Syngeneic Syrian hamster tumors feature tumor-infiltrating lymphocytes allowing adoptive cell therapy enhanced by oncolytic adenovirus in a replication permissive setting

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ABSTRACT
Adoptive transfer of tumor-infiltrating lymphocytes (TIL) has shown promising yet sometimes suboptimal results in clinical trials for advanced cancer, underscoring the need for approaches improving efficacy and safety. Six implantable syngeneic tumor cell lines of the Syrian hamster were used to initiate TIL cultures. TIL generated from tumor fragments cultured in human interleukin-2 (IL-2) for 10 d were adoptively transferred into tumor-bearing hamsters with concomitant intratumoral injections of oncolytic adenovirus (Ad5-D24) for the assessment of antitumor efficacy. Pancreatic cancer (HapT1) and melanoma (RPMI 1846) TIL exhibited potent and tumor-specific cytotoxicity in effector-to-target (E/T) assays. MHC Class I blocking abrogated the cell killing of RPMI 1846 TIL, indicating cytotoxic CD8+ T-cell activity. When TIL were combined with Ad5-D24 in vitro, HapT1 tumor cell killing was significantly enhanced over single agents. In vivo, the intratumoral administration of HapT1 TIL and Ad5-D24 resulted in improved tumor growth control compared with either treatment alone. Additionally, splenocytes derived from animals treated with the combination of Ad5-D24 and TIL killed autologous tumor cells more efficiently than monotherapy-derived splenocytes, suggesting that systemic antitumor immunity was induced. For the first time, TIL of the Syrian hamster have been cultured, characterized and used therapeutically together with oncolytic adenovirus for enhancing the efficacy of TIL therapy. Our results support human translation of oncolytic adenovirus as an enabling technology for adoptive T-cell therapy of solid tumors.

Abbreviations: Ad, adenovirus; ANOVA, analysis of variance; APC, allophycocyanin; CAR, chimeric antigen receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; E/T, effector-to-target; FBS, fetal bovine serum; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; IFN-γ, interferon gamma; IFNα2, interferon alpha-2; IL-2, interleukin-2; IL-15, interleukin-15; MHC, major histocompatibility complex; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium; NK, natural killer; OVA, ovalbumin; PBS, phosphate buffered saline; PE, phycoerythrin; RANTES, regulated on activation, normal T-cell expressed and secreted; REP, rapid expansion protocol; SE, standard error; TAA, tumor-associated antigen; TCC, American Type Culture Collection; TIL, tumor-infiltrating lymphocyte; TNFα, tumor necrosis factor alpha; VP, viral particle.

Introduction
Adoptive transfer of ex vivo expanded TIL has shown promising results in murine models of melanoma, and in humans. However, not all patients benefit from the treatment and significant toxicity accompanies the high doses of intravenous IL-2 and preconditioning lymphodepletion that have been considered a prerequisite to support the T-cell graft. Novel approaches for improving the efficacy and safety of adoptive transfer regimens are thus needed. Of note, a safe technology allowing the omission of pre- and post-conditioning, without reduction in efficacy, would be attractive.

Oncolytic viruses are genetically modified viruses that can infect and lyse tumor cells while leaving normal cell unharmed. Preclinical and clinical studies have revealed that in addition to a direct cytopathic effect, oncolytic viruses induce the release of tumor-associated antigens (TAAs) upon infection of tumor cells and can thereby promote antitumor immune responses. Oncolytic adenoviruses in particular possess a good safety profile and can be effectively combined with conventional cancer treatments. Not all oncolytic viruses are alike with regard to their effects on adaptive immunity but adenovirus might be especially attractive in this regard.

Unlike most rodent species, the Syrian hamster supports productive replication of human adenovirus. Several implantable tumor cell lines, with wide histological range, have been identified and utilized in cancer gene therapy studies with oncolytic adenovirus vectors in this fully immunocompetent model. Yet, immunological studies of Syrian hamster tumors are scarce, owing to general lack of hamster-specific reagents. With regard to TIL therapy, the features and therapeutic potential of TIL in hamsters are unknown.
Standard human protocols for generating sufficient numbers of TIL for adoptive transfer comprise lengthy and labor-intensive in vitro expansion steps and immunological screening assays to identify tumor-reactive cultures. In contrast, the use of "young" unselected TIL have simplified the production of large-scale numbers of lymphocytes that demonstrate comparable antitumor activity to standard TIL. Young TIL also display a favorable differentiation status with higher expression of costimulatory molecules and longer telomeres. Furthermore, recent clinical studies support the use of young unselected TIL in adoptive transfer trials. Therefore, young TIL may represent a preferred option for therapy.

