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Research Article

Replication-Competent, Tumor-Specific Immuno-Gene Vectors Allow for Exchange of Transgenes and Lead to Viral Persistence Following IV Administration

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ABSTRACT

Introduction: Enadenotucirev (EnAd) and successor transgene-armed Tumor-Specific Immuno-Gene (T-SIGn) vectors are replication-competent, blood-stable viral vectors that traffic to tumor sites following intravenous (IV) administration. Following initial proof of mechanism for this immunotherapy modality, there is a need to identify a safe dosing approach, understand whether transgenes affect tolerability, and determine how to measure exposure and pharmacodynamic effects. **Methods:** Safety data from multiple phase 1 trials were aggregated to assess various dosing regimens and toxicities, including those that may be associated with transgene arming. Viral delivery to tumors, peripheral viral persistence, transgene expression, and cytokine responses were also assessed and compared between the unarmed EnAd and armed T-SIGn vectors. **Results:** IV administration of EnAd and the armed T-SIGn vectors led to virus detection in epithelial tumors and prolonged peripheral virus persistence. Despite the short half-life of the viruses and cell-free nucleic acids, viral DNA or transgene messenger RNA (mRNA) remained detectable in blood at higher dose levels for at least 56 days, indicating sustained release of these molecules likely driven by ongoing viral replication and concomitant transcription of the transgene in tumors. Safety,

predominantly defined by the effects of IV administration and associated viremia, was managed by a well-tolerated dosing regimen. To date, no transgene-related or off-target toxicities have been observed. Transient monocyte chemoattractant protein 1 (MCP-1) and interleukin (IL)-6 elevations were associated with viremia. A second phase of elevations of proinflammatory cytokines such as IL-12p70, interferon (IFN) α2, IL-17A, and IFNγ without clinical symptoms started around day 12, suggesting that release of these cytokines was driven by localized pharmacodynamic effects within the tumor microenvironment (TME). **Conclusion:** Armed T-SIGn vectors exhibit the same high tumor selectivity and replicative activity as the unarmed parent EnAd and achieve consistent viral delivery to a broad range of epithelial tumor tissues. The aggregated safety analysis revealed that safety and tolerability are defined by systemic viremia but not off-target or transgene-related toxicities. Nucleic acid and cytokine release from tumors and changes in the TME (e.g., CD8+ T cells), indicative of the mechanism of action of replication-competent viral vectors, constitute potentially valuable data to support the definition of a recommended phase 2 dose and the assessment of the benefit of multicycle administration.

Keywords: oncolytic immunotherapies, immunotherapy, viral persistence, solid tumors, tolerability

INTRODUCTION

Although the concept of genetically engineered oncolytic immunotherapies was first described by Martuza et al[1] in 1991 (thymidine kinase gene mutation vector), the full potential of this approach has yet to be clinically realized, with only intratumorally (IT) delivered talimogene laherparepvec (T-Vec) having received US FDA approval based on an increase in durable response rate (Imlygic; Amgen 2023). The historical focus on IT administration of oncolytic immunotherapies was likely guided in part by the understanding that herpes simplex virus (HSV)-1 viruses such as T-Vec may not be well suited for intravenous (IV) delivery[2] and the perceived disadvantages of IV administration of oncolytic immunotherapies (e.g., low therapeutic index and high toxicity).[3]

With appropriate vector design and dosing regimens, IV administration can ensure systemic bioavailability and delivery to both primary and metastatic tumor sites. IV administration is also relatively noninvasive, allows for standardization of the drug dosage administered, and has the potential for multicycle administration. The IV delivery of experimental oncolytic immunotherapies has been investigated in several early-phase clinical trials, showing promising evidence of virus accumulation in tumors, viral replication, and early efficacy signals.[4–6]

Enadenotucirev (EnAd) is a group B chimeric Ad11/Ad3 adenovirus developed via directed evolution to replicate selectively in both primary and metastatic epithelial-derived solid tumors.[7] To enhance the therapeutic potential of EnAd, Tumor-Specific Immuno-Gene (T-SIGn) therapeutics were developed through the addition of transgenes, e.g., an anti–cluster of differentiation (CD) 40 agonist antibody, allowing potent immunostimulatory proteins to be expressed within tumor cells and released to the local tumor microenvironment (TME). Early-phase studies of EnAd and armed T-SIGn vectors have demonstrated their stability in systemic circulation and ability to selectively target tumors.[8,9] The tumor selectivity may be a result of a combination of factors, including genomic changes that render these viruses dependent on the phenotype of malignant cells to replicate[10] and the overexpression of viral entry receptors (e.g., of CD46) on tumor cells.[11] Multiple armed T-SIGn vectors have been developed, including: (1) NG-350A, our lead vector, which expresses a full-length human immunoglobulin G2 (IgG2) CD40 agonist antibody; (2) NG-641, which expresses a single-chain variable fragment (scFv) antibody recognizing human fibroblast activation protein and human CD3e (FAP-TAc), human IFNα, CXCL9, and CXCL10; and (3) NG-348, which expresses cell membrane–anchored human CD80 and membrane-anchored scFv antibody recognizing human CD3e. A head-to-head comparison of two alternative routes of administration for NG-350A demonstrated superior overall safety and pharmacodynamics profiles following IV administration, with no apparent disadvantages compared with IT injection.[9] Demonstration of proof of concept and a potentially high therapeutic index for this IV-administrated oncolytic immunotherapy is ongoing in a neoadjuvant study (FORTRESS; ClinicalTrials.gov Identifier: NCT06459869).

Here we set out to assess key elements for developing effective IV-administered oncolytic immunotherapies. These include (1) demonstration of long-term viral persistence and replication in tumor tissues; (2) adoption of a safe and well-tolerated IV dose regimen termed *low-high-high* (an initial lower dose of virus particles followed by two subsequent higher doses) to balance optimal delivery with tolerability; (3) demonstration that transgene arming does not affect the safety profile; and (4) identification of pharmacodynamic parameters. The demonstration of comparable replication profiles and oncolytic activity of the transgene-armed T-SIGn and unarmed EnAd vectors permitted these analyses (see online Supplemental Material).

METHODS

This study is based on safety and pharmcodynamic data for EnAd and T-SIGn from multiple clinical trials involving human participants that were approved by a

central institutional review board (Supplemental Table S1). All participants provided written informed consent to participate in the trials before taking part.

Armed T-SIGn Vector Generation

Armed T-SIGn vectors were generated through the insertion of transgenes in a noncoding region downstream of the EnAd virus major late promoter.[7] Transgenes for the heavy and light chain genes encoding the full-length, clinically tested CD40 agonist antibody selicrelumab of the IgG2 isotype were inserted into the lead armed T-SIGn vector, NG-350A. The additional T-SIGn vector NG-641 was generated through a similar process with a transgene cassette encoding a FAP-TAc bispecific as well as human IFNα, CXCL9, and CXCL10. The preclinical study methods are provided in the Supplemental Data.

