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Models and Technologies

Molecular Cancer Therapeutics

Oncolytic Adenovirus Expressing Monoclonal Antibody Trastuzumab for Treatment of HER2-Positive Cancer

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Abstract

Monoclonal anti-HER2 antibody trastuzumab has significantly improved the survival of patients with HER2-overexpressing tumors. Nevertheless, systemic antibody therapy is expensive, limited in efficacy due to physical tumor barriers, and carries the risk of severe side effects such as cardiomyopathy. Oncolytic viruses mediate cancer-selective transgene expression, kill infected cancer cells while mounting antitumor immune responses, and have recently demonstrated promising efficacy in combination treatments. Here, we armed an oncolytic adenovirus with full-length trastuzumab to achieve effective *in situ* antibody production coupled with progressive oncolytic cancer cell killing. We constructed an infectivity-enhanced serotype 5 oncolytic adenovirus, Ad5/3-Δ24-tras, coding for human trastuzumab antibody heavy- and light-chain genes, connected by an internal ribosome entry site. Infected cancer cells were able to assemble full-length functional antibody, as confirmed by Western blot, ELISA, and antibody-dependent cell-mediated cytotoxicity assay. Importantly, oncolysis was required for release of the antibody into tumors, providing additional spatial selectivity. Ad5/3-Δ24-tras showed potent *in vitro* cytotoxicity and enhanced antitumor efficacy over oncolytic control virus Ad5/3-Δ24 or commercial trastuzumab in HER2-positive cancer models *in vivo* (both $P < 0.05$). Furthermore, Ad5/3-Δ24-tras resulted in significantly higher tumor-to-systemic antibody concentrations ($P < 0.001$) over conventional delivery. Immunological analyses revealed dendritic cell activation and natural killer cell accumulation in tumor-draining lymph nodes. Thus, Ad5/3-Δ24-tras is an attractive anticancer approach combining oncolytic immunotherapy with local trastuzumab production, resulting in improved *in vivo* efficacy and immune cell activation in HER2-positive cancer. Moreover, the finding that tumor cells can produce functional antibody as directed by oncolytic virus could lead to many valuable antitumor approaches. *Mol Cancer Ther; 15(9); 2259–69. ©2016 AACR.*

Introduction

HER2 is a key tumor-associated molecule, providing growth signal and contributing to resistance to standard hormonal and chemotherapies, consequently associating with poor prognosis (1–3). HER2 is overexpressed in 20% to 30% of common female malignancies, breast and ovarian cancer, and up to 34% of gastroesophageal junction cancers (1, 2, 4, 5). Routine examination of tumor HER2 status and systemic therapy with anti-HER2 antibody trastuzumab have become standard oncology practice for HER2-positive cancers, owing to the significant survival benefits seen (2, 5).

Trastuzumab is a high-affinity humanized antibody targeting the extracellular domain of HER2-mediating several antitumor activities. Blocking receptor signaling induces G_1 cell-cycle arrest and inhibition of PI3K/Akt signaling pathways, which leads to induction of apoptosis and inhibition of angiogenesis (6). In addition, IgG class antibody possessing a human Fc region, such as trastuzumab, can prime target cells for attack by the immune system (7, 8).

Nevertheless, systemic trastuzumab therapy, similar to other antibody treatments, has major limitations: (i) Monoclonal antibodies need to be administered rather frequently and are expensive. (ii) Systemic administration of high molecular weight compounds results in poor penetration of tumor masses (9), as recently demonstrated in patients for another antitumor IgG antibody (10). (iii) Although monoclonal antibodies are highly specific, they carry the risk for serious adverse reactions caused by "on-target, off-tumor" toxicity where the antibody binds to its target at an unwanted site. With regard to anti-HER2 treatment, stressed cardiomyocytes also express HER2, which results in potentially fatal myocardial damage and congestive heart failure in 2% to 4% of trastuzumab-treated patients (11, 12). Furthermore, (iv) in order to achieve therapeutic concentrations at the tumor site, systemic administration requires high doses of antibody that increases off-tumor toxicities and hypersensitivity

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reactions (off-target, off-tumor) toward the murine components of trastuzumab (13). Finally, (v) impurities resulting from conventional production methods can contribute to toxicities and allergic reactions at least in theory.

To circumvent these issues and to improve antitumor efficacy, we engineer a cancer-selective oncolytic adenovirus that encodes trastuzumab antibody heavy- and light-chain genes connected by a linker sequence. Our approach allows production of monoclonal anti-HER2 antibody directly in tumors, by cancer cells, which are then lysed by the replicating virus, releasing both new virions and the assembled functional antibody into the tumor microenvironment, thus improving anticancer efficacy while reducing systemic exposure. Production by tumor cells of the patient circumvents any issues with product purity.

The oncolytic virus field is finally coming of age, with promising results in recent clinical trials (14, 15). On April 29, 2015, the FDA voted in favor of approving an oncolytic virus for treatment of melanoma (15). Tumor-targeted viruses have the capacity for direct cancer cell lysis and tumor debulking, but the greatest promise seems to lie in their immunotherapeutic effect: Oncolytic adenovirus in particular triggers immunogenic cancer cell death and release of tumor antigens for dendritic cell (DC) recognition and activation (14, 16). Indeed, strategies that further enhance DC maturation, antigen cross-presentation to cytotoxic T cells, and/or combat immunosuppression are becoming the central focus of combination strategies and virus arming (15, 17, 18).

Favorable immunological effects of IgG class antibody therapies have been recognized: Trastuzumab promotes killing of HER2-overexpressing cancer cells *via* antibody-dependent cellular cytotoxicity (ADCC), mainly mediated by natural killer (NK) cells (7), which possess the intriguing capacity for lysis of cancer cell clones expressing low levels of human leukocyte antigen (HLA). Also complement-dependent cytotoxicity and opsonization by antigen–antibody immune complexes can induce immune cell–mediated killing and DC activation (8). Furthermore, NK cells can enhance adaptive immune activation by editing out immature tolerogenic DCs, thus selecting for more activated DC phenotype (19).

To date, gene therapy approaches featuring monoclonal antitumor antibodies have focused on production of antibodies from normal cells (20–23). Meanwhile, it has remained unknown whether full-length antibodies can be produced directly from cancer cells because antibody production in humans is normally restricted to plasma cells (24). Here we show, for the first time, that arming of a cancer-selective oncolytic adenovirus with anti-HER2 antibody trastuzumab achieves sustained local antibody production at the tumor site, coupled with progressive replication, tumor debulking, and immune cell activation, resulting in efficacy superior to virus or antibody only in HER2-positive cancer *in vivo*.

Materials and Methods

Cell culture and viability assays

Breast cancer BT-474 (year obtained: 2009), CAMA-1, MDA-MB-435, MDA-MB-436, SKBR3 (2006, each), ovarian cancer CaOV3, OVCAR3, SKOV3 (2002, each), and gastric cancer N87 cells (2009) were purchased from the American Type Culture Collection (ATCC), esophageal cancer OE19 cells (2009) from European Collection of Cell Cultures (ECACC; Sigma-Aldrich), and transformed embryonic kidney 293 and retinoblast 911 cells (2009, both) from Microbix Biosystems. Breast cancer JIMT-1 (2007) cell line was a generous gift by Dr. Minna Tanner (Institute of Medical Technology, Tampere University Hospital, Finland), ovarian cancer HEY (2001) from Dr. Judy Wolf (M.D. Anderson Cancer Center, Houston, TX), and gastric cancer MKN-28 and MKN-45 (2006, both) from Dr. Hiroshi Yokozaki (Kobe University, Japan). The ATCC, ECACC, Microbix, and collaborators used morphology, karyotyping, and short tandem repeat profiling for cell line authentication, and PCR, Hoechst DNA stain, and microbiological cultures for ruling out microbial contaminants. All lines were cultured as recommended (25, 26) and used within 10 passages from resuscitation for experiments: For viability assays, triplicates of cells on 96-well plates were treated with adenoviruses as in (ref. 25; Supplementary Figs. S1 and S2) or trastuzumab [purified virus-produced, or Herceptin (Roche); Supplementary Figs. S1 and S3] and viability measured 6 to 9 days later by MTS reagent according to manufacturer's instruction (Promega). Background and mock readings were subtracted.

