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IL-12 encoding oNDV synergizes with CAR-T cells in orthotopic models of non-small cell lung cancer

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Systemic administration of oncolytic viruses (OVs) is a promising approach for targeting metastatic solid tumors, but their anti-tumor activity is limited by pre-existing neutralizing antibodies against common human viruses. Therefore, investigators have developed OVs derived from non-human host viruses. Successful implementation of this strategy requires that the viral vector selectively infects and replicates within human cancer cells. Newcastle disease virus (NDV) is an avian paramyxovirus that, as NDV-based OVs (oNDVs), has demonstrated safety and activity against multiple human tumors in clinical trials. Their use as a single agent, however, is insufficient to cure tumors. Similarly, chimeric antigen receptor-modified T cells (CAR-T cells) enable systemic targeting of cancer cells but have limited anti-tumor effects against bulky solid tumors, in part due to the immunosuppressive tumor environment. In this study, we evaluated the anti-tumor effects of combining systemic oNDV and CAR-T cell treatments. In models of non-small cell lung carcinoma (NSCLC), we found that oNDV itself and interleukin (IL)-12 derived from oNDVs enhance HER2-directed CAR-T cell anti-tumor activity and persistence *in vitro* and *in vivo*, leading to superior control of NSCLC tumors compared with either agent alone *in vivo*. Our data indicate that oNDV enhances the anti-tumor effects of HER2.CAR-T cells, thus controlling the growth of orthotopic NSCLC tumors.

INTRODUCTION

Systemic oncolytic virus (OV) treatment shows promise as an effective regimen that targets metastatic tumors compared with local OV treatments, which have limited anti-tumor activity against OV-untreated metastatic tumors. Modified OVs derived from human pathogens (e.g., adenovirus, herpesvirus) readily infect and replicate in human tumors. However, given the high seroprevalence of common pathogens, OVs delivered systemically prove to be short-lived due to inactivation by pre-existing memory immune responses, as most patients have neutralizing antibodies (NAbs) that impede systemic viral trafficking.[1]

Newcastle disease virus (NDV) is an avian paramyxovirus that does not naturally infect humans and therefore is not subject to NAb recognition, a trait that makes it an acceptable candidate for use in systemic OV treatments. Like other RNA viruses, NDV-derived OVs (oNDVs) activate innate anti-viral pathways (e.g., type I interferon [IFN] pathway) and have been tested as tumor vaccines in clinical trials.[2] However, prevailing concerns about the use of velogenic (highly virulent) and mesogenic (moderately virulent) NDV strains in humans, despite their robust lytic effects, surround the high communicability and virulence these strains exert among avian species and the potential for similar adverse effects upon treatment in patients. Common lentogenic (non-virulent) NDV strains, on the other hand, cause subclinical or mild symptoms in birds (including poultry), often presenting as respiratory or enteric disease. In patients, lentogenic NDV-based OVs have shown limited lytic effects against human cancer cells compared with other NDV strains but appear safe after systemic administration, without the attendant risks of the other strains.[3]

Adoptive immunotherapy with chimeric antigen receptor-modified T cells (CAR-T cells) likewise enables systemic targeting of cancer cells but has limited anti-tumor effects against bulky solid tumors, in part due to the immunosuppressive tumor environment that limits infiltration and favors exhaustion of effector cells.[4] This situation changes once OVs are partnered with CAR-T cell therapy. Evidence shows that OV-mediated damage to the tumor microenvironment (TME) enhances CAR-T cell recognition and destruction of malignant cells.[5–7] Therefore, we hypothesized that combination therapy in which systemic lentogenic oNDVs in conjunction with CAR-T cell treatment would enhance anti-tumor activity via disruption of the TME and pro-inflammatory stimulation, helping to promote

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anti-tumor CAR-T cell activity. As a proof of concept, we combined an oNDV encoding a pro-inflammatory cytokine (interleukin [IL]-12) with HER2-directed CAR-T cells in orthotopic models of HER2-positive non-small cell lung carcinoma (NSCLC). We found superior anti-tumor effects of the combination therapy compared with each agent alone *in vivo*. This beneficial effect is mediated in part by oNDV-derived IL-12, which attenuates terminal differentiation of tumor-infiltrated CAR-positive T cells.

RESULTS

Evaluation of oNDV lytic activity and transgene expression

We first assessed whether lentogenic oNDVs could express their transgene (EGFP, IL-12p70) while retaining lytic effect in infected NSCLC cells *in vitro*. While oNDV had a wide range of infectivity in a panel of NSCLC cell lines, we found dose-dependent transgene expression and oncolysis that increased concomitantly over time, demonstrating that transgene expression does not impair lytic effect (Figures 1A, 1B, and S1). As transgene expression is dependent upon oNDV replication, the levels of transgene expression are proportional to lytic activity, with A549 and H441 having the highest lytic activity and transgene expression, while H1703 showed the least lytic activity and lowest transgene expression (Figures 1A, 1B, and S1). We next examined whether systemic oNDV treatment controls NSCLC tumor growth *in vivo* in two orthotopic xenograft models using A549 and H1650 cells. Although we found dose-dependent IL-12p70 circulation in the blood of mice (Figure 1C), there was no anti-tumor effect against NSCLC tumors in either model (Figures 1D and 1E). While the untreated control group had a more scattered range of survival, this did not reach statistical significance. All animals, irrespective of group, were euthanized at >20% body weight loss and had tumor in their lungs and/or metastases, most commonly in the liver. These data suggest that lentogenic NDV-based oNDVs as single agents have limited anti-tumor effects *in vivo*.

