Cancer Gene Therapy (2015) 22, 154–162

www.nature.com/cgt

ORIGINAL ARTICLE

The addition of recombinant vaccinia HER2/neu to oncolytic vaccinia-GMCSF given into the tumor microenvironment overcomes MDSC-mediated immune escape and systemic anergy

CR de Vries[1], CE Monken[2] and EC Lattime[3]

Effective immunotherapeutic strategies require the ability to generate a systemic antigen-specific response capable of impacting both primary and metastatic disease. We have built on our oncolytic vaccinia a granulocyte-macrophage colony-stimulating factor (GM-CSF) strategy by adding recombinant tumor antigen to increase the response in the tumor microenvironment and systemically. In the present study, orthotopic growth of a syngeneic HER2/neu-overexpressing mammary carcinoma in FVB/N mice (NBT1) was associated with increased $Gr1^+CD11b^+$ myeloid-derived suppressor cells (MDSCs) both systemically and in the tumor microenvironment. This MDSC population had inhibitory effects on the HER2/neu-specific Th1 immune response. VVneu and VVGMCSF are recombinant oncolytic vaccinia viruses that encode HER2/neu and GM-CSF, respectively. Naive FVB mice vaccinated with combined VVneu and VVGMCSF given systemically developed systemic HER2/neu-specific immunity. NBT1-bearing mice became anergic to systemic immunization with combined VVneu and VVGMCSF. Intratumoral VVGMCSF failed to result in systemic antitumor immunity until combined with intratumoral VVneu. Infection/transfection of the tumor microenvironment with combined VVGMCSF and VVneu resulted in development of systemic tumor-specific immunity, reduction in splenic and tumor MDSC and therapeutic efficacy against tumors. These studies demonstrate the enhanced efficacy of oncolytic vaccinia virus recombinants encoding combined tumor antigen and GM-CSF in modulating the microenvironment of MDSC-rich tumors.

Cancer Gene Therapy (2015) **22,** 154–162; doi:10.1038/cgt.2015.2; published online 30 January 2015

INTRODUCTION

The goal of tumor immunotherapy is the development of modalities given alone or in combination that result in the generation of an effective systemic tumor-specific immune response by the host that can overcome immune escape mechanisms. The identification of immunogenic targets unique to or overexpressed by tumor cells is critical to achieving this goal. The *HER2/neu* oncogene encodes human epidermal growth factor receptor 2 (HER2/neu), a member of the epidermal growth factor receptor family of transmembrane tyrosine kinase receptors, which participates in processes including physiology, proliferation and differentiation of various human tissues.[1,2] Overexpression of HER2/neu is found in ~20% of invasive breast cancers, and is associated with a more invasive phenotype and a poorer prognosis.[3] Development of an active immune response using a vaccine targeting HER2/neu represents an attractive immunotherapeutic strategy for overcoming immune escape mechanisms induced by the tumor microenvironment.

Myeloid-derived suppressor cells (MDSCs), a population of immature myeloid cells that are increased systemically and in the tumor microenvironment of both murine cancer models and human malignancies, are prominent contributors to tumor immune escape.[4,5] This heterogeneous population is characterized phenotypically in mice by the cell surface antigens CD11b and Gr-1.[5] Gr-1 encompasses two subtypes, Ly-6C and Ly-6G, which have been used to further differentiate MDSCs into $CD11b^+Ly\text{-}6C^{high}$ $Ly\text{-}6G^-$ monocytic (mMDSC) and $CD11b^+Ly\text{-}6C^{low}Ly\text{-}6G^+$ granulocytic (gMDSC) subpopulations, respectively.[6,7] Consistent with their heterogeneous phenotype, MDSCs suppress the antitumor immune response through multiple mechanisms.[8] MDSCs interfere with lymphocyte proliferation via deprivation of essential amino acids, such as arginine and cysteine.[7,9,10] They also mediate oxidative stress via production of reactive oxygen species and peroxynitrate. This leads to nitration of tyrosine in CD8 and the T-cell receptor, ultimately changing the rigidity of the T-cell receptor.[11] Furthermore, MDSCs support induction of other immune inhibitory populations such as regulatory T cells (Tregs) through the production of transforming growth factor-β and interleukin-10.[12–15] Given these immune-suppressive effects, therapies that can overcome systemic anergy induced by MDSCs have generated great interest.

Studies from our group were the first to develop and test recombinant vaccinia vectors encoding the immune-enhancing granulocyte-macrophage colony-stimulating factor (GM-CSF) for

[1]Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA; [2]Rutgers Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA and [3]Department of Surgery, Rutgers Cancer Institute of New Jersey and Rutgers Robert Wood Johnson Medical School, New Brunswick, NJ, USA. Correspondence: Dr EC Lattime, Rutgers Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901, USA.
E-mail: e.lattime@rutgers.edu
Received 10 December 2014; revised 30 December 2014; accepted 30 December 2014; published online 30 January 2015

the localized treatment of solid tumors. In preclinical studies, we demonstrated that vaccinia and vaccinia recombinants were effective in infecting/transfecting tumors and, importantly, that despite the immunogenicity of the vaccinia vector, high levels of transfection could be obtained following repeated injections of tumor in mice[16] and subsequently in patients with recurrent superficial melanoma.[17] We developed and took clinical VVGMCSF into phase I trials in melanoma.[18] Subsequent to our studies, this recombinant (JX-594) was shown to have antitumor activity in preclinical models and clinical trials in a number of diseases.[19,20]

In the present study using orthotopic growth of an aggressive HER2/neu-expressing murine tumor characterized by high levels of $CD11b^{+}Gr\text{-}1^{+}$ MDSCs in the tumor microenvironment and systemically that suppressed HER2/neu-specific Th1, we show that intratumoral treatment with the oncolytic VVGMCSF is ineffective at reducing tumor growth nor does it lead to the development of a systemic tumor-specific immune response. However, when combined with a neu-encoding vaccinia VVneu and administered into the tumor microenvironment, mice develop systemic anti-neu immunity, significant reduction in tumoral and systemic MDSC and manifest a major antitumor response. The same virus combination (vaccine) fails to generate a similar response when given systemically in NBT1-bearing mice. We characterize the presence and function of the MDSC response and the resultant cytotoxic T lymphocyte (CTL) and interferon responses to HER2/neu. These results point to the ability of the tumor microenvironment to both promote immune escape and act as an effective vaccination site for tumor antigen-encoding oncolytic viruses that can result in a systemic immune response capable of mediating tumor regression.

MATERIALS AND METHODS

Cell culture

The HER2/neu-expressing NBT1 mammary tumor cell line was derived by serial cell culture of tumor cells obtained from a spontaneous breast tumor that arose in a FVB/neuT mouse transgenic for a HER2/neu receptor that is constitutively activated by a point mutation in the transmembrane region. NBT1 was maintained in media composed of CMRL-1066 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU ml^{-1} penicillin/streptomycin and 4 μM dexamethasone. Splenic cell cultures used in immune analyses were carried out using a supplemented RPMI-1640 media as described.[21] The Sf9 and Sf21 cell lines (Invitrogen) were maintained in Grace's supplemented medium (Invitrogen) supplemented with 10% fetal bovine serum and 50 IU ml^{-1} penicillin/streptomycin. HER2/neu Sf21 cell lysates used for *in vitro* restimulation of the CTL assay described below were prepared by resuspending cells in RPMI medium with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), lysing via four freeze/thaw cycles, sonicating for 2 min and centrifugating for 15 min at 13 500 rcf.