T-SIGn Clinical Studies

For the clinical assessment of armed T-SIGn vectors, 16 patients received NG-350A monotherapy and 10 patients received NG-350A in combination with a checkpoint inhibitor. NG-641 was assessed in 30 patients as monotherapy and in 12 patients in combination with a checkpoint inhibitor. Based on the results of the unarmed EnAd studies, armed T-SIGn vectors were administered only on days 1, 3, and 5. Studies conducted with T-SIGn vectors were compared with previously published outcomes for EnAd monotherapy[12] and EnAd in combination with a checkpoint inhibitor.[13] The comparison of adverse drug reactions (ADRs) across the different EnAd dosing regimens supported the inclusion of all regimens for the objectives of the analyses to (1) establish a safe and well-tolerated dose regimen and (2) assess the effect of the insertion of transgenes. A summary of all studies including dosing regimens and included patients per analysis objective is provided in Table 1 and Table S1.

Peripheral Viral Persistence and Immunogenicity

Predose and postdose whole blood samples were collected and analyzed by qPCR to detect viral genomic DNA (E3), as previously described.[9] A serum-based electrochemiluminescence ligand binding assay was used to assess immunogenicity against the virus, as previously described.[8]

Viral Delivery Analyses and Immunohistochemistry

Immunohistochemistry (IHC) staining was performed at CellCarta (Antwerpen, Belgium) on 4-μm thick sections of FFPE tissue. Following confirmation of tumor content by hematoxylin and eosin (H&E) staining, sections were baked for 2 hours at 60°C and IHC staining was performed for adenoviral hexon using the antiadenovirus antibody, clone 20/11 from Merck Millipore (Cat# MAB8052).

Transgene Analyses and Cytokine Measurements

The expression of transgene was assessed using serum samples by quantitative reverse transcriptase–polymerase chain reaction (RT-qPCR) with primers and probes from Eurofins Genomics, Ebersberg, Germany (primer sequences provided in the Supplemental Material). A multiplex Luminex assay was used to analyze 17 cytokines in serum samples from patients, as previously described.[9]

Clinical Safety Analyses

The safety and tolerability of NG-350A and NG-641 was a primary end point in all the previously described phase 1 studies. Events were reported according to National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. ADRs for NG-350A and NG-641 were compared with previously published outcomes for EnAd monotherapy[12] and EnAd plus nivolumab combination treatment.[13]

Statistical Analyses

Descriptive statistics were used for all analyses. Data were summarized and tabulated according to the relevant categorical groupings.

RESULTS

T-SIGn Clinical Study Summary

The unarmed EnAd and armed T-SIGn vectors exhibited comparable replication kinetics and oncolytic activity (Supplemental Figs. S1–S3). Clinical analyses described in this manuscript focus on IV administration of EnAd (94 patients) or armed T-SIGn vectors (68 patients) (Table 1). A total of 16 patients were treated with NG-350A monotherapy and 10 patients were treated with NG-350A plus pembrolizumab. For NG-641, a total of 30 patients were treated with monotherapy and 12 patients received NG-641 monotherapy plus nivolumab. In contrast, of the 94 patients treated with EnAd, 31 patients received monotherapy, 48 received EnAd plus nivolumab, three received EnAd plus pembrolizumab, and the 12 patients from the CEDAR[14] study were treated with EnAd in combination with chemoradiotherapy (CRT). Table 1 provides a summary of the included studies, incorporating both demographic characteristics and outcomes.

Establishment of a Safe and Well-Tolerated Low-High-High dose regimen

This analysis included patients receiving IV EnAd ($n = 51$) or T-SIGn ($n = 68$) on days 1, 3, and 5 or days 1, 3, 5, 8, 10, and 12 (Table 1). At these dose levels, tolerability of EnAd and armed T-SIGn vectors was determined primarily by acute reactions to viral particle (vp) infusion immediately following dosing. Table S2 summarizes selected acute ADRs following flat dosing (e.g., 3×10^{12} vp on days 1, 3, and 5 [3-3-3]) or low-high-high dosing (e.g., 1×10^{12} vp on day 1 followed by 3×10^{12} vp or

Table 1. Summary of clinical studies involving EnAd and T-SIGn

Study (Vector)	Pts (*n*)	Dosing Level and Regimen[a]	Patient Characteristics and Treatment	Safety Findings	Pharmacodynamic Effects	Clinical Outcomes
EVOLVE (EnAd)[12]	31	D1, 3, and 5: 1×10^{10} vp to 10-10-10	Metastatic cancer population with a median of 4 prior systemic therapies; Monotherapy	MTD is 3-3-3; ADRs associated with viremia ($2 \times$ G3 [pyrexia at 6-6-6]). Tolerability improved with prolonged infusion time.	Fast clearance of virus ($t_{1/2}$ = 16.7 min), yet virus remains detectable at D3 baseline and 8 wk postdosing. Conversion of most seronegative pts, AVA plateaued by D20. Transient dose-dependent increases in cytokines (e.g., MCP-1, IL-6) after first dose, lower responses on subsequent doses on D3 and 5. Viral hexon protein detected in skin metastasis 39 days after final dose (6-6-6).	SD > 12 wk in 40% of pts (RECIST v1)
SPICE (EnAd)[13]			45 CRC and 6 HNSCC pts who failed prior Tx: 65% had ≥ 3 and 20% had received > 5 regimens.	L-H-H schedule improved acute tolerability compared with flat dosing by minimizing effects of viremia; allowed for higher cumulative dose.	Viral persistence and AVA response similar to those observed in EVOLVE. Viral DNA detectable in tumor biopsies in at least 3 pts. Increased CD8+ cells in tumor posttreatment in 12/14 pts; 4/14 pts converted from "desert" to "inflamed." Attenuated acute cytokine responses with L-H-H dosing despite higher cumulative dosing.	One PR; 45% SD (including 3 pts with SD for ≥ 24 wk). Pts progression-freeat 6 and 12 mo: 31% and 10% (RECIST v1.1). Median (95% CI) OS was 16.0 (12.6, 28.8) mo.
	42	D1, 3, and 5: 1-1-1 to 1-6-6	One (20), 2 (19), or up to 6 cycles (3 pts) in combination with nivolumab			
	9	D1, 3, 5, 8, 10, 12: 3-3-3	One cycle in combination with nivolumab			
CEDAR (EnAd)[14]	2	D1, 3, and 5: 1-1-1	LARC, neoadjuvant CRT; EnAd administration pre-CRT	Mild to moderate ADRs associated with viremia.	Hexon protein detection in 1 pt in tumor tissue 13 wk and 7 mo after final dose (1-1-1 pre-CRT only).	Clinical or pathological CR 42.6% (5 of 10 pts with EnAd administration pre- and post-CRT)
	10	D1, 3, and 5: 1-1-1; 1-3-3	LARC, neoadjuvant CRT; EnAd administration pre- and post-CRT			
FORTITUDE (NG-350A)[9]	19	D1, 3, and 5: 1×10^{11}, 1-3-3 and 1-6-6	Metastatic cancer population (≥ 3 CRC, HNSCC, and PDAC, 60% with ≥ 3 metastatic sites) with a median of 3 prior lines of anticancer therapy. 3 pts in combination with pembrolizumab	MTD is 1-6-6; moderate ADRs associated with viremia and a lack of transgene-related or off-target viral toxicity.	Viral persistence and AVA response similar to those observed in EnAd trials. Transgene expression detected in 5/9 pts with higher doses. Conversion from "desert" to "inflamed" TME observed in 1 pt (0.1-0.1-0.1 or 10^{11}) with durable 10+ fold increase in CD8+ and GZMB+ cells. Sustained cytokine response (e.g., IFNγ, IL-2) observed post-D12.	BoR of SD of 46.7% in IV cohorts (22.2% in pts treated with IT NG-350A).

