Reverse Translation Identifies the Synergistic Role of Immune Checkpoint Blockade and IL15 to Enhance Immunotherapy of Ovarian Cancer

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ABSTRACT

Immune checkpoint blockade (ICB) has changed the standard of care for many patients with cancer, yet no ICB is approved for ovarian cancer. We hypothesized that maintenance therapy with an IL15 "superagonist" (N-803) and ICB in combination could induce potent immune activation in ovarian cancer. Using flow cytometry, cytometry by time of flight analysis, and cytotoxicity assays, we analyzed patient samples from women with advanced epithelial ovarian cancer treated with N-803 for indications of PD-1/PD-L1 upregulation with this treatment. In addition, ICB and N-803 were evaluated in preclinical studies to determine the functional impact of combination therapy on natural killer (NK) cells *in vitro* and *in vivo*. We observed that N-803 stimulated initial NK-cell expansion in patient samples; however, proliferation was not sustained beyond 2 weeks despite continued treatment. This result was reverse translated back to the laboratory to determine the functional relevance of this finding. The addition of ICB with an antibody-dependent cellular cytotoxicity IgG1 antibody against PD-L1 (avelumab) or an IgG4 antibody against PD-1 (pembrolizumab) enhanced N-803 induced NK-cell function *in vitro*. Using models of human ovarian cancer and NK-cell adoptive transfer in mice, we showed enhanced antitumor control with N-803 and ICB, as well as a combination effect that enhanced NK-cell persistence and expansion *in vivo*. This work suggests that PD-1/PD-L1 blockade combined with IL15 signaling may overcome resistance to cytokine therapy in ovarian cancer.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy. The estimated 5-year survival is 46% when considering patients diagnosed at all ovarian cancer stages and 28% for those diagnosed with distant disease. Notably, 62% of women with ovarian cancer present with stage III or IV disease, for which the rate of recurrence is 60% to 70% (1). Ovarian cancers are immunogenic and elicit spontaneous antitumor immune responses (2, 3). Some of the strongest evidence linking antitumor immunity and cancer has been in the ovarian setting (4–6). The first evidence of the role of immunosurveillance against human ovarian cancer was the discovery of tumor-infiltrating lymphocytes (TIL), the presence of which strongly correlated with patient survival (4). Patients whose tumors contained TILs had 5-year overall survival (OS) rates of 38%, whereas in patients whose tumors lacked TILs the OS was 4.5%.

In the past decade, several immune-based interventions have gained regulatory approval for multiple solid tumors and hematologic malignancies. Unfortunately, there are currently no FDA-approved immune therapies specifically for ovarian cancer. The importance of the immune system in ovarian cancer has been demonstrated by favorable prognostic implications, such as the presence of TILs, but contradictory outcomes have been reported with increased PD-L1 expression on ovarian cancer cells (4, 7, 8). Different immunotherapeutic approaches for the treatment of recurrent ovarian cancer (ROC) are currently being tested, including immune checkpoint blockade (ICB), autologous T-cell infusions, vaccines, and combinations of biologic and immune stimulatory agents (9–15). Recognizing that immunosuppression is a major hindrance for lymphocytes to kill cancer, approaches with various cytokines to induce inflammation have been tested. Preclinical and early-phase clinical trial data show that cytokines such as IL2 and others are often limited by toxicity and activation of regulatory T cells (Treg; refs. 16–18). The use of cytokines clinically has provided proof of concept that they can stimulate T cells and natural killer (NK) cells, which can impact tumor progression. One such strategy is the development of an IL15 "superagonist complex" called N-803 (formerly ALT-803). By broad consensus, the NCI Immunotherapy Workshop ranked IL15 as the #1 agent with "high potential for immunotherapy" (19).

The IL15 superagonist complex N-803 (IL15N72D:IL15RαSu/IgG1 Fc complex; ImmunityBio) was created to overcome some of the biologic, regulatory, and commercial limitations of unmodified *E. coli*–derived recombinant human IL15 (rhIL15). N-803 contains an IL15 mutant with a single substituted amino acid (rhIL15N72D) that has a 4-fold increase in biologic activity when compared with wild-type IL15 (20). Previous studies have shown that the biologic activity of IL15 can be increased 50-fold by administering preformed complexes of IL15 and soluble IL15R, which has a longer half-life than rhIL15 (21, 22). The IL15:IL15R complex increases activity at lower concentrations, and the fusion with IgG1 Fc increases serum half-life,

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providing more ideal pharmacokinetics with prolonged cytokine function (23). IL15, in contrast to IL2, does not support the expansion of Tregs (22). Thus, IL15 administration holds promise to specifically boost NK-cell cytotoxicity without the undesired stimulation of Tregs. Because a clinical study has correlated NK-cell numbers to positive outcomes in ovarian cancer, we have previously evaluated N-803 in preclinical ovarian cancer models, to determine whether it could enhance NK-cell expansion and thus outcomes, but noted that *in vivo* effects seemed restrained compared with *in vitro* findings (24, 25).

On the basis of preclinical nonhuman primate and early-phase clinical trial data, IL15 regimens can potently increase NK-cell numbers to augment immunotherapy (26–31). Given those findings, we analyzed samples from a pilot phase II prospective randomized clinical trial (NCT03054909) that investigated N-803 administered subcutaneously or intraperitoneally as maintenance therapy in advanced epithelial ovarian cancer following first-line chemotherapy. Using these specimens, we show here that N-803 treatment induced a negative feedback loop that resulted in restricted functionality and expansion of NK cells with subsequent N-803 treatments. Further immune monitoring and preclinical investigation of the mechanism behind this functional defect identified in the clinical trial samples pointed to induction of the PD-1/PD-L1 pathway upon N-803 treatment. On the basis of these findings, we set out to determine whether we could maximize NK-cell immune function in the setting of N-803 by the addition of ICB.

Materials and Methods

Patient samples

Blood and peritoneal washing samples were obtained from a pilot phase II study of N-803 given as maintenance therapy after the completion of first-line platinum-based adjuvant chemotherapy in stage III and IV high-grade serous ovarian, fallopian tube, and primary peritoneal cancer (clinical trial identifier: NCT03054909). All samples were collected under approved Institutional Review Board (IRB) number 00000013 at the University of Minnesota, Minneapolis, MN. Clinical investigation was conducted according to Declaration of Helsinki principles. Written informed consent was received from all participants prior to inclusion in the study.

In cycle 1, N-803 (10 mcg/kg) was administered either subcutaneously or intraperitoneally weekly for 4 weeks. After cycle 1, patients were administered N-803 subcutaneously weekly for an additional three cycles given on a 4-week on/4-week off schedule (four doses per cycle). Limited samples were obtained over cycle 2. Blood and peritoneal washings were processed, cryopreserved and stored by the Translational Therapy Shared Resource (Masonic Cancer Center, University of Minnesota, Minneapolis, MN).

Blood and peritoneal lymphocyte number, phenotype, and function were assessed prior to first N-803 treatment (pre–cycle 1) and at days 5, 8, 15, and 22 (prior to each subsequent dose). Detailed immune monitoring was performed and analyzed from specimens obtained from 7 patients. Immune monitoring of the peritoneal cavity was limited because of inability to obtain peritoneal samplings from each participant.

Blood and peritoneal washing (ascites fluid) were collected at the time of cytoreductive surgery from a pilot study of women undergoing surgery for ovarian cancer or benign conditions. No investigational agents were used. All samples were collected under approved IRB number 1610M96942 and 0702E01841 at the University of Minnesota, Minneapolis, MN. Clinical investigation was conducted according to Declaration of Helsinki principles. Written informed consent was received from all participants prior to inclusion in the study. The Translational Therapy Shared Resource (Masonic Cancer Center, University of Minnesota, Minneapolis, MN) lab processed, cryopreserved, and stored the samples. A total of 15 ascites samples from women with high-grade serous ovarian cancer were used to determine whether ascites mediated PD-L1 upregulation via IFNγ.

Immune cells and tumor cell lines

Healthy human donor blood was obtained from the Memorial Blood Centers (Minneapolis, MN) under the IRB-approved protocol 9709M00134, and processed by density gradient Ficoll-Paque (GE Healthcare) to isolate peripheral blood mononuclear cells (PBMC). PBMCs were cryopreserved in liquid nitrogen at a 90% mix of FBS (catalog no. 10437-028, Gibco) and 10% DMSO (catalog no. D2650-100 mL, Sigma). PBMCs were also processed and used without freezing to enrich NK cells (90%–95%) using the EasySep Human NK cell enrichment kit (catalog no. 19055, Stemcell Technologies) or to deplete $CD3^+$ and $CD19^+$ cells using positive selection kits (catalog nos. 17851 and 17854, Stemcell Technologies). All human samples, were obtained after approval from the IRB at the University of Minnesota (Minneapolis, MN), following written informed consent, received in compliance with guidelines by the Committee on the Use of Human Subjects in Research and in accordance with the Declaration of Helsinki.