Construction of recombinant vaccinia virus expressing HER2/neu (VVneu)

The rat *neu* complementary DNA (cDNA) was cloned from pSV2-neuNT (provided by RA Weinberg), ligated into pBluescript (Stratagene, La Jolla, CA, USA) using *Sal*I and *Hin*d III restriction sites, and then removed from pBluscript using *Sal*I and *Not*I. It was cloned into vaccinia recombination plasmid pSC11/9. VVGMCSF was prepared as previously described.[21–23] VVβGal was provided as a negative control by Dr Laurence Eisenlohr (Thomas Jefferson University, Philadelphia, PA, USA).

Construction of recombinant Baculovirus expressing rat HER2/neu

The rat *neu* cDNA-coding sequence was assembled in the pFASTBac/CT-TOPO recombination vector (Invitrogen). Rat *neu*-transforming cDNA was excised from a vaccinia recombination plasmid containing the full-length rat neuNT cDNA sequence[24] in the vector pSC11/9 using *Not*I and *Sal*I restriction enzymes. The 4727 bp rat *neu* DNA was gel purified and then ligated to pBluescript II SK(+) (Stratagene) at Not/Sal to produce plasmid BSratneuNT. This neuNT-containing plasmid was used to generate three DNA fragments which, when ligated together, prepared the rat neuNT-coding sequences for ligation into pFASTBac/CT-TOPO. pBSratneuNT was first digested with *Hin*cII, which cut at two sites: a *Hin*cII site at residue 1599 and the *Sal*I site at the 3'-end of the neuNT cDNA, residue 4622. *Hin*cII digestion released a 3023-bp fragment, residues 1599–4622, which contained two problematic *Nco*I sites. The *Hin*cII-cut vector was gel purified, religated and cloned and then digested with *Nco*I. Filling in the *Nco*I site, located at position −19, with T4 DNA polymerase followed by ligation transformed the *Nco*I site into a *Nsi*I site, creating plasmid BSNeuNsi. Plasmid BSNeuNsi was digested with both *Sbf*I and *Sal*I releasing a 1366-bp fragment and leaving 338 bp of cDNA containing 233 bp of the 5'-coding sequence. Plasmid pBSratneuNT was next digested with both *Sbf*I and *Sal*I releasing a 4389-bp fragment, residues 234–4622, which, after gel purification, was cut with *Nco*I to generate a 2775-bp middle fragment comprising residues 234–3008. Lastly, pBSratneuNT was PCR-amplified using primers NTNco (5'-gcatagcggccgccatggacagtaccttctaccgtt-3') and NTSal (5'-ctacg cgtcgacacaggtacatccaggcctaggtac-3') to produce a 768-bp fragment of the *neu*-coding sequence comprising residues 3009–3777. At its 3' end, this amplicon contained a *Sal*I site for cloning and a final residue codon mutation of GTA to GTG. The NTNco/NTSal PCR amplicon—residues 3009–3777—was used without subcloning in a three-way ligation, with the *Sbf*I/*Nco*I middle fragment—residues 234–3008—and the plasmid BSNeuNsi—residues −105 to 233—cut with *Sbf*I/*Sal*I. Following transformation, clone selection and DNA sequencing, a correct full-length neuNT cDNA clone was identified, pBSNeuNsiSal. This plasmid was cut with both *Nsi*I and *Sal*I to release the neuNT cDNA and was treated with mung bean nuclease to remove 3' and 5' overhangs, generating a blunt-end cDNA. Treatment with Antarctic alkaline phosphatase removed terminal phosphates allowing neuNT cDNA to be TOPO cloned into pFASTBac/CT-TOPO vector. The recombinant bacmid was transfected into the Sf9 cell line to produce P1baculovirus stock expressing HER2/neu (bac HER2/neu), which was further amplified with several rounds of infection of Sf21 cells. Bac HER2/neu was used to infect Sf21 cells. Infected HER2/neu Sf21 cells were pelleted and used to generate Sf21neu lysate for restimulation and enzyme-linked immunosorbent assay as described below.

Animal experiments

Four to 6-week-old FVB/N mice (Jackson Labs, Bar Harbor, ME, USA) were maintained in a high-efficiency particulate air-filtered cage system for at least 1 week prior to use. For *in vivo* experiments involving tumor-bearing mice, anesthetized FVB/N mice were injected using a 27-gauge tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ, USA) into the right second mammary fat pad with 2×10^{6} NBT1 cells suspended in Hanks balanced salt solution (Sigma-Aldrich) as previously described.[25] For *in vivo* vaccination studies in naive FVB/N mice, mice received 2 subcutaneous (s.c.) or intra-mammary fat pad (i.m.f.) injections, 2 weeks apart, using a cocktail of 1×10^{6} plaque-forming unit (p.f.u.) VVGMCSF plus either 1×10^{6} p.f.u. VVneu or VVβGal using a 27-gauge tuberculin syringe. In *in vivo* vaccination studies of NBT1 tumor-bearing mice, mice were treated with 2 s.c. or intratumoral (i.t.) injections, 2 weeks apart, of 1×10^{6} p.f.u. VVGMCSF plus 7.5 μg keyhole limpet hemocyanin (KLH) (Sigma-Aldrich) with either 1×10^{6} p.f.u. VVneu or VVβGal. For vaccinations, i.m.f. injections were made directly into the number 2 fat pad. All i.t. injections were made directly into the mammary fat pad tumors. S.c. injections were placed in the contralateral (left) groin. All viral injections used the same total of 2×10^{6} vaccinia p.f.u. Five animals per group were used for each independent *in vivo* experiment. Animal experiments were conducted in accordance with protocols approved by the Rutgers Institutional Animal Care and Use Committee.

CTL assays

CTL assays were performed as previously described,[21,26] with several modifications. Briefly, effector cells were prepared from splenocytes, vaccination site-draining lymph nodes (VDN) or tumor-draining lymph nodes of treated/vaccinated FVB/N mice. Spleens or lymph nodes were homogenized and red blood cells were lysed using ammonium chloride buffer (ACK buffer, 0.15M NH_4Cl, 1.0 mM $KHCO_3$ and 0.1 mM EDTA), washed with tissue culture medium (TCM) and filtered through a 70-μm nylon mesh (BD Biosciences, San Jose, CA, USA). Effector cells were resuspended at 7×10^{6} cells ml^{-1} and cultured with 3×10^{6} cells ml^{-1} of irradiated (25 Gray) splenocytes from naive FVB/N mice that had been incubated overnight in 50 ml conicals at 37 °C at 4×10^{6} cells ml^{-1} in TCM+2-mercaptoethanol with 300 μg ml^{-1} of the $RNEU_{420–429}$ (PDSLRDLSVF) (Genscript, Piscataway, NJ, USA) immunodominant epitope of the rat neu protein,[27] lymphocytic choriomeningitis virus nucleoprotein $NP_{118–126}$ (RPQASGVYM) control

peptide (Genscript) or indicated cell lysates. Restimulation cultures were in a total of 2 ml of TCM+2-mercaptoethanol in 24-well plates for 5 days at 37 °C, 5% CO_2. On day 3 of restimulation, 50 µl of supernatant was assayed for interferon-γ using an enzyme-linked immunosorbent assay, as described previously.[28] On day 5, stimulated effector cells were collected and cultured for 4 h with ^{51}Cr-labeled NBT1 cells as targets. Hundred µl of supernatant were removed and ^{51}Cr release of target cells was measured with a gamma counter (Packard Bioscience, Meriden, CT, USA). Percent-specific lysis was calculated from the formula [(experimental release − spontaneous release) × 100/(maximal release in 1% SDS − spontaneous release)].