Adenoviruses

Recombinant adenoviruses were cloned using AdEasy Vector System (Agilent Technologies): Trastuzumab cassette (Fig. 1A) was cloned into shuttle plasmid pTHSN replacing only the 6.7K/gp19K open reading frame of E3 region, and then recombined with pAdEasy-1.5/3Δ24 rescue plasmid containing the serotype 5 genome harboring 24-base-pair deletion in E1A and chimeric serotype 3 knob (26). For Ad5/3-tras and Ad5-tras, trastuzumab cassette was cloned into the multiple cloning site of a pShuttle-CMV plasmid and then recombined with either AdEasy-1.5/3 or AdEasy-1 rescue plasmids, respectively (both E1 and E3-deleted). Adenoviruses were propagated in 293 or 911 (oncolytic viruses) cells, confirmed by PCR, and tittered for VP/mL (Fig. 2) and functional titers (pfu/mL; Supplementary Fig. S2); Ad5-tras, Ad5/3-tras, Ad5/3-Δ24 (26), and Ad5/3-Δ24-tras had functional ratios (VP/pfu) of 136, 89, 24, and 203, respectively, reflecting the high genomic load of the transgene-packaged oncolytic virus.

Western and dot blot analysis

Supernatants containing trastuzumab were boiled in Laemmli SDS buffer (including 10% β-Mercaptethanol for reducing condition) for 6 minutes, electrophoresis-separated (not for dot blot) on PAGEr Gold (Lonza), transferred to Hybond-P PVDF membranes (GE Healthcare), blocked overnight in 5% milk, and incubated in primary polyclonal goat anti-human IgG (HC+LC, 1:1,500), and then secondary donkey anti-goat IgG-HRP (1:50,000) for full-length trastuzumab or goat anti-human IgG (HC)-HRP (1:1,500) and IgGκ-HRP (1:1,000) for the separate antibody chains (1 hour each; AbD Serotec). Proteins were visualized using the ECL PlusTM detection system (GE Healthcare).

ELISA

The human IgG ELISA (Immunology Consultants Laboratory) was performed according to the manufacturer's instructions.

Antibody purification and ADCC assay

Supernatants from virus-infected 293 cells were purified using the Montage antibody Kit and Centricon Plus-70, or

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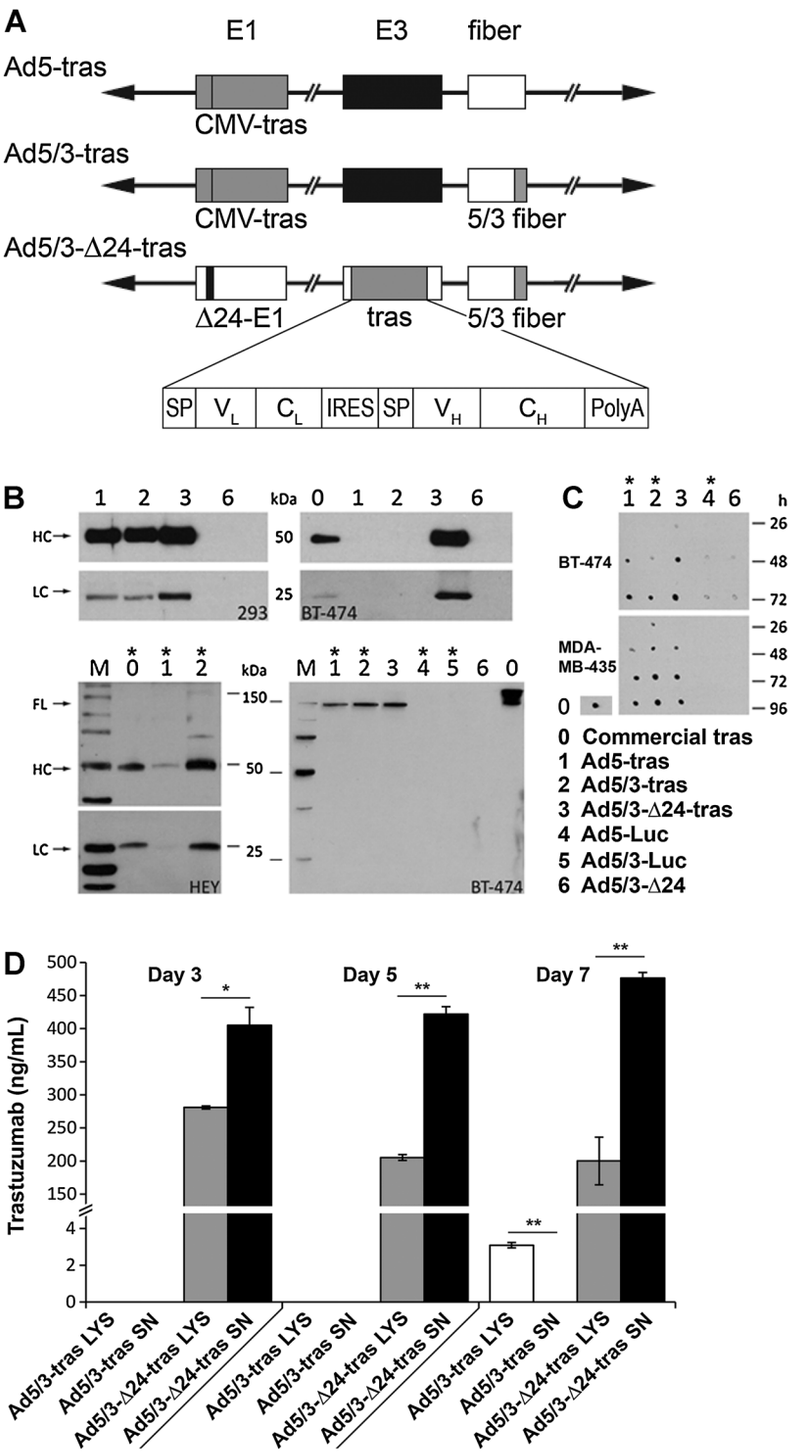

Figure 1.

