Biochemical, Lakewood, NJ, USA). After overnight incubation with the enzyme cocktail, the digestion product was filtered through a 100 µm mesh and treated with Ammonium-Chloride-Potassium (ACK-buffer). The resulting single-cell suspension was plated in medium containing RPMI-1640 medium supplemented with 10% FBS, L-Glutamine (2 mMol), penicillin (100U/mL), streptomycin (100 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5 % $CO_2$. After plating the cell suspension and an incubation always inferior than 6 hours, the cell cultures were used for various testings.

## 3.2 THERAPEUTIC APPROACHES

Individual treatment diagrams have been included as figures for individual *in vivo* experiments (described in studies I-IV). *In vitro* treatments were also detailed specifically for each experiment.

### 3.2.1 VIRUS TREATMENTS [I, II, III, IV]

This work is ultimately oriented towards the use of oncolytic adenoviruses and other oncolytic viruses as therapeutic tools for the treatment of human cancer. One of the limitations of preclinical *in vivo* studies in research animals is that they might not provide adequate conditions for those viruses to replicate the same way it occurs when the host cell is human. In that sense, some experimental conditions used in the present work allowed virus replication and others did not. Common murine models do not support replication of human adenovirus serotype 5, but it has historically been validated as a legitimated tool to model adenoviral drugs that were used similarly in humans.

#### *3.2.1.1 Non-replicative conditions*

Serotype 5 adenoviruses expressing murine TNFa (Ad5-CMV-mTNFa) and murine IL-2 (Ad5-CMV-mIL2) under the cytomegalovirus promoter, were mixed in a 1:1 proportion. The construction of those viruses has been described in a previous work [129]. 1 x $10^8$ viral particles (vp) were injected intratumorally in a total volume of 50 µL of PBS using 30G insulin needles. In mock groups, animals were injected with 50 µL of PBS to counter the possible

effect in tumours derived from the needle puncture and the physical placement of a volume intratumourally.

#### *3.2.1.2 Replicative conditions*

In study I, multiple viruses were used as treatment under replicative conditions as the hosts were Syrian hamsters. Those viruses were: Ad5/3-E2F-d24 (Adenoviridae), VVtd-tomato (JX-929 strain) (Poxviridae), HSV-1 (17+)Lox-PmCMVGFP (Herpesviridae; a kind gift from Beate Sodeik, Hannover Medical School, Germany), and Pelareorep (Reolysin) (Reoviridae; a kind gift from Oncolytics). Additionally, an engineered version of the adenovirus candidate, expressing TNFa and IL-2 (Ad5/3-E2F-d24-hTNFa-IRES-hIL2, also known as TILT-123) was used. The dose calculation method is explained in full detail in study I. All the viruses were administered in a total volume of 50 μL using PBS as diluent with 30G insulin needles. In the mock group, animals were similarly injected with 50 μL of PBS to counter the possible effect in tumours derived from the needle puncture and the physical placement of a volume intratumourally.

In study III, TILT-123 (Ad5/3-E2F-d24-hTNFa-IRES-hIL2) and an unarmed control virus (Ad5/3-E2F-d24) were used to infect human cancer cells using a multiplicity of infection (MOI) of 100, which means that for each cell used, 100 viral particles were added. In those conditions viruses were expected to replicate.

### 3.2.2 T-CELL RELATED THERAPIES

#### *3.2.2.1 Adoptive-cell therapy*

In study I, Adoptive-cell transfer of tumour infiltrating lymphocytes (ACT-TILs) was used as a way to study the effect of oncolytic viruses on T-cell therapies. To generate the ACT-TILs, Syrian hamsters were engrafted with four HapT1 subcutaneous tumours and let them to grow. When tumours were around 10 mm in maximum diameter, they were collected and cut into fragments of around 2 mm diameter. Tumour pieces were cultured in gas-permeable rapid-expansion wells (G-Rex, Wilson Wolf, Saint Paul, MN, USA). Growth media consisted of RPMI-1640 medium supplemented with 10% FBS, L-Glutamine (2 mMol), penicillin (100U/mL), streptomycin (100 mg/mL), HEPES (15 mMol), 2-mercaptoethanol (50 μMol), Sodium pyruvate (1 mMol), concanavallin A (1 μg/mL) (all of the above from Sigma-Aldrich, St. Louis, MO, USA), and human IL-2 (6 000 U/mL). On day 5, 7 and 9 after the culture was

started, half of the medium was extracted and replaced with fresh one. By day 10, when microscopically visible TIL growth had taken place, TILs were collected and used for ACT.

#### *3.2.2.2 Immune checkpoint inhibitors*

PD-1 and PD-L1 inhibitors have been used in several *in vivo* and *in vitro* experiments. For murine experiments, systemic treatments of 0.1 mg of anti-PD-1 (clone RMPI-14) or anti-PD-L1 (clone 10F.9G2), both from BioXCell (Lebanon, NH, USA) were administered intraperitoneally. Anti-human PD-L1 (Atezolizumab, Roche, Basel, Switzerland) was used at a 20 μg/mL final concentration.

## 3.3 EXPERIMENTAL OUTCOMES

### 3.3.1 BIOLOGICAL OUTCOMES [I, II, III, IV]

#### *3.3.1.1 Biodistribution analyses*

The ACT-TIL cell graft was produced as described previously. For *in vivo* biodistribution analyses, the T cells were labelled with $^{111}$Indium-oxine (4.82 ± 0.72 MBq). After labelling and washing the cells, they were intraperitoneally transferred into Syrian hamsters. The treated animals were studied using a whole-body scan (NanoScan SPECT/CT, Mediso, Budapest, Hungary) at days 1, 3 and 6 after engraftment. Using the CT images, tumours were delineated and the specific radioactivity for the tissue was calculated. Further TIL biodistribution analyses were performed *ex vivo*, with a gamma counter (Wizard 3, Perkin Elmer, Waltham, MA, USA). As on day 6 both *in vivo* and *ex vivo* measurements were available, validation calculi were performed comparing the values from both methods.

#### *3.3.1.1 Gene expression analyses*

Gene expression analyses were performed on tumour tissues on studies I and IV. In both cases, tumour tissue was collected from animals and stabilised in RNA-preserving solution (RNAlater, Sigma-Aldrich, St. Louis, MO, USA) until RNA was extracted. For RNA extraction, RNeasy kits (Qiagen, Hilden, Germany) were used following manufacturer's indications. Using a spectrophotometer (Biophotometer, Eppendorf, Westbury, NY, USA), RNA

concentration was adjusted for optimal sequencing conditions. In study I, a 96-gene custom panel designed to analyse T-cell signatures from Syrian hamster origin was ordered (NanoString Technologies, Seattle, WA, USA). Data collection and data analysis were outsourced (single-blind analysis). In short, gene expression was normalized. Then, quality-control checks were performed on all the samples and for all the gene probes included in the panel. Differential expression of genes of interests among experimental groups was represented in volcano plots based on the significance and fold-change in expression for each gene. In study IV, sequencing, data cleaning and quantitative analyses were outsourced (BGI Tech Solutions, Tai Po, Hong Kong).

