Attenuated *Toxoplasma gondii* Stimulates Immunity to Pancreatic Cancer by Manipulation of Myeloid Cell Populations

Kiah L. Sanders, Barbara A. Fox, and David J. Bzik

**Abstract**

Suppressive myeloid cells represent a significant barrier to the generation of productive antitumor immune responses to many solid tumors. Eliminating or reprogramming suppressive myeloid cells to abrogate tumor-associated immune suppression is a promising therapeutic approach. We asked whether treatment of established aggressive disseminated pancreatic cancer with the immunotherapeutic attenuated *Toxoplasma gondii* vaccine strain CPS would trigger tumor-associated myeloid cells to generate therapeutic antitumor immune responses. CPS treatment significantly decreased tumor-associated macrophages and markedly increased dendritic cell infiltration of the pancreatic tumor microenvironment. Resident macrophages and dendritic cells, particularly cells actively invaded by CPS, increased expression of costimulatory molecules CD80 and CD86 and concomitantly boosted production of IL12. CPS treatment increased CD8^{+} and CD8^{−} T-cell infiltration into the tumor microenvironment, activated tumor-resident T cells, and increased IFNγ production by T-cell populations. CPS treatment provided a significant therapeutic benefit in pancreatic tumor-bearing mice. This therapeutic benefit depended on IL12 and IFNγ production, MyD88 signaling, and CD8^{+} T-cell populations. Although CD8^{+} T cells exhibited activated effector phenotypes and produced IFNγ, CD8^{−} T cells as well as natural killer cells were not required for the therapeutic benefit. In addition, CD8^{−} T cells isolated from CPS-treated tumor-bearing mice produced IFNγ after re-exposure to pancreatic tumor antigen, suggesting this immunotherapeutic treatment stimulated tumor cell antigenspecific CD8^{+} T-cell responses. This work highlights the potency and immunotherapeutic efficacy of CPS treatment and demonstrates the significance of targeting tumor-associated myeloid cells as a mechanism to stimulate more effective immunity to pancreatic cancer. *Cancer Immunol Res; 3(8); 891–901. ©2015 AACR.*

**Introduction**

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive, lethal disease and is the fourth deadliest cancer for both men and women in the United States (1). Currently, early tumor resection is the optimal treatment. Unfortunately, few patients are eligible and there is a high incidence (~70%) of tumor recurrence (2–4). Classical cancer therapeutic approaches are relatively ineffective in eliminating PDAC. Consequently, immunotherapeutic approaches that boost the ability of the immune system to recognize and kill tumor cells may be necessary to combat pancreatic cancer.

Although antitumor CD8^{+} T-cell populations develop spontaneously to PDAC, active suppression mediated by tumor-associated myeloid cells negates the ability of T cells to eradicate tumor cells (5–7). Suppressive myeloid cells dampen the effectiveness of the antitumor immune response in multiple murine models of PDAC (8, 9), and higher levels of suppressive macrophages correlate to shorter overall survival of pancreatic cancer patients (10). Targeting suppressive myeloid cells in PDAC has emerged as a promising immunotherapeutic strategy to stimulate CD8^{+} T-cell populations with improved potential to kill pancreatic tumor cells (11, 12).

One remarkably potent microbial stimulator of the immune system is an attenuated strain of *Toxoplasma gondii*, an obligate intracellular parasite that uses secretory mechanisms and a specialized form of gliding motility to actively penetrate host cells (13). This avirulent nonreplicating vaccine strain (CPS) was developed by genetic disruption of the 19), CD4^{+} T cells actively invades cells but fails to replicate, making this vaccine strain avirulent and safe. CPS vaccination elicits strong induction of IL12 and local IFNγ that drives development of a potent CD8^{+} T-cell immunity and memory against *T. gondii* infection (14, 15, 17, 19–22). Immunotherapeutic CPS treatment of mice bearing established aggressive ovarian cancer or B16 melanoma recently was shown to stimulate potent antitumor responses and tumor-free survival (16, 23–25).