The cell line MA148 [established locally at the University of Minnesota (RRID: CVCL_AK47)] is a human epithelial high-grade serous ovarian carcinoma cell line we obtained in 2017. OVCAR8, OVCAR5, OVCAR3, and SKOV3 were obtained in 2017 from the DTP, DCTD Tumor Repository sponsored by the Biological Testing Branch, Developmental Therapeutics Program, NCI, NIH (Frederick, MD; RRID: CVCL_1629). K562 cells were purchased in 2015 from ATCC (catalog no. CCL-243, RRID:CVCL_0004). For the spheroid assay and the *in vivo* experiments, MA148 and OVCAR8 cell lines were transfected with a luciferase reporter construct (pKT2/PKG-Bsd:GFP CLP-Luc) using Invitrogen's Lipofectamine Reagent (catalog no. 11668030, Thermo Fisher Scientific). Selective pressure was applied with 10 μg/mL of blasticidin (catalog no. ant-bl-05, Invivogen) for 2 weeks. Cells were than sorted by GFP expression followed by single-cell cloning and expansion. All cell lines were maintained in RPMI1640 medium (catalog no. 11875-119, Life Technologies) supplemented with 10%–20% FBS (catalog no. 10437-028, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (catalog no. 15140-122, Gibco) and 2 mmol/L L-glutamine (catalog no. 25030-081, Gibco). Tumor cell lines were incubated at 37°C with 5% CO_2. Ovarian cells were passaged using 0.05% trypsin–Ethylenediaminetetraacetic acid (catalog no. 25300-054, Gibco) for detachment when cells reached more than 90% confluence. K562 cells were split down to 2×10^5 cells two times per week. The cell lines were not further authenticated, but were expanded and frozen at passage 1–3 and were used 2–6 passages after thawing. The cell lines were tested quarterly for *Mycoplasma* using the ATCC Universal *Mycoplasma* detection kit (catalog no. 301012K, ATCC).

In vivo models

All animal studies were performed under protocols approved by the University of Minnesota Institutional Animal Care and Use Committee, Minneapolis, Minnesota. Studies were conducted under guidelines for animal welfare provided by the NIH. NOD/SCID/$\gamma c^{-/-}$ (NSG) mice (Jackson Laboratories) were used for all *in vivo* experiments.

Female (NSG) mice were given 2×10^5 MA148 or 1×10^5 OVCAR8 luciferase-expressing tumor cells via intraperitoneal injection 3 days

prior (D-3) to the mice undergoing irradiation (225 cGy), and bioluminescent imaging (BLI; D0). BLI was used to ensure tumor engraftment. For the purposes of this study, NK cells were obtained from an unidentified donor from the Memorial Blood Centers (Minneapolis, MN), cells were CD3/CD19 depleted by magnetic beads (catalog nos. 17851 and 17854, Stemcell Technologies) and incubated overnight with 10 ng/mL NCI IL15 prior to administration into mice. Four days after tumor injection, 1×10^6 CD3/CD19-depleted human NK cells were given intraperitoneally to mice. On D1, pembrolizumab (Keytruda, Merck), N-803 (ImmunityBio), or avelumab (Bavencio, Pfizer) were administered simultaneously with the NK cells. BLI was used to track tumor growth weekly using the IVIS 100 Spectrum *in vivo* Imaging system (PerkinElmer). A total of 100 μL of 30 mg/mL Luciferin substrate (catalog no. Lucna-1G, Gold BioTechnology) was injected into mice 10 minutes prior to imagining. Images were analyzed with living Image 4.5 software (Xenogen Corporation) and readings were placed onto the same scale for comparison. Mice received intraperitoneal injections of N-803 (1.25 μg/injection) and either pembrolizumab (100 μg/injection) or avelumab (100 μg/injection) twice a week (Tuesdays and Fridays).

For the infiltration model, 14 days prior to NK-cell injection, female NSG mice were injected intraperitoneally with 1×10^6 firefly luciferase-expressing OVCAR8 tumor cells (D-13). On day 0 (D0), 24 hours prior to NK-cell injection, mice were sublethally irradiated (225 cGy) and analyzed for presence of tumor cells by BLI using the IVIS Spectrum In Vivo Imaging system (PerkinElmer). Day 1 (D1) mice were given intraperitoneal injections of NK cells simultaneously with N-803 (1.25 μg/injection) and/or pembrolizumab (100 μg/injection). Drug injections were administered twice a week for 3 weeks and BLI was used to track tumor growth weekly. After 3 weeks of treatment, mice were euthanized and tissues were harvested. The tumors were identified based on gross examination and cut to a thickness of 2 to 5 mm. They were fixed with 10% formalin (catalog no. 23-305510, Thermo Fisher Scientific) for 24 hours. Following fixation, samples were rinsed and placed in 80% ethanol (catalog no. 2801, Decon Laboratories) for processing by the BLS Histology and Research laboratory (University of Minnesota, Minneapolis, MN). Samples were processed on a Leica ASP300S tissue processor (Leica Biosystems) and then embedded into molds and paraffin blocks using Richard Allan Paraffin Type 6 (catalog no. 8336, Thermo Fisher Scientific). Leica RM2255 rotary microtome (Leica Biosystems) cut the slides 4 μm in thickness. Hematoxylin and eosin staining was performed using the H&E kit from Vector laboratories (catalog no. H-3502). The specimens involved by ovarian cancer cells were stained with anti-human granzyme B (Abcam, catalog no. ab208586, RRID: AB_2924920) and anti-human CD3ζ (Abcam, catalog no. 188850, RRID:AB_2910545). IHC staining was performed according to manufacturer's specifications using Horse anti-Mouse IgG ImPRESS Secondary Antibody (catalog no. MP-7402-15, Vector Laboratories) or Horse anti-Rabbit IgG ImmPRESS Secondary Antibody (catalog no. MP-7401-50, Vector Laboratories).

Pathology data analysis

The tissue sections stained with granzyme B and CD3ζ were evaluated for areas within the tumor with relatively highest content of infiltrating immune cells. The positively stained NK cells with granzyme B or CD3ζ were counted under the microscope, selecting 5 nonoverlapping microscopic fields of view with relatively highest content of NK-cell infiltrate in each animal. The intratumoral NK cells were at 100×10 magnification, corresponding to field of view diameter 0.022 mm (Olympus BX46 microscope). Extratumoral NK cells were counted per 40× field of view (corresponding to field of view diameter 0.055 mm).

ELISA for *in vitro* detection of IFNγ

Serum from patients treated with N-803 was tested by the Cytokine Reference Laboratory (University of Minnesota, Minneapolis, MN) for IFNγ production. Samples were assayed according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique (ELISA; catalog no. DIF50C, R&D Systems). The samples were run neat and absorbance was measured on the microtiter plate reader (EPOCH). Samples were tested in duplicate and values were interpolated from a LOC-LOG fitted standard curve.

Measuring PD-L1 expression in ovarian cancer cells

Tumor cells lines were passaged for 1 week before being treated with 500 U/mL IFNγ (catalog no. 285-IF-100/CF, R&D Systems) overnight in a 12-well plate to assess PD-L1 expression. For the IFNy inhibition assays, tumor cells were plated overnight in a 12-well plate. The next day tumor cells were treated with media only, 500 U/mL IFNγ (catalog no. 285-IF-100/CF, R&D Systems), or 500 U/mL IFNγ (catalog no. 285-IF-100/CF, R&D Systems) and 10 μg anti-hIFNγ (R&D Systems, catalog no. MAB2851, RRID:AB_2123305) overnight. For studies evaluating the effects of patient ascites fluid, tumor cells lines were incubated overnight with ascites fluid obtained from several patients with ovarian cancer at the time of cytoreductive surgery, excluding ascites cells (IRB number 1610M96942). For the IFNy inhibition assays with ascites fluid, tumor cells were plated overnight in a 12-well plate. The next day tumor cells were treated with 50% ascites fluid, media only, 500 U/mL IFNγ (catalog no. 285-IF-100/CF, R&D Systems) or 500 U/mL IFNγ (catalog no. 285-IF-100/CF, R&D Systems) and 10 μg anti-hIFNγ (R&D Systems, catalog no. MAB2851, RRID: AB_2123305) overnight. Following treatment with IFNy, IFNy blocking antibody or ascites fluid, tumor cells were stained with Live/Dead Fixable Aqua Staining Kit (catalog no: L-34966, Thermo Fisher Scientific), followed by APC-conjugated PD-L1 (catalog no. 329078, BioLegend, RRID:AB_940360). APC-conjugated IgG2b k Isotype control (MPC-11, BioLegend, catalog no. 400320) was used as the PD-L1 isotype control. Data acquisition was conducted on LSRII machines at 60 seconds/sample, at high setting. FlowJo version 10.8.1 was used to analyze the data.

