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Opposing Effects of Toll-like Receptor (TLR3) Signaling in Tumors Can Be Therapeutically Uncoupled to Optimize the Anticancer Efficacy of TLR3 Ligands

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Abstract

Many cancer cells express Toll-like receptors (TLR) that offer possible therapeutic targets. Polyadenylic-polyuridylic acid [poly(A:U)] is an agonist of the Toll-like receptor TLR3 that displays anticancer properties. In this study, we illustrate how the immunostimulatory and immunosuppressive effects of this agent can be uncoupled to therapeutic advantage. We took advantage of two TLR3-expressing tumor models that produced large amounts of CCL5 (a CCR5 ligand) and CXCL10 (a CXCR3 ligand) in response to type I IFN and poly(A:U), both *in vitro* and *in vivo*. Conventional chemotherapy or *in vivo* injection of poly(A:U), alone or in combination, failed to reduce tumor growth unless an immunochemotherapeutic regimen of vaccination against tumor antigens was included. CCL5 blockade improved the efficacy of immunochemotherapy, whereas CXCR3 blockade abolished its beneficial effects. These findings show how poly(A:U) can elicit production of a range of chemokines by tumor cells that reinforce immunostimulatory or immunosuppressive effects. Optimizing the anticancer effects of TLR3 agonists may require manipulating these chemokines or their receptors. *Cancer Res; 70(2); 490–500. ©2010 AACR.*

Introduction

Agonists of Toll-like receptors (TLR) are being evaluated for the treatment of cancer (1, 2). Preclinical studies revealed that systemic administration of TLR agonists can boost innate immunity, augment antibody-dependent effector functions, and enhance adaptive immune responses (1–3). TLR3 is the critical sensor of viral double-stranded RNA (4). The synthetic polyinosinic:polycytidylic acid [poly(I:C)] is a TLR3 ligand (TLR3L) that mediates potent adjuvant effects in thus far that it strongly enhances antigen-specific $CD8^+$ T-cell responses (5, 6), promotes antigen cross-presentation by dendritic cells (7), and directly acts on effector $CD8^+$ T and natural killer (NK) cells to augment IFN-γ release (8). Poly(I:C) is recognized by both the endosomal receptor TLR3 and cytosolic receptors, including RNA helicases such as RIG-I and the *melanoma differentiation–associated gene 5* (*MDA5*). In the poly(I:C)-induced immune responses *in vivo, MDA5* is critical for IFN-γ induction, whereas TLR3 is mandatory for IL-12p40 release (9).

Another synthetic double-stranded RNA, polyadenylic:polyuridylic acid [poly(A:U)], which only signals through TLR3, has also been widely used in preclinical and clinical studies. When combined with a candidate protein or viral antigen in mice, poly(A:U) can promote antigen-specific Th1-immune responses and boost antibody production (10, 11). Poly(A:U) has been safely used with moderate success for treating breast or gastric cancers as a monotherapy (12–14). Retrospective analyses highlighted that TLR3-expressing breast cancers may be selectively sensitive to the antitumor effects of poly(A:U). Indeed, TLR3 is not only expressed by immune cells but also by some epithelial (15) or endothelial cells (16). Intracellular staining for TLR3 was reported for human breast cancers (17) and melanoma (18) and its expression can be induced by type I IFNs. TLR3 signaling can directly inhibit the proliferation of carcinoma cells (19) or can induce apoptosis when combined with protein synthesis inhibitors or type I IFN (17, 18). Besides these beneficial effects on established cancers, TLR3 signaling may also

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participate in proinflammatory reactions contributing to tumorigenesis, suggesting that exploiting the TLR system in cancer might be a doubled-edged sword (20–22). Consequently, there is a need for a fine dissection of the direct (on tumor cells) versus the indirect (on immune cells) effects of TLR agonists as their potential anticancer effects are being evaluated.

Taking advantage of two murine tumor models expressing TLR3, we show that poly(A:U) acts not only in host cells but also in the tumor parenchyma to generate the opposite action of two chemokines, CXCL10 and CCL5, which are favorable and deleterious for the clinical outcome, respectively. These findings support the idea that manipulating TLR3 signaling for cancer therapy will benefit from uncoupling chemokine receptor signaling at the tumor/host interface.

Materials and Methods

Reagents. Poly(A:U) was from Innate Pharma. The murine type I IFN was produced by M. Ferrantini (Istituto Superiore di Sanità). Human IFN α2b and ELISA kits for CCL5 and CXCL10 were from R&D Systems. Ovalbumin was from Calbiochem. CpG oligodeoxynucleotide (ODN) 1668 was from MWG Biotech AG. Methionylated RANTES (MetRantes) was provided by Amanda Proudfoot (Merck Serono Geneva Research Center, Geneva, Switzerland).

Mice and cell lines. B16-OVA murine melanoma cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 IU/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acids, and 10 mmol/L HEPES. Murine GL26 glioma cells (H-2b) were maintained in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin/streptomycin, 10 mmol/L HEPES, and 50 μmol/L β-mercaptoethanol. Human breast cancer primary cultures were established at Institut Gustave Roussy from metastatic patients suffering from ascitis; patients provided informed consent. Cells were used after three passages of propagation in AIM-V culture medium.

C57BL/6 mice were purchased from Charles River. C57BL/6 nude mice were obtained from animal facility of Institut Gustave Roussy. *Trif*$^{-/-}$, *Cxcr3*$^{-/-}$, and *Ccr5*$^{-/-}$ green fluorescent protein (GFP) mice were bred at Centre National de la Recherche Scientifique IEM 2815, Orléans, France, and Institut National de la Santé et de la Recherche Médicale, U543, Paris, France. The experimental protocols were approved by the Animal Care and Use Committee of Institut Gustave Roussy.

In vitro *tumor stimulation assays.* B16-OVA (or GL26; 5×10^4) or primary human breast cancer cells (2×10^5) were seeded in 24-well plates, treated with 1,000 IU/mL of type I IFN for 18 h, and then treated with poly(A:U) for 48 h. Supernatants were collected to dose chemokine production.

Tumor models and immunotherapy. B16-OVA (3×10^5 or 6×10^5) and GL26 (6×10^5) cells were inoculated s.c. into the left flank of mice. Vaccines were injected into the right footpad [for CpG+OVA: CpG ODN 1668 (5 μg/mouse) plus ovalbumin (1 mg/mouse)] or right flank [or cell vaccines: 10^6 B16-OVA or GL26 pretreated with type I IFN (1,000 IU/mL) for 18 h and then doxorubicin (20 μmol/L) for 24 h for each mouse]. Chemotherapy (oxaliplatin) was applied i.p. at 5 mg/kg. Poly(A:U) was injected i.p. at 100 μg per mouse in B16-OVA model and at 500 μg per mouse in the GL26 model. MetRantes (10 μg/mouse) was injected i.p. daily for 3 wk to block CCL5. Necrotic cells (F/T) were obtained following two consecutive cycles of freezing (liquid nitrogen) and thawing (37°C). For preimmunization, OVA-CpG vaccine was injected into the right footpad 7 d before inoculation of tumor cells. To block CXCR3, anti–CXCR3-173 neutralizing monoclonal antibody (mAb) or the control mAb (PIP) were injected i.p. at 200 μg per mouse every other day for 12 d since 5 d before tumor cell inoculation.

