Armed Oncolytic Virus Enhances Immune Functions of Chimeric Antigen Receptor–Modified T Cells in Solid Tumors

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Abstract

The clinical efficacy of chimeric antigen receptor (CAR)-redirected T cells remains marginal in solid tumors compared with leukemias. Failures have been attributed to insufficient T-cell migration and to the highly immunosuppressive milieu of solid tumors. To overcome these obstacles, we have combined CAR-T cells with an oncolytic virus armed with the chemokine RANTES and the cytokine IL15, reasoning that the modified oncolytic virus will both have a direct lytic effect on infected malignant cells and facilitate migration and survival of CAR-T cells. Using neuroblastoma as a tumor model, we found that the adenovirus Ad5Δ24 exerted a potent, dose-dependent, cytotoxic effect on tumor cells, whereas CAR-T cells specific for the tumor antigen GD2 (GD2.CAR-T cells) were not damaged. When used in combination, Ad5Δ24 directly accelerated the caspase pathways in tumor cells exposed to CAR-T cells, whereas the intratumoral release of both RANTES and IL15 attracted CAR-T cells and promoted their local survival, respectively, increasing the overall survival of tumor-bearing mice. These preclinical data support the use of this innovative biologic platform of immunotherapy for solid tumors. Cancer Res; 74(18); 5195–205. ©2014 AACR.

Introduction

Adoptive transfer of T cells genetically modified to express a CD19-specific chimeric antigen receptor (CAR) coupled with costimulatory endodomains has shown significant clinical impact in lymphoid leukemias (1–3). In contrast, the clinical relevance of this approach has been more limited for solid tumors (4–6).

Insufficient migration of CAR-T cells to the tumor site and suboptimal persistence within the immunosuppressive tumor environment are all critical factors that limit the impact of T-cell immunotherapies in solid tumors. Insufficient migration of tumor-specific T cells may result from unfavorable chemokine gradients, as tumor-specific T cells may lack the appropriate chemokine receptors for chemokines secreted by tumor cells (7). Alternatively, tumor cells or stromal cells produce chemokines that preferentially attract T cells with regulatory function rather than T cells with antitumor activity (8, 9). Even if tumor-specific T cells reach the tumor environment, multiple mechanisms exploited by tumor cells themselves or by surrounding stromal cells can block an effective immune response. Among these mechanisms, downregulation of costimulatory molecules (CD28 and CD86) by tumor cells (10), abundant TGFβ production (11), and infiltration by regulatory T cells (Treg; ref. 9) significantly impair activation and proliferation of tumor-specific T cells, whereas overexpression of death receptor ligands by tumor cells, including FasL and PD-L1, may directly favor premature apoptosis of T lymphocytes (12, 13).

Engineering of CAR-T cells to include costimulatory endodomains can rescue T-cell activation through the autocrine production of IL2 (14–17), but this may still be insufficient to reverse the pro-nergic properties of the tumor environment (18, 19). CAR-T cells will also need to be further modified not only to migrate to the tumor site through co-expression of specific chemokine receptors matching relevant tumor-secreted chemokines such as Gro-α (20), CCL17 (21), and CCL2 (22) but also to specifically counter the local inhibitory milieu, for example, due to TGFβ (23) and CCL2 (24) and to make use of autocrine or administered growth factors (25–27). Because tumor cells likely use multiple inhibitory strategies, it is challenging to construct vectors able to accommodate and express in T lymphocytes all the immunomodulatory genes required to overcome tumor inhibition while increasing T-cell trafficking, survival, and safety (28).

To solve this complex task, we exploited oncolytic viruses that selectively infect, lyse, and replicate in malignant cells while leaving nonmalignant cells unaffected (29). Oncolytic viruses have sufficient cargo capacity to insert ectopic genes.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and have produced clinical responses in patients with solid tumors (30). We therefore armed an oncolytic adenovirus (Ad5Δ24) with the chemokine RANTES and the cytokine IL15 to enhance the subsequent trafficking and survival of T cells expressing a tumor-directed CAR.

**Materials and Methods**

**Tumor cell lines**

The neuroblastoma cell lines IMR-32, LAN-1, SKNLP, SK-N-SH, and SH-SY5Y and the lung carcinoma cell line A549 were obtained from the ATCC, whereas neuroblastoma cell lines LAN-5 and CHLA-255 were gifts from Dr. I.S. Metelitsa (Baylor College of Medicine, Houston, TX) and we verified that these lines retain the surface expression of the target antigen GD2. Cells were maintained in RPMI-1640 (IMR-32, LAN-1, LAN-5, SKNLP, SK-N-SH, and SH-SY5Y) or IMDM (CHLA-255 and A549). All media were supplemented with 10% FBS and 2 mmol/L L-glutamine (Invitrogen), with the exception of CHLA-255, which was supplemented with 20% FBS. Tumor cell lines were transduced with a gamma retroviral vector encoding enhanced GFP (eGFP) to obtain GFP-positive tumor cells.