Magnetic bead depletion of MDSCs

MDSCs were depleted from the splenocyte population of NBT1 tumor-bearing mice using a mouse MDSC isolation kit (Miltenyi Biotec, Auburn, CA, USA) as per the manufacturer's instructions. Briefly, 1×10^8 splenocytes were resuspended in 350 µl MACS buffer (phosphate-buffered saline, 0.5% bovine serum albumin and 2 mM EDTA) and blocked for 10 min with 50 µl FcR blocking reagent at 4 °C. Hundred µl of anti-Ly6G-biotin was added and incubated for 10 min at 4 °C. Cells were washed in 10 ml MACS buffer and resuspended in 100 µl MACS buffer. Two hundred µl anti-biotin microbeads were added and incubated at 4 °C for 15 min. Magnetic separation was performed using LS disposable column and MACS Magnetic separator (Miltenyi Biotec). The negative fraction was collected and restimulated as described above. MDSC depletion was validated using flow cytometry.

Flow cytometry

Flow cytometry for $RNEU_{420–429}$ tetramer (provided by the NIAID Tetramer Facility, Atlanta, GA, USA) was conducted as described previously.[21] Briefly, effectors from treated mice were restimulated for 5 days as described above. Effectors were collected, washed and resuspended in phosphate-buffered saline/5% fetal bovine serum with 0.1% w/v sodium azide at 1×10^6 cells per 100 µl. Cells were stained with anti-CD8α-fluorescein isothiocyanate at the manufacturer's recommended concentration (BD Biosciences) and the $RNEU_{420–429}$-specific tetramer labeled with phycoerythrin (1:100 dilution). Flow cytometry for MDSC was conducted on splenocytes, lymph node cells and tumor suspensions straight *ex vivo*. Tumor suspensions were prepared as described previously,[29] with modifications. Tumors were cut into small pieces and incubated at 37 °C with gentle shaking for 20 min in 5 ml RPMI with collagenase D (1 mg ml^{-1}, Roche, Diagnostics, Indianapolis, MO). Tumor pieces were dissociated with a metal strainer, washed in TCM+2-mercaptoethanol, red blood cells were lysed using ACK buffer, washed again with TCM and filtered through a 70-µm nylon mesh (BD Biosciences). Cells were stained using anti-Gr-1 PE (eBioscience, San Diego, CA, USA), CD11b fluorescein isothiocyanate (eBioscience), anti-Ly6G APC (BD Biosciences) and anti-Ly6C PE-Cy7 (eBioscience). Flow cytometry data were acquired using a FC-500 flow cytometer (Beckman Coulter, Miami, FL, USA) and analyzed using CXP (Beckman Coulter) provided by the CINJ flow cytometry shared resource.

Statistical analysis

Results were expressed as mean ± s.e. Significance for experiments with >2 conditions ($P < 0.05$) was determined by analysis of variance with the *post hoc* Tukey multiple comparisons test using the InStat software package (GraphPad Software, La Jolla, CA, USA). Significance for experiments with two conditions was determined using the Student's *T*-test, two-tailed with unequal variances in Microsoft Excel (Microsoft, Redmond, WA, USA). Kaplan–Meier survival curves were generated using Prism (GraphPad Software), with significance calculated using the log-rank (Mantel–Cox) test and correction for multiple comparisons using the Bonferroni method.

RESULTS

Vaccination of naive FVB/N mice results in a systemic HER2 MHC class I epitope-specific CTL response

We hypothesized that vaccination of a naive host with a vaccinia construct expressing HER2/neu would induce an antigen-specific Th1 immune response, regardless of the site of vaccination. We utilized i.m.f. injection or contralateral s.c. injection of VVneu +VVGMCSF in naive FVB/N mice. Two weeks after the final vaccination, splenocytes and draining lymph nodes of the vaccination site (VDN) were restimulated with irradiated splenocytes from naive female FVB/N mice that had been pulsed with immunodominant $RNEU_{420–429}$ peptide (Figure 1a). Both s.c. and i.m.f. vaccination with VVneu and VVGMCSF resulted in increased percent-specific lysis of ^{51}Cr-labeled NBT1 tumor cells by splenic CTLs *in vitro*, when compared with controls (Figure 1b).

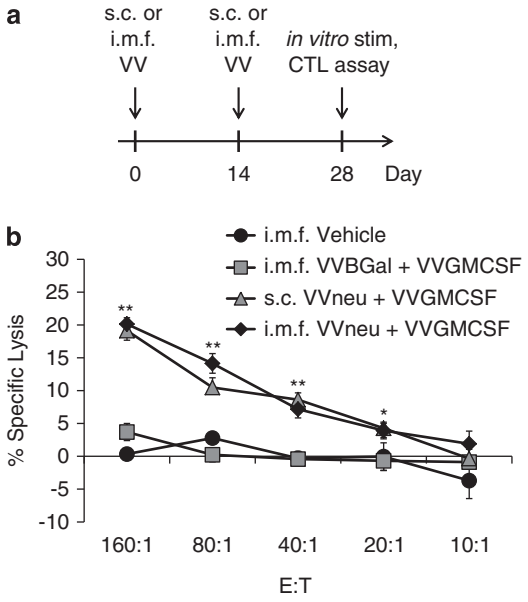

Figure 1. Vaccination of naive FVB/N mice with VVneu induces a systemic HER2-specific Th1 response. (**a**) Female FVB/N mice were injected twice, 2 weeks apart, with VVneu+VVGMCSF (either subcutaneous (s.c.) or intra-mammary fat pad (i.m.f.)), VVBGal +VVGMCSF (i.m.f.) or vehicle (i.m.f.). Two weeks after the second and final vaccination, spleens were restimulated with irradiated splenocytes from naive female FVB/N mice that had been pulsed with immunodominant $RNEU_{420–429}$ peptide. (**b**) NBT1 tumor-specific systemic (spleen) cytotoxic T lymphocyte (CTL) activity against NBT1 target cells after restimulation. Differences of both s.c. and i.m.f. VVneu+VVGMCSF groups compared with controls were significant (**$P < 0.01$, *$P < 0.05$). Results are representative of three independent experiments.

MDSCs suppress the systemic antitumor immune response

MDSCs are a heterogeneous population of immature granulocytic and monocytic cells that have inhibitory effects on tumor-specific T-cell activation and function.[5,6,30] Given previous studies demonstrating the importance of a type 1 antitumor response in effective immune therapies,[21,28] we hypothesized that MDSCs inhibited a systemic antitumor immune response in the NBT1 model. To determine whether the presence of orthotopic NBT1 tumor affected levels of MDSC in FVB/N mice, we injected FVB/N mice with NBT1 cells in the right number 2 mammary fat pad. After 4–5 weeks of tumor growth, we used flow cytometry to measure levels of Gr-1, Ly-6G, Ly-6C and CD11b on cells from spleen and tumor. The $CD11b^+Gr1^+$ population was significantly increased in the systemic population of tumor-bearing mice compared with naive mice (Figure 2a). A large population of $CD11b^+Ly\text{-}6C^{int}Ly6G^+$ cells was detected, a phenotype consistent with granulocytic MDSCs (gMDSCs, Figure 2b).