#### *3.3.1.2 Cell viability analyses*

Cell viability was assessed upon different conditions by using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), following the manufacturer's instructions. Every experimental condition was plated in quadruplicates and mock cell viability was set as reference (100 %) to compare viability with other conditions. In this colorimetric method, a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS) is metabolized by mitochondrial activity in viable cells. When the MTS is reduced, it turns into a coloured formazan product that can be quantitated at 490 nm. The amount of formazan that is produced is directly proportional to the number of viable cells in the culture.

#### *3.3.1.3 Cytokine analyses*

To measure cytokine concentrations, tumour biopsies and cell culture supernatants were used as starting materials. For tumour biopsies, tissues were collected, immediately snap-frozen on dry ice and stored at -80°C. In studies II and III, Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine kits (BD, Franklin Lakes, NJ, USA) were used to study the concentration of murine TNFa, IL-2, IFNg, IL-4, IL-6, IL-10 and IL-17a. In study III, cell culture supernatants were collected, centrifuged to remove any possible cell remaining and then assayed with a custom Legendplex panel and a Free Active/Total TGF-b1 detection kit (both from Biolegend, Dedham, MA, USA) in order to quantify the presence of IFNg, TNFa, IL-2, IFNb, granzyme B, CXCL10, IL-6, arginase, and TGF-b1. The measurements were performed by Accuri C6 Cytometer and analysed with FCAP Array Software (both from BD, Franklin Lakes, NJ, USA) under manufacturer's instructions. Cytokine concentrations were normalized by total protein concentration present in the

sample, after measurements were taken by spectrophotometry (Biophotometer, Eppendorf, Westbury, NY, USA) and relative concentrations calculated with Warburg-Christian method.

#### 3.3.1.4 Cytometric analyses

In studies II and III, fluorochrome based flow-cytometry was performed on different tissue samples and *in vitro* cultured cells. In study IV, metal-based flow cytometry was performed on tumour samples. For tissue analyses, samples were collected, disrupted with a handheld rotor–stator, and subsequently passed through a 70µm cell strainer to obtain a single cell suspension. Single cell suspensions were centrifuged and stored in media containing FBS (90%) and DMSO (10%) at -80°C until the analyses were performed. *In vitro* cultured cells used in flow cytometric analyses were washed and detached from the flasks prior to the staining. Cells for fluorochrome-based flow cytometry were stained, fixed in 1.6 % (v/v) paraformaldehyde and analysed with either Accuri C6 Cytometer (BD, Franklin Lakes, NJ, USA) or Sony SH800Z cytometer (Sony, Tokyo, Japan) under recommended instructions. Cells for metal-based flow cytometry were first stained with 5uM cisplatin as a viability marker. Then, cells were fixed with 1.6 % (v/v) paraformaldehyde. Subsequently samples were barcoded with 20 unique palladium isotopes as per manufacturer's protocol (Fluidigm Co, San Francisco, CA, USA). Afterwards, antibodies for cell surface staining were added first, followed by intracellular staining for nuclear proteins. Additionally, DNA was stained with 1:4000 Iridium and fix-perm buffer (Fluidigm Co, San Francisco, CA, USA) and stored overnight. Samples were acquired the following day on CyTOF3-Helios mass cytometer in double-distilled H2O spiked with 10% EQ four element Calibration Beads (Fluidigm Co, San Francisco, CA, USA). After acquisition, sample files were normalized over time with the use of the calibration beads and deconvoluted into individual sample files through Fluidigm debarcoding software (Fluidigm Co, San Francisco, CA, USA). Detailed tables of antibodies used in every experiment is provided in the materials and methods section of each publication. CD16 and CD32 antibodies were used to prevent unspecific binding of the antibodies of interest.

#### 3.3.1.5 Histopathology

In study III, histopathological analyses were performed on tissue samples of human and murine origin. After sample collection, the tissues were fixed in formalin (10 %) for 48 hours and preserved in ethanol (70 %) until further

paraffin embedding. When the paraffin-embedded tissue was ready 4-5 μm thick slides were cut and sent to the pathology services. Human tumour tissue was stained with Haematoxylin and eosin (H&E) for pathological analyses. Additionally CD8 (clone 4B11, CD8-4B11-L-CE-H, Leica Biosystems, Wetzlar, Germany) and PD-L1 (VENTANA SP142 assay, Roche, Basel, Switzerland) stainings were performed and analysed by a trained pathologist following routine parameters in the clinical set-up. Histopathological analyses from murine samples were carried out by a trained veterinary pathologist in a similar way.

### 3.3.2 DIRECT OUTCOMES [I, II, III, IV]

#### *3.3.2.1 Antitumour efficacy*

Across all studies, *in vivo* antitumour efficacy has been assessed by studying the impact of the treatments on the tumour volume. Tumour volume was calculated using a standardized formula:

$$Tumour\ volume = \frac{Maximum\ diameter\ \times\ Minimum\ diameter^2}{2}$$

Tumour volume records were created after repeated measurements that were acquired at different time intervals using a digital calliper. Animals with tumours that reached the threshold established as maximum ethically allowed size (according to the model and number of tumours growing in the same individual), were sacrificed and data collection stopped. When tumours shrank to zero, it was recorded as 0.0001 $mm^3$ to avoid mathematical limitations.

#### *3.3.2.2 Survival analyses*

Similarly, in all studies, *in vivo* antitumour efficacy was assessed by studying the impact of the treatments on overall and tumour specific survival. Animals were taken into the survival studies as soon as the treatments started and then followed until they reached the maximum tumour volume threshold, which was counted as a tumour associated death or for a reasonable time period after the animals had cleared the tumours. In some cases, animals presented necrotic wounds in the skin near the tumour area (independent of treatments, due to skin invasion of subcutaneous tumours), in those cases, non-tumour associated death was registered as they were killed to avoid the wound associated complications but not due to tumour progression.

## 3.4 STATISTICS

The results of *in vitro* experiments regarding tumour growth over time, was analysed by using a linear mixed-model using time-correlated logarithmically transformed volumetric measurements. For those analyses SPSS software (IBM, Armonk, NY, USA) version 24 for study I, and version 25 for studies II-IV. To assess synergy in tumour growth control, calculi based on fractional tumour volume were performed. Kaplan-Meier survival data was analysed for significance using log rank Mantel-Cox text using GraphPad Prism (GraphPad Software, San Diego, CA, USA) version 7 for study I, and version 8 for studies II-IV. The same software was used to establish treatment-related hazard ratios, correlation between variables (using Pearson's R test), comparison in different parameters between groups (using Mann-Whitney u test or unpaired t test with Welch's correction), as well as for the evolution of variables over time (two-way ANOVA). The threshold for significance was set at P values below 0.05 for all the studies.