**Oncolytic adenovirus**

Oncolytic adenovirus Ad5Δ24 and nonreplicable control adenovirus Ad5-Luc1 were kindly provided by Dr. Akseli Hemminki (University of Helsinki, Helsinki, Finland). Ad5Δ24/RANTES, Ad5Δ24/GD2.CAR, and Ad5Δ24/RANTESII.GD2.CAR were generated and amplified according to standard procedures (31). For Ad5Δ24, RANTESII.GD2.CAR, the two genes were linked together using a 2A-like sequence (25).

**Flow cytometry**

The following monoclonal antibodies conjugated with fluorochromes were used: Coxackie-adenovirus receptor, GD2, CD95 (Fas), CD80, CD86, CD40L, OX40L, CD25, CD69, IFNγ, CD3, granzyme B (BD Biosciences); TRAIL, TRAIL-R1, and CD95 (Fas), CD80, CD86, CD40L, OX40L, CD25, CD69, IFNγ, CD3, granzyme B (BD Biosciences); TRAIL, TRAIL-R1, and OX40 (Biolegend). Expression of GD2.CAR by T cells was detected using a specific anti-idiotype, 1A7 (TriGem, Titan), followed by staining with secondary antibody RAM-IgG1 (BD Biosciences). FACS data were collected with a FACSCalibur (Becton Dickinson) and analyzed using FlowJo software version 9.3 (Tree Star). For the caspase assay, CHLA-255 cells were seeded in 24-well plates (1 × 10⁴/well), infected with Ad5Δ24 or mock (100 vp/cell), and cultured for 4 days. Control and GD2.CAR-T cells (2.5 × 10⁵/well) were then added to the tumor cells. Active caspases in CHLA-255 cells were measured at 0, 2, and 4 hours by FACS. The apoptotic cells were stained using Vybrant FAM Poly Caspases Assay Kit (Molecular Probes) according to manufacturer’s instructions. The frequency of early apoptotic cells was determined as percentage of FAM⁺ (carboxyfluorescein as a reporter) cells excluding CD3⁻ and propidium iodide (PI)⁺ cells from the analysis (32).

**MTS assay**

Cells were seeded into 96-well plates (10⁴ cells per well), infected with or without viruses at the indicated doses and incubated at 37°C for 96 hours. Cell viability was analyzed by MTS assay according to manufacturer’s instruction (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega). Values of virus-infected cells were normalized to those of mock-infected cells (percent cell viability). The experiments were performed in triplicate and repeated three times.

**Retroviral production and CAR-T cell generation**

The vectors encoding the GD2-specific CAR (GD2.CAR), incorporating the CD28 and OX40 costimulatory endodomains, the fusion protein eGFP-firefly luciferase (FFLuc), and the methodology for the production of retroviral supernatant and CAR-T cells have been described previously (15, 16, 33). For the T-cell proliferation assay in vitro, we used a first generation GD2.CAR that lacks both CD28 and OX40 signaling domains.

**Coculture experiments**

Tumor cells were seeded in 24-well plates (5 × 10⁴/well for cytotoxicity assay and 1 × 10⁵/well for T-cell proliferation assay), infected with Ad5Δ24 (50–100 vp/cell), and then cultured for 3 days. Control and GD2.CAR-T cells (3 × 10⁵/well for cytotoxicity assay and 5 × 10⁵/well for T-cell proliferation assay) were then added and cultured for additional 3 days. Residual GFP⁺ neuroblastoma cells and T cells were then counted on the basis of GFP and CD3 expression, respectively, using microbeads (CountBright Absolute Counting Beads, Invitrogen). Normalized residual tumor cells were calculated as 100 × tumor cell counts with treatment/tumor cell counts without treatment (%).

**Confocal microscopic video imaging**

GFP-labeled CHLA-255 cells were seeded into 8-well chamber slide (Lab-TekII, Thermo Scientific) 10⁴ cells/well, infected with Ad5Δ24 (100 vp/cell), and cultured for 3 days. Control and GD2.CAR-T cells were then added to the well (10⁵ cells/well). GFP⁺ neuroblastoma cells stained with Annexin V (Invitrogen) were imaged using a spinning disk confocal microscope for 16 hours. Imaging data were acquired and analyzed using Zen software (Zeiss).

