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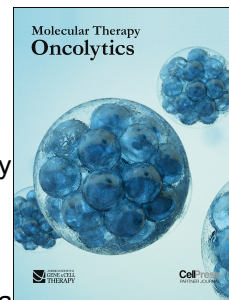
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Comparison of clinically relevant oncolytic virus platforms for enhancing T-cell therapy of solid tumors

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Short title (less than 50 characters including spaces): Comparison of OV's to enhance T-cell therapies

Abstract (no more than 200 words)

Despite some promising results, the majority of patients do not benefit from T-cell therapies, as tumors prevent T-cells from entering the tumor, shut down their activity, or downregulate key antigens. Due to their nature and mechanism of action, oncolytic viruses have features that can help overcome many of the barriers currently facing T-cell therapies of solid tumors. This study aims to understand how four different oncolytic viruses (adenovirus, vaccinia virus, herpes simplex virus and reovirus) perform in that task. For that purpose, an immunocompetent *in vivo* tumor model featuring adoptive tumor-infiltrating lymphocyte (TIL) therapy was used. Tumor growth control ($p < 0.001$) and survival analyses suggest that adenovirus was most effective in enabling T-cell therapy. The complete response rate was 62% for TILs+Adenovirus vs 17.5% for TILs+PBS. Of note, TIL biodistribution did not explain efficacy differences between viruses. Instead, immunostimulatory shifts in the tumor microenvironment mirrored efficacy results. Overall, the use of oncolytic viruses can improve the utility of T-cell therapies, and additional virus engineering by arming with transgenes can provide further antitumor effects. This phenomenon was seen when an unarmed oncolytic adenovirus was compared to Ad5/3-E2F-d24-hTNFa-IRES-hIL2 (TILT-123). A clinical trial is ongoing, where patients receiving TIL treatment also receive TILT-123 (NCT04217473).

Keywords: oncolytic virus, immunotherapy, T-cell therapy, tumor microenvironment, solid tumor.

Introduction

The fundamental idea of viral infections being beneficial for tumor control has been present for over a hundred years now¹, originating just a few years after viruses were discovered. Already on a theoretical level, it makes sense to use those agents to treat cancer as the cellular and molecular characteristics of tumor cells make them particularly vulnerable to viral infections. Fast growth and defects in apoptosis and immune mechanisms are some of the key flawed mechanisms in cancer cells, making it more difficult for them to stop virus infections². In 1949³, the first one using a virus as an antitumor therapy was carried out and in the following years, other studies using viruses for the treatment of different tumor types were conducted⁴⁻⁶. However, the overall inconclusive results, influenced by small patient numbers and poor “products” (mainly consisting of wild type viruses), reduced the interest of the scientific community in virotherapy of cancer. Later on, technological development allowed the refinement of the approach by the engineering of tumor-selective viruses⁷.

Because the efficacy of single agent virotherapy has generally been suboptimal, immunosuppressive chemotherapy has been studied preclinically for its ability to increase direct oncolytic activity⁸⁻¹². When it was realized that in humans oncolytic viruses trigger anti-tumor immunity, the approach was radically changed¹³. Instead of aiming at optimal oncolysis, viruses were now being designed to enhance immune activities. The first oncolytic virus approved in the United States and Europe¹⁴, Talimogene laherparepvec (Imlygic®), is a Herpes simplex virus engineered to express granulocyte-macrophage colony-stimulating factor (GM-CSF). Like Talimogene laherparepvec, other oncolytic viruses have also been designed to include immunostimulatory gene constructs, such as cytokines, ligands or antagonists¹⁵⁻¹⁷.

In the last decade, the T-cell component of the immune system has been drawing attention, as T-cell related therapies, such as chimeric antigen receptor T cells (CAR T)^{18, 19}, adoptive T-cell therapy with tumor-infiltrating lymphocytes (TIL)^{20, 21}, adoptive T-cell receptor therapy²², and checkpoint inhibitors²³ emerged as potent tools in clinical oncology. These approaches have been used in the clinics with promising results, as they can result in long-lasting responses, and in fact, appear to be able to cure a proportion of patients with metastatic cancer. Consequently, several products have been approved.

On the other hand, and especially regarding solid tumors, only a fraction of treated patients benefit from these therapies and only in particular indications. Overall response rates are below 10% for CAR T therapy²⁴, around 50% for adoptive T-cell therapy of melanoma²⁵ and 0-40% for checkpoint inhibitors²⁶. Most commonly, T-cell therapies fail because of immunosuppressive conditions in the tumor milieu^{27, 28}, the ability of some tumors to prevent T-cell infiltration^{29, 30}, and antigen loss for those treatments where specific targets were chosen for engineered T cells³¹.

Due to their mechanism of action, oncolytic viruses have been proposed as a valuable tool to overcome T-cell therapy limitations^{15, 32}, and actually tested by different groups^{33, 34}. Their ability to create an immunostimulatory signal increases immune cell trafficking towards the tumor^{35, 36}, reverses the immunosuppressive status of the microenvironment^{37, 38} and creates *de novo* adaptive immunity against the pool of tumor epitopes released upon oncolysis³⁹⁻⁴¹. Taking into account that different viruses have different properties, each virus will probably offer distinct therapeutic possibilities. In this study, four different viruses representing different families (*Adenoviridae*, *Poxviridae*, *Herpesviridae*, and *Reoviridae*), widely studied clinically, were chosen for study in the context of adoptive T-cell therapy.

Results

- *Selecting treatment dose for different viruses*

The Syrian hamster model was selected to study *in vivo* efficacy, as it is perhaps the only model permissive for the productive replication of all viruses used in the study⁴²⁻⁴⁵. The selected tumor model, HapT1 pancreatic

carcinoma, enables the isolation of tumor infiltrating lymphocytes (TILs) for *ex vivo* amplification, for use as an adoptive cell therapy (ACT)⁴⁶. It was also assessed that the selected virus had oncolytic activity on the cell line (Supplementary figure 1)

For the comparison of adenovirus, vaccinia virus, herpes simplex virus, and reovirus, weight-per-weight hamster doses were established based on the maximum tolerated dose used in human trials. At the beginning of the project (May 2016), a search for clinical trials investigating the abovementioned viruses was performed, and the maximum tolerated dose in humans was identified for each of the viruses (Table 1). Because there was no established maximum tolerated dose for an unarmed herpes simplex virus, that of Talimogene laherparepvec was selected for this study.⁴⁷

The dose extrapolation from human maximum tolerated dose to the one used in Syrian hamsters assumes 75 kg as standard human weight and 0.1 kg as standard Syrian hamster weight. Thus, maximum tolerated doses in humans were divided 750 times to achieve with the dose to be used in hamsters. To assess the feasibility of the extrapolated doses, ten times higher dose and ten times smaller dose were also tested *in vivo* (Supplementary figure 2). None of the groups treated with 10 times more virus showed better tumor growth control than the directly extrapolated dose (presumably because of virus replication decreasing the importance of input dose), supporting the rationale for using the extrapolated doses. Using the same units (vp, pfu or TCID₅₀) as had been published in human trials avoided the problem of different titering procedures.

- *Oncolytic adenovirus has the best antitumor efficacy when used as T-cell therapy enabler*

For the study of the antitumor efficacy of different viruses, HapT1 cells were subcutaneously engrafted in the lower right flank of Syrian hamsters. Ten days later, when tumors were palpable and measurable (mean volume: 205.63 mm³, standard error of the mean: 15.76 mm³), those animals were randomized into groups. All animals received an adoptive cell graft of *ex vivo* expanded TILs intraperitoneally at day 0. Depending on the group, the animals also received intratumoral virus treatments or PBS as a negative control. Intratumoral treatments were administered on days 0, 1, 3 and then once every three days until day 39 (Figure 1A). After day 39, animals were not treated, but regular measuring and health checkups were performed. Animals were kept alive until their tumor exceeded the allowed tumor dimensions (22 mm for the longest tumor diameter).

Individual values of normalized tumor volumes are displayed in Figures 1B-F. The only virus that provided a significant reduction in tumor volume compared to the effect of adoptive T-cell therapy alone was adenovirus ($p < 0.001$). The group treated with adenovirus also showed significantly better tumor growth control than those animals treated with herpes simplex virus ($p < 0.001$). None of the other viruses were able to provide significant tumor growth control compared to the PBS control or other viruses.

In line with tumor growth control results, the only group that showed significantly increased survival was the one treated with adenovirus (Figure 1G), when compared with the herpes simplex virus group ($p = 0.049$). It is also relevant to look at the proportion of complete responses by day 250: PBS (17.5%), adenovirus (62.5%), vaccinia virus (12.5%), herpes simplex virus (0%), and reovirus (12.5%). There were three times more complete responses in the adenovirus group than in the second best group.

- *Antitumor memory can be elicited by the use of oncolytic adenovirus and T-cell therapy*

After initial treatment, animals that showed no visible tumors by day 250 were included in a follow-up experiment, to investigate if they had gained specific antitumor memory able to reject a new graft of the same HapT1 tumors that they were cured from. For that purpose, the same amount of HapT1 cells were engrafted in the opposite side of the hamster's back (upper left flank). At the same time, a different cell line (DDT1-MF2, Syrian hamster leiomyosarcoma), for which the animals were naïve to, was engrafted in the upper right flank to

control the specificity of the antitumor memory. After this tumor rechallenge, the animals did not receive any treatment, as the purpose was to study the ability of the previous treatments to generate immunological memory. Hamsters that were never exposed to any cancer cell lines and/or treatments served as negative control (Figures 2A-B). After tumor engraftment, the animals were followed for 19 days. By that day, DDT1-MF2 tumors were reaching the regulatory tumor size limit in the majority of the animals.

As displayed in Figure 2C, all animals (with the exception of the animal from the vaccinia virus group) that showed a complete response from the previous treatments had lower tumor volumes on day 19 than naïve animals. The animals that had previously received adoptive T-cell therapy and either PBS or reovirus started developing the tumors faster in the first days of the experiment, but then showed noticeable partial responses by day 19. A much clearer result was observed from the animals previously treated with adoptive T-cell therapy and adenovirus, as those animals showed lower average tumor volume than naïve animals as early as by day 8. By day 19, 5 out of 6 animals in that group showed no visible HapT1 tumor. This result suggests that adenovirus was most effective in generating antitumor memory. The animal cured with vaccinia virus and T-cell therapy had a larger tumor than naïve animals, but drawing conclusions on just one animal is difficult. There were no animals cured with Herpes and thus rechallenge could not be performed.

DDT1-MF2 tumors took a longer time to start growing visibly, but then they increased their volume exponentially (Figure 2D). None of the animals included in the experiment showed protection against this cell line, validating the assumption that rechallenged complete responders had antitumor memory against HapT1 specifically.

- *Antitumor efficacy is not directly explained by trafficking of the T-cell graft*

Our next goal was to study the contribution of the transferred T-cell graft to the response and repolarization of the immune microenvironment. For that purpose, a new animal experiment was set up, following similar conditions as before. The main differences were the length of the study and the primary endpoint: tumors were collected 6 days after treatment start, to study the biodistribution of the adoptive T-cells and gene expression profiles (Figure 3A).

