Avirulent *Toxoplasma gondii* Generates Therapeutic Antitumor Immunity by Reversing Immunosuppression in the Ovarian Cancer Microenvironment

Jason R. Baird, Barbara A. Fox, Kiah L. Sanders, Patrick H. Lizotte, Juan R. Cubillos-Ruiz, Uciane K. Scarlett, Melanie R. Rutkowski, Jose R. Conejo-Garcia, Steven Fiering, and David J. Bzik

Microenvironment and Immunology

Abstract

Reversing tumor-associated immunosuppression seems necessary to stimulate effective therapeutic immunity against lethal epithelial tumors. Here, we show this goal can be addressed using *cps*, an avirulent, nonreplicating uracil auxotroph strain of the parasite *Toxoplasma gondii* (*T. gondii*), which preferentially invades immunosuppressive CD11c⁺ antigen-presenting cells in the ovarian carcinoma microenvironment. Tumor-associated CD11c⁺ cells invaded by *cps* were converted to immunostimulatory phenotypes, which expressed increased levels of the T-cell receptor costimulatory molecules CD80 and CD86. In response to *cps* treatment of the immunosuppressive ovarian tumor environment, CD11c⁺ cells regained the ability to efficiently cross-present antigen and prime CD8⁺ T-cell responses. Correspondingly, *cps* treatment markedly increased tumor antigen-specific responses by CD8⁺ T cells. Adoptive transfer experiments showed that these antitumor T-cell responses were effective in suppressing solid tumor development. Indeed, intraperitoneal *cps* treatment triggered rejection of established ID8-VegfA tumors, an aggressive xenograft model of ovarian carcinoma, also conferring a survival benefit in a related aggressive model (ID8-Defb29/Vegf-A). The therapeutic benefit of *cps* treatment relied on expression of IL-12, but it was unexpectedly independent of MyD88 signaling as well as immune experience with *T. gondii*. Taken together, our results establish that *cps* preferentially invades tumor-associated antigen-presenting cells and restores their ability to trigger potent antitumor CD8⁺ T-cell responses. Immunochemotherapeutic applications of *cps* might be broadly useful to reawaken natural immunity in the highly immunosuppressive microenvironment of most solid tumors.

Cancer Res; 73(13); 1–10. ©2013 AACR.

Introduction

Survival rates for patients with ovarian cancer have not significantly improved in the last few decades (1). Many tumors protect themselves from antitumor immune responses via multiple mechanisms that include recruitment of immunosuppressive dendritic and myeloid cell populations (2). This is a particularly acute problem in aggressive epithelial cancers because many of these cancers use the inflammatory cellular machinery to actively abrogate immune mechanisms that would otherwise act to exert effective immune pressure against tumors (3–6).

Immature CD11c⁺ dendritic cells accumulate in high numbers in solid epithelial ovarian tumors and deliver multiple proangiogenic and immunosuppressive mediators (3, 7, 8). While ovarian tumor-associated antigen-presenting cells (APC) are competent to phagocytose tumor antigens, these cells fail to efficiently cross-present tumor antigen to prime T cells (3). Ovarian tumor-associated APCs actively suppress T-cell function in the tumor microenvironment (9). Consistent with this mechanism of immune suppression, depletion of immunosuppressive CD11c⁺ dendritic cells from the ovarian tumor microenvironment delays tumor progression (10). Nonetheless, it is also clear that ovarian cancer does naturally trigger detectable antitumor responses (11, 12). Recent evidence indicates that these antitumor responses are more effective in the early stages of ovarian tumor development and diminish as the tumor microenvironment acquires increasingly immunosuppressive characteristics (13).

Antitumor responses to ovarian carcinoma can be increased via therapeutic interventions such as immune cocktail boosting (3), CD40 and TLR3 agonists (9), polyethyleneimine-based siRNA nanocomplexes (14), or pulsing tumor dendritic cells...
with tumor antigens (15). All of these therapeutic intervention strategies rely on stimulation of tumor-associated CD11c⁺ dendritic cells. Consequently, immune stimulation of CD11c⁺ cells in epithelial cancers may be a highly effective approach for therapeutic intervention (16).