**Migration assay**

Migration assays were conducted as previously described (21) with minor modifications using 5-μm pore 24-well Transwell plates (Corning Life Science). The percentage of migrating cells was calculated as follows: 100 × (cell count of experimental sample – cell count of negative control)/(cell count of positive control – cell count of negative control).

**ELISA and Milliplex assay**

To measure the in vitro production of chemokines and cytokines, tumor cells were plated at 5 × 10⁵ cells/mL in 24-well plates and infected with viruses (50–100 vp/cell). Supernatants were collected 72 hours later and analyzed for the production of RANTES, MIP-1α, MIP-1β, MCP-1, IP-10, and IL-15. To measure the in vivo production of RANTES and IL-15, tumor and blood samples were collected 14 to 18 days after virus inoculation. Tumor homogenates and serum were
neuroblastoma xenograft animal model

To assess antitumor effects and persistence of GD2.CAR-T cells, we used NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Laboratory). All mouse experiments were performed in accordance with Baylor College of Medicine Animal Husbandry and Institutional Animal Care and Use Committee guidelines. Mice were engrafted with CHLA-255 cells (3 \times 10^7) subcutaneously, and after 10 to 14 days, mice were inoculated intratumorally with PBS or oncolytic viruses (10^5–10^7 vp/mouse). Mice were then infused intravenously with either control or GD2.CAR-T cells (10^7 cells) 3 to 4 days after virus inoculation. Tumor volumes were monitored twice a week by caliper measurement (volume = length \times width^2/2). To track the migration and survival of GD2.CAR-T cells in vivo, control and GD2.CAR-T cells were labeled with eGFP::FFLuc (15). Biodistribution was assessed using the In Vivo Imaging System (Xenogen) as previously described (15).

Immunohistochemistry

Tumor samples were fixed, processed, and stained according to standard procedures. We performed hematoxylin and eosin staining and labeling of human T cells using polyclonal rabbit anti-human CD3 mAb (A0452, Dako). For detection, we used rabbit anti-human CD3 mAb (A0452, Dako). For detection, we used Dako LSAB + System-HRP (K0679, Dako).

Statistical analysis

ANOVA with Bonferroni correction and the 2-sided unpaired t test were used for comparison of 3 or more groups, or 2 groups, respectively, as stated in the figure legends. Mixed-model ANOVA was applied to compare tumor growth in different groups of mice. Survival curves were plotted using the Kaplan–Meier methods, and the differences in the survival between groups were assessed by log-rank test. Data are presented as mean ± SD or SEM as stated in the figure legends. Statistical significance was defined at \( P < 0.05 \). Statistical analysis was performed with Prism 5 (GraphPad Software).

Results

Coxackie-adenovirus receptor is functionally expressed in neuroblastoma cell lines but not by GD2.CAR-T cells

To determine whether Ad5Δ24 and CAR-T cells could be combined without toxicity to T lymphocytes in a neuroblastoma tumor model, we first compared the expression of the Coxackie-adenovirus receptor in seven neuroblastoma cell lines and CAR-T cells specifically targeting the GD2 antigen expressed by neuroblastoma cells. As shown in Fig. 1A, all neuroblastoma cell lines expressed the virus receptor as assessed by flow cytometry whereas CAR-T cells did not. Incubation with the oncolytic adenovirus Ad5Δ24 induced cellular toxicity in 5 of 7 neuroblastoma cell lines in a dose-dependent manner (Fig. 1B), whereas GD2.CAR-T cells were unaffected, even at the highest dose and for up to 7 days of culture (Fig. 1B). Thus, Ad5Δ24 is cytopathic for neuroblastoma cells but not for GD2.CAR-T cells.

