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Immune-Mediated Regression of Established B16F10 Melanoma by Intratumoral Injection of Attenuated Toxoplasma gondii Protects against Rechallenge

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Immune recognition of tumors can limit cancer development, but antitumor immune responses are often blocked by tumor-mediated immunosuppression. Because microbes or microbial constituents are powerful adjuvants to stimulate immune responses, we evaluated whether intratumoral administration of a highly immunogenic but attenuated parasite could induce rejection of an established poorly immunogenic tumor. We treated intradermal B16F10 murine melanoma by intratumoral injection of an attenuated strain of Toxoplasma gondii (cps) that cannot replicate in vivo and therefore is not infective. The cps treatment stimulated a strong CD8+ T cell–mediated antitumor immune response in vivo that regressed established primary melanoma. The cps monotherapy rapidly modified the tumor microenvironment, halting tumor growth, and subsequently, as tumor-reactive T cells expanded, the tumors disappeared and rarely returned. The treatment required live cps that could invade cells and also required CD8+ T cells and NK cells, but did not require CD4+ T cells. Furthermore, we demonstrate that IL-12, IFN-γ, and the CXCR3-stimulating cytokines are required for full treatment efficacy. The treatment developed systemic antitumor immune activity as well as antitumor immune memory and therefore might have an impact against human metastatic disease. The approach is not specific for either B16F10 or melanoma. Direct intratumoral injection of cps has efficacy against an inducible genetic melanoma model and transplantable lung and ovarian tumors, demonstrating potential for broad clinical use. The combination of efficacy, systemic antitumor immune response, and complete attenuation with no observed host toxicity demonstrates the potential value of this novel cancer therapy. The Journal of Immunology, 2013, 190: 000–000.

Despite considerable progress using surgery, chemotherapy, and radiation to treat cancer, the 5-y survival rates for many cancers are still very low and not improving. There is currently a great deal of interest in developing therapies that stimulate effective immune responses against cancer to establish another major therapeutic option and improve outcomes. The immune system is stimulated by microorganisms, and since the studies of Coley (1) over 100 y ago, the possibility of using microorganisms as adjuvants to stimulate antitumor immunity has been recognized. However, despite frequent efficacy against what were deemed to be incurable, often metastatic cancers, “Coley’s toxins” were not accepted clinically. Since Coley’s time, we have developed a detailed understanding of both the immune system response to tumors and the suppression of the immune system by tumors. Genetic manipulation enables the generation of microorganisms with reduced virulence that can still function as powerful immunologic adjuvants, and this has led to progress in developing microorganisms as immune-stimulating antitumor reagents. For example, the standard of care for treating superficial bladder cancer is instilling Bacillus Calmette-Guerin into the bladder (2), and a variety of other microorganisms, such as Listeria monocytogenes, Salmonella typhimurium, and multiple viruses, are in various stages of development as antitumor vaccines and treatments (3–5). Importantly, although each of the most heavily studied organisms has efficacy in various models and some show promise in clinical trials, none of these organisms has been shown to eliminate an established, poorly immunogenic tumor.

Toxoplasma gondii is a single-cell, obligate intracellular, eukaryotic parasite. cps, the strain used in this study, is a uracil auxotroph due to deletion of the carbamoyl phosphate synthetase II enzyme (6). Because the required enzyme is inactivated, there is little potential for reversion. The cps grows well in vitro in mammalian cells in uracil-supplemented medium. In vivo, cps is nonreplicative, but efficiently invades cells and generates a strong adaptive immune response, characterized by activation of APCs to stimulate CD8+ T cell maturation and expansion with associated generation of high levels of IL-12 and IFN-γ (7). Because such immune responses are associated with effective antitumor immunity, we evaluated whether cps could induce the specific antitumor immunity required to eliminate an aggressive, notoriously difficult-to-treat tumor.
B16F10 melanoma has been used extensively as a poorly immunogenic, highly aggressive model for murine tumor immunotherapy studies (8). Shrinkage of established B16F10 has not been achieved with an immune-based monotherapy. Established B16 tumors have been treated at low frequency by combination immunotherapies, such as adoptive transfer of Ag-specific transgenic T cells along with TLR agonist administration (9), or at high frequencies by combining adoptive transfer of Ag-specific transgenic T cells and recombinant viral infection and preconditioning the host through total body irradiation (10). These approaches are complex and will be difficult to accomplish in a widespread clinical context. However, they do provide encouragement for cancer immunotherapy. New approaches that are easy to apply and have high levels of efficacy and minimal side effects need to be developed to improve cancer immunotherapy outcomes.

Our hypothesis was that cps introduced into the tumor microenvironment would transform that environment from one that is predominantly immunosuppressive to an immunostimulatory environment in which the endogenous antitumor immune response would effectively eliminate the tumors. In the studies reported in this work, we tested that hypothesis by using cps monotherapy to treat established B16F10 melanoma. We show that treatment of established B16F10 dermal melanoma by intratumoral injection of cps not only regresses the primary tumor, but also establishes systemic and memory antitumor immune responses with significant ability to reject or slow the development of rechallenge. The efficacy of cps monotherapy against the primary tumor and development of systemic response and immune memory, in combination with its inability to replicate in vivo and associated lack of observed toxicity, establishes the cps strain of T. gondii as a potentially powerful reagent for immunotherapy of solid tumors.

