Adenovirus Improves the Efficacy of Adoptive T-cell Therapy by Recruiting Immune Cells to and Promoting Their Activity at the Tumor

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Abstract

Despite the rapid progress in the development of novel adoptive T-cell therapies, the clinical benefits in treatment of established tumors have remained modest. Several immune evasion mechanisms hinder T-cell entry into tumors and their activity within the tumor. Of note, oncolytic adenoviruses are intrinsically immunogenic due to inherent pathogen-associated molecular patterns. Here, we studied the capacity of adenovirus to overcome resistance of chicken ovalbumin-expressing B16.OVA murine melanoma tumors to adoptive ovalbumin-specific CD8+ T-cell (OT-I) therapy. Following intraperitoneal transfer of polyclonally activated OT-I lymphocytes, control of tumor growth was superior in mice given intratumoral adenovirus compared with control mice, even in the absence of oncolytic virus replication. Preexisting antiviral immunity against serotype 5 did not hinder the therapeutic efficacy of the combination treatment. Intratumoral adenovirus injection was associated with an increase in proinflammatory cytokines, CD45+ leukocytes, CD8+ lymphocytes, and F4/80+ macrophages, suggesting enhanced tumor immunogenicity. The proinflammatory effects of adenovirus on the tumor microenvironment led to expression of costimulatory signals on CD11c+ antigen-presenting cells and subsequent activation of T cells, thus breaking the tumor-induced peripheral tolerance. An increased number of CD8+ T cells specific for endogenous tumor antigens TRP-2 and gp100 was detected in combination-treated mice, indicating epitope spreading. Moreover, the majority of virus/T-cell–treated mice rejected the challenge of parental B16. F10 tumors, suggesting that systemic antitumor immunity was induced. In summary, we provide proof-of-mechanism data on combining adoptive T-cell therapy and adenovirotherapy for the treatment of cancer. Cancer Immunol Res; 3(8); 915–25. © 2015 AACR.

Introduction

Immunotherapy using ex vivo–expanded tumor-infiltrating lymphocytes (TIL) was pioneered by Steven Rosenberg in the 1980s (1), and adoptive T-cell therapy (ACT) is currently gaining ground in the form of receptor-engineered immune cell therapy. Although CD19–expressing hematologic malignancies in particular seem amenable to adoptive chimeric antigen receptor (CAR) T-cell therapy (2), efficacy in solid tumors is lacking possibly due to T-cell hypofunction (3–5). Recently, several approaches have been developed to improve the activity of genetically redirected antitumor T cells in solid tumors, with the main focus being on modification of T-cell costimulatory genes, receptor affinities, and optimal target molecules (3, 4). Nevertheless, these advances alone may not be sufficient to reverse the effects of the immune-suppressive nature of the tumor microenvironment (TME). Thus, sensitization of tumor milieu for T-cell therapy may prove to be crucial to achieve optimal clinical responses.

Tumor immunogenicity is the sum of several phenotypic hallmarks, including the degree and type of tumor-infiltrating immune cells, stromal cells, and expression of MHC molecules on tumor cells. During cancer development, several immune evasion tactics are employed by tumor cells. Immunoediting leading to escape variants can result in failure of the host immune system to mount a sufficient immune response against the tumor (6). Peripheral tolerance of tumor-specific T cells due to insufficient costimulation by professional antigen-presenting cells (APC) may result in T-cell deletion or anergy (7). Furthermore, the highly immunosuppressive TME typically renders infiltrating T cells incapable of killing their target (tumor) cells (8). For successful cancer immunotherapy, the TME has to be immunogenic enough in order to accomplish T-cell–mediated cytotoxicity and subsequent antitumor responses.

Oncolytic virotherapy is the use of cancer cell–specific, conditionally replicating viruses in the treatment of cancer. Adenovirus–based oncolytic viruses have been shown to trigger potent innate and adaptive antiviral and antitumor immune responses (9),...
while still maintaining good safety profile in patients (10). Adenovirus infection of tumors results in chemokine secretion, release of tumor-associated antigens (TAA) from infected dying tumor cells, and activation of APCs, which recognize the multiple pathogen-associated molecular patterns (PAMP) of the virus (9). Importantly, infection also results in changes in immune cell composition of the tumor, supporting the argument that local immunosuppression can be overcome (11). As the efficacy of adoptive antitumor immunotherapy may depend on support by innate immune responses (12), combination with oncolytic virus therapy appears an attractive approach that warrants testing.