The obligate intracellular protozoan parasite Toxoplasma gondii (T. gondii) preferentially invades dendritic cells and macrophages (17). This eukaryotic organism actively manipulates APCs in multiple ways via direct stimulation of response activators such as toll-like receptor (TLR) and by secretion of parasite molecules directly into the host cell cytoplasm that actively intercept cellular signaling and transcriptional pathways (18). Our previous studies have shown that the invasive but avirulent T. gondii uracil auxotroph vaccine strain cps (19) provokes a locally strong Th1 immune response and elicits lifelong CD8⁺ T-cell immunity against rechallenge with T. gondii (20). We reasoned that the cps vaccine strain would selectively invade and phenotypically modify immunosuppressive myeloid cell types in the tumor environment and that parasite manipulation of tumor-associated CD11c⁺ APC could stimulate immunity to tumors. Here, we show that the cps vaccine strain invades CD11c⁺ APC in the ovarian tumor environment and activates antitumor CD8⁺ T cells thereby stimulating the therapeutic rejection of established ovarian cancer.

Materials and Methods

Cell lines

ID8 cells were provided by Katherine Roby (Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS) and transduced with Defb29 and Vegf-A using retroviruses (3). ID8-Defb29/Vegf-A or ID8-Vegf-A orthotopic peritoneal tumors were established in mice as described (3). Flank tumors were established using ID8-Defb29/Vegf-A (14).

Parasite culture

Replicating tachyzoites of the cps strain of T. gondii (19) were grown in human foreskin fibroblast cells supplemented with 0.2 mmol/L uracil. Parasites were purified by filtration through 3.0 μm nucleopore membranes and washed with PBS before use.

Mice

C57BL/6, IL-12p40⁻/⁻ (Jax, 002693), IL-12p35⁻/⁻ (Jax, 002692), IL-17A⁻/⁻ (Jax, 016879), MyD88⁻/⁻, OT-1 (Jax, 003831), and Foxp3GFP (21) mice were purchased from the National Cancer Institute or The Jackson Laboratory (Jax). Mouse studies were approved by the Dartmouth Institutional Animal Care and Use Committee.

Treatment of ovarian tumors

Mice were intraperitoneally injected with 2 × 10⁶ ID8-Defb29/Vegf-A or 1 × 10⁶ ID8-Vegf-A cells, and were treated intraperitoneally at 8 days and again at 20 days with 2 × 10⁶ freshly isolated tachyzoites of cps. For analysis of cps-invaded cells, ID8-Defb29/Vegf-A tumors were established for 19 or 34 days and treated intraperitoneally with 1 × 10⁵ of cps-YFP expressing yellow fluorescence protein (22).

Adoptive transfer

CD3⁺ T cells were harvested from cps treated or PBS treated ID8-Defb29/Vegf-A tumor bearing mice on day 31 posttumor challenge, or from naive mice (PBS treated). Treated mice were injected with cps 8 days and again 20 days after tumor challenge. A total of 2 × 10⁶ splenic T cells were separated using MACs kit (cat. no. 130-095-130) and intravenously injected into naive recipient mice that received 300 rad of radiation 2 hours before transfer of T cells. Twenty-four hours later, 15 × 10⁶ ID8-Defb29/Vegf-A tumor cells were mixed in Matrigel and injected subcutaneously into the flank of the T-cell–treated recipient mice to establish flank tumors.

Antibodies, tetramers, and flow cytometry

Anti-mouse antibodies: CD16/CD32 (93, eBioscience), CD45 (30-F11, eBioscience), CD69 (H1.2F3, eBioscience), CD11c (HL3, BD Biosciences), DEC205 (NLdendritic cells145, Serotec), MHC-II (NMR-4, eBioscience), CD3e (eBio500A2, eBioscence), CD8b (YTS156.7.7, eBioscience), CD44 (KM201, Southern Biotech), CD62L (MEL-14, eBioscience), CD80 (16-10A1, eBioscience), CD86 (YTS156.7.7, eBioscience), CD11b (M1/70, eBioscience), B220 (RA3-6B2, eBioscience), NK1.1 (PK136, Biolegend), F4/80 (BMS, eBioscience), IL17A (TC11-18H10, BD Pharmingen), and CD4 (GK1.5, Biolegend). Tetramer Class I iTag MHC Tetramer (ATVTNV/SLG) specific for CD8⁺ T cells recognizing ovarian ID8-Defb29/Vegf-A (Beckman Coulter; ref. 14). Red blood cell lysis (eBioscience, RBC lysis reagent). Unfixed cell populations were stained with antibodies before analysis by flow cytometry. Flow cytometry used a FACS-Canto (BD Biosciences). Data were analyzed using Flowjo software (version 7.6). Cell populations from peritoneal washes were sorted using a FACS Aria sorter (BD Biosciences).