Engineered adenoviruses mediate trastuzumab production in cancer cells. **A,** schematic illustration of adenovirus constructs with modifications of E1, E3, and fiber regions. Black indicates deletion, and gray indicates modification/insertion. The trastuzumab expression cassette encodes heavy- and light-chain cDNAs, both preceded by signal peptides (SP) to endoplasmic reticulum, linked with an IRES, and followed by a poly-adenine tail (polyA). The Ad5/3-tras and Ad5/3-Δ24-tras viruses were also infectivity-enhanced by replacing the serotype 5 fiber knob with serotype 3 knob. CMV, cytomegalovirus promoter; tras, trastuzumab; $V_{L/H}$, variable domain light/heavy chain; $C_{L/H}$, constant domain light/heavy chain. **B–D,** cells were infected with indicated adenoviruses at 100 or 1,000 (*) VP/cell, and supernatants were analyzed for trastuzumab by Western blot (**B**), dot blot (**C**), and ELISA (**D,** both supernatants and cell lysates). **B,** each trastuzumab-coding adenovirus showed production of trastuzumab from 293 cells that allow replication of also E1-deleted viruses. In cancer cells (BT-474 and HEY), however, only Ad5/3-Δ24-tras produced the antibody at 100 VP/cell, whereas 10-fold higher titer was required for nonreplicating viruses (*). Reducing conditions resulted in dissociation of heavy-chain (HC) and light-chain (LC) proteins, whereas nonreducing conditions (BT-474 below) showed only the full-length (FL) antibody. **C,** kinetics of antibody production from cancer cells was tested by dot blot: Each trastuzumab-coding adenovirus mediated trastuzumab production by 48 hours, which remained stable thereafter. **D,** antibody binding, release, and concentration were assessed by ELISA in SKOV-3 cells infected with 100 VP/cell. At each time point, oncolytic Ad5/3-Δ24-tras showed high levels of functional trastuzumab in both cell lysates (LYS; gray bars) and importantly in supernatant (SN; black bars). In contrast, nonreplicating Ad5/3-tras failed to release antibody in the supernatant, despite the presence of antibody in lysate at day 7 (viable cells in microscopy; white bar). Cropped blots (**B,** above) show important bands. Bars in **D** represent the mean ±SEM.

Amicon Ultra-4 100K (each Millipore) and UV-inactivated (Supplementary Figs. S1 and S3). ADCC assay: 5×10^3 target cells/well were incubated in trastuzumab (containing supernatant) or irrelevant IgG with/without human blood mononuclear cells as in Fig. 3 and Supplementary Fig. S3. Lactate-dehydrogenase release was assayed 6 hours later according to the manufacturer's instructions (CytoTox 96-assay; Promega), and background and immune cell–only signals were subtracted.

Animal experiments

Animal experiments were approved by the University of Helsinki and Provincial Government of Southern Finland Animal

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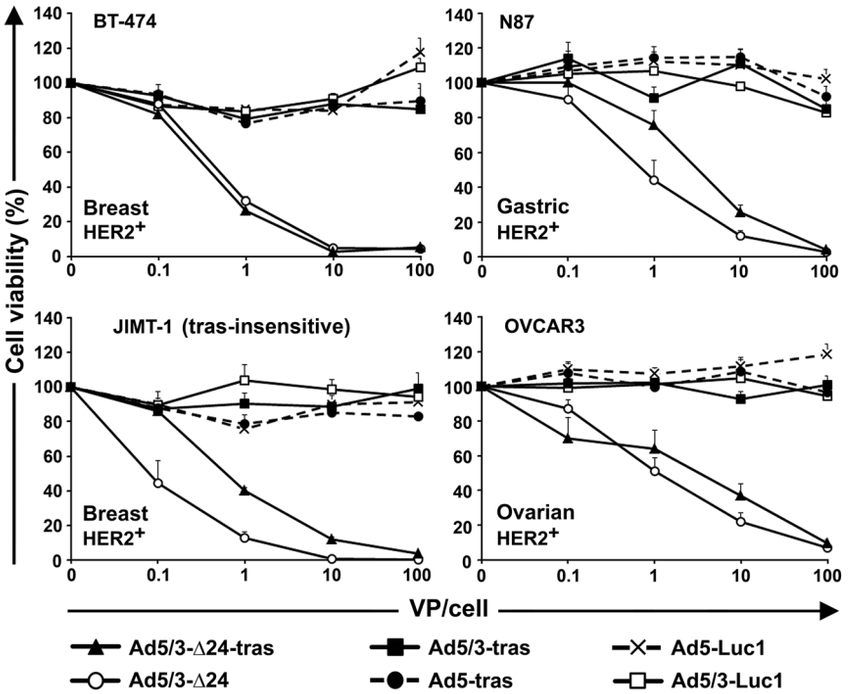

Figure 2.
Trastuzumab-coding oncolytic adenovirus mediates *in vitro* cytotoxicity. HER2-positive breast cancer BT-474 and JIMT-1, gastric cancer N87, and ovarian cancer OVCAR3 cells were infected with indicated viruses at increasing titers of virus particles per cell (VP/cell). Ad5/3-Δ24-tras mediated efficient cell killing in all cancer cell lines, comparable with oncolytic control virus Ad5/3-Δ24 at 100 VP/cell dose. Trastuzumab-coding Ad5/3-Δ24-tras showed also total eradication, although slower cytotoxicity, in breast cancer–initiating JIMT-1 cells, which are resistant to trastuzumab therapy (Supplementary Fig. S1) and other conventional therapies (32). When trastuzumab-sensitive breast cancer BT-474 cells were tested, Ad5/3-Δ24-tras showed equal cell killing potency to the oncolytic control virus. None of the replication-deficient control viruses showed cytotoxicity, as expected due to their low transgene production and release (see Fig. 1).

Committees. Three- to 4-week-old nude/NMRI mice (Taconic/Harlan) were used, with health monitored daily and euthanized according to guidelines: Subcutaneous xenograft-bearing mice received intratumoral injections of indicated virus (Figs. 4 and 5; Supplementary Figs. S4–S6) or saline, with/without commercial trastuzumab intraperitoneally.

Flow cytometry analysis

Single-cell suspensions were stained with FITC-conjugated anti–HER-2/neu antibody or isotype control (mouse IgG1κ; both BD biosciences) to determine HER2 expression, or anti-mouse antibodies (Fig. 5 and Supplementary Fig. S6): hamster anti-CD11c IgG (cat.# 25-0114-81), hamster anti-CD80 IgG (cat.# 110801-81) and hamster anti-CD40 IgG2a,κ (cat.# 170401-81), and rat anti-CD49b IgM (cat.# 175971-81; each eBioscience), and analyzed by FACSAria (BD Biosciences) and FlowJo v10.0.7 (TreeStar). HER2 overexpression was determined as greater than 75% HER2 expression. To assess the binding of *in vivo*–produced trastuzumab (Supplementary Fig. S5), 1×10^6 N87 cells were incubated with 100 μL serum from Ad5/3-Δ24-tras–treated mice (1:1 mixture from 2 individual mice) or Herceptin (positive control, c = 21 mg/mL) for 1 hour, washed, and then incubated in FITC-conjugated anti-human IgG secondary antibody (Rockland Immunochemicals; 1:500) for 1 hour, washed twice, and analyzed by flow cytometry. Of note, low circulating trastuzumab levels (Fig. 4B) likely achieved only partial saturation of HER2 binding sites (vs. positive control), as previously determined for N87 cells (27).

Statistical analysis

Cytotoxicity, ADCC, and immune cell assays were analyzed by the Student *t* test (Microsoft Excel), and tumor growth by the Mann–Whitney test (GraphPad Prism v.5.0). IC_{50} values were calculated by fitting dose–response curves in GraphPad Prism. $P < 0.05$ was considered statistically significant.

Results

Oncolytic adenovirus mediates effective monoclonal antibody production, assembly, and release from cancer cells

We constructed an oncolytic adenovirus Ad5/3-Δ24-tras, and two nonreplicating control viruses Ad5/3-tras and Ad5-tras, each coding for trastuzumab heavy- and light-chain genes linked together by an internal ribosome entry site (IRES) sequence (28), which allows for translation of both mRNAs without the need for additional promoters. The trastuzumab transgene was inserted into E3-region of the adenovirus genome, replacing gp19k/6.7k reading frames, thus utilizing the endogenous E3-promoter (25). In the nonreplicating viruses, we substituted the E1A-region with trastuzumab, thus enabling early transgene expression without virus replication (Fig. 1A). Replication-competent Ad5/3-Δ24, lacking the trastuzumab transgene, was used as an oncolytic control virus.