We set out to study whether intratumoral adenovirus injections would benefit adoptive T-cell therapy. We used the syngeneic C57BL/6 mouse B16.OVA melanoma tumor model, which is poorly immunogenic (13), and thus representative of advanced human melanomas (which are often initially immunogenic but then become severely immunosuppressive and immunoedited at the advanced metastatic stage). We observed superior antitumor efficacy and increased levels of tumor-infiltrating immune cells, enhanced maturation of APCs, and higher numbers of activated T cells in the combination-treated group compared with the group treated with T-cell therapy alone. In addition, induction of endogenous antitumor T cells was seen in mice treated with adenovirus and adoptive T-cell therapy, suggesting that the combination approach leads to epitope spreading and systemic antitumor immunity.

Materials and Methods

Cells

Mouse melanoma B16 cells expressing ovalbumin were a kind gift of Professor Richard Vile (Mayo Clinic, Rochester, MN; September 30, 2010) and have been tested to be pathogen-free (by Surrey Diagnostics Ltd). Mouse melanoma B16.F10 cells were obtained from the American Type Culture Collection (November 25, 2014). B16.OVA and parental B16.F10 were maintained in RPMI, 10% FBS, 1% L-Glutamine, 1% penicillin/streptomycin solution and propagated at 37°C and 5% CO₂, G418 (5 mg/mL; Roche) was added to the culture medium for B16.OVA cells.

Viruses

The treatment viruses Ad5/3-D24 and Ad5/3-D24-hGMCSF have been described (10, 14). Briefly, the virus consists of a human adenovirus serotype 5 (Ad5) nucleic acid backbone, a 5/3 chimeric fiber knob, and a 24-bp deletion (D24) in the Rb binding constant region 2 of adenoviral E1. Although the Ad5/3-D24-hGMCSF contains a transgene, it actually resembles an unarmed, replication-incompetent virus, as human GM-CSF is not biologically active in mouse cells (15), nor does the human adenovirus replicate productively in mice (16). Replication-incompetent Ad5-Luc1 is an E1-deleted serotype 5 vector expressing firefly luciferase (17), and it was used in the preimmunization experiment to model Ad5-based immunity.

Animal experiments

All animal protocols were approved by the experimental animal committee of the University of Helsinki (Helsinki, Finland) and the Provincial Government of Southern Finland. Four- to 7-week-old C57BL/6 immunocompetent female mice (Harlan Laboratories) were implanted subcutaneously with 2.5 × 10⁵ B16.OVA cells in 50 μL RPMI, 0% FBS, in the right flank, one tumor per mouse. Roughly 10 days after tumor implantation (when tumors became injectable, ~3 mm minimum diameter), mice were divided into groups and treated on 6 consecutive days with intratumoral injections of either 50 μL PBS or 1 × 10⁵ viral particles (VP) of oncolytic adenovirus in 50 μL PBS. Tumor growth of mice was monitored every 2 to 3 days by using electronic calipers, and volume was calculated as 0.52 × length × width². For Ad5 preimmunization, mice were injected twice intramuscularly with replication-incompetent 1 × 10⁵ VPs of Ad5-Luc1 in 20 μL of PBS 3 weeks before intratumoral virus treatments. For B16. F10 challenge, B16.OVA-bearing mice (treated with oncolytic adenovirus and adoptive transfer of 2 × 10⁵ OT-I T cells) were implanted with 2.5 × 10⁵ B16-F10 cells in 50 μL RPMI, 0% FBS, in the left flank on day 13 after transfer. B16.F10-challenged mice were followed for 14 additional days for tumor emergence or tumor growth.