In this study, we investigated CPS immunotherapy using a highly aggressive, nonimmunogenic disseminated peritoneal PDAC model. We demonstrated CPS treatment prolonged survival of mice bearing disseminated pancreatic tumors and examined the mechanisms underlying this effective
immunotherapeutic treatment. CPS treatment rapidly increased expression of costimulatory molecules and IL12 production by tumor-associated macrophages and dendritic cells (DC), particularly in myeloid cells actively invaded by CPS. Subsequently, T-cell populations exhibited activated phenotypes, and CD8+ T cells produced IFNγ in response to pancreatic tumor antigens. The therapeutic benefit of CPS treatment relied on invasive parasites, IL12 and IFNγ production, MyD88 signaling, and CD8+ T cells. Our findings demonstrate immunotherapy with the attenuated CPS vaccine strain neutralized suppressive myeloid cell mechanisms in PDAC and stimulated effective antitumor T-cell responses. These results highlight the significance of targeting suppressive myeloid cell populations as an effective immunotherapeutic mechanism to combat pancreatic cancer.

Materials and Methods

Mice and cell lines

Six- to 8-week-old female C57BL/6 (000664), IL12p35−/− (002692), IFNγ−/− (002287), MyD88−/− (009088), and CD8α−/− (002665) were purchased from The Jackson Laboratory. All animal work was performed at the Dartmouth Hitchcock Medical Center animal facility with Dartmouth Institutional Animal Care and Use Committee approval. The mice were maintained in high glucose RPMI-1640 media. ID8-GFP cancer Treatment Tumor Repository (NCI). Pan02 cells known as Panc02 (26), was acquired from the Division of Laboratory. All animal work was performed at the Dartmouth Hitchcock Medical Center animal facility with Dartmouth Institutional Animal Care and Use Committee approval. The murine pancreatic adenocarcinoma Pan02 cell line, also known as Panc02 (26), was acquired from the Division of Cancer Treatment Tumor Repository (NCI). Pan02 cells were maintained in high glucose RPMI-1640 media. ID8-GFP cells (27) were maintained in high glucose DMEM. Human foreskin fibroblasts (HFF; ref. 28) cultures were maintained in Eagle's Minimum Essential Medium. All cell culture media were supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin.

Parasites

Tachyzoites of the CPS vaccine strain were grown in HFF cells supplemented with 300 μmol/L of uracil (14, 15). Tachyzoites were purified through a 0.4-μm nucleopore membrane and washed twice with PBS before treatment of tumor-bearing mice. For experiments tracking cell types invaded by CPS, tachyzoites were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; ref. 16).

Tumor inoculation and treatment of pancreatic tumors

All studies used 105 Pan02 cells injected i.p. in 200 μL of PBS. All CPS treatments used 2.0 × 106 tachyzoites injected i.p. For survival studies, mice were treated with CPS using a 2-dose (7 and 19 days), 3-dose (7, 19, and 31 days), or 6-dose (7, 8, 11, 12, 24, and 36 days) schedule. For cytokine analysis, mice were treated once at 7 days. For all cellular analysis studies, mice were treated with CPS at 14 days.

Tissue and cell isolation

For spleen and mesenteric lymph node isolations, tissues were homogenized with DMEM in 10% FBS, and single-cell suspensions were obtained by disrupting the organs using a cell strainer (40 μm). Peritoneal cells were harvested by lavage at the time of sacrifice. Red blood cells were lysed in cell suspensions using red blood cell lysis buffer (eBioscience). Serum and peritoneal fluid were stored at −80°C.