T-regulatory cell assay

Foxp3GFP reporter mice were previously described (21). C57BL/6 or Foxp3GFP reporter mice were intraperitoneally injected with 2 × 10⁶ ID8-Defb29/Vegf-A cells in 0.2 mL PBS and intraperitoneally treated 8 days later with 2 × 10⁶ cps. Spleen and peritoneum samples were collected 12 days after cps treatment. Flow cytometry was conducted to determine the frequency of CD45⁻CD3⁻CD4⁺Foxp3⁺ cells in cps-treated and PBS-treated groups.

IL-17 intracellular assay

Mice were intraperitoneally injected with 2 × 10⁶ ID8-Defb29/Vegf-A cells and were intraperitoneally treated 8 days later with 2 × 10⁶ cps. Staining and flow cytometry analysis of cell populations in the peritoneum and spleen were conducted 12 days after intraperitoneal cps treatment. Collected samples were cultured in brefeldin A (GolgiStop; BD Biosciences; cat. no. 555028) for 5 hours at 37°C. Cells were washed in PBS/1% FBS + GolgiStop. All subsequent manipulations were carried out in the dark. Cells were stained for CD45, CD3, and CD4 before fixation. Samples were fixed in PBS + GolgiStop and 2% paraformaldehyde, washed in PBS twice, and stained with anti-IL-17A antibody in permeabilization buffer (0.2% saponin in PBS + 1% FBS) before FACS analysis.
Cytokine assay
Peritoneal fluid was used for the detection of cytokines in the tumor environment. ELISA was used for detection of interleukin (IL)-12p70 (Biolegend, cat. no. 433604) and IL-23 (Biolegend, cat. no. 433704). IL-12p40 was determined using individual Luminex (Bio-Rad cat. no. 5015198). IL-2, IL-4, IL-6, Ifn-γ, IL-1b, and IL-17 were determined using mouse 32-plex Luminex (Millipore).

ELISpot
CD8+ T cells were isolated from peritoneal samples, purified using anti-CD8 MACS magnetic beads (Miltenyl Biotec), and were cocultured with bone marrow–derived dendritic cells (BMDC; 10 BMDC:1 tumor cell) for 48 hours in coated and blocked ELISpot plates. The BMDCs were previously pulsed (overnight) with doubly irradiated (Gamma and UV) ID8-Defb29/Vegf-A cells (3). Control samples were pulsed with sterile media. Analysis was done according to the manufacturer’s protocol for detection of granzyme B–expressing T cells (eBioscience).

OT-1 antigen presentation assay
ID8-Defb29/Vegf-A tumor bearing mice were intraperitoneally injected with 0.6 mg of full-length endotoxin-free OVA (Sigma, grade VII) 21 days posttumor challenge. Four hours later, mice received 1 × 10^7 cps intraperitoneally and 18 hours later, mice were injected intraperitoneally with 2 × 10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-I CD3+ cells (purified using MACS magnetic beads, Miltenyl Biotec) that were harvested from spleens and lymph nodes of OT-I mice. For ex vivo antigen presentation to OT-1 cells, CD45+ CD11c+ DEC205+ MHC-II+ cells were sorted from terminal tumor ascites, pulsed or not with 50 μg/mL of full-length OVA (Sigma) for 2 hours, and then activated with αCD40 (1 μg/mL) and poly(I:C) (2 μg/mL). After 3 hours, negatively selected CFSE-labeled OT-I splenocytes were added (2:1 ratio; 1 × 10^6/mL, 5 × 10^6 total cells). T-cell expansion was quantified by flow cytometry 5 days later.

Immunity to T. gondii
To generate mice immune to Toxoplasma, mice were vaccinated with cps as described previously. Mice from the immune group were shown to be immune by surviving lethal (LD = 1,000) T. gondii strain RH challenge as described (19, 20), whereas age-matched naive mice did not survive this challenge.