Materials and Methods

Mice

C57BL/6 were purchased from the National Cancer Institute. IL-12p35−/− (002692), IFN-γ−/− (002287), and NOD/SKID/IL-2Rγ−/− (005557) mice were purchased from The Jackson Laboratory. Tumors were generated in Tyr-CreERT2:BrafCA:Ptenlox/lox transgenic mice, on a mixed genetic background by intradermal (i.d.) injection of 4-hydroxy tamoxifen in the flank. Animal experiments were approved by the Institutional Animal Care and Use Committee at Dartmouth Medical School.

Tumor cell inoculations

The B16F10 mouse melanoma cell line was originally obtained from I. Fidler (MD Anderson Cancer Center, Houston, TX) and passaged i.d. in C57BL/6 mice seven times to ensure reproducible growth. Tumor cells were cultured in RPMI 1640 containing 7.5% FBS and inoculated into mice only if viability exceeded 96%. A total of 1.25 × 10^6 live B16F10 cells was inoculated i.d. in the right flank. Tumor diameters were measured three times weekly, and mice were euthanized when tumor diameters reached 15 mm. To determine T cell recall capacity, B16F10-bearing mice were treated as seen in Fig. 1A and then euthanized on day 20 of tumor challenge, and CD8 T cell responses were assessed by flow cytometry or ELISPOT, as described below. The UpK10 tumor line was generated from C57BL/6 mice, as described (11). A total of 5 × 10^5 live UpK10 cells was injected i.d. Lewis lung tumors were generated by injecting 1 × 10^6 live cells, i.d. on the right flank.

Blocking Abs

Depleting anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) were produced as bioreactor supernatants and administered i.p. in doses of 250 μg 1 day prior to treatment and then once weekly. Greater than 95% depletion of target T cell populations was confirmed by flow cytometry. Depleting anti-NK1.1 (clone PK136) was produced similarly and administered i.p. in doses of 200 μg for four treatments on days −2, 0, 2, and 9 relative to initial cps tumor treatment. CXCXR3 blocking Ab (clone CXCXR3-173) was purchased from BioLegend, administered i.p. in doses of 100 μg beginning 1 d before cps treatment, and continued every third day after for four total treatments. CXCXR3 blockade was also performed at 200 μg, yielding similar results.

Abs and gating strategies used for flow cytometry

CD45 (30-F11), CD3e (145-2C11), CD8b (YTS165.7.7), CD44 (IM7), CD11c (N418), and CD11b (M1/70) were purchased from BioLegend. NK1.1 (14B11) and IFN-γ (XMG1.2) were purchased from eBioscience. Fig. 2A–F flow cytometry samples were gated on CD45+ cells after the forward light scatter (FSC) and side light scatter (SSC) gate was set. Then Fig. 2B was gated on CD11c+ CD11b+ cells, whereas Fig. 2C was gated on CD11c+CD11b+ cell populations. Fig. 2D was gated on CD3+ cells, whereas Fig. 2E was gated on NK1.1+ populations and Fig. 2F was gated on Gr1+, CD11b+ . The initial gating strategy for Fig. 2G was the same strategy used for Fig. 2A–F; however, a final gate was made that defined the population of cells infected. Infected cells were defined by the invaded presence of CSFE-stained cps parasites. Fig. 2H–L samples were first gated on CD45+ cells after the FSC and SSC gate was set. Fig. 2I was gated on CD3+CD8+ cell populations, and Fig. 2J was also gated on CD3+CD8+ cell, and then a histogram gate was made defining IFN-γ+ CD8+ cells. Fig. 2K was gated on NK1.1+ cells, and Fig. 2L was first gated on NK1.1+ and then gated on IFN-γ+ NK1.1+.

Fig. 3A and 3B flow cytometry samples were first gated on CD45+ cells after the FSC and SSC gate was set. Then Fig. 3A was gated on CD3+CD8+ cells, followed by gating on the CD8+CD44+ cell population. Fig. 3B was gated on CD3+CD8+ cells, followed by gating on the CD8+CD44+ cell population.

Cytokine detection

Tumor explants were manually homogenized in T-Per Lysis Buffer (Thermo Scientific/Pierce Protein Research Products) with complete protease inhibitor mixture (Roche Applied Science), and total protein was quantified using Life Technologies/Invitrogen Qubit Protein Assay kit and an Invitrogen Qubit 2 fluorometer. IFN-γ, CXCL9, and CXCL10 protein were quantified using specific ELISA kits (R&D Systems) with SuperAquaBlue substrate (eBioscience); absorbance was measured using a BioTek Epoch microplate spectrophotometer. Cytokine and chemokine concentrations were calculated from a specific standard curve.