Table 1 continues on next page

Table 1. Continued

Study (Vector)	Pts (*n*)	Dosing Level and Regimen[a]	Patient Characteristics and Treatment	Safety Findings	Pharmacodynamic Effects	Clinical Outcomes
FORTIFY (NG-350A)[b]	7	D1, 3, and 5: 1-3-3 and 1-6-6	Metastatic cancer population (5 of 7 pts HNSCC and GC) with a median of 3 prior lines of anticancer therapy. Combination with pembrolizumab	MTD is 1-6-6; moderate ADRs associated with viremia and lack of transgene-related or off-target viral toxicity.	Viral persistence and AVA and cytokine responses similar to those observed in FORTITUDE. Transgene expression detected in 6/6 pts.	BoR of SD in 2 of 7 pts (1 SD of 6 months).
STAR (NG-641)[b]	23	D1, 3, and 5: 1×10^{11} to 1-10-10	Metastatic cancer population (11 CRC, 4 HNSCC, and 3 PDAC) with a median of 4 prior systemic therapies. Monotherapy.	MTD is 1-6-6 (CRS was DLT at 1-10-10); lack of transgene-related or off-target viral toxicity.	Viral persistence and AVA and cytokine responses similar to those observed in EnAd/NG-350A. Transgene expression detected in 1 pt (1-6-6).	BoR of SD in 7 of 20 evaluable pts.
MOAT (NG-641)[b]	7	D1, 3, and 5: 1×10^{11} to 1-3-3	Recurrent clinical stage III–IVb HNSCC pts: monotherapy prior to surgical resection (2 pts had recurrent disease following CRT)	Mild to moderate ADRs associated with viremia.	Viral persistence, AVA, and cytokine not analyzed. Transgene expression not detected (1-1-1 or 1-3-3).	No primary or secondary end points related to efficacy.
NEBULA (NG-641)[b]	12	D1, 3, and 5: 1-1-1 to 1-6-6	Metastatic cancer population (4 HNSCC and 3 NSCLC) with a median of 4 prior systemic therapies. Combination with nivolumab.	Mild to moderate ADRs associated with viremia; no DLTs or related SAEs. One pt with PIGN (1-3-3).	AVA and cytokine responses similar to those observed in EnAd/NG-350A. Virus persistence not analyzed. Transgene not detected.	BoR of SD in 3 of 5 evaluable pts.

[a]Definition of IV dose levels to allow for cross study comparisons: 1-1-1 = 1×10^{12} vp on D1, 3, and 5; 1-3-3 = 1×10^{12} vp on D1 followed by 3×10^{12} vp on D3 and 5; 3-3-3 = 3×10^{12} vp on D1, 3, and 5; 1-6-6 = 1×10^{12} vp on D1 followed by 6×10^{12} vp on D3 and 5; 1-10-10 = 1×10^{12} vp on D1 followed by 10×10^{12} vp on D3 and 5; 10-10-10 = 10×10^{12} vp on D1, 3, and 5.

[b]Unpublished data.

ADR: adverse drug reaction; AVA: antiviral antibody; BoR: best overall response; CD8: cluster of differentiation 8; CR: complete response; CRC: colorectal cancer; CRT: chemoradiotherapy; D: day; EnAd: enadenotucirev; DLT: dose-limiting toxicity; GC: gastric cancer; G3: Grade 3; GZMB: granzyme B; HNSCC: head and neck squamous cell carcinoma; IFNγ: interferon γ; IL-2: interleukin 2; IL-6: interleukin 6; IT: intratumoral; IV: intravenous; LARC: locally advanced rectal cancer; L-H-H: low-high-high; MCP-1: monocyte chemoattractant protein 1; MTD: maximum tolerated dose; NSCLC: non–small cell lung cancer; OS: overall survival; PDAC: pancreatic ductal adenocarcinoma; PIGN: postinfectious glomerulonephritis; PR: partial response; pt: patient; RECIST: Response Evaluation Criteria in Solid Tumors; SAE: Serious Adverse Event; SD: stable disease; T-SIGn: Tumor-Specific Immuno-Gene; Tx: treatment; vp: viral particle.

6×10^{12} vp on days 3 and 5 [1-3-3 or 1-6-6]) across multiple clinical trials involving EnAd, NG-641, and NG-350A. In the flat dosing regimen (3-3-3; $n = 22$), four patients (18%) receiving 3×10^{12} vp on day 1 experienced grade 2 or higher ADRs of pyrexia, chills, or influenza-like illness following dosing on day 1. By contrast, when patients received low-high-high dosing, for example, among patients in the 1-3-3 dosing regimen ($n = 27$), only one out of 27 patients (4%) experienced similar ADRs, suggesting a desensitization (Table S2) and improved tolerability to the regimen.

Consistently, peak levels of proinflammatory cytokines such as monocyte chemoattractant protein 1 (MCP-1) and IL-6 during days 1–5 were found to be highest in patients dosed at 3-3-3, and these cytokine responses were attenuated with low-high-high dosing regimens (1-3-3 and 1-6-6), leading to a superior tolerability without affecting pharmacodynamic activity (Fig. S4; cytokine data available for 107 of 119 patients).

Safety and Tolerability of EnAd Following the Insertion of Transgenes

The safety and tolerability profile of single-cycle IV dosing (Fig. 1) was similar with the armed T-SIGn (NG-350A and NG-641) and unarmed EnAd vectors. Tolerability of all three vectors was determined primarily by reactions to vp infusion during the days immediately following dosing, with little evidence of any additional delayed toxicity that may have been associated with the armed T-SIGn vectors due to transgene expression (Fig. 1). The most common grade 1–3 ADRs following single-cycle IV dosing were similar with armed T-SIGn (pyrexia, chills, and nausea) and unarmed EnAd (pyrexia, chills, and fatigue). A full breakdown of the most common ADRs observed with EnAd or armed T-SIGn vectors is provided in Table 2. Notably, to date no evidence of safety signals that might be associated with the encoded CD40 agonist transgene in NG-350A has been observed. For example, there was no evidence of increased liver toxicity or cytokine response syndrome (CRS), a previously reported safety signal with systemically administered CD40 agonists.[15,16]

Other than the inflammatory events following dosing (e.g., chills, fever, influenza-like illness), two safety signals thought to be related to reactions to vps but occurring at delayed time points were observed across the armed T-SIGn vectors and EnAd: (1) a previously described finding of prolonged activated partial thromboplastin time (aPTT) associated with the transient presence of antiphospholipid antibodies[17] and (2) acute kidney injury (AKI). Serious cases of AKI occurred in 3.7% of patients treated across the platform (242 patients at all dosing regimens and routes of administration), typically 20–28 days after first dosing. For one patient an ADR of a postinfectious glomerulonephritis (PIGN) following viral dosing was reported (without IHC confirmation), which may be aggravated by coadministration of immune checkpoint inhibitors. This PIGN seemed to respond to the use of corticosteroids. The risk of AKI was managed by frequent preventative monitoring of proteinuria, creatinine, estimated glomerular filtration rate (eGFR), complement C3 and C4 levels, and blood pressure during dosing to allow for timely identification and treatment initiation.