Trastuzumab production was tested in several cancer cell lines by Western and dot blot analysis of infected supernatants. All trastuzumab-encoding adenoviruses were able to express and assemble the full-length antibody in cancer cells (Fig. 1B). Importantly, no other bands were detected under normal conditions, indicating that antibodies were dimerized correctly and the ratio of heavy- and light-chain production was balanced. Under reducing conditions, disulfide bonds were properly broken and the antibody dissociated into its two heavy and two light chains, as expected (Fig. 1B and C).

Oncolytic Ad5/3-Δ24-tras showed high trastuzumab production by 48 hours after infection in all tested cell lines, including breast cancer BT-474 and MDA-MB-435, and ovarian cancer SKOV-3 and HEY, at dose of 100 virus particles (VP)/cell

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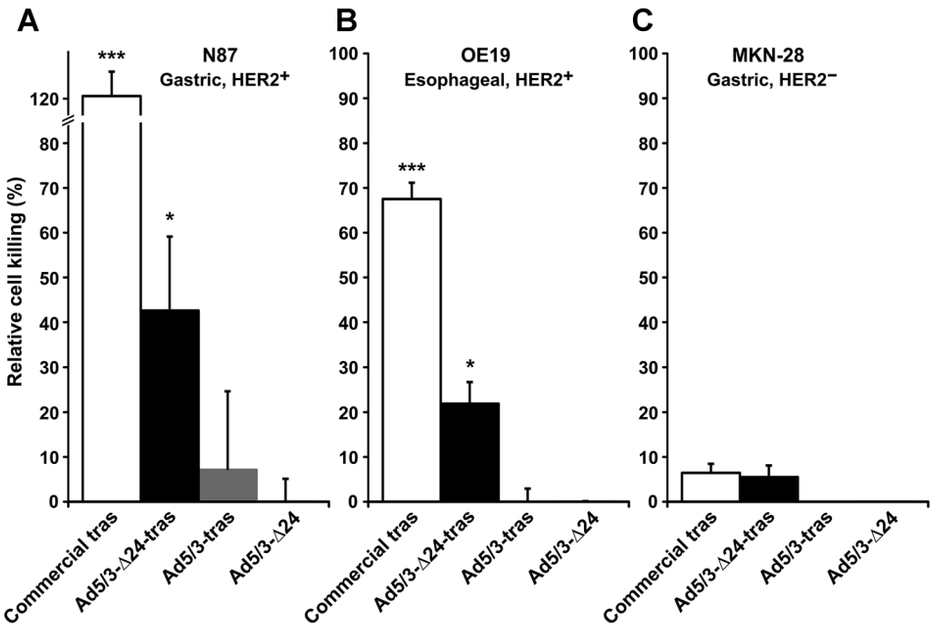

Figure 3.

Trastuzumab produced by oncolytic adenovirus induces ADCC. Purified and concentrated supernatants from mock or virus-infected 293 cells (filtered virus-free, but antibody-containing) were added on N87 gastric (**A**), OE19 esophageal (**B**), or MKN-28 gastric (**C**) cancer cells. Then, human peripheral blood mononuclear cells were added (60:1 effector-to-target ratio), and cell viability was measured by lactate dehydrogenase release assay. Ad5/3-Δ24-tras supernatant that contained virus-produced trastuzumab (black bars) resulted in significant immune cell–mediated target cell killing of HER2-positive N87 (**A**) and OE-19 (**B**), but not of HER2-negative MKN-28 cancer cells, as compared with immune cells only that are subtracted here ($P < 0.05$ in **A** and **B**). Commercial trastuzumab was used as a positive control (white bars), which seemed more effective in mediating ADCC than supernatant from virus-infected cells likely due to more meticulous pharmacologic purification process. Data represent two independent experiments. Bars, mean + SEM. *, $P < 0.05$; **, $P < 0.01$; all Student *t* tests.

(Fig. 1B and C). Meanwhile, nonreplicating viruses required up to 1,000 VP/cell, although strong bands were detected at 100 VP/cell in 293 cells that allow their replication by trans-complementing the deleted viral E1A-region (29). These data indicated that virus replication is crucial for effective *in situ* antibody production for two reasons: (i) it allows much higher levels of transgene expression, because the genome harboring the transgene amplifies, and (ii) because tumor cells lack the antibody secretion machinery of plasma cells (24), lysis caused by the virus releases the antibody from the infected cells.

We next assessed antibody concentration, release from cancer cells, and binding to human HER2 antigen and secondary anti-IgG by ELISA. Supernatants and lysates of SKOV-3 cells were collected at different time points after infection with 100 VP/cell of viruses. Importantly, virus-produced trastuzumab present in either compartment could bind to HER2 and secondary anti-human IgG antibody, confirming biological functionality. After day 3 after infection, oncolytic Ad5/3-Δ24-tras presented gradual accumulation of trastuzumab in the supernatant over cell lysate ($P < 0.01$; Fig. 1D), at concentrations up to 476.1 ng/mL, which coincided with progressive cytopathic effect observed in microscopy. In striking contrast, nonreplicating Ad5/3-tras showed much lower degree of antibody production and failed to release any antibody in the supernatant. Thus, both the antibody production and its release were dependent on oncolysis.

Breast, ovarian, and gastric cancer cell lines were screened for HER2 expression by FACS, and assessed for sensitivity to commercial trastuzumab in a dose-escalating manner (Supplementary Fig. S1). Three of seven HER2-positive cell lines, breast BT-474, gastric N87, and esophageal OE-19, were found susceptible to trastuzumab treatment, with IC_{50} values at 80.3, 196.3, and 239.1 ng/mL, respectively. Lack of trastuzumab sensitivity, as seen with the other four HER2-positive lines, has been reported to result from mutations/alterations in the HER2-signaling pathway (30–32). Because oncolytic virus-produced trastuzumab levels remained constantly above the IC_{50} concentrations ($P < 0.05$ vs. all IC_{50} values; Fig. 1D), we conclude that oncolytic adenovirus is a feasible platform for antitumor antibody production, and clearly superior to nonreplicating virus.

Potent oncolytic capacity is retained by the trastuzumab-armed replicating adenovirus

We evaluated cytotoxicity mediated by engineered viruses in standard MTS cell killing assays. At 100 VP/cell, oncolytic Ad5/3-Δ24-tras showed efficient cell killing, comparable with the potency of oncolytic control virus Ad5/3-Δ24 in cell lines sensitive to trastuzumab (BT-474 and N87; Fig. 2). In JIMT-1, which is not sensitive to trastuzumab (32), the unarmed virus was more potent. As expected, nonreplicating viruses Ad5/3-tras and Ad5-tras failed to cause significant oncolysis, and neither did we find evidence of antibody-mediated cytotoxicity (Figs. 2 and 3) because these viruses lacked sufficient trastuzumab production at 100 VP/cell, as indicated in Fig. 1.