Mass cytometry assay

Cells from patients in the phase II pilot study were counted and viability was measured using trypan blue exclusion. Two hundred thousand cells from each donor were aliquoted into 5 mL polystyrene U-bottom tubes for barcoding and cytometry by time of flight (CyTOF) staining. Cells were stained with Cisplatin (Fluidigm Product# 201064) followed by barcoding using the Cell-ID 20-Plex Pd Barcoding Kit (FluidigmProduct# 201060). After barcoding, all cells were combined into a single 5 mL polystyrene U-bottom tube and incubated in the surface marker antibody cocktail (Supplementary Table S1) for 30 minutes at 4°C. Following surface staining, cells were then fixed using 2% paraformaldehyde. For intracellular staining, cells were permeabilized by incubation with Triton X 0.1% for 5 minutes at room temperature, followed by incubation with intracellular antibody cocktail (Supplementary Table S1) for 30 minutes at 4°C. Stained cells were then incubated overnight with Cell-ID Intercalator (Fluidigm Product# 201192A). The following morning cells were washed and run on the CyTOF 2 instrument (Fluidigm). Wash steps were completed using either Maxpar PBS (Fluidigm Product# 201058), Maxpar Cell Staining Buffer (Fluidigm Product# 201068), or Millipure Water at

1600 RPM for 4 minutes. Data were visualized in viSNE (Cytobank Inc. RRID:SCR_014043).

Measuring CD107a degranulation and IFNγ cytokine production via functional assay

Healthy donor PBMCs, PBMCs from patients in the pilot phase II study, or enriched NK cells from healthy donors, obtained by following the EasySep Negative Human NK Selection kit (catalog no. 19055, Stem Cell), were treated with 1 nmol/L of N-803 overnight. Targets treated with recombinant human IFNγ (catalog no. 285-IF-100, R&D Systems) received 500 U/mL IFNγ overnight. The following day, effectors and targets were cocultured using a 2:1 effector to target ratio, with either addition of 10 μg/mL pembrolizumab (KEYTRUDA, Merck), 10 μg/mL avelumab (Bavencio, Pfizer), or no treatment (NT). A 10:1 effector to target ratio was used to evaluate CD107a degranulation in PBMCs from patients in the pilot phase II study against K562 cells, a myelogenous leukemia target cell line commonly used as a tool to evaluate NK-cell function. K562 cells were used at passage 3 to robustly activate the NK cells and induce potent degranulation. Cells were stained with FITC-conjugated anti-CD107a (catalog no. 328606, BioLegend RRID:AB_1186036) and left for an 1-hour incubation period. One hour after the addition of anti-CD107a, cells were exposed to Golgi Stop and Golgi Plug (catalog no. 554724 RRID: AB_2869012, catalog no. 555029 RRID:AB_2869014BD Biosciences), and were incubated for 4 hours. At the end of the 5-hour incubation period, cells were stained with Live/Dead Fixable Aqua Staining Kit (catalog no. L-34966, Thermo Fisher Scientific), then stained with PE-CY7–conjugated anti-CD56 (catalog no. 318318, BioLegend RRID: AB_604107), PE-CF594–conjugated anti-CD3 (catalog no. 562280, BD BioSciences RRID:AB_11153674), and APC-conjugated anti-CD8 (catalog no. 344722, BioLegend) with an optional APC-CY7–conjugated anti-CD16 (catalog no. 302018, BioLegend RRID:AB_314218) staining. The cells were then fixed, and permeabilized. Permeabilized cells were stained with BV650-conjugated IFNγ (catalog no. 502538, BioLegend RRID:AB_256360). Degranulation and cytokine production were assed for live NK-cell ($CD56^+CD3^-$) and $CD8^+$ T-cell ($CD8^+CD3^+$) populations. Data acquisition was conducted on LSRII machines at 60 seconds/sample, at high setting. FlowJo version 10.8.1 was used to analyze the data.

Spheroid assay

GFP-expressing OVCAR8 cells were seeded at 2×10^4 target cells in 100 μL/well in a 96-well U-bottom low adhesion plate (ULA plate, catalog no. 7007, Corning). The plates were spun at 40 g (500 RPM) in one orientation and then 80 g (700 RPM) in the opposite orientation without any brake applied to the centrifuge, to allow for a uniform monolayer to form. Images were then acquired using the IncuCyte live cell analysis system and quantified using the Incucyte S3 imager software (Sartorius Inc.) to determine spheroid formation by fluorescence detection of the spheroid mass. After 72 hours, spheroids formed and CellTrace labeled healthy donor peripheral NK cells were added with either addition of 1 nmol/L of N-803, 10 μg/mL avelumab (Bavencio, Pfizer) or NT to the culture. Images were taken every hour for 5 days to determine experiment outcome. The size of each spheroid was measured for each timepoint and normalized to the size of the spheroid at the time effectors and drugs were added.

Ki67 proliferation assay

Peripheral blood from 7 patients in the pilot phase II study were surface stained using APC-eFluor–conjugated anti-CD19 (Thermo Fisher Scientific, catalog no. 47-0199-42, RRID:AB_1582230), PE-Texas Red–conjugated anti-CD3 (Thermo Fisher Scientific, catalog no. MHCD0317, RRID:AB_10376002), APC-conjugated anti-CD25 (BD Pharmingen, catalog no. 555434, RRID:AB_398598), PerCP/Cyanine5.5-conjugated anti-CD4 (BioLegend, catalog no. 300530, RRID:AB_893322), BV500-conjugated anti-CD45 (BD Horizon, catalog no. 560777, RRID:AB_1937324), BV605-conjugated anti-CD56 (BD Pharmingen, catalog no. 562780, RRID:AB_2728700), and BV650-conjugated anti-CD8a (BioLegend, catalog no. 301042, RRID:AB_2563505). Cells were then treated with FoxP3 fixation/permeabilization solution and washed with permeabilization buffer (catalog no. 00-5223-00, eBioScience) using the protocol suggested by company. After cells were stained with Pacific Blue–conjugated anti-Foxp3 (BioLegend, catalog no. 320216, RRID:AB_2104902) and PE-conjugated anti-Ki67 (BD Pharmingen, catalog no. 556027, RRID: AB_2266296). As the Ki67 isotype control, the accompanying PE-conjugated IgG1 k Isotype control (BD Pharmingen, catalog no.556027 RRID:AB_2266296) was used. Data were acquired using LSRII machine. FlowJo version 10.8.1 was used to analyze the data.

Statistical analysis

Data presented are mean ± SEM unless otherwise noted. All graphs were produced and statistically analyzed using Prism version 6.0, GraphPad. We used unpaired *t* tests to compare the averages between two groups. We used an ANOVA and performed multiple comparisons by comparing the mean of each group with the mean of every other group for all of the experiments that had multiple treatments or timepoints. Experiments that were missing timepoints were analyzed by a mixed-effects model. Image analysis was performed using Living Image 4.5 software (PerkinElmer), and BLIs were calculated. We chose an α of 0.05 (95% confidence interval) and data were deemed significant if $P < 0.05$.

Data availability

The data generated in this study are available within the article and its Supplementary Data or from the corresponding author upon reasonable request.

Results

NK cells in patients with N-803–treated ovarian cancer demonstrate early expansion and function during treatment that is not sustained at later stages of treatment. Samples from 7 patients with ROC, treated with either subcutaneous ($n = 3$) or intraperitoneal administration ($n = 4$) of N-803, were used to evaluate the effect of N-803 treatment on the patient's immune system over time. Peripheral blood and peritoneal washings, collected at baseline and set timepoints after N-803 treatment, were evaluated in this analysis. In the peripheral blood of patients receiving N-803, we observed an increase in lymphocyte proliferation by flow cytometric analysis, depicted by a rise in Ki67 expression on NK cells and marginally on $CD8^+$ T cells from baseline (collected prior to first N-803 treatment) until day 8, and this started subsiding at day 15 (**Fig. 1A**; Supplementary Fig. S1A). Proliferation levels continued to drop through day 22, despite weekly dosing of N-803, and did not recover in the second cycle of dosing.

Because proliferation appeared to increase from baseline to day 8 on NK cells from patients treated with N-803, and subsided thereafter, we evaluated whether endogenous NK-cell cytolytic capabilities also displayed similar kinetics using an indirect measure of killing, NK-cell degranulation (surface CD107a expression). PBMCs collected prior to first N-803 treatment (pre–cycle 1) and on days 5, 8, 15, and 22 of cycle 1 were incubated with K562 cells, showing peak NK-cell

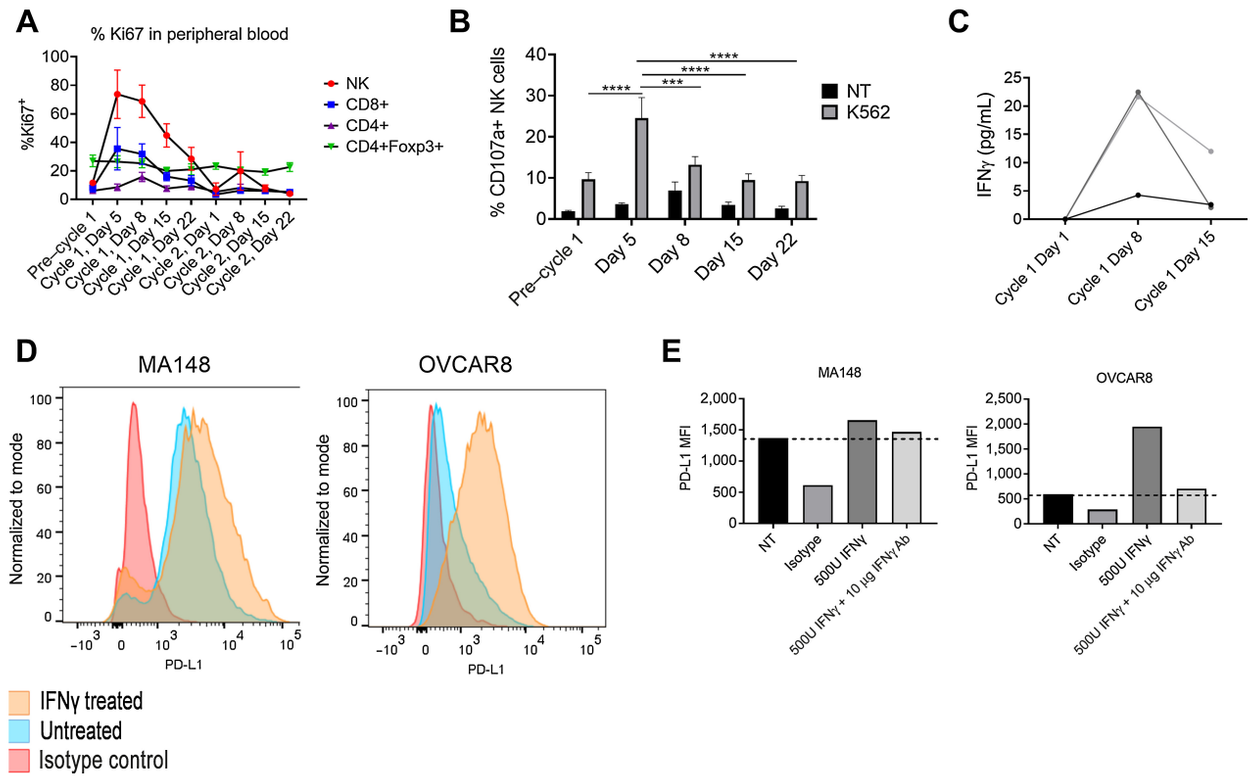

Figure 1.