IFN-γ ELISPOT

CD8 T cells were isolated from spleens and draining inguinal lymph nodes and purified using anti-CD8 MACS magnetic beads (Miltenyi Biotech). CD8+ T cells were then plated at a 10:1 ratio with irradiated EL-4 B16F10 melanoma cell targets (American Type Culture Collection) that had been pulsed with 1 μg/ml MHC-I–restricted peptide epitopes TRP-2 180–188, or OVA257–264, or heat-killed cps.

Flow cytometry and cytokine assay

Flow cytometry was performed on a FACS-Canto system (BD Biosciences). Data were analyzed using FlowJo software (version 7.6).

For survival experiments

Mice were i.d. injected with 1.25 × 10^6 B16 cells and treated when tumor size reached 3.5–5 mm in diameter (unless otherwise stated). Tumors were injected four times with 1.5 × 10^7 tachyzoites of cps suspended in PBS, as outlined in Fig. 1A. Mice were monitored daily and sacrificed when tumors reached 1.5 cm in diameter.

Intracellular cytokine staining

Cells from tumors were cultured for 5 h at 37°C in RPMI 1640 containing brefeldin A (10 μg/ml). Following incubation, cells were washed and stained with Abs against surface markers and then fixed, permeabilized, and stained intracellularly with anti–IFN-γ PE (clone XMG1.2; BioLegend). Flow cytometry was performed, as described above.

Identifying cps-infected cells

The cps was stained in 7.5 μM CFSE for 5 min at 37°C. After staining, cps was washed in PBS, and then 1.5 × 10^6 tachyzoites were intratumorally injected into B16F10 tumor. Eighteen hours after second CSFE-labeled cps injection, cells were harvested from the tumor by surgically removing the tumor and disassociating with a scalpel, followed by flushing through a sterile cell strainer with 100-μm pores (catalog 22363549; Fisher Scientific). Strained cells were Ab stained using provider-suggested staining protocols.

T. gondii lines and culture

The cps is identical to csps2-1 (6). T. gondii strains were maintained as tachyzoites by serial passage in human foreskin fibroblast cell monolayers. To grow cps, tissue culture medium was supplemented with 0.2 mM uracil. For mouse infections with T. gondii, parasites were purified by filtration.
through a 3.0-μm filter (Nuclepore; Steriltech, Kent, WA) and washed with medium or PBS.

Statistics and experimental repeats

All experiments were repeated at least twice with similar results between experiments. Survival experiments used between four and nine mice per group. Figures denote statistical significance of *p < 0.05, **p < 0.01, and ***p < 0.001. Statistical analysis was performed with GraphPad Prism 4 software. The p values <0.05 were considered significant. Survival experiments used log-rank Mantel Cox test for survival analysis. Data for bar graphs, including for the mean fluorescence of flow cytometry, are from a normal distribution applied to two independent groups, and the confidence interval was calculated using two-tailed unpaired Student t test. 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.

Results

Live cps regresses established dermal B16F10 melanoma by immune mechanisms

B16F10 was inoculated i.d. into C57BL6 mice, and palpable 3.5- to 5-mm tumors (day 9–11 after inoculation) were treated with intratumoral cps injection (treatment schematic; Fig. 1A). The cps-treated tumors initially stopped growing and then rapidly regressed and were undetectable within 12 d of the first treatment in 100% of the treated mice (Fig. 1B, 1C). In comparison, by day 21, at which all tumors had disappeared in cps-treated mice, all mice treated with PBS had large tumors that constituted the experimental endpoint. The vast majority of the treated tumors (83%) did not return after the treatment (Fig. 1C), establishing, to our knowledge, cps treatment as the first immune-based monotherapy that can dependably

FIGURE 1. Live cps treatment causes regression of established dermal B16F10 melanoma by immune effects. (A) The cps treatment strategy: each treatment was 1.5 × 10^7 tachyzoites of cps injected intratumorally that began when tumors reached 3.5- to 5-mm diameter. (B) Photos of tumors 21 d postchallenge. The cps-treated mice are top panel; PBS-treated mice are bottom panel. (C) Individual mouse tumor growth kinetics (left) and survival curve (right) of established primary B16F10 tumor-bearing mice treated with cps or PBS. (D) Tumor growth and survival curve of B16F10 tumor-bearing mice treated with cps or PBS after established latent infection by T. gondii (PRU). (E) Tumor growth and survival curve of primary tumor treatment with cps killed by heating at 65˚C prior to injection. (F) Tumor growth and survival curve of tumors in NOD/SCID/IL-2γR KO mice. Error bars are SEM; *p < 0.05, ***p < 0.001; n/s is not statistically significant; n = minimum of 6 per group; data are representative of at least two independent experiments.
regress established tumors of this very widely studied melanoma model.

Although "cps" does not express any tumor Ags, we also tested whether "cps" monotherapy could be therapeutic if injected into nontumor sites. The "cps" was administered to mice with 3.5- to 5-mm i.d. B16F10 both i.p. and i.v. Although intratumoral "cps" injection regressed the tumor, as expected, i.v. and i.p. "cps" injection did not slow tumor growth or improve survival in mice with established B16F10 melanoma (Supplemental Fig. 1). This demonstrates that, for the treatment of a primary tumor to be effective, "cps" must be in the tumor microenvironment, because i.v. or i.p. applied "cps" would not have accumulated in the tumor.