Lentivirus-based short hairpin RNA construction. The lentivirus construction and viral particles were designed and produced by Vectalys SA. As for the lentivirus carrying the short hairpin RNA (shRNA) knocking down CCL5, the forward primer 5′-CGCGACGTCAAGGAGTATTTCTATTCAAGAGATAGAAATACTCCTTGACGTTTTTTTGCA-3′ and the reverse primer 3′-TGCAGTTCCTCATAAAGATAAGTTCTCTATCTTTATGAGGAACTGCAAAAAA-5′ were annealed and ligated into vector [pLV-H1-EF1-PURO-IRES-GFP (pV2.3.127)] by cohesive *Mlu*I/*Nsi*I ligation. A similar approach was used to knockdown Lamin A/C and TRIF expression targeting sequences 5′-GAAGGAGGGTGACCTGATA-3′ and 5′-GGAAAGCAGTGGCCTATTA-3′, respectively.

Flow cytometry. Cells from tumor, tumor draining lymph node (DLN), or vaccine DLN were isolated by mechanical dissociation and filtered through a 70-μm cell strainer. CD3ε-PerCP, CD8-FITC (BD Pharmingen), CXCR3-PE (R&D System), NK1.1-Pacific Blue (eBioscience), and isotype control antibodies (2.5 μg/mL) were used for the surface staining at 4°C for 30 min. Hydroxystilbamidine (Molecular Probes, Invitrogen) was used to exclude dead cells. For intracellular staining, freshly isolated cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate, 1 μg/mL ionomycin, and Golgi-stop (BD Pharmingen) for 4 h at 37°C in RPMI containing 2% mouse serum (Janvier). Cells were then fixed, permeabilized, and stained with IFN-γ–allophycocyanin (BD Pharmingen) with fixation/permeabilization kits (BD Bioscience).

Protein extraction. Tumors were mechanically dissociated with lysis buffer (T-PER Tissue Protein Extraction Reagent, Pierce) containing a protease inhibitor (complete Mini EDTA-free, Roche). Tumor lysate was then centrifuged at 10,000 × *g* for 5 min at 4°C to obtain supernatant. Alternatively, tumors were digested with 400 U/mL Collagenase IV and 150 U/mL DNase I for 30 min. Single-cell suspension was sorted using AutoMACS (Miltenyi Biotec) to obtain CD45$^+$ and CD45$^-$ fractions, and whole-cell protein was extracted using lysis buffer (1×10^6 cells/100 μL buffer).

Statistical analyses. Comparison of continuous data and categorical data were achieved by the Mann-Whitney *U* test and by χ^2 as appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curves. Statistical analyses were performed using GraphPad Prism 5.0. All *P* values are two-tailed. All *P* values <0.05 were considered statistically significant for all experiments. *, **, and *** indicated *P* values of <0.05, <0.01, and <0.001, respectively.

Results

Synergistic effects between vaccines, chemotherapy, and poly(A:U). To characterize the relative importance of direct effects of poly(A:U) on tumor parenchyma versus indirect, immune-mediated effects, we took advantage of the B16-OVA, which expresses TLR3 (data not shown), such as the parental cell line B16F10 (Supplementary Fig. S1) as well as the model antigen ovalbumin (OVA). Albeit mediating significant cytostatic effects on B16-OVA tumor cells *in vitro* (Supplementary Fig. S2), oxaliplatin-based chemotherapy failed to hamper tumor progression *in vivo* when it was administered alone or combined with the poly(A:U) (Fig. 1*B*), following the protocol detailed in Fig. 1*A*. However, the administration of a vaccine composed of OVA plus the adjuvant CpG before the combination of oxaliplatin and poly(A:U) significantly retarded tumor growth (Fig. 1*B*) and prolonged the survival of tumor-bearing C57BL/6 mice (Fig. 1*C*). This vaccine, when applied in the

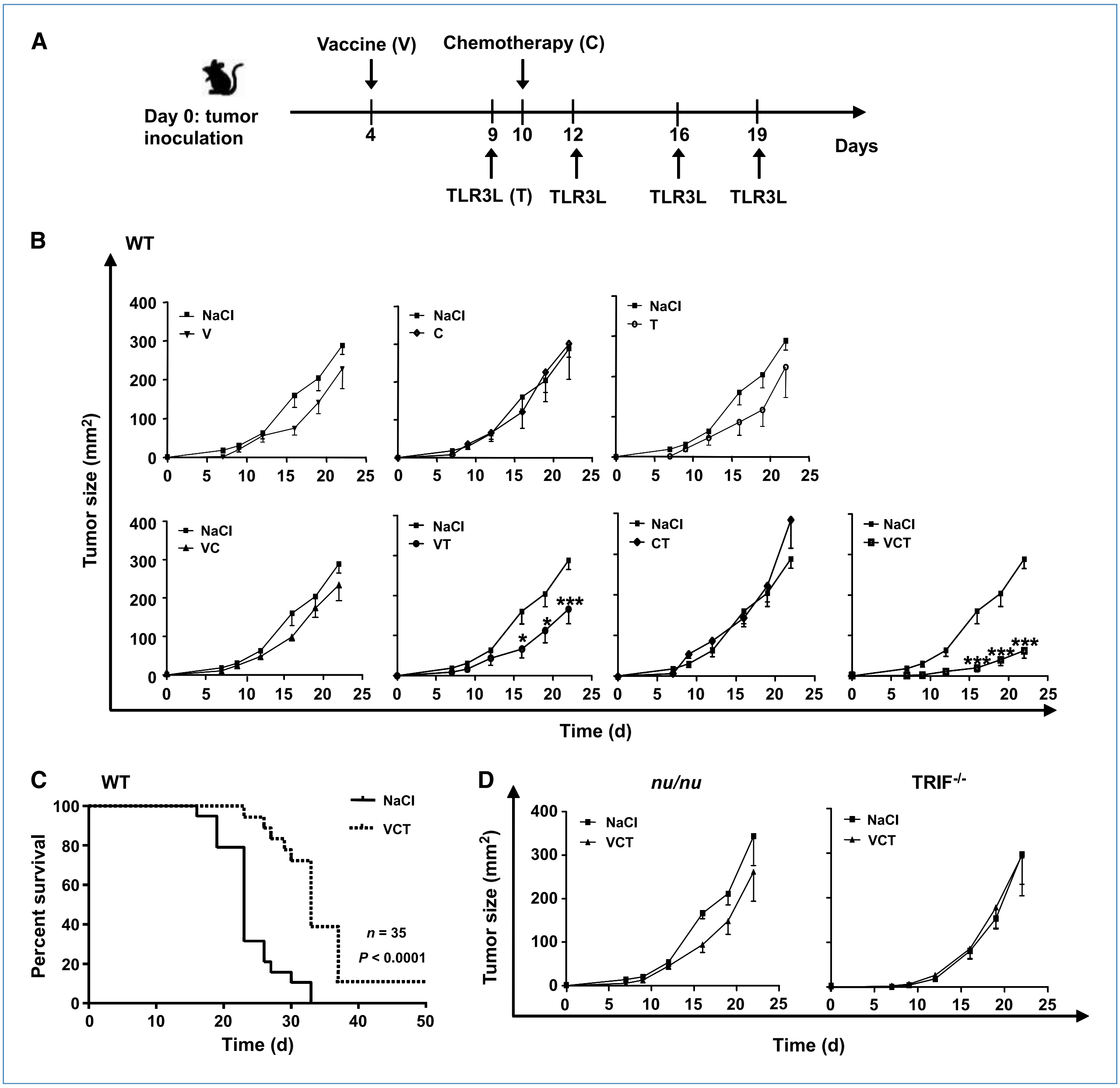

Figure 1. Sequential immunochemotherapy is efficient against established melanoma. *A,* therapeutic setting of VCT treatment is shown as a scheme. *B* and *C,* B16OVA tumor growth was monitored in WT mice receiving single-agent therapy (*V, C,* or *T*), two agent–based therapy (*VC, VT,* or *CT*), or sequential tritherapy (*VCT*). *Points,* mean of tumor size from one representative experiment out of five ($n = 5$ mice per group); *bars,* SEM (*B*). The survival curve shows 35 mice in each group (*C*). *D,* tumor growth curve in *nu/nu* (*left*) or TRIF$^{-/-}$ (*right*) C57Bl/6 mice treated with or without VCT.

footpad opposite to the flank where the tumor was growing, stimulated an OVA-specific Th1 immune response in the DLN (Supplementary Fig. S3). It is noteworthy that B16-OVA did not express TLR9 and did not respond to CpG ODN *in vitro* (data not shown). The antitumor effects of the sequential administration of a vaccine followed by oxaliplatin and TLR3L was well reproducible in immunocompetent wild-type (WT) C57BL/6 mice, yet failed to be observed in *nu/nu* and $Trif^{-/-}$ mice (Fig. 1*D*), indicating the obligate contribution of T cells and TRIF-dependent signals to the therapeutic effect. Altogether, 11% of WT mice were completely protected from melanoma by the sequential therapeutic regimen (Fig. 1*C*), and 67% among the tumor-free mice developed long-term protective immunity and hence became resistant to a later challenge with live tumor cells (data not shown).