## 3.5 ETHICS

All experiments included in this work have been performed following Finnish and European ethics guidelines for research.

Following the instructions gathered in the "Act on the Protection of Animals Used for Scientific or Educational Purposes" (497/2013) and the "Protection of Animals Used for Scientific or Educational Purposes" (564/2013), as well as the European Directive 2010/63/EU, methods and practical procedures were drafted for evaluation and approval by the ethical committee of the Animal Experimental Board (ELLA) of the Regional State Administrative Agency of Southern Finland. Following that framework, animals were observed daily and overall health assessed.

Similarly, studies performed on patient samples (HUS/850/2017), were reviewed and approved by the Central Hospital Operative Ethics Committee. Before samples were collected for subsequent analyses, patients signed an informed consent form accepting to contribute in these studies.

# 4 RESULTS AND DISCUSSION

Despite promising results provided with a variety of immunotherapies, currently, the percentage of patients benefitting from those therapies is low. T-cell centred immunotherapies, and immune checkpoint inhibitors such as PD-1 or PD-L1 inhibitors are the most implemented type of immunotherapies in cancer clinics but even in those approaches the majority of patients do not benefit from their use [316, 317]. Among those patients that benefit the most from immune checkpoint inhibitors, it has been observed that their tumours have an immunologically "hot" status, meaning that there is an inflammatory cytokine signature [259, 260, 318] and there is presence of tumour infiltrating lymphocytes [319, 320]. The inherent mechanism of action of oncolytic viruses can favour the transition of immunologically "cold" tumours (lacking tumour infiltrating lymphocytes and immunostimulatory signals) into "hot" ones, making them an interesting approach when trying to expand the benefit of immune checkpoint inhibitors to a larger percentage of the cancer patient population. Moreover, the use of an oncolytic virus tailored around the idea of enabling T-cell responses against cancer could be particularly useful when trying to enable T-cell responses against solid tumours. Herein, oncolytic viruses were studied for their ability to stimulate T-cell responses in a context of PD-1 pathway immune checkpoint inhibition.

## 4.1 ONCOLYTIC ADENOVIRUS ENGAGES T-CELL RESPONSES AGAINST SOLID TUMOURS BETTER THAN OTHER ONCOLYTIC VIRUSES (I)

As the aim for this work is to study how oncolytic viruses are able to improve T-cell responses against cancer, it was particularly relevant to understand if there is major impact derived from the selection of the viral background. Due to their dominant presence in clinical trials regarding oncolytic viruses, adenovirus, herpes simplex virus, vaccinia virus and reovirus were the four candidates subject of comparison in study I. In this case the use of the different oncolytic viruses was coupled with adoptive-cell therapy of tumour infiltrating lymphocytes as an embodiment of T-cell therapy. Overall, the adenovirus candidate delivered better results in terms of therapeutic benefit for the animals suffering from cancer. It is relevant to understand that the results should be understood in terms of adenoviruses being better at triggering T-cell responses than the other viruses tested, but there is a possibility for other viruses interacting better with other compartments of the immune system and thus being potentially better candidates if the form of immunotherapy is changed.

### 4.1.1 DIRECT ANTITUMOUR EFFICACY

When studying the interaction between different viruses and the T-cell compartment, it is relevant to study the interaction at the molecular level but the ultimate goal is always oriented towards tumour regression and curative effect. In figure 1 (Study I), it is addressed how different viruses manage or not to deliver tumour regression and the impact of those into overall survival. All the animals were treated with both adoptive cell transfer of tumour infiltrating lymphocytes (performed only once, intraperitoneally), and 15 rounds of oncolytic virotherapy or PBS (intratumourally).

Tumours treated with oncolytic adenovirus were the only tumours significantly smaller than those tumours injected with PBS. By day 21, after 8 rounds of the virus being administered into the tumour, 3/8 animals had displayed complete responses. Additionally, 2/8 animals had managed to control the tumour growth making the tumour size comparable to the size that they had prior to the treatments. A couple of weeks later, those two animals that had controlled the disease, also showed complete responses to the treatments. With a total of 62.5 % of complete responders, the adenovirus group is the only group with a cure rate above the 12.5 % of the animals that were cured with T-cell therapy and PBS intratumorally. The reovirus-treated group, managed to control the disease for a longer time than the mock group but the number of complete responders was the same. Vaccinia virus and HSV treated tumours seemed to show no benefit from the virotherapy treatments. In other experiment performed in this study, direct antitumour efficacy was one of the secondary end-points (Figure 3b, study I). In that case, the same conclusion was validated as the adenovirus treated group displayed the best tumour growth control among all the virus candidates. Interestingly, significant differences in antitumour efficacy were ablated when the animals were not simultaneously administered with T-cell therapy (supplementary figure 2, study I).

Because oncolytic viruses have mechanisms of action that include both immune mediated and a direct lytic effect of cancer cells, it was relevant for the study to understand if the better performance in the adenovirus group was influenced by a potentially faster lytic activity than in the rest of the groups treated with other viruses. Supplementary figure 1 (study I) indicates that adenovirus lytic activity is not faster than that of other viruses. Complete cell lysis of the same number of tumour cells treated with a medium dose of oncolytic adenovirus required 11 days, while for reovirus required 6, for example. It is very likely the case, that for some viruses direct cell lysis plays a bigger role in antitumour efficacy than other viruses where immune mediated cell lysis is the main driver of antitumour efficacy [321].

Currently the only approved oncolytic virus for use in cancer by EMA and FDA is Talimogene laherparepvec, an oncolytic HSV-1 coding for GM-CSF. Even if that is the most studied virus in terms of clinical trials registered in clinical trials.gov, the number of clinical trials testing "oncolytic herpes virus" is 16 while for "oncolytic adenovirus" is 33 (complete search criteria described in "1.2 Virotherapy" section). It is possible that the state-of the art regarding immunotherapy for cancer has changed in 2015, when Talimogene laherparepvec was first approved as it is also the year of the first approval for immune checkpoint inhibitors. While talimogene laherparepvec is mainly oriented towards the engagement of the myeloid compartment [322], PD-1 and PD-L1 inhibitors are rather oriented towards the lymphoid compartments of the immune system. In that sense, it seems that the lymphoid approach has conquered the clinical practice far beyond its myeloid alternatives. For that reason, the lymphoid-oriented oncolytic viruses might receive more attention in the near future for combination with currently dominant immunotherapies than talimogene laherparepvec.