"T. gondii" latently infects between 10 and 70% of people in most countries (12). To determine how latent infection with "T. gondii" affects treatment efficacy, we infected mice with the Prugniaud (PRU) strain of "T. gondii" to establish a latent infection in mice (13) and tested the efficacy of treating dernal B16F10 with "cps" in these latently infected mice. Latent infection with PRU did not interfere with the ability of "cps" treatment to elicit an effective antitumor response (Fig. 1D). These data indicate that the widespread latent infection of humans by "T. gondii" is not likely to affect the efficacy of "cps" as a clinical antitumor treatment.

Tumors were treated with heat-killed "cps" to determine whether live "cps" is required for efficacy, or whether "cps" constituents act by direct stimulation of the innate immune response through recognition of molecular patterns of pathogens. Using the same treatment regimen associated with regression, there was a slight delay in tumor growth and a modest increase in survival time (Fig. 1E; p < 0.05). However, treatment with heat-killed "cps" failed to halt tumor growth or cause tumor regression in any mice, demonstrating that antitumor efficacy requires live organisms and suggesting that the organism must actively invade cells for efficacy.

A potential problem with live organism therapy is the possibility of establishment of active infection, especially in immunocompromised patients. To rigorously demonstrate that "cps" would be innocuous regardless of immune status, we administered the organism to NOD/SCID/IL-2Rγ chain knockout (KO) mice (NSG). These mice lack B, T, and NK cells (14). Intraperitoneal injection of 100,000 "cps" tachyzoites had no observable effect on immunodeficient mice (Supplemental Fig. 2), despite the fact that 100 tachyzoites of the parental RH strain of "T. gondii" are lethal to wild-type mice within 2 wk (15). These studies demonstrate that even in an immunocompromised host, the "cps" strain of "T. gondii" poses no safety risk as a treatment modality.

A previous study using i.p. injection of a nonattenuated strain of "T. gondii" demonstrated the ability to slow the growth of B16F10 and concluded that the effect was not adaptive immune system mediated, as tumor growth reduction could be achieved in immunodeficient mice (16). To conclusively prove that immune effector function is required for "cps"-mediated elimination of established B16F10 tumors, we evaluated "cps" treatment of B16F10 in severely immunodeficient NSG mice. The "cps" treatment failed to affect tumor growth in NSG mice (Fig. 1F), proving that "cps"-mediated tumor regression is mediated by the immune system.

The "cps" treatment rapidly increased leukocyte infiltration of tumors, and leukocytes were invaded by "cps"

Leukocyte distribution was assessed in untreated and "cps"-treated tumors by immunohistochemistry and flow cytometry. Both methodologies showed that "cps" treatment rapidly increased CD45+ leukocyte infiltration of the tumors by 2- to 3-fold after treatment (Fig. 2A). CD11b+CD11c+ macrophages were significantly increased in approximately the same proportions as total CD45+ cells (Fig. 2A, 2B). CD11c-expressing myeloid dendritic cells

**FIGURE 2.** The "cps" treatment rapidly increased leukocyte infiltration of tumors, and leukocytes were invaded by "cps." (A–F) The 4-mm tumors were treated twice 24 h apart and harvested 18 h later to assess cell infiltration into tumor and assayed for the following: (A) percentage of CD45+ cells in the tumor; (B–F) the percentage of major leukocyte subsets within the CD45+ cells in the tumor; (B) CD11b+CD11c+ cells; (C) CD11b+CD11c+ cells; (D) CD3+ cells; (E) NK1.1+ cells; and (F) Gr1+CD11b+. White bars are control PBS treated; black bars are "cps" treated. (G) A total of 1.5 × 107 CFSE-stained tachyzoites of "cps" was injected twice 24 h apart into 4-mm tumors that were harvested 18 h later. Cells were assayed for percentage of each cell type invaded by "cps" in the tumor. (H–L) The 4-mm tumors were treated as previously on consecutive 2 d and then harvested 3 d later to assess cell infiltration into tumors. White bars are PBS treated, and black bars are "cps" treated. (H) Percentage of CD45+ cells in tumor; (I) percentage of CD45+ cells that are CD8+ cells; (J) percentage of CD8+ cells that are expressing IFN-γ; (K) percentage of CD45+ cells that are NK1.1+; (L) percentage of NK1.1+ cells that are expressing IFN-γ. Error bars are SEM; *p < 0.05, **p < 0.01, ***p < 0.001. n = minimum of 4 mice per group; data are representative of two independent experiments.
Dendritic cells (DCs), NK1.1-expressing NK cells, and CD11b-Gr1+ neutrophils were not significantly changed as a proportion of CD45+ cells, although the increase in CD45+ cells means that the total cell numbers of these leukocytes did increase (Fig. 2C, 2E, 2F). Perhaps surprisingly, the percentage of CD3+ T cells in the tumor was significantly reduced in the early stages of the treatment. Overall this shows that the initial response to treatment is recruitment of myeloid, but not lymphoid cells.