To demonstrate the immune-suppressive nature of the MDSCs induced by NBT1, we used a MDSC magnetic bead isolation kit

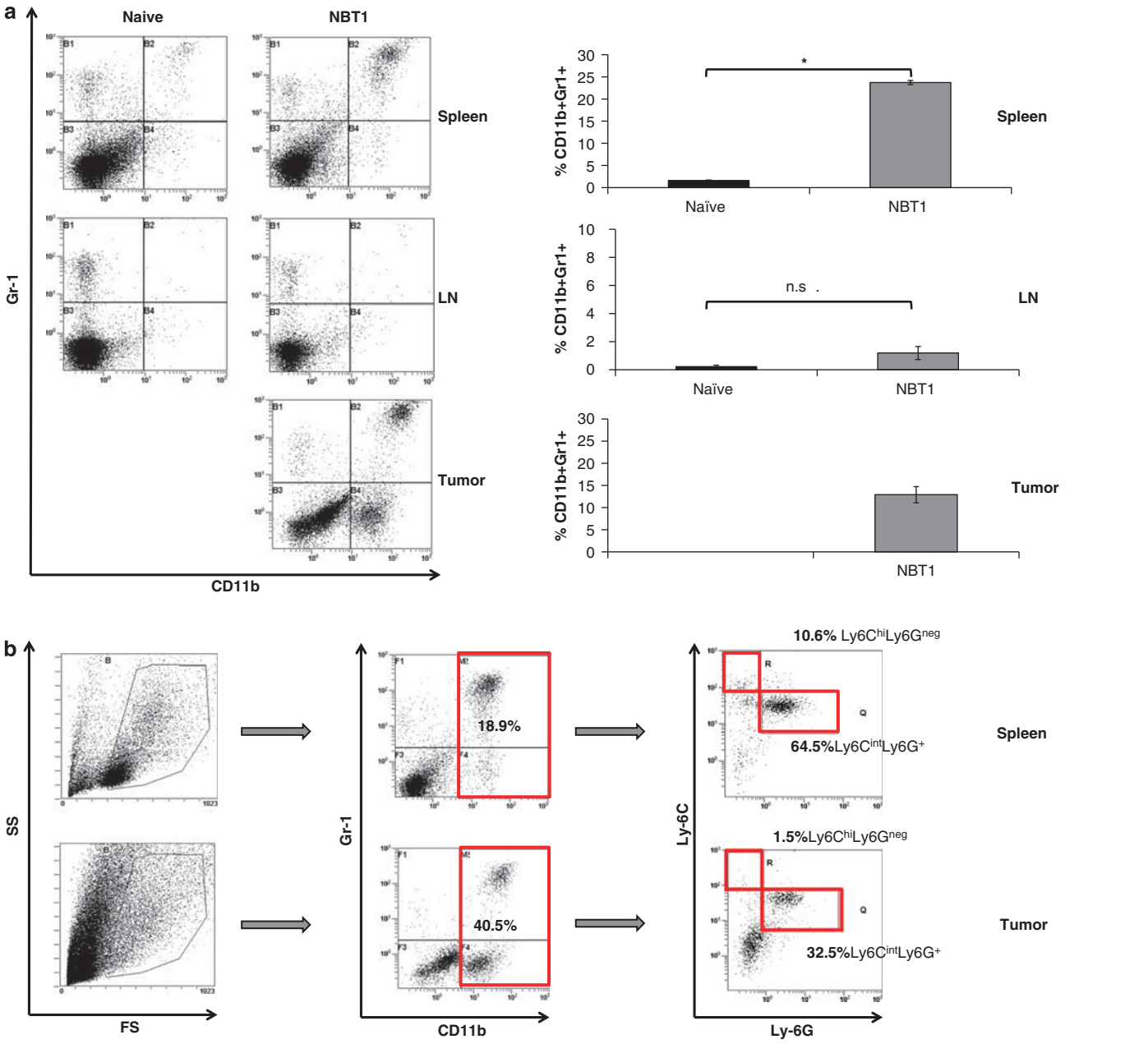

Figure 2. NBT1 growth in female FVB/N mice leads to increased levels of Ly6G$^+$ gMDSC, both systemically and in the tumor microenvironment. (**a**) Flow cytometry was performed on spleen, tumor-draining lymph node (TDN) and tumor samples stained for Gr-1, CD11b, Ly-6C and Ly-6G. Levels of CD11b$^+$Gr1$^+$ cells in spleen, TDN and tumor were compared with levels in spleen and axillary lymph node (LN) of naive female FVB/N mice. Cumulative results are presented from five independent experiments (*$P < 0.05$). (**b**) Cells from spleen and tumor were gated to include monocytic and granulocytic populations for evaluation of Gr-1 and CD11b. This population was then further gated for CD11b$^+$ cells, which were evaluated for Ly-6G and Ly-6C. Results are representative of three independent experiments. NS, not significant.

(Miltenyi Biotec, Auburn, CA) to deplete Ly6G$^+$ cells from the splenocyte population from NBT1 tumor-bearing mice. We validated depletion of MDSCs from the negative fraction using flow cytometry staining for CD11b, Gr-1, Ly6G and Ly6C (Figure 3a). We then restimulated this MDSC-depleted splenocyte population with irradiated splenocytes from naive female FVB/N mice that had been pulsed with the immunodominant major histocompatibility complex (MHC) class I RNEU$_{420–429}$ peptide or HER2/neu protein in Sf21 neu baculovirus-infected lysate, with NP$_{118–126}$ peptide and unmodified Sf21 cells used as restimulation controls. Interferon-γ production by MDSC-depleted splenocytes was increased in most conditions compared with pre-depletion controls, with HER2/neu-restimulated effectors significantly increased compared with all other conditions (Figure 3b).

Vaccination with VVneu+VVGMCSF into the tumor microenvironment, but not VVGMCSF alone, results in a systemic HER2-specific CTL response, decreased MDSCs and tumor regression

We asked whether vaccination with HER2/neu-antigen-encoding VVneu and VVGMCSF could overcome MDSC-associated anergy against HER2/neu induced by the NBT1 model. After 2 weeks of