At lower titers, Ad5/3-Δ24-tras exhibited lower cytotoxicity than the oncolytic control virus, most likely reflecting its slower replication rate due to insertion of the large antibody sequence

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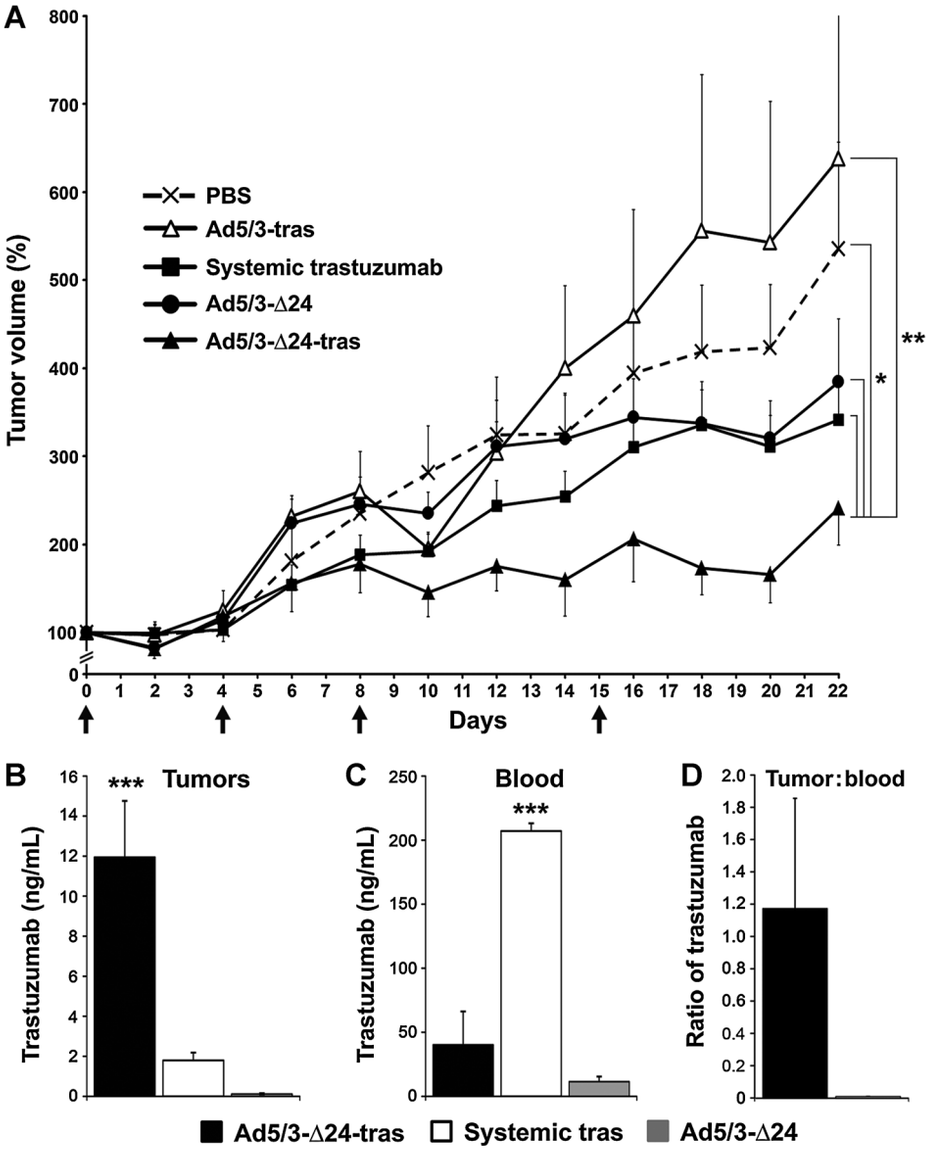

Figure 4.
Oncolytic adenovirus coding for trastuzumab inhibits HER2-positive tumor growth and improves tumor-to-systemic antibody distribution. Subcutaneous N87 gastric cancer xenograft-bearing nude/NMRI mice (Taconic) received intratumoral injections of indicated virus (2×10^8 VP/tumor), or saline (PBS) with/without systemic commercial trastuzumab intraperitoneally (0.3 μg/g) on indicated days (arrows). **A,** Ad5/3-Δ24-tras treatment resulted in significant tumor growth inhibition as compared with all other groups ($P < 0.05$). In contrast, none of the other treatments resulted in significant antitumor effect as compared with mock-treated animals. **B,** endpoint tumors and blood samples were measured by human IgG ELISA to assess trastuzumab concentration: Ad5/3-Δ24-tras–treated mice showed significantly higher trastuzumab concentrations (**B**) in tumors ($P < 0.001$), while presenting (**C**) much lower circulating levels ($P < 0.001$), than systemic trastuzumab-treated mice. **D,** tumor-to-systemic ratio of trastuzumab in each evaluable animal revealed strikingly higher average ratio (>1.0) after Ad5/3-Δ24-tras treatment than after systemic antibody therapy (<0.01). Bars, mean + SEM, $n = 5$ per group. *, $P < 0.05$l; **, $P < 0.01$; ***, $P < 0.001$; Mann–Whitney test in **A,** Student *t* test in **B** and **C**.

(higher genomic load). In most of the HER2-positive cell lines, however, trastuzumab-coding Ad5/3-Δ24-tras and its nontransgenic counterpart were equally effective, suggesting that antibody activity compensates for replication rate and may potentiate its cytotoxic capacity, even in the absence of an immune system (Supplementary Fig. S1). Notably, we achieved 100% cytotoxicity with the trastuzumab-coding oncolytic virus also in cancer cell lines that are highly resistant to trastuzumab, for example, JIMT-1 breast cancer initiating/stem cells (32) and triple-negative MDA-MB-436 (33), indicating that adenoviral oncolysis can eradicate trastuzumab-resistant clones, which may have clinical significance. Finally, to normalize for the slower replication rate caused by the larger genome of the armed virus, we also compared cytotoxicity based on functional virus titers (Supplementary Fig. S2). In this setting, Ad5/3-Δ24-tras showed enhanced cytotoxicity over oncolytic control virus Ad5/3-Δ24, especially in HER2-positive trastuzumab-sensitive cancers.

Virus-produced trastuzumab exhibits direct antitumor activity and triggers ADCC by immune cells

Blocking the HER2-receptor signaling of HER2-overexpressing cancer leads to induction of apoptosis (6). We demonstrated direct anticancer activity by purified virus-produced trastuzumab against HER2-positive N87 gastric cancer (Supplementary Fig. S1C), which emerged equally effective as commercial trastuzumab. We then focused on ADCC, which is triggered by immunoglobulin class G1 antibodies, such as trastuzumab. The variable region binds to the cell surface molecule, herein HER2, whereas NK cells are recruited *via* antibody Fc-tail. We studied the ability of virus-produced trastuzumab to induce ADCC of cancer cells by human peripheral blood mononuclear cells *ex vivo*.