To determine what cells are being invaded by cps, cps was labeled with CFSE, injected intratumorally on consecutive days, and assayed 18 h after the second cps injection. Flow cytometry revealed that ~50% of CD45+ cells were CFSE positive and >80% of leukocytes were invaded (CD45+ cells; Fig. 2G). This demonstrates that leukocytes are first rapidly recruited to the tumor by cps injection (Fig. 2A) and preferentially invaded by cps. Macrophages, DCs, and NK cells were all 70–80% invaded, but only 20–30% of T cells and neutrophils were invaded (Fig. 2G). This shows that cps can invade a variety of nucleated cell types, but preferentially invades phagocytes, as is consistent with wild-type T. gondii.

To further investigate the kinetics of the adaptive immune response, we performed 2 d of cps injection, and then 3 d later analyzed the leukocyte populations in the tumor (Fig. 2H–L). Leukocytes (CD45+) were 2- to 3-fold higher in treated tumors (Fig. 2H, Supplemental Fig. 3), CD8+ T cells increased 3-fold (Fig. 2I), and 8% of these CD8+ T cells were producing IFN-γ as compared with 3% in the controls (Fig. 2J). Interestingly, whereas the fraction of NK cells was similar between treated tumors and the now much larger control tumors, there is a marked increase of NK cells producing IFN-γ in treated tumors (Fig. 2L). Together, these data show that cps treatment drives a rapid and durable leukocyte infiltration of the tumor (changes expressed in cell numbers rather than percentages are shown in Supplemental Fig. 4). At this later stage of treatment, there is an increase in CD8+ T cells and increased expression of IFN-γ by both CD8+ T cells and NK cells.

Tumor Ag-specific responses were enriched in draining lymph nodes and spleens (Fig. 3A, 3B). IFN-γ ELISPOT showing tumor Ag TRP-2 and T. gondii (cps) Ag-specific CD8+ T cells are enriched in draining lymph nodes (G) and spleen (D). OVA was an irrelevant peptide control. (E) B16F10 tumor-bearing mice treated with cps or PBS, after CD8+ T cell depletion; left are average growth curves for the groups, and right are survival curves. Similar for (F) CD4+ T cell depletion or (G) depletion of NK (NK1.1+) cells. Error bars are SEM; *p < 0.05, **p < 0.01, ***p < 0.001. Growth curves show mean value with no error bars for visual clarity. n = minimum of 6 mice per group; data are representative of at least two independent experiments.
mine whether the treatment generated T cells that recognize specific tumor Ags in the peripheral lymphoid organs. This was assessed by ELISPOT assay of CD8+ IFN-γ–producing T cells in response to presentation of the melanoma Ag TRP-2. There was a dramatic increase of IFN-γ–secreting CD8+ T cells recognizing the Kb-restricted epitope TRP-2 180–188 in both draining lymph nodes and spleens of treated versus untreated animals (Fig. 3C, 3D). These data demonstrate a systemic tumor Ag-specific CD8+ T cell response after cps treatment. Taken together with the results in Fig. 2, this indicates that cps monotherapy causes an early robust recruitment of innate immune leukocytes, followed by generation of activated tumor-specific lymphocytes. We and collaborators have recently shown that cps induces a strong Th1 immune response and elicits a lifelong CD8+ T cell immunity (7). Given that CD8+ T cell responses are capable of eliminating tumor masses, we next determined whether T cells were involved in tumor elimination.

To determine the relative roles of CD8+ and CD4+ T cells in tumor elimination, we evaluated cps-mediated tumor eradication and survival in conjunction with administration of depleting Abs to remove CD4+ or CD8+ T cells. Interestingly, depletion of CD8+ cells, but not CD4+ cells, abrogated the antitumor efficacy of cps (Fig. 3E, 3F), demonstrating that CD8+ T cells are required for the success of cps monotherapy, but CD4+ T cells and associated T cell help are dispensable. The potential role of NK cells was also determined by administering Abs to deplete NK1.1-expressing cells. As occurred with depletion of CD8+ T cells, tumor growth was similar in untreated animals and animals that were cps treated in combination with NK1.1 depletion, revealing a requirement for NK cells for successful cps monotherapy (Fig. 3G).

Treatment efficacy requires IFN-γ, IL-12, and CXCR3 signaling
Tumor-specific IFN-γ–expressing CD8+ T cells are increased in the tumor following cps treatment (Fig. 2G). To determine the changes in IFN-γ levels after cps treatment, cytokines in tumor lysates were assayed at either 18 h or 5 d after the initial cps treatment. IFN-γ is increased 5- to 10-fold at both time points (Fig. 4A). We therefore tested the functional importance of IFN-γ by cps treatment of B16F10 tumors in IFN-γ KO mice (17). The treatment has no effect on the tumor in these mice (Fig. 4B), demonstrating a requirement for host IFN-γ for treatment efficacy.