NK cells from patients with N-803–treated ovarian cancer demonstrate increased expansion and function from baseline to day 15 that was not sustained at later stages of treatment. **A,** Average % of endogenous peripheral blood NK cells, $CD8^+$ T cells, $CD4^+$ T cells, and Tregs that are expressing Ki67, a marker of proliferation in patients treated with N-803 ($n = 7$). **B,** Evaluation of endogenous NK-cell degranulation (expression of surface CD107a) against K562 cell line at an effector to target ratio of 10:1 in PBMCS after N-803 treatment ($n = 7$). **C,** Systemic IFNγ production in the serum of 3 patients treated with N-803 (each line represents a patient). **D,** Representative plots of PD-L1 expression in ovarian cancer tumor cell lines incubated overnight with or without IFNγ. **E,** Representative quantification of PD-L1 expression in MA148 and OVCAR cell lines incubated overnight with or without IFNγ and treated with or without IFNγ neutralizing antibody ($n = 3$ independent experiments). One-way ANOVA was used to compare samples. *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P < 0.0001$. Error bars represent SEM.

degranulation at day 5; however, with repeat weekly dosing of N-803 on days 8, 15, and 22, there was a decrease in CD107a expression (**Fig. 1B**). In addition, we evaluated IFNγ levels in the serum of 3 patients at pre–cycle days 1, 8, and 15 of N-803 treatment. Systemic IFNγ production increased at day 8, indicating that N-803 was inducing its production, but decreased by day 15 of dosing (**Fig. 1C**).

Together, the data indicated that treatment of patients with ovarian cancer with N-803 initially gave rise to NK-cell expansion and cytolytic function, but that enhancement quickly subsided despite ongoing treatment. To evaluate the root cause of this, CyTOF analysis was performed on peritoneal washing samples obtained from 1 patient over multiple timepoints to simultaneously determine expression of several antigens at once (Supplementary Fig. S1B). Concordant with the PBMC flow cytometric data, Ki67 expression peaked at day 8 on peritoneal NK cells and then decreased. PD-1, an inhibitory checkpoint receptor, displayed low expression in the beginning of cycle 1, but steadily increased from days 15 to 29. NKG2D, an NK cell–activating receptor involved in natural cytotoxicity, peaked on day 15 and displayed downregulated expression by day 22 of treatment, albeit levels were still higher than at baseline. Perforin, a functional protein involved in cytotoxicity, did not seem to follow this pattern, and it stayed elevated from day 8 to day 29, providing evidence that the cytolytic machinery remains primed with N-803. Several of these changes in expression were also present, albeit to a lower extent for some proteins (Perforin), on cytolytic $CD8^+$ T cells. These data provide a hypothesis of why N-803 treatment only temporarily induces an enhancement of NK-cell function in patients: the upregulation of inhibitory PD-1 signaling shuts down the NK-cell response despite ongoing stimulus through N-803.

IFNγ induces PD-L1 expression on ovarian cancer cells

Because of the observed upregulation of PD-1 expression on immune cells following N-803 treatment, we reverse translated back into the laboratory to understand the significance of this finding. Our previous preclinical studies demonstrated that N-803 can potently induce IFNγ production on NK cells (24), a finding that is further supported by the systemic clinical data (**Fig. 1C**). Because this cytokine has been shown to induce PD-L1 upregulation on some tumor settings, we next explored the effect of IFNγ on ovarian cancer cells (32). PD-L1 expression was evaluated on several ovarian cancer cells lines incubated overnight with media alone or media supplemented with IFNγ (**Fig. 1D**; Supplementary Fig. S1C). IFNγ stimulation resulted in increased expression of PD-L1 in multiple ovarian cancer cell lines tested. In addition, blockade of IFNγ signaling using an IFNγ-specific neutralizing antibody resulted in prevention of PD-L1 upregulation beyond basal levels for each tumor, as a result of IFNγ inducing PD-L1

expression to different extents in our tumor models (**Fig. 1E**). Furthermore, ascites fluid, obtained from 9 patients with ovarian cancer at the time of cytoreductive surgery, was used to evaluate how the ovarian cancer soluble inflammatory environment affects PD-L1 expression. Incubation of ascites fluid, excluding ascites cells, with ovarian cancer cell lines induced PD-L1 expression on all the cell lines tested (Supplementary Fig. S1D), indicating the soluble microenvironment in patients with ovarian cancer, in the absence of N-803 treatment, has the ability to trigger expression of checkpoint ligands. To determine whether the patients with ovarian cancer ascites mediated PD-L1 upregulation via IFNγ, we cocultured MA-148 cells with ascites and IFNγ-blocking antibody (Supplementary Fig. S1E). The results indicated that in four of six ascites samples tested, the IFNγ blockade prevented PD-L1 upregulation, but in the remaining two it did not, highlighting the heterogenous nature of ovarian cancer and a possible different mechanism at play for PD-L1 upregulation in some tumor microenvironments. These results show that although N-803 induced an initial immune response based on the samples collected from patients in our clinical trial, induction of IFNγ secretion and inflammatory factors in the tumor microenvironment can lead to upregulation of checkpoint ligands.

Preclinical evaluation of checkpoint inhibitor in combination with N-803 treatment demonstrates amplification in NK-cell activity. On the basis of the clinical and preclinical results showing upregulation of checkpoint ligands, we wanted to determine whether checkpoint inhibitors can amplify the effect of N-803 on NK-cell activity *in vitro*. Antibodies that interfere with the PD-1/PD-L1 axis were tested in combination with N-803 to evaluate NK-cell degranulation (surface CD107a expression) and inflammatory cytokine (intracellular IFNγ) production in healthy donor peripheral blood against ovarian cancer cells. N-803 treatment alone resulted in increased CD107a and IFNγ production compared with NT (**Fig. 2A** and **B**). However, the combination of N-803 and the checkpoint inhibitor avelumab, which blocks PD-L1 but also can induce NK cell–mediated