Assessment of Virus Delivery to Tumor Sites

Following the low-high-high dosing regimens, E3 DNA of the viral vector could be detected in core needle tumor biopsies in patients with metastatic cancer treated with both EnAd and armed T-SIGn vectors, including four out of eight patients treated with EnAd, 11 of 12 patients treated with NG-350A, and 10 of 10 patients treated with NG-641. Notably, these patients had a variety of epithelial tumor types, including colorectal cancer, pancreatic cancer, liver cancer, prostate cancer, head and neck cancer, and other cancers, suggesting that EnAd and armed T-SIGn vectors were successfully delivered to multiple types of primary or metastatic tumor sites via IV administration (Fig. 2A).

Additional evidence supporting selective viral delivery and persistence was provided by an investigator-initiated study of EnAd plus CRT in locally advanced rectal cancer (LARC) (CEDAR[14]; Table 1). In this neoadjuvant study, the presence of virus in tumor samples was assessed using IHC in a patient who underwent total mesorectal excision 14 weeks posttreatment due to a lack of clinical complete response. In addition, a surgical specimen of a resection of a lung metastasis (present at study enrollment) 7 months posttreatment was tested for presence of the virus hexon protein (patients with oligometastatic disease were permitted so long as they were considered suitable for neoadjuvant CRT). Notably, positive virus hexon protein staining was observed in both the primary rectal tumor tissue and the lung metastasis resection (Fig. 2B iii and iv). By contrast, hexon staining was not observed in healthy tissue within surgical resections or in archival biopsies following CRT in a patient not treated with EnAd (Fig. 2B i and ii). Additionally, even within the primary tumor resection that was positive for hexon staining, little or no staining was observed within healthy cryptlike areas, consistent with the expected epithelial tumor selectivity for EnAd (Fig. 2B iii).

Monitoring of Virus Persistence and Pharmacodynamic Activity

Virus persistence in circulation

Similar systemic virus persistence (determined as E3 viral DNA) was observed across multiple independent studies when armed T-SIGn and unarmed EnAd vectors (Fig. 3A) were given at the same dose level (1-3-3 and 1-6-6; see Table 1). Viral concentration increased rapidly in blood after each IV administration, whereas significantly lower levels were observed prior to the next administration on day 3 or 5. However, sustained detection of the viral DNA was observed irrespective of the

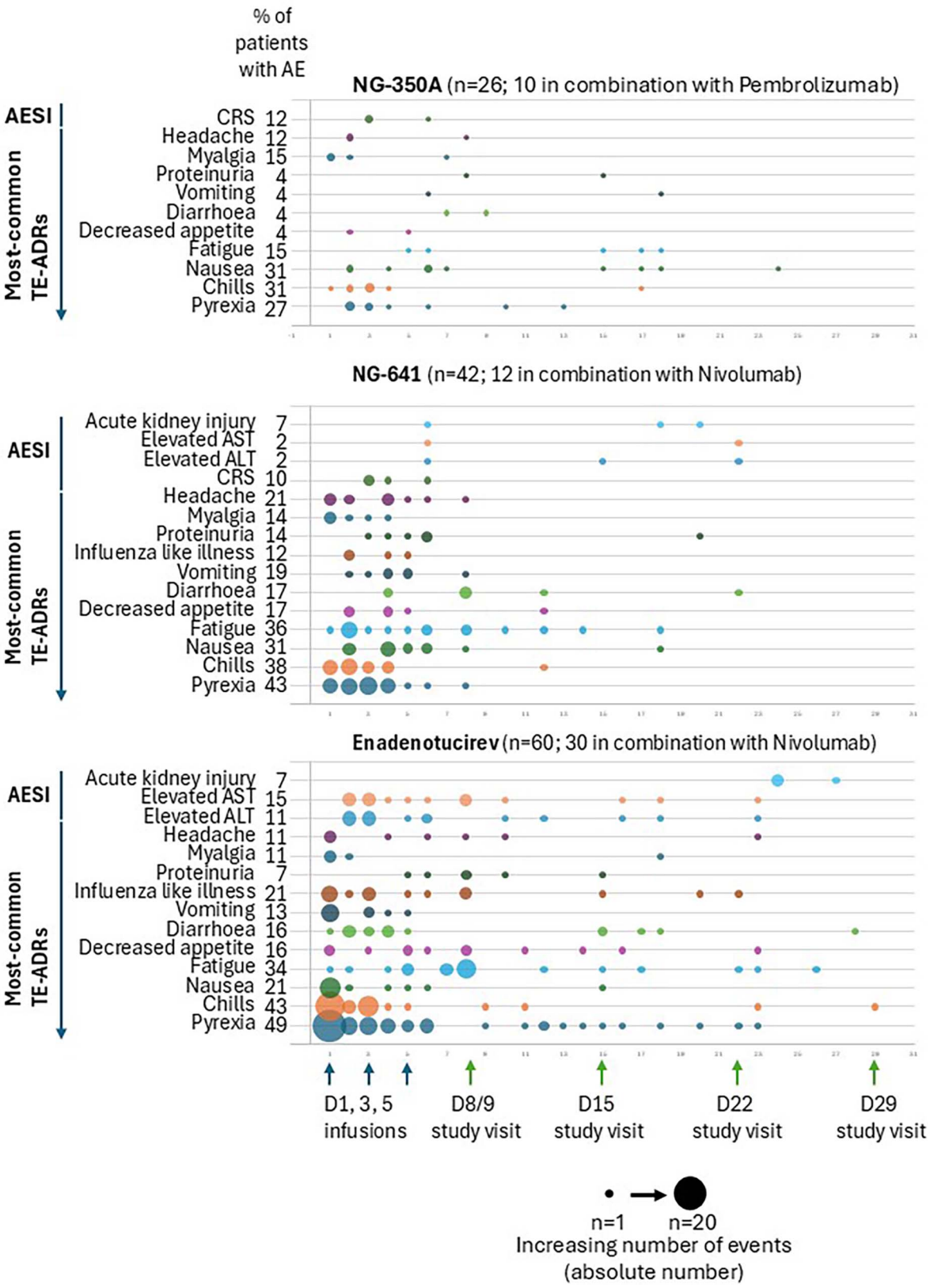

Figure 1. T-SIGn and EnAd tolerability with single-cycle IV dosing. Unarmed EnAd and armed T-SIGn vectors demonstrated similar tolerability profiles during the first 30 days postdosing (based on References 9, 12, 14, and unpublished data).

AE: adverse event; AESI: adverse event of special interest; ALT: alanine transaminase; AST: aspartate transferase; CRS: cytokine release syndrome; D: day; EnAd: enadenotucirev; IV: intravenous; TE-ADR: treatment-emergent adverse drug reaction; T-SIGn: Tumor-Specific Immuno-Gene.

