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Editor's Summary

Immune Cells and a Virus Teaming Up to Fight Cancer

Immunotherapy, or harnessing the patient's own immune system to help fight cancer, is becoming increasingly popular as researchers discover newer and more successful approaches focused on different aspects of the immune system. Two of these approaches include oncolytic viruses and therapies that block immune checkpoints and thus stimulate the antitumor activity of T cells. One virus that is known to have oncolytic activity is Newcastle disease virus (NDV), an avian virus that is not pathogenic in humans. Although previous studies have successfully demonstrated the antitumor effects of NDV, these were thought to require direct injection of this virus into every tumor, thus greatly limiting its effectiveness against metastatic disease.

Now, Zamarin and coauthors used mouse models of cancer with multiple tumor sites to demonstrate that NDV can be used even in the setting of distant spread of disease. Although the virus had to be injected into a tumor to have any effect, the subsequent tumor killing was not limited to the injected tumor. Additional masses that were distant in s pace and time, such as tumors implanted at different body sites and at later time points, were targeted by the immune system in animals that had been treated with NDV injection into the primary tumor. This immunostimulatory effect of N DV was particularly pronounced when the virotherapy was combined with immune checkpoint blockade by an anti–CTLA-4 antibody.

The current study was performed in mice bearing tumors derived from established cell lines, and the findings will need to be confirmed in the setting of spontaneously arising metastatic tumors. Nevertheless, the results of this work raise the tantalizing possibility that two therapies known to be safe for human use could be combined and work with the immune system to eradicate tumors throughout the body.

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CANCER IMMUNOTHERAPY

Localized Oncolytic Virotherapy Overcomes Systemic Tumor Resistance to Immune Checkpoint Blockade Immunotherapy

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Preexisting lymphocytic infiltration of tumors is associated with superior prognostic outcomes in a variety of cancers. Recent studies also suggest that lymphocytic responses may identify patients more likely to benefit from therapies targeting immune checkpoints, suggesting that therapeutic efficacy of immune checkpoint blockade can be enhanced through strategies that induce tumor inflammation. To achieve this effect, we explored the immunotherapeutic potential of oncolytic Newcastle disease virus (NDV). We find that localized intratumoral therapy of B16 melanoma with NDV induces inflammatory responses, leading to lymphocytic infiltrates and antitumor effect in distant (nonvirally injected) tumors without distant virus spread. The inflammatory effect coincided with distant tumor infiltration with tumor-specific $CD4^+$ and $CD8^+$ T cells, which was dependent on the identity of the virus-injected tumor. Combination therapy with localized NDV and systemic CTLA-4 blockade led to rejection of preestablished distant tumors and protection from tumor rechallenge in poorly immunogenic tumor models, irrespective of tumor cell line sensitivity to NDV-mediated lysis. Therapeutic effect was associated with marked distant tumor infiltration with activated $CD8^+$ and $CD4^+$ effector but not regulatory T cells, and was dependent on $CD8^+$ cells, natural killer cells, and type I interferon. Our findings demonstrate that localized therapy with oncolytic NDV induces inflammatory immune infiltrates in distant tumors, making them susceptible to systemic therapy with immunomodulatory antibodies, which provides a strong rationale for investigation of such combination therapies in the clinic.

INTRODUCTION

The discovery of T cell regulatory receptors provided targets for immunotherapies aiming to enhance activation of antitumor immune responses or to reverse immunosuppressive mechanisms governing tumor resistance to immune surveillance and destruction (*1*). Targeting of the latter with antibodies to immunologic checkpoints such as CTLA-4 and PD-1 demonstrated durable tumor regressions, though the therapeutic efficacy in patients and in poorly immunogenic animal models has not been universal (*2*–*5*). These findings call for identification of biomarkers predictive of response and development of combinatorial strategies that could make therapy beneficial for a larger patient population and a broader range of tumor types.

Data from clinical trials identified preexisting tumor-infiltrating lymphocytes (TILs) and an immune-active tumor transcriptional profile as strong predictors of response to immunotherapy (*6*, *7*), with type I interferon (IFN) emerging as an important pathway in CD8-mediated tumor rejection (*8*, *9*). These findings provide a strong incentive to explore strategies that could activate the type I IFN pathway and enhance tumor immune infiltration as a means to render tumors sensitive to therapy with immune checkpoint blockade.

Oncolytic viruses (OVs) represent another class of promising emerging cancer therapeutics, with viruses from several families currently being evaluated in clinical trials (*10*). Although in many studies OVs appeared to be effective antitumor agents with locoregional administration, very few studies have demonstrated therapeutic efficacy or characterized immune responses in established distant or metastatic lesions (*11*–*13*), which presents an obvious impediment to clinical investigation.

To address the limitations of these two therapeutic approaches, we explored whether the inflammatory responses generated by OVs with local administration could be harnessed to improve therapeutic efficacy of agents targeting immunologic checkpoints, which would, in turn, eliminate the need for viral delivery to all tumor sites. To this end, we used the nonpathogenic Newcastle disease virus (NDV), an avian paramyxovirus with robust type I IFN–inducing and oncolytic properties and strong clinical safety record (*14*–*18*). We initially set out to characterize the effects of NDV on the microenvironment of the virus-injected tumors and distant tumors, modeling metastatic disease. Unexpectedly, we find that intratumoral administration of NDV results in distant (nonvirally injected) tumor infiltration with activated lymphocytes in the absence of distant viral spread. Conversion of distant tumors to an inflammatory phenotype made them susceptible to therapy with systemic CTLA-4 blockade, leading to tumor rejection and long-term survival in most mice treated with the combination approach. These findings demonstrate an attractive strategy to enhance therapeutic efficacy of immunotherapeutic antibodies and to overcome the limitations of oncolytic virotherapy, providing a strong rationale for exploration of such combination strategies in a clinical setting.

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RESULTS

NDV replication is restricted to the injected tumor site

In an attempt to use NDV for therapy that would be active against metastatic disease, we initially characterized the viral distribution kinetics with intratumoral and systemic administration. Intratumoral injection of recombinant NDV expressing firefly luciferase reporter (NDV-Fluc) resulted in sustained luciferase signal in the injected flank tumor, whereas systemic administration of the virus resulted in no detectable luciferase signal in the tumor (fig. S1A). Because limited systemic virus delivery was unlikely to induce sufficient tumor lysis and immune response, we explored the intratumoral NDV injection as a means to elicit an antitumor immune response that could potentially overcome the limitations of systemic OV therapy. Hence, for our further studies, we modeled metastatic disease by using the bilateral flank B16-F10 tumor model (Fig. 1A). NDV-Fluc administration into the right flank tumor resulted in viral replication within the injected tumor, with the luciferase signal detectable for up to 96 hours (fig. S1, B to D). No virus was detected in the contralateral (left flank) tumor by luminescent imaging (fig. S1, B to D), passage in embryonated eggs, or reverse transcription polymerase chain reaction. This system thus allowed us to characterize the immune responses in both virus-injected and distant tumors, which were not directly affected by NDV.

NDV therapy increases local and distant tumor lymphocyte infiltration and delays tumor growth

Analysis of the virus-injected tumors revealed an inflammatory response as evidenced by increased infiltration with cells expressing leukocyte common antigen CD45 (fig. S2, A and B). The immune infiltrates were characterized by increase in innate immune compartment, including myeloid cells, natural killer (NK) cells, and NKT cells (fig. S2C), and the adaptive compartment, including $CD8^+$ and conventional $CD4^+FoxP3^-$ (T_{conv}) T cells, leading to significant increase of CD8 and T_{conv} to regulatory (T_{reg}) T cell ratios ($P = 0.0131$ and $P = 0.0006$, respectively) (fig. S2, D to F). Remarkably, analysis of the contralateral tumors revealed a similar increase in the inflammatory infiltrates (Fig. 1, B and C, and table S1), characterized by increased numbers of both innate immune cells (Fig. 1D and table S1) and effector T cells (Fig. 1, E and G, and table S1). Notably, although there were no major changes in the absolute number of T_{regs} (Fig. 1G), there was a substantial decrease in their relative percentages (Fig. 1, E, F, and H, and table S1), with significant enhancement of the CD8 and T_{conv} to T_{reg} ratios ($P = 0.002$ and $P = 0.0021$, respectively) (Fig. 1I and table S1). Effector T cells isolated from the distal tumors expressed increased activation, proliferation, and lytic markers ICOS, Ki-67, and granzyme B, respectively (Fig. 1, J and K, and table S1). As previously, we were unable to isolate the virus or viral RNA from the distant tumors, suggesting that the observed changes in the distant tumor microenvironment were not due to direct viral infection. To further exclude the possibility of undetectable local viral spread, we implanted the tumors at other distant sites, such as bilateral posterior footpads, which generated similar findings (fig. S3).