Cellular analysis and flow cytometry

For intracellular staining studies, cells were incubated with Brefeldin A for 5 hours at 37°C. Antibody reagents were obtained from Biologend: AF647-conjugated anti-mouse CD45 (30-F11), phycoerythrin (PE)-conjugated and AF647-conjugated anti-mouse CD11b (M1/70). Brilliant Violet 421–conjugated anti-mouse CD11c (N418), PE-Cy7–conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD4 (GK1.5), AF647-conjugated anti-mouse CD19 (6D5), PE-Cy7–conjugated anti-mouse F4/80 (BM8), Brilliant Violet 421–conjugated anti-mouse CD3 (17A2), PE-Cy7–conjugated anti-mouse Gr-1 (RB6-8C5), PE-conjugated and AF647-conjugated anti-mouse CD8b (YTS156.7.7), AF488-conjugated anti-mouse/human CD44 (IM7), PE-Cy7–conjugated anti-mouse CD62L (MEL-14), AF647-conjugated anti-mouse CD69 (H1.2F3), PE-conjugated anti-mouse IFNγ (XMG1.2), PE-conjugated anti-mouse IL12/IL23p40 (C15.6), PE-conjugated anti-mouse CD86 (GL-1), PE-conjugated anti-mouse CD80 (16-10A1), AF647-conjugated anti-mouse FoxP3 (MF-14), PE-conjugated anti-mouse CD25 (PC61), and CD16/32 blocking antibody (93). IL12p35 (4D10p35) eflour660 was obtained from eBioscience. FACs analysis was performed using a Miltenyi 8-color MACSQuant, and data were analyzed using FlowJo (TreeStar).

Depleting antibodies

Purified anti-CD4 (GK1.5), anti-CD8 (2.43), and isotype control (rat IgG2a) antibodies were purchased from BioXCell. Antibody (500 μg) was administered i.p. 1 day before, and 250 μg of antibody was administered i.p. 0 and 3 days after each CPS treatment. Anti-NK1.1 (50 μg; PK136) was administered i.p. 2 days before and 0 and 3 days after CPS treatment. In all experiments, target-cell populations were depleted by greater than 99%.

Cytokine measurements

IFNγ, IL12p40, and IL12p70 levels in serum and peritoneal fluid were determined using OptEIA ELISA kits and reagent sets (BD Biosciences).

IFNγ ELISPOT

CD8+ T cells were isolated from splenic tissue 10 days after CPS treatment. CD8+ T cells were purified using the EasySep Mouse CD8+ T cell Enrichment Kit (Stem Cell Technologies). Target cells (Pan02, HFF, or ID8-GFP) and splenocytes (5.0 × 104) were irradiated (300 Rads). CD8+ T cells were plated at a 1:1 ratio of T cells to irradiated target cells in the presence of 5.0 × 104 irradiated splenocytes.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 software. The log-rank Mantel–Cox test was used for survival analysis. Bar graph samples were compared using the Student t test. Error bars show the SEM. P values of less than 0.05, 0.01, 0.001, or 0.0001 are indicated by *, **, ***, ****, respectively, and statistically nonsignificant differences are indicated by “n.s.”

Results

CPS treatment alters myeloid cell populations in the tumor microenvironment

Myeloid cell suppression of immune responses systemically and within the pancreatic tumor neutralizes the effectiveness of
antitumor CD8+ T cells (9). The nonreplicating Toxoplasma CPS vaccine strain is a potent stimulator of DCs and macrophages (20, 23–25, 29). We established disseminated, peritoneal PDAC tumors by injection of Pan02 cells, a model that establishes pancreatic tumor on the pancreas and in distal locations throughout the peritoneum (30), and asked whether CPS treatment of disseminated pancreatic tumors would stimulate tumor-associated myeloid cells. The cellular compositions of the local tumor microenvironment (TME), the spleen, and the mesenteric lymph node were analyzed at 1, 2, 4, 7, and 10 days after CPS treatment. Treatment significantly increased the population of DCs (CD11c+ CD11b− CD14+ F4/80−) in the TME for at least 4 days (Fig. 1A). In addition, the number of DCs at systemic locations in the mesenteric lymph node and spleen was increased significantly in the first few days after CPS treatment (Supplementary Fig. S1A and S1C). In contrast, macrophage populations (CD11b− CD11c+ F4/80−) in the TME sharply decreased immediately following CPS treatment (Fig. 1B). This reduction in macrophages was not observed systemically (Supplementary Fig. S1B and S1D).