Table 2. Most common adverse drug reactions (ADRs)

	EnAd (n = 60)*		T-SIGn (n = 68)*	
	Grade 1/2	Grade 3	Grade 1/2	Grade 3
Pyrexia	49	0	37	0
Chills	43	0	35	0
Fatigue	34	0	27	2
Nausea	21	0	31	0
Decreased appetite	21	0	12	2
Headache	11	0	16	2
Influenza-like illness	21	0	7	0
Diarrhea	15	2	12	0
Myalgia	11	0	15	0
Vomiting	13	0	13	0
aPTT prolonged	3	2	15	6
Dyspnea	8	2	7	3

Values are percentage of patients with drug-related TEAE occurring in $\geq$ 10% of patients (single-cycle IV dosing on days 1, 3, and 5 or days 1, 3, 5, 8, 10, and 12).
*See Supplemental Table S1 for patient populations used in this analysis.
ADR: adverse drug reaction; aPTT: activated partial thromboplastin time; EnAd: enadenotucirev; IV: intravenous; TEAE: treatment-emergent adverse event; T-SIGn: Tumor-Specific Immuno-Gene.

vector used, with at least approximately 1×10^4 vp/mL detected in blood up to 50 days after the last dosing of virus (day 5). Notably, this sustained and persistent detection of viral DNA was observed despite the presence or induction of antivirus antibodies with all three vectors (Fig. 3B). Some degrees of preexisting immunity to the chimeric adenovirus were observed in 10–20% of patients across studies, and the majority of patients exhibited seroconversion after initial doses of the viruses. In either case, antivirus antibody titers increased after dosing before plateauing at approximately day 15 and persisting until the final assessments at day 57.

Transgene expression

Serum samples from patients treated with NG-350A and NG-641 were analyzed for the presence of transgene messenger RNA (mRNA). Across two independent studies (FORTITUDE[9] and FORTIFY [unpublished data]), four out of six (dose level 1-3-3) and seven of nine (dose level 1-6-6) patients treated with low-high-high IV NG-350A regimens had detectable aCD40 transgene mRNA at one or more time points (Fig. S5). In the NG-641 studies (STAR, MOAT, and NEBULA [unpublished data]), zero out of six (dose level 1-3-3), zero out of three (dose level 1-3-3) and one of four (dose level 1-6-6) treated patients had detectable transgene mRNA levels (OKT3-VH).

Delayed and sustained cytokine elevations following treatment

Increases in a subset of proinflammatory cytokines such as IL-12p70, IFNα2, IL-17A, and IFNγ were also detected in serum following treatment (Fig. 4; Fig. S6). In contrast to the increases of MCP-1 and IL-6, which happened immediately after dosing and were a transient response to the viremia, these proinflammatory cytokine increases were observed from approximately day 12 onwards and were sustained until the final assessments at day 57 (except for IFNγ, which also showed a transient elevation after dosing).

DISCUSSION

Here we report the successful generation of multiple transgene-armed T-SIGn vectors, each designed to target different aspects of resistance to current anticancer therapies. We show that the insertion of transgenes did not affect core biological properties of the vector such as the oncolytic potency and tumor-selective replication (Figs. S1–S3), permitting the aggregation of clinical data to assess virus persistence, safety, and tolerability.

Clinically, the intentions to improve acute vector tolerability in order to establish a well-tolerated outpatient regimen and to increase the dose intensity per cycle led to the testing of low-high-high dose regimens, in which a lower dose of 1×10^{12} vp is administered on day 1, followed by 3×10^{12} or 6×10^{12} vp on days 3 and 5 (1-3-3 or 1-6-6). As EnAd and armed T-SIGn vectors have similar biological properties and safety profiles (primarily defined by the initial viremia following IV dosing), pooled adverse events across studies were analyzed to compare different dosing regimens and further assess the effect of low-high-high dosing in a larger number of patients, including patients treated with armed T-SIGn vectors. This pooled analysis supports the notion of a desensitization following an initial low dose (Table S2). Importantly, a low-high-high dose of 1-6-6, which is a numerically higher cumulative dose than the previous flat maximum tolerated dose (3-3-3), was tolerated in both EnAd and armed T-SIGn clinical studies (Fig. 1). Attenuated acute cytokine responses were also observed with the low-high-high regimens, in which lower peak values of MCP-1 and IL-6, proinflammatory cytokines often associated with CRS, were observed (Fig. S4), confirming unpublished preclinical data.

Notably, the pooled safety analysis also demonstrates that there was no evidence of virus-related off-target toxicity and that the arming with potent immunostimulatory transgenes does not appear to adversely affect safety and tolerability (Fig. 1). The safety profiles of the armed T-SIGn vectors (NG-350A and NG-641) remained consistent with that of the unarmed vector EnAd and were predominantly defined by mild inflammatory responses to administration of vps. Indeed, when comparing safety outcomes, the highest rates of inflammatory adverse events were reported for EnAd, a finding likely related to the faster infusion rates and higher day 1 dose used in early EnAd studies.[12] Overall, these data suggest that optimizing the viral delivery regimen (i.e., through lower infusion rates and low-high-high dosing) has improved acute tolerability by minimizing viremia-related effects and consequently may allow for a potentially high therapeutic index following IV administration. Our analyses included a substantial proportion of patients who received oncolytic immunotherapies in