Consistent with the observed inflammatory effect, intratumoral administration of NDV resulted in growth delay not only of the injected but also of the contralateral tumors, resulting in prolonged animal survival (Fig. 1, L and M, and table S2). To determine whether this effect was transient and whether durable antitumor protection was possible, we intratumorally treated single-flank B16-F10 tumor-bearing mice with NDV, and injected long-term survivors with B16-F10 cells on the opposite flank. Most animals demonstrated tumor growth delay, and 30% of the animals completely rejected rechallenged cells, suggesting that intratumoral therapy with NDV can indeed induce protective antitumor memory responses (fig. S4).

NDV induces tumor infiltration and expansion of tumor-specific lymphocytes

To determine whether the antitumor immune response was dependent on the NDV-injected tumor type or a result of nonspecific inflammation generated by NDV infection, we performed the experiment with heterologous tumors (MC38 colon carcinoma and B16-F10 melanoma) implanted at the opposite flanks (Fig. 2A and table S3). To track tumor-specific lymphocytes, we adoptively transferred T cell receptor–transgenic congenically marked $CD8^+$ (Pmel) cells or luciferase-marked $CD4^+$ (Trp1) cells recognizing the melanoma differentiation antigens gp100 (Pmel) and Trp1 (*19*, *20*). Bioluminescent imaging was used to measure the distribution and expansion kinetics of the adoptively transferred Trp1 cells. Transfer of Trp1 cells into phosphate-buffered saline (PBS)–treated tumor-bearing animals failed to result in Trp1 accumulation in the tumors, highlighting the highly immunosuppressive nature of the tumor microenvironment in this model (Fig. 2, B to D, and table S3). NDV injection into B16-F10 tumors resulted in significant increase in the luciferase signal within the injected tumors (Fig. 2, B to D, and table S3), indicating Trp1 T cell expansion [area under the curve (AUC); $P = 0.0084$]. Remarkably, similar expansion was seen in the contralateral tumor, albeit at a delay ($P = 0.0009$) (Fig. 2, B to D, and table S3). In contrast, NDV injection into MC38 tumors failed to induce substantial Trp1 infiltration into the injected MC38 tumors or distant B16-F10 tumors (Fig. 2, B to D, and table S3), suggesting that the distant tumor-specific lymphocyte infiltration is likely dependent on the antigen identity of the injected tumor. Similarly, intratumoral injection of NDV resulted in increased infiltration of Pmel cells in distant tumors, which was more pronounced when the injected tumor was B16-F10 rather than MC38 (Fig. 2E and table S4).

Although infiltration of distant B16-F10 tumors with adoptively transferred lymphocytes was dependent on the injected tumor identity, distant tumors did demonstrate increased immune infiltration even when the primary injected tumor was MC38 (Fig. 2F), suggesting that a nonspecific inflammatory response component may also play a role. Indeed, serum from NDV-treated animals, treated with ultraviolet (UV) irradiation to inactivate any potential virus, induced tumor leukocyte infiltration when injected intratumorally into naïve B16-F10 tumor-bearing mice (Fig. 2, G and H, and table S4), with most of the increase seen in the NK and $CD8^+$ compartments ($P = 0.0089$ and $P = 0.0443$, respectively) (Fig. 2I and table S4).

NDV and CTLA-4 blockade synergize to reject local and distant tumors

Despite the prominent inflammatory response and growth delay seen in distant tumors, complete contralateral tumor rejection with long-term survival was only seen in about 10% of animals (Fig. 1M and table S2), suggestive of active immunosuppressive mechanisms in the tumor microenvironment. Characterization of NDV-injected and distant tumors revealed up-regulation of CTLA-4 on tumor-infiltrating T cells (fig. S5). We thus hypothesized that NDV-induced tumor inflammation would make the tumors sensitive to systemic therapy with CTLA-4 blockade, as was previously suggested in patients with preexisting tumor inflammatory infiltrates (*6*, *7*). Remarkably, combination therapy of

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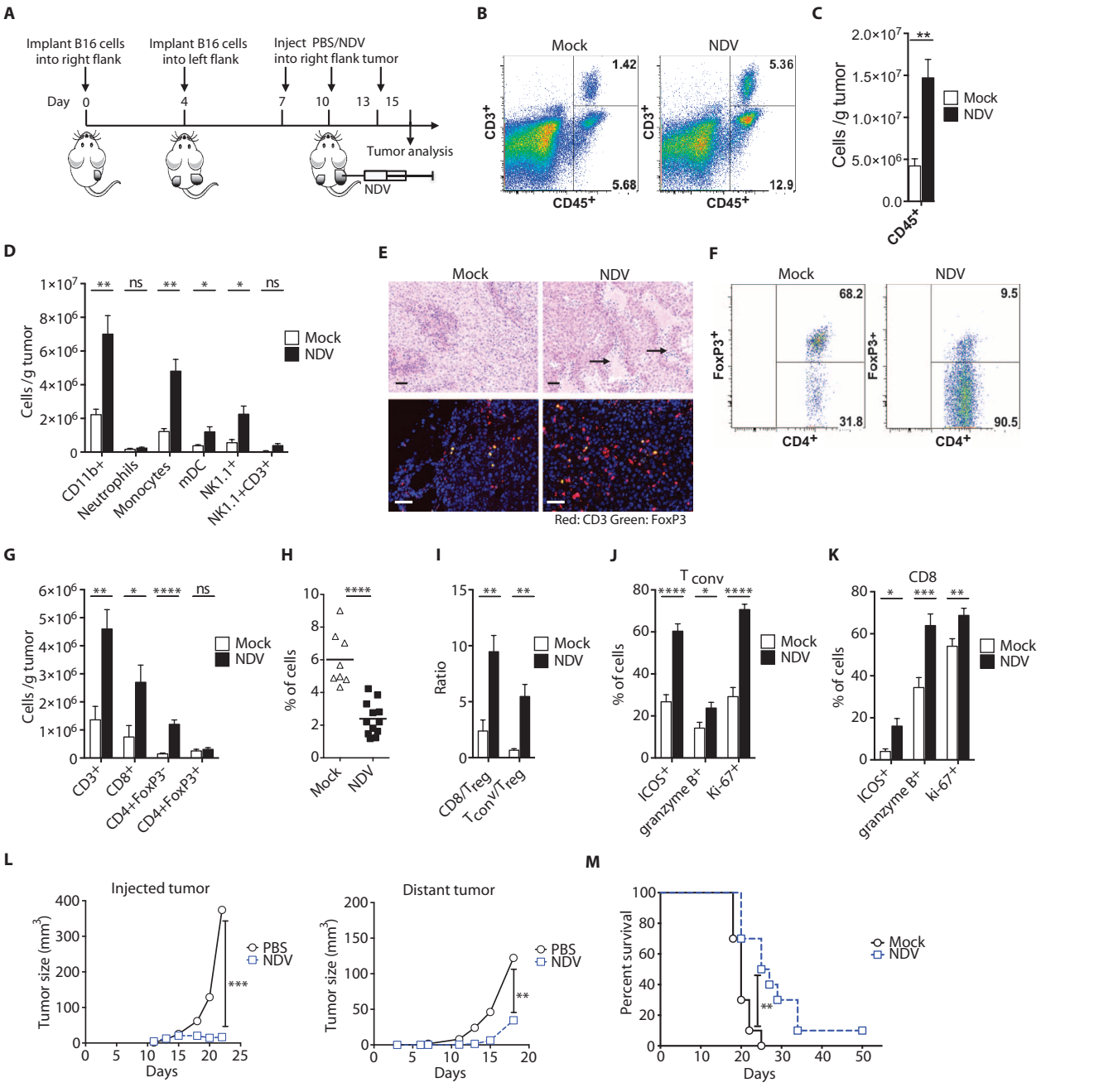