CPS activates invaded myeloid cells in the TME

CPS was shown recently to stimulate strong Th1 and CD8+ T-cell responses by preferential invasion and activation of myeloid cells (20). To track CPS-invaded cells in the pancreatic TME, CPS was CFSE-labeled to identify actively invaded myeloid cells (CFSE+), in contrast with exposed but not actively invaded myeloid cells (CFSE−). Remarkably, approximately 25% of DCs and approximately 45% of macrophages present in the TME were actively invaded by CPS (Supplementary Fig. S2A and S2B). CPS-invaded cells were rarely observed at systemic locations in the

![CPS treatment alters numbers and activation of dendritic cells and macrophages in the pancreatic TME. Pan02 cells (1 x 10^6) were injected i.p., and mice were CPS treated (n = 4) or PBS was administered (n = 4) 14 days after tumor inoculation. A, at indicated time points, peritoneal cells were analyzed by flow cytometry staining for CD45+/CD11c+/CD11b− F4/80− dendritic cells. B, peritoneal cells isolated from the same mice as in A were analyzed at the same time for CD45+/CD11c−/F4/80+ macrophages. C and D, dendritic cells and macrophages from CPS-treated and untreated Pan02 tumor-bearing mice were analyzed 1 day after treatment for expression of CD80 and CD86 (mean fluorescence index, MFI). CPS-invaded and CPS-treated noninvaded cells were tracked using CFSE-labeled CPS parasites. *, P < 0.05; **, P < 0.01; ***, P < 0.001. n.s., not statistically significant.](www.aacrjournals.org)

### Figure 1.

CPS treatment alters numbers and activation of dendritic cells and macrophages in the pancreatic TME. Pan02 cells (1 x 10^6) were injected i.p., and mice were CPS treated (n = 4) or PBS was administered (n = 4) 14 days after tumor inoculation. A, at indicated time points, peritoneal cells were analyzed by flow cytometry staining for CD45+/CD11c+/CD11b− F4/80− dendritic cells. B, peritoneal cells isolated from the same mice as in A were analyzed at the same time for CD45+/CD11c−/F4/80+ macrophages. C and D, dendritic cells and macrophages from CPS-treated and untreated Pan02 tumor-bearing mice were analyzed 1 day after treatment for expression of CD80 and CD86 (mean fluorescence index, MFI). CPS-invaded and CPS-treated noninvaded cells were tracked using CFSE-labeled CPS parasites. *, P < 0.05; **, P < 0.01; ***, P < 0.001. n.s., not statistically significant.

Production of IL12 is required for CD8+ T-cell–dependent immunity to T. gondii (17, 22). In cancer patients, systemic administration of IL12 slows tumor growth but is associated with severe systemic toxicity (32, 33). Recruitment and activation of myeloid cells in the TME suggested that CPS treatment could activate production of IL12 by DCs and macrophages. We examined IL12 production locally, systemically, and at the cellular level in Pan02 tumor–bearing mice following CPS treatment. IL12p40 and IL12p70 levels were significantly increased in the TME after CPS treatment (Fig. 2A and B), and IL12p70 was induced at a higher level than IL12p40. In addition, systemic IL12p70 levels increased following CPS treatment (Supplementary Fig. S3B), whereas systemic IL12p40 levels were unchanged (Supplementary Fig. S3A).