Combined therapy of GD2.CAR-T cells with Ad5Δ24 enhances apoptosis of neuroblastoma cells in vitro

We next explored whether combining Ad5Δ24 and GD2.CAR-T cells enhanced the elimination of neuroblastoma cells in coculture experiments. When CHLA-255 cells were incubated with 75 vp/cell of Ad5Δ24 and a low ratio of GD2.CAR-T cells (effector:target = 3:5), tumor cells were effectively eliminated by day 7 of culture compared with tumor cells incubated with each agent alone. Residual tumor cells were significantly decreased to less than 5% in the presence of Ad5Δ24 together with GD2.CAR-T cells, compared with 30% ± 7% and 33% ± 7% in the presence of GD2.CAR-T cells (\( P = 0.01 \)) and Ad5Δ24 combined with control T cells (\( P = 0.01 \); Fig. 2A). Comparable results were obtained when 2 other neuroblastoma cell lines IMR-32 and SKNL were used (Fig. 2A). To clarify the mechanism behind the superior antitumor effects of the combined treatment, we explored whether Ad5Δ24 potentiated the function of T cells by upregulating in infected tumor cells the GD2 antigen, death receptors (Fas and TRAIL R1/R2), or costimulatory molecules and ligands (CD80, CD86, OX40L, and CD40L). However, no upregulation of any of these molecules by neuroblastoma cells was detected upon infection with Ad5Δ24 (Supplementary Fig. S1). We also investigated whether Ad5Δ24-infected neuroblastoma cells could indirectly increase the activation or effector function of CAR-T cells. However, neither CD25, CD69, IFNγ, granzyme B nor TRAIL was significantly upregulated by GD2.CAR-T cells exposed to Ad5Δ24-infected neuroblastoma cells (Supplementary Fig. S2). In contrast, we found that Ad5Δ24-infected neuroblastoma cells underwent more rapid apoptosis when exposed to GD2.CAR-T cells. As illustrated in Fig. 2B and Supplementary Movie S1, we conducted sequential confocal microscope imaging of GFP+ CHLA-255 cells cocultured with GD2.CAR-T cells. We found that neuroblastoma cells preincubated for 72 hours with Ad5Δ24 became Annexin-V+ within 4 hours after exposure to GD2.CAR-T cells, whereas noninfected CHLA-255 cells exposed to GD2.CAR-T cells required more than 16 hours to bind detectable levels of Annexin-V. We quantified the active caspases in neuroblastoma cells and found in response to GD2.CAR-T cells a consistent increase in the percentage of early apoptotic cells when neuroblastoma cells were preincubated with Ad5Δ24 compared with neuroblastoma cells that had not been exposed to Ad5Δ24 (FAM+/PI- cells were 20% ± 2% vs. 15% ± 1% at 2 hours, \( P < 0.05 \); 24% ± 2% vs.18% ± 2% at 4 hours, \( P < 0.05 \); Fig. 2C and D). These results indicate that the caspase activity and apoptosis in neuroblastoma cells are more pronounced after incubation with CAR-T cells if neuroblastoma cells are infected with Ad5Δ24.
Combined GD2.CAR-T cells and Ad5Δ24 have more robust antitumor activity in vivo

We examined whether the combination of GD2.CAR-T cells and Ad5Δ24 was also effective in a neuroblastoma xenograft mouse model. Mice engrafted subcutaneously with the neuroblastoma cell line CHLA-255 were inoculated intratumorally with either PBS or Ad5Δ24 and then infused intravenously with either control or GD2.CAR-T cells (10⁷ cells). Control mice, inoculated with PBS and control T cells, had rapid tumor progression and were euthanized within 20 days (Supplementary Fig. S3). When mice were inoculated with a high dose of Ad5Δ24 (10⁶ vp), tumor growth was controlled irrespective of T-cell transfer (Fig. 3A). However, when mice were inoculated with lower doses of Ad5Δ24, the addition of CAR-T cells improved tumor control compared with Ad5Δ24 plus control T cells ($P = 0.04$ in $10^6$ vp; Fig. 3A).

We also found that GD2.CAR-T cells showed enhanced, although still transient, persistence in vivo at the tumor site when tumors were inoculated with Ad5Δ24. As shown in Fig. 3B and C, FFLuc-labeled GD2.CAR-T cells localized and persisted up to day 6 in Ad5Δ24-inoculated tumors compared with PBS-inoculated tumors ($1.8 \times 10^6 \pm 0.63 \times 10^6$ p/s/cm²/sr vs. $1.5 \times 10^3 \pm 0.2 \times 10^3$ p/s/cm²/sr, $P < 0.01$) and then became undetectable. These findings raise the possibility that Ad5Δ24-infected neuroblastoma cells may release T-cell chemoattractant factors. However, measurement of the chemokines RANTES, MIP-1α, MIP-1β, MCP-1, and IP-10 in the culture of neuroblastoma cells infected with Ad5Δ24 showed no significant production of these cytokines before and after infection, with the exception of the SK-N-SH neuroblastoma cell line that spontaneously produced a high level of MCP-1 (Supplementary Table S1). In vitro migration assays confirmed that supernatants collected from neuroblastoma cells infected with Ad5Δ24 did not promote T-cell migration, excluding the possibility that other unknown factors that favor T-cell migration are released by Ad5Δ24-infected neuroblastoma cells (Fig. 3D). Thus, the transient beneficial effects of the combined Ad5Δ24 and GD2.CAR-T cells support the need for the engineering of Ad5Δ24 to increase the antitumor benefits.