### 4.1.2 INTERACTION WITH THE T-CELL COMPARTMENT DRIVES POSITIVE OUTCOME OF THE THERAPEUTIC APPROACH

When focusing on the interaction between different oncolytic viruses and the T-cell compartment of the immune system, it was interesting to see no direct correlation between trafficking of the T-cell graft and tumour growth control (Figure 3, study I). While adenovirus-treated tumours showed the best antitumour efficacy *in vivo*, those animals did not show significantly higher number of transferred tumour infiltrating lymphocytes trafficking into the tumour. While there is room for technical limitations in terms of explaining the lack of correlation (supplementary figure 4, study I), it is also possible that the unarmed adenovirus is unable to efficiently increase the number of those transferred T-cells trafficking into the tumour. As a note, in a different experiment of the same study it was shown, how arming the adenovirus with TNFa and IL-2 (figure 5F, study I) can overcome this limitation, as it seemed to be the case in other similar studies [129, 139]. Another important factor to take into account is that trafficking of transferred T-cells into the tumour does not necessarily mean that those cells will be able to exert antitumour functions [323]. In that sense a tumour microenvironment can be able to suppress the activity of the cell graft soon after it enters the tumour niche. For that reason, as relevant as it is to study the amount of transferred T cells able to get in the tumour, it is to understand what the suppressive/stimulatory status of the tumour is after the oncolytic virus treatment.

In this case, it was particularly relevant to understand the molecular signature of those genes related with T-cell activity. For that reason a 96-gene

panel designed around T-cell responses was built (supplementary table 1, study 1). The results of those analyses (figure 4, study I) showed a clear picture as the tumours treated with adenoviruses were the only ones with a significant regulation in the T-cell signature of those tumours, while HSV, vaccinia virus and reovirus had few subsignificantly upregulated genes but no significant variations in gene expression. If the list of genes had included genes associated with a wider variety of immune components, it is likely the number of upregulated genes would have been different. For example, vaccinia virus clearance mainly occurs via macrophages and neutrophils [324], but as those are not cell types which functions have been integrated in the panels used for this study, there is a clear lack of significant results. In the case of adenovirally treated tumours, they show an upregulation in the proinflammatory cytokine and chemokine network (one of the features of immunologically "hot" tumours). The overall effect of the adenoviral treatment seems to have an impact in both the innate and the adaptive immune system. In that sense, not only the adoptively transferred T cells but also the endogenous T cells (and other endogenous immune components) could input for the overall antitumour effect. Previous studies already showed the importance of the T-cell compartment in the physiological clearance of adenoviral infections [88, 210, 211], and this work shows how the same is an advantage when using oncolytic adenoviruses to enable T-cell therapies against cancer.

### 4.1.3 GENERATION OF ANTITUMOUR MEMORY

To further understand the effect of the different oncolytic viruses combined with adoptive-cell therapy of tumour infiltrating lymphocytes, an experiment was conducted to further investigate the presence of immune memory against the tumours. For that reason, animals that were able to show complete responses (*i.e.* no tumours visible to the naked eye, after 250 days of the original tumour was established [>4 mm]) were rechallenged again with the originally engrafted tumour cell line (HapT1, pancreatic carcinoma) and left untreated to assess the protection offered by the hosts immune system. To test the specificity of the immune memory, animals were additionally engrafted with a tumour cell line (DDT1-MF2, leiomyosarcoma) for which they were naïve. While all the animals grew the tumours from the cell line they were not exposed to, the level of protection against the previously rejected tumour varied among different groups (figure 2, study I). Even if the number of animals that got complete responses is low, by day 19, those animals that were treated with reovirus or PBS intratumourally and expanded tumour infiltrating lymphocytes systemically, seemed to control the tumour growth better than naïve animals that were never exposed to the HapT1 cell line. On the other hand, the vaccinia virus survivor seemed to have no antitumour memory against the HapT1 tumour that was cured once. On the other side of the spectrum, adenovirally treated tumours had the best control of the

rechallenging, as 80% of the animals rejected the tumours by day 9, achieving a second complete response without additional treatments. In terms of improved antitumour memory, 100% of the survivors showed complete responses when the tumours were treated with a further engineered adenovirus, coding for TNFa and IL-2 (figure 5D, study I). Havunen R. et al showed similar results in terms of providing antitumour memory after the administration of oncolytic adenoviruses coupled to T-cell therapy [128].

From a conceptual point of view, these results are another paramount pillar when choosing which virus to select to support antitumour T-cells. As the animals are tumour free for over a hundred days, it is very unlikely that the animals have any (tumour selective) functional virus at the moment of rechallenge. If animals are able to achieve complete responses without virus being present, the most likely explanation for the selective antitumour effect is the antitumour memory generated after the treatment. These results legitimate the idea that oncolytic adenoviruses are an efficient tool to train the immune system to recognize and attack tumours. In a broader sense, this same concept could be applied in a situation where a patient is treated locally with the virus but it triggers a systemic protection against tumours of similar origin. That immunity could also exert antitumour actions uninjected, distant metastases in a so called “abscopal effect”. That phenomenon has been studied preclinically by Havunen R et al in a similar set-up [222].

## 4.2 T-CELL THERAPIES CAN BE ENHANCED WITH TNFA AND IL-2 VIROTHERAPY (I, II)

As mentioned before, the use TNFa and IL-2 armed adenoviruses was developed to enhance adoptive T-cell therapies in our group [128, 129, 131], and new aspects of this approach were also studied in this work (studies I and II). Initially, it was one of the possibilities to maintain the adoptive-cell therapy and virotherapy as the main therapy and include checkpoint inhibition on the top to achieve better results. To our surprise, the triple combination rendered worse results in the long run than virotherapy combined with either of the other therapies (Figure 1, study II). Only when the dose of virus is reduced a 100 fold, the triple combination delivered better result than the double combination groups (Figure 2, study II). From the same animals, a look at the tumour microenvironment was taken by sacrificing some animals randomly from each group (Figure 3, study II). Those biological studies hinted that the triple combination (even in suboptimal dose) was triggering additional suppressive mechanisms exemplified by CTLA-4 and TIM-3 expression on CD8 T cells. Going back to the first experiment, where full doses were experimented, it was possible to observe that TNFa and IL-2 virotherapy plus anti-PD-1 performed as well as virotherapy plus adoptive cell

therapy, but the biological data from the second study showed better T-cell characteristics when virotherapy was combined with adoptive cell therapy. Therefore, it was decided to continue to further experiments taking virotherapy and anti-PD-1 as the best approach. Shim KG et al, arrived to similar conclusions using a vesicular stomatitis based virotherapy in two different models: The addition of PD-1 blockade or TIM-3 blockade did not produce additional benefit beyond the virotherapy plus adoptive cell therapy effects [325].

## 4.3 TNFA AND IL-2 ARMED ADENOVIRUSES RESHAPE THE TUMOUR MICROENVIRONMENT TOWARDS IMMUNE ACTIVATION IN THE CONTEXT OF PD-1 PATHWAY INHIBITION (I, II, III)

The process by which tumour selective oncolytic adenoviruses were armed with TNFa and IL-2 has been described before [128, 129, 131] but briefly, it was a data driven process based on multiple parameters assessing how the those cytokines could improve the antitumour efficacy of adoptively transferred tumour infiltrating lymphocytes. Wheter those changes that favoured cell-based T-cell therapies would occur in the context of immune checkpoint inhibitors or not, was unknown.