**FIGURE 4.** Treatment efficacy requires IFN-γ and IL-12, and is supported by CXCR3. (A) Assay of intratumoral IFN-γ at 18 h and 5 d after initial cps treatment. (B) Treatment of established B16F10 with cps or PBS in IFN-γ KO mice. (C) B16F10 tumor-bearing mice treated with cps or PBS in IL-12p35 KO mice; middle panel is an expanded view of day 5–30 of 100-d growth curves. (D and E) Assay of intratumoral CCL9 (D) and CCL10 (E) at 18 h or 5 d after cps treatment. (F) Mean tumor size of B16F10 tumor-bearing mice treated with PBS, cps, or cps plus anti-CXCR3 blocking Ab. Error bars are SEM; *p < 0.05, **p < 0.01, ***p < 0.001. n = 3 for cytokine and chemokine; n = minimum of 4 per group for survival; and data are representative of two independent experiments.
Along with IFN-γ, IL-12 is part of the cps-stimulated immune response (7) and is strongly associated with cell-mediated cytotoxic immune responses, including antitumor responses. A requirement for IL-12 for treatment efficacy was tested using IL12p35 KO mice (18). The cps treatment did not regress established tumors in IL-12 KO mice, and the resultant tumor growth rate was not statistically different from controls over the full experimental course (Fig. 4C), demonstrating the clear requirement of IL-12 for the normal treatment response. Interestingly, the treatment did stall tumor growth during the early treatment phase when cps was most likely still present in the tumor (see days 11, 16; Fig. 4C, middle panel), a pattern not seen with IFN-γ KO mice or mice with CD8+ T or NK cell depletion. This shows that there are two distinct immunological phases of the treatment, an initial phase in which cps is abundant and does not require host IL-12, and a subsequent phase that no longer requires the presence of high levels of cps, but does require IL-12, reflecting an ongoing antitumor adaptive immune response.

To further dissect mechanisms by which cps treatment shrinks established tumors, we investigated chemokines by which lymphocytes could be recruited into the tumor following cps injection. Recruitment of effector T cells to tumor locations has been reported to involve interaction of the CXCR3 chemokine receptor expressed on T cells with its chemokine ligands, CXCL9, 10, or 11, generated in the tumor microenvironment (19). We assayed for expression of CXCL9 and CXCL10 in the tumors themselves 18 h or 5 d after cps treatment was initiated and found that cps treatment generated robust increases of both cytokines at each time point (Fig. 4D, 4E). To determine whether the CXCR3 axis is functionally relevant in cps-mediated tumor treatment, we assessed the efficacy of cps treatment in the presence of CXCR3 blocking Ab. CXCR3 blockade reduced, but did not eliminate the antitumor efficacy of cps treatment (p < 0.05). Although survival of CXCR3-blocked animals was significantly better than untreated controls, only 20% of the treated mice exhibited complete regression of the primary tumor when CXCR3 was blocked (Fig. 4F). Taken together, these results indicate that cps monotherapy induces elevated levels of both CXCL9 and CXCL10, and this response is rapid (apparent at 18 h) and durable (still present at 5 d). The presence of these cytokines is consistent with the partial requirement of CXCR3 signaling for efficacy.

Responses against T. gondii are reported to involve the TLR adaptor molecule MyD88 (20). The requirement for MyD88 for efficacy of treatment with cps was tested in mice lacking MyD88 (21). Surprisingly, tumors in MyD88 KO mice responded normally to the treatment (data not shown), and therefore MyD88 is not required for response to cps monotherapy. CCR5 is similarly thought to play an important role in the immune response to T. gondii by its involvement in stimulating IL-12 production (22, 23). Again, contrary to expectation, CCR5 KO mice (24) responded identically to wild-type animals to treatment of established B16F10 tumors with cps (data not shown), indicating that CCR5 signaling does not influence the therapy’s efficacy.

**FIGURE 5.** Regression of dermal melanoma by cps treatment stimulates vitiligo and supports systemic and memory antitumor immune responses. (A) Picture of mice exhibiting vitiligo after elimination of primary dermal B16F10 melanoma by cps treatment. (B) Graph of percentage of mice that developed vitiligo; 22 of 29 (76%) of cps-treated mice got detectable vitiligo (among wild-type mice with no other manipulation). (C) Treatment strategy for rechallenge during primary tumor treatment. (D) Growth curve of B16 i.d. rechallenge, same and opposite flank. (E) Survival curves for flank rechallenges. (F) Dermal rechallenge on day 40 (left) or day 120 (right) after establishment of primary tumor. The cps groups had the primary tumor regressed by cps; PBS-treated groups had the primary tumor surgically resected on day 13. Error bars are SEM; ***p < 0.001. n = minimum of 5; data are representative of two independent experiments.
Mice treated by cps injection exhibit vitiligo, systemic antitumor immunity, and antitumor immune memory

Across many individual experiments, 76% of wild-type mice treated with cps for dermal B16F10 melanoma developed localized or disseminated vitiligo (Fig. 5A, 5B). This indicates that the immune response that eliminated the melanoma also initiated an immune response against normal melanocytes. The vitiligo suggested that the antitumor response was systemic and may be associated with system-wide immunity and immunological memory against the tumor. To determine whether cps treatment of a primary tumor would impact a secondary tumor, mice were challenged with a secondary tumor inoculation on the day after the second injection of cps or PBS into the primary tumor. Secondary challenge inoculation was done into the skin of the flank with the primary tumor or the other flank, and treatment of the primary tumor was completed (Fig. 5C). The cps-treated mice rejected the rechallenge in every case, whereas all tumors grew on PBS-treated tumor-bearing mice (Fig. 5D, 5E) that had the primary tumor surgically removed on day 13 to better compare with standard clinical therapy and limit the size of the rapidly growing primary tumor.