Statistical analysis
Unless noted otherwise, all experiments were repeated at least 2 times and results were similar between repeats. A P < 0.05 was considered to be statistically significant. Statistical analysis was done with GraphPad Prism 4 software. Data for bar graphs (except ELISpot analysis) was calculated using 2-tailed unpaired Student t test. Student t tests were conducted under the assumption of equal variance. Error bars represent SEM from independent samples assayed within the represented experiments. ELISpots used pooled cells from multiple mice and the values presented are from triplicate assay on the pools that were compared by one-way ANOVA analysis (GraphPad Prism 4). Survival experiments used the log-rank Mantel–Cox test for analysis.

Results
CD11c+ cells from the ovarian tumor microenvironment are preferentially invaded and are activated by the avirulent cps strain of T. gondii
Hyperaggressive ID8-Defb29/Vegf-A ovarian tumors have been modified to express β-defensin (Defb29) and VEGF (Vegf-A). Prior reports have shown that the ID8-Defb29/Vegf-A ovarian tumor environment recruits CD8α+ dendritic cells that express CD11c/DEC205/MHCII and mediate immunosuppression and vascularization (3, 9). Consistent with these reports, we sorted the CD8α+ CD11c+ DEC205+ MHCII+ population from the ascites of tumor bearing mice, pulsed them or not with ovalbumin (OVA) antigen, stimulated with CD40 ligand/polyI:C, and assessed the expansion of CFSE-labeled cocultured OT-1 cells, confirming that this immunosuppressive dendritic cell population may be induced to cross-present antigen to CD8+ T cells upon activation (Supplementary Fig. S1). While these dendritic cells are the predominant CD11c+ cell type in the microenvironment of this ovarian cancer model (3), further work was typically conducted with cells characterized by CD11c expression only and we define this population broadly as CD11c+ APCs.

Tachyzoites of T. gondii preferentially invade myeloid cells during acute infection (17). To confirm preferential invasion of CD11c+ cells in the tumor microenvironment, we tracked cps invasion of cells in established orthotopic peritoneal ID8-Defb29/Vegf-A tumors that were intraperitoneally treated using a cps strain expressing yellow fluorescent protein (cps-YFP). We found that CD45+ cells in the peritoneum accounted for the vast majority of cells invaded by cps-YFP (86.4% ± 8.6%; Fig. 1A) and among CD45+ leukocytes, CD45+ CD11c+ APC were the most significant population of the invaded cell types (78.5% ± 8.0%; Fig. 1A). As shown in the right panel, total CD45+ cells were preferentially invaded (18.7% ± 2%) as compared with the CD45+ cells, which are predominantly tumor cells (2.3% ± 0.5%). Within the CD45+ population, the CD45+CD11c+ cells were preferentially invaded (49.4% ± 3.5%) as compared with CD45+ CD11c+ cells (3.6% ± 0.3%; Fig. 1A). These data show that cps is preferentially invading CD11c+ cells in the tumor microenvironment.

Confirming previous reports (3, 9, 14), immunosuppressive CD11c+ cells in the ovarian cancer microenvironment expressed moderate levels of CD86 and low levels of CD80 costimulatory molecules essential for the activation of T-cell immunity (Fig. 1B). CD11c+ cells (either invaded or bystander) exposed to cps in vivo rapidly increased their expression of both CD80 and CD86 compared with tumor bearing mice not exposed to cps (Fig. 1B). This pattern occurred in mice that had been bearing tumors for 19 days (Fig. 1C) or 34 days (Fig. 1D). Tumor-associated CD45+CD11c+ cells invaded by cps exhibited higher levels of CD80 and CD86 expression as compared with non-invaded cps-exposed CD45+CD11c+ bystander cells (Fig. 1C and D).
This in vivo experiment cannot differentiate the effects of cps on APC already present in the tumor microenvironment from the recruitment of new APC due to the effects of cps treatment. It is likely that both resident and recruited APC play a role in the improved ability to present antigens following cps treatment. To directly test the effect of cps treatment.
on immunosuppressive CD11c+ cells present in the tumor microenvironment, terminal tumor ascites were harvested from tumor bearing mice and exposed to cps ex vivo. Eighteen hours later, the ex vivo cps-treated tumor-associated CD11c+ APC exhibited increased levels of CD80 and CD86 (Supplementary Fig. S2A and S2B).