To study the biodistribution of the T-cell graft, the cells were labeled with $^{111}\text{Indium-oxine}$. $^{111}\text{Indium-oxine}$ is a radioactive compound that allows the tracking of cells *in vivo* with SPECT/CT measurements as well as *ex vivo* radioactivity measurements after organs are collected. At days 0, 1, 3, and 6 tumors were measured with a digital caliper (Figure 3B). In addition, tumor volume was determined on day 6 based on CT images and validated by correlating with the tumor mass after harvesting (Figure 3C). The *in vivo* radioactivity measurements observed with SPECT/CT were also validated by comparing the values to the radiation measured by a gamma counter after tissue harvesting (Figure 3D). A clear correlation between *in vivo* and *ex vivo* radiation uptake was observed ($p < 0.0001$). Thus, SPECT/CT measurements can be used reliably when evaluating the trafficking of adoptively transferred T-cells in different groups.

In vivo measurements of the animals were performed approximately 24 (day 1), 72 (day 3) and 144 (day 6) hours after the labeled cells were transferred to the animals. A graphic representation of the amount of radioactive signal is shown in Figure 3E. For days 1 and 3, there was no statistically significant increase in the radioactivity levels, but at day 3 a trend indicating reovirus recruiting a higher number of radiolabeled T cells in tumors was observed. At day 6, a significant increase of radioactivity signals were found in tumors treated with reovirus ($p = 0.028$) and with herpes simplex virus ($p = 0.031$) when compared with PBS. On the other hand, the vaccinia virus treated group had lower radioactivity signals than any other group ($p < 0.01$). The reovirus treated group also showed significantly higher radioactive intensity signals than the adenovirus treated one ($p = 0.042$). However, the groups having the highest T-cell trafficking did not match the groups having the best antitumor response or immunological memory against the tumors.

Besides tumors, other tissues including blood, kidney, spleen, and muscle, were analyzed *ex vivo* to have a deeper understanding of the biodistribution of the T-cell graft. In addition, we wanted to assess if any of the viruses caused extratumoral accumulation of adoptively transferred T cells (Figure 3F). Kidneys in the adenovirus group had lower radioactivity than all the other groups, and the difference was statistically significant when compared with vaccinia and herpes simplex virus groups ($p < 0.05$). Also, a considerable proportion of the T-cell graft could be found in spleens for all the groups regardless of the treatments.

- *Immune repolarization is seen in the groups with the best antitumor responses*

Animals receiving a T-cell transfer might obtain an improved outcome from the therapy, if the tumor microenvironment is altered to favor the development and exertion of immune responses against malignant cells. To study this hypothesis, tumors collected at day 6 (Figure 3A) were analyzed for gene-expression levels. RNA was extracted and studied by a multiplexed immune panel, specifically designed for Syrian hamsters (Supplementary table 1). From all the designed probes, only those that passed quality control were included in the comparison of expression profiles (Figure 4). Adjusted p values and fold changes are described in Supplementary table 2.

Gene expression profiles after treatment with virotherapy and T-cell therapy showed that the only candidate able to induce significant changes in the studied genes was adenovirus. In adenovirus-treated tumors, genes related to the following functions were upregulated: production of proinflammatory cytokines (*IL1B*, *TNF*, *GZMM*), proinflammatory cytokine receptors (*IFNGR1*), innate immune system (*TLR9*, *TLR2*), myeloid stimulatory markers (*CD80*, *CD40*), chemokines (*CCL3*, *CCL4*), chemokine receptors (*CCR1*), adaptive immunity cell markers (*CD4*, *CD3E*) and immune signal mediators (*HCK*, *SYK*). This points to a broad immune activity being stimulated at the tumor niche, covering a wide range of immune mechanisms. In addition, an upregulation of *ARG1* (a gene coding for immunosuppressive arginase 1) was also witnessed, possibly indicating a counter-reaction to immunostimulation.

The other three viruses studied in this experiment did not produce any significant upregulation in the genes included in the panel, although sub-significant gene expression variations were seen in reovirus and herpes simplex virus groups (Supplementary table 2). For the tumors treated with vaccinia virus, there were barely any gene expression alterations even at a sub-significant level.

- *An engineered adenovirus armed with TNF α and IL-2 increases efficacy*

As adenovirus appeared the best candidate to enhance ACT among the studied viruses, we studied differences between an unarmed virus and a cytokine-armed virus (Figure 5A). TILT-123 is armed with TNF α and IL-2 (Ad5/3-E2F-d24-hTNF α -IRES-hIL2)^{51, 52}, selected for their ability to boost antitumor activity in the T-cell compartment⁵¹.

When comparing antitumor efficacy in the experimental set up described in Figure 1A, TILT-123 performed better in terms of tumor growth control than the unarmed adenovirus (Adenovirus vs PBS $p = 3.41 \times 10^{-4}$. TILT-123 vs PBS $p = 1.15 \times 10^{-9}$) (Figure 5B). Survival data (Figure 5C) shows how the armed version of the virus provided better survival for the first 40 days, but after treatment discontinuation, tumor progression led to the death of many animals (non-significant difference).

Complete responders were rechallenged as described before (Figure 2A). Although the number of animals was limited ($n = 2$), TILT-123 treated animals showed total rejection, specifically for HapT1 tumors (Figures 5D-E). These data seem to indicate improved antitumor efficacy and immunological memory with cytokine-armed oncolytic adenovirus over the unarmed adenovirus.

When studying the impact of the arming device on the trafficking of the adoptive T-cell graft, there was statistically significant increased trafficking when compared to mock ($p < 0.046$), in contrast with the unarmed adenovirus (Figure 5F).

We also studied how the arming device affects the tumor's gene expression profile. When the expression patterns of armed and unarmed adenovirus tumors were directly compared, the only genes significantly downregulated in TILT-123 treated group were *CCL4* (adjusted p value = 0.045, Fold change = 0.436) and *TNF* (adjusted p value = 0.045, Fold change = 0.201) (Supplementary figure 3). When comparing the changes induced by each of those viruses as opposed to the PBS injection, some of the changes were seen with both viruses (*IL1B*, *CD4*, *CD3E*, *GZMM*), while other genes that were unaffected by the unarmed virus were upregulated (*IL2RA*, *PRF1*) or downregulated (*LBP*, *IL-10*, *CCL7*, *CASP3*, *CASP8*) upon TILT-123 treatment (Figures 5G-H). Additionally, several of the upregulated genes with the unarmed virus were not induced with the armed virus (*CCL4*, *CCL3*, *HCK*, *IFNGR1*, *CD40*, *CD80*, *TLR2*, *TLR9*, *CCR1*, *SYK*, *ARG1*, *TNF*).

Discussion

The implementation of immunotherapies such as checkpoint inhibitors and various immune-cell therapy based platforms has mediated a therapeutic revolution in oncology. At the same time, there is clear room for improvement as the patients responding to immunological treatments are still the minority^{26, 53}, with some exceptions (e.g. CAR T therapies in some hematological malignancies or anti-PD1 in selected indications). Oncolytic viruses offer a rational alternative to solve the limitations of those immunotherapies, but the lack of head-to-head comparisons between different virus platforms has slowed optimization of the approach. In addition, given major differences in the biology of popular oncolytic viruses, there might be different optimal uses for each. For that reason, this study focuses in a direct comparison of some of the most relevant candidates in the context of TIL therapy.

When comparing different therapeutic options, the challenge is to select the optimal dosing for each treatment. One possible option is to use the same dose for all of the viruses. For that approach, the first limitation is that different viruses are usually titered by different parameters: adenoviruses in viral particles (vp), herpes simplex and vaccinia virus in plaque forming units (pfu), and reovirus in median tissue culture infectious dose (TCID₅₀). Although these differences could, in theory, be sorted out by assembling a set of viruses and measuring their properties, the conversion between units would not be applicable to multiple virus batches, thus invalidating references to previous work. Another critical limitation when using the same dose for all the viruses is the fact that different viruses have different effects in the host, which is translated into different therapeutic windows. Those virus-caused effects occur at various levels, including at the immune compartment. For this reason, we narrowed down the comparison of the viruses only in the context of T-cell therapies and not going into a deep characterization of the viruses as monotherapies.

The approach almost universally used in oncology is that drugs are used at their maximum tolerated dose. Different oncolytic viruses are used in the clinics at different therapeutic ranges, so taking this information into account also helps obtaining more realistic results with regards to safety versus efficacy. In that sense, and together with the results showing that the 10-fold increase in dose did not produce stronger antitumor effect, we considered that the doses extrapolated weight per weight from the maximum tolerated human dose were appropriate.

Adenovirus emerged as the strongest candidate for enabling adoptive T-cell therapy. Tumors responding to treatment within the first 21 days (5/8) did not progress afterwards, and eventually showed complete responses. The large amount of complete responders (62.5% of all the animals receiving TILs and adenovirus compared to 17.9% of those receiving TILs alone) highlights the potential of adenoviruses to enable T-cell antitumor functions. Other viruses did not provide significant improvements in tumor growth control or

survival. However, some parameters like median survival, were increased when compared with the PBS control (108 days for reovirus versus 67 days for PBS, not statistically significant).

In vaccinia virus, herpes simplex virus and reovirus treated groups, responses to the treatment were observed in individual animals, but unlike in the adenovirus group, some of the tumors that shrank to barely palpable volume, eventually progressed and reached the maximum tumor dimensions allowed. In this particular experiment, the herpes simplex group was the only one that did not show any complete responses. Further engineering of the HSV could help to have higher local antigen presentation (ICP47 deletion) and higher local immunostimulatory effect (gamma34.5 deletion) in the tumor milieu⁵⁴.

As oncolytic viruses have a dual mechanism of action (direct oncolysis and immune-mediated antitumor response), we wanted to assess what is the immune outcome of each oncolytic virus. For that purpose, animals that developed complete responses against pancreatic carcinoma tumors were challenged again with the same cell line and left untreated. The only animals that completely rejected the tumors were those belonging to the adenovirus treatment group. Eighty percent of the animals once cured by oncolytic adenovirus and TIL therapy showed fully protective antitumor-specific memory, while none of the other animals (previously cured with TILs alone or in combination with vaccinia virus or reovirus) managed to reject the graft. Other studies focused on the systemic effect after the use of oncolytic adenoviruses and T-cell therapies, showing that metastatic processes can be avoided⁵⁵ and that uninjected established tumors benefit from the treatment⁵⁶. These results enabled us to hypothesize that oncolytic viruses can deliver complete responses by either direct oncolysis or by the development of immune-related effects. While the development of adaptive responses against the tumor is enhanced by oncolysis^{39, 40}, meaning that the variables are not really independent, it is at least theoretically possible that some oncolytic viruses deliver antitumor responses mainly by direct oncolysis. This would imply that cell lysis by those viruses would not enhance effective generation of antitumor-specific immune responses. The dual mechanism of action does not need to be seen from a binary perspective, but to be understood in a sense that each virus generates both mechanisms to different extents. Of note, our data suggests that there are important differences between viral strains in this regard.