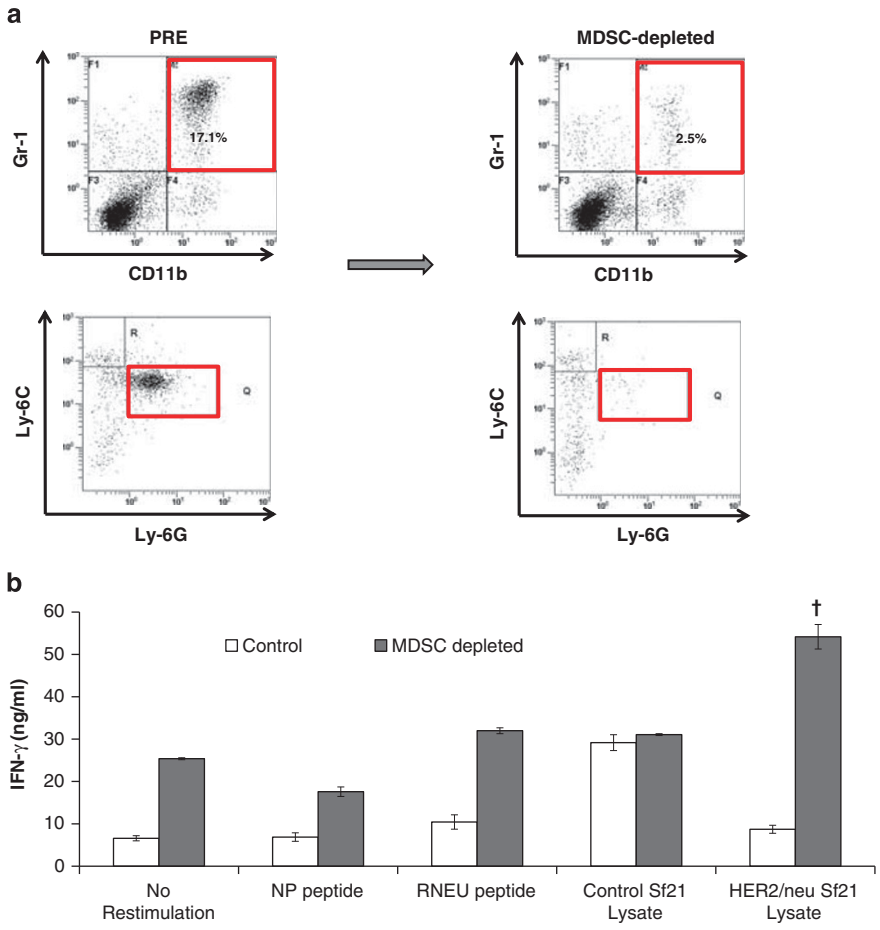

Figure 3. Myeloid-derived suppressor cells (MDSCs) suppress a HER2/neu-specific Th1 response in NBT1 tumor-bearing mice. MDSCs from spleen of NBT1 tumor-bearing mice were depleted using a MDSC ($Ly6G^+$) magnetic bead isolation kit. (**a**) Flow cytometry was performed on pre- and post-depletion samples stained for Gr-1, CD11b, Ly-6C and Ly-6G. (**b**) The MDSC-depleted negative fraction was restimulated with irradiated naive splenocytes pulsed with control $NP_{118–126}$ peptide, $RNEU_{420–429}$ peptide, lysate from Sf21 insect cells that had been infected with baculovirus expressing HER2/neu and control Sf21 cell lysate. Interferon (IFN)-γ enzyme-linked immunosorbent assay was performed in triplicate on supernatant from MDSC-depleted effectors. Results are representative of three independent experiments. ($^{\dagger}P < 0.001$ compared with all other stimulation conditions.).

orthotopic NBT1 tumor growth, two doses of VVneu+VGMCSF +KLH, 2 weeks apart, were administered subcutaneously or by injection into the tumor microenvironment (i.t., Figure 4a). KLH was added to all treatment groups and controls, as previous studies by our group and others had demonstrated its effectiveness as a vaccine adjuvant.[21,31,32] Two weeks after the final vaccination, splenocytes and VDN were restimulated with irradiated splenocytes from naive female FVB/N mice that had been pulsed with immunodominant $RNEU_{420–429}$ peptide. Splenocytes from mice vaccinated with i.t. VVneu+VVGMCSF+KLH showed a significantly increased ability to lyse target NBT1 cells after *in vitro* restimulation when compared with both equivalent s.c. vaccination and i.t. VVBGal+VVGMCSF+KLH (Figure 4b). Similarly, cytolytic activity of the VDN population was highest in mice given i.t. VVneu+VVGMCSF+KLH (Figure 4c). Increased CTL activity was specific to the $RNEU_{420–429}$ MHC class I epitope, as restimulation with control $NP_{118–126}$ did not result in increased cytolytic activity (not shown). The percentage of restimulated $CD8^+$ lymphocytes that were RNEU tetramer-positive from both spleen and VDN was significantly higher in mice that were treated with i.t. VVneu+VVGMCSF+KLH than in all other groups (Figure 4d).

MDSC levels were evaluated using flow cytometry. In mice treated with i.t. VVneu+VVGMCSF+KLH, MDSC levels in spleen on day 28 were significantly decreased compared with i.t. vehicle (Figure 5). Systemic MDSC levels were not significantly decreased in mice treated with s.c. VVneu+VVGMCSF+KLH. Notably, tumor-bearing mice treated with i.t. VVBGal+VVGMCSF+KLH exhibited a significant increase in intratumoral, although not systemic, MDSC levels (Figure 5). A potential explanation may be that, in addition to its enhancing effects on antigen presentation, GM-CSF is one of multiple cytokines that support development of MDSCs.[30]

Primary tumors in mice vaccinated with i.t. VVneu+VVGMCSF +KLH regressed from peak size and were significantly smaller than all other treatment conditions on day 42, including equivalent s.c. VVneu and i.t. VVGMCSF without antigen (Figures 6a–c). S.c. VVneu +VVGMCSF+KLH vaccination or i.t. VVBGal+VVGMCSF+KLH did not inhibit NBT1 growth.

DISCUSSION

The studies presented here demonstrate, using an aggressive orthotopic model of HER2/neu-driven mammary tumor (NBT1), that growth of the tumor results in a significant infiltration of intratumor and systemic MDSC, resulting in the lack of