Here, we describe the use of minimally cultured TIL of the Syrian hamster as a novel platform for adoptive immunotherapy studies. Hamster TIL antitumor CD8⁺ T-cell activity was dependent on the tumor model, as validated by flow-cytometric cell labeling and negating of CD8⁺ cytolytic activity using blocking anti-mouse major histocompatibility complex (MHC)-I antibody. Synergistic antitumor efficacy in vivo was achieved when unselected bulk TIL (young TIL), generated in 10 d from tumor fragments cultured in high-dose human IL-2, were administered in combination with intratumoral injections of oncolytic adenovirus Ad5-D24. The Syrian hamster TIL models and the immunological methods described here will facilitate functional studies on hamster immune cells in the future. Our data strongly supports investigation of adenovirus-enhanced TIL therapy in human cancer patients.

Results

CD4⁺ to CD8⁺ ratio in Syrian hamster tumors

For immunophenotypic studies of Syrian hamster tumors, subcutaneously implanted tumors (representing various tumor types) were collected and analyzed by flow cytometry. CD4 and CD8 staining of single-cell suspensions revealed a tumor type-dependent pattern of lymphocyte infiltration (Fig. 1A–F). HapT1 (pancreas), RPMI 1846 (melanoma), HaK (kidney) and PC1 (pancreas) tumors contained CD8⁺ lymphocytes ranging from 0.08% to 0.93%, while DDT1-MF2 (leiomyosarcoma) and HMAM5 (breast) did not...
contain detectable levels of CD8. CD4+ lymphocytes were observed in all tumor types and ranged from 0.17% to 3.25%.

**CD4⁺ lymphocytes predominate over CD8⁺ in hamster TIL cultures**

To obtain TIL, we cultured tumor fragments in high-dose recombinant human IL-2 for 10 d. With all tumor types studied, TIL outgrowth was visible by Day 5 of culture. On Days 5 and 10, CD4/CD8 FACS was performed on individual TIL cultures (i.e. TIL/fragment/well) and on a pooled sample (Fig. 2). Interestingly, in all tumor types studied, CD4⁺ cells predominated over CD8⁺ cells to varying degrees. RPMI 1846 and DDT1-MF2 TIL consisted of 40% CD4⁺ cells while the CD8⁺ cells constituted 13.5% and 0.2% of total, respectively. The portion of CD4⁺ cells in HapT1, HaK, HMAM5 and PC1 cultures reached 12–24%, with CD8⁺ lymphocytes being observed mainly in HapT1 (9.6%) and possibly in HMAM5 (0.2%). It is noteworthy that the presence of natural killer (NK) cells, a likely component of IL-2-stimulated TIL cultures, could not be determined due to the lack of cross-reactive antibodies against Syrian hamster NK cells.

**HapT1 and RPMI 1846 TIL exhibit tumor-specific cytolytic activity**

The cell killing ability of Syrian hamster TIL was studied in E/T assays where autologous tumor cells, non-related hamster tumor cells or human cells were co-cultured with effector TIL (Fig. 3). HapT1 TIL efficiently killed autologous cells (HapT1), but not human cells (A549) or other hamster cells (DDT1-MF2), even at low E/T ratios (Fig. 3A). RPMI 1846 TIL exhibited a similar pattern of tumor-specific lysis (Fig. 3B). TIL from DDT1-MF2 and HaK killed target cells non-specifically as A549 and other hamster cells were also lysed at the higher E/T ratios (10:1 and 20:1) (Fig. 3C–D). HMAM5 and PC1 TIL did not effectively kill either autologous tumor cells or other cells (Fig. 3E–F).