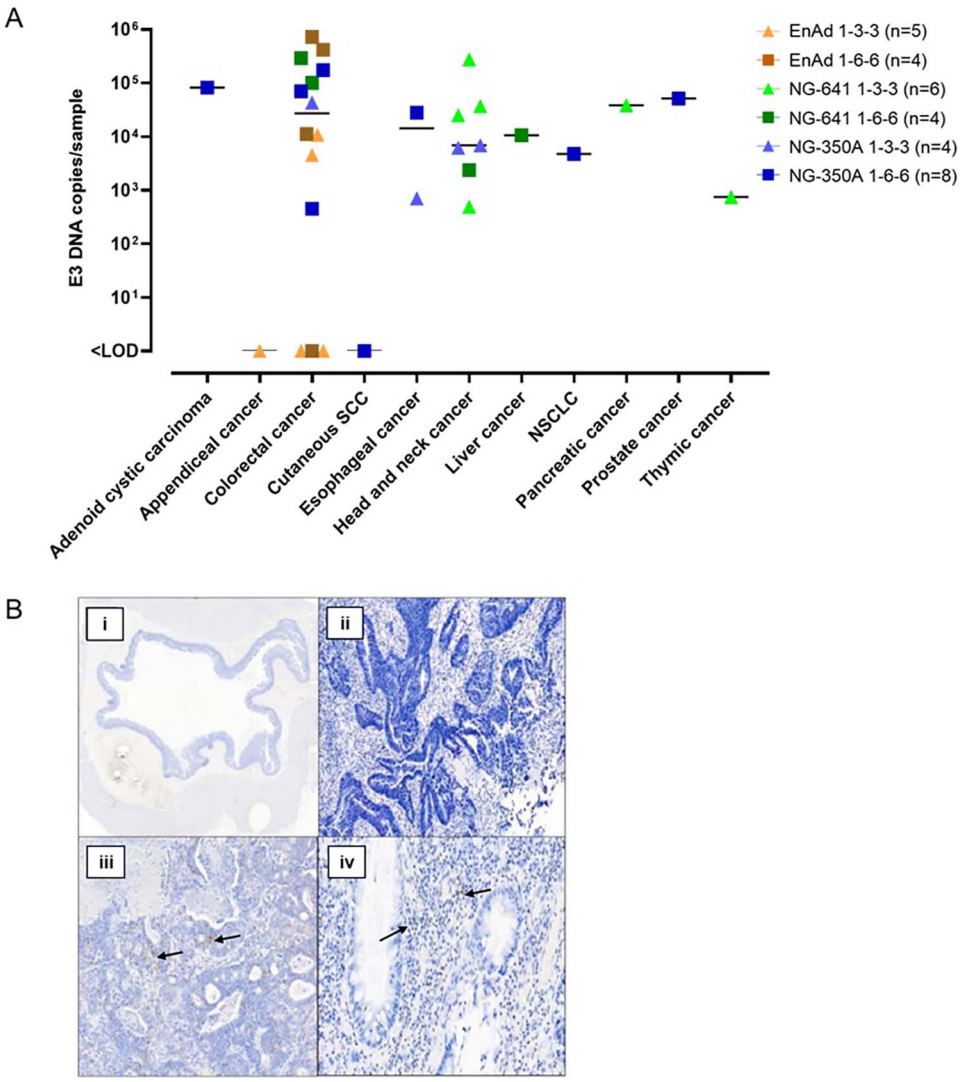

Figure 2. Virus delivery to tumor sites following IV administration. (**A**) Detection of E3 viral DNA in tumor samples. (**B**) Detection of viral hexon protein in specimen from surgical resection. Representative hexon IHC staining (brown) for patient CED-101. (**i**) Negative control primary rectal resection sample containing no tumor. (**ii**) Archival rectal tumor biopsy scored negative for virus hexon protein (some focal aspecific staining observed in plasma cells). (**iii**) Lung metastasis resection taken 7 months posttreatment. Scored positive for hexon protein with Allred proportion of tumor cells score of 1 and intensity score of 3; 1 potential focus of viral hexon staining (equivocal). (**iv**) Primary rectal tumor resection taken 14 weeks posttreatment. Scored positive for hexon protein with Allred proportion of tumor cells score of 1 and intensity score of 1. Little or no staining in healthy cryptlike areas.

EnAd: enadenotucirev; IHC: immunohistochemistry; IV: intravenous; LOD: limit of detection; NSCLC: non–small cell lung cancer; SCC: squamous cell carcinoma.

combination with an immune checkpoint inhibitor. The initiation of immune checkpoint inhibitor therapy occurred no earlier than day 15 and did not influence the outcomes presented in Figure 1. As detailed in Table 1, one case of PIGN was identified in combination with an immune checkpoint inhibitor.

We have demonstrated across multiple phase 1 trials with EnAd and armed T-SIGn vectors that these replication-competent viruses can be successfully delivered to and persist within tumor tissues of various epithelial origins following IV administration (Fig. 2A and Table 1). Data from CEDAR[14] provided further evidence of selective virus persistence in tumor tissues (but not in adjacent healthy tissue) as shown in specimens from surgical resections up to 7 months postdosing (Fig. 2B). These findings demonstrate that IV-administered T-SIGn vectors can achieve sufficient tumoral uptake and sustained replication. Although viral replication in healthy tissues cannot be entirely excluded, the observed safety profiles (Fig. 1) and lack of virus detection in nontumor tissues (Fig. 2B) are consistent with tumor-selective viral replication. Consistently, systemic detection of E3 DNA (Fig. 3A) and transgene mRNA (Fig. S5) in blood at higher dose levels (1-3-3 and 1-6-6) is likely a result of ongoing viral replication and subsequent release of these molecules from tumor cells. These pharmacodynamic signals may originate from tumor-derived exosomes[18] or oncolytic activity, and are consistent with the known short, dose-independent