Neuroblastoma cells infected with Ad5Δ24 engineered to release RANTES and IL15 produce functional amounts of both proteins

We armed Ad5Δ24 with RANTES and IL15 (Ad5Δ24. RANTES.IL15) to induce migration and prolong persistence of GD2.CAR-T cells at the tumor site (Fig. 4A) and demonstrated that neuroblastoma cells infected with this virus release RANTES (709–2,247 pg/ml at 72 hours) and IL15 (864–2,131 pg/ml at 72 hours) in vitro (Fig. 4B). Both factors...
were functional because the supernatant of neuroblastoma cells infected with Ad5Δ24.RANTES.II15 improved the migration (19% ± 2% in Ad5Δ24.RANTES.II15 vs. 9% ± 2% in mock; P = 0.002, and vs. 13% ± 3% in Ad5Δ24; P = 0.01) and expansion (1.6 ± 0.1-fold in Ad5Δ24.RANTES.II15 vs. 0.71 ± 0.07 fold in mock; P = 0.01, and vs. 0.55 ± 0.04-fold in Ad5Δ24; P = 0.005) of GD2.CAR-T cells in vitro (Fig. 4C and D) without impairing the oncolytic property of the virus (Fig. 4E). Thus, neuroblastoma cells infected with Ad5Δ24.RANTES.II15 produce functional levels of both proteins.

**Ad5Δ24.RANTES.II15 enhances the persistence of GD2.CAR-T cells in vivo**

Mice engrafted with CHLA-255 cells were inoculated intratumorally with either Ad5Δ24 or Ad5Δ24.RANTES.II15 (10⁶−10⁷ vp) followed by one single intravenous infusion of FLuc-labeled GD2.CAR-T cells (10⁵ cells). As shown in Fig. 5A and B, bioluminescence signals of GD2.CAR-T cells were higher at the site of tumors inoculated with Ad5Δ24.RANTES.II15 than in tumors inoculated with unmodified Ad5Δ24 (2.1 × 10⁵ ± 1.0 × 10⁴ p/s/cm²/sr vs. 5.6 × 10⁵ ± 4.1 × 10⁴ p/s/cm²/sr at day 12, P < 0.001). RANTES and IL15 secretion by Ad5Δ24.RANTES.II15-infected tumor did not promote accumulation of control T cells lacking CAR expression, indicating that antigen specificity of T cells is also necessary for T-cell persistence at the tumor site (Fig. 5C). The increase in photon intensity corresponded to an increased numeric infiltration of human T cells within the tumor as demonstrated by both flow cytometry and immunohistochemistry (Fig. 5D and E). Quantification of RANTES and IL15 in the sera and tumor biopsies confirmed that these two factors were produced only in mice inoculated with Ad5Δ24.RANTES.II15 (Fig. 5F). Moreover, both RANTES and IL15 were predominantly detected at the tumor site (5.890 ± 1.055 and 1.762 ± 0.360 pg/mL, respectively) rather than in serum (111 ± 46 and 357 ± 162 pg/mL, respectively;
RANTES; \( P < 0.001 \) and IL15; \( P < 0.01 \); Fig. 5F). Quantification of other human and mouse cytokines in serum by a Milliplex assay as indicated in Materials and Methods demonstrated no significant modifications except for an equal elevation of mouse granulocyte colony-stimulating factor in both Ad5Δ24 and Ad5Δ24.RANTES.II15-treated mice. Combination therapy with GD2.CAR-T cells and Ad5Δ24.RANTES.II15 significantly enhanced the survival of mice compared with GD2.CAR-T cells and Ad5Δ24 (73% vs. 44% at day 45, \( P = 0.03 \); Fig. 5G). Finally, we found that the intratumoral injection of the Ad5Δ24.RANTES.II15 is essential to sustain the persistence of CAR-T cells. When mice were indeed engrafted with tumor cells in both flanks, CAR-T cells significantly persisted and promoted antitumor activity only at the virus-inoculated tumor site (Supplementary Fig. S4).