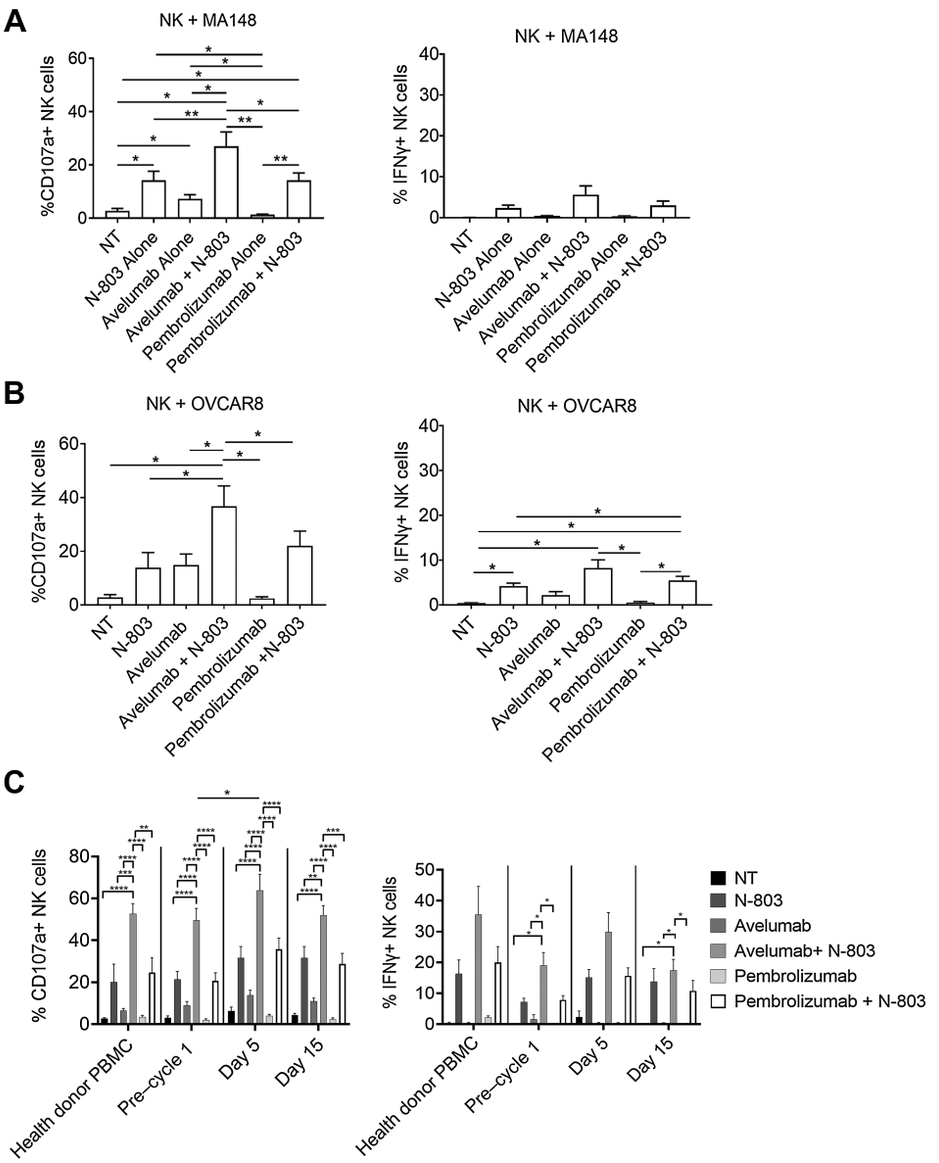

Figure 2.

Preclinical evaluation of checkpoint blockade and N-803 treatment induces enhanced activity in healthy donor peripheral NK cells. Healthy donor peripheral NK cells were incubated with or without N-803 and with or without MA148 (**A**) and OVCAR8 (**B**) tumor cells lines for 5 hours in the presence or absence of checkpoint inhibitors. The percentage of surface CD107a and intracellular IFNγ was measured ($n = 6$–9). **C,** Healthy donor and patient samples from the N-803 trial were treated the same as (**A** and **B**) with OVCAR8 tumor cells ($n = 3$–7). One-way ANOVA was used to compare all the treatments in samples in **A** and **B**. In **C**, a mixed-effect analysis was performed to evaluate the significant difference for each treatment within a time point (bracket) and to evaluate for difference between time points (bar). *, $P \leq 0.05$; **, $P \leq 0.01$, stats were not provided on $P \geq 0.05$ (**A**). Error bars represent SEM.

antibody-dependent cellular cytotoxicity (ADCC) against PD-L1–expressing targets, resulted in the highest CD107a and IFNγ expression on NK cells for all conditions tested. Combination of N-803 and pembrolizumab (PD-1–specific blocking antibody), did not significantly impact degranulation compared with N-803 alone, and only showed a mild increase in NK-cell production of IFNγ against OVCAR8 cells (**Fig. 2B**). IFNγ treatment of OVCAR8 cells overnight, which we showed upregulated PD-L1 expression on the tumor cells (**Fig. 1D**), resulted in more significant differences in degranulation between the N-803 alone group and the N-803 + checkpoint (avelumab or pembrolizumab) groups (Supplementary Fig. S2A, left), as well as a higher overall production of IFNγ by the Avelumab + N-803 group (Supplementary Fig. S2A, right). Similar results were observed when this assay was carried out with fresh PBMCs instead of enriched NK cells (Supplementary Fig. S2B–S2D). We also evaluated the combination of N-803 and checkpoint inhibitors on patients treated with N-803 and observed significant differences between the different treatments and the N-803 + Avelumab group (**Fig. 2C**). There was also a significant difference in CD107a degranulation between pre–cycle 1 (before first N-803 treatment) and day 5, similar to what we saw in **Fig. 1B**. Given the possible role of IL15 in $CD8^+$ T-cell activation, we evaluated $CD8^+$ T-cell degranulation and IFNγ production but saw no significant differences between dosing strategies (Supplementary Fig. S2E). In summary, the combination of N-803 with checkpoint inhibitors demonstrated maximal activity, with avelumab combination showing the strongest response, which may be due to an additive effect via ADCC.

Healthy donor peripheral NK cells demonstrate enhanced killing when treated with both N-803 and avelumab *in vitro*

To test NK-cell killing of ovarian cancer over time, a spheroid assay was carried out. GFP-expressing OVCAR8 cells were incubated on an ultralow adhesion plate for 72 hours to allow spheroid formation. Following incubation, CellTrace-labeled healthy donor peripheral NK cells were added to the culture at a 2:1 ratio (calculated from original spheroid seeding). Images were taken every hour for 5 days. NK cells without treatment or avelumab alone demonstrated little to no killing (**Fig. 3A**). While the N-803 treatment alone eventually induced some spheroid killing, the kinetics of killing were considerably faster with N-803 plus avelumab (**Fig. 3A–C**). Pooled analysis at different time points (72 and 96 hours) demonstrated that N-803 and avelumab provided maximal spheroid killing (**Fig. 3D** and **E**).

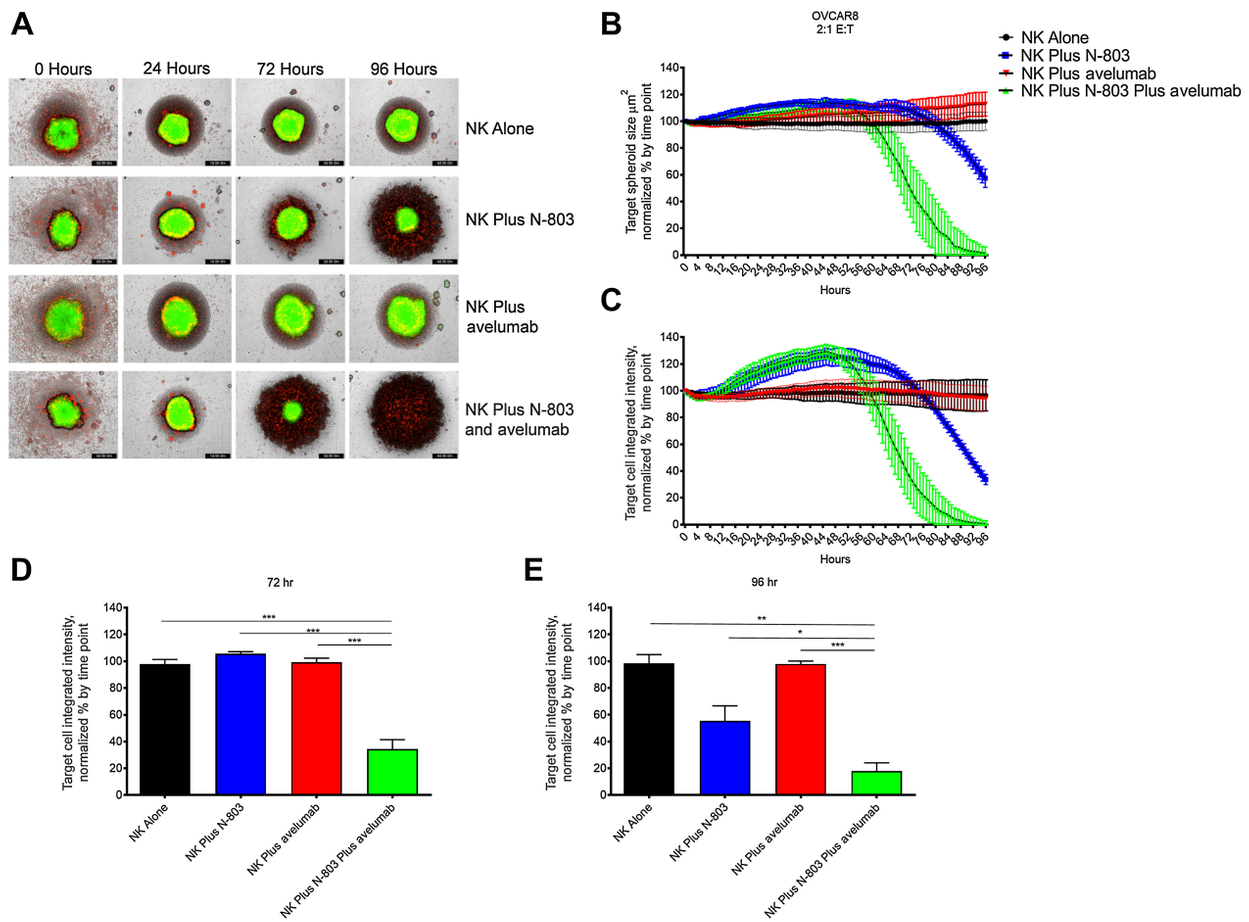

Figure 3.
Avelumab and N-803 treatment enhanced NK-cell killing of OVCAR8 tumor cells. GFP-expressing OVCAR8 cells were incubated with CellTrace-labeled healthy donor peripheral NK cells and imaged at the noted time points. **A,** Representative image of spheroid tumor killing at 0, 24, 72, and 96 hours. **B,** Representative quantification of spheroid size (technical replicates = 7). **C,** Representative quantification of spheroid intensity (technical replicates = 7) and pooled quantification of spheroid intensity at 72 (**D**) and 96 hours (**E**; $n = 5$). One-way ANOVA was used to compare samples. *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P < 0.0001$. Error bars represent SEM.