CD45+CD11c+ cells in the ID8-Defb29/Vegf-A ovarian tumor environment actively phagocytose tumor material in the microenvironment, but rather than stimulating antitumor immunity, these immunosuppressive APC actively suppress T-cell function (3, 9, 14). We speculated that the rapid increase in expression of CD80 and CD86 in the population of CD11c+ cells in cps-treated mice signified a transition from an immunosuppressive to an immune-activated state that could prime more effective T-cell immunity. To determine whether cps treatment activated CD8+ T-cell responses in the immunosuppressed tumor environment, we injected full-length ovalbumin antigen into the tumor environment and measured proliferation of adoptively transferred CFSE-labeled OT-I T cells that recognize the ovalbumin peptide SINEFKL presented on MHC class I in H-2Kb-restricted C57BL/6 mice (23). While APC in the PBS-treated tumor environment weakly cross-presented antigen to OT-I CD8+ T cells, APC in the cps-treated tumor environment efficiently cross-presented antigen to OT-I CD8+ T cells (Fig. 1E). Preferential targeting of tumor-resident CD45+CD11c+ cells by cps rapidly activated the priming of CD8+ T-cell responses to antigen present in the tumor environment, reversing active immunosuppression and reawakening natural immunity.

cps treatment promotes leukocyte recruitment into the tumor microenvironment and spleen of mice bearing ovarian carcinoma

Peritoneal treatment with cps was previously shown to stimulate strong local Th1 cytokine and cellular responses (20) that elicited exceptional CD8+ T-cell responses in the peritoneum and in the spleen (24). To investigate the impact of cps on leukocytes in the ovarian tumor microenvironment and spleen, we treated 8-day established peritoneal ID8-Defb29/Vegf-A tumors by intraperitoneal cps injection. Twelve days after treatment, mice exhibited splenomegaly (Fig. 2A) and CD45+CD11c+ cells (Fig. 2B), macrophages (Fig. 2C), B cells (Fig. 2D), natural killer (NK) cells (Fig. 2E), CD8+ T cells (Fig. 2F), and CD4+ T cells (Fig. 2G) were significantly increased in spleens of cps-treated mice (flow cytometry gating strategies for Fig. 2 data are in Supplementary Fig. S3A–S3)). At the local tumor site, the number of peritoneal CD45+ leukocytes was significantly increased in cps-treated tumor bearing mice compared with PBS-treated tumor bearing mice (Fig. 2H). Tumor-associated CD45+CD11c+ cells (Fig. 2I), CD8+ T cells (Fig. 2J), and CD4+ T cells (Fig. 2K) were significantly increased. Treatment with cps significantly increased the percentage of peritoneal CD45+CD11c+ cells (Fig. 2L) and CD8+ T cells (Fig. 2M) among tumor-associated leukocytes. Foxp3+ T regulatory (Treg) CD4+ cells have been shown to be an adverse prognostic factor in human ovarian cancer (25, 26), and infection with wild-type T. gondii is associated with a strong reduction in CD45+CD3+CD4+Foxp3+ Treg cells (27, 28). We observed a reduction in the percentage of Treg...
(CD45⁺CD3⁺CD4⁺Foxp3⁺) cells among the CD4⁺ cells (Fig. 2N), suggesting that the influence of suppressive T-reg cells is reduced by cps treatment.

**Cps treatment does not increase T_{H17} cells or IL-17-associated cytokines**

Increased IL-17 levels have been shown to correlate with patient survival in ovarian cancer (29, 30) and therefore we investigated whether IL-17 levels changed and whether T_{H17} cells play a role in cps treatment in this model. In the ID8-Defb29/Vegf-A model, IL-17-expressing CD4⁺ cells as assessed by intracellular staining for IL-17A are below 1% of the CD4⁺ population in spleen and peritoneum and this frequency is not increased by cps treatment (Supplementary Fig. S4E and S4F). This correlates with no detectable change after treatment with cps in cytokine levels of IL-17 and the IL-17-associated cytokines, IL-1β, IL-6, and IL-23 (Supplementary Fig. S4B and S4D). In addition, while we observed a modest increase in Ifn-γ, there was no change in IL-2 or IL-4 cytokines measured 18 hours after cps treatment of terminal tumor ascites in vivo (Supplementary Fig. S4A and S4C). To further investigate any potential role of IL-17, we conducted survival experiments in IL-17A knockout mice and in those mice the treatment had normal efficacy (Supplementary Fig. S4G). The therapeutic efficacy of cps treatment does not seem to depend on the expression of IL-17A or modulation of T_{H17} cells.