Evaluation of HER2.CAR-T cell killing against NSCLC

The HER2-specific Ab trastuzumab deruxtecan is approved for the treatment of inoperable or metastatic NSCLC harboring activating HER2 mutations and for those who have received a prior systemic therapy,[8] and we have safely treated patients with osteosarcoma and glioblastoma with HER2-specific CAR-T cells (HER2.CAR-T cells).[9] We hypothesized that such HER2-directed CAR-T cells could also be used to recognize and kill HER2-positive NSCLC. In *in vitro* killing assays of NSCLC lines using HER2.CAR-T cells derived from multiple healthy donors, we confirmed consistently effective killing of co-cultured tumor cells (Figure 2A). We next examined whether HER2.CAR-T cells recognize and kill HER2-positive NSCLC tumors *in vivo* (Figure 2B). We found that HER2.CAR-T cells control NSCLC tumor growth, significantly prolonging survival compared with vehicle control in the H1650 cell model ($p = 0.0142$), with a trend toward prolonged survival in the A549 cell model ($p = 0.0602$) (Figure 2C). However, tumors inevitably recurred in both models, with evidence of reduced antigen expression (Figure S2). We isolated residual T cells from recurrent tumors for phenotypic analysis and, as we previously reported in other models, demonstrated that these bulk, CD3+ T cells were mostly CAR− (Figures 2D and S3).[5,10] Analysis of markers of T cell exhaustion of residual CAR-positive T cells after 12 weeks showed a drastic increase in PD-1 expression. These data suggest that CAR-T cells may require additional signal(s) for sustained CAR expression and/or anti-tumor activity.

Combination with oNDV enhances the anti-tumor activity of HER2.CAR-T cells

OV-mediated damage to the tumor, including the TME, may increase CAR-T cell access to malignant cells within a bulky tumor mass thereby leading to enhanced tumor cell killing.[7] To assess this in our NSCLC models, we evaluated whether infection with oNDV sensitizes NSCLC cells to HER2.CAR-T cells (Figure 3A). Treatment with control oNDV (expressing EGFP) or oNDV*IL12* significantly improved HER2.CAR-T anti-tumor effects against A549 ($p < 0.0001$), H441 ($p < 0.0001$), and H1650 ($p = 0.0002$) cell lines. No significant difference was seen between oNDV*GFP*- or oNDV*IL12*-infected cells except in the H1703 cell line. The degree of killing correlates with the oNDV infectivity and replication in each cell line, so the additive anti-tumor effect was least effective against H1703 cells, which have the most limited oNDV infectivity/replication and, therefore, IL-12 transgene expression (Figure 1). By contrast, H441 and A549 had the highest oNDV transgene expression and showed the greatest benefit when combined with CAR-T cells. Since previous studies with oncolytic adenoviruses expressing IL-12 demonstrated that intratumoral IL-12 production increased the persistence of CAR-positive T cells, we combined oNDV*IL12* and HER2.CAR-T cells *in vivo* (Figure 3B). We found that this combination produced superior tumor control compared to HER2.CAR-T alone ($p = 0.0013$). We next monitored HER2.CAR-T cell expansion and persistence in this mouse model using HER2.CAR-T cells labeled with *EGFP_ffLuc* (ffLuc, firefly luciferase; Figure S4). The combination did not prolong the *in vivo* persistence of adoptively transferred T cells (Figure 3C), but mice treated with the combination showed significantly higher plasma levels of IFNγ compared to mice treated with CAR-T cells alone (Figure 3D). This effect correlated with improved tumor control and overall survival ($p = 0.0258$) (Figure 3E). These results indicate that systemic oNDV treatment improves adoptively transferred CAR-T anti-tumor activity *in vivo*.

IL-12 derived from oNDV improves HER2.CAR-T cell persistence *in vivo*

To address how the combination of oNDV*IL12* and HER2.CAR-T improves *in vivo* anti-tumor activity, we collected whole lungs from mice with xenografted orthotopic NSCLC tumors at different time points after treatment and performed RNA sequencing on these samples (Figure 4A). We found that the Th1 cytokine IFNγ and Th1-related molecules granzyme B and perforin are highly expressed in the lungs of NSCLC-bearing mice 2 weeks after receiving the combination treatment compared to mice receiving only CAR-T cells. This Th1-related expression profile correlates with the high CD8 gene signature seen in mice receiving the combination treatment. There was no significant difference in the expressions of the senescence