**Effect of MHC class I blocking on the cytolytic activity of TIL**

The TIL that exhibited specific lytic activity in E/T assays (HapT1 and RPMI 1846) were selected for further analyses. HapT1 and RPMI 1846 target tumor cells were pre-incubated with a cross-reactive anti-MHC-I antibody before the
addition of TIL (Fig. 4). In the presence of the antibody the cytolytic activity of RPMI 1846 TIL was abrogated, indicating that cytotoxic CD8⁺ T cells are responsible for the observed cytolytic activity (anti-MHCI vs. no inhibition, \( p = 0.01 \)) (Fig. 4A). For HapT1, MHC I blocking only partially inhibited cell killing (68% vs. 60% viability between blocked and non-blocked target cells, respectively; \( p = 0.02 \)) (Fig. 4B). HapT1 cells were also confirmed to express MHC

![Figure 3](image-url)  
**Figure 3.** Cytolytic activity of TIL *ex vivo*. Pooled Day 10 TIL were co-cultured with either autologous cancer cells, non-related hamster cells (DDT1-MF2 or equivalent) or human cells (A549) with different effector-to-target ratios (E/T). Target cell viability was determined 24 h later by MTS.

![Figure 4](image-url)  
**Figure 4.** Effect of MHC Class I blocking on cytolytic activity of TIL. (a) RPMI 1846 and (b) HapT1 target cells were pre-incubated for 2 h with anti-MHC Class I antibody (50 μg/mL), isotype control (50 μg/mL) or left untreated (no inhibition) before adding TIL. Target cell viability was determined 24 h later by MTS. Error bars, SE. \(^*\)\( p < 0.05, \text{ns} = \text{not significant.} \)
Ad5-D24 enhances the antitumor efficacy of adoptive TIL transfer

The antitumor activity of TIL and oncolytic adenovirus combination was studied in hamsters bearing established HapT1 tumors. Intratumoral injection of 1.5 × 10⁶ HapT1 TIL and Ad5-D24 (1 × 10⁵ VP/tumor) together were able to reduce tumor size significantly compared with the single agents (combination vs. TIL, p = 0.009; combination vs. Ad5-D24, p = 0.007; combination vs. mock, p = 0.046) (Fig. 6). The treatment regimen consisted of two virus injections given on Days 1 and 8 with one local administration of TIL on Day 2.

Combination therapy-derived splenocytes show increased cell killing capability

Tumors collected at the in vivo experiment end-point (Day 24) were analyzed for CD4⁺ and CD8⁺ T cells with flow cytometry. An increase in CD8⁺ lymphocytes was observed in the combination-treated tumors over TIL-treated tumors with a p value approaching significance (p = 0.05) (Fig. 7B). No significant differences were seen in CD4⁺ between treatment groups (Fig. 7A). Finally, splenocytes collected from end-point animals were co-cultured with HapT1 target cells. Interestingly, splenocytes from combination-treated animals killed HapT1 cells more efficiently than the splenocytes from mock-treated or monotherapy-treated animals (combination vs. mock, p <0.0001; combination vs. Ad5-D24, p = 0.0015; combination vs. TIL, p = 0.0018) (Fig. 7C).

Discussion

Adoptive transfer of TIL represents a promising approach for the treatment of advanced cancer. However, the approach has not been successfully used beyond melanoma, and even in this disease the therapy only works when combined with highly toxic pre- and post-conditioning regimens. We hypothesize that the anti-immunosuppressive effects of adenoviruses could be a key feature in enabling the full potential of adoptive cell therapies.18-20 However, assessing these questions has been plagued by the lack of a model system where the oncolytic potential of adenoviruses would be present in the context of an immunocompetent animal, taking into account that human adenoviruses do not produce new virions in mouse tissues, even if the genome does replicate.10 Since the danger signaling relevant for anti-immunosuppression depends on oncolysis,21 the lack thereof renders murine models suboptimal at best.

Tumors of various histological origin from the Syrian hamster were immunophenotyped with currently available cross-reactive antibodies. A common feature between the different tumor types was the preponderance of CD4⁺ TIL over CD8⁺ TIL. However, the specific subtypes of these CD4⁺ lymphocytes remain unknown given the shortage of antibodies against key molecules. Since CD4⁺ T cells can obtain "helper" or "regulator" functions depending on the cytokine milieu,22,23 further investigations (pending hamster-specific reagents) could be performed to elucidate the phenotypic and functional characteristics of tumor-associated CD4⁺ T cells of the Syrian hamster. Some tumor types (such as HaK, HMAM5 and PC1 in
hamsters) with notable CD4+ infiltration in the total absence of CD8+ TIL suggests that these CD4+ cells might consist mainly of T regulatory cells (Tregs), which have been reported to inhibit CD8+ T-cell infiltration into tumors. On the other hand, the CD4+ cells in other tumor types (such as HapT1 and RPMI 1846) might in fact represent T helper cells (Th) and thus participate in the activation of CD8+ CTLs. Importantly, as bulk TIL were used in the adoptive transfer experiments instead of CD8+ enriched cells, the potential immunostimulatory role of CD4+ lymphocytes cannot be discarded.