Adoptive transfer of OT-I cells

On the first day of the virus treatment, the mice also received 5 × 10⁵ to 2 × 10⁶ CD8a-enriched and expanded splenocytes from 4- to 8-week-old C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice (ref. 18; The Jackson Laboratories), genetically engineered to have only ovalbumin-specific CD8 T-cell receptors (TCR), in 100 μL RPMI and 0% FBS. CD8a enrichment was performed by depletion of nontarget cells with mouse CD8a (Ly-2) MicroBeads as per the manufacturer’s instructions (Miltenyi Biotech). Enriched cells were expanded in numbers for 5 days in lymphocyte medium (RPMI, 10% FBS, 20 mmol/L L-glutamine, 1 × penicillin/streptomycin solution, 15 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, 1 mmol/L Na pyruvate) in the presence of 160 ng/mL recombinant murine IL2 (R&D Systems) and 0.3 μg/mL soluble anti-mouse CD3e antibody clone 145-2C11 (Abcam). Administration of the intraperitoneal cavity was based on the notion that total number and accumulation kinetics of adoptively transferred OT-I cells into B16.OVA tumors are independent of injection route (19).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software Inc.) using unpaired, two-tailed Student t test. Tumor volume data were analyzed by repeated measures ANOVA on log-transformed values with SPSS version 21 (SPSS IBM). Differences were considered statistically significant when P values were less than 0.05.

Results

Infection with adenovirus results in low-level antitumor immune responses

To study whether adenovirus infection results in antitumor immune responses in mice with melanoma, subcutaneous B16.OVA tumors were injected intratumorally with either PBS or 5/3 fiber-chimeric adenovirus for 6 consecutive days. As murine cells are poorly permissive to human adenovirus (16), multiple intratumoral virus injections were used to mimic virus replication–induced inflammation. Relative tumor volumes in Fig. 1A show that, compared with the PBS-treated mock group, Ad-treated mice showed minor tumor growth control (5.495% ± 1.679% vs. 2.067% ± 329%, respectively). A declining copy number of virus genomes in the tumors over time suggests...
induction of antiviral immune responses and confirms previous reports regarding the absence of productive amplification of human adenovirus in mice (Fig. 1B). These results are in line with previous reports showing that intratumoral administration of adenovirus can induce antitumor immunity (20, 21). Correspondingly, we saw virus-induced increase in secretion of intratumoral IFNγ (Fig. 1C) which was associated with a trend toward upregulation of IFNγ-inducible chemokines RANTES, MIP-1α, and MCP-1 on day 10 (Supplementary Fig. S1A–S1C). Flow cytometric analysis of the tumors revealed that adenovirus injections increased the number of TILs (Fig. 1D) and induced a low-level endogenous antitumor response in the form of CD8⁺ T cells specific for melanoma antigen glycoprotein 100 (gp100) but not for tyrosinase-related protein 2 (TRP-2; Fig. 1E and F). Adenovirus treatment enhances the efficacy of adoptive T-cell therapy despite inducing antiviral immunity

To assess the impact of adenovirus treatment on adoptive T-cell therapy, mice bearing subcutaneous B16.OVA tumors were given intraperitoneal injections of polyclonally activated (with anti-CD3ε and IL-2) 5 × 10⁶ CD8⁺-enriched splenocytes from OT-1 mice (Supplementary Fig. S2A), in which all CD8⁺ T cells carry MHC-I–restricted ovalbumin peptide SIINFEKL-specific TCRs. Beginning on the same day, tumors were either left noninjected or injected with PBS or 5/3 fiber-chimeric adenovirus for 6 consecutive days (Supplementary Fig. S2B). Interestingly, superior tumor growth control was observed in the group treated with the combination of virus + T cells compared with control groups receiving PBS injections and OT-I cells or OT-I cells alone (631% ± 20% vs. 4,646% ± 827% vs. 5,565% ± 1,221%, respectively; Fig. 2A). Increasing OT-I dose to 2 × 10⁶ cells (roughly equivalent to 1 × 10⁶ cells/kg) resulted in somewhat higher efficacy in the virus/T-cell group versus the PBS/T-cell group (198% ± 36% vs. 3,451% ± 1,620%, respectively) but did not alter the shape of the tumor growth curves (Fig. 2B). According to earlier reports (22, 23), using even higher cell numbers in adoptive transfer does not translate into long-term survival of B16.OVA-bearing mice, highlighting the immunosuppressive nature of the model, which is in accord with clinical observations suggesting a lack of efficacy in T-cell therapies used as single agents for the
treatment of solid tumors. Keeping in mind that human adenovirus does not productively replicate in or lyse murine B16.OVA cells, the observed synergy between adenovirus and the transferred T cells was likely the result of favorable immune responses instigated by infection per se.