Logically, trastuzumab derived from oncolytic adenovirus was found to induce immune cell–mediated ADCC in HER2-positive cancer ($P < 0.05$ in N87 and OE-19 cells, Fig. 3A and B), but not in HER2-negative cancer (Fig. 3C) or without immune cells in this

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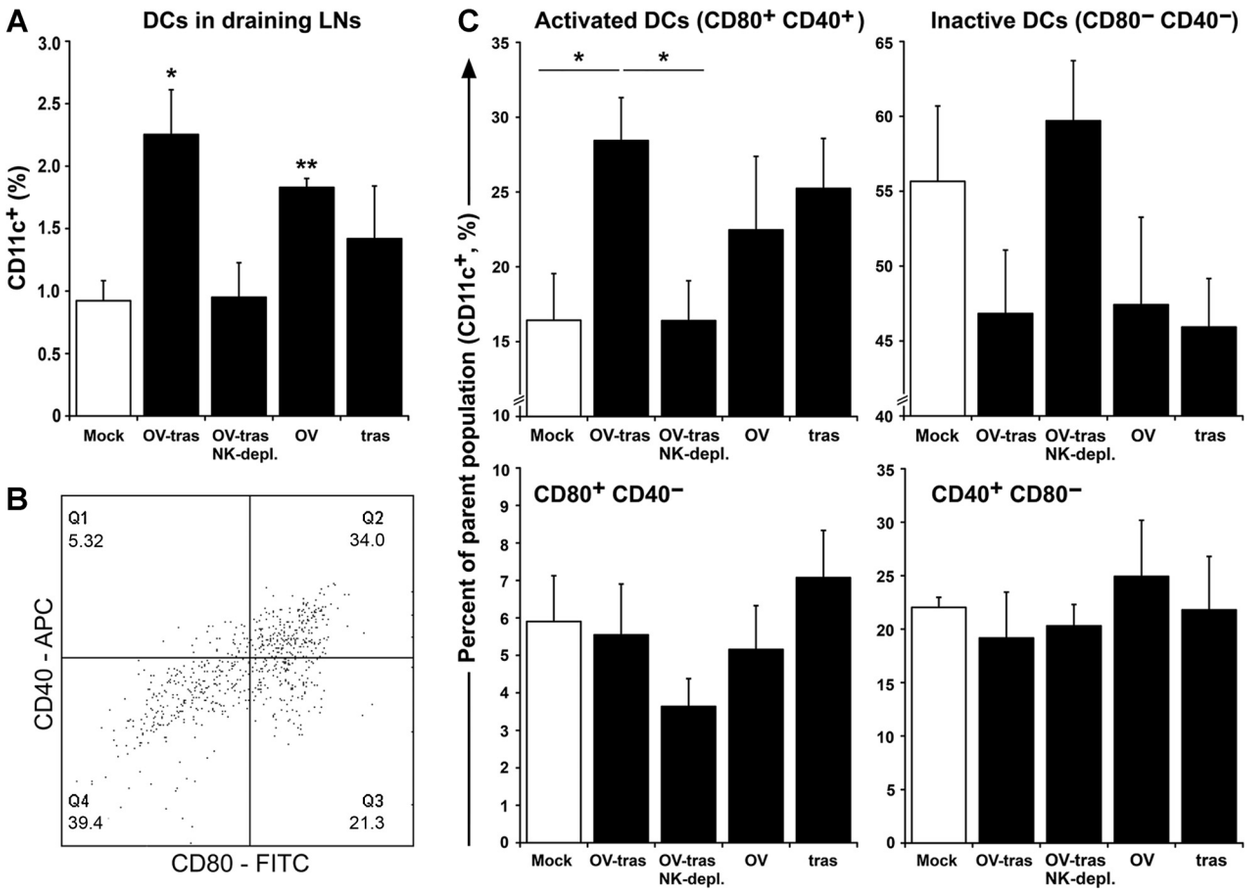

Figure 5.

Treatment with trastuzumab-expressing oncolytic adenovirus results in activation of DCs in tumor-draining lymph nodes. Tumor-draining lymph nodes (LN) of animals treated as in Fig. 4, but with adjusted doses, were collected on day 20 and analyzed by flow cytometry. **A,** total amount of CD11c-positive DCs were significantly more frequent in mice treated with oncolytic Ad5/3-Δ24-tras (OV-tras; $P = 0.03$), as well as oncolytic control virus (OV; $P = 0.01$), as compared with mock. **B** and **C,** subpopulation analysis of $CD11c^+$ DCs was performed using CD40 and CD80 maturation markers, with **B** showing a representative gating strategy. **C,** in the subset analysis, OV-tras group showed the only significant accumulation of activated $CD80^+CD40^+$ DCs ($P = 0.047$, vs. mock). NK cell depletion by anti-asialo antibody prior to OV-tras therapy (OV-tras NK-depl.) totally reversed the DC-activating effect ($P = 0.037$, vs. OV-tras). Analogously, NK cell–depleted animals trended for higher levels of immature $CD80^-CD40^-$ DCs that include highly immunosuppressive subtypes ($P = 0.092$, vs. OV-tras). Bars, mean + SEM, $n = 3$ mice/group. *, $P < 0.05$; **, $P < 0.01$; all Student t tests.

6-hour assay (Supplementary Fig. S3). Also commercial trastuzumab (used as a positive control) exhibited efficient ADCC in HER2-positive cancer, whereas the nonreplicating adenovirus-derived trastuzumab could not be produced and separated enough to achieve the effect, again underlining the utility of the oncolytic platform. Hence, trastuzumab produced by Ad5/3-Δ24-tras could bind to HER2 on human cancer cells, and was recognized by human immune cells such as NK cells, leading to efficient ADCC, confirming full functionality. Moreover, this experiment provided a powerful example of immunological therapeutic efficacy of the approach in a fully human system.

Trastuzumab-coding oncolytic adenovirus shows enhanced inhibition of HER2-positive tumor growth *in vivo*

We focused on HER2-positive gastric cancer *in vivo* because trastuzumab has been approved for this indication, while there is room for improvement given the high rate of HER2 overexpression but relatively low response rate to systemic trastuzumab therapy (5, 34). Established N87 gastric cancers were treated with four intratumoral injections of Ad5/3-Δ24-tras, Ad5/3-Δ24, Ad5/3-tras, or saline (mock-treated), whereas one saline-treated group received also commercial trastuzumab (0.3 μg/g) intraperitoneally to simulate systemic administration of the antibody. Systemic trastuzumab therapy failed to exhibit tumor-growth inhibition in this aggressive model, and the same applied for the oncolytic virus Ad5/3-Δ24 (Fig. 4A). In striking contrast, trastuzumab-coding Ad5/3-Δ24-tras virus resulted in significant tumor growth inhibition in comparison to all other groups ($P < 0.05$; Fig. 4A). It is noteworthy that enhanced antitumor efficacy was seen despite the fact that Ad5/3-Δ24-tras had a slower replication rate than the oncolytic control virus *in vitro* (likely due to high genome size), suggesting utility of antibody production *in vivo*.

In order to solidify the importance of HER2-specific effects of trastuzumab in our strategy, we conducted a control *in vivo* experiment using HER2-negative breast cancer MDA-MB-436: After similar treatment schema to the previous experiment, both

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oncolytic adenoviruses inhibited tumor growth over mock treatment ($P < 0.001$ for Ad5/3-Δ24, $P < 0.05$ for Ad5/3-Δ24-tras), but trastuzumab transgene did not add to efficacy against HER2-negative cancer (Supplementary Fig. S4). We further confirmed the capacity of *in vivo* virus-produced trastuzumab to bind to extracellular HER2 on cancer cells by incubating HER2-positive N87 cells first in serum derived from Ad5/3-Δ24-tras–treated mice and then in secondary anti-human IgG antibody, which resulted in high fluorescence signal of these cells over background in flow cytometry (Supplementary Fig. S5). These assays further highlighted the antitumor/immunostimulatory role of anti-HER2 trastuzumab in our approach.

Trastuzumab-coding oncolytic adenovirus results in high antibody concentrations at the tumor, while systemic levels remain low

We postulated that local production from a vector platform would result in increased local trastuzumab concentrations, contributing to the enhanced antitumor effect. Indeed, indicative of continuous oncolysis and transgene expression, Ad5/3-D24-tras–treated mice showed significantly higher trastuzumab levels in endpoint tumors (ranging from 4.0 to 25.4 ng/mL), as compared with other groups ($P < 0.001$; Fig. 4B), whereas circulating concentrations remained strikingly lower than after systemic trastuzumab treatment ($P < 0.001$; Fig. 4C).