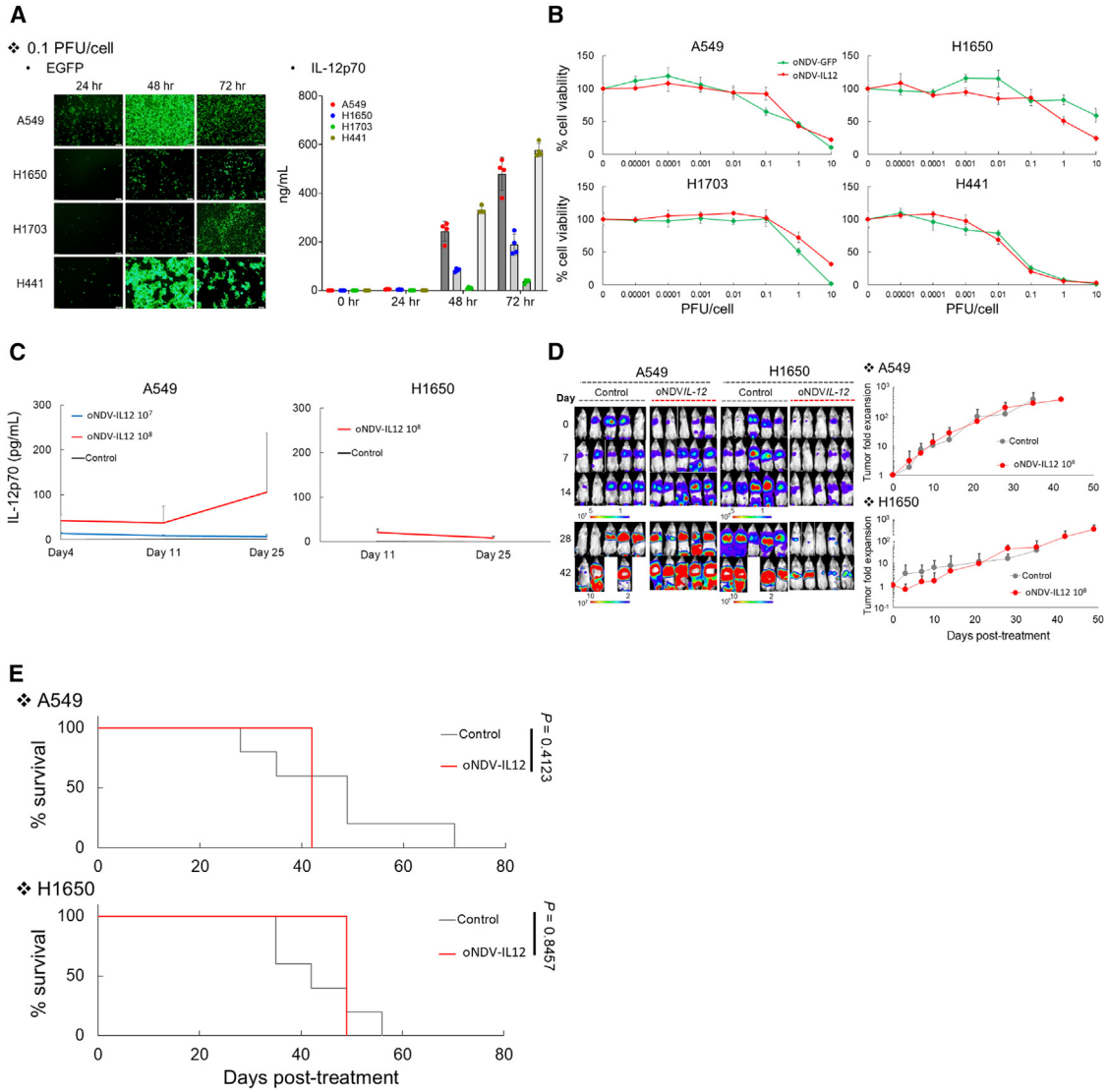

Figure 1. oNDV transgene expression and lytic effect in NSCLC

(A) A panel of NSCLC cell lines (A549, H1650, H1703, and H441) were infected with 0.1 PFU/cell oNDV*EGFP* or oNDV*IL12*. GFP expression in live cells was monitored via fluorescent microscopy, and medium was collected for IL-12p70 ELISA 24, 48, and 72 h post-infection. Data are presented as means ± SD ($n = 4$, representative images shown). (B) NSCLC cells were infected with increasing doses of oNDV*EGFP* or oNDV*IL12*. Viable cells were analyzed at 72 h by MTS assay. Data are presented as means ± SD ($n = 4$). (C) A549 and H1650 cells expressing firefly luciferase (ffluc) were orthotopically transplanted via tail vein injection to NSG mice. A total of 1×10^7 or 1×10^8 PFUs of oNDV*IL12* or vehicle control were systemically administered 14 days after tumor inoculation. Serum samples were collected 4, 11, and 25 days after injection of oNDV, and IL-12p70 levels in serum were measured by ELISA. Data are presented as means ± SD ($n = 5$). (D) Bioluminescence of A549 or H1650 tumor cells was monitored at different time points. Data are presented as means ± SD ($n = 5$). (E) Kaplan-Meier survival curve after administration of oNDV. The humane endpoint was established as body weight <80% from baseline ($n = 5$). p values were determined using the log rank Mantel-Cox test. Statistical significance set at $p < 0.05$, ns > 0.05. The experiment was repeated for purposes of tissue collection, with similar trends for all replicates.

markers CD57 and KLRG-1 between combination treatment and CAR-T cells alone. In contrast, mice treated only with HER2.CAR-T cells showed higher expression of the immunosuppressive marker CD38 and an angiogenesis gene signature than mice treated with the combination at all time points (Figure 4A). These signatures are associated with tumor progression in mice treated with HER2.CAR-T cells alone (Figure 3).

To address whether these gene signatures correlate with the residual CAR-T cell phenotype in the lung, we repeated the aforementioned experiment, harvesting lung tissue at the indicated time points for phenotypic analysis of residual T cells in the lung (Figures 4B and S5). We found that CAR-positive CD4 and CD8 T cells persist in mice receiving the combination treatment, similar to our observations in previous studies with adenovirus (Ad) gene therapy