As most humans have preexisting memory T cells against several adenovirus serotypes (especially Ad5), we also studied the role of preimmunization by injecting a group of mice twice with $1 \times 10^5$ VPs of Ad5-Luc1 intramuscularly 3 weeks before the aforementioned treatment regimen. As shown in Fig. 2C, preexisting antiviral immunity did not hinder the efficacy of virus/T-cell combination therapy. Furthermore, similar levels of antiviral T cells, characterized by IFNγ production of CD8⁺ CD44⁺ splenocytes upon HAdV-5 peptide stimulation, were generated by both preimmunization and treatment regimens (Fig. 2D).

Traffic of transferred T cells into tumor is not significantly enhanced by adenovirus injection

To investigate the mechanisms of adenovirus-facilitated boosting of adoptive T-cell therapy, the number and localization of OT-I cells in tumors and periphery were studied ex vivo by flow cytometry and in vivo by SPECT/CT imaging. Surprisingly, no statistically significant differences in the levels of tumor-infiltrating OT-I cells between groups at any time point were detected (Fig. 2E). Overall, the amount of OT-I infiltration decreased over time in all treatment groups (on average from 3.8% to 1.1%). These results were further

Figure 2. Combining oncolytic adenovirus injections with adoptive transfer of T cells improves treatment efficacy but not through enhanced tumor trafficking of transferred cells. B16.OVA-bearing mice were adoptively transferred with (A, E) $5 \times 10^5$ or (B) $2 \times 10^5$ CD8α⁺-enriched OT-I lymphocytes intraperitoneally, and tumors were either left noninjected or injected with 50 μL PBS or $1 \times 10^5$ VPs of 5/5 fiber chimeric oncolytic adenovirus in PBS ($n = 13$ on day 0). Tumor growth was monitored every 2 to 3 days with an electronic caliper. C and D, to study the effect of preexisting adenoviral immunity on the efficacy of combination treatment, mice were preimmunized intramuscularly with $1 \times 10^7$ VPs of serotype 5 adenovirus (Ad5) 3 weeks before intratumoral virus treatments ($n = 5$). D, antiviral T-cell responses were evaluated by the frequency of CD8⁺ CD44⁺ splenocytes positive for IFNγ following peptide stimulation. E, levels of transferred ovalbumin-specific OT-I cells in the tumors were quantified on days 1, 7, and 14 after transfer using SIINFEKL-H-2Kb pentamer and flow cytometry ($n = 3$–6). F, CD8α⁺-enriched OT-I cells ($6 \times 10^5$) were labeled with 111In-oxine and adoptively transferred into B16.OVA-bearing mice to quantify early biodistribution of transferred cells using nanoSPECT/CT ($n = 3$). Percentage of total radioactivity normalized to tumor size (mm³) on days 4 and 7 after transfer. Data, mean ± SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.0001$ by unpaired t test (D) or by repeated measures ANOVA (A–C). ns, not statistically significant; OVA, ovalbumin.
Corroborated by an imaging experiment, where accumulation of 111In-oxine–radiolabeled OT-I cells into tumors was quantified by SPECT/CT in vivo and by gamma counting ex vivo (Fig. 2F, Supplementary Fig. S3). Data on early biodistribution showed presence of transferred OT-I lymphocytes in the tumor, spleen, axillary lymph nodes, and the lymphatic system (Supplementary Fig. S3C). Despite a slight increase in radioactivity in virus-treated tumors, signal levels detected between treatment groups at different time points after transfer did not reach statistical significance (Fig. 2F, Supplementary Fig. S3D).