After observing tumor-specific immune responses, it was interesting for us to understand the contribution of the T-cell graft and the overall immune microenvironment to those responses. T-cell graft biodistribution data did not support a hypothesis where the outcome of the therapies could be solely explained by trafficking. A major limitation of this technique could arise from the intratumoral proliferation of infiltrated radiolabeled T-cells. For example, the percentage of the initially injected dose measured by SPECT/CT would be the same regardless of whether T cells newly arrived to the tumor would remain non-proliferative or expand several rounds. Because the radioactivity inside cells cannot increase, a non-proliferative cell would give the same absolute radioactive signal as the sum of *e.g.* 8 cells resulting from 3 rounds of mitosis of the initial cell, while the actual amount of T-cells is 8 times higher (Supplementary figure 4). Still, taking this possibility into account, the values and significances from the early time points in the biodistribution assay do not endorse trafficking as the main mechanistic explanation on why the tumors treated with oncolytic adenovirus respond better to adoptive T-cell therapy than other groups. It is also relevant to emphasize that the trafficking of endogenous T cells to the tumor was not measured, and that can play an important role in terms of antitumor efficacy. Moreover, we did not measure possible *de novo* adaptive responses, also known as epitope spreading, which are known to occur following adenovirus injection into tumors⁵¹.

Investigating expression levels of immunologically relevant genes from tumors complements the T-cell graft trafficking data. Previous studies highlighted the ability of adenoviruses to engage successfully with the immune system, in particular with the T-cell compartment⁵⁷⁻⁵⁹. Following those ideas, the results presented in this study show how adenovirus – uniquely among the studied viruses - triggers a wide immunostimulatory response. Genes upregulated by treatment related to innate immunity elements (*TLR9*, *TLR2*), myeloid cell markers of activation (*CD80*, *CD40*), lymphocyte markers (*CD3E*, *CD4*) and their cytotoxic effector components (*GZMM*), diverse extracellular and intracellular messengers of immune stimulation (*IL1B*, *TNF*, *IFNGR1*, *HCK*,

SYK) and proinflammatory chemokines for the attraction of lymphocytes (*CCL3*) and macrophages (*CCL4*). This molecular network points to a circumstance where it is very likely that innate immunity promotes an eventual appearance of adaptive immunity against the tumor. In this sense, the use of oncolytic viruses seem to have a broad effect in the tumor microenvironment that include not only interactions with the T-cell compartment of the immune system but a diverse number of cell types. Dissecting the effect of the virus on the different cell types in the tumor would be a way to study further the mechanism on how oncolytic viruses can enhance T-cell therapies.

The study of the whole tumor expression patterns was relevant to understand the overall situation at the tumor although it would be valuable to have a deeper understanding on the contribution of those changes specifically by different cell types (*i.e.* tumor cells, dendritic cells, T cells, etc)).

In addition, we saw upregulation of *ARG1*, which codes for arginase, an enzyme that attenuates T-cell activity⁶⁰. The induction of *ARG1* can be a homeostatic response to balance the upregulation of the other 15 immunostimulatory genes^{61,62}. One of the limitations of this experiment is that it has been set to a specific time point (day 6 after treatment started), which might give an incomplete view of the immune effects triggered earlier or later. However, day 6 should be an adequate time point to study the coexistence of both innate and adaptive immune processes, as adaptive responses usually take 4-7 days to be developed. These results go in line with previous work by others⁶³⁻⁶⁵, where they show importance of having a favorable tumor microenvironment that supports antitumor immune responses.

Modern oncolytic virotherapy is gaining relevance as a therapeutic approach due to the possibilities it offers in terms of immune modulation. Several oncolytic viruses that are currently under clinical and preclinical development are engineered to express immune mediators, such as cytokines, immune ligands or antagonists. TILT-123 is a 5/3 chimeric adenovirus, designed specifically to enable the T-cell compartment of the immune system⁵². Because adenovirus performed better than the other viruses as a T-cell enabler, it was particularly attractive to compare the treatment outcome with an adenovirus engineered to enhance specifically such a cell population. The arming device had a positive impact on reducing tumor size, increasing antitumor specific memory and increasing T-cell trafficking towards the tumor. Interestingly, there was less significant upregulation of immunostimulatory genes as compared with the unarmed adenovirus, as previously described by Havunen *et al*⁵⁶. In addition, treatment induced some immunosuppressive genes, such as *IL-10*, which again might be a homeostatic response.

Reovirus and herpes simplex virus showed the fastest direct oncolysis of tumor cells but delivered sub-significant tumor microenvironment modifications, which, if reinforced or optimized, could end up improving the overall outcome of the therapy. Vaccinia seemed to have a less visible impact at the immunological level, maybe because even if tumor selective, it is a virus naturally armed with a considerable armamentarium for immune evasion^{66, 67}. Another possibility for the absence of changes in the immune microenvironment after some of the viruses is that the intrinsic tumor immune suppressivity could dampen the signals produced by those viruses and only the most potent ones would be able to overcome that suppressivity threshold. Nevertheless, vaccinia viruses together with herpes simplex viruses have the largest genome space for the insertion of transgenes. Thus, comparison of viruses armed with immunostimulatory molecules could yield different results. Another layer of complexity is added when taking into account that different viruses have faster or slower oncolytic cycles, which affects both immunogenicity and direct tumor cell killing.

An important issue regarding the use of oncolytic viruses is the length of treatment. In the case of the comparison between armed and unarmed adenovirus it seems that as long as the therapy was ongoing, a higher proportion of tumors were under control with TILT-123 treatment. When the therapy was discontinued, survival decreased to a similar level as with the unarmed group. One interpretation of this phenomenon could be linked to the fact that the virus is designed to express the transgenes only while the virus is actively replicating⁵². If tumor fate is still undecided in terms of antitumor vs protumor forces, having ongoing cytokine production there might play an important role that is ablated as soon as the virus is cleared. This would not be

the case for other sources of antitumor effects, such as the immune responses generated after the oncolytic cycle. It can be argued that long term dosing could be required in clinical trials using viruses with an arming device. This approach was employed in a Phase 3 trial with Talimogene laherparepvec, when treatment continued for up to 18 months if efficacy is seen⁶⁸. Another rationale supporting the multiple administration of the virotherapy relates to the fact that as antiviral immunity builds up, it reduces the persistence of the virus but it can be helpful to boost immune activity inside the tumor⁶⁹. In summary, armed and unarmed adenoviruses appear appealing for enabling T-cell therapy.

This study did not intend to compare the direct oncolytic activity of the different viruses, but instead how they could be used in the context of T-cell therapy. For the use of oncolytic viruses to enable other types of therapies, a different experimental design would be required. In this study's specific context, oncolytic adenoviruses seemed the strongest candidate among those tested. Higher antitumor effects correlated with changes in the tumor microenvironment. Interestingly, modification of the microenvironment appeared more important than effects on T-cell trafficking. The results also make evident the opportunities arising from oncolytic viruses interacting with the immune system to favor antitumor responses. Tailoring a T-cell specific arming device in an adenovirus (such as TNF α and IL-2 used here), can help improve the immunostimulatory capacities of these viruses even further. TILT-123 is now being studied in melanoma patients receiving a TIL therapy (NCT04217473).

Materials and Methods

- Oncolytic viruses

Oncolytic viruses from four different virus families were included in this study: Ad5/3-E2F-d24 (*Adenoviridae*), VVtd-tomato (JX-929 strain) (*Poxviridae*)⁷⁰, HSV-1 (17+)Lox-PmCMVGFP (*Herpesviridae*, a kind gift from Beate Sodeik, Hannover Medical School, Germany)^{71, 72} and Pelareorep (Reolysin[®]) (*Reoviridae*, a kind gift from Oncolytics). An engineered version of the adenovirus candidate (Ad5/3-E2F-d24-hTNF α -IRES-hIL2, A.K.A. TILT-123) was also used in the experimental phase. Intratumoral administration of the viruses in 50 μ l of PBS (or PBS alone in the control groups) was performed by direct injection with 30G insulin needles according to the schedule established for each experiment.

- Animal and tumor model

Male Syrian hamsters (French colony) were used as an animal model for the *in vivo* experimentation. They were obtained as 4-6 week old from Charles River Laboratories (Wilmington, Massachusetts, USA). Syrian hamster model was selected to study *in vivo* efficacy, as it is one of the few models permissive for the replication of all of the viruses used in the study⁴²⁻⁴⁵. The tumor model selected is the syngeneic HapT1 pancreatic carcinoma as it allows growing tumor infiltrating lymphocytes (TILs) *ex vivo*, to be used as a model for adoptive cell therapy (ACT). A syngeneic pancreatic carcinoma cell line (Hap-T1) was used to studying the antitumor efficacy of the treatments in the study. For that purpose, 2×10^6 cells were subcutaneously delivered into the lower lateral flank(s) of the hamsters. 5-6 days later, when tumors were palpable and measurable, they started receiving treatments. Animals, whose tumors surpassed the maximum tolerated tumor dimensions, were euthanized and marked as dead. Animals developing ulcers were marked as censored in survival studies and euthanized. When studying antitumor memory, those animals showing complete responses from the originally engrafted tumor (lower right flank) were rechallenged with 2×10^6 HapT1 cells in the upper left flank and 2.5×10^5 DDT1-MF2 (syngeneic leiomyosarcoma cell line) in the upper right flank to evaluate the specificity of the antitumor memory. For the tumor volume records, a digital caliper was used to measure tumor dimensions and transformed into volume by using a standardized formula ($0.5 \times \text{longest diameter} \times \text{shortest diameter}^2$).

- *Adoptive cell therapy treatments*

Tumor-infiltrating lymphocytes (TILs) were generated out of HapT1 tumors and used as adoptive cell therapy graft as described before⁴⁶. Briefly, HapT1 tumor were grown until they reach a diameter close to 20 mm. At that point, tumors are harvested and cultured in immunostimulatory conditions (IL-2 and Concanavalin A) to expand the TILs present in the tumor. After expansion, T cells are collected, and 4×10^7 cells are intraperitoneally administered to the animals on what is considered day 0 of the experiments.

- *Biodistribution analyses*

TILs were labeled with ¹¹¹In-oxine as described earlier and administered intraperitoneally into Syrian hamsters⁵⁶. The injected dose was 4.82 ± 0.72 MBq. The hamsters were bearing two HapT1 tumors (n = 3-4/group). Animals were imaged with NanoScan SPECT/CT (Mediso, Budapest, Hungary) at 24, 72, and 144 hours after the administration of the radiolabeled cells. For *in vivo* measuring of the TIL trafficking and accumulation, tumors were delineated by using the co-registered CT images. The results were calculated as percentage of activity in the tumor from the injected dose. Corresponding values from the biodistribution data at day 6 were divided by the tissue mass (g). On day 6, tumors were harvested after *in vivo* imaging and the radioactivity was measured *ex vivo* by a gamma counter (Wizard 3, Perkin Elmer, Waltham, Massachusetts, USA) for validation. Similarly, CT-defined tumor volumes were correlated with *ex vivo* mass to validate the approach. Other tissues such as blood, kidney, spleen, and muscle were also harvested for *ex vivo* measurement of radioactivity.