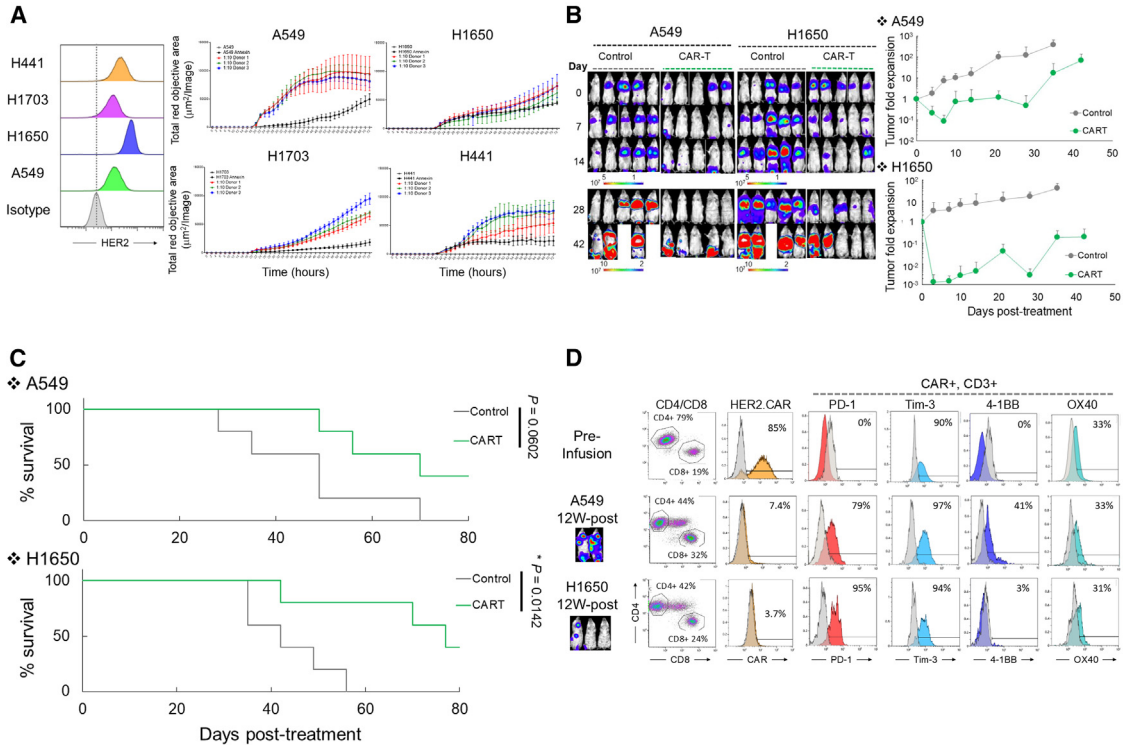

Figure 2. HER2.CAR-T cells control NSCLC growth both *in vitro* and *in vivo*

(A) HER2 expression was analyzed by flow cytometry on NSCLC cell lines. Isotype antibody staining served as a negative control. NSCLC cell lines were seeded into 48-well plates. After 24 h, HER2.CAR-T cells derived from three healthy donors were co-cultured with tumors cells (effector-to-target ratio [E:T] = 1:20). The co-culture was then stained with Annexin, and the total red objective area was measured to quantify cell death via Incucyte over 72 h. Data are presented as means ± SD (n = 3). (B) A549 and H1650 cells expressing firefly luciferase (ffluc) were orthotopically transplanted via tail vein injection to NSG mice. This experiment was performed concomitantly with the study shown in Figure 1, and the control animals are the same. A total of 1×10^6 HER2.CAR-T cells or vehicle control was systemically administered 14 days after tumor inoculation. Bioluminescence of A549 or H1650 tumor cells was monitored at different time points. Data are presented as means ± SD (n = 5). (C) Kaplan-Meier survival curve after administration of HER2.CAR-T cells. The humane endpoint was established as body weight <80% from baseline (n = 5). p values were determined using the log rank Mantel-Cox test. Statistical significance set at $p < 0.05$, ns > 0.05. The experiment was repeated for purposes of tissue collection, with similar trends for all replicates. (D) T cells were isolated from the lungs of 2–3 mice 12 weeks post-infusion to ensure sufficient T cell numbers for analysis, and HER2.CAR expression and markers of T cell activation/exhaustion from bulk HER2.CAR-positive T cells were analyzed by flow cytometry.

expressing IL-12p70. These data suggest that forced expression of IL-12p70 can maintain CAR-positive T cells at the tumor site regardless of the type of viral vector used to deliver the therapeutic transgene. We next analyzed activation and exhaustion markers on these CAR-positive T cells (Figures 4C and S6). Although CAR-T treatment alone resulted in terminally differentiated CD8 T cells 2 weeks post-administration, combination treatment instead increased effector memory CD8 T cells, which improved the persistence of CAR-positive CD8 T cells, unlike mice treated with CAR-T alone. We previously reported that IL-12p70 gene therapy maintained a CAR-positive CD4 T cell subset.[5] In this study, we also found that IL-12p70 derived from oNDV attenuates exhaustion of CAR-positive CD4 T cells, leading to better and more persistent anti-tumor effects through CAR-T cells. Since we did not find any toxicity (e.g., weight loss) in mice treated with combination therapy, these data indicate that local expression of IL-12 through systemically delivered oNDV is safe and attenuates CAR-T cell differentiation/exhaustion at the tumor site.

DISCUSSION

In this study we demonstrated that lentogenic oNDV expressing IL-12p70 enhances the anti-tumor activity of adoptively transferred HER2.CAR-T cells in orthotopic models of NSCLC. Compared with CAR-T cell administration alone, the adjunct use of oNDV-derived IL-12 led to long-term CAR-T cell persistence with tumor control because of its attenuation of terminal differentiation and exhaustion.

OVs based on human pathogens readily infect and replicate in human tumors, and systemic OV treatment has shown safety and anti-tumor efficacy in some patients.[11] Broader applicability is stymied by NAbs that are triggered by these OVs, impeding their desired systemic trafficking. For example, the OV enadenotucirev, constructed from the human pathogen adenovirus, was safely administered intravenously to patients with advanced solid tumors in a phase 1 clinical trial in which 5 of 61 patients achieved stable disease. However, increased NAb activity after the first treatment cycle inhibited oncolytic activity