MHC-blocking experiments revealed that TIL cultured from RPMI 1846 tumors exhibited MHC-restricted cytoxicity, indicative of cytotoxic CD8+ T-cell presence. The cytolytic activity of HapT1 TIL was only partially inhibited by MHC-blocking, suggesting that the HapT1 TIL population contained NK cells in addition to CD8+ and CD4+ T-cells. However, E/T assays performed with HapT1 TIL showed highly specific cell killing, contradicting the presence or tumor cell-killing activity of NK cells. Intriguingly, some tumor-reactive CD4+ T-cells can develop cytotoxic activity and many tumors express functional MHC-II. We hypothesized that CD4+ T cells may have acquired this function in the HapT1 TIL population but not in RPMI 1846 TIL, which killed autologous tumor cells in a completely MHC-I-restricted manner. Indeed, we found that HapT1 cells express MHC Class II molecules, indicating that the highly specific cytoxicity of HapT1 TIL that was (seemingly counter-intuitively) not abrogated by MHC-blocking can be explained by the presence of cytotoxic CD4+ T-cells.

The TIL generated from RPMI 1846 melanoma tumors initially seemed to represent the most appealing option for adoptive transfer studies together with Ad5-D24. RPMI 1846 TIL grew more robustly in hIL-2 than other TIL and had the highest percentage of CD8+ lymphocytes that were also responsible for cytotoxicity. However, as reported before, these melanoma cells do not support adenovirus replication and were therefore excluded from combination studies. Yet, our data on RPMI 1846 TIL may be of interest to other investigators and potentially useful for immunotherapeutic approaches involving melanoma-derived TIL from a species other than mouse or human.

Ad5-D24 and HapT1 TIL acted additively in tumor cell killing assays in vitro. Previous research has shown that oncolytic adenoviruses can be successfully combined with more conventional cancer treatments i.e., chemotherapy and radiotherapy. Further studies are needed to elucidate the underlying mechanism behind the increased efficacy of the combination of TIL and oncolytic adenovirus in vitro. Superior tumor cell killing with the combination of CAR T-cells and Ad5-D24 was recently reported to be associated with accelerated caspase pathways in human tumor cells. However, upregulation of death receptors or costimulatory molecules on infected tumor cells (rendering tumor cells more sensitive to lymphocyte-mediated killing) was not observed. Speculatively, these mechanisms may play a role in hamster tumor cells, warranting further investigation of the in vitro setting, if the appropriate reagents can be generated.

To study the in vivo efficacy of the combination treatment, TIL expanded in hIL-2 for 10 d were injected locally into established HapT1 tumors with or without adenovirus treatment. These TIL comprised 15% CD4+ and 10% CD8+ cells. In comparison, human TIL preparations exhibit highly variable CD4/CD8 T-cell ratios even between different fragments from the same tumor. The remaining cells could not be characterized, but MHC-blocking experiments suggest at least the presence of NK cells and/or cytotoxic CD4+ T cells. Our TIL culture protocol did not include the rapid-expansion phase (REP) typically used in adoptive transfer trials. NK cells expand alongside T cells in initial TIL cultures with IL-2, but are subsequently lost when anti-CD3 stimulation is introduced during the REP. Due to lack of cross-reactive anti-CD3 antibody, the presence and/or expansion of NK cells in the hamster TIL cultures is rather likely. The role of NK cells in TIL products is unclear, but could prove valuable as shown in murine B16 melanoma, where the depletion of NK cells and CD8+ T cells together (but not either alone) abrogated the therapeutic efficacy of high-dose IL-2 and CTLA-4 blockade. Similarly, the importance of CD4+ T cells in TIL products remains poorly understood since some patients have responded well to TIL treatments dominated by CD4+ T cells. Preclinically, cotreatment with tumor-specific CD4+ and CD8+ T cells resulted in synergistic efficacy with indications that the CD4+ T cells reduced CD8+ T-cell exhaustion. Furthermore, while the REP allows large numerical expansion of TIL it affects the survival and proliferative capacity of T cells after transfer, which is linked to decrease in telomere length and the loss of cell-surface CD28 expression. Taken together, the heterogeneous “bulk” TIL product used for adoptive transfer into hamsters might represent the ideal TIL population.