- *Gene expression analyses*

Tumors harvested during *in vivo* experimentation were stabilized in RNAlater (R0901, Sigma-Aldrich, St. Louis, Missouri, USA) and stored at -20°C. RNA was purified from those tumors following RNeasy (74104, Qiagen, Hilden, Germany) kit manufacturer's guide. Total RNA concentration was measured in all the samples with a Biophotometer (Eppendorf, Wesbury, New York, USA) to ensure the presence of sufficient RNA concentration. For quantitative assessment of the expression of 96 genes, we designed a custom nCounter panel (NanoString technologies, Seattle, Washington, USA) that was run for the samples as indicated by manufacturer. Data analysis was performed by Nanostring's Data Analysis service (single blind analysis) where normalization of gene expression based on housekeeping genes was performed. Quality control check-ups were performed for all the samples and all the target genes included in the panel. Differential expression in genes between oncolytic virus treated tumors versus PBS treated tumors was represented in volcano plots based on the significance and fold change for each gene.

- *Oncolytic activity measurements*

HapT1 cells were cultured *in vitro* up to 14 days in the presence of the above mentioned viruses at different concentrations. The concentrations used are relative to the viral doses described before. Cell viability was assessed with Cell Titer 96 AQueous One Solution Cell Proliferation Assay (G3582, Promega, Madison, Wisconsin, USA), following manufacturer indications. The viability of mock-treated cells was set to 100%.

- *Statistics*

SPSS Statistics 25 software (IBM, Armonk, New York, USA) was used to perform a mixed-model analysis for tumor growth evolution based on the tumor volumes (logarithmic transformation of the volumes normalized on day 0 volumes) as described before⁷³. GraphPad Prism 8 (GraphPad Software, San Diego, California, USA) was used for log rank Mantel-Cox test on Kaplan-Meier survival curves, Pearson's *r*, linear regression, as well as the graphic representation of the data. P values under 0.05 were considered statistically significant

- *Ethical statement*

Based on the recommendations included in the Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013) and the Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013) as well as the European Directive 2010/63/EU, experimental protocols and procedures were established and then approved by the ethical committee of the Animal Experimental Board (ELLA) of the Regional State Administrative Agency of Southern Finland.

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Author contributions

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- Development of methodology: VCC, JMS, DCAQ, JHAC, TK, DL, MV, MA.
- Acquisition of data: VCC, EK, DCAQ, JHAC, JMS, RH, TK, DL, MA.
- Analysis and interpretation of data: VCC, DCAQ, JMS, RH, MS, TK, AA, AH.
- Writing, review and/or revision of the manuscript: VCC, DCAQ, RH, JMS, EK, JHAK, MS, SZ, CH, TK, DL, MV, AG, AA, SS, MA, VH, AK, AH.

- Administrative, technical, or material support: AG, AA, MA, VH, AK, AH.

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Figure information

Figure 1. Antitumor efficacy after the use of different oncolytic viruses to enable T-cell therapy. (A) Experimental design: forty-one Syrian hamsters carrying subcutaneous HapT1 tumors were randomized into groups and treated with TILs intraperitoneally, and PBS or one of the studied viruses intratumorally. After the treatment period, the animals were followed up to day 250. (B-F) Individual normalized tumor volume lines for the different groups (n=8-9). Tumor size at the beginning of the treatments was set to 100% (Mixed model analysis, ***p<0.001). (G) Overall survival of the treated animals. (Kaplan-Meier, Log rank Mantel-Cox test; *p<0.05).

Figure 2. Study of antitumor memory in complete responders. (A) Experimental design: animals treated with ACT and different oncolytic viruses (or PBS) experiencing complete responses were rechallenged with HapT1 and challenged with DDT1-MF2 to study antitumor specific memory (no additional treatments given). In addition, naïve animals were simultaneously engrafted with HapT1 and DDT1-MF2 tumors. (B) Groups included in the experiment: Naïve animals group (n=3), PBS + TILs group (n=1), Adenovirus + TILs group (n=5), Vaccinia + TILs (n=1) and Reovirus + TILs (n=1). The number of animals depended on how many had been cured in the first part of the experiment. (C) Mean tumor volumes for the HapT1 tumors. (D) Mean tumor volumes for the DDT1-MF2 tumors.

Figure 3. Tracking of systemically administered TILs after oncolytic virus intratumoral injection. (A) Experimental design: thirty-four Syrian hamsters carrying subcutaneous HapT1 tumors were randomized into

groups and treated with $^{111}\text{Indium}$ labelled TILs intraperitoneally, and PBS or one of the studied viruses intratumorally. During the experiment, animals were imaged with SPECT/CT to quantify the biodistribution of the injected TILs. At the end of the experiment, animals were euthanized and different organs were harvested for *ex vivo* $^{111}\text{Indium}$ measurement or, in the case of tumors also for multiplexed RNA sequencing. (B) Normalized tumor volumes for the different groups ($n=6-8$). (C) Correlation between tumor volumes measured using CT and the weight of those tumors after they were harvested. (D) Correlation between the *in vivo* radiation signal measured with SPECT/CT and the *ex vivo* samples measured by gamma counting. (E) TIL-associated radiation of tumors measured *in vivo* with SPECT/CT on days 1, 3 and 6. (F) TIL-associated radiation measured *ex vivo* on different tissues by gamma counting on day 6 ($*p<0.05$; $**p<0.01$).

Figure 4. Impact of different oncolytic viruses in the tumor microenvironment. Comparisons were made between RNA expression profiles from the different virally treated groups vs PBS treated group. Viral treatment used in the group compared to PBS treated animals: (A) Adenovirus. (B) Vaccinia Virus. (C) Herpes simplex virus. (D) Reovirus. The plots indicate the names of those genes for which there is a statistically significant difference (adjusted p value <0.05) and an expression change of at least double or half compared to reference group ($-1 > \text{Log}_2 \text{fold change} > 1$).

Figure 5. Antitumor and immunological effects of arming an adenovirus with immunostimulatory cytokines. (A) Summary of unarmed and armed virus constructs. (B) Grouped normalized tumor volume values for the different groups ($n=6-9$ per group) after receiving TIL therapy (intraperitoneally) and virotherapy or PBS (intratumorally). Treatment schedule is presented in Figure 1A. (C) Overall survival data. Gray dashed line marks discontinuation of the treatments. (D) Mean tumor volume at day 19 after HapT1 rechallenge in complete responders from HapT1 tumors. (E) Mean tumor volume at day 19 after DDT1-MF2 challenge in complete responders from HapT1 tumors (Naïve $n=3$, PBS+TILs $n=1$, Adenovirus+TILs $n=5$, TILT-123+TILs $n=2$). (F) 13 Syrian hamsters carrying subcutaneous HapT1 tumors were randomized into groups and treated with $^{111}\text{Indium}$ labelled TILs intraperitoneally, and with PBS ($n=4$), unarmed adenovirus ($n=5$), or armed adenovirus ($n=4$). At day 6 of the experiment, tumors were harvested for *ex vivo* $^{111}\text{Indium}$ measurement by a gamma counter ($*p<0.05$). Comparisons were made between the RNA expression profiles from each of the two adenovirus treated groups vs PBS treated group. Animals carrying these tumors were treated as described in Figure 3A. Viral treatment used in the group compared to PBS treated animals: (G) Adenovirus. (H) TILT-123. The plots indicate the names of those genes for which there is a statistically significant difference (adjusted p value <0.05) and an expression change over double or below half to those in the reference group ($-1 > \text{Log}_2 \text{fold change} > 1$).

Tables

VIRUS	REFERENCE	YEAR	MAXIMUM DOSE (HUMAN)	EXTRAPOLATED DOSE (HAMSTER)
ADENOVIRUS	Small EJ ⁴⁸	2006	6×10^{12} vp	8×10^9 vp
VACCINIA	Zeh HJ ⁴⁹	2015	3×10^9 pfu	4×10^6 pfu
HERPES SIMPLEX	Andtbacka RH ⁴⁷	2015	4×10^8 pfu	5.3×10^5 pfu
REOVIRUS	Karapanagiotou EM ⁵⁰	2012	3×10^{10} TCID ₅₀	4×10^7 TCID ₅₀

Table 1. Viral dose extrapolation according to maximum tolerated doses in humans. *vp*; viral particles. *pfu*; plaque forming units, $TCID_{50}$; median tissue culture infectious dose.

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List of Changes after Reviewers comments

Reviewer #1 (Remarks to the Author):

This is a convincing manuscript that provides critical data supporting viro-immune therapy of cancer. The studies were done in an adequate hamster model and employ state-of-the art techniques to document the oncolytic and immune-stimulatory effects of adenovirus, vaccinia virus, herpes simplex virus, and reovirus in combination with adoptive T-cell therapy. A critical caveat of such a comparative study, i.e. selecting the treatment dose for the different viruses, has been addressed satisfactory.

Minor comments:

-Figure 1: Viruses were injected every three days until day 39. Please comment on the effect of developing adaptive immune responses against the virus on anti-tumor efficacy of the treatment.

The impact of antiviral immune responses was added into the discussion:

“Another rationale supporting the multiple administration of the virotherapy relates to the fact that antiviral as antiviral immunity builds up, it reduces the persistence of the virus but it can be helpful to boost immune activity inside the tumor”.

-Please discuss the relevance of the approach for metastatic disease, when only selected tumor lesions can be injected with oncolytic virus.

It has been included a sentence (and its respective references) regarding the relevance of the approach on uninjected lesions and metastatic disease.

“Other studies focused on the systemic effect after the use of oncolytic adenoviruses and T-cell therapies showing that metastatic processes can be avoided and that uninjected established tumors benefit from the treatment.”

-Please provide references for successful anti-PD1 CAR-T therapy.

References including the success of CAR T-cell therapy and anti-PD-1 were added into the first paragraph of discussion.

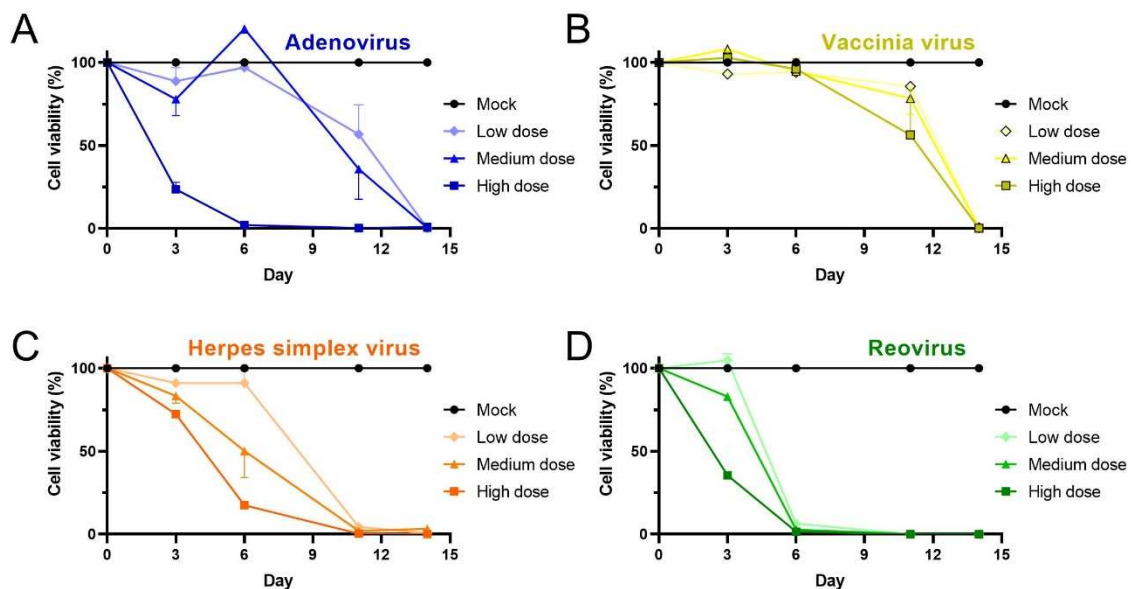
Reviewer #2 (Remarks to the Author):

Authors compare four different oncolytic viruses in terms of antitumor activity when combined with TIL therapy against one immune-competent hamster tumor model. The idea of comparing different oncolytic viruses is seldom presented in papers and if properly done would direct clinical research toward the most effective virus type. In general terms although the concept is really relevant, a sound conclusion in favor of one virus type against the others would require research in different conditions and models.