Fig. 1. NDV increases distant tumor lymphocyte infiltration and delays tumor growth. (**A**) Treatment scheme. (**B**) Representative flow cytometry plots of percentages of tumor-infiltrating $CD45^+$ and $CD3^+$ cells. (**C**) Absolute numbers of $CD45^+$ cells per gram of tumor. (**D**) Absolute numbers of innate immune cells per gram of tumor. (**E**) Tumor sections from distant tumors were stained with hematoxylin and eosin (H&E) (upper panels) or labeled for CD3 and FoxP3 (bottom panels) and analyzed by microscopy. Areas denoted by arrows indicate areas of necrosis and inflammatory infiltrates. Scale bars, 200 μm. (**F**) Representative flow cytometry plots of percentages of $CD4^+FoxP3^+$ (T_{reg}) and $CD4^+FoxP3^-$ (T_{conv}) cells. (**G**) Absolute numbers of conventional and regulatory $CD4^+$ cells and $CD8^+$ cells per gram of tumor calculated from flow cytometry. (**H**) Relative percentages of T_{regs} out of $CD45^+$ cells. (**I**) Calculated T_{conv}/T_{reg} and $CD8^+/T_{reg}$ ratios. (**J** and **K**) Up-regulation of ICOS, granzyme B, and Ki-67 on tumor-infiltrating T_{conv} (J) and $CD8^+$ cells (K). (**L**) Growth of NDV-injected and distant tumors. (**M**) Overall animal survival. Data represent cumulative results from three (B to K) or two (L to M) independent experiments with $n = 3$ to 5 per group. Mean ± SEM is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ns, not significant.

NDV with anti–CTLA-4 antibody (Fig. 3A and table S5) resulted in rejection of bilateral tumors and long-term survival in most animals, an effect that was not seen with either treatment alone (Fig. 3, B to D, and table S5). To determine the durability of the observed protection, we injected the surviving animals in the right flank on day 90 with B16-F10 cells without any further therapy. Animals treated with NDV

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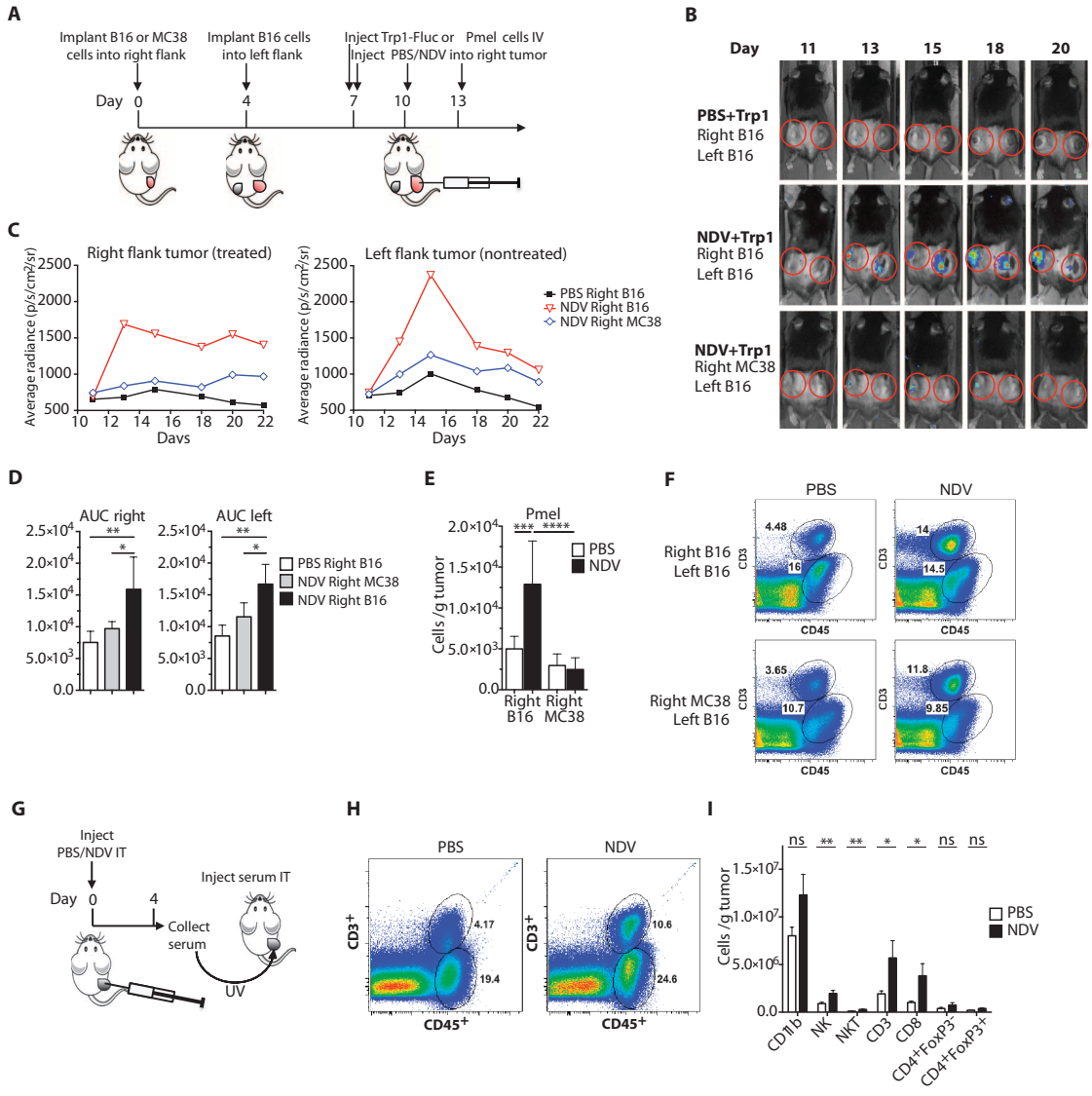

Fig. 2. NDV induces infiltration of tumor-specific lymphocytes and facilitates tumor inflammation. (**A**) Treatment scheme. IV, intravenously. (**B**) Representative luminescence images from animals treated with NDV and adoptively transferred Trp1-Fluc lymphocytes. (**C**) Quantification of average luminescence from the tumor sites. (**D**) AUC calculated from the data in (C). (**E**) Absolute number of Pmel lymphocytes from distant tumors calculated from flow cytometry. (**F**) Representative flow cytometry plots of percentages of $CD45^+$ and $CD3^+$ cells infiltrating distant tumors of animals treated per treatment scheme in (A). (**G**) Experimental scheme for serum transfer from animals treated intratumorally (IT) with single injection of NDV or PBS. (**H**) Representative flow cytometry plots of percentages of $CD45^+$ and $CD3^+$ cells infiltrating serum-injected tumors. (**I**) Absolute numbers of the indicated cell subsets in serum-injected tumors calculated from flow cytometry. Data for (B) to (E) represent one of three experiments with $n = 4$ to 5 per group. Data for (G) to (I) represent pooled data from two independent experiments with $n = 5$ per group. Mean ± SEM is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

and anti–CTLA-4 combination therapy demonstrated more than 80% protection against tumor rechallenge compared with 40% protection in the animals treated with single-agent anti–CTLA-4 antibody (Fig. 3E and table S5).