To demonstrate antitumor immune memory, we rechallenged mice that had successful treatment of the primary tumor 40 or 120 d after inoculation of the primary tumor (20 or 100 d after elimination of palpable primary tumor). The majority of these mice rejected the secondary tumor challenge at day 40, and the tumors that did grow in these mice grew more slowly than in the control mice whose primary tumor was surgically removed on day 12 (Fig. 5F). Although all secondary challenge tumors grew in the 120-d mice, they grew at a significantly slower rate than surgically treated control mice (Fig. 5F).

Intratumoral injection of cps has efficacy against other tumors

The ability of cps, which lacks melanoma-associated Ags, to mediate antitumor immunity against B16F10 suggests that cps treatment induces tumor-specific immune responses directed against tumor Ags. Therefore, we hypothesized that cps treatment would demonstrate efficacy against other tumors. The ability of cps to treat other tumors was tested using melanomas generated in an inducible genetic melanoma system that utilizes Braf mutation and Pten loss (25), syngeneic C57BL/6 mouse-derived Lewis lung carcinoma (26), and syngeneic C57BL/6 mouse-derived UpK10 ovarian cancer (11). Using a treatment approach similar to that described for B16F10, intratumoral cps administration either significantly slowed tumor growth or led to complete regression, depending on the model (Fig. 6). Thus, intratumoral cps injection is a generalizable approach that has efficacy against a variety of established tumors.

Discussion

Intratumoral injection of cps is an effective immunotherapy with no toxicity

To our knowledge, this study provides the first example of the use of cps for tumor immunotherapy and the first demonstration of high-frequency successful immunological treatment of established B16F10 melanoma using either an attenuated pathogen or an immunological monotherapy. Importantly, cps accomplishes this with no infection-associated toxicity because it cannot replicate in vivo. This combination of efficacy and lack of toxicity is the essential definition of a treatment with an outstanding therapeutic index.

Approximately 20% of the world population is latently infected with T. gondii, with latent infection much higher in some areas. Latent infection with T. gondii does not impact the efficacy of intratumorally administered cps. Thus, this approach could be used to effectively treat patients, regardless of past exposure to T. gondii. This finding suggests that Abs induced by infection with wild-type T. gondii do not interfere with subsequent initial or repeated cps-based therapies. This is an advantage over viral vector-based cancer therapy strategies that often induce Abs that block infectivity following repeated administrations (27).

T. gondii has been studied previously in connection with cancer therapy. Latent infection with T. gondii slowed tumor development in mice (28). Mouse studies using acute toxoplasmosis from i.p. injection to treat cancer retarded tumor growth, but, unlike treatment with cps, it was not used to treat an established tumor, the adaptive immune system was not required, the primary tumors were not eliminated, and the approach itself was limited by toxicity of the infection (16). T. gondii extracts have been used to mature DCs in vitro to improve T cell adoptive transfer efficacy (29). This effect of T. gondii extracts is reflected in our studies using heat-killed cps, in which there was a discernable but modest slowing of tumor growth. The results reported in this study require live metabolically active cps and are not attributable to microbial constituents.

These studies show that leukocytes are preferentially, but not exclusively invaded by cps and there is a highly effective specific immune response both against the tumors and against cps. Interestingly, efficacy was achieved even though cps does not express any tumor Ags. Although it is possible that there is a Toxoplasma Ag that cross-reacts with an Ag in B16F10, the efficacy in lung and ovarian tumors argues against such a possibility. One explanation for apparent Ag spreading is that cps treatment and associated immunostimulation release adaptive immune responses that are present, but being suppressed by the tumors prior to treatment. In studies using L. monocytogenes (30–32) or S. typhimurium (4)
to stimulate antitumor immunity, it was shown that expression of tumor Ags by pathogens significantly improves the antitumor immune response generated. The cps can be engineered to express Ags to increase tumor-Ag–specific responses that may further improve efficacy and immune memory development (33).

It is clear that treating dermal melanoma by immunotherapy is only superior to surgical resection if it stimulates systemic antitumor immune responses that can eliminate occult metastases. Vitiligo is generated by cps therapy and reveals an immune response against normal melanocytes. Development of vitiligo is a predictor of positive outcome in melanoma patients (34–36) and has been shown to be required for long-term T cell memory against B16F10 melanoma (37). The ability to reject or markedly impair subsequent challenges either during treatment of the primary tumor, or after the initial effector phase of the response is complete and the memory phase has begun, supports the potential for this approach to clinically impact early-stage metastatic disease. This therapeutic approach may be effective in the crucial stage in the course of the disease when it may have metastasized, but is not yet so disseminated as to be virtually incurable.