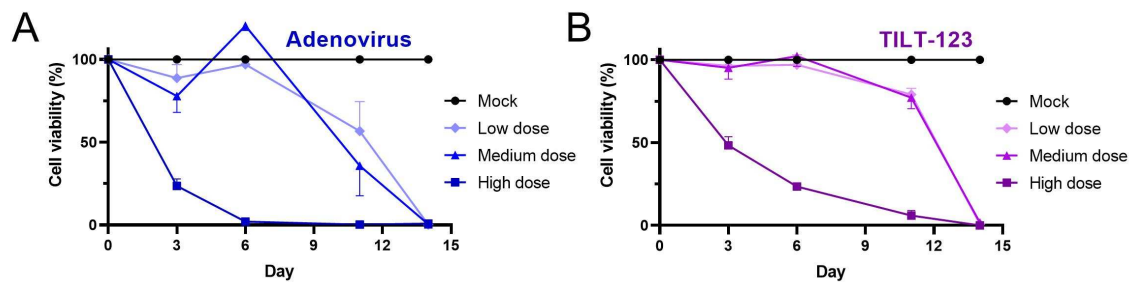
Although the authors discuss that they did not intend to compare the direct oncolytic activity of the four viruses but how they behave in the context of T-cell therapy, a virus-alone immunotherapy should be the basis to further explore combination immunotherapies (such as the TIL-virus combo presented in the current paper). One hamster tumor model (HapT1) is selected based on a putative replication permissiveness to the four viruses. It would be nice to see a comparison of such permissiveness *in vitro*, and also to study the supposed intratumoral virus replication *in vivo*. A central question remains as to whether replication is needed at all, in particular when so many intratumoral injections are performed. Replication in tumors is not evaluated. Although the main aim is not to compare oncolytic activity, this parameter should be investigated to understand the results.

Direct oncolysis ability from all the viruses was studied on HapT1 cells. A new figure was created (supplementary figure 1), and two graphs were added to supplementary figure 3. Results text description for figure 1 was also amended to include the new data:

“The selected tumor model, HapT1 pancreatic carcinoma, enables the isolation of tumor infiltrating lymphocytes (TILs) for *ex vivo* amplification, for use as an adoptive cell therapy (ACT). It was also assessed how the different viruses have different oncolytic dynamics on this cell line, even if all of them showed complete tumor cell lysis by day 14 (Supplementary figure 1).”



Supplementary figure 1. Oncolytic capability of the viruses. HapT1 cells were cultured with three concentrations of viruses extrapolated from in previously described treatments (using each virus standard units) and cell viability was measured at days 3, 6, 11 and 14. (A) Adenovirus: 10^2 , 10^3 and 10^4 vp/cell. (B) Vaccinia virus: $5 \cdot 10^{-2}$, $5 \cdot 10^{-1}$ and 5 pfu/cell. (C) Herpes simplex virus: $7 \cdot 10^{-3}$, $7 \cdot 10^{-2}$ and $7 \cdot 10^{-1}$ pfu/cell. (D) Reovirus: $5 \cdot 10^{-1}$, 5 and $5 \cdot 10^1$ TCID₅₀/cell.



Supplementary figure 3. Impact of the TNF α and IL-2 arming device on oncolytic activity and gene expression. (A-B) HapT1 cells were cultured with three concentrations of both adenoviruses and cell viability was measured at days 3, 6, 11 and 14. 10^2 , 10^3 and 10^4 vp/cell of each virus was used.

This data was generated to check that the viruses are able to trigger oncolysis on HapT1 cells and to understand if there are differences in the lysis kinetics between viruses. Further studies (refs 42-45) on the use of those viruses *in vivo* in solid tumor models strengthen the conclusion.

Going deeper into the question of the relevance of multiple administration of the virus was not something we focused in on this paper as it is generally accepted in the field, even in clinical practice with talimogene laherparepvec that multiple administration is beneficial for the treatment's outcome. In addition, other possible benefits of the multiple administration scheme might rely on the antiviral immunity boosting the antitumor one. A study trying to challenge single vs multiple oncolytic virus administration should be thorough and probably an article by itself. Discussion was added with ideas about this topic:

“Another rationale supporting the multiple administration of the virotherapy relates to the fact that antiviral as antiviral immunity builds up, it reduces the persistence of the virus but it can be helpful to boost immune activity inside the tumor”

Of course, the relevance of the results would increase if more than just one model is tested. Other hamster tumor cell lines have been reported to be permissive to adenovirus.

While we understand the benefits of adding more models and more cell lines, we were not able to confirm adenoviral replication from additional models for which there are also available TIL production possibilities.

Even for Syrian hamster tumor models, creation of a TIL graft of quality is challenging and only optimized to our knowledge for HapT1 (Siurala M et al. Oncoimmunology 2016) while other tumor models failed to provide meaningful results.

Another parameter is the genetic modifications in the compared viruses. In this paper vaccinia seems to have tomato fluorescent protein and herpes seem to express GFP, compared to adenovirus and reovirus which are devoid of transgenes. Could this affect the results?

The use of bioluminescent tags such as the tomato fluorescent protein and GFP, has been widely tested in different vectors and models without noticeable impact in their functionality. Their lytic ability could be affected if the cloning of the fluorescent tags would be incorrect but the new Supplementary figure 1 is the evidence that they are still fully replicative (besides the fact that it has been used by our collaborators, the ones that created those viruses).

A different discussion would be to around the topic of the immunogenicity of those fluorescent proteins. While some studies showed that those proteins are immunogenic and can cause adaptive

immune responses against GFP+ or tomato fluorescent protein+ cells, in this study we don't see major immunogenic processes developing in those groups. As they are the groups with the lower modifications in gene expression, we do not think hypothetically less immunogenicity (by removing the fluorescent tags) would change the results in the comparison of the viruses.

The adenovirus selected has the chimeric 5/3 capsid. How does this compares to the unmodified capsid?

Different adenovirus serotypes have been used both preclinically and clinically (Koski, A., et al. 2013 Human Gene Therapy, Cervera-Carrascon V et al. 2019. Expert opinion in biological therapy).

As mentioned in previous comments differences could be expected from different viruses, but we understand that 5/3 serotype is one relevant candidate to study in the scope of the present study. Furthermore, the comparison between 5/3 serotype versus purely 5, are likely to be minimal under the experimental conditions used as the model is only semi-permissive to adenoviruses.

Are the results only applicable to the four particular viruses studied, and in only the HapT1 model, and only when combined with TIL therapy?

About the concerns regarding the presence of one model only, those were replied in the reviewer question #3 ("Of course, the relevance of the results would increase if more than just one model is tested. Other hamster tumor cell lines have been reported to be permissive to adenovirus.").

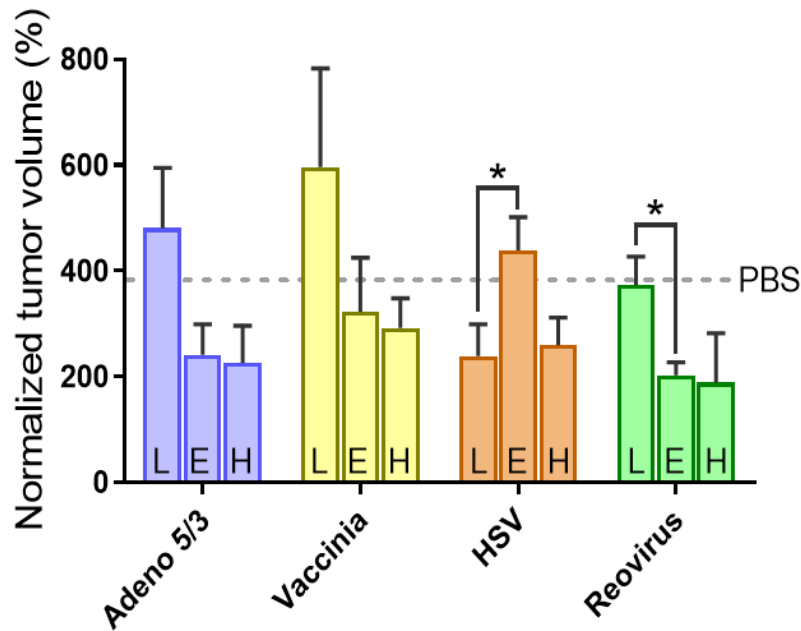
Yet again, we understand the point that testing more viruses and not only the particular ones would help to have a broader view on the topic but having representative viruses from four different virus families gives a sense on how different virus related biologies impact the ability to enable immune responses.

Regarding TIL therapy, it comes back again to the fact that HapT1 is the only suitable model to generate TILs. There are no CAR T cell therapies or Checkpoint inhibitors for Syrian hamster, so it is not possible to test further alternatives of T cell therapies.

Why the MTD is taken from studies where the injection route is different (systemic for adenovirus and reovirus vs intratumoral for herpes and vaccinia)?

The maximally tolerated dose for each virus was chosen regardless of the route of administration as the extrapolated doses were going to be tested with a 10 fold increase and a 10 fold decrease for a confirmation that the dose/effect had reached plateau (Supplementary figure 2).

In those confirmatory studies we saw how for all the viruses, a 10 fold increase did not produce an improved outcome in terms of antitumor efficacy. That finding, together with the daily check-up on the health of the animals where no visible side effects were assessed after the treatments, allowed us to support the doses selected.



Supplementary figure 2. Assessment of the dose-effect differences for different oncolytic viruses. 39 Syrian Hamsters engrafted with bilateral subcutaneous HapT1 tumors were randomized and assigned to different groups treated intratumorally with PBS or different oncolytic viruses at different doses (L; 0.1x extrapolated dose. E; 1x extrapolated dose. H; 10x extrapolated dose). Each group comprised 6 tumors. Mean normalized tumor volume for each group is shown (with SEM). The grey dashed line represents mean normalized tumor volume for a negative control group.

Do TILs alone (intraperitoneally administered) provide any therapeutic effect compared to no treatment?

