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

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**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 auxotrophic 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.

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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 cytokines (18). T. gondii is avirulent in healthy animals but 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 (21).

Parasite culture

Replicating tachyzoites of the cps strain of T. gondii (19) were grown in human foreskin fibroblast cell cultures 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 (30-F11, eBioscience), CD69 (H1.2F3, eBioscience), CD11c (HL3, BD Biosciences), DEC205 (Noindritic cells145, Serotec), MHC-II (NIMR-4, eBioscience), CD3e (eBio500A2, eBiosience), 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 (ATVTNVSGL) specific for CD8+ T cells recognizing ovarian ID8-Defb29/Vegf-A (Bennett 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 CD4+ 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 (Milteny 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 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+ APCs 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

Figure 1. cps preferentially invades CD45+CD11c+ tumor-associated APC, activates costimulatory molecule expression, and increases antigen presentation. A, mice (n = 5) bearing ID8-Defb29/ Vegf-A tumors for 19 days were injected intraperitoneally with cps-YFP and peritoneal cells were isolated and analyzed by FACS 18 hours later. A, left and middle, cps-invaded cells (YFP+) were CD45+CD11c+. A, right, the percentage of invaded cells that are CD45+, CD45+, CD45+CD11c+, and CD45+CD11c-. B, PBS-treated and cps-treated tumor-bearing mice (n = 5), treated as in A, analyzed for expression of CD80 and CD86 in the cps-invaded (YFP+, dotted line histogram) and cps-exposed noninvaded (solid line histogram) CD45+CD11c+ population. C, PBS- and cps-treated tumor-bearing mice (n = 5) treated as in A, analyzed for CD80 and CD86 (mean fluorescence index, MFI) in cps-invaded (YFP+) and cps-treated noninvaded CD45+CD11c+ population. D, PBS- and cps-treated mice (n = 5) bearing tumors for 34 days treated as in B, analyzed for CD80 and CD86 in cps-invaded (YFP+) and cps-treated and noninvaded CD45+CD11c+ populations. E, mice bearing tumors for 21 days were injected with ovalbumin and 4 hours later with cps (n = 6) or PBS (n = 4). Mice intraperitoneally injected with CFSE-stained OT-I T cells 18 hours later and proliferation determined by FACS 48 hours later by dilution of CFSE in CD45+CD3+CD8+ T cells (cps-treated, dotted line; PBS-treated, solid gray). Representative of 2 independent experiments.
on immunosuppressive CD11c<sup>+</sup> 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<sup>+</sup> APC exhibited increased levels of CD80 and CD86 (Supplementary Fig. S2A and S2B).

CD45<sup>+</sup>CD11c<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T-cell responses in the immunosuppressed tumor environment, we injected full-length ovalbumin peptide SIINFEKL-presented on MHC class I in H-2K<sup>b</sup>-restricted C57BL/6 mice (23). While APC in the PBS-treated tumor environment weakly cross-presented antigen to OT-I CD8<sup>+</sup> T cells, APC in the cps-treated tumor environment efficiently cross-presented antigen to OT-I CD8<sup>+</sup> T cells (Fig. 1E). Preferential targeting of tumor-resident CD45<sup>+</sup>CD11c<sup>+</sup> cells by cps rapidly activated the priming of CD8<sup>+</sup> T-cell responses to antigen present in the tumor environment, reversing active immunosuppression and reawakening natural immunity.

cps<sup>+</sup> 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 T<sub>1</sub>H cytokine and cellular responses (20) that elicited exceptional CD8<sup>+</sup> 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<sup>+</sup>CD11c<sup>+</sup> cells (Fig. 2B), macrophages (Fig. 2C), B cells (Fig. 2D), natural killer (NK) cells (Fig. 2E), CD8<sup>+</sup> T cells (Fig. 2F), and CD4<sup>+</sup> 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–S3J). At the local tumor site, the number of peritoneal CD45<sup>+</sup> leukocytes was significantly increased in cps-treated tumor-bearing mice compared with PBS-treated tumor-bearing mice (Fig. 2H). Tumor-associated CD45<sup>+</sup> CD11c<sup>+</sup> cells (Fig. 2I), CD8<sup>+</sup> T cells (Fig. 2J), and CD4<sup>+</sup> T cells (Fig. 2K) were significantly increased. Treatment with cps significantly increased the percentage of peritoneal CD45<sup>+</sup>CD11c<sup>+</sup> cells (Fig. 2L) and CD8<sup>+</sup> T cells (Fig. 2M) among tumor-associated leukocytes. Foxp3<sup>+</sup> T-regulatory (Treg) CD4<sup>+</sup> 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<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells (27, 28). We observed a reduction in the percentage of Treg