**Figure 7.** T-cell infiltration and splenocyte cell killing activity following treatment with TIL and Ad5-D24. (a) CD4+ and (b) CD8+ T cells were analyzed from Day 24 tumors by flow cytometry. (c) Splenocytes (collected from hamsters sacrificed on Day 24) were co-cultured with HapT1 cells at the effector-to-target ratio of 50:1. Target cell viability was determined 24 h later by MTS. Error bars, SE. "p < 0.01, "p < 0.0001.
It remains unclear whether the borderline significant $p$ value (0.05) observed in CD8$^+$ cells between TIL vs. combination tumors after treatment has true biological significance, i.e. increased tumor-infiltration or local expansion of CD8$^+$ lymphocytes. Specifically, it is not known if increases in CD8$^+$ are necessary or useful for efficacy. Mouse studies performed in our group do not necessarily support these hypotheses in the context of combination therapy. Instead, other mechanisms such as the activation status of T cells are suggested and discussed below. In contrast to mouse studies, the available human evidence suggest that accumulation of T cells does correlate with efficacy endpoints. Further, for most tumor types there is a large body of data indicating that the concentration of T cells in tumors (TIL) correlates with favorable outcome.

In addition to antitumor efficacy in vivo, increased tumor cell killing activity of combination treatment-derived splenocytes ex vivo suggest the presence of active antitumor T cells on a systemic level. Previously, we have shown that adenovirus improved the efficacy of adoptive T-cell transfer in mice by increasing the number of IFN$\gamma$-expressing CD8$^+$ T cells as well as inducing endogenous CD8$^+$ T cells against the TAA TRP-2 and gp100. Unfortunately, these hypotheses cannot be studied in the hamster model due to lack of hamster-specific (or acceptably cross-reactive) reagents for the aforementioned markers. In addition, the actual mechanism-of-action might differ from mouse studies due to active oncolysis of hamster tumor cells, a scenario that cannot be studied in murine models but is much closer to the clinical situation in human patients.

The Syrian hamster model we describe has several advantages related to adenovirotherapy and adoptive cell transfer. First, Syrian hamsters are permissive for human adenovirus replication as opposed to mice, enabling the safety and efficacy evaluation of adenovirus vectors in a model that more accurately mimicks the setting in human cancer patients. Secondly, immunodeficient mouse models lack stromal cells that affect tumor growth and response to therapy. Currently, these aspects need to be dissected by using several mouse models. In this regard, the hamster model reported here represents a significant improvement.

Future studies could explore oncolytic adenoviruses armed with transgenes that support the transferred T cells in situ, e.g. cytokines and chemokines. Recently, this approach was used in a preclinical immunodeficient model of neuroblastoma with CAR T-cells and adenovirus coding for RANTES and IL-15. Other candidates might include IL-2, TNF$\alpha$, IFN$\alpha$2 and IFN$\gamma$. Of note, many human cytokines are biologically active in Syrian hamsters, highlighting the value of this immunocompetent rodent model for future research. With regard to weaknesses of the model, key laboratory reagents are lacking and the growth of tumors over 2 weeks do not recapitulate the complexity which develops over a decade or more in human patients. Additionally, the route of administration of TIL in our study (intratumoral) was not typical of current TIL protocols that often employ intravenous administration, although also intratumoral and intracavitary have been used. Unfortunately, since a stimulatory hamster anti-CD3 antibody is lacking, a true REP growth step could not be performed, which limited the available options to local injection. Moreover, the lack of a tail vain in hamsters complicates intravenous injection in this animal. The low efficacy of single-agent therapies observed in the in vivo experiment could be explained by the absence of concomitant pre- and post-conditioning treatments (TIL alone group), which are prerequisite for efficacy in humans, and the low virus dose (Ad5-D24 alone group). Also, the virus used here was not armed with a transgene. It is generally thought that armed oncolytic viruses are more potent than unarmed viruses and therefore the former are typically employed in clinical development.

In conclusion, we have shown that oncolytic adenovirus can be used to improve the efficacy of adoptive TIL transfer. Also, this is the first time TIL of the Syrian hamster have been cultured, characterized and used therapeutically. Our model fulfills the key requirement for an effective preclinical combination therapy platform: adenoviral replication comparable to humans in a fully immunocompetent environment. These results support the further development of oncolytic adenoviruses as an enabling technology for adoptive T-cell therapies including but not limited to TIL therapy.