Development of TIL therapy for this specific model have been developed (Siurala M et al. 2016. Oncoimmunology) and repeatedly tested, showing limited antitumor efficacy (see snip below from Santos JM et al. 2016. International Journal of Cancer)

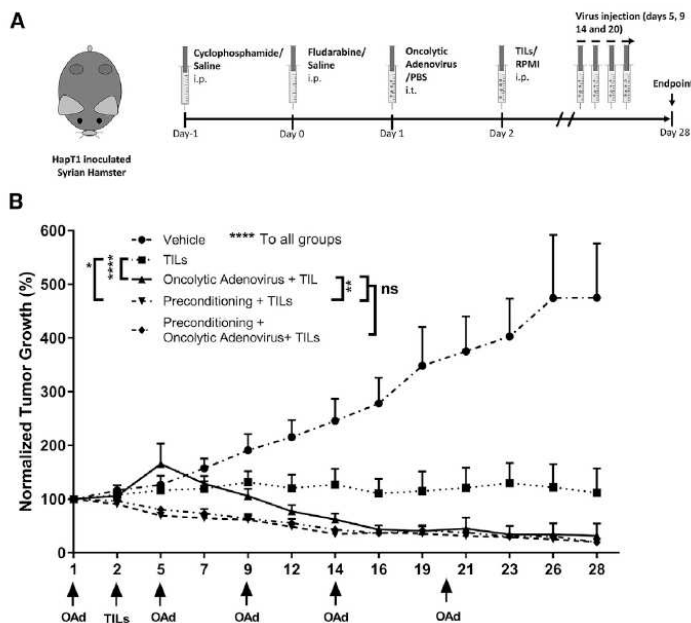


Figure 1. Antitumor Efficacy and Systemic Effects of Local TIL and Oncolytic Adenovirus Therapy of Hamsters

(A) Hamsters with 5-day-old subcutaneous HapT1 tumors received a lymphodepleting regimen consisting of cyclophosphamide (550 mg/kg) and fludarabine (100 mg/kg) or saline intraperitoneally (i.p.). 1×10^8 virus particles (VPs) oncolytic adenovirus (OAd) coding for human IL-2 and TNF- α or PBS was injected intratumorally (i.t.) on day 1 and repeated 4 times. Tumor-infiltrating lymphocytes (TILs) or RPMI was injected i.t. a day after the first virus injection. (B) Tumor growth curves from different treatment groups (n = 5–6). Tumors were measured every 2–3 days after virus treatment initiation and followed until day 28 when animals were sacrificed and tumors and organs were collected for further analysis. (C) Ratio of CD4/CD8+ cells obtained by flow cytometric analysis on endpoint tumors. (D) Mitochondrial activity corresponding to cell viability obtained by performing MTS assay on co-cultures of endpoint-pooled splenocytes from each therapeutic group and tumor cell lines at a ratio of 20:1 splenocytes:tumor cells. Experiment was performed in triplicates. Data are presented as mean + SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Is the multiple intratumoral injection (every three days) effective? (Produced NABs preclude intratumoral infection?)

Besides the comments provided to the reviewers comments on point #1 (“A central question remains as to whether replication is needed at all, in particular when so many intratumoral injections are performed”), it is highly likely that the administration of the viruses triggers generation of adaptive responses against the viruses themselves. As the multiple administration is given at the same time, we do not believe this would be a point to change the endpoint result of the comparisons.

From a different angle, the impact of antiviral has also been understood as a factor with a positive outcome arising from oncolytic virotherapy in the sense that antitumor responses will be indirectly boosted when the tumor microenvironment (Ricca JM et al. 2018. Mol Ther, Li X et al. 2017. Clin Can Res).

Considering this information, we decided to focus on the comparison of the viruses using the same scheme rather than trying to optimise regimen of administrations of different viruses.

Rechallenging results (Fig2) for vaccinia and reovirus were based on one animal (and none for herpes) and as mentioned no conclusions can be drawn. However it would be really of interest to know if these viruses induce antitumor immunity. The same very limited number of animals applies to the rechallenging experiment with TILT123 (Fig5).

As the reviewer acknowledged, we are not drawing conclusions regarding the statistical significance of the rechallenging experiments with a low number of subjects. On the other hand, the low number of animals that showed complete responses from the initial tumor is informative on the whole efficacy of the treatment.

Even if it would be interesting to have a deeper understanding on the antitumor memory for those groups and have significant results, the amount of animals per group should be around 40-50 to end up with 5-6 complete responders. Following those indications will result in an animal experiment with 250 animals, which is considerably demanding.

Similar studies regarding adaptive memory of adenovirally treated tumors have been shown in other studies (Havunen R et al. 2018. Mol Ther Oncolytics).

The T cell biodistribution study (Fig 3) would have been more informative if T cells had been labeled with a tracer that can be seen in tissue (tumor) sections (several fluorescent tracers are available for this) and an in situ biodistribution had been studied.

The use of radioactive tracers required for in vivo tracking of the TILs transferred to the animals made logistically challenging many subsequent analyses of the tumors *ex vivo*. As each virus could have effects on T cell biodistribution at different times, in this study we considered more informative to have multiple measurements of the T cell biodistribution rather than having an *ex vivo* evaluation at a particular time point.

It is really intriguing that multiple injections of herpes, vaccinia or reovirus intratumorally do not induce significant changes of intratumoral gene expression of inflammatory genes and other innate immune genes (Fig 4, and suppl table 2, with vaccinia even not sub-significant changes). One would expect that a virus in a tumor induces some inflammatory changes. Does this match with results described for those viruses? Has this been corroborated in multiple experiments?

Results from Figure 4 have been confirmed in two different experiments. Statistical analyses of the gene expression were performed blindly by the Data analysis team at Nanostring, and even if one would expect significant inflammatory changes after oncolytic virus administration the significance is not present with this sample size. With this sample size we could not obtain significance for those viruses but most likely with a higher n, statistical significance would be achieved.

In this sense, we consider informative that at the current sample size adenovirus shows multiple significant upregulations while the other viruses do not. Those kind of observations are the whole point of the study as what we are aiming to compare the viruses with each other. We do not claim that Herpes simplex viruses or Vaccinia viruses do not cause inflammation but with the current data we can just conclude that Adenovirus achieves it better.

We did not intend to go deep on the biology of each virus to try to explain the results but for example poxviruses (such as Vaccinia virus) as mentioned in discussion:

“Vaccinia seemed to have a less visible impact at the immunological level, maybe because even if tumor selective, it is a virus naturally armed with a considerable armamentarium for immune evasion”.

Reviewer #3 (Remarks to the Author):

Major comments:

1. Although the authors found that combination with Onc.Ad and TILs had the best anti-tumor effects among Onc.Vs tested in this study, they did not address their overall hypothesis that Onc.V can improve the utility of adoptive T-cell therapy because they injected Onc.Vs every 3 days up to day 40 (15 times) in contrast to single TIL infusion at day 0. To better address their stated hypothesis, the authors should evaluate/compare the anti-tumor effects of injections of Onc.Vs prior to TIL infusion as in their prior study (Molecular Therapy Oncolytics 2017).

An alternative and interesting approach for the regimen of administration would be as proposed in this comment when a number of OV treatments would be given prior to the adoptive cell transfer. In our 2017 study, we used such approach only Adenoviruses, but as it was the only virus used there we don't know if the same conclusions could be extrapolated for other viruses. There is the possibility that different viruses would have different optimal regimen of administrations. As the regimen of administration presented in this manuscript is the same for all the viruses we think it is a fair approach.

2. Following the above, since the authors stated that Onc.V treatments induce immunostimulatory signals and reverse immunosuppression, the authors should address how these Onc.V treatments change the cytokine/chemokine profile of tumors as well as the tumor microenvironment (immune cell infiltration) prior to TIL infusion.

Having an understanding on how these different viruses affect the tumor without the TIL infusion would be of relevance but the present work aims to understand this phenomenon in the specific context of T-cell therapy. For that purpose it is stated in discussion that:

“...this study focuses in a direct comparison of some of the most relevant candidates in the context of TIL therapy.”

3. If authors aim to compare the direct impact of Onc.V treatments on adoptively transferred TILs,

they should isolate adoptively transferred TILs (e.g., tagged with EGFP) and profile their RNA expression, especially in the experiment directly comparing Unarmed Onc.Ad to TILT-123.

The use of oncolytic viruses to enable TILs is a process most likely enabled by a wide range of effects triggered in the tumor, including direct effects on TILs but not exclusively. In this sense, the interaction of the viruses with the microenvironment can be as critical or even more to TIL functionality than the direct interaction between viruses and TILs. Even if we prioritized on the overall changes in the tumor,

“the use of oncolytic viruses seem to have a broad effect in the tumor microenvironment that include not only interactions with the T-cell compartment of the immune system but a diverse number of cell types. Dissecting the effect of the virus on the different cell types in the tumor would be a way to study further the mechanism on how oncolytic viruses can enhance T-cell therapies.”

Minor comments:

1. In the Abstract and Discussion, the authors mention that a clinical trial ongoing with combination of adoptively transferred TILs and TILT-123, but there is no NCT number in manuscript. The authors should provide NCT number.

NCT number from clinical trial is now included in the text where it is mentioned.

“A clinical trial is ongoing, where patients receiving TIL treatment also receive TILT-123 (NCT04217473).”

“TILT-123 is now being studied in melanoma patients receiving a TIL therapy (NCT04217473).”

2. In the Introduction, the history of viruses for cancer treatment (first paragraph) is redundant. The authors can remove this paragraph and emphasize how other groups (University of Pennsylvania, Baylor College of Medicine, IDIBELL) combine Onc.Vs with T-cell therapy to improve adoptively transferred T cell activity.

Paragraph 1 has been edited to avoid redundancy.

References including international efforts using oncolytic viruses to enable T-cell therapies were added in the last paragraph of introduction.

3. The authors should clarify that adoptively transferred T-cell therapies have succeeded in patients with hematologic malignancies but have had limited success in patients with solid tumors. The current statement in the manuscript misleads readers, and these sentences should be changed.

Modifications in the text were performed following reviewer's indications.