We observed a similar antitumor effect when chemotherapy and poly(A:U) injections were combined with a cell-based anticancer vaccine. The freeze-thawing technique aimed at mediating the nonimmunogenic cell death (necrosis) in contrast to anthracycline-induced tumor cell death that generates an endoplasmic reticulum stress response (23). In accordance with our previous reports, type I IFN and doxorubicin induced immunogenic cell death of B16-OVA cells and injection of dying cells induced a protective immunity against later rechallenge with live B16-OVA cells (Fig. 2*A* and *B*). This cell-based vaccine boosted the antitumor activity of the combination of oxaliplatin plus poly(A:U) (Fig. 2*C*) and enhanced survival (Fig. 2*D*) when used in a therapeutic setting after the implantation of tumors. Very similar results were obtained when B16-OVA melanoma cells were replaced by another TLR3-expressing cell line, GL26 glioblastoma (Supplementary Fig. S1), which only bears natural tumor antigens. Vaccination of immunocompetent mice with GL26 cells that were dying in response to type I IFN and doxorubicin was efficient in preventing tumor outgrowth in the prophylactic setting (Fig. 3*A*) and also in the therapeutic setting only if the vaccination was combined with oxaliplatin and TLR3L following a regimen identical to that presented in Fig. 1*A* (Fig. 3*B*). To further show the importance of the TLR3 agonist on tumor parenchyma during vaccine+chemotherapy +TLR3L (VCT) therapy, we selectively knocked down the TRIF adaptor molecule in GL26 glioblastoma (Lamin as a negative control). Interestingly, VCT therapy failed to control the tumor outgrowth of TRIF knockdown GL26 *in vivo* (Fig. 3*C*).

Altogether, it seems that poly(A:U) could mediate synergistic antitumor effects with chemotherapy against established TLR3-expressing tumors, provided that this combined therapy was preceded by anticancer vaccination. For the sake of brevity, we will refer to this therapeutic schedule as "immunochemotherapy."

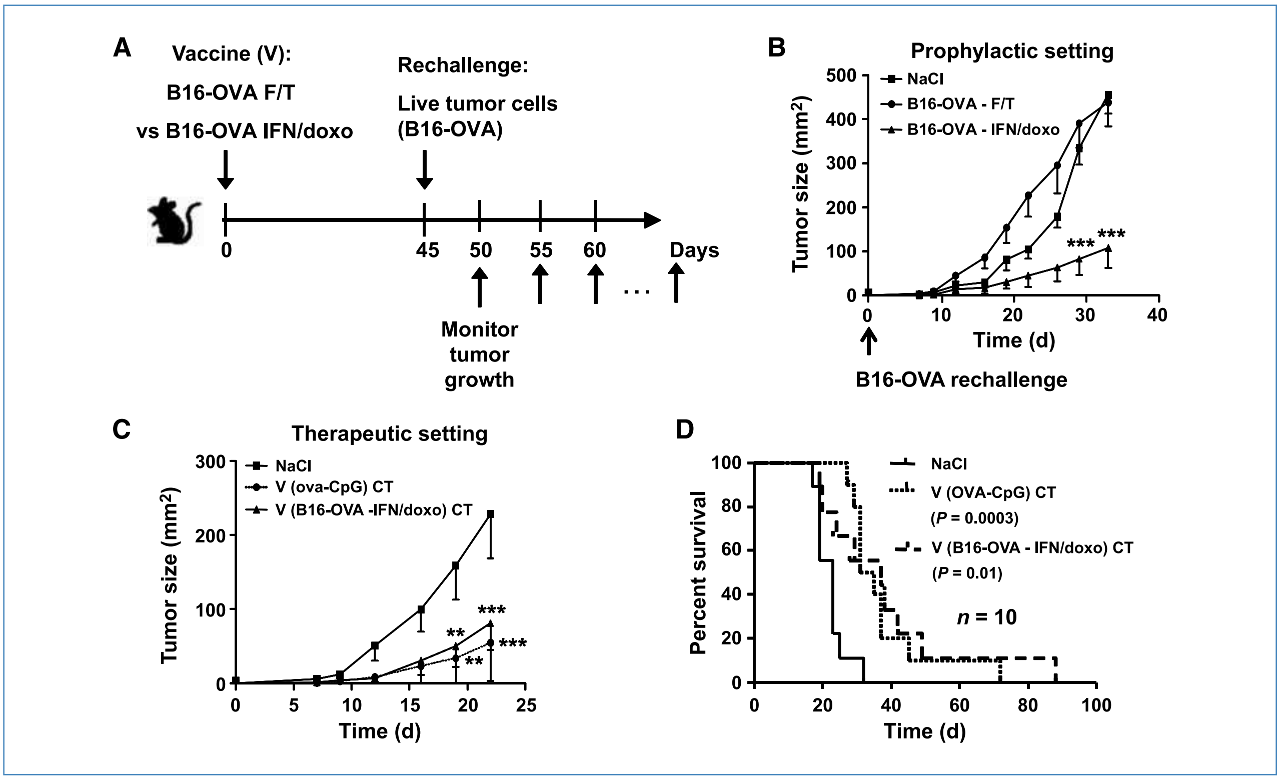

Figure 2. Immunochemotherapy of melanoma with cell-based vaccines inhibits tumor outgrowth. *A*, prophylactic setting in a schematic view. Naïve C57b/6 mice were vaccinated with B16-OVA pretreated with type I IFN plus doxorubicin (*doxo*) or freeze-thawed (*F/T*). Forty-five days later, mice were rechallenged with live syngeneic tumor cells. *B*, tumor growth is depicted with five mice per group following prophylactic setting. *C* and *D*, the therapeutic regimen depicted in Fig. 1*A* was performed with two different vaccines, OVA-CpG or the cell-based vaccine (same as in *A*), and tumor growth was monitored (*C*). Survival curve with 10 mice per group; the *P* value indicates the comparison between each treated and control group (*D*).