### 4.3.1 CHANGES IN IMMUNE SIGNALLING

First approaching the topic from an angle focused on the changes triggered at the cytokine, chemokine and immune ligand level, the most obvious changes in that sense are related to TNFa and IL-2. As it can be expected from viruses engineered to express TNFa and IL-2 in tumours, the concentration of TNFa and IL-2 in tumours treated with those viruses is higher than in control groups (Figures 3A-B, [study II], Figures 2B-C, 6B-C [study III]). Even if those cytokines are expressed by the virus, the effect on the host is the same as if they were produced physiologically. In that sense, the artificial production of TNFa and IL-2 can mean a kick-start stimulation in immunosuppressive tumours that would repolarize towards a wider stimulatory status once the adequate changes follow. These two cytokines had also shown the importance for attracting T cells into the tumour and thus making them immunologically hotter (Figure 5F, study I) [139]. Studying the indirect production of stimulatory signals can provide further information about the development of immune responses. In opposition to TNFa and IL-2 that can be found in the tumour by the mere presence of the virus, the production of interferons, granzymes and other immune mediators require functional immune

mechanisms in place to be produced. When *in vivo* treated tumours (with TNFa and IL-2 coding viruses and PD-1 axis inhibitors) are analysed, IFNg production is favoured (Figure 5 [study II], Figure 6 [study III]). In study III, human tumour histocultures were assayed for this exact purpose; understanding the immune signals triggered after the treatment with TILT-123 (Ad5/3-E2F-d24-hTNFa-IRES-hIL2) together with a PD-L1 inhibitor (Figure 2, study III). The results of those tests showed that other relevant immunostimulatory signals, such as CXCL10 and Granzyme B were upregulated after the use of the treatments. At the same time, it is important to understand that immunostimulatory treatments can trigger a counter immunosuppressive signalling. For that purpose, the studies aiming at understanding the implication of the combination therapy in the immune status, always compared stimulatory signals versus suppressive ones. In general, it was assessed that the double therapy achieved higher immune stimulation with no increases (or some decreases) in suppressive signals (Figure 5H [study II], Figures 3C and 6G [study III]).

Other immune signals relevant for the effective development and activity of antitumour responses are co-stimulatory and co-inhibitory signals. The experiments conducted in this work are centred on T-cells and those particular signals will be discussed later in this chapter.

### 4.3.2 GRANTING A FUNCTIONAL T-CELL PRESENCE IN THE TUMOUR

When studying the changes triggered from the virotherapy and immune checkpoint blockade from a cellular point of view, we were primarily focused on understanding the impact of the approach on the T-cell compartment as it it is the main target, directly or indirectly, of PD-1 axis blockade [326]. As mentioned before, the addition of TNFa and IL-2 transgenes in the virus play a major role in T-cell recruitment into the tumour (Figure 5F, study I), and T cells being present at the tumour is a requirement of PD-1 blockade mediated antitumour efficacy [319, 320]. In the context of concomitant PD-1 axis inhibition, tumour samples collected from in vivo experiments also showed that the treatment with TNFa and IL-2 coding viruses managed to increase the presence of CD8 T cells (Figure 3A [study II], Figure 5A [study III]) and non-regulatory CD4 T cells (Figure 3I, study II). Increasing the presence of T cells in the tumour microenvironment is desirable but the transferred cells need to be active to be able to exert antitumour functions [327]. Besides the previously described presence of cytokines associated with T-cell activity (such as IFNg and Granzime B), flow cytometric analyses of tumour resident T cells revealed that they are more active when the double treatment is provided (Figures 5 B-C, study III). Conceptually these findings make sense as the therapies work in parallel towards that aim: the virotherapy attracts the T cells towards the

tumour and provide stimuli for them to be active while the immune checkpoint inhibitors prevent suppressive signals to turn those T cells anergic. Another desirable treat from the T cells arriving to the tumour is that they are able to recognize tumour cells. The cancer models used to study the efficacy of the treatments allowed assessing the presence of tumour specific T cells. In figure 3F of study II, increased number of tumour specific CD8 T cells were seen in all groups treated with virotherapy and anti-PD-1 antibodies. The importance of these tumour specific T-cell clones was highlighted when it was seen that animals that developed complete responses had a significantly higher proportion of those tumour specific T cells than the animals that were sacrificed at earlier time points (Figure 6, study II).

Interaction between immune cells and other cells can also influence the outcome of the therapeutic approach. For that reason, it was interesting to study the expression of T cell surface proteins such as PD-1, CTLA-4 or TIM-3. The surface expression of PD-1 on T cells occurs around 24 hours after the T cell is activated [328], and was strongly correlated with the presence of active T cells (Supplementary figure 4A, study III). While it can be understood as an activation marker, it also allows the T cell to be suppressed if it binds its ligands (PD-L1 or PD-L2). In that regard, the double treatment increased the expression of PD-1 (Figure 3B [study II], Figure 3E [study III]). Those results more than anything, legitimate the combined use of virotherapy with PD-1 blockade, as if there is an effective inhibition of those PD-1 receptors with anti-PD-1 antibodies, the cells will not suffer inhibition. A similar conclusion can be drawn from PD-L1 expression; if there is effective PD-1 blockade it will not render T-cell anergy. While the treatment with viruses coding for TNFa and IL-2 induced PD-L1 expression (Figures 5 I-J, supplementary figures 6A-D [study III]), it is likely related to the ongoing antitumour responses, as the virus itself, neither the cytokines are able to induce PD-1 ligands expression in tumour cells (Supplementary figure 5, study III). The expression of other sources of potential anergy triggered in the tumour infiltrating lymphocytes such as CTLA-4 and TIM-3 were also studied (Figures 3D-E, study II). While CTLA-4 was significantly upregulated on T cells from tumours treated with both the virotherapy and immune checkpoint inhibition, only 1% of the total CD8 T cells do it. For TIM-3, the double treatment approach did not increase the surface expression of the marker and again below 0.1% of total CD8 T cells express it. Comparing the minimal expression of CTLA-4 and TIM-3 with the majority of CD8 T cells expressing PD-1, justifies the idea of using PD-1 blockade instead of CTLA-4 or TIM-3 inhibitors.

### 4.3.3 OTHER IMMUNE CELLS AFFECTED

While the focus of this work is around the lymphoid compartment, there are hints of the impact of the approach in other cell subsets. For example in

Study I, it was seen how adenoviruses are also able to upregulate stimulatory elements expressed mainly outside the T-cell compartment [329, 330] such as CD80 and CD40 (Supplementary table 2, study I). Another particularly interesting cell population to study is the MDSC compartment, due to their powerful suppressive activity against antitumour responses [331]. One of the possible downsides of having immunostimulatory signals in the tumour is the possibility of attracting regulatory components to grant tissue homeostasis [332]. In that sense, cytometric analyses from tumour samples revealed that virotherapy used as monotherapy increases the MDSC presence, but when virotherapy is combined with anti-PD-1 the percentage of MDSCs in the tumour is the same as in control groups (Supplementary figures 6G-H, study III). This might be another example to legitimate the combination of virotherapy and anti-PD-1, as MDSCs, due to their inherent suppressive nature, express high levels of PD-L1 and PD-L2 (Supplementary figures 6E-F and I-J, study III).