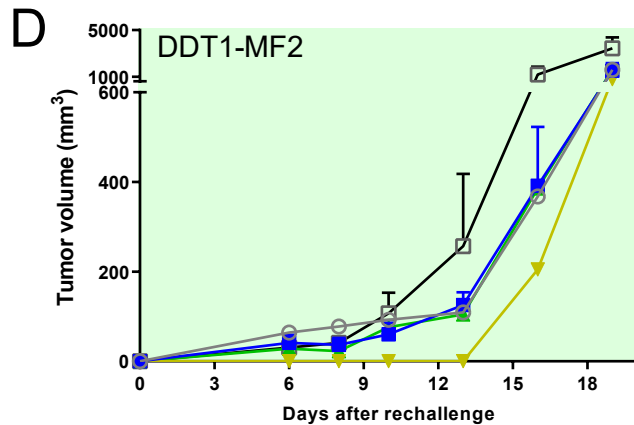
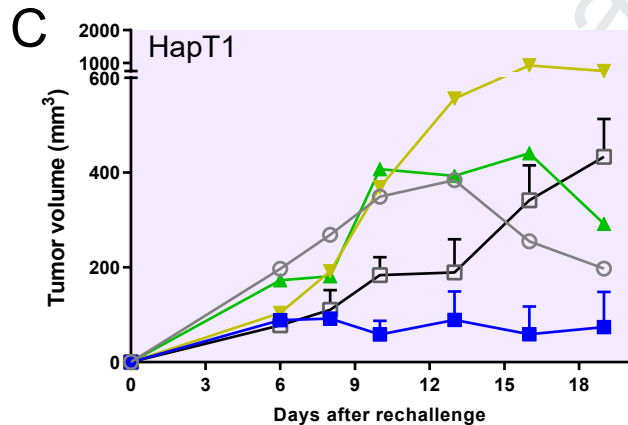
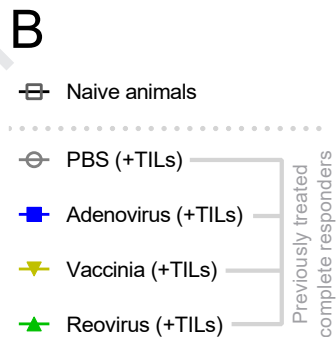
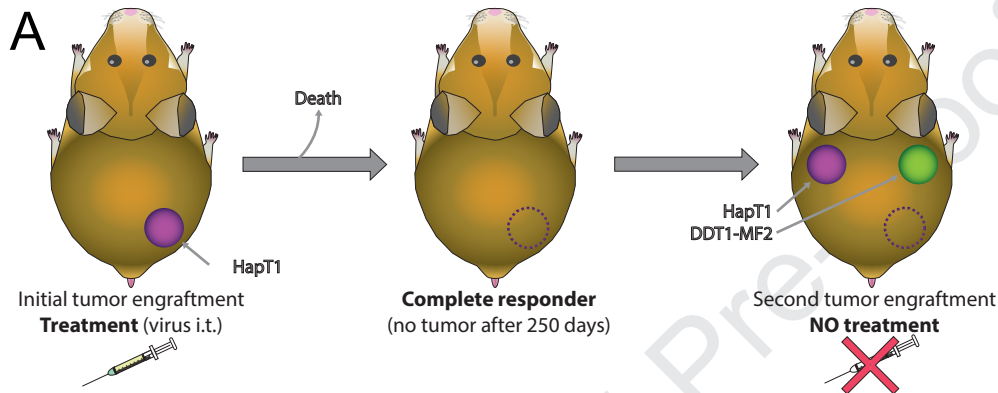
“The implementation of immunotherapies such as checkpoint inhibitors and various immune-cell therapy based platforms has mediated a therapeutic revolution in oncology. At the same time, there is clear room for improvement as the patients responding to immunological treatments are still the minority, with some exceptions (e.g. CAR T therapies in some hematological malignancies or anti-PD1 in selected indications).”

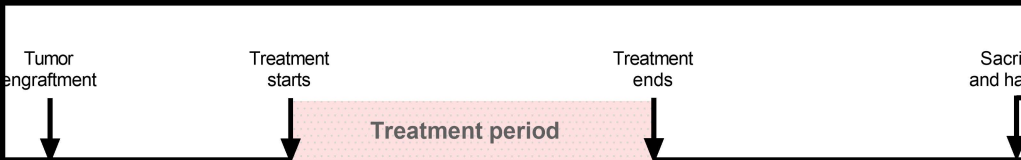
4. The authors should evaluate whether Onc.V agents tested in this manuscript similarly infect and lyse HapT1 cells in vitro and put that result in Fig. 1.

A new figure was created after the study of the direct lytic capability of the different viruses used in the rest of the manuscript. Those results have been included as a separate new figure and not as part of Figure 1 as it could mislead the reader.

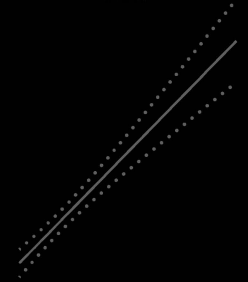
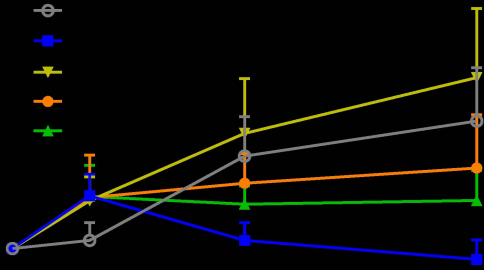
While the new figure relates to the direct lytic ability of each virus *in vitro*, figure 1 describes the method to calculate the dose after the maximum doses used *in vivo* (in humans). For that reason we believe it is more adequate to have figure 1 for dose calculation and supplementary figure 1 as a test on how different viruses have different oncolytic velocities.

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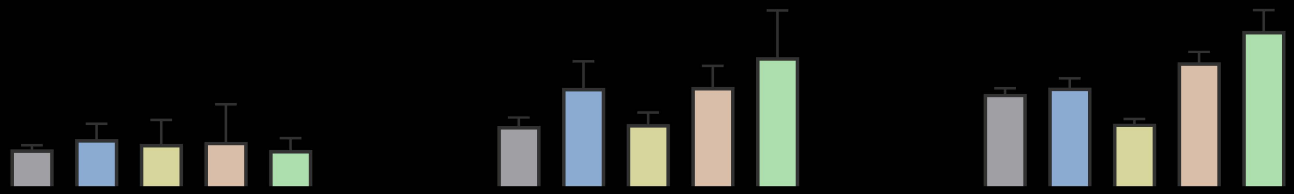




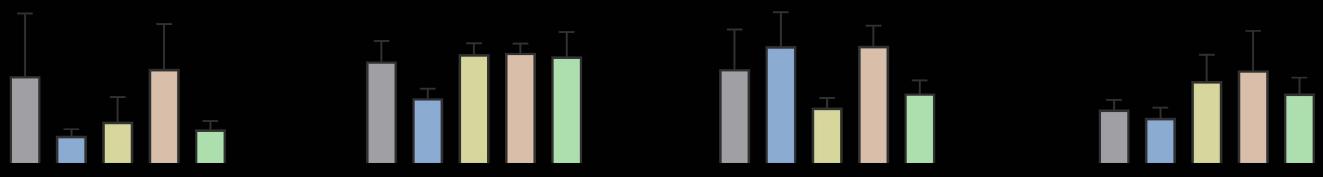
- RNA profiling**
 - 1/2 tumor
- Gamma counting**
 - 1/2 tumor
 - blood
 - kidney
 - spleen
 - muscle



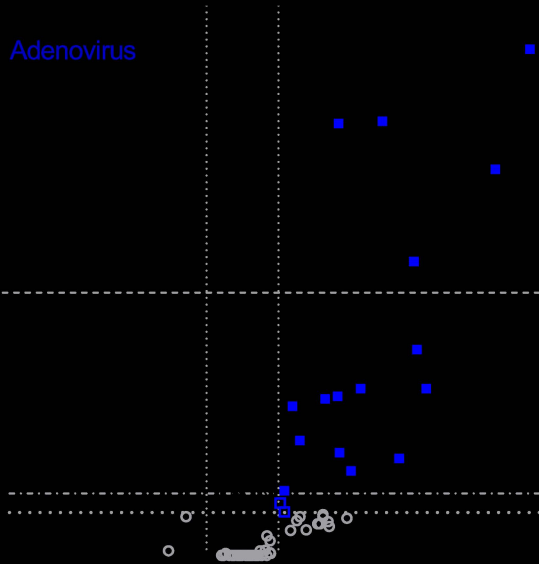
..... SPECT/CT (*in vivo*)



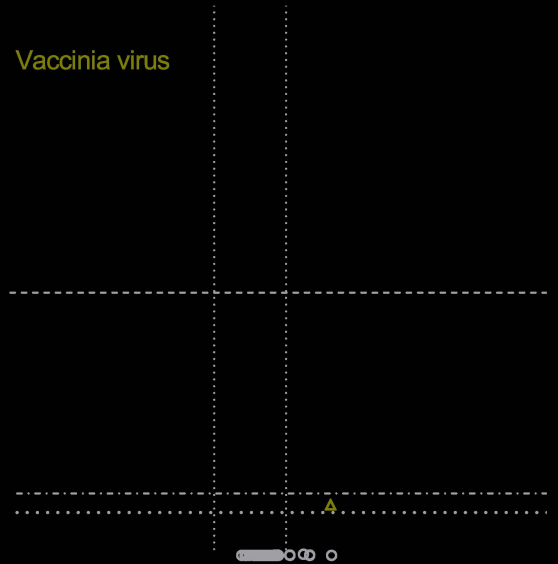
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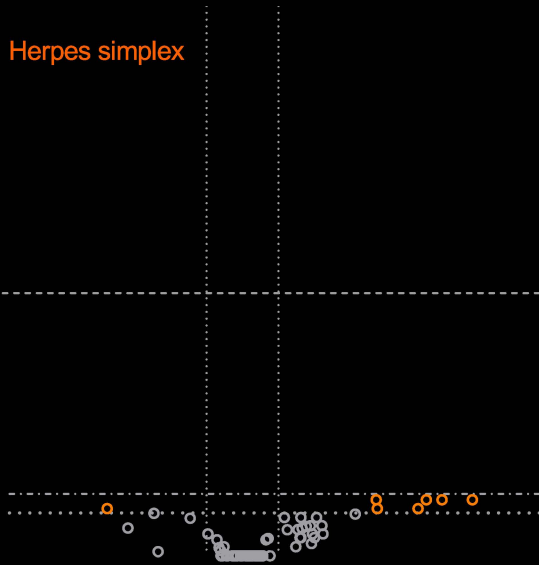
Adenovirus



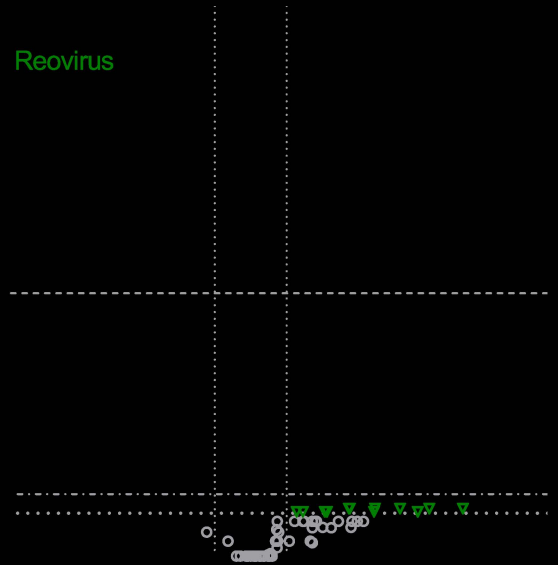
Vaccinia virus

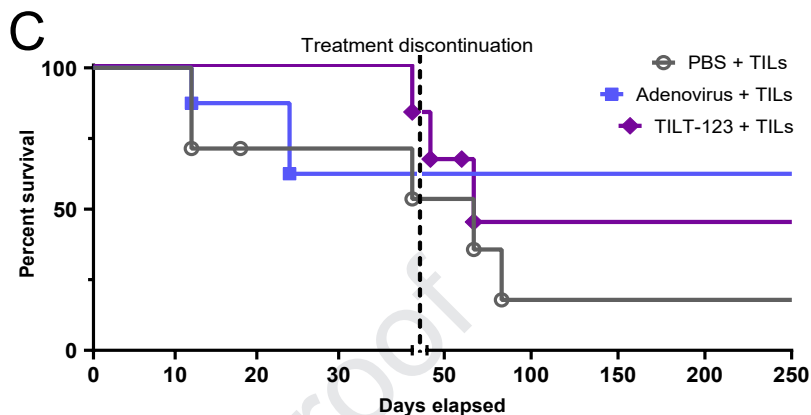
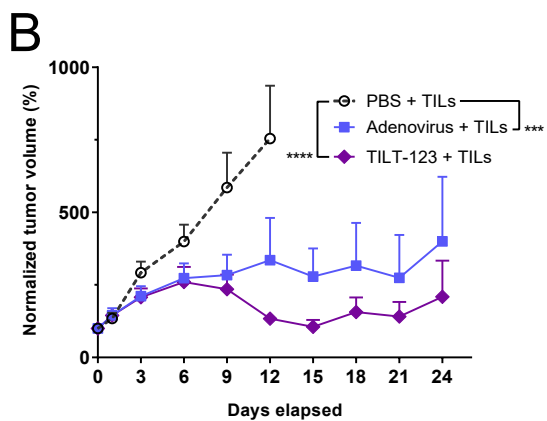
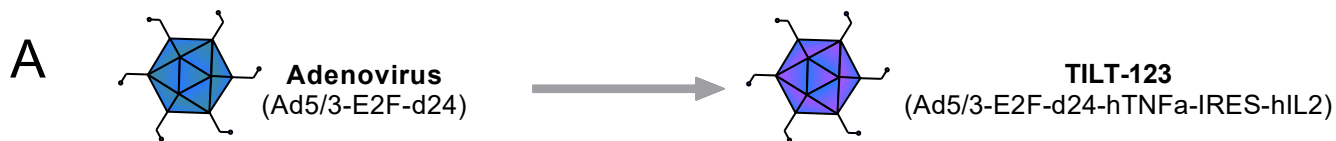