The cps monotherapy orchestrates multiple immune pathways to achieve tumor regression

These studies demonstrate that treatment efficacy requires CD8+ T cells, which is not surprising, but less predictably shows that CD4+ T cells are not required for full efficacy. This demonstrates that the mechanism does not depend on classic T cell help. T cell help for cytotoxic T cells is associated with Th1-type Th cells and the classic Th1 cytokines IFN-γ and IL-12, which are in fact both required for efficacy using cps. This implies that other cell types are taking over the role of Th1 CD4+ T cells when cps is present. It is likely that one of those cell types is NK cells, which are also required for efficacy and can be a major source of IFN-γ in cancer (38).

The cps treatment efficacy required host production of IL-12, and whereas T. gondii has been previously reported to increase IL-12 levels through CCR5 signaling (22, 23), CCR5 KO mice have normal efficacy with this treatment approach. Therefore, other pathways are manipulated by cps to increase IL-12 or the IL-12 is generated through the CCR5 pathway by the genetically normal B16F10 tumor cells. Such pathways may be functional normally or may require specific changes that are associated with the presence of tumors. Similarly, Myd88 signaling has been implicated in the immune response against T. gondii, but is not needed for treatment efficacy. It appears that this therapy is working through as yet unrecognized pathways of T. gondii interaction with the immune system. It should be noted that MyD88 is broadly expressed, and because tumor cells are invaded by cps, it is possible that MyD88 in tumor cells does play a role in the treatment.

Detailed examination of tumor growth curves from the IL-12 KO mouse studies shows that treatment initially inhibits growth of the tumor while cps is present. However, the tumor again overcomes the immune response once cps is less prevalent. By contrast, other conditions that block efficacy, such as elimination of NK or CD8+ T cells or treatment of IFN-γ KO mice, lost all treatment impact and were identical to PBS controls. This suggests that the early response, in which cps is present in the tumor and the tumor growth halts, depends on different mechanisms than the later response during which the tumor is eliminated despite the fact that cps is no longer present. Potentially, the early response breaks immunosuppression and activates innate immune functions that support already existing, but suppressed adaptive responses, and the later response depends on expansion and recruitment of naive tumor-specific T cells.

The data show that the CXCR3 axis plays an important role in cps-mediated antitumor immune responses and is required for full treatment efficacy, but tumor growth is still significantly slowed when CXCX3 is blocked on host cells. Although this suggests that the CXCR3 axis is not the only mechanism by which effector CD8+ T cells are recruited to the tumor, its importance is underlined by the finding that, in humans, the presence of circulating tumor Ag-specific CXCX3+ CD8+ T cells was associated with enhanced tumor-free survival of melanoma patients (39).

The cps treatment has efficacy as a monotherapy against an orthotopic poorly immunogenic tumor. Immunological monotherapy of any sort has rarely exhibited significant efficacy, and most tumor immunotherapy approaches involve multiple, distinct immune manipulations. The demonstration of monotherapy effectiveness presented in this study is likely to be further improved by coupling with other immunological approaches, such as adoptive T cell therapy and blocking of immune checkpoints that suppress T cells. Monotherapy cps treatment of B16F10 also stands out from many other immunotherapy approaches by relying totally on manipulation of the endogenous immune response in vivo.

The efficacy against established B16F10, lack of dependence on specific Ag expression that is subject to immunoediting, response regardless of pre-exposure to T. gondii, and systemic and memory responses establish cps monotherapy of tumors as a unique potential clinical therapy. Additionally, the simplicity, safety, and ability to effectively treat multiple solid tumor types suggest that cps can be developed into a valuable clinical tool for stimulation of therapeutic antitumor immunity.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental figure 1

IV and IP cps injection do not inhibit tumor growth. (a) Intratumoral injection done as previously. (b) Intravenous injection on same schedule as intratumoral treatment. (c) Intraperitoneal injection on same schedule. (e) Survival curves.
**Supplemental figure 2**

A. Survival of NOD/SCID/IL2Rγ-/- mice after CPS inoculation

B. Blood levels of AST and ALT.

**cps, is not toxic even in the immune deficient NSG mice.** a.) Survival curve of NOD/SCID/IL2/gamma receptor KO mice after inoculation with 1x10^5 cps parasites (n=8). b.) Blood levels of alanine aminotransferase, aspartate aminotransferase, and creatinine (below detectible levels, not shown) were measured 60 days after inoculation.
Supplemental figure 3

$cps$ treatment causes recruitment of large numbers of white blood cells into tumors.
Mice with 5 mm B16F10 tumors were treated as previously on 2 consecutive days and then harvested 3 days later for assay of CD45 cell infiltration into tumors. Shown are immunohistochemistry of treated and untreated tumors stained with anti-CD45 (top) and quantification of assay for CD45 cells in histology sections (bottom).
Supplemental figure 4

**Figure 4 Relative cell numbers for percentages displayed in figure 2.** (A) Show the relative cell numbers for the cell types presented in figure 2a-f. (B) shows the relative cell numbers for the cell types presented in figure 2h-l.