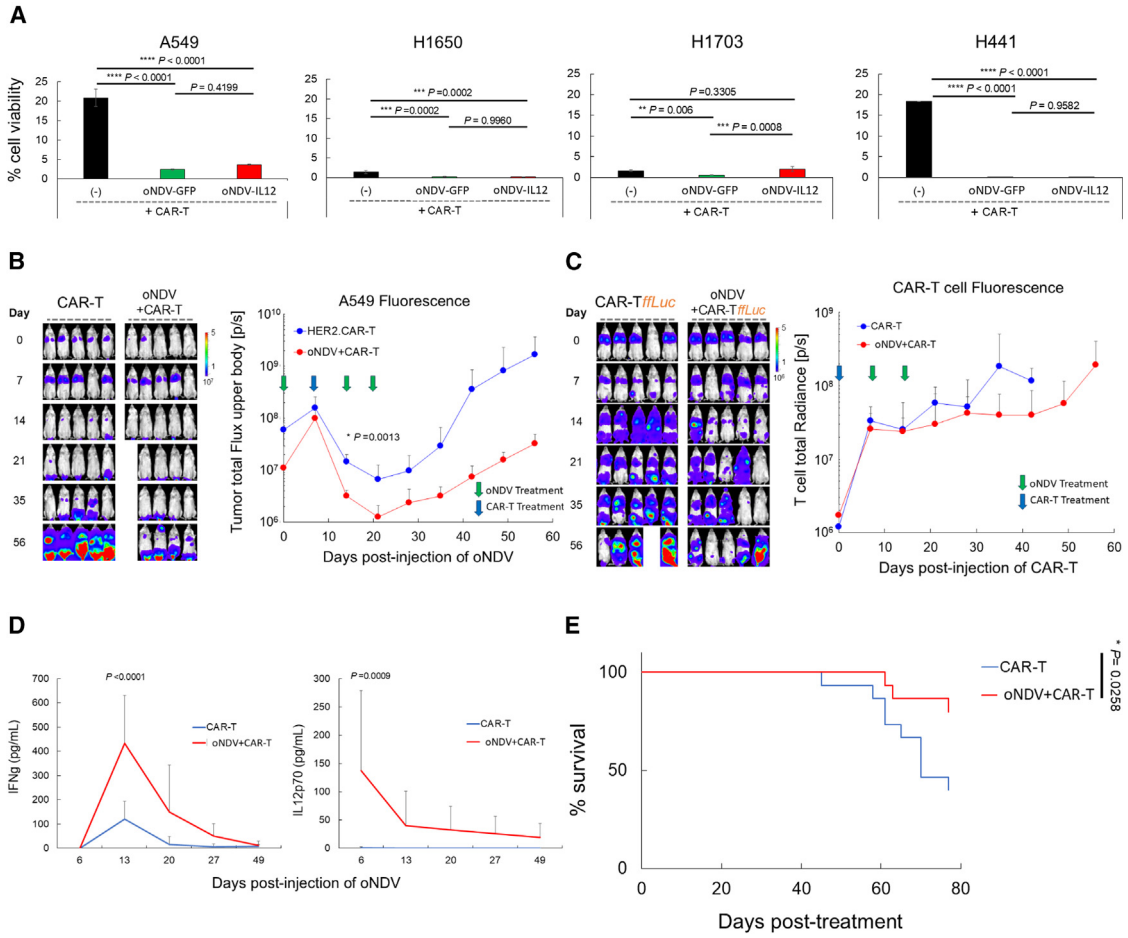

Figure 3. Combination of oNDV*IL12* and HER2.CAR-T cell enhances anti-tumor activity of HER2.CAR-T cells

(A) NSCLC cell lines expressing firefly luciferase (ffluc) were infected with 0.1 PFU/cell of oNDV*EGFP* or oNDV*IL12*. HER2.CAR-T cells were added to all conditions 24 h post-infection (E:T = 1:20; uninfected cells [–] served as a negative control). Cells were harvested 72 h post-co-culture, and viable cancer cells were analyzed by luciferase assay. Data are presented as means ± SD ($n = 4$), relative to untreated tumor cells. (B) A549 cells expressing ffluc were orthotopically transplanted via tail vein injection to NSG mice. On day 10 after tumor inoculation, 1×10^8 PFU oNDV*IL12* was administered via tail vein injection, with subsequent doses given at weeks 2 and 3 (total of 3×10^8 PFUs) (green arrows). HER2.CAR-T cells were systemically administered 7 days after the first dose of oNDV (blue arrow), and mice without oNDV treatment served as controls. Bioluminescence of A549 tumor cells was monitored at different time points. Data are presented as means ± SD ($n = 5$). (C) A549 cells were orthotopically transplanted via tail vein injection to NSG mice. On day 10 after tumor inoculation 1×10^8 PFU oNDV*IL12* was administered via tail vein injection, with subsequent doses given at weeks 2 and 3 (total of 3×10^8 PFUs). HER2.CAR-T cells expressing ffluc were systemically administered 7 days after the first dose of oNDV, and mice without oNDV treatment served as controls. Bioluminescence of HER2.CAR-T cells was monitored at different time points. Data are presented as means ± SD ($n = 5$). (D) Serum samples were collected 6, 13, 20, 27, and 49 days after initial injection of oNDV, and IL-12p70 and IFNγ levels in serum were measured by ELISA. Data are presented as means ± SD ($n = 15$, data were combined from three independent experiments). (E) Kaplan-Meier survival curve after treatment. The humane endpoint was established as body weight <80% from baseline ($n = 15$, data were combined from three independent experiments). p values were determined using the log rank Mantel-Cox test. Statistical significance set at $p < 0.05$, ns > 0.05.

during subsequent treatment cycles.[12] In contrast, several studies have shown that NDV-based OV induces favorable anti-tumor activity in a range of human cancer types *in vitro* and *in vivo*, indicating that the use of OVs derived from such non-human pathogens should reduce the potential for early systemic neutralization.[2] As observed in a phase 1 trial evaluating oNDVs, among 32 patients with advanced solid tumors, only one patient had detectable NAbs upon initial treatment, which differs from results typically observed in seroprevalence studies of adenoviruses.[13] Although most patients ultimately developed NAbs over the course of treatment, the Ab titer plateaued at a moderate level even after multiple courses of intravenous oNDV.[13] Nonetheless, oNDV administered as a single agent is insufficient to cure solid tumors.[14]

As an alternative, CAR-modified T cells show promise as cancer immunotherapy agents for hematologic malignancies, but the immunosuppressive solid TME is a lingering obstacle that inhibits T cell anti-tumor activity.[15] We previously demonstrated that treatment