N-803 and avelumab combine to reduce tumor burden in a xenogeneic model of ovarian cancer

The combined benefits of N-803 and avelumab were tested in a xenogeneic model of ovarian cancer containing human cancer cells (MA148lucs) and human NK cells. In this mouse model, ovarian cancer growth is not localized and spreads throughout the peritoneal cavity and mimics several characteristics of ovarian cancer in humans, including formation of ascites in the peritoneum of the mice. NSG mice were injected with MA148luc tumor cells 3 days prior to irradiation and 4 days prior to injection of an enriched (CD3/CD19-depleted) NK-cell product (**Fig. 4A**). Mice were treated with two doses per week of N-803 (50 μg/kg), avelumab (100 μg/injection) or a combination of N-803 and avelumab. BLI was performed over a 35-day period to assess tumor growth, and it showed mice that received NK cells and N-803, particularly N-803 plus avelumab, had significantly reduced tumor burden compared with mice that received tumors and NT (**Fig. 4B** and **C**; Supplementary Fig. S3A and S3B). Furthermore, mice that received N-803 and avelumab had reduced tumor burden compared with tumor alone and avelumab alone. While NK-cell expansion in the presence of N-803 alone was substantially limited, as seen in a previous study (24), N-803 and avelumab overcame the deficit in expansion, resulting in a 13-fold increase in NK-cell numbers (**Fig. 4D**). Taken together, these data illustrate the benefit of combining IL15 cytokine therapy (N-803) with a PD-L1–targeting/blocking antibody capable of ADCC (avelumab).

Combination of N-803 and avelumab results in increased tumor infiltration

The combined benefits of N-803 and avelumab *in vivo* were further tested in a second xenogeneic model of ovarian cancer, OVCAR8luc. Unlike the MA148 model, which presents with ascites and diffuse tumor spread within the peritoneal cavity, the OVCAR8 model forms solid tumors in the ovaries and thus allows for better exploration of immune infiltration at that site. To determine characteristics of tumor infiltration in the context of N-803 and/or avelumab, mice were injected with OVCAR8 tumor cells 14 days prior to NK cells, irradiated and injected with NK cells (**Fig. 5A**). Mice were treated with N-803, avelumab, or a combination of N-803 and avelumab for 3 weeks and were then euthanized. Formalin-fixed and paraffin-embedded ovary sections were stained for human granzyme B and human CD3ζ (due to poor staining specificity with CD56). Given the xenogeneic model system, the only cells that stain positive for human granzyme B and human CD3ζ are the human NK cells injected into the mice. Staining showed that the combination of N-803 and avelumab, increased intratumoral NK cells (**Fig. 5B**; arrows), with the largest increase of NK-cell infiltration observed with the N-803 plus avelumab treatment (**Fig. 5C**). Similar results were seen peritumorally for N-803 and avelumab, with a small increase in NK cells (granzyme B+) in the N-803–treated group (**Fig. 5D**). N-803 and pembrolizumab showed an increase in intramural and peritumorally NK cells but not to the extent of N-803 and avelumab (Supplementary Fig. S4A and S4B).

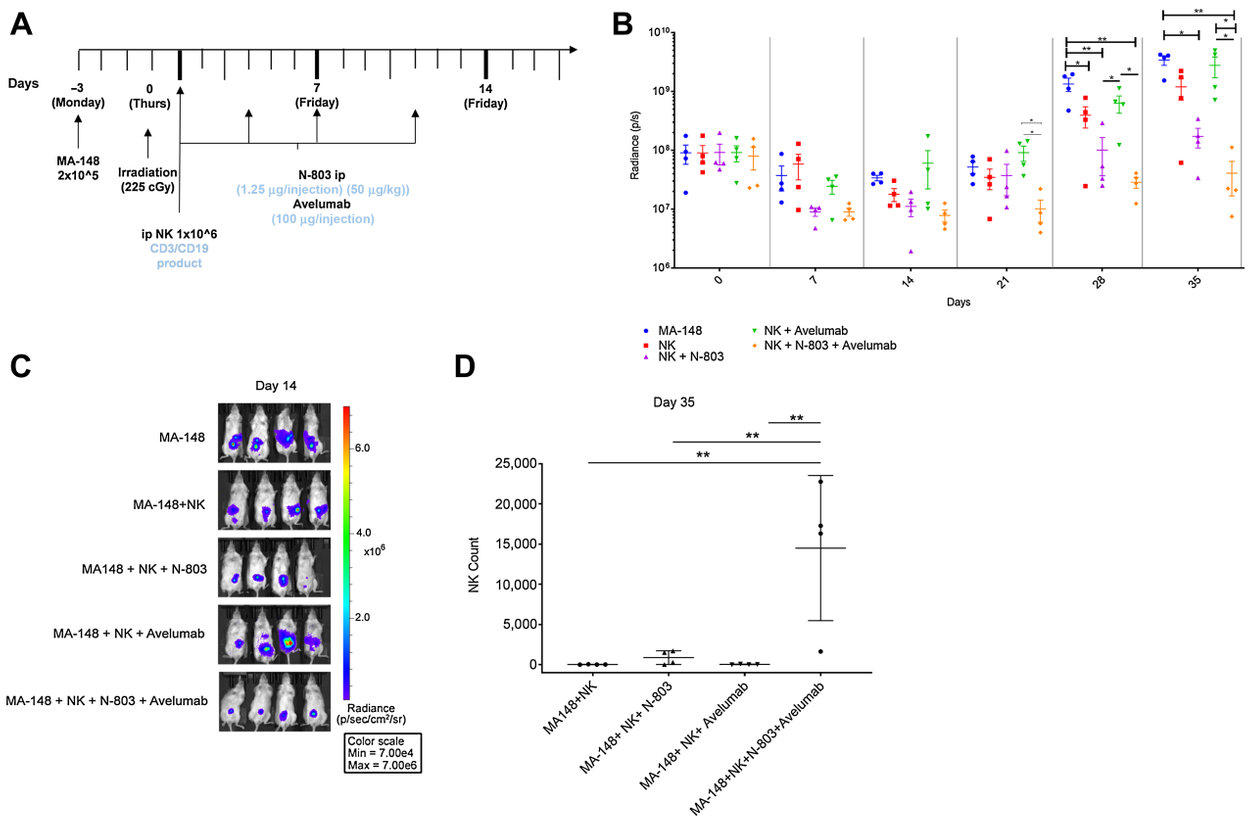

Figure 4.
Combined treatment of avelumab and N-803 induced an additive effect in mice with MA148 tumor cells. **A,** Schematic of MA148luc tumor model in NSG mice ($n = 4$ per group). **B,** Bioluminescence was used to assess tumor load in mice over a 35-day period. **C,** Day 14 bioluminescence of mice engrafted with tumor alone, tumor plus NK cells, tumor plus NK cells plus avelumab, tumor plus NK plus N-803, and tumor plus NK plus N-803 plus avelumab. **D,** NK-cell number in peritoneal cavity at day 35. Samples were compared using one-way ANOVA (¬) and unpaired *t* test (–). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P < 0.0001$. Error bars represent SEM.