**Figure 2.** Treatment with cps increases cellular infiltration into the spleen and the peritoneum. Mice (n = 4) bearing ID8-Defb29/Vegf-A tumors for 8 days were treated with cps or PBS and leukocytes in the spleen (A–G) or peritoneum (H–N) assayed 12 days later by FACS (see Supplementary Fig. S3 for gating strategies). Number of splenocytes (A), CD45<sup>+</sup>CD11c<sup>+</sup> cells (B), macrophages (F4/80<sup>+</sup>; C), B cells (B220<sup>+</sup>; D), NK cells (NK1.1<sup>+</sup>; E), CD3<sup>+</sup>CD8<sup>+</sup> T cells (F), CD3<sup>+</sup>CD4<sup>+</sup> T cells (G), CD45<sup>+</sup> cells (H), CD45<sup>+</sup>CD11c<sup>+</sup> cells (I), CD3<sup>+</sup>CD8<sup>+</sup> T cells (J), CD3<sup>+</sup>CD4<sup>+</sup> T cells (K), CD45<sup>+</sup>CD11c<sup>+</sup> cells (L) as percentage of total peritoneal leukocytes. M, CD3<sup>+</sup>CD8<sup>+</sup> T cells as percentage of total peritoneal leukocytes. N, CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> T-reg cells as a percentage of total peritoneal CD4<sup>+</sup> T cells. Representative of 2 independent experiments.
(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 TH17 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 TH17 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-1b, 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 TH17 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, 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 TH17 cells.

**Figure 3.** Treatment with cps increases tumor antigen-specific CD8^+ T-cell responses. Mice (n = 6) bearing ID8-Defb29/Vegf-A tumors for 8 days treated with cps or PBS and CD8^+ T-cell populations in spleen (A–D) and peritoneum (E–H) analyzed by FACS (A–G) or assessed by granzyme B ELISpot (H) 12 days later. A, number of splenic CD8^+ expressing CD44 and CD62L. B, same as in A, as percentage of total CD8^+ T cells. C, number of splenic CD3^+CD8^+ tetramer^+ tumor antigen–specific T cells. D, same as in C, as percentage of total CD8^+ T cells. E, total of CD3^+CD8^+ tetramer^+ peritoneal CD8^+ T cells. F, same as E, as percentage of total CD8^+ T cells. G, same as in E, except tetramer-positive CD8^+ T cells were assessed for coexpressing CD44 and CD69. H, mice (n = 6) bearing ID8-Defb29/Vegf-A tumors for 8 days were treated with cps or PBS and peritoneal CD8^+ T cells assayed for granzyme B 12 days later. CD8^+ T cells were purified and cocultured with BMDCs either pulsed with tumor antigen or unpulsed before assay. Representative of 2 independent experiments.
Vegf-A ovarian tumors 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 eliminates an ID8 model of ovarian carcinoma that expresses VEGF (Vegf-A) and the therapeutic benefit is independent of immune status to T. gondii**

Figure 4. T cells from cps-treated tumor bearing mice suppress growth of ID8-Defb29/Vegf-A ovarian flank tumors. Mice (n = 4) bearing ID8-Defb29/Vegf-A flank tumors were injected intravenously with 2 x 10^6 total CD3^+ T cells purified from mice bearing ID8-Defb29/Vegf-A tumors treated with cps 8 days and 20 days after tumor challenge, from mice bearing untreated ID8-Defb29/Vegf-A peritoneal tumors or from naive mice. Flank tumors were removed and measured 2 months later. Representative of 2 independent experiments.

Figure 5. Rejection of established ID8-Vegf-A tumors is independent of immune status to T. gondii. A, mice bearing ID8-Vegf-A tumors treated with cps (n = 6) or PBS (n = 8) on day 8 and day 20 and survival was monitored. B, same as in A, except groups of mice were vaccinated with cps 90 days earlier to establish a life-long (19, 20) immunity to Toxoplasma before tumor challenge. Representative of 2 independent experiments.


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 therapeutic role in the tumor environment by promoting antitumor immunity (33) and also by inhibiting angiogenesis (34). Mice bearing late-stage (35 days) ovarian tumors markedly extended the survival (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

![Figure 6. The cps antitumor response requires IL-12 but not MyD88. A, mice (n = 8) bearing ID8-Defb29/Vegf-A treated with cps or PBS on days 8 and 20 and survival is shown. B, mice (n = 7) bearing ID8-Defb29/Vegf-A tumors were treated with cps and peritoneal IL-12p40 was evaluated 18 hours later. C, mice (n = 4) same as in B, except assay for IL-12p70. D, IL-12p40−/− or C57BL/6 mice (n = 4) bearing ID8-Defb29/Vegf-A tumors were treated with cps or PBS on days 8 and 20. E, IL-12p35−/− or C57BL/6 mice (n = 6) bearing ID8-Defb29/Vegf-A tumors treated with cps or PBS on days 8 and 20. F, MyD88−/− or C57BL/6 mice bearing ID8-Defb29/Vegf-A tumors were treated with cps (n = 6) or PBS (n = 6) on days 8 and 20 (P = 0.8694, cps-treated C57BL/6 compared with cps-treated MyD88−/−). Representative of 2 independent experiments.](image-url)
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-Deff29/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 adaptor 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.

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References
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