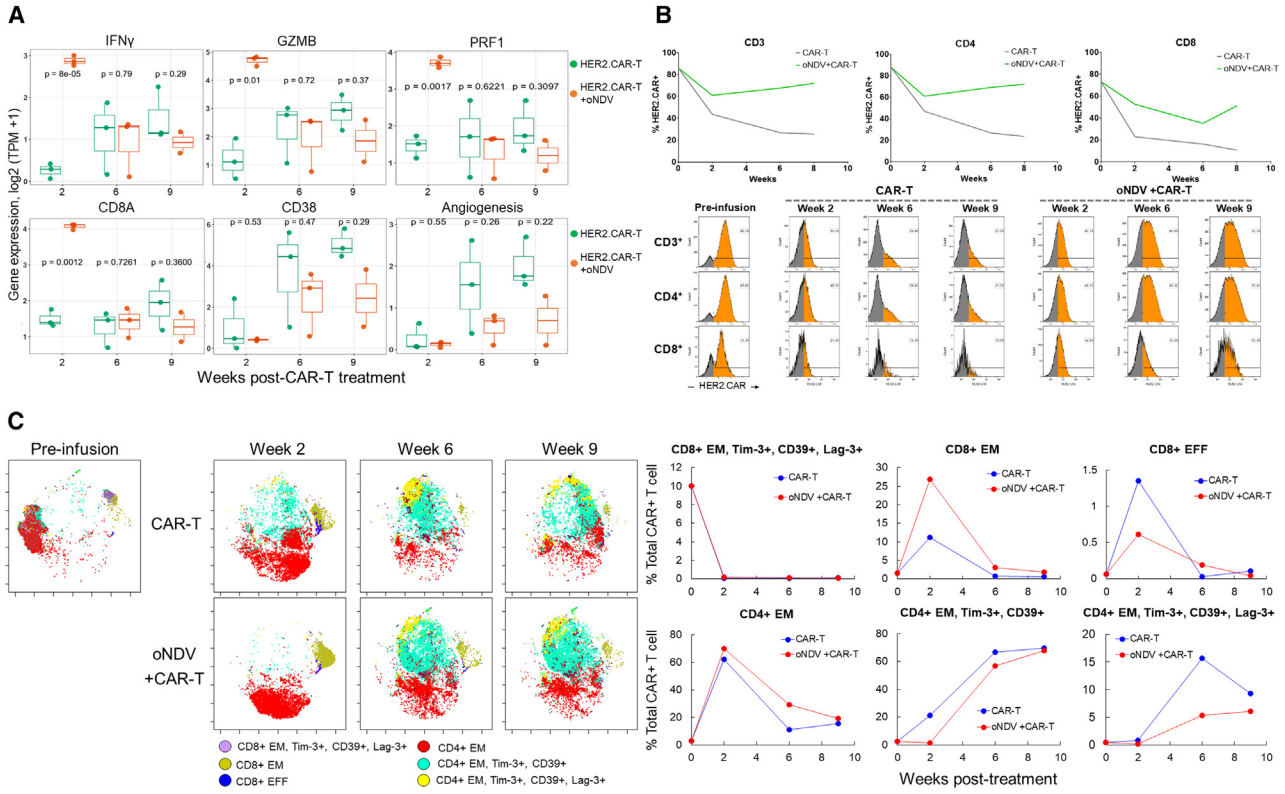

Figure 4. IL-12 derived from oNDV improves CAR-positive T cell persistence *in vivo*

(A) A549 cells were orthotopically transplanted via tail vein injection to NSG mice. On day 10 after tumor inoculation, 1×10^8 PFU oNDV*IL12* was administered via tail vein injection, with subsequent doses given at weeks 2 and 3 (total of 3×10^8 PFUs). HER2.CAR-T cells expressing firefly luciferase (ffluc) were systemically administered 7 days after the first dose of oNDV, and mice without oNDV treatment served as controls. Lungs from treated mice were collected 2, 6, and 9 weeks post-administration of CAR-T cells and subjected to RNA sequencing. Lungs from untreated mice and pre-infusion CAR-T cells served as controls ($n = 3$ for each condition). (B and C) A549 cells were orthotopically transplanted via tail vein injection to NSG mice. On day 10 after tumor inoculation, 1×10^8 PFU oNDV*IL12* was administered via tail vein injection, with subsequent doses given at weeks 2 and 3 (total of 3×10^8 PFUs). HER2.CAR-T cells were systemically administered 7 days after the first dose of oNDV, and mice without oNDV treatment served as controls. T cells were harvested from lungs of 2–3 mice 2, 6, and 9 weeks post-administration of CAR-T cells to ensure sufficient T cell numbers and stained for flow cytometry and phenotyping.

with local oncolytic adenovirus expressing IL12p70 enhances the subsequent activity and persistence of CAR-positive T cells in several solid tumor models.[5,6,10,16] IL-12p70 is known to polarize T cells toward a T_H1 subset, which enhances CAR-T anti-tumor activity and proliferation.[17] Although systemic administration of recombinant IL-12p70 is toxic,[18] we hypothesized that the systemic delivery of oNDV expressing IL-12p70 to tumor sites would locally enhance CAR-T cell activity.

Using a panel of human NSCLC cell lines, we confirmed that lentogenic oNDV is infectious and expresses transgenes. Although lentogenic NDV is known to have limited lytic capacity compared with other NDV strains, we demonstrated dose-dependent oncolysis in four NSCLC lines *in vitro*; oNDV alone, however, had no significant anti-tumor activity in xenograft mouse models. In comparison, HER2.CAR-T cells were able to effectively target NSCLC xenograft tumors and prolong animal survival. We confirmed surface expression of HER2 on the NSCLC cell lines used in this study and previously demonstrated that our HER2-directed CAR-T cells cannot control HER2 knockout cancer cell growth,[10] consistent with anti-tumor activity mediated by HER2 specificity. Although the HER2.CAR-T cells initially controlled tumor growth, tumors eventually relapsed, and phenotypic analysis of adoptively transferred T cells revealed the absence of CAR-positive cells. While residual tumors also show reduced antigen expression, we have previously demonstrated that HER2.CAR-T cells can target these low-antigen-expressing cells.[5] Together, these data demonstrate that the anti-tumor activities of either oNDV or CAR-T cells as single agents are insufficient to cure tumors in xenograft mouse models.

To exploit the benefits of each individual agent (i.e., direct oncolysis and local transgene expression from oNDVs and systemic tumor targeting by CAR-T cells), we demonstrated the complementary potential of this combined immunotherapy. Infection of NSCLC cells with a sub-therapeutic dose of oNDV sensitized them to subsequent HER2.CAR-T cell killing *in vitro*. The enhanced tumor cell killing

in the combination setting is independent of the transgene expressed by the oNDV, as both oNDV*GFP* and oNDV*IL12* resulted in similar levels of tumor cell death when combined with CAR-T cells. Since oNDV infection leads to caspase activation in infected cells,[2] a sub-therapeutic dose may activate this pathway within infected cells, sensitizing cancer cells to cytotoxic molecules (e.g., granzyme) derived from CAR-T cells.