Combination therapy with NDV and CTLA-4 blockade is effective against virus nonpermissive tumors

To determine whether this treatment strategy could be extended to other tumor types, we evaluated it in the poorly immunogenic TRAMP

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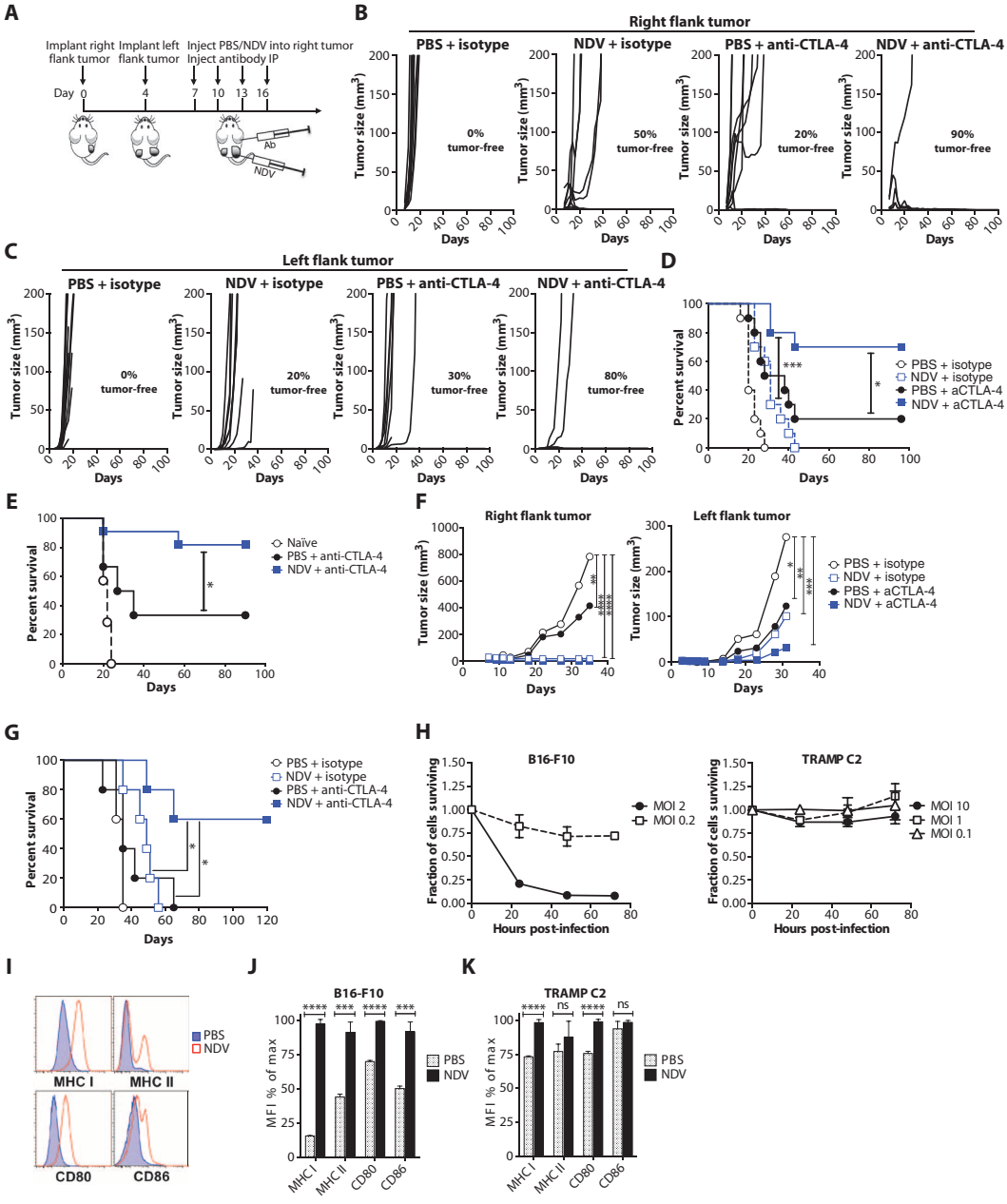

Fig. 3. NDV and CTLA-4 blockade synergize to reject local and distant tumors. (**A**) Treatment scheme. IP, intraperitoneally; Ab, antibody. (**B**) Growth of virus-treated (right flank) B16-F10 tumors. (**C**) Growth of distant (left flank) B16-F10 tumors. (**D**) Long-term survival in the B16-F10 model. (**E**) Surviving animals were injected with 1×10^5 B16-F10 cells in the right flank on day 90 and followed for survival. Data represent cumulative results from three experiments with $n = 6$ to 11 per group. (**F**) Growth of virus-treated (right flank) and distant (left flank) TRAMP C2 tumors. (**G**) Long-term survival in the TRAMP C2 model. (**H**) In vitro sensitivity of B16-F10 and TRAMP C2 cells to NDV-mediated lysis at different MOIs. (**I** to **K**) Up-regulation of MHC I, MHC II, CD80, and CD86 in B16-F10 and TRAMP C2 cells infected with NDV. Representative flow cytometry plots from B16-F10 cells (I) and calculated average median fluorescent intensities (MFI) for B16-F10 (J) and TRAMP C2 (K) cells are shown. Mean ± SEM is shown. Data represent results from one of three (B to E) or one of two (F and G) independent experiments with $n = 5$ to 10 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

C2 prostate adenocarcinoma model. Similarly to the B16-F10 model, combination therapy caused regression of the injected tumors (Fig. 3F and table S6) and either delayed the outgrowth of distant tumors or led to complete distant tumor regression with prolonged long-term survival (Fig. 3, F and G, and table S6). Whereas B16-F10 cells were susceptible to NDV-mediated lysis in vitro, TRAMP C2 cells were strongly resistant, with low cytotoxicity observed at a multiplicity of infection (MOI) of up to 10 (Fig. 3H). In both cell lines, NDV infection in vitro resulted in surface up-regulation of major histocompatibility complex (MHC) and costimulatory molecules (Fig. 3, I to K). MHC class I was up-regulated uniformly in all cells, even though not all cells get infected with NDV at the MOI of 1. In our previous studies, we demonstrated that NDV induces type I IFN expression in B16-F10 cells (*14*). Both type I IFN (*21*) and IFN-γ (*22*) are known to up-regulate MHC class I on B16-F10 cells, suggesting that within the context of the infected tumors, these mechanisms may play an additional role in enhancement of tumor immunogenicity. These results thus suggest that in vitro sensitivity to virus-mediated lysis is not necessary for sensitivity to NDV therapy in vivo, and further highlight the importance of a virus-generated inflammatory response, rather than direct oncolysis, in the observed antitumor efficacy. A similar therapeutic effect was observed in the CT26 colon carcinoma model, which also showed poor in vitro sensitivity to NDV-mediated lysis (fig. S6).

Systemic antitumor effect is antigen-restricted to the injected tumor type

To determine whether the observed antitumor effect in the distant tumor was specific to the injected tumor type, we evaluated the combination therapy in animals bearing a unilateral distant B16-F10 tumor and in animals with heterologous tumor types (MC38 colon carcinoma and B16-F10 melanoma) implanted at the opposite flanks (Fig. 4A). Although administration of the virus intradermally into the non–tumor-bearing