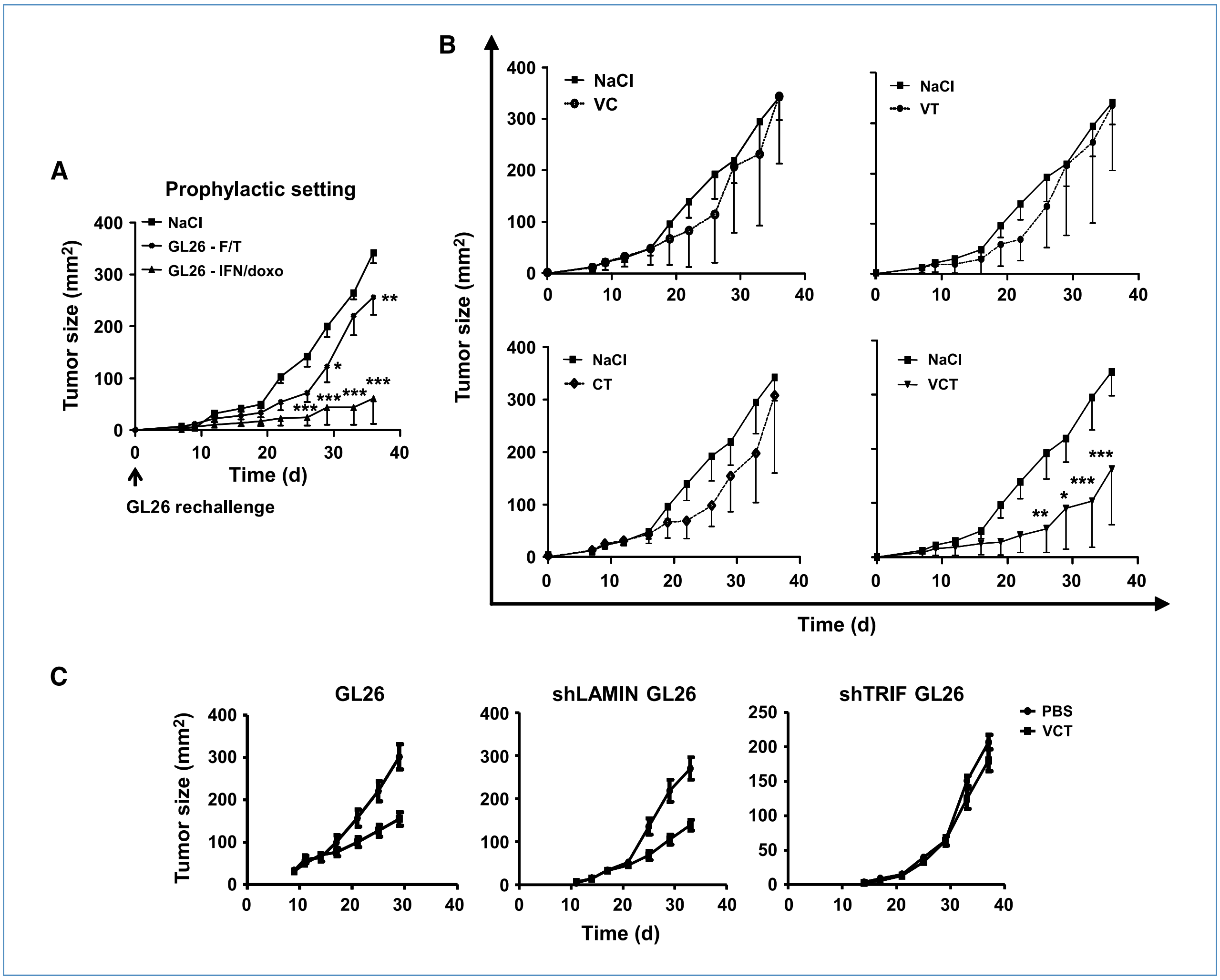

Figure 3. Immunochemotherapy is efficient against established glioblastoma. *A,* naïve C57bl/6 mice were vaccinated with GL26 tumor cells pretreated with type I IFN plus doxorubicin and rechallenged with live syngeneic tumor cells 7 d later. The kinetics of tumor outgrowth are monitored. *B,* the cell-based vaccine was then assessed for its therapeutic efficacy in the VCT setting outlined in Fig. 1*A*. *C,* after knockdown TRIF expression in GL26 (Lamin as a control), established tumors were treated with the VCT protocol starting from day 15. Tumor growth kinetics are shown from one representative experiment with five mice per group.

TLR3-expressing tumors directly responded to poly(A:U). The finding that TRIF must be intact both in the host's immune system and the tumor parenchyma for full antitumor effects (Figs. 1*D* and 3*C*) suggested that poly(A:U) might exert direct effects on the tumor parenchyma. When added to B16-OVA cells *in vitro*, poly(A:U) induced the secretion of copious amounts of both CCL5/RANTES and CXCL10/IP-10. This effect could be further enhanced by preincubation with type I IFN (Fig. 4*A*). Type I IFN plus poly(A:U) showed an additive effect on CCL5 secretion by both GL26 (Fig. 4*B*) and human breast cancer cells (in three of four primary cultures; Supplementary Fig. S4). GL26 cells also secreted more CXCL10 when treated with type I IFN plus poly(A:U) compared with either treatment alone (Fig. 4*B*). TRIF knockdown GL26 cells lost their response to poly(A:U) stimulation, whereas Lamin knockdown GL26 behaved like parental cells (Supplementary Fig. S5). Interestingly, the secretion of CXCL1 by B16-OVA was abolished by poly(A:U) (Fig. 4*A*).

To validate these findings *in vivo*, we studied the concentration of CCL3/MIP-1α, CCL5, and CXCL10 within tumor beds at each single step of the tritherapy in B16-OVA model. We observed a significant production of CCL5 at baseline before chemotherapy. This CCL5 production dropped after the first TLR3L injection but increased again after the third injection of poly(A:U) (Fig. 4*C, top left*), whereas no CCL3 was produced (data not shown). In accordance with *in vitro* data, the tissular concentration of CXCL10 paralleled that of CCL5 *in vivo* after oxaliplatin injection and the third injection of poly(A:U) (Fig. 4*C, bottom right*). To further dissect whether chemokine production originated from leukocytes or tumor cells, we sorted CD45$^+$ versus CD45$^-$ cells from dissociated tumor beds after each poly(A:U) injection and observed that