We and others have demonstrated the advantages of IL-12 for adoptive cell therapy, including persistence and maintenance of CAR expression on adoptively transferred T cells in various cancer types.[5,13,16,17] Although clinical trials using systemic recombinant IL-12p70 were halted due to severe toxicity with high doses (500 ng/kg),[19] later evidence indicates that using OVs to express cytokines directly at the tumor site reduces the circulating concentration of the cytokine and its associated toxicities and provides support to effector cells where it is needed. We recently demonstrated that local treatment of our binary oncolytic adenovirus expressing IL-12p70 shows minimal circulation of IL-12p70 in the blood of patients, but this treatment led to durable anti-tumor activity in our study subjects.[20] As a single agent, oNDV*IL12* provides no survival advantage; however, its combination with a sub-curative dose of HER2.CAR-T cells significantly prolonged animal survival. To investigate the mechanism of this synergy, we performed RNA sequencing of NSCLC-bearing whole lung at different post-treatment time points. We found that a combination treatment of oNDV with HER2.CAR-T cells increases the Th1 cytokine IFNγ and Th1-related genes (e.g., granzyme B) at an early time point (2 weeks post-treatment). This correlates with CAR-positive CD8 T cell numbers, specifically the effector memory population, in the lung of mice treated with the combination. We also found delayed differentiation of CAR-positive CD4 subsets in the terminally differentiated/exhausted subset in animals receiving oNDV. Although IL-12 has been shown to hinder the formation of CD8 T cell memory precursors in a bacterial infection mouse model,[21] we and others demonstrated that IL-12 promotes the persistence of CAR-positive T cells with long-term anti-tumor effects in numerous tumor models.[5,17] In this study, we showed that IL-12 attenuates CAR-positive T cell differentiation (both CD4 and CD8 subsets) in orthotopic NSCLC tumors. These studies in immunodeficient mice are limited in their ability to evaluate the contribution of endogenous immune responses and "on-target, off-tumor" or immune-mediated toxicities. We have previously demonstrated in immune humanized mouse models that while the immunosuppressive TME reduces the effectiveness of CAR-T cell treatment, combination therapy with IL-12 gene therapy safely enhances the anti-tumor activity of adoptively transferred CAR-T cells and endogenous immune responses.[16] We expect that CAR-T and oNDV combination therapy would provide similar results in future studies. We are currently conducting a clinical trial investigating the combination of local oncolytic Ad gene therapy expressing IL-12 and systemic HER2.CAR-T cells (ClinicalTrials.gov: NCT03740256) to discover whether IL-12 expression at the tumor site can also promote the persistence of CAR-T cells in patients. To date, no on target, off-tumor toxicities have occurred in patients, providing further evidence of the safety of this approach.

Overall, we have demonstrated that while systemically administered oNDV alone has little anti-tumor activity, oNDV sensitizes tumor cells to CAR-T cell-mediated killing, and oNDV*IL12* provides support to HER2.CAR-T cells to further enhance their persistence *in vivo*. Patients with advanced disease often present with multiple metastatic lesions, which cannot all be targeted with direct intratumoral administration. Systemic gene and cell therapies allow the treatment of multiple tumors with a single administration and provide a way to target inaccessible or uninjectable tumors. Given the data presented here, the combination of systemic oNDV with CAR-modified T cells presents a potential alternative approach as either a different modality or treatment vehicle for patients with metastatic disease.

MATERIALS AND METHODS

oNDVs

Lentogenic NDV-based oNDVs expressing EGFP or IL-12 were obtained from AstraZeneca. These transgenes were cloned between P and M genes, and viruses were amplified in embryonated chicken eggs as described previously.[22]

Cell lines

Human NSCLC lines A549, H1650, H1703, and H441 were obtained from ATCC (Manassas, VA). Cell lines were authenticated utilizing short tandem repeat (STR) profiling by ATCC. Cells were cultured under the conditions recommended.

Primary cells

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus according to the manufacturer's instructions (Axis-Shield, Norton, MA). The vector encoding the HER2-directed CAR incorporating the CD28 costimulatory endodomain (second-generation HER2.28ζ.CAR), the fusion protein EGFP-*ffLuc*, and the methodology for the production of retrovirus and CAR T-cells have been described previously.[5,6] Briefly, PBMCs were activated with OKT3 (1 μg/mL) (Ortho Biotech, Bridgewater, NJ) and CD28 Abs (1 μg/mL) (Becton Dickinson, Franklin Lakes, NJ) and fed every 2 days with media supplemented with 10 ng/mL recombinant human IL-7 and 5 ng/mL IL-15. On day 3 post-OKT3/CD28 T blast generation, activated T cells (0.125×10^6/mL) were added to HER2.28ζ. CAR retroviral coated plate and centrifuged at $400 \times g$ for 5 min.

Co-culture experiments

NSCLC cell lines expressing GFP*ffLuc* were seeded and infected with 0.01 plaque-forming unit (PFU) per cell of oNDVs as described in the figure legends. HER2.CAR-T cells were added 48 h post-infection at a 1:20 effector-to-target ratio and cultured for 3 additional days. Residual cancer cells (*ffLuc* activity) were measured using the Luciferase Assay System (Promega, Madison, WI) and a plate reader (BMG LABTECH, Cary, NC).