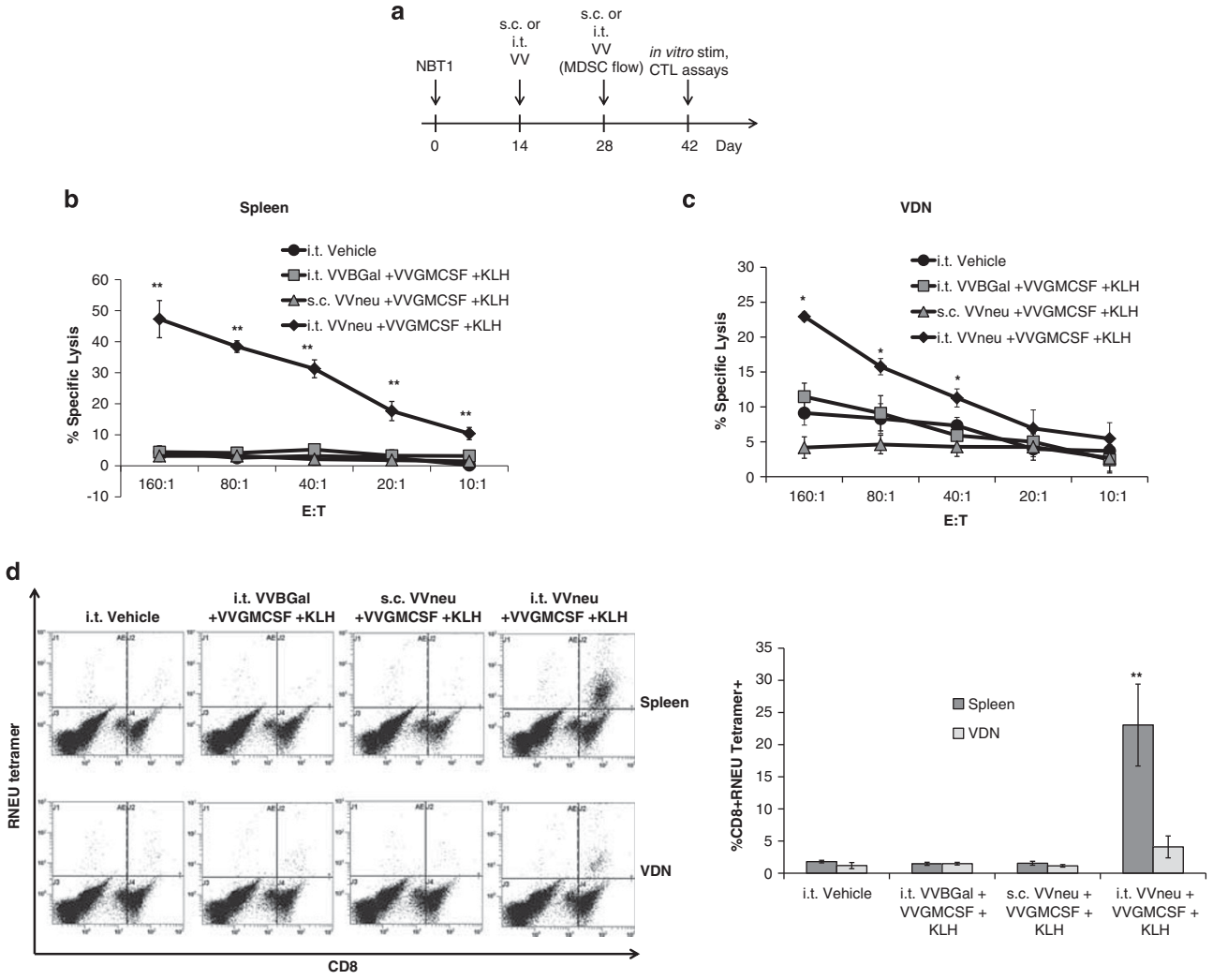

Figure 4. Vaccination with VVneu into the tumor microenvironment generates a systemic antitumor cytotoxic T lymphocyte (CTL) response. (**a**) Female FVB/N mice were injected with 2×10^6 NBT1 tumor cells into the right second mammary fat pad. Mice were treated twice (days 14 and 28) with VVneu+VVGMCSF+KLH (subcutaneous (s.c.) or intratumoral (i.t.)), VVBGal+VVGMCSF+KLH (i.t.) or vehicle control (i.t.). On day 42, spleens and vaccination site-draining lymph nodes (VDN) were restimulated with irradiated naive splenocytes that had been pulsed with $RNEU_{420–429}$ peptide. (**b**) Ability of splenocyte effectors to lyse target NBT1 cells was measured by the ^{51}Cr release assay. Difference between i.t. VVneu+VVGMCSF+KLH and all other conditions was significant (**$P < 0.01$). (**c**) Tumor-specific CTL activity of VDN effector cells was measured by ^{51}Cr lysis. Differences between i.t. VVneu+VVGMCSF+KLH and all other groups were statistically significant (**$P < 0.01$, *$P < 0.05$). (**d**) Restimulated effectors were evaluated for RNEU tetramer-positive CD8+ cells using flow cytometry. The percent of CD8+ cells that are RNEU tetramer positive was measured, with representative dot plots and cumulative results of three independent experiments presented (**$P < 0.01$). KLH, keyhole limpet hemocyanin.

development of systemic immunity and anergy to peripheral s.c. immunization using a neu-encoding vaccinia vaccine that is effective in naive (non-tumor bearing) mice. Treatment of tumor-bearing mice using i.t. oncolytic VVGMCSF fails to induce systemic immunity nor does it lead to tumor regression. In fact, intratumoral VVGMCSF and resulting GM-CSF expression in tumor leads to a significant expansion of tumor resident MDSC, as suggested by the Vonderheide group.[33] However, when tumor antigen-encoding VVneu is combined with VVGMCSF, thus providing a virally expressed tumor antigen to the tumor microenvironment, the combination results in the generation of systemic neu-specific immunity, a significant reduction in tumor and systemic MDSC and a significant antitumor response. We confirmed the contribution of MDSCs to immune escape in our model using flow cytometry to identify a population of $CD11b^+$ $Ly\text{-}6C^{int}Ly\text{-}6G^+$ cells. We also demonstrated that these cells have suppressive effects by finding, despite an overall increased background of IFN-γ in MDSC-depleted cultures, increased HER2/neu-specific IFN-γ production of the systemic splenocyte population after depletion of $Ly\text{-}6G^+$ cells.

The goal of tumor immunotherapy is the development of modalities given alone or in combination that result in the generation of an effective systemic tumor-specific immune response by the host that can overcome immune escape mechanisms. Original studies from our laboratory using a murine bladder cancer model demonstrated the unexpected expansion of antigen-specific (tetramer positive) $CD8^+$ T cells in the tumor microenvironment, but not systemically.[21] This led us to immunize intratumorally using a vaccinia-encoding vaccine with resultant induction of systemic T-cell immunity.[21] Coincidently, studies from the Schlom group using a colon cancer model demonstrated the induction of significant antitumor immunity, when priming or

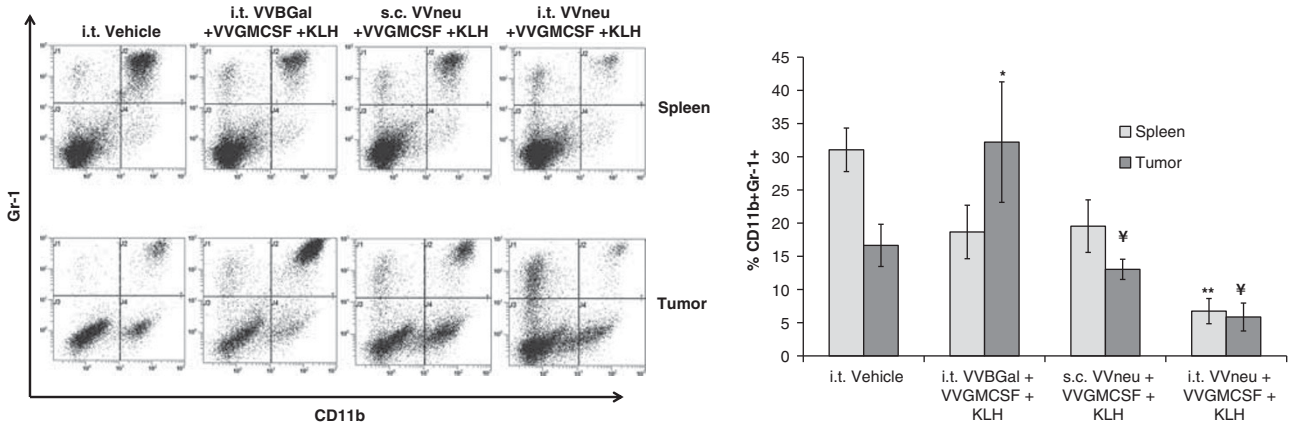

Figure 5. Vaccination with VVneu into the tumor microenvironment results in decreased levels of systemic and tumor microenvironment myeloid-derived suppressor cells. Splenocytes and tumor were stained *ex vivo* for Gr-1 and CD11b using flow cytometry on day 28. Representative dot plots and combined mean %CD11b$^+$Gr-1$^+$ cells from three independent experiments are shown (*$P < 0.05$ compared with intratumoral (i.t.) vehicle tumor, **$P < 0.01$ compared with i.t. vehicle spleen, ¥$P < 0.05$ compared with i.t. VVBGal+VVGMCSF+KLH tumor; all other comparisons are not significant). KLH, keyhole limpet hemocyanin; s.c., subcutaneous.