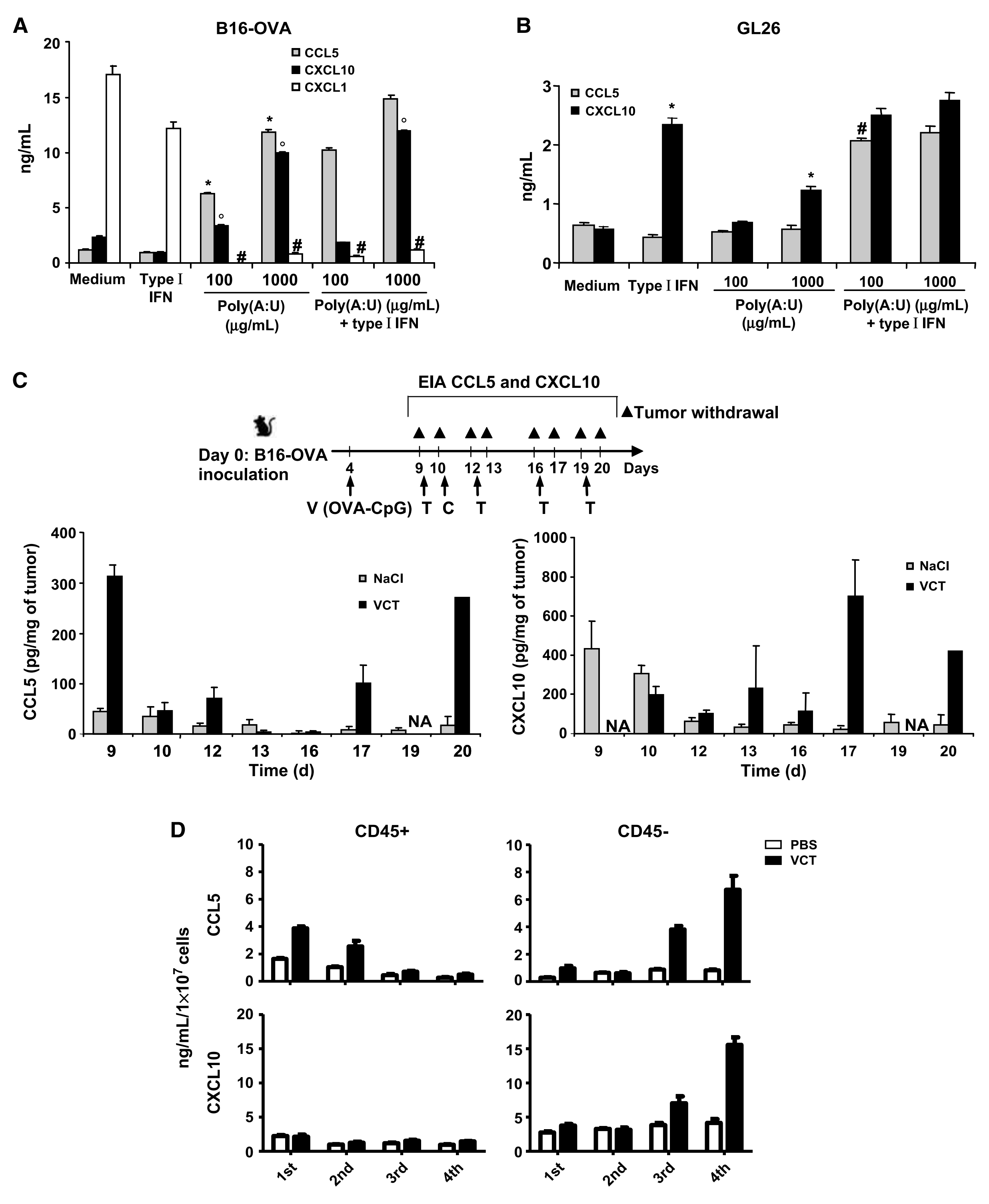

Figure 4. CXCL10 and CCL5 release upon stimulation with poly(A:U). B16-OVA (*A*) and GL26 (*B*) were treated with type I IFN and poly(A:U) and the supernatants were harvested to dose the chemokine secretion. *Columns,* mean of two triplicated experiments (#, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$); *bars,* SEM. Established B16-OVA tumors from the NaCl and VCT groups were harvested at various time points and either were dissociated to measure their contents of CCL5 and CXCL10 (*NA,* not available due to limited tumor size; *C*) or cell sorted after tumor dissociation on the basis of CD45 staining to monitor their chemokine content 36 h after each poly(A:U) injection (*D*). *Columns,* means of chemokine per milligram of tumor (*C*) or per milliliter per 1×10^7 cells (*D*); *bars,* SEM.

the accumulating source of chemokines resided in the tumor parenchyma (Fig. 4*D*).

Altogether, these results indicate that poly(A:U) can directly act on tumor cells to stimulate the production of chemokines, both *in vitro* and *in vivo*.

Deleterious role of CCL5 and CCR5. TLR3 stimulation can trigger the release of a variety of chemokines, including CCL5 (24, 25), as confirmed for the tumors studied in this article, whereas the role of CCR5 (CCL5 receptor) in cancer remains controversial. CCR5 expression in tumor epithelia has been associated with tumorigenesis (26) although some cancer immunotherapies require a functional CCR5 pathway (5, 27, 28). Therefore, we investigated the impact of CCR5 on the synergistic effects of our immunochemotherapy. Surprisingly, the tritherapy was more efficacious when it was applied to *Ccr5*$^{-/-}$ mice rather than to WT mice (Fig. 5*A*

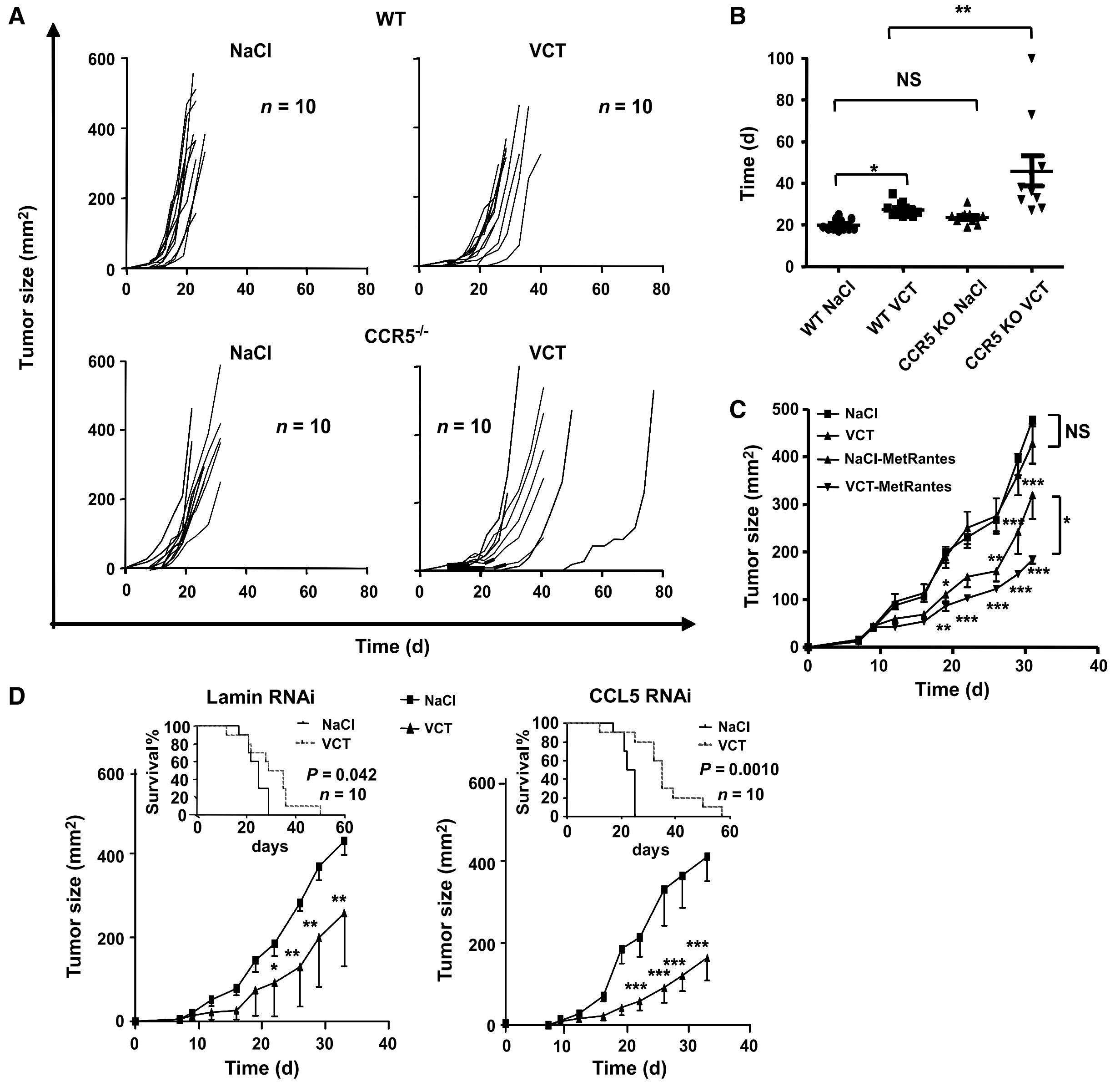

Figure 5. CCR5 signaling antagonized the efficacy of immunochemotherapy. B16-OVA tumor growth was compared in WT versus *Ccr5*$^{-/-}$ mice with or without VCT treatment. Each curve features one single animal (*A*); *NS,* not significant. The time needed for tumors to reach the size of 200 mm^2 was shown for each group (*B*). *C,* 0.6 × 10^6 B16-OVA were inoculated and VCT was performed along with daily administration of MetRantes for 3 wk. *D,* the efficacy of VCT was compared between CCL5 and Lamin knockdown B16-OVA. All experiments were conducted with five mice per group at least twice, yielding identical results.

and *B*). We corroborated these data using a pharmacologic inhibitor recombinant MetRantes that could inhibit agonist-induced activities (29). MetRantes significantly improved tumor growth retardation caused by the immunochemotherapy in the B16-OVA model (Fig. 5*C*). This result was also confirmed in the GL26 glioblastoma (data not shown).