Materials and methods

Tumor cell lines and oncolytic virus

The following Syrian hamster tumor cell lines were used: HapT1 (courtesy of Dr Hernandez-Alcoceba, Pamplona, Spain); HMAM5 (courtesy of Dr Michael Mathis, Louisiana State University, LA, USA); DDT1–MF2, PC1, HaK (courtesy of Prof. William Wold, St. Louis, MO, USA); RPMI 1846 (American Type Culture Collection, ATCC). B16.OVA mouse melanoma cells were kindly gifted by Prof. Richard Vile (Mayo Clinic, Rochester, MN, USA). A549 (human lung adenocarcinoma) was purchased from ATCC. All cell lines were cultured in recommended conditions. Ad5-D24 has been described previously.

TIL culture

5–6-week-old Syrian hamsters (Mesocricetus auratus) were purchased from Harlan Laboratories. Subcutaneously implanted tumors were excised when they reached approximately 1 cm in size. Tumors were cut into fragments of 1–3 mm$^3$ and cultured in individual wells of a 24-well plate (2 mL/well). Culture medium consisted of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, 15 mM HEPES, 50 $\mu$M 2-mercaptoethanol, 1 mM Na-pyruvate and 6,000 IU/mL human IL-2 (hIL-2) (PeproTech). Half of the medium was renewed 5 d after culture initiation and every 2 d after that. On Day 10 of culture, wells with visible TIL growth were collected and pooled for E/T assays or adoptive transfer.

Flow cytometric analyses

Single-cell suspensions of excised tumors (Day 0) or TIL cultures (Days 5 and 10) were stained with the following cross-reactive antibodies: anti-rat CD8b PE (clone 341, eBioscience) and anti-mouse CD4 APC (clone GK1.5, eBioscience) for 30 min, fixed in 4% paraformaldehyde, and analyzed with BD Accuri C6 (BD Biosciences) collecting at least 50,000 events per
sample. HapT1 cells were incubated with FITC-conjugated anti-mouse/rat MHC Class II antibody (I-Ek; clone 14-4-4 S, eBioscience) for 1 h and fixed in 4% paraformaldehyde for 5 min, and analyzed on BD Accuri C6.

**Co-culture and blocking experiments**

E/T assays were performed by seeding 96-well plates with autologous tumor cells, other hamster tumor cells or human A549 cells (1 × 10^4 target cells/well). Effector TIL were added at different E/T ratios and cell viability was determined 24 h later by MTS assay (Promega). In combination experiments with TIL and Ad5-D24, target tumor cells were infected with 100 viral particles/cell (VP/cell) and TIL (2:1 E/T ratio) were added 3 d later. Cell viability was determined 24 h after addition of TIL. In blocking experiments, target cells on 96-wells plates were pre-incubated with 50 μg/mL of anti-MHC-I monoclonal antibody (clone 2G5, Novus Biologicals) or isotype control (mouse IgG2b, Novus Biologicals) for 2 h at 37°C before adding effector cells and determining cell viability 24 h later. The 2G5 clone is reported to cross-react with Syrian hamster MHC-I and to block MHC-I in functional assays. The blocking capability was confirmed by pre-incubating B16.OVA cells with the antibody (or isotype control) before adding OVA-specific CD8^+^-enriched OT-I T-cells (1:1 E/T ratio) and determining cell viability as above (Fig. S2).

**Adoptive TIL transfer and virus treatments**

Hamsters bearing established HapT1 tumors (five animals per group) were treated with intratumoral injection of 1 × 10^7 viral particles of Ad5-D24 on days 1 and 8 in 50 μL of PBS. HapT1 TIL were administered on day 2 by intratumoral injection in 50 μL of plain RPMI (1.5 × 10^6 TIL per tumor). All treatments were performed under isoflurane anesthesia. On day 24, all animals were killed and selected organs were collected for biological analyses. All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

**Statistical analyses**

Statistics were performed with GraphPad Prism 6 (GraphPad Software Inc.). Student’s t-test and repeated measures two-way ANOVA were used; p values of <0.05 were considered significant.

**Disclosure of potential conflicts of interest**

A.H. is shareholder in Targovax AS. A.H. is employee and shareholder in TILT Biotherapeutics Ltd. M.S. and S.P. are employees of TILT Biotherapeutics Ltd.

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