We also constructed Ad5Δ24 encoding either RANTES alone (Ad5Δ24.RANTES) or IL15 alone (Ad5Δ24.II15) to determine the specific role of each single component (Fig. 6A). Both viruses were functional, as infected neuroblastoma cells produced either RANTES or IL15 in vitro (Fig. 6B), and both viruses retained oncolytic activity (Fig. 6C). When tested in vivo, GD2.CAR-T cell persistence was superior in mice that received Ad5Δ24.RANTES.II15 (\( 1.9 \times 10^7 \pm 0.9 \times 10^7 \) p/s/cm²/sr), compared with those that received either Ad5Δ24.RANTES (\( 1.0 \times 10^6 \pm 0.2 \times 10^6 \) p/s/cm²/sr, \( P < 0.01 \)) or Ad5Δ24.II15 (\( 3.9 \times 10^5 \pm 2.8 \times 10^5 \) p/s/cm²/sr, \( P < 0.05 \); Fig. 6D and E). Thus, the combination of intratumoral injection of Ad5Δ24.RANTES.II15 and intravenous infusion of GD2.CAR-T cells produces significant improvements in the control of tumor growth.

**Discussion**

We have demonstrated that the Ad5Δ24 armed with RANTES and IL15 increases the number of T cells infiltrating a solid tumor, likely through the enhanced trafficking and prolongation of T-cell survival that these agents respectively induce. These effects were achieved without compromising the intrinsic lytic activity of the oncolytic virus or CAR-T cells, and the combination of both agents resulted in better control of the tumor growth and prolonged survival of tumor-bearing animals.

The rationale for combining oncolytic viruses and CAR-T cells for the treatment of solid tumors stems from two main experimental evidences. First, Ad5Δ24 engineered to accommodate genes supporting T-cell function remain toxic to tumor cells without damaging or compromising CAR-T cell activities, even when the viruses were used at high concentrations. Second, Ad5Δ24-infected neuroblastoma cells become more susceptible to the lytic effects of CAR-T cells. It was previously described that oncolytic adenoviruses induce
activation of caspase-3 in infected tumor cells (34). Here we support this observation and demonstrated that Ad5Δ24 further accelerates and amplifies the occurrence of caspase-induced cell death of neuroblastoma cells mediated by CAR-T cells. In turn, the faster lysis of tumor cells promoted by CAR-T cells may facilitate the spread of the virus, which in solid tumors is usually limited to the surrounding area of the virus inoculation (35). As a consequence, when unmodified Ad5Δ24 and CAR-T cells are combined, better control of the neuroblastoma tumor growth is observed in animals. Despite the superior cytolytic activity observed with the combined treatment described above, we found that the overall infiltration and persistence of CAR-T cells at the tumor site was not robustly improved, as Ad5Δ24-infected neuroblastoma cells did not release factors that promote T-cell migration and survival. Even though the GD2-specific CAR we used is engineered to co-express 2 costimulatory molecules such as CD28 and OX40 (16, 22), this combination is evidently insufficient to sustain the persistence of the few CAR-T cells that reach the tumor microenvironment. These limitations strongly support our strategy to engineer oncolytic virus to produce not only a T-cell chemoattractant factor but also a T-cell growth factor.

For optimized T-cell trafficking and survival, we selected the chemokine RANTES and the cytokine IL15. While we and others have previously focused on identifying chemokines specifically produced by tumor cells and on engineering CAR-T cells with the cognate receptor (20–22), here we selected RANTES as a broadly applicable chemokine because its receptors CCR1, CCR3, and CCR5 are retained by ex vivo expanded T cells (22, 36). This assures that upon adoptive transfer, T cells will migrate to multiple types of tumors if they are forced to release RANTES. We selected IL15 as a T-cell growth factor because in addition to its...
Of note, the detection of RANTES and IL15 in our model was consistent with the potential toxicity of oncolytic viruses (40, 41). Of note, the detection of RANTES and IL15 in our model was consistent with the potential toxicity of oncolytic viruses (40, 41).