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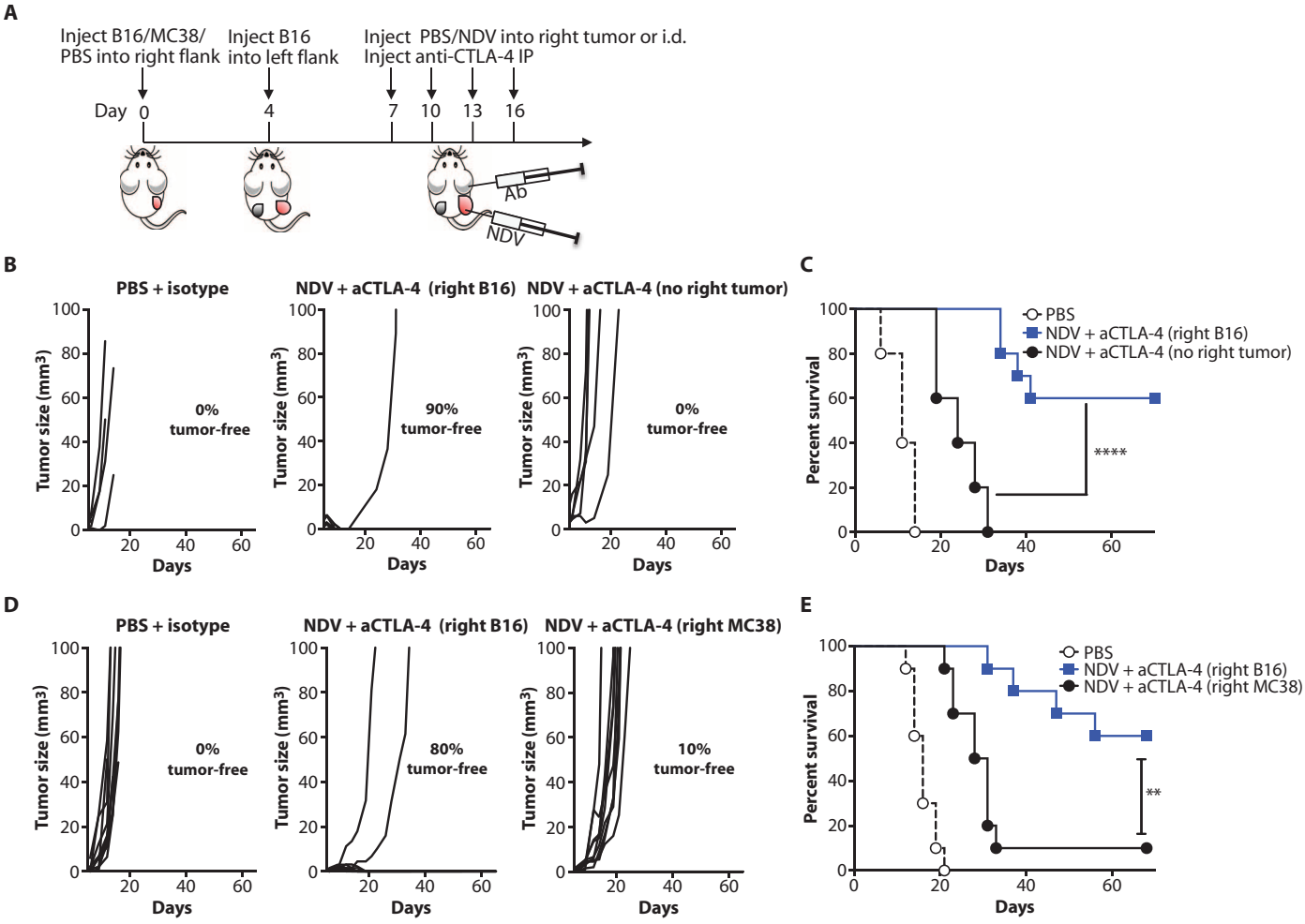

Fig. 4. Systemic antitumor effect is restricted to the injected tumor type. (**A**) Animals were injected intradermally (i.d.) in the right flank with B16-F10 melanoma, MC38 colon carcinoma, or PBS and in the left flank with B16-F10 cells and treated as outlined in the scheme. (**B** and **C**) Growth of distant tumors (B) and overall survival (C) of animals that received right B16-F10 or no right flank tumors. Data show representative results from one of two independent experiments with 5 to 10 mice per group. (**D** and **E**) Growth of distant tumors (D) and overall survival (E) of animals that received right B16-F10 or MC38 tumors. Data represent results from one of two independent experiments with $n = 10$ per group. $^{**}P < 0.01$, $^{****}P < 0.0001$.

right flank resulted in delayed left flank tumor outgrowth, it failed to result in long-term protection and tumor rejection seen in the animals bearing bilateral B16-F10 tumors (Fig. 4, B and C, and table S7). Similarly, injection of NDV into the right flank MC38 tumors of the animals bearing left flank B16-F10 tumors failed to induce B16-F10 tumor rejection (Fig. 4, D and E, and table S7), suggesting that the NDV-induced antitumor immune response is likely antigen-restricted to the injected tumor.

Combination therapy with NDV and anti–CTLA-4 induces tumor infiltration with activated lymphocytes

To examine the B16-F10 tumor microenvironment in the treated animals, we collected and processed bilateral tumors for analysis of infiltrating cells. Analysis of the injected and distant tumors from the treated animals revealed prominent inflammatory infiltrates and large areas of tumor necrosis in the animals treated with combination therapy (Fig. 5A, fig. S7, and table S8). This correlated with increased numbers of $CD45^+$ cells and T cells in the combination therapy group (Fig. 5, A to C, fig. S7, A to C, and table S8). As previously, the observed increase in TILs was primarily due to infiltration of $CD8^+$ and T_{conv}, but not T_{reg}, cells, leading to enhanced effector to T_{reg} ratios (Fig. 5, D to F, fig. S7, C to E, and table S8). Phenotypic characterization of $CD4^+$ and $CD8^+$ TILs from animals receiving the combination treatment demonstrated up-regulation of ICOS, granzyme B, and Ki-67 over the untreated and anti–CTLA-4–treated animals (Fig. 5, G to I, and table S8) and a larger percentage of IFN-γ–expressing $CD8^+$ cells in response to restimulation with dendritic cells (DCs) pulsed with B16-F10 tumor lysates (Fig. 5J and table S8).

Antitumor activity of NDV combination therapy depends on $CD8^+$ cells, NK cells, and type I and II IFNs

To determine which components of cellular immunity were responsible for the observed therapeutic effect, we repeated the treatment in the presence of depleting antibodies for $CD4^+$, $CD8^+$, or NK cells. Adequate cell depletion of each cell subset was confirmed by flow cytometry of peripheral blood (fig. S8). Depletion of either $CD8^+$ or NK cells resulted

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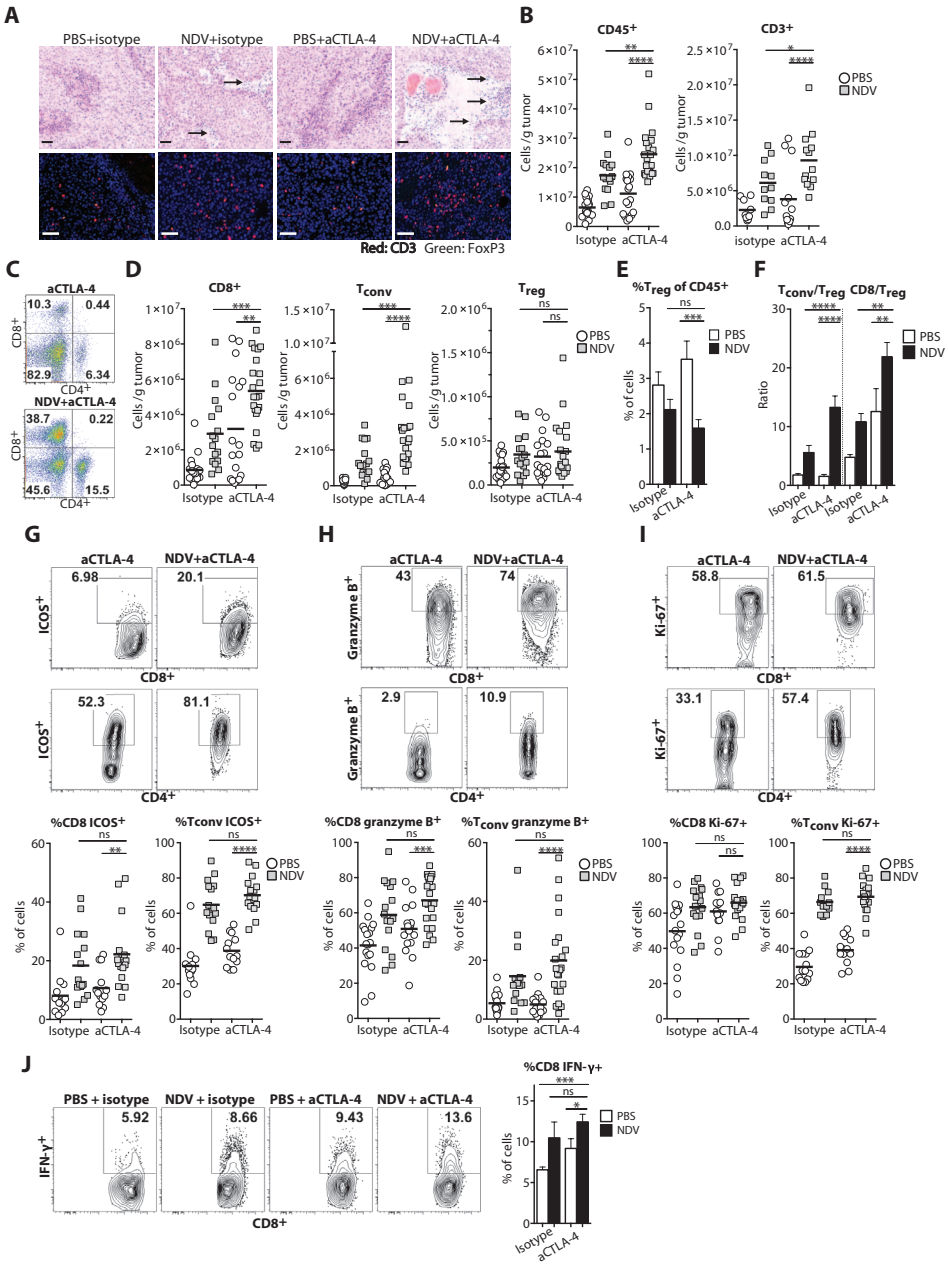