**Cps treatment stimulates a population of activated tumor antigen-specific CD8⁺ T cells in the spleen and in the tumor microenvironment**

The increased numbers and activation of tumor-associated CD45⁺CD11c⁺ cells (Fig. 1) and increases in CD8⁺ cells in the peritoneum and the spleen following cps treatment (Fig. 2) could reflect immune activation of antitumor CD8⁺ T-cell responses. Tumor antigen–specific CD8⁺ T-cell responses are strongly associated with increased survival in the ID8-Defb29/Vegf-A ovarian and other epithelial tumor models (9, 14, 16). We initially assessed CD8⁺ T-cell responses in the spleens of tumor bearing mice and found the total number (Fig. 3A) and the percentage of CD8⁺ T cells (Fig. 3B) with markers of central memory differentiation (CD44⁺CD62L⁺) were significantly increased by cps treatment. To examine the hypothesis that cps treatment activated tumor antigen–specific CD8⁺ T-cell responses.
responses, we used a tetramer that specifically identifies ovarian tumor antigen-specific CD8\(^+\) T cells (14). Treatment with cps significantly increased the total number (Fig. 3C) and the percentage (Fig. 3D) of tetramer-positive CD3\(^+\)CD8\(^+\) T cells present in the spleen. We confirmed that at the peritoneal tumor location, tetramer-positive CD3\(^+\)CD8\(^+\) T cells were markedly increased in absolute number (Fig. 3E) and were also increased as a percentage (Fig. 3F) of total CD8\(^+\) T cells after cps treatment. These peritoneal tumor antigen–specific CD8\(^+\) T cells exhibited markers for activation and antigen experience (CD69\(^+\)CD44\(^+\); Fig. 3G). In addition, ex vivo cps treatment of terminal tumor ascites also increased expression of CD69 on CD3\(^+\)CD8\(^+\) T cells (Supplementary Fig. S2C). Furthermore, ELISpot analysis of cps-treated mice showed a significant increase in the number of activated CD8\(^+\) T cells that expressed cytolytic granzyme B in response to tumor antigens (Fig. 3H). Collectively, these results reveal that treatment of the tumor microenvironment with cps spontaneously elicits a significant increase in tumor antigen–specific CD8\(^+\) T cells present in the spleen and present at the peritoneal ovarian tumor location.

**cps treatment elicits T-cell responses that actively suppress solid tumor development**

To investigate the functional consequence of increased tumor antigen-specific CD8\(^+\) T-cell responses, we used a flank model of ectopic ID8-Defb29/Vegf-A implanted subcutaneously (14) and treated with adoptive transfer of total splenic T cells from naive, untreated tumor bearing, or cps-treated tumor bearing mice. Mice that received T cells from naive mice developed large tumors (1,956 ± 323 mm\(^3\)) and adoptive transfer of T cells from untreated tumor bearing mice transferred a measurable antitumor activity based on a reduction in tumor volume (587 ± 127 mm\(^3\)). This result is consistent with previous evidence showing that the ID8-Defb29/Vegf-A ovarian tumor is immunogenic and spontaneously generates measurable, although suboptimal, anti-tumor T-cell responses (3, 9, 13). In contrast, adoptive transfer of T cells from tumor bearing mice previously treated with cps significantly reduced the tumor volume (97 ± 19 mm\(^3\)), showing that cps-elicted T-cell populations in tumor bearing mice effectively suppressed ovarian tumor development (Fig. 4).

**cps treatment eliminates an ID8 model of ovarian carcinoma that expresses VEGF (Vegf-A) and the therapeutic benefit is independent of immune status to T. gondii**

We assessed the ability of cps treatment to elicit the rejection of established aggressive ID8-Vegf-A ovarian tumors that accelerate tumor development by increasing vasculogenesis (3). Established disseminated intraperitoneal ID8-Vegf-A ovarian tumors were eliminated by cps treatment (Fig. 5A). Furthermore, tumor rejection elicited by cps treatment was independent of whether the mice had been previously exposed and were immune to T. gondii (Fig. 5B). These therapeutic results show that previous exposure and development of immunity to T. gondii, as is common in humans and animals, did not significantly reduce the therapeutic antitumor effects of cps treatment.
**cps treatment provides an IL-12–dependent and MyD88-independent therapeutic benefit in hyperaggressive ID8-Defb29/Vegf-A ovarian tumors**