Figure 5. Ad5Δ24.RANTES.IL15 improves persistence of GD2.CAR-T cells. A and B, NSG mice engrafted subcutaneously with CHLA-255 cells were inoculated intratumorally with oncolytic viruses (10⁵–10⁶ vp) by day 10. Four days later, mice were infused intravenously with FFuclabeled GD2.CAR-T cells. T-cell bioluminescence was then measured. Data represent mean ± SEM. *** P < 0.001; **** P < 0.0001 by Student t test. C, NSG mice engrafted subcutaneously with CHLA-255 cells were inoculated intratumorally with oncolytic viruses (10⁶ vp) by day 10. Four days later, mice were infused intravenously with FFuclabeled control T cells or FFuclabeled GD2.CAR-T cells diluted with control T cells at 6:10 or 2:10 ratios. T-cell bioluminescence was then measured. Data represent mean ± SEM. D and E, T cells infiltrating the tumors were assessed by FACS and IHC. F, detection of RANTES and IL15 in serum and tumor homogenates collected from mice 14 to 18 days after inoculations of oncolytic viruses. Data represent mean ± SEM in 8 mice for each virus. * P < 0.05; ** P < 0.01; *** P < 0.001 by Student t test. G, survival curves of NSG mice bearing CHLA-255 cells and treated with single and combined agents. *, P < 0.05 by log-rank test.

Table 1. Levels of RANTES and IL15 in serum and tumor homogenates collected from mice 14 to 18 days after inoculations of oncolytic viruses.

<table>
<thead>
<tr>
<th>Group</th>
<th>RANTES (ng/mL)</th>
<th>IL15 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control T cells</td>
<td>0.000%</td>
<td>0.002%</td>
</tr>
<tr>
<td>Ad5Δ24</td>
<td>5.72%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Ad5Δ24.RANTES.IL15</td>
<td>5.27%</td>
<td>2.0%</td>
</tr>
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Although the immunodeficient mouse model we have used has limits in assessing the effect of the inhibitory components of the immune system such as Tregs, tumor-associated macrophages, and myeloid-derived suppressor cells, it is nevertheless relevant for a clinical translation. It demonstrates indeed that the infection of tumor cells with RANTES- and IL15-engineered oncolytic virus is sufficient to enhance the overall antitumor activity of CAR-T cells generated following the manufacturing procedures currently applied for the production of CAR-T cells for clinical use (33). There is also additional evidence that tumors infected with oncolytic virus can trigger danger signals by dendritic cells and promote cross-antigen presentation, ultimately leading to the elicitation of innate and adaptive immune responses (42). Thus, the positive effects of armed oncolytic viruses observed in our own and other models (36, 43) may be further amplified in patients due to the recruitment of other components of the immune system.

multiple beneficial effects on T cells (19, 37), this cytokine is not produced by CAR-T cells upon activation and thus the oncolytic virus provides a growth factor that is generally absent in the tumor environment (25). In addition, we previously demonstrated that IL15 is preferentially used by effector T cells rather than Tregs (19) in the unfortunate event that RANTES also attracts this cell subset (38). We found that only Ad5Δ24 co-expressing RANTES and IL15 produced substantive increases in CAR-T cell numbers at tumor sites, which lead to improved tumor control in vivo. Of note, the detection of RANTES and IL15 in our model was mostly confined to the tumor site, indicating a preferential local expression of both factors and thereby circumventing the toxicities associated with systemic administration of cytokines (39). To further potentiate the safety of this strategy for the clinical application, antiviral agents, such as cidofovir, or the inclusion of the thymidine kinase gene of the human herpes virus type I can be used to abort any potential toxicity of oncolytic viruses (40, 41).
Conception and design: N. Nishio, I. Diaconu, V. Cerullo, G. Dotti
Development of methodology: N. Nishio, I. Diaconu, I. Carnana, V. Hoyos, B. Savoldo, G. Dotti

In our model, we selected to administer the oncolytic virus intratumorally, as this is the preferential route of infusion of oncolytic adenoviruses in clinical trials. We found that the local presence of the virus is essential in supporting the persistence of CAR-T cells. Thus, to be effective for metastatic tumors, our proposed combined approach will likely require the use of CAR-T cells. Thus, to be effective for metastatic tumors, our approach will likely require the use of CAR-T cells. Thus, to be effective for metastatic tumors, our approach will likely require the use of CAR-T cells.

Disclosure of Potential Conflicts of Interest
G. Dotti and B. Savoldo have ownership interest (including patents) in the field of T cell and gene-modified T-cell therapy for cancer. The Center for Cell and Gene Therapy has a collaborative research agreement with Celgene and Bluebird bio.