Combination of adoptive T-cell therapy and adenovirus increases the level and activation of endogenous anti-melanoma T cells

As we did not see clear evidence of increased trafficking of adoptively transferred T cells into tumors, we investigated tumor localization of endogenous T cells that may cooperate with adoptively transferred T cells for antitumor efficacy (24), as well as other immune cell subsets, such as natural killer (NK) or CD4+ cells, which may in other experimental systems control B16 tumors (25, 26). On day 14 after transfer, virus-treated tumors contained more CD45+ leukocytes, CD3+ T lymphocytes, and...
CD8⁺ cytotoxic T lymphocytes than control tumors (Fig. 3A–C), whereas the levels of CD19⁺ B lymphocytes, CD4⁺ T lymphocytes, and NK1.1⁺ NK cells did not differ significantly between the groups (Supplementary Fig. S4A–S4C). Upon pentamer analysis, we detected a statistically significant increase in tumor-infiltrating CD8⁺ T cells specific for MHC-I–restricted endogenous TRP-2 and gp100 antigens in the virus-treated group compared with that of controls (Fig. 3D–F). To establish the level of...
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activation of these cells, we performed a flow cytometric analysis of intracellular IFNγ and found that virus-treated tumors were enriched for IFNγ-expressing CD8+ T cells compared with control tumors (Fig. 4A). In addition, an IFNγ ELISPOT assay with splenocytes incubated with mouse CD8-specific peptides for ovalbumin, human adenovirus, and melanoma antigens gp100 and TRP-2 revealed that T-cell–mediated immune reactions to all studied peptide epitopes were stronger in virus-treated mice compared with control mice treated with PBS (Fig. 4B and C).

Finally, as a dramatic reduction of draining lymph node tumor burden was observed in adenovirus-treated mice compared with PBS-injected animals (Fig. 4D), we wanted to study whether these mice could reject parental B16.F10 tumors not expressing the target molecule ovalbumin. Indeed, when B16.OVA-bearing mice were treated with the virus/T-cell combination, 67% of mice rejected the challenge of B16.F10 tumors, whereas only 10% of naive mice were tumor-free on day 14 after implantation (Fig. 4E). This observation led us to conclude that combination treatment can result in systemic tumor control, despite local injection of virus. Overall, the data indicate that combination of adenovirus and adoptive cell therapy resulted in induction of specific antitumor immunity to a degree that could not be achieved by either adenovirus or T-cell transfer alone.

Adoptive T-cell therapy and virotherapy specifically result in simultaneous upregulation of T-cell activation markers and reduction of anergy markers

Induction of endogenous antitumor CD8+ T-cell activity in the virus/T-cell–treated tumors implied that the melanoma-induced T-cell immunosuppressive mechanisms were perturbed. To assess this, we compared activation markers CD25 and CD69 with known anergy markers CTLA-4, PD-1, and TIM-3. Virus-treated mice had a higher level of CD8+ CD25+ CD69+ TILs 14 days after transfer compared with that of the PBS control group (Fig. 5A). At the same time, while the expression levels of immune checkpoint molecules CTLA-4 and PD-1 were equal in all treatment groups, TIM-3 expression was significantly reduced in Ad- and PBS-treated tumors compared with noninjected control tumors (Fig. 5B, Supplementary Fig. S5). Thus, simultaneous T-cell activation and reduction of anergy markers occurred only in the virus/T-cell combination group.

Adenovirus-mediated inflammation enhances antigen cross-presentation

To study how tumor immunosuppression may have been overcome in the virus/T-cell combination group, we assessed the degree of APC activation in relation to putative immunosuppressive stromal cell presence. Notably, although total levels of CD11c+ dendritic cells did not increase postvirus in either the tumor or tumor-draining lymph node (dLN), a higher number of CD11c+ cells expressing maturation marker CD86 was observed in the virus-treated group (Fig. 6A–D), arguing for virus-triggered activation of APCs. In addition, the frequency of CD11b+ myeloid cells and CD11b+ F4/80+ macrophages was increased in virus-treated tumors compared with the PBS group (Fig. 6E and F).