To further show that the source of the deleterious CCL5 was indeed the tumor cells stimulated by poly(A:U) during our sequential therapy, we carried out CCL5 knockdown in B16-OVA by lentiviruses carrying a shRNA-targeting CCL5 (Lamin as a control). This infection induced a significant suppression of the poly(A:U)-induced CCL5 production *in vitro* (Supplementary Fig. S6). The tritherapy mediated enhanced antitumor activity and long-term survival against established B16-OVA–shRNA CCL5 compared with established B16-OVA–shRNA Lamin control (Fig. 5*D*), whereas the spontaneous growth of each transfectant was comparable *in vitro* (data not shown).

Altogether, these results support the idea that the interaction between CCL5 that originated from tumors and CCR5 that was expressed in the host-derived immune effector has a negative impact on the outcome of immunochemotherapy.

CXCR3 as a positive mediator of immunochemotherapy. The OVA-CpG vaccine, which elicited potent IFN-γ–polarized T-cell responses in WT mice (Supplementary Fig. S3), failed to promote the tumoricidal activity when combined with chemotherapy and TLR3L in *nu/nu* C57BL/6 mice (Fig. 1*D*), suggesting that IFN-γ–producing T lymphocytes are required for the antitumor effects. Knowing that IFN-γ–polarized T cells express CXCR3 (30) and TLR3L promotes CXCL10 secretion (a CXCR3 ligand) by tumor cells (31), we compared the efficacy of the immunochemotherapy in WT versus *Cxcr3*$^{-/-}$ mice carrying B16-OVA tumors. In contrast to WT littermate controls, in which immunochemotherapy yielded a significant delay in tumor growth, no beneficial effect was observed for the control of tumors growing in *Cxcr3*$^{-/-}$ mice (Fig. 6*A*). Therefore, the chemokine receptor CXCR3, which is widely expressed in NK cells and activated Th1 and CTLs, is mandatory for the therapeutic success of the combined therapy. Accordingly, functional immunophenotyping revealed that immunochemotherapy induced augmented recruitment of CD8$^+$ CXCR3$^+$ T lymphocytes in the vaccine DLN but not in the tumor DLN (data not shown). These lymphocytes were able to produce IFN-γ upon restimulation with OVA (Supplementary Fig. S3; Fig. 6*B*). NK cells did not express CXCR3 in these settings (data not shown). Importantly, the percentage of CD8$^+$ CXCR3$^+$ T cells increased among tumor-infiltrating lymphocytes (TIL) after immunochemotherapy (Fig. 6*B*), supporting the notion that this T-cell subset contributes to the anticancer efficacy of immunochemotherapy.

Next, we incubated B16-OVA with type I IFN and poly(A:U) (which both mediated cytostatic effects on B16-OVA *in vitro* as shown in Supplementary Fig. S2) and inoculated these tumor cells into WT animals. This pretreatment reduced the minimal tumorigenic dose (the number of cells that had to be inoculated to generate a tumor; Fig. 6*C*). This gain of tumorigenicity was lost when the animals were immunized with the OVA-CpG vaccine (Fig. 6*D*), indicating that the direct effect of poly(A:U) stimulation of the tumor cells is beneficial only when the host has been immunized (when specific CTL against tumoral antigen are present within the host). The beneficial effect of prophylactic immunization with OVA-CpG was abrogated if the tumor cells were injected together with an anti-CXCR3 neutralizing antibody (Fig. 6*D*). Altogether, these results underscore the importance of the chemokine receptor CXCR3 for allowing immune effectors to control tumor growth *in vivo*.

Discussion

Although TLR agonists may contribute to the activation of anticancer responses, they may also directly increase the tumorigenic potential of TLR-expressing tumor cells (3, 15). The aim of this study was to weigh the relative impact of individual components of the chemokine cascade resulting from chronic stimulation of the tumor epithelium with the TLR3L *in vivo*. Our findings revealed that poly(A:U) triggers the concomitant secretion of both CCL5 and CXCL10 from TLR3-expressing tumor *in vitro* and *in vivo* (Fig. 4), and interfering with CCR5 engagement on host hematopoietic cells enhanced the efficacy of an immunogenic treatment that stimulated a T-cell– and CXCR3-dependent anticancer immune response (Figs. 1*D* and 6*A* and *D*). These results suggest that the optimization of anticancer therapies relying on TLR adjuvants may require uncoupling of the chemokine cascade.

It is known that systemic administration of poly(A:U) can exert immunoadjuvant effects through TLR3 and TLR7 (32). Although both TLR3 and TLR7 were required for the clonal expansion of antigen-specific CD8$^+$ T cells, only TLR3 was mandatory to generate IFN-γ–producing CD8$^+$ T cells (32). Our biweekly administration of poly(A:U) was not able to trigger potent immunoadjuvant effects when poly(A:U) was given alone. However, combined with vaccines and chemotherapy, poly(A:U) triggers an efficient T-cell–dependent and TRIF-dependent antitumor response. TRIF signaling leads to type I IFN production by host allophycocyanin, which might directly act on tumor cells to upregulate TLR3 expression (33, 34) and/or synergize with TLR3 to stimulate the release of chemokines (Fig. 4). Of note, we could measure increased levels of CXCL10 and CCL5 in tumor beds only after three systemic administrations of poly(A:U), supporting that host factors (such as type I IFN) may cooperate with poly(A:U) to stimulate the induction of chemokines by tumor cells.

As shown by other groups (35), combinations of specific tumor vaccines with chemotherapy may significantly ameliorate progression-free survival. Surprisingly, although two different vaccines could elicit prophylactic antitumor effects (Figs. 2*B* and 3*A*) and IFN-γ–producing T cells on their own (Supplementary Fig. S3 or data not shown), we could not achieve significant synergistic effects by associating such vaccines with taxanes or oxaliplatin for the treatment of melanoma (Fig. 1 and data not shown). One possible explanation for this absence of synergy might be the failure of tumor beds to produce chemokines that attract polarized effector CD8$^+$