Fig. 5. Combination therapy with NDV and CTLA-4 blockade induces inflammatory changes in distant tumors. Animals were treated per schema in Fig. 3A. Tumors were harvested on day 15 and analyzed for infiltrating immune cells. (**A**) Tumor sections from distant tumors were stained with H&E (upper panels) or for CD3 and FoxP3 (lower panels) and analyzed by light and fluorescence microscopy, respectively. Areas denoted by arrows indicate necrosis and inflammatory infiltrates. Scale bars, 200 μm. (**B**) Absolute number of tumor-infiltrating $CD45^+$ and $CD3^+$ cells per gram of tumor calculated from flow cytometry. (**C**) Representative flow cytometry plots of percent of tumor-infiltrating $CD4^+$ and $CD8^+$ cells gated on $CD45^+$ population. (**D**) Absolute numbers of T_{conv}, T_{reg}, and $CD8^+$ cells per gram of tumor. (**E**) Relative percentages of tumor-infiltrating T_{regs} out of $CD45^+$ cells. (**F**) Calculated T_{conv}/T_{reg} and $CD8^+/T_{reg}$ ratios. (**G** to **I**) Up-regulation of ICOS, granzyme B, and Ki-67 on tumor-infiltrating $CD8^+$ and T_{conv} lymphocytes. Representative flow cytometry plots (upper panels) and cumulative results (bottom panels) are shown. (**J**) TILs were restimulated with DCs pulsed with B16-F10 tumor lysates, and IFN-γ production was determined by intracellular cytokine staining. Representative flow cytometry plots (left panel) and cumulative results (right panel) are shown. Data represent cumulative results from five (A to I) or two (J) independent experiments with $n = 3$ to 5 per group. Mean ± SEM is shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

in abrogation of therapeutic effect in both virus-injected and distant tumors (Fig. 6, A and B, and table S9), with significant reduction in long-term survival ($P < 0.0001$ for CD8 and $P = 0.0011$ for NK depletion) (Fig. 6C and table S9). Consistent with these findings, treatment of the animals with an anti–IFN-γ–neutralizing antibody also decreased therapeutic efficacy. In contrast, depletion of $CD4^+$ cells did not result in appreciable change in antitumor effect, though these results must be interpreted with caution because anti-$CD4^+$ depletion also results in concurrent depletion of T_{regs}.

Type I IFN has been previously demonstrated to play an important role in priming of $CD8^+$ cells for antitumor immune response (*8*, *9*). To investigate the role of type I IFN in tumor rejection by NDV, we repeated the experiments in the type I IFN receptor knockout ($IFNAR^{-/-}$) mice. The $IFNAR^{-/-}$ mice demonstrated rapid progression of both injected and contralateral tumors and were completely resistant to the combination therapy (Fig. 6, D to F, and table S9). Overall, these findings highlight the important role of both innate and adaptive immune responses for the systemic therapeutic efficacy of the virus observed in this study.

DISCUSSION

The presence of TILs has been shown to be a favorable prognostic indicator in a number of cancers, and gene expression profiling demonstrated that patients with high baseline tumor expression of genes related to both innate and adaptive immune response were more likely to favorably respond to immunotherapy (*7*, *23*, *24*). The presence of TILs in tumors has been shown to be associated with type I IFN transcriptional profile, and additional studies demonstrated the critical role for type I IFN in $CD8a^+$ DC-mediated antigen cross-presentation and priming of tumor-specific $CD8^+$ T cells (*8*, *9*). These findings provide a strong rationale to explore tumor therapeutic strategies that activate the type I IFN pathway. Indeed, combination therapy using intratumoral CpG oligonucleotides with antibodies targeting immune checkpoints has been shown to be an effective therapeutic strategy resulting in depletion of T_{regs} at the injected tumor site and in regression of distant tumors (*25*).

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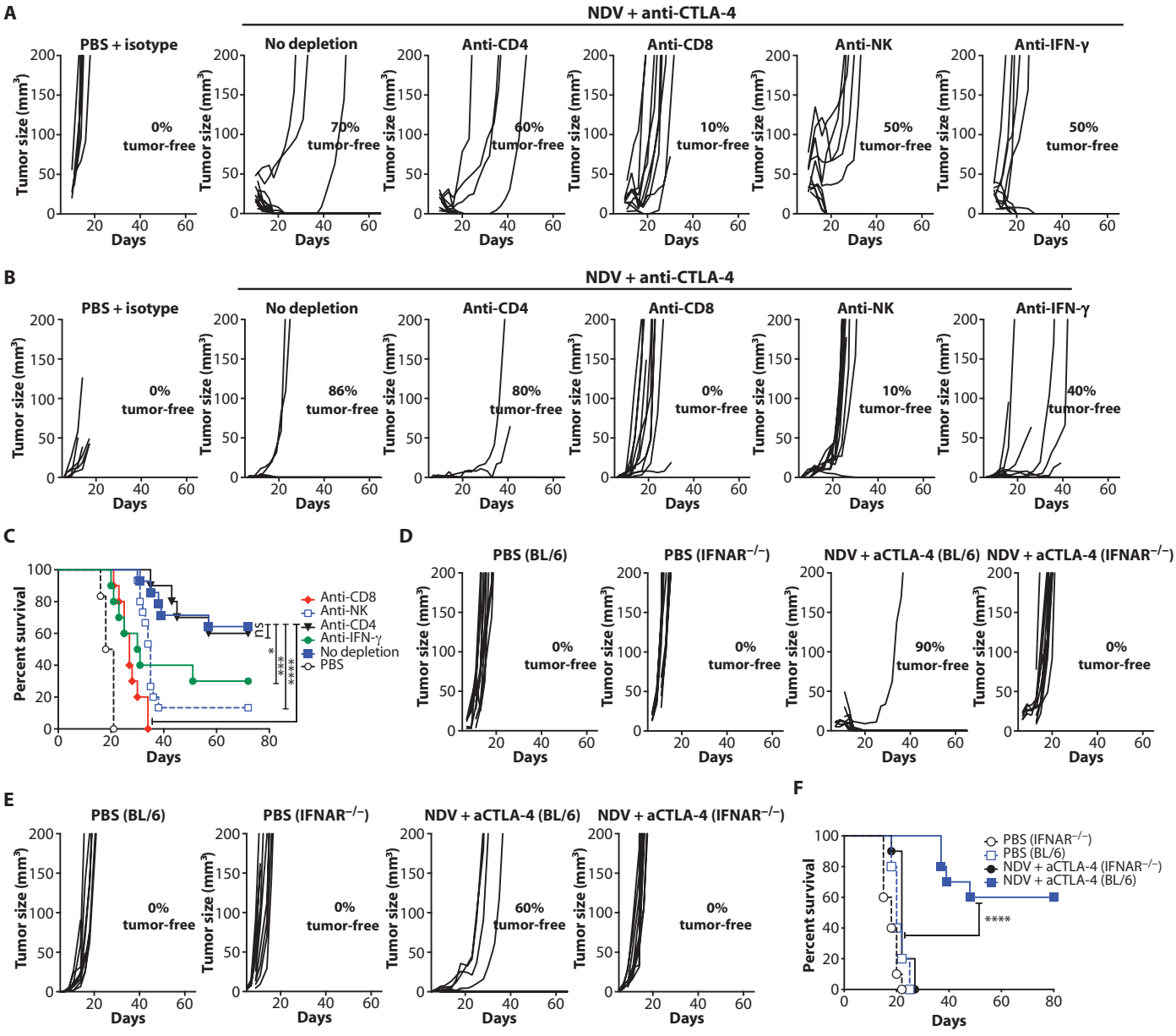