However, these cells did not exhibit putative cell surface markers of immunosuppressive myeloid-derived suppressor cells (MDSC) or M2 macrophages, cell types that have been proposed to be able to interfere with T-cell induction (refs. 27, 28; Supplementary Fig. S4D–S4G). Moreover, because antigen presentation occurs in the lymphatic system, which is subverted by melanoma cells to an immunosuppressive phenotype (29, 30), we enumerated lymphatic endothelial cells (LEC), presented as CD45+ CD31+ gp38+ cells, in the dissociated tissues. We observed significantly fewer LECs in adenovirus-injected tumors and dLNs compared with those in PBS-injected tumors (Fig. 7A and C). In addition, levels of CD45+ CD31+ gp38+ fibroblastic reticular cells (FRC) were significantly higher in the adenovirus-treated mice compared with both control groups (Fig. 7B and D), possibly facilitating interactions between APCs and T cells (31, 32). Taken together, our results suggest that B16 immunosuppression, which normally prevents APC-mediated T-cell activation and leads to T-cell tolerance, was overcome by the combined action of transferred CD8+ T cells and adenovirus infection.

**Discussion**

Considering the good safety profile of oncolytic adenovirus in patients and the potential of tumor-specific T cells, merging these two forms of immunotherapy is an appealing approach for cancer treatment. In a recent study, Nishio and colleagues reported that
adoptive T-cell therapy can be enhanced by oncolytic adenovirus armed with chemokine RANTES and cytokine IL15, thus improving the trafficking and survival of CAR T cells in an immunodeficient mouse model (33). Although the results from these authors are valuable, the lack of an immune system overlooks many key attributes of ACT. We set out to study the immunologic aspects of the combination of adenovirus and adoptive T-cell therapy in a fully immunocompetent setting because tumor-infiltrating immune cells and components of stroma can have a major impact on the treatment outcome. For this, we used the B16 model, a highly immunosuppressive murine model of melanoma, which has been referred to as the ultimate preclinical test for immunotherapies (13). B16 cells expressing xenoantigen ovalbumin are generally considered immunogenic (based on tumor incidence rate after implantation and the induction of NK cells), but this model also exerts nonimmunogenic properties (very low expression of MHC class I, highly aggressive growth, and resistance to OT-I T cells). Anti–CTLA-4 monoclonal antibodies, which have been approved by the FDA for treatment of human melanoma, lack efficacy in this model (34).

Reflecting the shortcomings of single-agent immunotherapies in advanced and immunosuppressive tumors, the treatment effect of either adenovirus or tumor-specific T cells alone in the B16.OVA model remained poor, whereas prominent tumor suppression was achieved by combining these two treatments (Figs. 1A and 2A). Unexpectedly, the number of adoptively transferred cells present at the tumor could not explain the superior antitumor efficacy, as tumor trafficking was not significantly enhanced by adenovirus (Fig. 2E and F).

Instead, activation of tumor-infiltrating T cells was increased...
following adenovirus treatment (Figs. 4A and 5A), indicating that the phenotypic activation status is essential in T-cell effector function and a key to successful immunotherapy.

Although resulting in significant tumor growth control, the unaltered status in tumor expression of immune checkpoint molecules CTLA-4 and PD-1 (Supplementary Fig. S5) may explain why combination treatment with ACT and adenovirus was incapable of curing mice from established tumors. Although systemic antitumor immunity contributed to antitumor effects and reduced dLN metastasis (Figs. 2B and 4D and E), the microenvironment remained highly immunosuppressive even in adenovirus-treated tumors. On the other hand, adenovirus infection did not increase the intratumoral levels of immunosuppressive cells (Supplementary Fig. S4D–S4G). Currently several immunotherapies are being combined with immune checkpoint inhibitors (35), raising the question of whether checkpoint blockade in our approach would enhance efficacy further.