In conclusion, our preclinical studies have shown increased antitumor efficacy of combining CAR-T cell and Ad5Δ24 armed with RANTES and IL15 to improve migration and persistence of the infused T cells. Our data support further exploration of this platform of biologic agents for therapy of solid tumors.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Nishio, V. Hoyos, I. Bouchier-Hayes, B. Savoldo, G. Dotti
Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): N. Nishio, I. Diaconu, H. Liu, I. Carnana, V. Hoyos, B. Savoldo, G. Dotti
Writing, review, and/or revision of the manuscript: N. Nishio, I. Diaconu, B. Savoldo, G. Dotti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Nishio, I. Diaconu, G. Dotti
Study supervision: N. Nishio, I. Diaconu, G. Dotti

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References

Armed Oncolytic Virus Enhances Immune Functions of Chimeric Antigen Receptor–Modified T Cells in Solid Tumors

Nobuhiro Nishio, Iulia Diaconu, Hao Liu, et al.


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Supplementary Table 1. Chemokine production of NB cells (pg/ml/5x10^5 cells).

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Figure S1

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Figure S2

CD25 CD69 IFNγ Granzyme B TRAIL

CHL A-255IMR SKNLP

CD25 (MFI) CD69 (MFI) IFNγ (%)

Granzyme B (%)

TRAIL (MFI)

no virus Ad5Δ24

% of maximum

Figure S2
Figure S3

Tumor volume (mm$^3$)

PBS+ Control T cells

Days after Ad5$\Delta^24$ injection

0 5 10 15 20

Tumor volume (mm$^3$)

Days after Ad5$\Delta^24$ injection
Figure S4

A

D5

D9

D14

B

PBS (n=5)

Ad5Δ24.RANTES.IL-15 (n=5)

Days after T cell infusion

Bioluminescence (p/s/cm²/sr)

Luminescence (x10⁶)

0 2

0 1

0.5

2x10⁷

4x10⁷

6x10⁷

0 5 10 15

*
Supplementary Legends to Tables, Figures, and Movies

Supplementary Table S1. Chemokine production of NB cells. Tumor cells were plated at 5 x 10^5 cells/ml in 24-well plates and infected with Ad5Δ24 (50-100 vp/cell). Supernatants were collected 72 hrs later and analyzed for the production of RANTES, MIP-1α, MIP-1β, MCP-1 and IP-10. Data represent mean ± SD of two experiments.

Supplementary Fig S1. NB cells infected with Ad5Δ24 do not upregulate GD2, Fas, TRAIL receptors and costimulatory molecules. NB cells infected with control or Ad5Δ24 (50-100 vp/cell) were assessed at day 3 after infection. Presented are histograms of 3 NB cell lines (CHLA-255, IMR-32, and SKNLP). The gray filled curve, blue line and red line correspond to isotype control, no-virus treatment and Ad5Δ24 infection, respectively.

Supplementary Fig S2. Ad5Δ24 does not increase the effector function of GD2.CAR-T cells. IFNγ, granzyme B, CD25, CD69 and TRAIL expression was assessed in T cells after co-culture with NB cells (CHLA-255, IMR-32 and SKNLP) preincubated for 72 hrs with or without Ad5Δ24 (50-100 vp/cell). Presented are representative histograms (top) and quantification (bottom) from 3 different preparations of T cells. The gray filled curve, blue line and red line correspond to T cells incubated without tumor cells and T cells incubated with tumor cells without or with Ad5Δ24, respectively.

Supplementary Fig S3. Growth of CHLA-255 cells in control mice. NSG mice were injected s.c. on the right flank with CHLA-255 cells and inoculated i.t. by day 14 with PBS followed by i.v. infusion of control T cells (10^7 cells) 4 days later. Tumor growth was assessed by caliper measurement. Data represent mean ± SEM.

Supplementary Fig S4. Growth of CHLA-255 cells in mice inoculated in both flanks. NSG mice were injected s.c. in both flanks with CHLA-255 cells. By day 12 these animal were inoculated i.t. with PBS on the left side and Ad5Δ24.RANTES.IL-15 on the right side. Four days later, they were infused i.v. with FFLuc labeled GD2.CAR-T cells (10^7 cells). T-cell bioluminescence at each tumor site was measured. Mice were euthanized by day 15 after T-cell infusion due to the growth of the tumor on the non-viral inoculated tumor. Data represent mean ± SEM. *P < 0.05 by Student’s t test.
Supplementary Movie. Sequential confocal microscope imaging. Co-culture experiments of GFP⁺ CHLA-255 cells preincubated with Ad5Δ24 for 72 hrs and then co-cultured with either control or GD2.CAR-T cells. Cells were imaged for 16 hrs after adding T cells. Apoptotic CHLA-255 cells binding Annexin-V appear in Orange. (A) Control T cells, (B) GD2.CAR-T cells, (C) Ad5Δ24 and control T cells, (D) Ad5Δ24 and GD2.CAR-T cells.