Fig. 6. Antitumor activity of NDV combination therapy depends on CD8$^+$ and NK cells and type I and II IFNs. (**A** to **C**) Animals were treated as described in Fig. 3A with or without depleting antibodies for CD4$^+$, CD8$^+$, NK cells, or IFN-γ. (A) Growth of injected tumors. (B) Growth of distant tumors. (C) Long-term survival. (**D** to **F**) IFNAR$^{-/-}$ or age-matched C57BL/6 mice (BL/6) were treated as described in Fig. 3A and monitored for tumor growth. (D) Growth of injected tumors. (E) Growth of distant tumors. (F) Long-term survival. Data for all panels represent cumulative results from two independent experiments with $n = 3$ to 10 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Here, to trigger immunogenic tumor cell death and an inflammatory response, we used nonpathogenic NDV, which, despite its relatively weak lytic activity, has been demonstrated to be a potent inducer of type I IFN and DC maturation (*26*, *27*). For our studies, we chose a bilateral flank melanoma model with staggered implantation of tumors at a schedule that was previously demonstrated not to be affected by concomitant immunity (*28*). We find that intratumoral injection of NDV results in distant tumor immune infiltration in the absence of distant virus spread. Notably, this effect was associated with relative reduction in the number of T_{regs} and marked enhancement of CD4 and CD8 effector to T_{reg} ratios, which has been previously demonstrated to be a marker of a favorable immunological response to immunotherapy (*29*, *30*). At present, it is unclear what contributes to the relative reduction in the number of T_{regs}, although NDV-induced inflammatory cytokines have been previously suggested to have functions that could counter T_{reg} activity (*31*). There is also a possibility that not all of the infiltrating FoxP3$^-$ CD4$^+$ cells exhibit effector functions, as FoxP3$^-$ regulatory T cells have been previously reported (*32*).

We further demonstrated that NDV enhances tumor infiltration with tumor-specific lymphocytes, an effect that was dependent on the

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identity of the virus-injected tumor. The enhanced tumor infiltration and expansion of adoptively transferred lymphocytes further suggest that there may be potential synergy between OV therapy and therapeutic approaches using adoptive T cell transfer. It is plausible that the tumor-specific lymphocytes undergo activation and expansion at the site of the initial viral infection, followed by their migration to other tumor sites, which is likely dependent on chemokines and lymphocyte homing receptors (*33*). We also observed that distant tumor immune infiltration was, in part, nonspecific and could be induced by NDV infection of a heterologous tumor or by transfer of serum from treated animals to naïve tumor-bearing mice. We speculate that increased vascular permeability induced by inflammatory cytokines such as interleukin-6 may strongly contribute to activation of tumor vasculature and lymphocyte recruitment into the tumors (*34*). Although it would be useful to identify the cytokines and other factors that mediate this inflammatory effect, it is unlikely that they alone will provide the same degree of therapeutic efficacy as that seen with intratumoral NDV injection. Indeed, although injection of MC38 tumor induced inflammatory infiltrates in the contralateral B16-F10 tumors, it failed to induce tumor infiltration with adoptively transferred T cells or increase therapeutic efficacy in combination with CTLA-4 blockade.

Despite the pronounced increase in TILs, therapeutic effect in distant tumors was rather modest with NDV monotherapy, highlighting the immunosuppressive nature of the microenvironment of these tumors (*6*). Remarkably, combination of systemic anti–CTLA-4 antibody with intratumoral NDV led to rejection of distant B16-F10 tumors with long-term animal survival. The animals were also protected against further tumor rechallenge, suggestive of establishment of long-term memory, though we did not specifically assess whether the memory lymphocytes resided in the lymphoid organs or the bone marrow (*35*). Therapeutic efficacy was also seen with TRAMP C2 and CT26 tumor models, which exhibit poor sensitivity to NDV-mediated cell lysis in vitro. These findings highlight the importance of the NDV-induced antitumor immune/inflammatory response, rather than direct lysis, as the primary mechanism driving the antitumor efficacy in this model. Indeed, analysis of NDV-injected and distant tumors treated with combination therapy demonstrated prominent infiltration with innate immune cells and activated $CD8^+$ and $CD4^+$ effector cells, whereas depletion of $CD8^+$ and NK cells abrogated the therapeutic efficacy. Furthermore, the combination strategy was completely ineffective in $IFNAR^{-/-}$ mice, which support the role of the type I IFN pathway in the induction of antitumor immunity in this system (*8*, *9*, *36*).

Our study model does present several limitations. First, we start therapy on day 3 after contralateral tumor challenge, where CTLA-4 blockade is ineffective as monotherapy. Although we are optimistic that the current combination approach will translate into improved clinical efficacy of CTLA-4 blockade, the development of approaches effective against larger B16 tumors is certainly warranted. Second, in our studies, we demonstrate increased immune infiltration and regression of distant soft tissue tumor sites with long-term survival without evidence of disease recurrence in the lungs, despite the known propensity for B16-F10 melanomas for early spontaneous metastases (*37*). Similarly, previous studies demonstrated that therapy of flank 3LL-D122 lung carcinoma with NDV protected the animals from spontaneous lung metastases (*38*). It would, however, be important to evaluate whether a similar effect is present in animal models with preestablished visceral metastases. Third, whereas our study focused primarily on characterization of the adaptive immune responses, it would be interesting to determine the role of NK cells and monocytes, which are also increased in the distant tumors of NDV-treated animals. Although depletion of NK cells resulted in accelerated early tumor outgrowth, the established tumors grew at a slower rate than was seen with CD8 depletion. This finding suggests that NK cells may play an early role in the antitumor effect of NDV, but the CD8 cells are required for long-term tumor control. Further experiments will be needed to elaborate on this observation.

In our studies, we find that systemic administration of NDV-Fluc fails to reach the tumors, although evidence of viral infection in the lungs can be seen by the presence of luciferase signal. Previous studies demonstrated that although NDV is capable of infection and protein expression in normal lung epithelial cells, virus progeny produced from such infection is minimal (*38*). This suggests that the virus likely undergoes a single-cycle replication with persistence of the luciferase reporter expression for several days. Notably, in clinical trials with systemically administered virulent NDV, which has a higher replicative capacity, no pulmonary toxicities were observed, with the exception of patients with large pulmonary tumor burden who developed inflammatory responses in the lung tumors (*18*). Nevertheless, a single-cycle infection could still potentially generate an inflammatory response in normal tissues, which could be amplified by immune checkpoint blockade. Because our strategy uses intratumoral administration of NDV, this might not be as relevant, but the potential for systemic toxicity certainly needs to be taken into account when designing clinical trials using combinations of immune checkpoint blocking agents with systemically administered OVs.