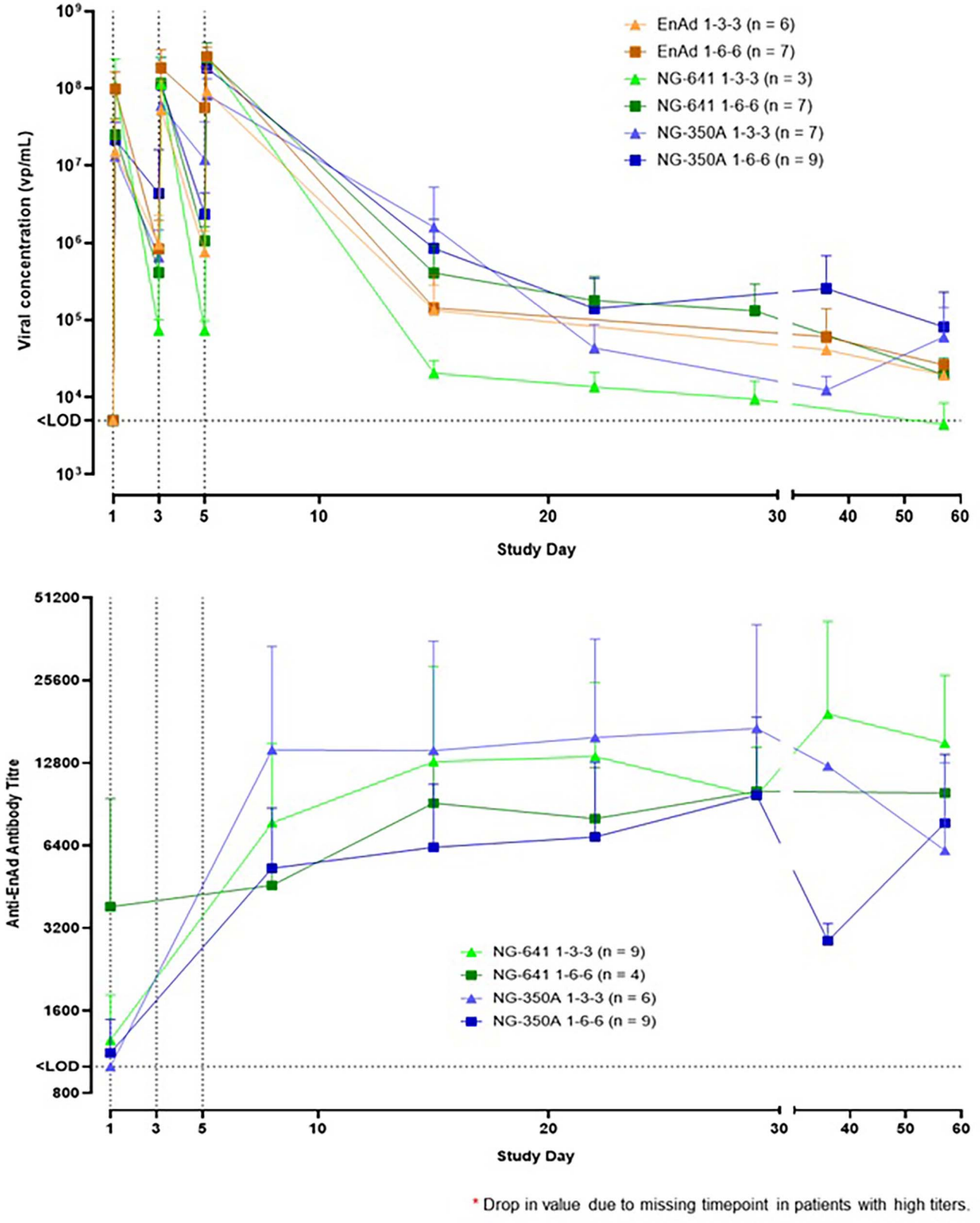

Figure 3. Peripheral virus persistence and immunogenecity (based on References 9, 13, and unpublished data). (**A**) Peripheral viral persistence following 1-3-3 or 1-6-6 single-cycle dosing. NG-350A data from the FORTITUDE and FORTIFY study, NG-641 data from the STAR and NEBULA studies, EnAd data from the SPICE study. (**B**) Antidrug antibodies response following 1-3-3 or 1-6-6 single-cycle dosing. NG-350A data from the FORTITUDE and FORTIFY studies, NG-641 data from the STAR and NEBULA studies. Antibody response against unarmed EnAd was published previously.[13]

EnAd: enadenotucirev; LOD: limit of detection; vp: viral particle.

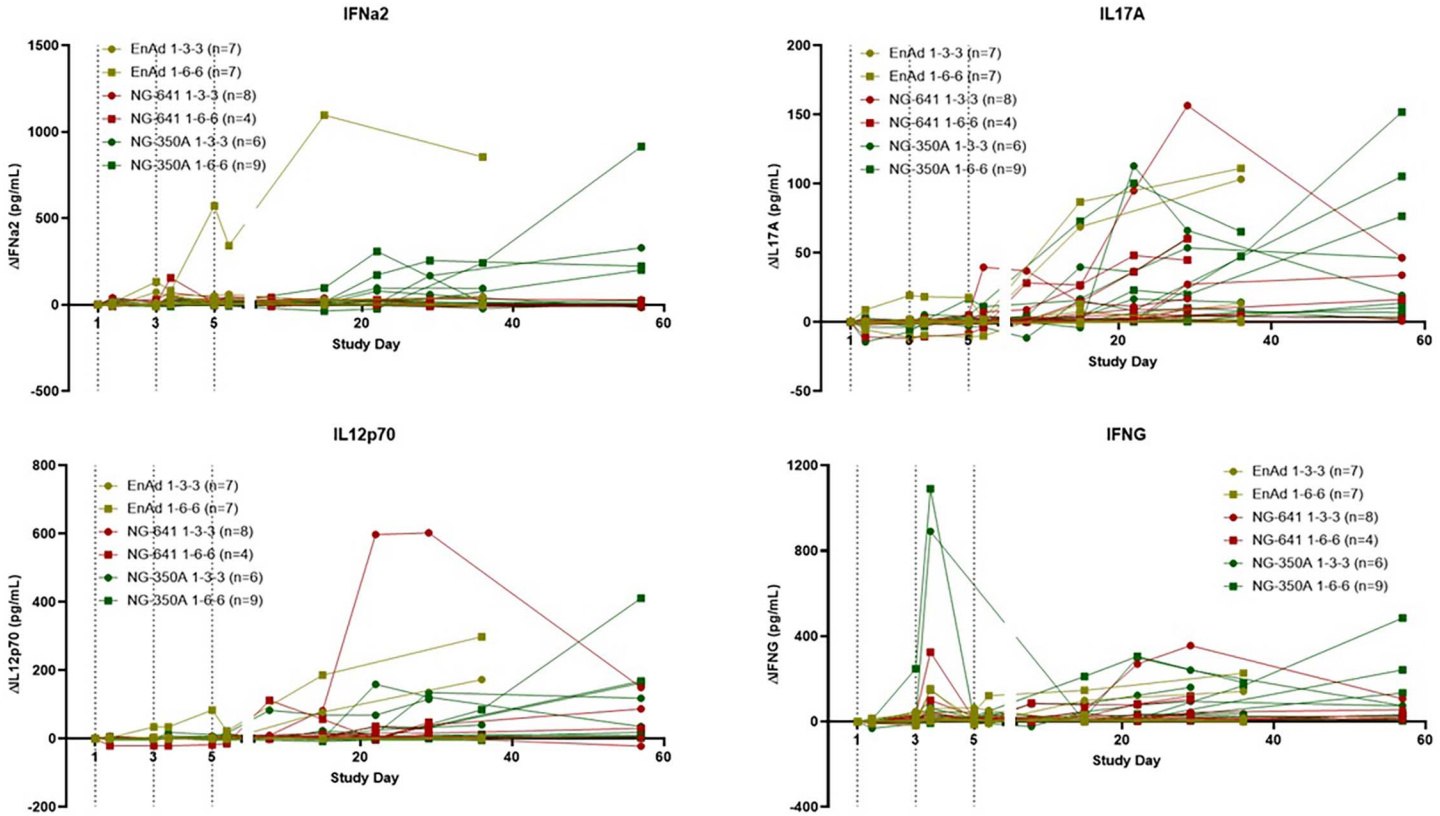

Figure 4. Delayed systemic cytokine elevations. Cytokine measurements in patients receiving 1-3-3 or 1-6-6 single-cycle dosing.
EnAd: enadenotucirev; IFNa2: interferon $\alpha 2$; IFNG: interferon γ; IL12p70: interleukin 12 p70; IL17A: interleukin 17a.

half-life (~17 min) for EnAd[12] and the rapid clearance of cell-free nucleic acids in blood.[19] Collectively, the kinetics of viral E3 DNA, presence of transgene mRNA, and favorable safety profile support the hypothesis of selective virus proliferation and localized production of transgenes (e.g., a CD40 agonist) in tumor tissues,[7] potentially leading to a high therapeutic index for the T-SIGn vectors.

Importantly, viral E3 DNA and transgene mRNA could represent promising, noninvasive pharmacodynamic biomarkers to monitor virus persistence and replication in tumor tissues. These markers may also guide the selection of a recommended phase 2 dose (RP2D) and inform future strategies involving multicycle administration of armed T-SIGn vectors.

Lastly, the delayed, systemic detection of proinflammatory cytokines likely represents another pharmacodynamic effect of EnAd and armed T-SIGn vectors within the TME. For example, elevations of IL-12 (as observed in Fig. 4) are expected, as the cytokine is primarily produced by activated dendritic cells to stimulate the Th1 immune response critical for antitumor immunity.[20] The previously reported dramatic changes in the TME (durable 10-fold increases in CD8+ and granzyme B+ cytotoxic T-cell infiltration in a patient with cholangiocarcinoma) represent a downstream pharmacodynamic effect[9] and occurred at a low dose level of armed NG-350A (1×10^{11}, i.e., 0.1-0.1-0.1) that did not allow for the systemic detection of E3 DNA and transgene mRNA in blood. Increases in CD8+ cells and an inflammatory gene signature were also observed in a 63-year-old patient with $BRAF^{mut}$ colorectal cancer who achieved a confirmed partial response following treatment with another armed T-SIGn vector NG-348 at the single-agent flat dose level of 3×10^{10} vp (ClinicalTrials.gov Identifier: NCT03363776, unpublished data).