CPS was labeled with CFSE to track the production of IL12p40 by invaded DC and macrophage populations. Percentages of IL12-producing DCs were unchanged in the total cell population
Figure 2.
Treatment with CPS increases IL12 production by myeloid cells. Pan02 cells (1.0 x 10^5) were injected i.p., and tumors were established for 7 days in mice. A and B, mice were CPS treated (n = 4) at 7 days [untreated mice (n = 4) received PBS control]. Peritoneal supernatant was collected at the indicated days after CPS treatment. IL12p40 and IL12p70 production was measured by ELISA. C and D, 18 and 48 hours after CPS treatment, peritoneal cells were harvested from CPS-treated (n = 4) and untreated (PBS) mice (n = 4). Cells were analyzed via flow cytometry with CD45, CD11b, CD11c, F4/80, and IL12p40 to identify IL12-producing dendritic cell and macrophage populations (representative panels are shown). Quantification for each experiment is shown below. E, CD45^-CD11b^-CD11c^-F4/80^- dendritic cells were analyzed via flow cytometry for expression of IL12p40 following treatment with CFSE-labeled CPS parasites to track invaded and noninvaded subpopulations. F, CD45^-CD11b^-CD11c^-F4/80^-Gr-1^- macrophages were analyzed via flow cytometry for expression of IL12p40 following treatment with CFSE-labeled CPS parasites to track invaded and noninvaded subpopulations. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
18 and 48 hours after CPS treatment (Fig. 2C). In contrast, the total peritoneal macrophage population rapidly increased IL12p40 production within 18 hours after CPS treatment, and this marked increase in IL12 in the TME was also present at 48 hours after treatment (Fig. 2D). The percentage of IL12-producing DCs significantly increased within 48 hours after treatment specifically in the population of CPS-invaded DCs (Fig. 2E). Similarly, CPS-invaded macrophages exhibited dramatic increases in the percentage of IL12p40-expressing macrophages at both 18 and 48 hours after treatment (Fig. 2F). In addition, CPS-invaded myeloid cells highly upregulated expression of IL12p35 (Supplementary Fig. S3C and S3D). These results highlight the potent ability of CPS to invade myeloid cell populations, stimulating biologically active IL12 production in the pancreatic TME.

Active invasion and IL12 production are required for the therapeutic benefit

To investigate whether myeloid cell responses triggered by CPS provided a therapeutic benefit, we established Pan02 tumors for 7 days, and tumor-bearing mice were treated using a 3-dose or a 6-dose strategy according to the schedule shown in Fig. 3A. CPS treatment resulted in significantly increased survival time compared with that of untreated tumor-bearing mice (Fig. 3B). Remarkably, 15% of CPS-treated tumor-bearing mice survived long term.

In view of increased IL12 production by CPS-activated DCs and macrophages, we examined whether IL12 production was essential for the therapeutic benefit (Fig. 3C). As well, CPS-treated IL12p35−/− mice failed to gain any detectable therapeutic benefit (Fig. 3C). Because MyD88 signaling plays a crucial role in protection against T. gondii infection through IL12 production (34), we examined whether MyD88 signaling was required for the therapeutic benefit. MyD88−/− mice exhibited no detectable increase in survival following CPS treatment (Supplementary Fig. S4). We also examined whether active parasite invasion of host cells, as opposed to phagocytosis of CPS by phagocytic cell types, was required for the therapeutic benefit. Heat-inactivated CPS tachyzoites incapable of cellular invasion (data not shown) failed to elicit any detectable therapeutic benefit (Fig. 3D). Collectively, these results revealed mechanistic requirements for active invasion of cells by CPS, IL12 production by CPS-invaded myeloid cells, and MyD88 signaling for stimulation of the therapeutic benefit provided by CPS treatment.