Notably, Ad5/3-Δ24-tras–treated mice had better tumor control and could thus be followed up longer (median of 48 days) than systemic trastuzumab-treated mice (median of 40 days), but nevertheless the trastuzumab levels were higher, indicating remarkably stable and effective local antibody expression. To further study the biodistribution of trastuzumab, we analyzed individual tumor-to-systemic antibody ratios in endpoint mice (Fig. 4D): Treatment with systemic antibody led to a dismal average ratio of 0.009 [±0.003 (SEM)], whereas Ad5/3-Δ24-tras treatment showed a more promising ratio of 1.17 [±0.68 (SEM)], in favor of tumor accumulation.

Thus, improved intratumoral and lower circulating antibody levels after Ad5/3-Δ24-tras treatment coincided with, and likely contributed to, the enhanced antitumor efficacy in HER2-positive cancer.

Treatment with trastuzumab-coding oncolytic adenovirus results in DC activation and favorable NK cell distribution *in vivo*

Activation of antigen-presenting and NK cells is considered critical for the immunotherapeutic activity of both oncolytic virus and antitumor antibody treatment in cancer patients (7, 14, 35). We found *in vitro* that HER2-bound trastuzumab on N87 cancer cell surface induces rapid ADCC by blood mononuclear cells (Fig. 3), which is mediated mainly by NK cells (7).

To address immunological effects *in vivo*, we analyzed innate immune cell populations in tumors, draining lymph nodes, and spleens. In order to study the impact of NK cells in immune activation, we included a group of mice receiving Ad5/3-Δ24-tras virus, which were pretreated with anti-asialo antibody that selectively depletes the NK cells.

Mice sacrificed on day 20 after treatment showed an increase in total amount of CD11c-positive DCs in tumor-draining lymph nodes when treated with oncolytic Ad5/3-Δ24 or trastuzumab-coding oncolytic Ad5/3-Δ24-tras virus ($P = 0.01$ and $P = 0.03$ vs. mock, respectively, Fig. 5A). Subpopulation analysis using DC-maturation markers CD80 and CD40 revealed that Ad5/3-Δ24-tras induced significant DC activation (Fig. 5C): Triple-positive $CD11c^+CD80^+CD40^+$ cells capable of antigen presentation were the most abundant in the Ad5/3-Δ24-tras group, representing over 25% of the total population ($P = 0.047$, as compared with mock). Interestingly, NK cell depletion by anti-asialo antibody completely blunted this effect ($P = 0.037$, as compared with Ad5/3-Δ24-tras), indicating a central role for NK cells in editing/stimulating DCs, as has been previously suggested (36). A similar trend was observed with regard to CD80-single–positive DCs.

Immature DCs lacking activation markers can abrogate antigen presentation in lymph nodes and antitumor T-cell responses in tumors (37, 38). Potentially counteracting this effect, Ad5/3-Δ24-tras showed the highest ratio of activated versus immature DCs (0.63 vs. 0.31 in mock), and lower absolute amounts of immature $CD11c^+CD80^-CD40^-$ DCs seemed to again depend on the presence of NK cells that mediate their editing ($P = 0.092$, both comparisons). Highlighting the biological significance of cross-presentation of tumor-derived antigens in draining lymph nodes, DC frequencies did not differ between the treatment groups in tumors or spleens of the animals.

Because NK cells are the main immune cell type mediating ADCC functions, besides promoting antigen presentation (7, 19), we next analyzed matured $CD49b^+$ NK cell frequencies in tumors, draining lymph nodes, and spleens of the animals. Absolute NK cell numbers did not differ between the groups in any compartment. However, Ad5/3-Δ24-tras treatment led to the highest ratio of NK cells in tumors and draining lymph nodes over spleens of the animals (Supplementary Fig. S6), thus suggesting redistribution of matured NK cells toward the site featuring virus replication and antibody production. Of note, we used NK cell marker CD49b that is highly expressed only at the expansion and matured stages of NK cell development in mice (39), indicating effector capacity of the observed NK cells.

Taken together, Ad5/3-Δ24-tras treatment was associated with significant accumulation of activated DCs that are capable of cross-presentation in draining lymph nodes, together with relative reduction of immature DC subsets. This beneficial phenomenon was dependent on NK cells (Fig. 5C), which seemed to redistribute toward the tumor and draining lymph nodes after Ad5/3-Δ24-tras treatment (Supplementary Fig. S6).

To summarize our results, we generated a tumor-targeted oncolytic adenovirus Ad5/3-Δ24-tras that mediates efficient production of monoclonal antibody trastuzumab directly from cancer cells. Trastuzumab antibody chains were produced in appropriate quantities from a single expression cassette, folding into a full-length functional antibody that was able to bind to HER2-positive cancer cells, to induce direct cytotoxicity, and to trigger ADCC by human immune cells. Treatment with Ad5/3-Δ24-tras resulted in a therapeutic level of antibody production, together with direct oncolysis that was comparable with replicative control virus in several cancer cell lines. Moreover, Ad5/3-Δ24-tras exhibited improved antitumor efficacy in a HER2-expressing cancer model, and presented higher local and lower systemic trastuzumab concentrations than systemic antibody therapy. Finally, treatment with Ad5/3-Δ24-tras induced significant accumulation of activated DCs in tumor-draining lymph nodes, which seemed to associate with, and depend on, the presence of NK cells.

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Discussion

Monoclonal anti-HER2 antibody therapy with trastuzumab has significantly improved the survival of HER2-expressing breast and, more recently, of gastric cancer patients (2, 5). Nevertheless, more than 80% of HER2-positive gastric tumors are resistant to systemic trastuzumab, while continuing to depend on the HER2 oncogene, suggesting that local antibody concentrations achieved with systemic therapy are insufficient for therapeutic efficacy (34, 40). Indeed, low tumor penetration and rapid clearance of monoclonal antibodies when given intravenously are major obstacles (9, 10), which cannot be overcome by increasing doses due to the risks associated with on-target, off-tumor toxicity as exemplified by the fatal cardiomyopathy seen in trastuzumab-treated patients (11, 12).

Gene therapy has the potential to circumvent issues of systemic antibody therapy, owing to the capacity to produce antibody *in situ* for prolonged periods. A key safety vis-à-vis efficacy aspect could be improved tumor-to-systemic distribution of the antibody, which could improve antitumor effects while reducing unwanted sequelae. Additional advantages include guaranteed absence of impurities and cost-effectiveness when the antibody is produced by tumor cells. Previous approaches have, however, focused on targeting normal cells (e.g., hepatocytes and myocytes) for production of anticancer antibodies encoded by nonreplicating viral vectors. In such approaches, nontarget organs are used for production of an antibody into the systemic circulation, from which it must diffuse into tumors, just like with systemic delivery. In addition, these attempts have been hampered by low transduction, short-term expression, and/or immunological eradication of input virus, thus resulting in low antibody levels, including those found in the tumor (20–23). Moreover, high-dose viral transduction of the liver is not without its risks (41).