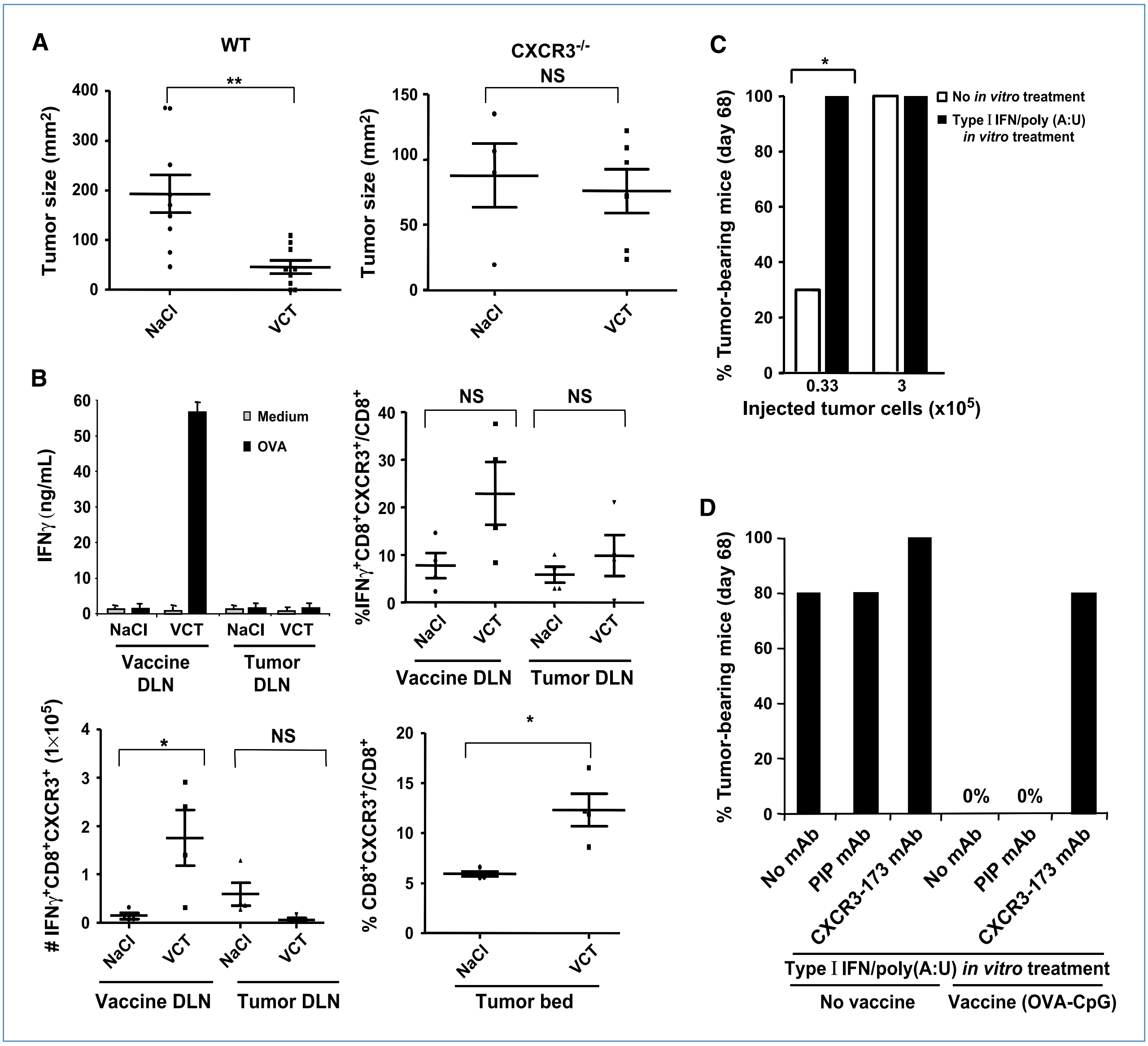

Figure 6. CXCR3-dependent antitumor effects mediated by VCT therapy associated with CXCR3$^+$ TILs. *A,* the mean tumor size at day 19 in VCT or control group is compared between *Cxcr3*$^{-/-}$ versus WT mice. *B,* DLNs from the vaccine site or the contralateral site were collected at day 13 from VCT or control group. Cells were restimulated with OVA protein (or PBS) either for 48 h to monitor the OVA-specific IFN-γ production in the supernatants by ELISA (*top left*) or for 12 h before intracellular stainings showing IFN-γ production by CD8$^+$CXCR3$^+$ T cells (*percentages* and *absolute numbers*). Tumors from NaCl versus VCT-treated mice were dissociated at day 16 and analyzed for the percentage of CXCR3$^+$ cells among CD8$^+$ cells (*bottom right*). *C,* B16-OVA cells were pretreated with type I IFN followed by poly(A:U) and the minimal tumorigenic dose of B16-OVA cells was determined. The percentages of tumor-bearing mice at day 68 are depicted. *D,* mice were preimmunized with OVA-CpG and challenged 7 d later with the minimal tumorigenic dose of B16-OVA tumor cells (0.33 × 10^5) presensitized with poly(A:U) and type I IFN. CXCR3-173 neutralizing mAb or control PIP mAb were applied. The graph depicts the percentages of tumor-bearing mice at day 68 in one of two experiments.

T cells (Fig. 4*C*). Indeed, some reports (36, 37) supported the notion that intratumoral chemokines (such as lymphotactin/XCL1 or CXCL10) could enhance the trafficking of effector T cell to tumors and ameliorate the anticancer efficacy of adoptively transferred T lymphocytes.

Although highly activated CD8$^+$ T cells can coexist with autoantigen-expressing hepatocytes without causing overt tissue damage (38), engagement of TLR3 could break this immunoprivileged state by triggering IFN-γ and tumor necrosis factor-α–dependent CXCL9 expression in the liver and by recruiting CXCR3$^+$ autoreactive CTLs (38). Indeed, a TLR3 agonist could induce the VLA-4–dependent homing of specific CTL into central nervous system tumors (39). Accordingly, several reports described that TLR3 signaling in astrocytes or glioma induced multiple proinflammatory cytokines and chemokines, including IP-10, IL-8, or GROα (39, 40). However, the theory that TLR3 agonists augment trafficking of CTL into tumor beds has been challenged by a recent report showing that injections of double-stranded RNA [poly(I:C)] into mesotheliomas did not stimulate the recruitment of newly primed antitumor T cells and rather reactivated local CD8$^+$ T cells in a type I IFN–dependent manner (41). However, it has not been clarified whether mesothelioma cells express TLR3 and it remains formally possible that poly(I:C) may activate TLR3-independent signaling pathways that improve clinical outcome by alternative mechanisms of action.

Secretion of CC chemokines is a major determinant for chemoattraction of macrophages, neutrophils, and lymphocytes into tumor beds in human carcinogenesis (42). In breast cancer for instance, mesenchymal stem cells produce CCL5, which enhances the metastatic potential of tumors and correlates with disease progression (43, 44). Moreover, tumor-infiltrating leukocytes may express high levels of the CCL5 receptors CCR1 and CCR5 (45). Injection of a CCL5 antagonist can reduce the migration of macrophages to tumor beds and facilitate tumor regression (45). In WT animals, CXCR3 expression in tumor-specific IFN-γ–producing T cells was enhanced, which facilitates their trafficking to the tumor beds (Fig. 6*B*), whereas in *Ccr5*$^{-/-}$ mice, we failed to observe an exaggerated accumulation of Tc1 cells (data not shown). Although concanavalin A–treated *Ccr5*$^{-/-}$ mice suffered from severe hepatitis related to pronounced recruitment and activation of IFN-γ–producing NK cells into the liver (46), we failed to monitor an enhanced proportion of CXCR3$^+$ NK cells in the tumor or DLNs (data not shown). It remains conceivable that CXCR3 can be downregulated in NK cells upon engagement with local chemokines. Therefore, the beneficial effect of CCR5 inhibition may be most likely related to the disappearance of subsets of immunosuppressive cells rather than to the recruitment or activation of effector IFN-γ–producing CD8$^+$ T cells.

Within the hematopoietic system, CCR5 is expressed in regulatory T cells (47) and myeloid-derived suppressor cells (MDSC; ref. 5), making them potential candidates for immune suppressors. However, we failed to improve the efficacy of the immunochemotherapy either by using metronomic dosages of cyclophosphamide that reduce functionally active regulatory T cells (48), or by administering sildenafil, a phosphodiesterase-5 inhibitor known to downregulate the principal immunosuppressive effectors (arginase-1 and NOS-2) of MDSC (data not shown; ref. 49). These results suggested that Treg and MDSCs may not be the CCR5$^+$-immunosuppressive subsets to be identified.