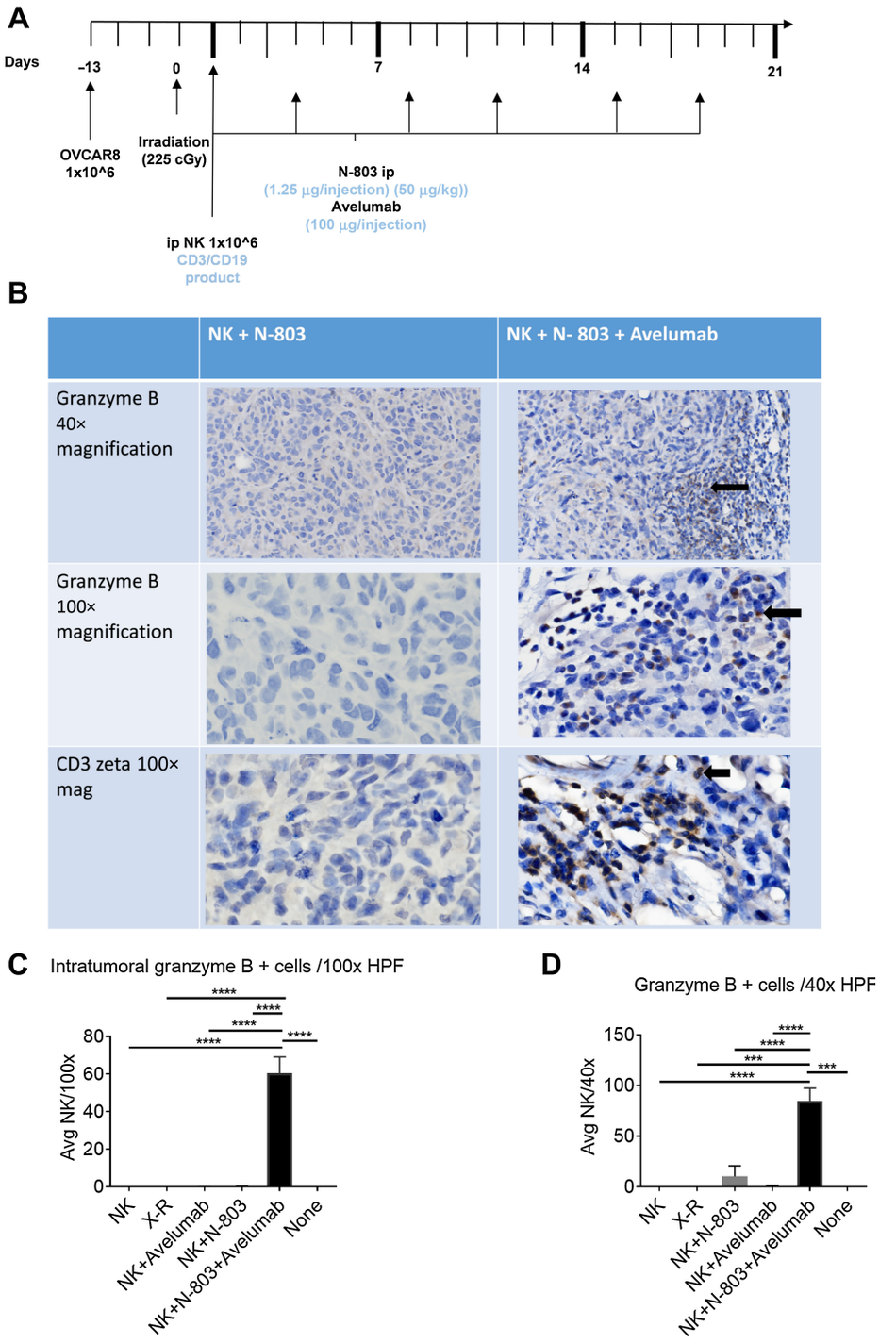

Figure 5.

N-803 and avelumab increased NK-cell infiltration into the tumor of mice injected with OVCAR8 tumor cells. **A,** Schematic of OVCAR8 tumor model in NSG mice with avelumab. **B,** Histology of tumors collected 3 weeks after treatment as shown in **A**. Ovaries were fixed with formalin and paraffin embedded to cut sections. Slides were cut and stained with anti-granzyme B and anti-CD3ζ to identify NK cells. Arrows point to intratumoral granzyme B^+ NK cells. Average intratumoral NK cells (granzyme B^+) at 100x HPF (**C**) and average peritumoral NK cells at 40x HPF (**D**). A total of 5 microscopic fields were counted per animal/tumor, selecting fields with high NK cells (n = 4–5 mice). One-way ANOVA was used to compare samples. *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P < 0.0001$. Error bars represent SEM.

Altogether, the data showed that the combination of N-803 and avelumab work together to induce a robust infiltration of NK cells into the tumor.

N-803 and pembrolizumab work together to reduce tumor burden without mediated priming of ADCC

Because avelumab could be driving the increase in tumor killing through N-803–mediated priming of ADCC, the question of whether checkpoint signaling is involved in the synergy with IL15 signaling remains open. To approach this question, pembrolizumab, which blocks PD-1 but does not drive ADCC, was tested in combination with N-803. NSG mice were injected with MA148luc or OVCAR8luc tumor cells 3 days prior to irradiation and 4 days prior to injection of an enriched (CD3/CD19-depleted) NK-cell product (**Fig. 6A**). The combination of N-803 and pembrolizumab yielded enhanced MA148 tumor control (**Fig. 6B** and **C**; Supplementary Fig. S5A and S5B),

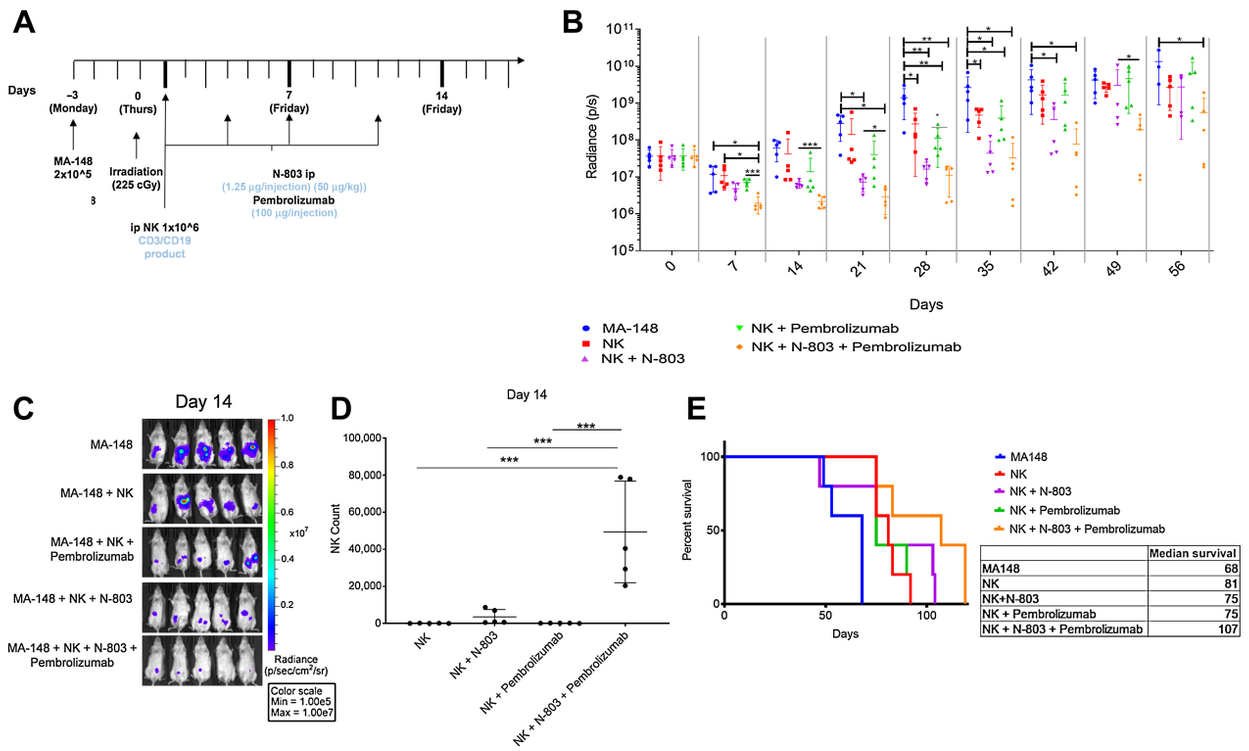

Figure 6.

Pembrolizumab and N-803 induced an additive effect in an *in vivo* model of MA148 without driving ADCC. **A,** Schematic of MA148 tumor model in NSG mice ($n = 5$ per group). **B,** Bioluminescence was used to assess tumor load in mice over a 56-day period. **C,** Day 21 bioluminescence of mice engrafted with tumor alone (MA148), tumor plus NK cells, tumor plus NK cells plus pembrolizumab, tumor plus NK plus N-803 and tumor plus NK plus N-803 plus pembrolizumab. **D,** NK-cell number in peritoneal cavity at day 14. **E,** Kaplan–Meier survival curve of mice treated with N-803 and pembrolizumab. NK + N-803 versus tumor only, $P = 0.0522$ and NK + N-803 + pembrolizumab versus tumor only, $P = 0.003$. Samples were compared using one-way ANOVA (¬) and unpaired t test (–). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P < 0.0001$. Error bars represent SEM.

similar to the combination of N-803 and avelumab (**Fig. 4B**). Data with an OVCAR8 model resulted in similar findings indicating that N-803 and pembrolizumab induce enhanced effects in OVCAR8 ovarian cancer tumor control *in vivo* (Supplementary Fig. S5C–S5E). As seen before with the combination of N-803 and avelumab, N-803 and pembrolizumab induced a 15.5-fold difference in NK-cell expansion over N-803 alone (**Fig. 6D**). To evaluate whether N-803 induced PD-1 expression on the NK cells themselves, we looked at an earlier timepoint, day 7, when the untreated NK cells are still present. The data show that N-803 treatment induced PD-1 expression on human NK cells *in vivo* (Supplementary Fig. S5F). To evaluate the long-term effects of N-803 treatment with checkpoint inhibitors, mice were followed for survival. The combination of N-803 and pembrolizumab resulted in a trend toward longer survival, with a median survival of 107 days compared with a median survival of 75 days in the N-803 alone group ($P = 0.07$; **Fig. 6E**). These data indicate that the interaction between cytokine signaling (N-803) and checkpoint blockade antibodies goes beyond ADCC and is present as a joint action between IL15 signaling and interruption of the PD-1/PD-L1 inhibitory signaling pathway.

Discussion

Samples obtained from a pilot clinical trial using an IL15 superagonist complex (N-803) as maintenance immunotherapy in women with advanced ovarian cancer gave us the unique opportunity to evaluate the effects of cytokine treatment on NK cells in the blood and in sequential peritoneal samples collected from the tumor microenvironment. While N-803 administration in patients induced initial robust NK-cell proliferation and functional priming, the effect was short-lived despite continued N-803 administration. This result was reverse translated back into the laboratory to understand the significance of this finding. Our data indicate that IL15 induces a negative feedback loop driven by upregulation of the PD-1/PD-L1 checkpoint axis in part through IFNγ, which is known to be produced by NK cells primed by IL15 signaling (24). This knowledge can be exploited by adding ICB, which synergizes with N-803, to improve cytokine driven immunotherapy in ovarian cancer.