We demonstrate that production of anticancer antibody from cancer cells is feasible by an oncolytic adenovirus, leading to its local release at therapeutic levels and remarkable improvement in the tumor-to-systemic antibody ratio. As shown by ELISA and immunoblotting assays *in vitro*, antibody was released over the course of progressive oncolysis (at least 7 days), and found in high quantities in endpoint tumors (median day 48), indicating stable expression *in vivo*, although pharmacokinetics of trastuzumab after Ad5/3-Δ24-tras treatment warrants further studies. By studying also nonreplicating counterparts of the trastuzumab-coding virus, we confirm that tumor cells do not actively secrete antibody as plasma cells do (24), but instead require full oncolysis for the antibody to be released at high concentrations. Because Ad5/3-Δ24-tras only replicates in cancer cells (42) and because the replication-linked transgene expression (E3-inserted) cassette amplifies during virus replication, our strategy uniquely combines oncolysis and antibody production at the tumor site which could lead to gains in both efficacy and safety. Reduced systemic exposure is expected to decrease cardiotoxicity, while also hypersensitivity reactions remain important to address, given that approximately 0.3% of patients receiving intravenous trastuzumab experience serious reactions that are linked to IgE-mediated responses to mouse components of the humanized antibody (12).

Although locally produced trastuzumab can be effective against susceptible HER2-positive cancer cells, the possible outgrowth of trastuzumab-resistant clones can be killed by the oncolytic action of the virus (42, 43). In this regard, the ability of 5/3 chimeric oncolytic adenoviruses to efficiently infect and lyse also cancer initiating/stem cells could be relevant (26, 42), as suggested by our results on JIMT-1 breast cancer cells (ref. 32; Fig. 2). Additional *in vivo* studies on other aggressive HER2-overexpressing cancer types are therefore warranted. Finally, a third mode of cell killing would be activation of immune cells by virus-produced trastuzumab recognizing HER2. Given the costimulatory potential of oncolytic adenovirus (14, 16, 44), this effect could be more prominent when antibody and virus are physically proximal.

Oncolytic adenoviruses are a potent form of active immunotherapy, able to stimulate both the innate and adaptive arms of the immune system (14, 16). Effective oncolysis, release of danger signals, and shedding of tumor-associated antigens can elicit DC activation, increased antigen uptake and presentation, and induction of antitumor T-cell responses (14, 18). Interaction of target-bound trastuzumab with the host immune system, in turn, occurs *via* its immunoglobulin G1 Fc domain. Such immunotherapeutic effects have been mainly attributed to NK cells that are responsible for ADCC (7). We demonstrate in a fully human system that trastuzumab derived from Ad5/3-Δ24-tras–infected cancer cells induces effective ADCC (Fig. 3). In this *ex vivo* experiment, commercial antibody seemed to mediate even higher ADCC, which is likely due to the pharmacologically optimized purification process, whereas only a Montage kit was used for the supernatant containing virus-produced trastuzumab. Alternatively, supernatant from the infected 293 human embryonic kidney cells may have contained inhibitory molecules limiting activity of human immune cells to mediate ADCC. Nonetheless, *in vivo* effects are not expected to depend on initial product integrity but rather on tumor delivery and clearance, as indicated by superior antibody presence, efficacy, and immunological effects after Ad5/3-Δ24-tras treatment of HER2-positive tumors (Figs. 4 and 5).

IgG class antibodies bound to target molecules (immune complexes) can also activate Fcγ receptors present on DCs and macrophages, thereby facilitating antigen processing and cross-presentation (8, 45). Trastuzumab has also been shown to promote HER2-specific $CD8^+$ T-cell responses (46), which may contribute to its efficacy especially against tumor variants that are mutated downstream of the HER2 oncogene and thus not susceptible to signaling inhibition by the antibody (40). Chen and colleagues recently reported promising results of a phase II trial addressing a whole-cell cancer vaccine with HER2-antigen-trastuzumab immune complexes and cyclophosphamide for treatment of metastatic HER2-positive breast cancer (47). The immunotherapy approach induced HER2-specific T-cell responses in patients and was previously shown *in vivo* to enhance DC maturation and antigen cross-presentation in draining lymph nodes, in a Fc-domain–dependent manner (35), thus corroborating our immunological findings (Fig. 5). Because antitumor immune activation has been regarded relevant for both modalities (trastuzumab and oncolytic adenoviruses), there is strong rationale for improving immunotherapeutic efficacy by a "combination" approach which in this case is a single virus.

Costimulatory signals on DCs are crucial for eliciting effective $CD4^+$ and $CD8^+$ T-cell priming, proliferation, and differentiation (44). Ad5/3-Δ24-tras treatment induced a population of activated DCs ($CD11c^+CD80^+CD40^+$) in tumor-draining lymph nodes (Fig. 5), which may be due to antigen–antibody complexes directly stimulating DC activation and cross-presentation (8, 35, 48). Meanwhile, effective ADCC as seen *in vitro* (Fig. 3) may have contributed to NK cell redistribution observed *in vivo* (Supplementary Fig. S6). In fact, all of these observations are likely to

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be connected since emerging evidence indicates intimate crosstalk between DCs and NK cells (36).

Matured NK cells can kill immature DCs, while sparing activated DCs, thus favoring an immunostimulatory DC subset during induction of antitumor immune responses (19). Such "DC-editing" by NK cells occurs both in peripheral tissues and in secondary lymphoid organs (36). This is well in accord with our observations of Ad5/3-Δ24-tras–treated mice showing redistribution of NK cells toward tumors and draining lymph nodes, coinciding with accumulation of activated DCs and reduction in immature DCs. In striking contrast, NK cell–depleted animals (pretreated with anti-asialo antibody) exhibited exactly the opposite phenomenon (Fig. 5). Of note, immature/tolerogenic DCs are known to mediate immunosuppressive functions by expressing, e.g., inhibitory programmed death (PD)-ligands 1 and 2 that negatively regulate T-cell priming (38, 49).

Matured NK cells also secrete TNFα and IFNγ that induce DC maturation and migration, and polarize primed $CD4^+$ T cells into T-helper 1 (Th1) phenotype, thus promoting cytotoxic T-cell responses (36). Importantly, also oncolytic adenoviruses skew the adaptive immunity toward Th1 phenotype, both preclinically and in cancer patients (17, 50), which suggests further combinatorial benefits with trastuzumab. Finally, both activated NK cells and oncolytic viruses can augment antigen processing of DCs by lysing tumor cells and spreading tumor epitopes (14, 36). Our approach will be interesting to assess in clinical trials, without limitations of thymic deficiency of nude mice, allowing both antitumor adaptive immune responses and adenovirus replication. Although systemic antibody therapy often appears insufficient in mounting an effective antitumor immune response, perhaps chiefly due to physical (poor tumor penetration) and immunosuppressive (tumor counteracts immune detection) issues, all of these known effects are expected to synergize with oncolytic immunotherapy in an immunocompetent host (14, 16, 47).

To conclude, Ad5/3-Δ24-tras appears a promising anticancer agent that combines oncolytic potency with high local trastuzumab production, resulting in improved *in vivo* efficacy and activation of the immune system against HER2-positive cancer. Our results demonstrate the feasibility of oncolytic adenovirus–mediated local antibody production at the tumor, which may have several future applications for cancer therapy, given the emergence of unprecedentedly effective, but expensive and rather toxic, immune checkpoint–blocking antibodies. Local production could improve efficacy/toxicity ratios in a cost-effective manner. Moreover, curing patients is priceless.

Disclosure of Potential Conflicts of Interest

A. Hemminki has ownership interest (including patents) in TILT Biotherapeutics Ltd and Targovax ASA. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: I. Liikanen, S. Tähtinen, T. Gutmann, P. Savola, A. Kanerva, A. Hemminki
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Molecular Cancer Therapeutics

Oncolytic Adenovirus Expressing Monoclonal Antibody Trastuzumab for Treatment of HER2-Positive Cancer

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