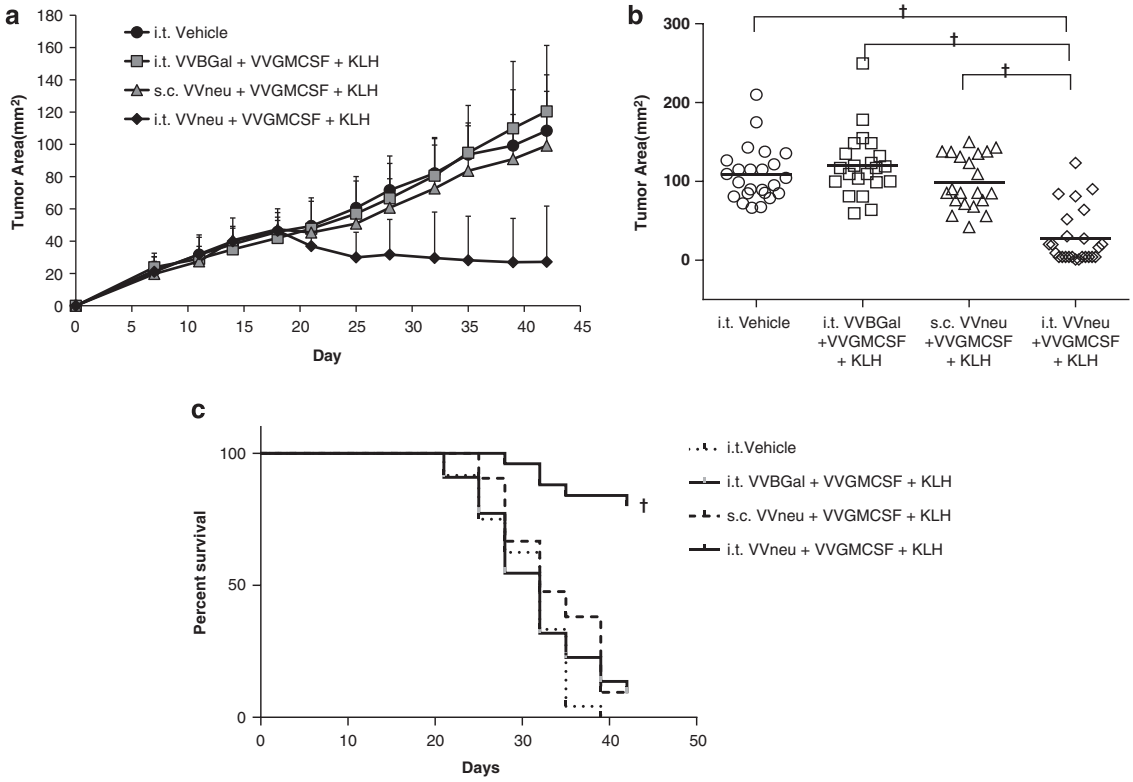

Figure 6. Vaccination with VVneu into the tumor microenvironment results in regression of NBT1 tumor. (**a**) Mean tumor size and s.d. from five combined independent experiments are shown. (**b**) Day 42 tumor size from five combined experiments, each point representing one mouse from the indicated treatment condition (†$P < 0.001$). (**c**) Kaplan–Meier survival curve from five combined experiments (†$P < 0.001$). KLH, keyhole limpet hemocyanin; i.t., intratumoral; s.c., subcutaneous.

boosting immunizations were given intratumorally.[34] These initial responses to intratumoral poxvirus-based vaccines have been translated to phase I trials in prostate cancer[35] and by us in pancreatic cancer[36] using antigen-encoding non-replicating fowlpox vectors.

The strategy of delivering an oncolytic virus to a tumor has long been studied with the overall hypothesis being that virus-mediated oncolysis could have primary antitumor effectiveness and as a sequellae of tumor lysis, antigen released into the tumor microenvironment could lead to a consolidating systemic antitumor response active against metastases. As recently reviewed by Lichty *et al*,[37] preclinical and clinical studies are ongoing that use a variety of oncolytic vectors and encoded immune regulatory molecules. Our initial studies focused on the

potential of generating systemic immunity using the oncolytic VVGMCSF, which would have the potential of lysing tumors, eliciting antigens and enhancing antigen presentation via the GM-CSF. This agent studied by others as JX-594 was subsequently characterized as to its bioavailability, and antitumor and immune responses.[19,20,38] Of particular note in these studies, JX-594 was shown to preferentially persist and replicate in the tumor microenvironment following intravenous administration in preclinical models and in patients.[19,20] Given that accessibility of tumor for local administration is a limitation to the use of these approaches, this finding suggests that the uniqueness of the vaccinia platform may allow systemic delivery of virus, immune-modulating encoded cytokines, as well as antigen as we present here. Clinical studies using another DNA viral vector HSV-1-GMCSF construct (OncoVEX$^{GM\text{-}CSF}$) have also demonstrated local infectivity, antitumor activity and local and systemic immune responses[39,40] further supporting the potential of this approach. Ongoing preclinical and clinical studies combining the oncolytic virus with other modalities such as chemotherapy and immune checkpoint inhibitors are also under development and ongoing by a number of investigators.[41,42]

In conclusion, the studies presented here provide support for delivering to the tumor microenvironment a combination of virally encoded tumor antigen and immunomodulating GM-CSF, both encoded by the oncolytic vaccinia vector. Our studies demonstrate the requirement for antigen delivered to the microenvironment in overcoming MDSC-based immune escape and subsequent development of a systemic antitumor immune response in our model. Our and others' studies have proven the feasibility of using DNA viral vectors to deliver multiple gene constructs to the tumor microenvironment via direct injection[18–20,38–40] and in some cases following intravenous administration.[19,20] These combined studies support the conclusion that this approach has the potential of impacting tumor alone, and supports future approaches combining this with added immune targeted and antitumor approaches.

CONFLICT OF INTEREST

Dr Lattime is an inventor of the patented recombinant Vaccinia-GMCSF that has been licensed to Sillajen and is being studied as JX-594. As such, he derives royalties and licensing fees from the Thomas Jefferson University where the patent is held. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Studies were supported by NCI R01 CA42908, and the CINJ shared resources were supported by NCI P30 CA72720.