Our results support two important conclusions. First, TLR3 agonists can promote TLR3$^+$ tumor cells to produce chemokines that accumulate locally to physiologically relevant concentrations. Second, these intratumoral chemokines likewise are not neutral in their clinical significance and need to be uncoupled to boost the efficacy of immunochemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Krieg AM. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. Oncogene 2008;27:161–7.
2. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 2007;13:552–9.
3. Killeen SD, Wang JH, Andrews EJ, Redmond HP. Exploitation of the Toll-like receptor system in cancer: a doubled-edged sword? Br J Cancer 2006;95:247–52.
4. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801.
5. Umemura N, Saio M, Suwa T, et al. Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. J Leukoc Biol 2008;83:1136–44.
6. Cui Z, Qiu F. Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. Cancer Immunol Immunother 2006;55:1267–79.
7. Schulz O, Diebold SS, Chen M, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005;433:887–92.
8. Tabiasco J, Devevre E, Rufer N, et al. Human effector CD8+ T lymphocytes express TLR3 as a functional coreceptor. J Immunol 2006;177:8708–13.
9. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101–5.
10. Wang L, Smith D, Bot S, Dellamary L, Bloom A, Bot A. Noncoding RNA danger motifs bridge innate and adaptive immunity and are potent adjuvants for vaccination. J Clin Invest 2002;110:1175–84.
11. Bot A, Smith D, Phillips B, Bot S, Bona C, Zaghouani H. Immunologic control of tumors by *in vivo* Fc γ receptor-targeted antigen loading in conjunction with double-stranded RNA-mediated immune modulation. J Immunol 2006;176:1363–74.
12. Lacour J, Lacour F, Spira A, et al. Adjuvant treatment with polyadenylic-polyuridylic acid (Polya.Polyu) in operable breast cancer. Lancet 1980;2:161–4.
13. Laplanche A, Alzieu L, Delozier T, et al. Polyadenylic-polyuridylic acid plus locoregional radiotherapy versus chemotherapy with CMF in operable breast cancer: a 14 year follow-up analysis of a randomized trial of the Federation Nationale des Centres de Lutte contre le Cancer (FNCLCC). Breast Cancer Res Treat 2000;64:189–91.
14. Jeung HC, Moon YW, Rha SY, et al. Phase III trial of adjuvant 5-fluorouracil and Adriamycin versus 5-fluorouracil, Adriamycin, and

polyadenylic-polyuridylic acid (poly A:U) for locally advanced gastric cancer after curative surgery: final results of 15-year follow-up. Ann Oncol 2008;19:520–6.

15. Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. Cancer Immunol Immunother 2008;57:1271–8.

16. Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 2008;452:591–7.

17. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006; 176:4894–901.

18. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like receptor 3 expressed by melanoma cells as a target for therapy? Clin Cancer Res 2007;13:4565–74.

19. Stewart WE II, De Clercq E, Billiau A, Desmyter J, De Somer P. Increased susceptibility of cells treated with interferon to the toxicity of polyriboinosinic-polyribocytidylic acid. Proc Natl Acad Sci U S A 1972;69:1851–4.

20. Huang B, Zhao J, Li H, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. Cancer Res 2005;65:5009–14.

21. Huang B, Zhao J, Shen S, et al. *Listeria monocytogenes* promotes tumor growth via tumor cell toll-like receptor 2 signaling. Cancer Res 2007;67:4346–52.

22. Kelly MG, Alvero AB, Chen R, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res 2006;66:3859–68.

23. Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 2007;13: 54–61.

24. Orinska Z, Bulanova E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. Blood 2005;106:978–87.

25. Takahashi N, Yamada T, Narita N, Fujieda S. Double-stranded RNA induces production of RANTES and IL-8 by human nasal fibroblasts. Clin Immunol 2006;118:51–8.

26. Azenshtein E, Luboshits G, Shina S, et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res 2002;62:1093–102.

27. Lavergne E, Combadiere C, Iga M, et al. Intratumoral CC chemokine ligand 5 overexpression delays tumor growth and increases tumor cell infiltration. J Immunol 2004;173:3755–62.

28. Nakazaki Y, Hase H, Inoue H, et al. Serial analysis of gene expression in progressing and regressing mouse tumors implicates the involvement of RANTES and TARC in antitumor immune responses. Mol Ther 2006;14:599–606.

29. Proudfoot AE, Power CA, Hoogewerf AJ, et al. Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. J Biol Chem 1996;271:2599–603.

30. Chen J, Vistica BP, Takase H, et al. A unique pattern of up- and down-regulation of chemokine receptor CXCR3 on inflammation-inducing Th1 cells. Eur J Immunol 2004;34:2885–94.

31. Loos T, Dekeyzer L, Struyf S, et al. TLR ligands and cytokines induce CXCR3 ligands in endothelial cells: enhanced CXCL9 in autoimmune arthritis. Lab Invest 2006;86:902–16.

32. Sugiyama T, Hoshino K, Saito M, et al. Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7. Int Immunol 2008;20:1–9.

33. Hoebe K, Janssen EM, Kim SO, et al. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nat Immunol 2003;4:1223–9.

34. Siren J, Pirhonen J, Julkunen I, Matikainen S. IFN-α regulates TLR-dependent gene expression of IFN-α, IFN-β, IL-28, and IL-29. J Immunol 2005;174:1932–7.

35. Belardelli F, Ferrantini M, Parmiani G, Schlom J, Garaci E. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? Cancer Res 2004;64:6827–30.

36. Huang H, Li F, Gordon JR, Xiang J. Synergistic enhancement of antitumor immunity with adoptively transferred tumor-specific CD4+ and CD8+ T cells and intratumoral lymphotactin transgene expression. Cancer Res 2002;62:2043–51.

37. Huang H, Liu Y, Xiang J. Synergistic effect of adoptive T-cell therapy and intratumoral interferon γ-inducible protein-10 transgene expression in treatment of established tumors. Cell Immunol 2002; 217:12–22.

38. Lang KS, Georgiev P, Recher M, et al. Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling. J Clin Invest 2006;116:2456–63.

39. Zhu X, Nishimura F, Sasaki K, et al. Toll like receptor-3 ligand poly-ICLC promotes the efficacy of peripheral vaccinations with tumor antigen-derived peptide epitopes in murine CNS tumor models. J Transl Med 2007;5:10.

40. Park C, Lee S, Cho IH, et al. TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: differential signaling mechanisms of TLR3-induced IP-10 and IL-8 gene expression. Glia 2006;53:248–56.

41. Currie AJ, van der Most RG, Broomfield SA, Prosser AC, Tovey MG, Robinson BW. Targeting the effector site with IFN-αβ-inducing TLR ligands reactivates tumor-resident CD8 T cell responses to eradicate established solid tumors. J Immunol 2008;180:1535–44.

42. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.

43. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449: 557–63.

44. Luboshits G, Shina S, Kaplan O, et al. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Res 1999; 59:4681–7.

45. Robinson SC, Scott KA, Wilson JL, Thompson RG, Proudfoot AE, Balkwill FR. A chemokine receptor antagonist inhibits experimental breast tumor growth. Cancer Res 2003;63:8360–5.

46. Ajuebor MN, Wondimu Z, Hogaboam CM, Le T, Proudfoot AE, Swain MG. CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. Am J Pathol 2007;170:1975–88.

47. Moreira AP, Cavassani KA, Massafera Tristao FS, et al. CCR5-dependent regulatory T cell migration mediates fungal survival and severe immunosuppression. J Immunol 2008;180:3049–56.

48. Taieb J, Chaput N, Schartz N, et al. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. J Immunol 2006;176:2722–9.

49. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 2006;203:2691–702.