Intraperitoneal *cps* treatment of established hyperaggressive ID8-Defb29/Vegf-A tumors markedly extended the survival of tumor-bearing mice (Fig. 6A). On the basis of the immune responses triggered by *cps* in naive mice (20, 24, 31, 32), we predicted that *cps* treatment would stimulate production of IL-12 in the peritoneum of ovarian tumor-bearing mice. IL-12 has a beneficial role in the tumor environment by promoting antitumor immunity (33) and also by inhibiting angiogenesis (34). Mice bearing late-stage (35 days) ovarian cancer exhibited a significant increase in peritoneal IL-12p40 (Fig. 6B) and in functional IL-12p70 (Fig. 6C) within 18 hours following *cps* treatment. To assess whether IL-12 was necessary for treatment efficacy, we showed that IL-12p40 knockout mice (Fig. 6D) and IL-12p55 knockout mice (Fig. 6E) failed to gain any protection from *cps* treatment of established ID8-Defb29/Vegf-A tumors. These results are consistent with previous reports that *T. gondii* induces strong IL-12 responses that are required for development of a potent CD8+ T-cell–dependent immunity against *T. gondii* (20, 35–37). The majority of IL-12 production induced by *T. gondii* is dependent on the TLR adaptor molecule MyD88 (18). However, IL-12 is also induced by *T. gondii* in a MyD88-independent manner (18, 38). We previously showed that vaccination with *cps* elicited an IL-12–dependent and CD8+ T-cell–dependent immunity in MyD88−/− mice (35). Therefore, to define the importance of MyD88 signaling in the antitumor response, we showed that MyD88 knockout mice respond as well (measured by overall survival) to *cps* treatment as wild-type mice and exhibit a significant survival advantage in comparison with PBS-treated tumor bearing MyD88−/− mice (Fig. 6F, *P* = 0.0019). While MyD88 signaling was not essential to the therapy, IL-12 signaling was required for the therapeutic benefit of *cps* treatment.

**Discussion**

The *cps* strain’s inability to replicate in vivo (19) and its lack of observed toxicity (19, 39) make it a potential reagent for immunotherapy. The efficacy of *cps* immunotherapy in ovarian cancer and our recent work in melanoma models (39) show the ability of *cps* to treat multiple solid tumor types when introduced into the tumor microenvironment, suggesting that *cps* can be developed into a valuable clinical tool for stimulation of therapeutic antitumor immunity.

The potential for *T. gondii* to slow tumor growth has been investigated previously. Chronic *T. gondii* infection slows tumor development in mice (40). Acute toxoplasmosis in mice slowed the growth of melanoma if applied before the melanoma challenge in mice, but this approach was limited by toxicity of unchecked infection and surprisingly did not require an intact adaptive immune system for efficacy (41). *T. gondii* extracts have also been used to mature dendritic cells in vitro to improve T-cell adoptive transfer efficacy (42).

Ovarian cancer is an aggressive and frequently occurring epithelial cancer (1). The most abundant leukocyte population in the ovarian carcinoma microenvironment is the CD45−CD11c+ dendritic cell that exhibits proangiogenic...
and immunosuppressive activity (3, 7, 9, 10, 13). Here, we show that the avirulent cps strain of *Toxoplasma gondii* invades and transforms immunosuppressive ovarian cancer–associated CD45+ CD11c+ cells into immunostimulatory cells that, along with newly recruited CD45+ CD11c+ cells, trigger therapeutic antitumor immunity via an IL-12–dependent and MyD88–independent mechanism. CD45+ CD11c+ cells invaded by cps strongly upregulate the costimulatory molecules CD80 and CD86 and the cps-treated tumor microenvironment regains the ability to cross-present antigen to prime tumor antigen–specific CD8+ T-cell responses.

Treatment with cps resulted in the rejection of otherwise lethal peritoneal ID8–Vegf-A ovarian tumors, and provided a significant survival advantage in mice bearing established hyperaggressive ID8–Deffb29/Vegf-A tumors. The therapeutic benefit was not diminished in mice that had existing CD8+ T-cell–dependent immunity to *Toxoplasma*, or in mice that do not express the TLR adapter molecule MyD88. Our results show that cps treatment is highly effective in targeting and activating tumor-associated APC and in stimulating therapeutic CD8+ T-cell antitumor immunity.