Cytokine treatment, to induce inflammation and immune cell expansion, has been used clinically to try to bypass immunosuppression. Preclinical and early-phase clinical trial data show that cytokines such as IL2 and others are often limited by toxicity and activation of Tregs (16–18). Clinical use of cytokines has, however, provided proof of concept that they can stimulate T cells and NK cells, which can impact tumor progression. Development of analogs of cytokines for local delivery may provide the required specificity to bring cytokines safely into clinical use.

Prior studies have examined the use of intraperitoneally administered cytokines. In 1997, Edwards and colleagues pioneered the use of IL2 given intraperitoneally in a phase I/II study using two dosing

schedules: intermittent weekly infusions of 24 hours' duration or alternating continuous 7-day infusions followed by 7-day intervals without therapy (33). This was given to women with ovarian cancer who had received ≥six courses of prior platinum-based chemotherapy and had undergone a laparotomy to confirm persistent or ROC. Among 35 assessable patients, there were six laparotomy-confirmed complete responses and three partial responses, for an overall response rate of 25.7% (nine of 35). The median survival for this cohort was 13.7 months. As most efforts today are focusing on systemic immunotherapy, these outcomes in a heavily pretreated population speak to the potential of intraperitoneally delivered cytokines. More recently, there was a case report of a patient with BRCA2$^+$ ovarian cancer who, following her second relapse, received platinum-based chemotherapy followed by IL2 intraperitoneally 900,000 units per week × 16 doses (34). The patient was disease free for 14 years following this regimen. This case raises the question as to whether the higher mutational load observed in tumors with BRCA mutations could increase responsiveness to such immunotherapy.

On the basis of preclinical nonhuman primate and early-phase clinical trial data, IL2 and IL15 regimens can potently increase NK-cell numbers to augment immunotherapy (26–31). Given IL15's promise in NK-cell immunotherapy, we analyzed both blood and peritoneal washings following N-803 administration to determine whether we could amplify NK-cell function in the ovarian cancer setting. Our published preclinical findings indicate that N-803 induces proliferation of NK cells from healthy donor peripheral blood, but more importantly, this complex was able to amplify proliferation of NK cells derived from the peritoneal ascites of patients with ovarian cancer (24). Furthermore, we showed that IL15 was able to significantly increase NK-cell degranulation and inflammatory cytokine production against ovarian cancer targets and rescue the function of NK cells from the ascites of patients. On the basis of these data, we hypothesized that N-803 could rescue the function of NK cells in the ovarian cancer tumor microenvironment to drive NK-cell expansion. While we found N-803 was able to induce NK-cell proliferation, we also found that IFNγ production by IL15-primed NK cells could mediate induction of PD-L1 on ovarian cancer creating a negative feedback loop on NK-cell function. We also found that in some instances, in the absence of cytokine treatment, the inflammatory soluble tumor microenvironment in patients with ovarian cancer at the time of debulking surgery can induce PD-L1 on ovarian cancer cells via IFNγ. Furthermore, our data indicate that N-803 treatment induces upregulation of PD-1 on patient's peritoneal NK cells and CD8$^+$ T cells as well as peritoneal NK cells in our preclinical models.

The PD-1/PD-L1 pathway is implicated in the evasion of immune recognition and tumoricidal activity of CD8$^+$ T cells in many cancer types. Immune checkpoint inhibitor therapy has now been approved for multiple cancers due to deep and durable responses that have been observed. However, ovarian cancer has not seen the response rates to checkpoint inhibitors realized by other solid tumors. Contradictory outcomes have been reported with increased PD-L1 expression on ovarian cancer cells (4, 7, 8). Despite promising results in other solid tumors, single-agent ICB trials in ROC populations have demonstrated low objective response rates of <15% (9, 10, 15). Several synergistic mechanisms are being proposed for immunostimulatory agents plus checkpoint blockade combination therapy. Tumor cells can escape immunosurveillance through upregulation of PD-L1 expression, which inhibits the proliferation and antitumor activity of T cells while immune cells can upregulate PD-1 expression. Pembrolizumab is an FDA-approved IgG4 mAb against PD-1 and avelumab is an FDA-approved human IgG1 PD-L1–specific mAb that has the ability to enhance tumor cell lysis by NK cells via ADCC (35). We show that IL15-mediated immune cell activation and IFNγ release induce PD-L1 expression by tumor and PD-1 expression by effector cells, resulting in subsequent attenuation of immune responses in N-803–treated patients. Thus, while some of these checkpoint antibodies might not offer a benefit on their own in the setting of ovarian cancer, and other cancer settings, expression of PD-1 and PD-L1 are dynamic and induced by immune strategies so they should be evaluated in combination approaches like N-803 and beyond.

The topic of PD-1 expression on NK cells remains controversial. A recent study supporting the presence of PD-1 on NK cells shows a potent antitumor role for PD-1 blockade in animal models that is substantially reduced with NK-cell depletion (36). In contrast, a separate study showed that many of these effects are mediated by T cells expressing NK1.1 and find little to no expression of PD-1 on mouse NK cells in the tumor microenvironment (37). Using CRISPR editing of PD-1 on expanded human NK cells, we have shown an enhancement of function of NK cells against tumors that express PD-L1 (38). Furthermore, using a potent single-chain variable fragment against PD-1, we have shown that human NK cells express low levels of PD-1 capable of potent suppression in the presence of a PD-L1 ligand (39). While the amplification of NK-cell function and antitumor control when using N-803 combined with IgG1 avelumab could be mediated by ADCC rather than checkpoint blockade, the same cannot be claimed for the enhanced function seen when N-803 is combined with pembrolizumab, as this IgG4 antibody is not capable of mediating ADCC. In addition, our models used enriched NK cells, so that T cells could not confound our results. Besides enhancing tumor control, our findings indicate a robust enhancement of NK-cell expansion *in vivo* when N-803 is combined with either form of checkpoint blockade supporting the role of PD-1/PD-L1 axis in human NK-cell therapy.

Three previous studies have evaluated combining IL15 with checkpoint blockade. One study evaluated a novel anti-PDL1-mIL15 mutein protein designed to deliver IL15 to TILs while not activating circulating NK cells or T cells (40). Given low expression of PD-1 on NK cells (39) and the mutein IL15 that is directed toward the TILs, this study differed significantly from ours in that this molecule would not be inducing NK-cell signaling, much less synergy, unlike what we are seeing with the combination of N-803 and checkpoint blockade in our study. The second study (41) evaluated combination of IL15 and PD-L1/CTLA-4 checkpoint blockade in a murine model. This study focused on the CD8$^+$ T cells and there are significant differences between mouse and human NK-cell biology (42) that preclude several comparisons, including comparisons in ADCC and cytokine signaling, and thus perhaps explain why NK cells were not important in this model. The last study evaluated combination of N-803 and nivolumab in patients with non–small cell lung cancer (28), and while this study was carried out in the clinic, the setting differed significantly from ovarian cancer and the synergistic aspect of the combination specifically on NK cells was not fully evaluated, thus we believe our study demonstrates a novel interaction between IL15 signaling and checkpoint blockade in the context of NK-cell immunotherapy.

Following disappointing results from two phase III clinical trials testing avelumab in first-line (Javelin Ovarian 100) and in platinum-resistant/refractory ovarian cancer (Javelin Ovarian 200), further investigation of avelumab in this disease has been put to the wayside (43, 44). Our *in vitro* and *in vivo* assays confirm the lack of activity of avelumab alone against ovarian cancer but demonstrate potent synergy in tumor control and NK-cell expansion when avelumab is combined with N-803. This combination approach in ovarian cancer

may prove to be beneficial in a population that has not yet experienced the successes of checkpoint blockade that other solid tumors have. The QUILT 3.055 clinical trial (NCT03228667) tested the combination of checkpoint inhibitors with N-803 for tumors that have progressed on or following prior checkpoint inhibition. Preliminary data showed promising efficacy of cessation of progression and induction of response and durable stable disease in patients who had previously progressed on a checkpoint inhibitor–containing regimen in multiple tumor types and various checkpoint inhibitors (45). As ovarian cancer does not have an FDA-approved checkpoint inhibitor this tumor is not included in this trial. Our data provide the impetus to investigate various NK cell–directed therapies in combination with ICB for women with ovarian cancer.

Authors' Disclosures

M. Felices reports personal fees from GT BioPharma outside the submitted work. J.S. Miller reports grants and personal fees from Fate Therapeutics, GT Biopharma, Vycellix; personal fees and other support from ONK Therapeutics; and other support from Wugen outside the submitted work. M.A. Geller reports grants from Department of Defense, American Cancer Society, NCI, Minnesota Ovarian Cancer Alliance, and Randy Shaver Cancer Research and Community Fund during the conduct of the study; other support from Fate Therapeutics, HCW Biologics, Sanofi, and Merck outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

M. Felices: Conceptualization, resources, data curation, formal analysis, supervision, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **E. Wesley:** Data curation, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **L.E. Bendzick:** Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology. **B. Kodal:** Data curation, formal analysis, validation, investigation, visualization, methodology. **R. Hopps:** Data curation, formal analysis, validation, investigation, visualization, methodology. **B. Grzywacz:** Data curation, formal analysis, investigation, visualization, methodology. **P. Hinderlie:** Data curation, formal analysis, validation, investigation, visualization, methodology. **J.S. Miller:** Conceptualization, validation, writing–review and editing. **M.A. Geller:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, visualization, writing–original draft, project administration, writing–review and editing.

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Note

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

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