Activated T cells accumulate in the TME following CPS treatment

Activation of myeloid cells and increased IL12 production mediated by CPS treatment suggested increased infiltration of the TME by T cells, which is a positive prognostic marker for antitumor responses in the premalignant phase of cancer (35, 36). Rapid recruitment of CD4+ and CD8+ T cells is also a key feature of the host response to CPS vaccination (17). We examined the recruitment of T cells to the spleen, mesenteric lymph node, and pancreatic TME at 1, 2, 4, 7, and 10 days after CPS treatment of tumor-bearing mice. The numbers of CD4+ and CD8+ T cells in the TME were significantly increased by CPS treatment (Fig. 4A and B). CD8+ T-cell populations were also increased in the mesenteric lymph node (Supplementary Fig. S5B) but were relatively unchanged in the spleen (Supplementary Fig. S5D). In contrast, CD4+ T-cell numbers fluctuated only modestly in the mesenteric lymph node and in the spleen (Supplementary Fig. S5A and S5C).

To determine whether recruited CD4+ and CD8+ T cells were activated to effector phenotypes, we examined tumor-resident CD4+ and CD8+ T cells 7 days after CPS treatment for expression levels of CD62L, CD44, and CD69. Resident CD4+ and CD8+ T cells...
Figure 4.

T cells accumulate and are activated within the TME. Pan02 cells (1.0 × 10^6) were injected i.p., and mice were treated 14 days after tumor inoculation with CPS or PBS (n = 4 for each group). A, at indicated times after CPS treatment, peritoneal cells were analyzed by flow cytometry staining for CD4^+ T cells (CD3^+CD4^+CD8b^−). B, at indicated times after CPS treatment, peritoneal cells were analyzed by flow cytometry staining for CD8^+ T cells (CD3^+CD8^+CD8b^+). C, 7 days after CPS treatment, peritoneal cells from CPS-treated and PBS mice were stained for CD3, CD4, CD62L, CD44, and CD69. Totals gated on CD3^+CD4^+ T cells. Shaded histogram, CPS-treated mice; white histogram, PBS-untreated mice. Quantification of activated cells is shown below. D, 7 days after CPS treatment, peritoneal cells from CPS-treated and PBS mice were stained for CD3, CD8, CD62L, CD44, and CD69. Totals gated on CD3^+CD8^+ T cells. Shaded histogram, CPS-treated mice; white histogram, PBS-untreated mice. Quantification of activated cells is shown below. *P < 0.05; **P < 0.001.
in CPS-treated tumor-bearing mice displayed a CD62Llo phenotype, exhibiting a downregulation of CD62L (Fig. 4C and D). Further evaluation of the CD62Llo CD4+ and CD62Lhi CD8+ T-cell populations revealed an increase in T-cell populations coexpressing CD44 and CD69, markers of phenotypic antigen-experience and T-cell activation (Fig. 4C and D). In addition, CPS treatment suppressed regulatory T cells (CD4+CD25+Foxp3+) and markedly increased the CD8+ T cell to regulatory T-cell ratio in the TME (Supplementary Fig. S5E and S5F). These results indicate that CPS treatment activated CD4+ and CD8+ T-cell populations in the pancreatic TME.

**IFNγ production is required for the therapeutic benefit**

IFNγ plays a significant role in the generation of antitumor responses (37, 38). Therefore, we investigated IFNγ levels within the TME and systemically. Interestingly, systemic IFNγ levels were not significantly altered following CPS treatment (Supplementary Fig. S6A). In contrast, IFNγ levels were elevated significantly in the TME within 7 days after CPS treatment (Fig. 5A). We hypothesized that the increased IFNγ arose from activated T cells present in the TME, although IFNγ may also arise from activated natural killer (NK) cells in response to T. gondii (39). NK-cell populations did not significantly change following CPS treatment, and depletion of NK cells did not diminish the therapeutic benefit of CPS treatment (Supplementary Fig. S7).