High-level activation of CD86 in macrophages was recently shown to depend on active invasion by *T. gondii* and subsequent activation of a host cell JNK signal transduction pathway (43). *Toxoplasma* also manipulates cellular signaling cascades such as STAT3 and STAT6 by injecting parasite-derived effector molecules directly into the host cell cytoplasm during invasion (44, 45) as well as by injecting these novel molecules into bystander cells that are not invaded (46). These injected effector molecules extensively reprogram host cell transcription (47). The early activation of ovarian tumor-associated CD45+ CD11c+ cells and the tumor microenvironment by cps treatment seems to be an active process driven by parasite biology. The cps vaccine strain provides a unique approach to tumor therapy by using a nonreplicating parasite that invades tumor-associated cells without lysing them, as well as by exposing tumor-associated cells to parasite molecules that influence the invaded cell and the local tumor microenvironment. Immunotherapy using cps provides a potent and unique therapeutic opportunity for ovarian cancer and other lethal epithelial tumors.

**Disclosure of Potential Conflicts of Interest**

J.R. Conejo-Garcia is a consultant/advisory board member of TTT Study section. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

**Conception and design:** J.R. Baird, B.A. Fox, J.R. Cubillos-Ruiz, J.R. Conejo-Garcia, S.N. Fiering, D.J. Bzik

**Execution of methodology:** J.R. Baird, B.A. Fox, J.R. Conejo-Garcia, S.N. Fiering, D.J. Bzik

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.R. Baird, B.A. Fox, K.L. Sanders, P.H. Lizotte, J.R. Cubillos-Ruiz, U.K. Scarlett, M.R. Rutkowski, J.R. Conejo-Garcia, S.N. Fiering, D.J. Bzik

**Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis):** J.R. Baird, B.A. Fox, K.L. Sanders, P.H. Lizotte, J.R. Cubillos-Ruiz, M.R. Rutkowski, J.R. Conejo-Garcia, S.N. Fiering, D.J. Bzik

**Writing, review, and/or revision of the manuscript:** J.R. Baird, B.A. Fox, P.H. Lizotte, M.R. Rutkowski, J.R. Conejo-Garcia, S.N. Fiering, D.J. Bzik

**Study supervision:** J.R. Conejo-Garcia, S.N. Fiering, D.J. Bzik

**Acknowledgments**

The authors thank Dr. David Roos (University of Pennsylvania, Philadelphia, PA) for providing cps–VFP and Li Wang (Geisel School of Medicine at Dartmouth, Lebanon, NH) for providing Foxp3–GFP mice. The authors also thank the Immune Monitoring Lab at the Geisel School of Medicine at Dartmouth.

**Grant Support**

This work was supported by grants from the NIH CA124515 (J.R. Conejo-Garcia), NIH CA157664 (J.R. Conejo-Garcia), NIH A804930 (D.J. Bzik), by pilot funding (S. Fiering) from The Friends of the Norris Cotton Cancer Center and NCI award 5P30CA023108 and by Host-Parasite Interaction at Dartmouth (D.J. Bzik), NIH training grant T32 AI007363 (J.R. Baird), NIH training grant T32 A8007519 (K.L. Sanders), National Research Service Award F31CA134188 (to U.K. Scarlett), and 2009-2010 John H. Copenhaver, Jr. and William H. Thomas, MD 1952 Fellowship (J.R. Cubillos-Ruiz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 21, 2012; revised March 26, 2013; accepted April 21, 2013; published OnlineFirst May 23, 2013.

**References**


Avirulent *Toxoplasma gondii* Generates Therapeutic Antitumor Immunity by Reversing Immunosuppression in the Ovarian Cancer Microenvironment

Jason R. Baird, Barbara A. Fox, Kiah L. Sanders, et al.

*Cancer Res* Published OnlineFirst May 23, 2013.

Updated version  
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-1974

Supplementary Material  
Access the most recent supplemental material at: [http://cancerres.aacrjournals.org/content/suppl/2013/05/23/0008-5472.CAN-12-1974.DC1](http://cancerres.aacrjournals.org/content/suppl/2013/05/23/0008-5472.CAN-12-1974.DC1)

E-mail alerts  
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

Permissions  
To request permission to re-use all or part of this article, contact the AACR Publications Department at [permissions@aacr.org](mailto:permissions@aacr.org).