## 4.4 TNFA AND IL-2 BASED ADENOVIROTHERAPY INCREASES THE THERAPEUTIC EFFICACY OF IMMUNE CHECKPOINT INHIBITORS (II, III)

The previously described alteration of the tumour microenvironment in favour of T-cell responses can be regarded as a valuable outcome of the use of virotherapy to enable checkpoint inhibitors. Ultimately, more important than proving the changes in the tumour microenvironment is to achieve consistent results in terms of tumour growth control or survival rates. For that reason, it is paramount in the evaluation of the combination of therapies, to assess of the presence of additive or synergistic benefit in terms those parameters.

### 4.4.1 ENABLING ANTI-PD-1 THERAPY

The early results from study II motivated the development of TNFa and IL-2 virotherapy to enable anti-PD-1 therapy. Once the therapeutic strategy was defined, an optimized regimen of administration of the therapies was sought. As the reduction of the virus dose rendered a negative effect, it was inferred that administering the virotherapy treatment multiple times (up to 15), as it is usually done in the clinical set-up, can result in better results. Increasing the rounds of virotherapy treatments, rendered better tumour growth control and better overall survival than previously studied regimens of administration, and in a group receiving both therapies, all subjects experienced complete responses (Figure 4, study II). It was mentioned that, to our knowledge, this study was the first time where a treatment was able to cure all the individuals with this aggressive melanoma cell line. While virotherapy alone group

performed better than the control group, anti-PD-1 monotherapy group did not. The use of both therapies together rendered a synergistic effect in controlling the tumour growth (Supplementary figure 3, Study II). As it was described before, it will not be repeated here, but the benefit of combining both therapies was seen in flow cytometric and cytokine based assays (Figures 3 and 5, study II).

### 4.4.2 ENABLING ANTI-PD-L1 THERAPY

Study III focuses on the validation of the results of study II, in a way that seeks to reproduce the conditions used when using virotherapy to enable PD-1 inhibition but in this case, instead of blocking PD-1, PD-L1 is the target of the antibody blockade. Adequately matching results were obtained also in the case of combination with PD-L1 blockade, where again it was achieved a 100% complete responses in a group of animals receiving both drugs (Figure 4, study III). Also this time, a synergistic benefit was assessed from the combination (Supplementary figure 2, Study III). In study III, already discussed biological analyses revealed molecular and immunological patterns supporting the combination of both therapies (Figures 5 and 6 study III).

In this study it was also investigated the potential benefit obtained after combining TNFa and IL-2 coding adenoviruses with anti-PD-L1 in *ex vivo* tumour histocultures. Independently of the PD-L1 expression and CD8 T cell presence at the tumour, the double therapy did not provide additional decreases in cell viability over virotherapy alone (Figure 1D, study III). Actually, the anti-PD-L1 by itself had a minimal effect on cell viability. The most likely explanation for that is the lack of a fully operative immune system. On a plate there are no options to recruit further immune cells after the treatments even if lymphoattracting chemokines were produced. At a glance, it is difficult to conclude anything from the combination strategy but when grouping together immunostimulatory signals and immunosuppressive ones, a trend of added value appears when anti-PD-L1 is added to virotherapy treatments (Figure 3, study III).

## 4.5 DIFFERENT IMMUNE CHECKPOINT INHIBITORS REQUIRE DIFFERENT REGIMENTS OF ADMINISTRATION FOR OPTIMAL THERAPEUTIC OUTCOME (II, III)

A topic unaddressed so far regarding the use of checkpoint inhibitors together with virotherapy is the regimen of administration. Even if, in principle, each therapy has a non-overlapping mechanism of action, the

immune system is a complex network that is affected by both approaches. In studies II and III, one of the aims was to determine whether it is more adequate to administer simultaneously both drugs or if it is best to first administer one drug only and include the second treatment afterwards. The second approach, could be oriented in two ways: start with the sole administration of virotherapy and then include immune checkpoint inhibitors or the other way around. While in principle both could provide insights, we consider more interesting to start with the virus and after certain number of monotherapy administrations include the PD-1 axis inhibitors. Previous experiences with TNFa and IL-2 viruses showed that recruitment of T cells into the tumour becomes evident only between 2 and 4 days after the first virus administration [129]. Because of the relevance of having T cells present at the tumour with regards of therapeutic benefit, it was sought to favour unleashing the effector activity of T cells only after they are in the tumour. For that reason it was decided to test an approach where tumours would be primed with tumour stimulatory signals (the virus) first and only starting from the third round of treatment, boost T-cell activity with the immune checkpoint inhibitor, in a "prime and boost" type of administration. At the same time, PD-1 axis blockade has been efficaciously used three to five times per week in similar preclinical models [308, 333]. Those premises led to the direct comparison of a simultaneous administration of viruses and antibodies ("Simultaneous" approach) versus the administration of the virus only to which PD-1 axis inhibiting antibodies were added from day 3 onwards ("Prime and boost" approach)(Figure 4).

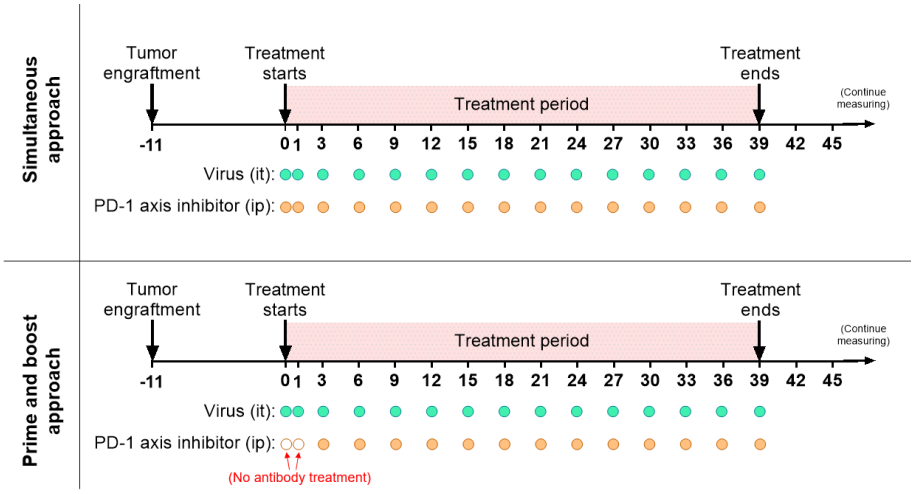


*Figure 4. Schematic plans for treatment schedules using "Simultaneous" approach and "Prime and boost" approach.*

When the use of TNFa and IL-2 coding adenoviruses together with anti-PD-1 monoclonal antibodies was studied, both of the previously described approaches were included as different experimental groups. Significantly

better tumour growth control and better overall survival (100%) was achieved with the Prime and boost approach (Figure 4, study II). As explained before, it made sense conceptually to first attract the immune effector cells to the tumour with the virus and then keep active and protected from anergy by the use of anti PD-1. Additionally, even if T-cells could arrive faster to the tumour than day 3, it still takes about 24 hours to have PD-1 upregulated on the cell surface after cell activation [334]. Both clinical and preclinical studies have shown that PD-1 mediated T-cell inhibition includes the risk of triggering immune adverse events associated with the loss of self-tolerance [335]. In that sense, it is particularly relevant to skip anti-PD-1 treatments if there are no ongoing T-cell activity in the tumour as it could create off-tumour immune activation in immunologically complex areas such as the gut or the lungs.