A potential limitation of the aggregated safety analysis comparing EnAd and armed T-SIGn vectors is the variety of dosing regimens and duration of IV administration in EnAd studies compared with those involving T-SIGn candidates. The assessment of pharmacodynamic effects associated with persistent viral replication within tumor tissue—including systemic proinflammatory cytokine response—was conducted in a cohort that included a substantial proportion of patients who initiated immune checkpoint inhibitor therapy on day 15. The inclusion of these patients was considered appropriate, as no significant difference was observed on parameters indicative of viral persistence, nor was there evidence of systemic cytokine elevation in patients receiving EnAd in combination with an immune checkpoint inhibitor. Importantly, the early clinical development of armed T-SIGn vectors benefited from the foundational clinical data acquired in EnAd studies, enabling a reduced number of required dose-escalation cohorts. Ongoing and future studies will continue to assess whether the safety and

tolerability of armed T-SIGn vectors is indeed primarily defined by viremia-related effects.

The limited efficacy observed in early-phase trials remains a key challenge for the T-SIGn programs and continues to be a central focus of the ongoing clinical development. This limitation may, in part, be attributable to the enrollment of heavily pretreated patient populations. To address this, the ongoing FORTRESS study (NCT06459869) is evaluating the therapeutic approach in a neoadjuvant setting in combination with CRT, a strategy that has demonstrated promising efficacy in the CEDAR trial (NCT03916510).[14] One critical consideration for future development is the identification of a synergistic combination partner, as all regimens to date have involved coadministration with an immune checkpoint inhibitor. Notably, the only approved and IT-delivered oncolytic virus (Imlygic) failed to meet its coprimary end points of progression-free survival (PFS) and overall survival (OS) in a placebo-controlled phase III trial in combination with pembrolizumab.[21] The FORTRESS study includes comprehensive translational end points designed to assess potential barriers to efficacy, including delivery efficiency and the effect of preexisting antiviral immunity.

CONCLUSION

Here we demonstrate that the unarmed EnAd vector can be armed with a variety of transgenes without affecting vector characteristics important for activity, including replication, oncolytic potency, and replication-dependent transgene expression. Successful delivery of these viral vectors to achieve persistent virus replication has been demonstrated across numerous epithelial tumor types. Notably, no evidence of transgene-related toxicity or off-target viral toxicity was observed, suggesting tumor-selective replication. We describe immunological and pharmacodynamic effects representing proof of mechanism that may support the definition of RP2D and further clinical development for this modality. These include viral DNA and transgene mRNA, likely resulting from viral replication and the persistence of replication-competent T-SIGn vectors in tumor sites, and seem to require a threshold dose level before these parameters are detectable in circulation (see Supplemental Graphical Abstract). Hence, pharmacodynamic effects in blood may not capture the pharmacodynamic effects in the TME because dramatic changes to the TME itself were previously observed at a dose level (0.1-0.1-0.1) far below the low-high-high regimens of 1-3-3 or 1-6-6. Additional pharmacodynamic effects include the delayed systemic cytokine release without clinical symptoms and changes in TME such as tumor-infiltrating lymphocytes.

CONFLICTS OF INTEREST

Lee S. Rosen reports receiving research funding (to institution) from Akamis Bio Ltd. *Christian Ottensmeier* reports receiving research funding (to institution) from Akamis, Biontech, Corbus Pharmaceuticals, Grey Wolf, Delcath Systems, Kisoji, Merck Sharpe and Dohme, Psivax, Scancell, Touchlight Genetics, Transgene, and Verastem; and is on the advisory boards and/or receives consulting fees from Immutep, Neuvogen, and Transgene. *Aung Naing* reports receiving research funding from NCI, EMD Serono, MedImmune, Healios Onc. Nutrition, Atterocor/Millendo, Amplimmune, ARMO BioSciences, Karyopharm Therapeutics, Incyte, Novartis, Regeneron, Merck, Bristol-Myers Squibb, Pfizer, CytomX Therapeutics, Neon Therapeutics, Calithera Biosciences, TopAlliance Biosciences, Eli Lilly, Kymab, PsiOxus, Arcus Biosciences, NeoImmuneTech, Immune-Onc Therapeutics, Surface Oncology, Monopteros Therapeutics, BioNTech SE, Seven & Eight Biopharma, SOTIO Biotech AG, and GV20 Therapeutics; is on the advisory boards and/or receives consulting fees from CTI, Deka Biosciences, Janssen Biotech, NGM Bio, Akamis Bio Ltd., Immune-Onc Therapeutics, STCube Pharmaceuticals, OncoSec KEYNOTE-695, Genome & Company, CytomX Therapeutics, Nouscom, Merck Sharp & Dohme Corp, Servier, Lynx Health, AbbVie, and PsiOxus; has received travel and accommodation expenses from ARMO BioSciences, NeoImmuneTech, NGM Biopharmaceuticals; and has received honoraria for speaking engagements from AKH Inc., The Lynx Group, Society for Immunotherapy of Cancer (SITC), Korean Society of Medical Oncology (KSMO), Scripps Cancer Care Symposium, ASCO Direct Oncology Highlights, European Society for Medical Oncology (ESMO), and CME Outfitters. *Guru Sonpavde* reports receiving research funding (to institution) from EMD Serono, Jazz Therapeutics, Bayer, Sumitomo Pharma, and Blue Earth Diagnostics; is on the advisory boards of EMD Serono, BMS, Merck, Seattle Genetics, Astellas, Janssen, Bicycle Therapeutics, Pfizer, Gilead, Scholar Rock, Eli Lilly, Loxo Oncology, Vial, Aktis, and Daiichi-Sankyo; has served as consultant and/or Scientific Advisory Board (SAB)/trial steering committee member for Syapse, Merck, Servier, Syncorp, and Ellipses; has spoken at Seagen, Gilead, Natera, Exelixis, Janssen, Astellas, Bayer, Aveo, Pfizer, and Merck; serves on the Mereo Data safety monitoring committee (honorarium); has received travel expenses from BMS and Astellas; and reports spouse employment by Myriad and Exact Sciences. *Brian A. Van Tine* reports receiving research grants from Polaris, Pfizer, Merck, Tracon Pharma, and Glaxo Smith Kline; personal consulting fees from Bayer, Cytokinetics Inc., Deciphera Pharmaceuticals, Daiichi Sankyo, Inc., EcoR1, Advenchen, Putnam, Salarius Pharmaceuticals, Boxer Capital LLC, Acuta Capital Partners LLC, Aadi Biosciences, Race Oncology Limited, Hinge Bio, Inc., and Kronos Bio, Inc.; honoraria for educational talks from Itertion Therapeutics, Inc., and Total Health Conference; personal fees for attending Data/Safety Advisory Board with Apexigen, Inc., Daiichi Sanko, Epizyme, Bayer US Medical Affairs, PTC Therapuetics, Aadi Biosciences, Boehringer Ingelheim, Agenus, Regeneron

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Data Availability

Data underlying the results reported in this article may be requested by contacting the corresponding author.

Supplemental Material

Supplemental materials are available online with the article.

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