Cytokine analysis

We collected medium samples from cultured plates and blood samples from xenograft mouse model at several time points (see results and the figure legends). Cytokine levels in media were measured using

BD Cytokine Bead Array kits according to the manufacturer's instructions (BD Biosciences, San Jose, CA).[23]

Flow cytometry

We used the following fluorochrome-conjugated monoclonal Abs: anti-human HER2 (24D2), CD3 (clone UCHT1), CD4 (SK3), CD8 (RPA-T8), CD25 (2A3), TIM-3 (7D3), CD278 (DX29), LAG-3 (T47-530), CTLA-4 (BNI3), CD134 (ACT35), CD137 (4B4-1), CCR7 (3DI2), CD45RO (UCHL1), PD-1 (MIH4), CD39 (TU66), recombinant HER2-Fc chimera, and anti-Fc (for detection of the HER2.CAR) (BD Bioscience; Beckman Coulter, Brea, CA; BioLegend, San Diego, CA). Cells were stained with these Abs for 30 min at 4°C. We determined live/dead discrimination via exclusion of 7AAD-positive cells (BD Pharmingen) or BD Horizon Fixable Viability Stain 780-positive cells (BD Bioscience). Stained cells were analyzed using a Gallios flow cytometer (Beckman Coulter) or BD Symphony (BD Bioscience) at the Flow Cytometry Core of Texas Children's Hospital. We analyzed data with Kaluza software v.2.2.1 (Beckman Coulter) and Cytobank (Cytobank, Mountain View, CA) according to the manufacturer's instructions.

Animal experiments

The Baylor College of Medicine Institutional Animal Care and Use Committee approved all animal experiments.

For the orthotopic xenograft models, 0.5×10^6 A549 cells or 1×10^6 H1650 cells expressing ffLuc were resuspended in a volume of 200 μL PBS and systemically injected via the tail vein of 7- to 8-week-old NSG male and female mice. Two weeks post-transplant, oNDV-IL12 and/or HER2.CAR-T cells were injected in a volume of 100 μL via the tail vein as described in Figures 1C, 2B, 3C, and 4. Cancer cells and CAR-T cells expressing *ffLuc* were assessed using the IVIS Imaging System (Xenogen, Alameda, CA). The humane endpoint was established as body weight <80% from pre-treatment baseline. Blood was collected retro-orbitally at the indicated time points using a microhematocrit capillary tube (Fisher Scientific) and placed in a 1.5 mL microfuge tube containing 3.2% sodium citrate. Plasma was separated by centrifugation and stored at −80°C until use.

RNA sequencing

Lung samples were collected and snap frozen as described in Figure 4. RNA was isolated from tissue samples using MagNA Lyser Green Beads (Roche; 03358941001) and the PicoPure RNA Isolation Kit (Thermo Fisher Scientific; KIT0204). MagNA Lyser Green Beads were pre-chilled on dry ice while tissue was cut into small pieces. Tissue was then added to bead tubes and kept on dry ice until it was ready for lysis. 450 μL of extraction buffer from the PicoPure RNA Isolation Kit was added to each bead tube. Bead tubes were vortexed for 3 min and then chilled on wet ice for 1 min. This was repeated a total of 4 times. The bead tubes were centrifuged at 8,000 × *g* for 3 min. Supernatant was transferred from the bead tubes to Qiashredder columns and centrifuged at 16,000 × *g* for 2 min. Flow-through was transferred from Qiashredder column to a 1.5 mL RNase-free tube and kept on ice until it was ready for incubation at 42°C for 30 min. The PicoPure RNA Isolation Kit manufacturer protocol was then followed for RNA isolation with a modification of step b. 70% EtOH was added at a volume equal to that of the lysate and mixed well by pipetting. The optional DNase treatment included in the protocol was performed at the designated step. RNA validation was performed with a Nanodrop spectrophotometer and Agilent TapeStation.

Libraries for RNA sequencing were generated using the Kapa RNA HyperPrep Kit with RiboErase (HMR) Globin (Roche; 08308241702) according to the manufacturer's protocol, with the following specificities. An input of 100 ng of RNA per sample calculated from Agilent TapeStation DV200 values was used for cDNA generation. Final libraries were assayed with Agilent TapeStation D5000 to determine the quality and average fragment lengths. Final libraries were then assayed with the KAPA Library Quantification Kit to determine the final concentrations. Library sequencing was completed on an Illumina NovaSeq 6000 with a target pair-end read count of 100 M per sample. All samples achieved at least a total of 100 M reads.

Paired-end reads from the RNA sequencing were aligned to human hg38 and mouse mm38 reference genomes with STAR v.2.6.1d[24] using bcbio bulk RNASeq pipeline v.1.2.9 (https://bcbio-nextgen.readthedocs.io/en/latest/contents/bulk_rnaseq.html). Gene-level read counts and transcript per million (TPM) gene expression values were generated using Salmon v.1.6.0[25] and tximport.[26] Welch's t test was used to compare gene expression levels between mice treated with HER2 CAR-T vs. combination treatment at three time points.

Statistics and reproducibility

Numerical results are presented as means of two or more independent experiments (biological replicates). Data from three or more groups were analyzed by ordinary one-way ANOVA. The Wilcoxon matched-pairs test was used to compare two groups of paired data. Data were analyzed with GraphPad Prism 9.5.1.

DATA AND CODE AVAILABILITY

The data presented in this study are available upon request from the corresponding author.

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AUTHOR CONTRIBUTIONS

M.S., M.K.B., and N.D. conceptualized the study; M.S., A.R.S., D.M., C.E.P., G.W.B., E.T., S.A., and N.D. generated and analyzed data; M.S., N.D., and M.K.B. supervised the study and acquired funding; A.R.S. and M.S. drafted the original manuscript; and M.S., N.D., and M.K.B. reviewed and edited the manuscript. All authors read and approved the submitted manuscript.

DECLARATION OF INTERESTS

E.T., S.A., and N.D. are employed by AstraZeneca. This study received funding from AstraZeneca. M.K.B. serves on advisory boards for Marker Therapeutics, Allogene, Walking Fish, Abintus, Athenex-Kuur, Onk Therapeutics, Coya Therapeutics, Triumvira, Adaptimmune, Vor Therapeutics, and Tscan. M.K.B. has equity in Allovir, Tessa Therapeutic, Ltd., and Marker Therapeutics. M.S. received research funding from Tessa Therapeutic, Ltd., and AstraZeneca. M.K.B. has royalties from Takeda and Bellicum. M.S. was a scientific consultant and C.E.P. a consultant for Tessa Therapeutic, Ltd.

SUPPLEMENTAL INFORMATION

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