Herpes simplex

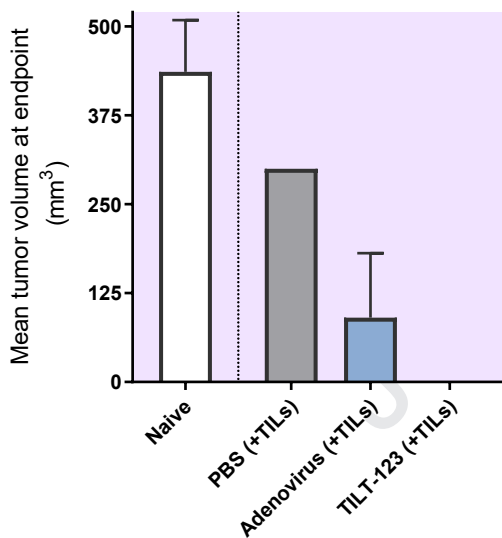


Reovirus

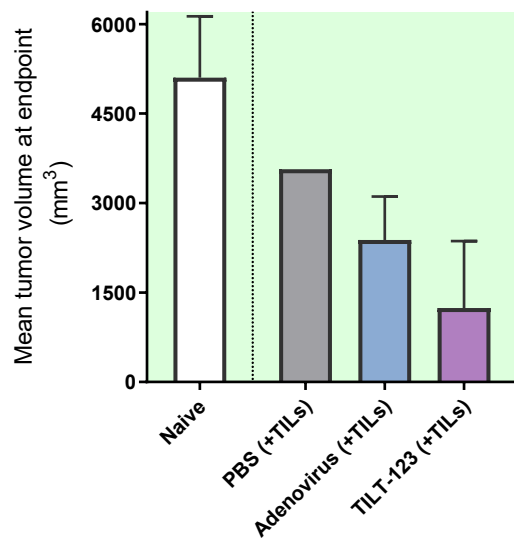




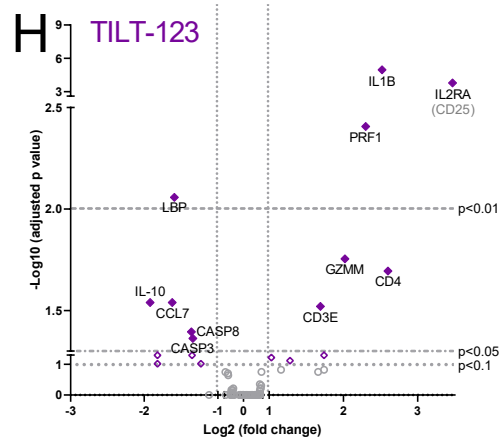
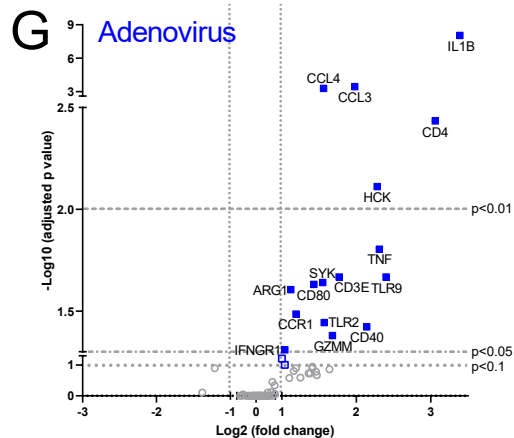
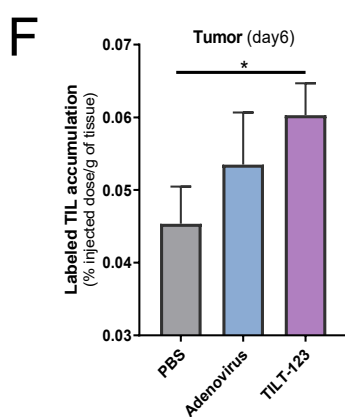
D HapT1 (rechallenge)



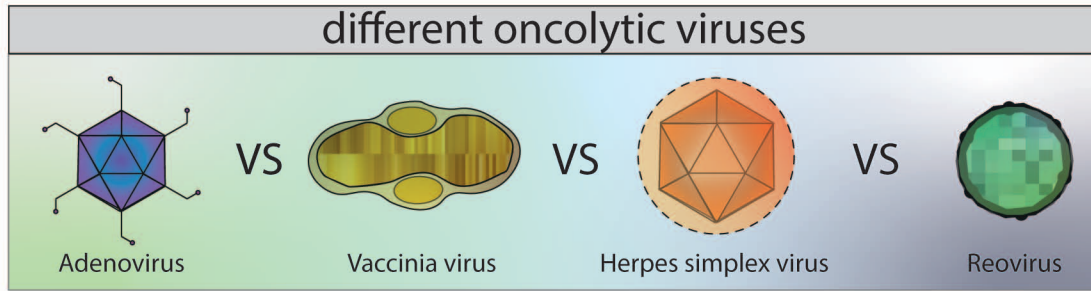
E DDT1-MF2 (control)



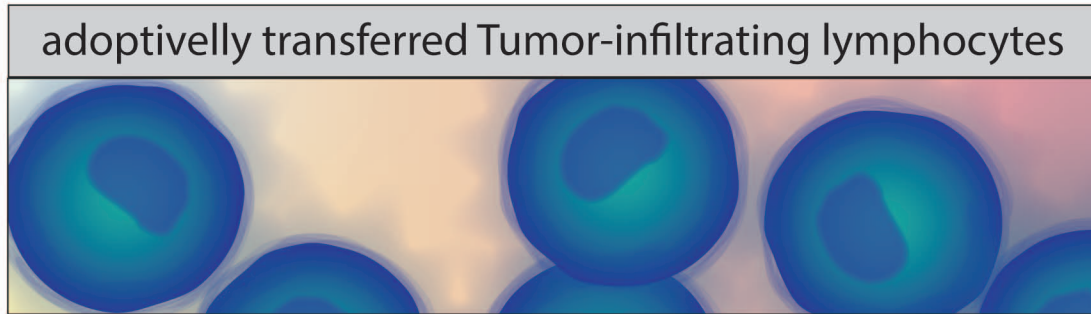
Second tumor engraftment
NO treatment



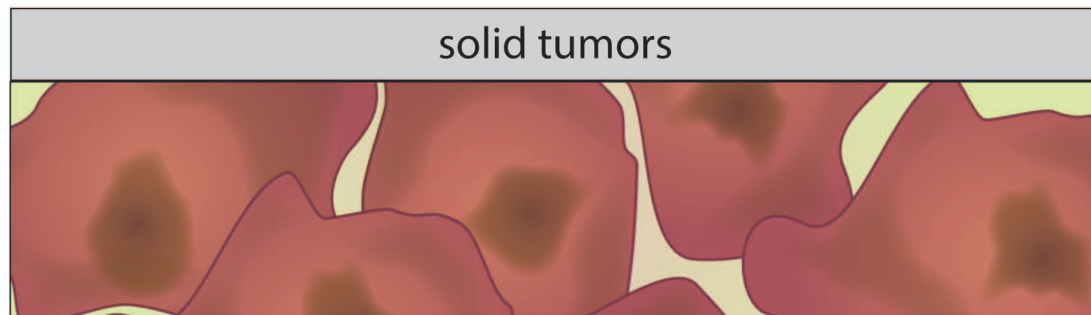
Comparison of...



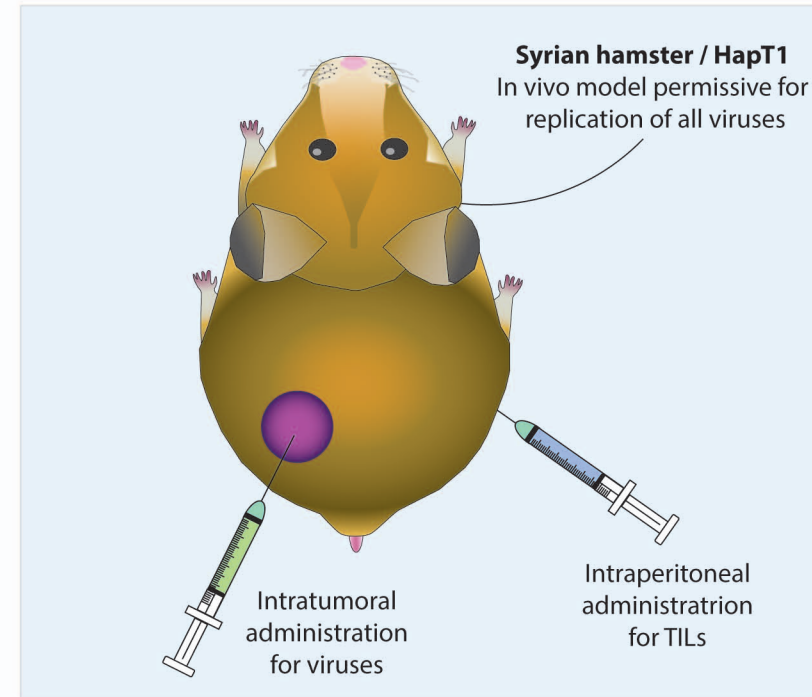
...used to enable...



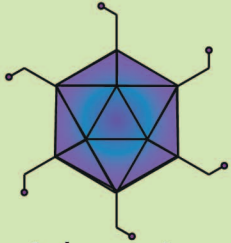
...against



Model used



Best virus to enable TILs



Adenovirus

Conclusion after studying:

- Tumor growth control
- Survival
- Antitumor immunememory
- Trafficking of the TILs
- Tumor microenvironment repolarization