In contrast with the results provided for anti-PD-1 and virotherapy, the optimal administration schedule, for the use of PD-L1 inhibitors, proved to be the simultaneous one. Better tumour growth control, and better overall survival (again 100%) was observed when both therapies were administered concomitantly from the beginning of the treatment period (Figure 4, study III). Additionally, in this study there was a possibility of assessing the status of the T-cell compartment early after treatments started (Figure 5, study III). The tumour samples originated in animals where simultaneous administrations were performed showed a better T-cell signature: more presence of CD8 and CD4 T cells as well as higher activation status (based on CD69 positivity) among those CD8 T cells. Zamarin D et al, used a Newcastle Disease Virus in combination with anti-PD-L1, also arriving to a similar conclusion [264]. PD-L1 in tumours can be constitutively expressed or induced after different immune signals [336]. B16.OVA, the model used in this experimental set seemed to have certain degree of baseline PD-L1 expression but can be increased further after exposure to IFNg (Supplementary figure 5, study III). In that sense, PD-L1 is already present in the tumour microenvironment prior to the interventional administration of the treatment, and thus it would make sense to start blocking those ligands from the beginning. Also, some recent studies on PD-L1 mechanisms of action highlight the importance of the cis interaction of PD-L1 and B7.1 (CD80) in APCs [337]. When the virus arrives to the tumour it stimulates the recruitment of existing T cells to the tumour but their mechanism of action also promotes de novo development of antitumour adaptive responses [307], which would be enhanced if the PD-L1/B7.1 interaction in cis of antigen presenting cells was inhibited. In the same line, it is possible that the first administration of virotherapy in the tumour triggers a series of adaptive immune responses that include IFNg production [338]. Those antiviral immune responses are also part of the actions that repolarize the tumour microenvironment towards a situation more favourable to develop antitumour responses [85, 339, 340].

Preparing the tumour by simultaneous immune stimulation and inhibition of the present suppressive agents makes sense for PD-L1 blockade but not as much for PD-1 blockade. Nevertheless, this does not mean that the "Simultaneous" approach does not work with the combination of virotherapy and PD-1 or the "Prime and boost" for the PD-L1, (as those strategies delivered better efficacy results than monotherapy controls). Instead, it is understood that in the search for therapeutic strategies to fight cancer great is always prefered over good.

## 4.6 ANTI-PD-1 RESISTANCE IN SOLID TUMOURS INVOLVES DEFICIENCIES IN PRESENCE AND PERFORMANCE OF THE T-CELL COMPARTMENT (IV)

The discovery and subsequent clinical development of immune checkpoint inhibitors for the treatment of cancer led to the present situation, where those antibodies (especially those targeting the PD-1 pathway) are the first or among the first lines of treatments for multiple indications [341, 342]. Unfortunately, only 10-40% of the patients respond to the therapy [316, 317]. Even among those patients that initially respond, a large number of them end up progressing, exposing an urgent unmet clinical need as the majority of patients do not experience any long term benefit after checkpoint inhibition treatments [255, 343]. There are multiple theories explaining possible mechanisms behind PD-1 resistance, both intrinsic [58, 344, 345] and extrinsic [346-348], and it is likely that both types of causes are present in different types of tumours. Described in figure 1 (study IV) is the development of an *in vivo* model of anti-PD-1 resistance, where tumours were treated with anti-PD-1 until they reached a certain tumour volume after which tumours will be harvested and compared with tumours of equal size that were not treated with PD-1 inhibitors. While there was a heterogeneous response to the therapy (as it is the case in the clinical set up), there was a similar gene signature among those tumours that were progressing after anti-PD-1 therapy (Figure 2, study IV) compared to untreated tumours of relatively same size. The gene expression analysis revealed 19 genes that were strongly associated with the immune system. From that list of genes the majority of downregulated genes (75%) could be associated with the T-cell compartment, while the only T-cell related gene that was upregulated in those PD-1 resistant tumours was *FOXP3*, a master regulator of regulatory T cells [349]. Those results legitimate the idea that the lack of benefit from anti-PD-1 therapy is associated to poorer effector T-cell activity as there is a decrease in granzymes and co-stimulatory factors directly associated with this cell-subset and there is an increase of the differential marker for regulatory T cells. In the model generated here, the type

of resistance seems to go in line with the resistance mechanisms described in the other work, associated to extrinsic factors [346-348]. The main aim of this study was not to delve into the reasons driving PD-1 resistance but to generate a model that resembles a naturally occurring PD-1 resistance.

## 4.7 TNFA AND IL-2 ADENOVIRUSES CAN REVERT ANTI-PD-1 RESISTANCE AND DELIVER COMPLETE RESPONSES IN SOLID TUMOURS (IV)

Using the PD-1 resistance model described above, the studies moved into the main aim, which is to understand if TNFa and IL-2 coding adenoviruses can deliver antitumour efficacy in those patients failing to respond to PD-1 blockade therapies. To that extend, TNFa and IL-2 coding have shown in multiple studies the ability to repolarize the tumour microenvironment towards a point favourable to develop and sustain antitumour T-cell responses (Studies I-III, [128, 129, 139, 222]). When virotherapy was administered to tumours that had progressed notably after anti-PD-1 administration, antitumour responses were assessed (Figures 3 and 4B, study IV). While tumours that were considered resistant to anti-PD-1, did not respond to anti-PD-1 and all the animals reached euthanizing criteria (due to excessive tumour volume) in less than 20 days, a number of animals treated with virotherapy displayed complete responses. In particular in the group treated with virotherapy and anti-PD-1, the efficacy results were particularly promising as 50% of the animals were cured. A third group treated with virotherapy but not further anti-PD-1 therapy also displayed some complete responses but the efficacy was not as good as for the double therapy treatment. These results already hint that virotherapy is not only able of exerting its antitumour effect in tumours that are resistant to the other immunotherapy but also that the virotherapy is able to revert the resistance and allow anti-PD-1 antibodies to deliver therapeutic efficacy again. It is also relevant to understand that even if the animals that were treated with virotherapy alone, did not receive further antibodies, the half-life of the antibody in adult mice is between 6 and 8 days [350]. That means that it is likely that clearer differences between double therapy and virotherapy alone would be achieved if the animals had no anti-PD-1 in them, rendering worse efficacy in the virotherapy alone group. This creates interesting possibilities for the application of TNFa and IL-2 viruses in patients undergoing PD-1 inhibitory treatment who do not show signs of efficacy. In that case, viruses like TILT-123 could be added directly along the regimen of administration of anti-PD-1 or anti-PD-L1 therapy.