Besides TCR engagement, costimulatory signals on APCs are needed to induce T-cell activation. After encountering and ingesting tumor antigens, APCs such as dendritic cells and macrophages undergo maturation and migrate to local lymph nodes. Combination of peptide:MHC complexes and costimulatory surface molecules (such as CD80 or CD86) on APCs activates T cells, which, in turn, triggers T-cell proliferation, differentiation, and migration (36). Without APC costimulation, TCR interaction with specific peptide:MHC causes antigen-specific tolerance (7). Indeed, upregulation of CD86 maturation marker on dendritic cells was observed after virus treatment in both tumor and dLN (Fig. 6B and D). This enhanced DC activation through pathogen alarm signals may have contributed to the induction of endogenous antitumor T cells (Figs. 3D–F and 4B and C).

Macrophages are highly plastic immune cells, and their phenotype, ranging from protumor to antitumor, is strongly dependent on the signals from the TME (37). Whether the macrophages detected as shown in Fig. 6F acted as professional APCs (38) or if their accumulation was part of antiviral response (39), their immunosuppressiveness was not increased (Supplementary Fig. S4F–S4G). Despite growing evidence that tumor-associated macrophages (TAM) are linked to poor prognosis in several cancer types (37), further experiments would be needed to confirm the actual role of these macrophages detected in the adenovirus-treated tumors.

An important aspect is the immune response against adenovirus (Figs. 2C and D and 4B and C), which might have contributed to overall efficacy through immune targeting of virus-infected tumor cells (21). In human patients, a correlation between antiviral and antitumor T-cell immunity has been reported (40). As human adenoviruses do not lyse mouse cells, the oncolytic effect is not accounted for in our B16.OVA model. It is tempting to speculate whether active oncolysis would have resulted in further efficacy, as adenoviral replication would result in continuous viral spread, tumor debulking activity, subsequent reduction in mass-related immunosuppressive mechanisms, and more pronounced levels of antiviral and possibly antitumor responses. Unfortunately, current preclinical animal models do not allow us to study this possibility in the context of adoptive T-cell transfer.
Many previous studies combining virotherapy and ACT have focused on oncolytic viruses encoding TAAs, thus relying mostly on oncolysis and vaccination effect against a particular tumor epitope (8, 41–43). In our hands, the mere adenoviral backbone acted as an adjuvant and boosted the endogenous antitumor immunity elicited by ACT. The increase in frequency of TRP-2 and gp100-specific T cells in the adenovirus-treated mice (Figs. 3D–F and 4B and C) indicated that intratumoral adenosine treatment combined with ACT can induce a potent antitumor response and lead to epitope spreading even in the absence of active oncolysis. Our results with adoptive transfer of ovalbumin-targeted T cells are in accord with previous immunotherapy reports, in which epitope spreading and broadened antitumor responses have been detected after targeting a single defined TAA (44–47). Furthermore, T-cell transfer has been shown to enable repertoire expansion through induction of endogenous antitumor T cells (48). From the clinical perspective, the importance of epitope spreading cannot be overstated. Tumors have tremendous capacity for adaptation under selective pressure, such as immune response against a single epitope, or blocking of a single pathway, explaining the failure of many cancer vaccines and targeted therapies. In this aspect, epitope spreading and systemic antitumor immunity may be a prerequisite of successful immunotherapy of advanced tumors.

In conclusion, the results described here focus on the immunologic synergy between adoptive T-cell transfer and adenovirus treatment. Importantly, these two therapies are not merely an attractive combination, but could represent an effective multimodal approach to treat solid tumors. Conversely, because it has been seen that single-agent oncolytic adenovirus lacks curative power in most patients with advanced and metastatic tumors (10, 49), the best use of the technology could well be related to the tremendous capacity for immunostimulation and breaking of tumor-associated tolerance (9, 40). We propose that adding an adenoviral danger signal can enhance the efficacy of T-cell–based approaches and provide the means to immunologically target and destroy otherwise nonimmunogenic solid tumors. Given the encouraging safety of oncolytic adenovirus in patients, this combination represents a highly feasible translation into clinical trials.

Disclosure of Potential Conflicts of Interest

M. Suurala is a staff scientist at TILT Biotherapeutics Ltd. A. Hemminki is CEO at TILT Biotherapeutics Ltd., reports receiving a commercial research grant from Oncos Therapeutics, and has ownership interest (including patents) in TILT Biotherapeutics and Oncos Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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