REFERENCES

1 Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DW Jr. *et al.* Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* 2003; **421**: 756–760.
2 Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001; **2**: 127–137.
3 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; **235**: 177–182.
4 Ostrand-Rosenberg S. Myel oid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer Immunol Immunother* 2010; **59**: 1593–1600.
5 Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 2009; **182**: 4499–4506.
6 Youn J-I, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol* 2008; **181**: 5791–5802.
7 Youn J-I, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *J Leuk Biol* 2012; **91**: 167–181.
8 Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 2012; **12**: 253–268.
9 Li Z, Pang Y, Gara SK, Achyut BR, Heger C, Goldsmith PK *et al.* Gr-1+CD11b+ cells are responsible for tumor promoting effect of TGF-beta in breast cancer progression. *Int J Cancer* 2012; **131**: 2584–2595.
10 Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res* 2009; **70**: 68–77.
11 Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L *et al.* Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 2007; **13**: 828–835.
12 Takaku S, Terabe M, Ambrosino E, Peng J, Lonning S, McPherson JM *et al.* Blockade of TGF-[beta] enhances tumor vaccine efficacy mediated by CD8+ T cells. *Int J Cancer* 2009; **126**: 1666–1674.
13 Goulart MR, Pluhar GE, Ohlfest JR. Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. *PloS One* 2012; **7**: e33274–e33274.
14 Mundy-Bosse BL, Young GS, Bauer T, Binkley E, Bloomston M, Bill Ma *et al.* Distinct myeloid suppressor cell subsets correlate with plasma IL-6 and IL-10 and reduced interferon-alpha signaling in $CD4^+$ T cells from patients with GI malignancy. *Cancer Immunol Immunother* 2011; **60**: 1269–1279.
15 Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD *et al.* Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* 2003; **198**: 1741–1752.
16 Lee SS *et al.* Intravesical gene therapy: vaccinia virus recombinants transfect murine bladder tumors and urothelium. *Proceedings of the American Association for Cancer Research* 1993; **34**: 337. abstract 2005.
17 Mastrangelo MJ, Maguire HC, McCue PA, Lee SS, A A, LN N. A pilot study demonstrating the feasibility of using intratumoral vaccinia injections as a vector for gene transfer. *Vaccine Res* 1995; **4**: 55–69.
18 Mastrangelo MJ, Maguire HC Jr., Eisenlohr LC, Laughlin CE, Monken CE, McCue PA *et al.* Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther* 1999; **6**: 409–422.
19 Parato KA, Breitbach CJ, Le Boeuf F, Wang J, Storbeck C, Ilkow C *et al.* The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther* 2012; **20**: 749–758.
20 Breitbach CJ, Burke J, Jonker D, Stephenson J, Haas AR, Chow LQM *et al.* Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 2011; **477**: 7102–7362.
21 Yang AS, Monken CE, Lattime EC. Intratumoral vaccination with vaccinia-expressed tumor antigen and granulocyte macrophage colony-stimulating factor overcomes immunological ignorance to tumor antigen. *Cancer Res* 2003; **63**: 6956–6961.
22 Chakrabarti S, Sisler JR, Moss B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* 1997; **23**: 1094–1097.
23 Eisenlohr LC, Yewdell JW, Bennink JR. Flanking sequences influence the presentation of an endogenously synthesized peptide to cytotoxic T lymphocytes. *J Exp Med* 1992; **175**: 481–487.
24 Bargmann CI, Hung M-C, Weinberg RA. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* 1986; **45**: 649–657.
25 Rusciano D, Burger MM. Cancer metastasis: experimental approaches (laboratory techniques in biochemistry and molecular biology). In: Welch DR (ed.) *Cancer Metastasis: Experimental Approaches*. Elsevier Science Publishing Co.: Amsterdam, Netherlands, 2000.
26 McAveney KM, Gomella LG, Lattime EC. Induction of TH1- and TH2-associated cytokine mRNA in mouse bladder following intravesical growth of the murine bladder tumor MB49 and BCG immunotherapy. *Cancer Immunol Immunother* 1994; **39**: 401–406.
27 Ercolini AM, Machiels JP, Chen YC, Slansky JE, Giedlen M, Reilly RT *et al.* Identification and characterization of the immunodominant rat HER-2/neu MHC class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice. *J immunol* 2003; **170**: 4273–4280.
28 Nikitczuk KP. PLGA-polymer encapsulating tumor antigen and CpG DNA administered into the tumor microenvironment elicits a systemic antigen-specific IFN-γ response and enhances survival. *J Cancer Ther* 2013; **04**: 280–290.
29 Sinha P, Chornoguz O, Clements VK, Artemenko KA, Zubarev RA, Ostrand-Rosenberg S. Myeloid-derived suppressor cells express the death receptor Fas and apoptose in response to T cell-expressed FasL. *Blood* 2011; **117**: 5381–5390.
30 Abe F, Dafferner AJ, Donkor M, Westphal SN, Scholar EM, Solheim JC *et al.* Myeloid-derived suppressor cells in mammary tumor progression in FVB Neu transgenic mice. *Cancer Immunol Immunother* 2010; **59**: 47–62.
31 Riggs DR, Jackson B, Vona-Davis L, McFadden D. In vitro anticancer effects of a novel immunostimulant: keyhole limpet hemocyanin. *J Surg Res* 2002; **108**: 279–284.

32 Sabbatini PJ, Ragupathi G, Hood C, Aghajanian CA, Juretzka M, Iasonos A *et al.* Pilot study of a heptavalent vaccine-keyhole limpet hemocyanin conjugate plus QS21 in patients with epithelial ovarian, fallopian tube, or peritoneal cancer. *Clin Cancer Res* 2007; **13**: 4170–4177.
33 Bayne LJ, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ *et al.* Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer Cell* 2012; **21**: 822–835.
34 Kudo-Saito C, Schlom J, Hodge JW. Intratumoral vaccination and diversified subcutaneous/ intratumoral vaccination with recombinant poxviruses encoding a tumor antigen and multiple costimulatory molecules. *Clin Cancer Res* 2004; **10**: 1090–1099.
35 Gulley JL, Heery CR, Madan RA, Walter BA, Merino MJ, Dahut WL *et al.* Phase I study of intraprostatic vaccine administration in men with locally recurrent or progressive prostate cancer. *Cancer Immunol Immunother* 2013; **62**: 1521–1531.
36 Repaka A, Ea Poplin, August Da, Ben-Menachem T, Michael H, Artymyshyn R *et al.* 350 Phase I trial of endoscopic ultrasound (EUS) guided intratumoral vaccination with recombinant Panvac-F and systemic Panvac-V in patients with locally advanced pancreatic cancer. *Gastrointest Endosc* 2013; **77**: 43.
37 Lichty BD, Breitbach CJ, Stojdl DF, Bell JC. Going viral with cancer immunotherapy. *Nat Rev Cancer* 2014; **14**: 559–567.
38 Park BH, Hwang T, Liu TC, Sze DY, Kim JS, Kwon HC *et al.* Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol* 2008; **9**: 533–542.
39 Hu JC, Coffin RS, Davis CJ, Graham NJ, Groves N, Guest PJ *et al.* A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin Cancer Res* 2006; **12**: 6737–6747.
40 Kaufman HL, Kim DW, DeRaffele G, Mitcham J, Coffin RS, Kim-Schulze S. Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding GM-CSF in patients with stage IIIc and IV melanoma. *Ann Surg Oncol* 2010; **17**: 718–730.
41 Lee SY, Kang TH, Knoff J, Huang Z, Soong RS, Alvarez RD *et al.* Intratumoral injection of therapeutic HPV vaccinia vaccine following cisplatin enhances HPV-specific antitumor effects. *Cancer Immunol Immunother* 2013; **62**: 1175–1185.
42 Zamarin D, Holmgaard RB, Subudhi SK, Park JS, Mansour M, Palese P *et al.* Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. *Sci Translat Med* 2014; **6**: 226ra32.