In summary, here, we have characterized the systemic tumor inflammatory effects induced by localized oncolytic NDV therapy and demonstrated that such inflammatory responses could be harnessed to enhance therapeutic efficacy of CTLA-4 blockade. Several distinguishing features of NDV, such as lack of preexisting immunity in humans, lack of recombination and genomic integration, strong induction of type I IFN, strong clinical safety record, and the ubiquitous nature of the NDV receptor (sialic acid), define potential advantages of this virus over some other OVs (*39*), though it is unknown whether combination of other OVs with CTLA-4 blockade would elicit similar therapeutic responses. Indeed, recent clinical studies with engineered herpes simplex virus-1 expressing granulocyte-macrophage colony-stimulating factor (talimogene laherparepvec) demonstrated response in distal tumors, with evidence of enhanced TILs (*13*, *40*), whereas preclinical studies with systemically administered oncolytic vesicular stomatitis virus expressing a library of tumor antigens demonstrated rejection of established melanomas (*41*). These findings suggest that the polyclonal antitumor immune response generated during the localized OV infection can be used to drive systemic antitumor immunity and provide a strong rationale for clinical exploration of combinations of immunoregulatory antibodies with NDV or other OVs. Studies using combination of talimogene laherparepvec with ipilimumab are currently under way and will provide a clinical proof of concept for the efficacy and safety of such combinations in humans (*42*).

MATERIALS AND METHODS

Study design

The primary research objective was to characterize the antitumor immune responses induced by oncolytic NDV and to evaluate combinatorial strategies of NDV with CTLA-4 blockade. Our prespecified hypothesis suggested that therapy with NDV would result in tumor inflammation,

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increasing tumor sensitivity to immunotherapy with anti–CTLA-4. The overall study design was a series of controlled laboratory experiments in mice, as described in the sections below. In all experiments, animals were assigned to various experimental groups in random. For survival studies, sample sizes of 10 to 15 mice per group were used. With 15 mice per group, 90% power, and a 5% significance level, we could detect differences in tumor-free survival from 20 to 80%. Survival analyses were performed with the log-rank test. The experiments were replicated two to three times as noted, and the final analysis included either pooled data or representative experiments where indicated. For the experiments reporting isolation of TILs, five mice per group were used for each experiment, with three to five replicates. All outliers were included in the data analysis.

In vitro infection experiments

For cell surface labeling, cells were infected in six-well dishes at MOI 1 (B16-F10) or MOI 5 (TRAMP C2) in triplicate. Twenty-four hours later, the cells were harvested by scraping and processed for surface labeling and quantification by flow cytometry. For in vitro cytotoxicity experiments, cells were infected at the indicated MOIs and incubated at 37°C in serum-free medium in the presence of TPCK-trypsin (250 ng/ml). At 24, 48, 72, and 96 hours after infection, the cells were washed and incubated with 1% Triton X-100 at 37°C for 30 min. Lactate dehydrogenase activity in the lysates was determined with the Promega CytoTox 96 Assay kit, according to the manufacturer's instructions.

Tumor challenge survival experiments

All mouse procedures and experiments for this study were approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee. Treatment schedules and cell doses were established for each tumor model to achieve 10 to 20% tumor clearance by NDV or anti–CTLA-4 as single agents. For the B16-F10 model, tumors were implanted by injection of 1×10^5 cells in the right flank intradermally on day 0 and 5×10^4 cells in the left flank on day 4. On days 7, 10, 13, and 16, the mice were treated with intratumoral injections of 1×10^7 plaque-forming units (PFU) of NDV in PBS in a total volume of 100 μl and intraperitoneally with anti–CTLA-4 antibody (100 μg in 100 μl). Control groups received a corresponding dose of isotype antibody intraperitoneal and intratumoral injection of PBS. The animals were euthanized for signs of distress or when the total tumor volume reached 1000 mm^3. For depletion of immune cells, mice were injected intraperitoneally with 500 μg of monoclonal antibodies to $CD8^+$, $CD4^+$, NK1.1, or IFN-γ 1 day before and 2 days after tumor challenge, followed by injection of 250 μg every 5 days throughout the experiment. For the TRAMP C2 model, animals received 1×10^6 cells in the right flank and 5×10^5 cells in the left flank on days 0 and 4, respectively. Treatment was performed on days 7, 10, 13, and 16 in a similar fashion to above. For the CT26 model, animals received 1×10^6 cells in the right flank and 1×10^6 cells in the left flank on days 0 and 2, respectively. Treatment was performed on days 6, 9, 12, and 15 in a similar fashion to above.

Serum transfer experiments

Groups of tumor-bearing mice were treated intratumorally with single injection of NDV or PBS. On day 4, blood was collected by terminal bleeding and serum was isolated by centrifugation. Sera were pooled from each group and UV-treated in Stratalinker 1800 with six pulses of UV light (300 mJ/cm^2) to inactivate any virus that could be potentially present. Undiluted 100 μl of serum was injected intratumorally into naïve B16-F10 tumor-bearing mice for a total of three injections given every other day. Tumors were removed 3 days after the last injection and processed for isolation of TILs.

Isolation of TILs

B16-F10 tumors were implanted by injection of 2×10^5 B16-F10 cells in the right flank intradermally on day 0 and 2×10^5 cells in the left flank on day 4. On days 7, 10, and 13, the mice were treated with intratumoral injections of 2×10^7 PFU of NDV, and 100 μg of intraperitoneal anti–CTLA-4 antibody where specified. The rare animals that died from tumor burden (always in untreated control groups) or animals that completely cleared the tumors (always in treatment groups) were not used for the analysis. On day 15, mice were sacrificed and tumors were processed as discussed in Supplementary Materials and Methods.

Statistics

Data were analyzed by two-tailed unpaired Student's *t* test (for comparisons of two groups in studies comparing NDV to control) and analysis of variance (ANOVA) (for comparison of multiple groups in studies where combinations of NDV with anti–CTLA-4 were used). Data for survival were analyzed by log-rank (Mantel-Cox) test. Two-sided $P < 0.05$ was considered statistically significant ($^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). The numbers of animals included in the study are discussed in each figure.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. NDV infection is restricted to the injected tumor.
Fig. S2. NDV infection increases tumor leukocyte infiltration in the virus-injected tumors.
Fig. S3. NDV therapy increases distant tumor lymphocyte infiltration in bilateral footpad melanoma model.
Fig. S4. Intratumoral NDV provides protection from tumor rechallenge.
Fig. S5. Tumor-infiltrating $CD8^+$ lymphocytes up-regulate CTLA-4 in response to NDV therapy.
Fig. S6. NDV and CTLA-4 synergize to reject local and distant tumors in the CT26 colon carcinoma model.
Fig. S7. Combination therapy with NDV and anti–CTLA-4 enhances tumor infiltration with innate and adaptive immune cells.
Fig. S8. Antibodies to CD8, CD4, and NK1.1 deplete the cells of interest in vivo.
Table S1. Raw data and statistical testing for Fig. 1, C to K.
Table S2. Raw data and statistical testing for Fig. 1, L and M.
Table S3. Raw data and statistical testing for Fig. 2, C and D.
Table S4. Raw data and statistical testing for Fig. 2, E to I.
Table S5. Raw data and statistical testing for Fig. 3, B to E.
Table S6. Raw data and statistical testing for Fig. 3, F and G.
Table S7. Raw data and statistical testing for Fig. 4.
Table S8. Raw data and statistical testing for Fig. 5.
Table S9. Raw data and statistical testing for Fig. 6.

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Society of Clinical Oncology Conquer Cancer Foundation. **Author contributions:** D.Z. designed and performed the experiments, analyzed the data, and prepared the manuscript. R.B.H., S.K.S., J.S.P., and M.M. performed the experiments and assisted in manuscript preparation. P.P., T.M., J.D.W., and J.P.A. assisted in experimental design, data interpretation, and manuscript preparation. **Competing interests:** J.P.A. is the primary inventor of intellectual property "Blockade of T lymphocyte down-regulation associated with CTLA-4 signaling" licensed by the University of California to Bristol-Myers Squibb. Memorial Sloan Kettering Cancer Center and Icahn School of Medicine at Mount Sinai filed a joint patent for the use of NDV for cancer immunotherapy. J.D.W. is an advisory board member to Bristol-Myers Squibb and MedImmune.

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