When tumour samples were analysed for a mechanistic explanation of this efficacy, the data pointed out to the same direction as the genomic analysis

result pointed when understanding the mechanism of resistance: the T-cell compartment (Figures 4,5 and supplementary figure 2, study IV). Virotherapy administered after PD-1 resistance, managed to increase the intratumoural frequency of multiple CD8 T-cell subpopulations with different developmental and functional statuses. Signs of T-cell activation and proliferation together with low levels of suppressive inhibitory pathways, resemble the opposite circumstance that the one linked with tumours able to resist anti-PD-1 functions. While the strong interaction between oncolytic adenoviruses and the T-cell compartment has been assessed across this work, (especially in study I) the technical possibilities offered by mass-cytometry allowed to study the effect of the treatments in other populations. Encompassing the general notion of reshaping the status of the immune microenvironment towards stimulatory conditions, downregulation of other immune cell populations that exert immune suppression, such as MDSCs [351, 352] and M2 macrophages [353, 354] were evidenced in those tumours. In this study, it is evidenced how TNFa and IL-2 virotherapy is able to refurbish the tumours with a variety of T-cell populations and to reduce the presence of immunosuppressive elements, which overall means the chance of responding to anti-PD-1 therapy.

# 5 SUMMARY AND CONCLUSIONS

In this work the possibilities of using oncolytic viruses as a tool to expand the percentage of patients that benefit were explored and optimised. When compared with other relevant oncolytic virus platforms such as the herpes simplex virus, vaccinia virus or reovirus, adenoviruses provide the best background to engage T-cell responses in solid tumours. The responses triggered in the tumour after the adenovirus administration include innate and adaptive immune mechanisms, that are able to support the activity of T-cell immunotherapy but also to promote *de novo* endogenous antitumour responses. Further benefits from the oncolytic adenoviruses can be gained if they are armed with TNFa and IL-2, as those cytokines stimulate T-cell trafficking into the tumour and support their activity leading to a better tumour control and a stronger antitumour memory. While the TNFa and IL-2 adenovirotherapy approach was designed around the concept of cell based T-cell therapies, it also provides synergistic benefit in antitumour efficacy when used together with immune checkpoint inhibitors of the PD-1 axis. Because the mechanism of action of PD-1 and PD-L1 inhibiting antibodies revolves around T cells, the repolarization of the tumour microenvironment towards a status supportive of their effector functions against cancer. The use of TNFa and IL-2 armed adenoviruses with anti-PD-1 or anti-PD-L1 antibodies rendered, in both cases, complete responses in an aggressive solid tumour *in vivo* model. Nevertheless, the optimal sequence of administration of the antibodies and the virus changes depending on the antibody due to the particular target protein expression patterns and presence on different cell types. Due to the widespread use of immune checkpoint inhibitors in the clinics, a particularly relevant application of this therapeutic strategy comes for those cases where the tumour does not respond to the therapy. In those cases, TNFa and IL-2 armed adenoviruses can revert tumour resistance against PD-1 blockade and provide meaningful antitumour responses.

Overall, oncolytic adenoviruses coding for TNFa and IL-2 (such as TILT-123) and PD-1 axis inhibitors are non-overlapping immunotherapies that proved synergistic antitumour efficacy due to their ability to reinforce each other weaknesses: the virus makes the tumour immunologically more active for the immune checkpoint blockade to be effective, while those immune checkpoint inhibitors support the activity of antitumour immune responses by preventing suppression of T cells mediated by PD-1 signalling. Two phase I clinical trials using TILT-123 and anti-PD-1 or anti-PD-L1 have been planned to be conducted in solid tumour patients.

# 6 FUTURE PROSPECTS

The constantly evolving cancer cells are a difficult target to eliminate. To compete with cancer's plasticity, it is an appealing idea to use the immune system as it is also a tool able to adapt to new circumstances and conditions. Unfortunately, it is difficult to outcompete cancer in terms of evolution rate. For that reason our immune system on its own is an insufficient resource to tackle advanced malignancies. Backing already existing immune defence mechanisms or implementing new ones is the strategy followed by immunotherapy, trying to push the balance towards immune elimination of the tumours.

Even if immunotherapies hold the potential of curing patients from cancer, it is a relatively novel approach and additional knowledge is required to fully grasp the opportunities offered in this therapeutic path. Both clinical and preclinical efforts are being carried out by researchers and healthcare professionals worldwide to that aim. The clinical development data on anti-PD-1/anti-PD-L1 is an example of the need and interest to understand their potential: the first human trial started in 2006, and by the end of 2018, more than 2600 trials were registered for the study of those drugs [355]. Other promising immunotherapeutic approaches should follow the immune checkpoint path and show that preclinical proof of concept data can be applied in the human setting and continue delving into the interactions of these immunotherapies, the immune system, the tumour, and the rest of the patient.

The immune system is formed by a complex network that uses multiple feedback loops and regulation system affecting and being affected by other components of the organism. Going deeper and wider on the understanding of the immune system and its relation with cancer might be only possible by using novel technologies that on one hand generate large amounts of data from study samples (such as single cell omics) and that are able to compute the humongous sets of information to make sense out of them. Previous therapeutic strategies like targeted therapies could be coupled to relatively simple biomarker study that reveals the presence or not of a specific mutation. That type of simple biomarker studies have been also coupled to immunotherapies, like the companion tests for checkpoint blockade testing PD-L1 expression. A complex network like the immune system cannot be simplified into PD-L1 positive or negative question, not even to PD-L1 positivity over a certain threshold because it provides incomplete assessments of the immunological status of the patient and the suitability for the therapy. In that sense, the next big step of immunotherapy should be bound to the development of complex biomarker systems able to properly assess the status of the patient based on a large number of parameters (presence, distribution and phenotype of immune cells, immune inhibitory pathways, mutations,

neoantigens, tumour associated antigens, tumour heterogeneity, metabolic status, vasculature, permeability of the tumour...) that would render into a suggestion for therapeutic approach. The algorithm for treatment selection should also integrate interaction between therapies and aiming for non-overlapping antitumour efficacy, including (but not restricted only to) immunotherapies to achieve the pursued long term benefit. In that sense it would mean an expansion from the current approach and not targeting an individual element but aiming to target tumour cells, supporting the immune system and remodelling the tumour microenvironment.

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