To identify the potential T-cell source of IFNγ production, we tracked cellular production of IFNγ by CD4+ and CD8+ T cells. The number and frequency of IFNγ-producing CD4+ T cells significantly increased following CPS treatment (Fig. 5B and C; Supplementary Fig. S6B). While the number of IFNγ− CD8+ T cells significantly increased following CPS treatment, the frequency of IFNγ+ CD8+ T cells was unchanged (Fig. 5D and E; Supplementary Fig. S6C). To address the functional importance of IFNγ in the TME, we examined CPS treatment of tumor-bearing IFNγ−/− mice. The therapeutic benefit of CPS treatment was fully dependent on IFNγ production (Fig. 5F). These results suggested that active production of IFNγ by infiltrating T cells in CPS-treated tumor-bearing mice played a critical role in stimulating potent antitumor responses.

**CD8+ T cells are specifically required for the therapeutic benefit**

To investigate the functional importance of recruited and activated T-cell populations, tumor-bearing mice were treated by depleting with rcCD4 or rcCD8 antibody before and following each CPS treatment (Fig. 6A). The absence of CD4+ T cells did not significantly affect the therapeutic benefit (Fig. 6B). In contrast, the absence of CD8+ T cells via antibody depletion (Fig. 6C) or genetic knockout (Fig. 6D) led to a complete loss of the therapeutic benefit. In addition, tumor-bearing 897 mice succumbed faster to pancreatic cancer than wild-type mice, suggesting that CD8+ T cells naturally respond to this tumor (Fig. 6D). Collectively, these findings point to the key functional significance of CD8+ T cells in controlling pancreatic cancer and expose a key role of the CD8+ T-cell population in mediating the therapeutic benefit provided by CPS treatment.

**CD8+ T cells produce IFNγ in response to pancreatic tumor antigen**

We next analyzed the capacity of CD8+ T-cell populations in CPS-treated tumor-bearing mice to produce IFNγ in response to restimulation by Pan02 tumor cells. CD8+ T cells harvested from the spleens of mice were re-exposed to irradiated Pan02 cells for 48 hours, and cellular production of IFNγ was measured by ELISPOT analysis (Fig. 7). CD8+ T cells from CPS-treated mice produced significantly higher levels of IFNγ in response to Pan02 antigens. To determine if this response was driven by Pan02 tumor-antigen specificity, an ovarian cancer cell line (ID8-GFP) or an unrelated human fibroblast (HFF) cell line was used as the target cell. The IFNγ response by CD8+ T cells to Pan02 cells was...
Cancer Immunol Res; 3(8) August 2015

Discussion

In this study, we explored therapeutic vaccination with attenuated *T. gondii* to disrupt pancreatic tumor-associated immune suppression. The complex network of immuno-suppression established by pancreatic cancer abrogates natural immunity and hinders the development of successful immunotherapeutic strategies (9). In murine genetic models of pancreatic cancer as well as in the Pan02 model of PDAC explored here, suppressive populations of myeloid cells infiltrate the TME and promote tumor growth while inhibiting immune responses that could halt tumor growth (5, 40-43). Previous therapeutics based on enzyme activation (44), magnetic nanoparticles (45), drug-eluting beads (46), and stem cells (47) in the disseminated intraperitoneal Pan02 PDAC model failed to provide significant long-term survival benefits. CPS immunotherapy is the first therapeutic treatment that has provided a long-term survival benefit.

Monocytes and myeloid cells are recruited quickly in response to malignancy, and these cell types are rapidly reprogrammed to suppress or be suppressed within the pancreatic TME (9). Treatment of Pan02 tumor-bearing mice with CPS altered the dynamics and functions of myeloid cells within the TME. CPS therapy did not simply alter the numbers of myeloid cells types. CPS selectively accessed myeloid cells in the TME and reprogrammed these cells to promote cellular activation and dynamic changes in tumor-associated cell populations. Compared with the dormant/protumor myeloid cells in untreated tumor-bearing mice, DCs and macrophages in CPS-treated mice upregulated expression of costimulatory molecules CD80 and CD86, particularly in CPS-invaded cells.

Although the highest levels of costimulatory molecule expression were found on CPS-invaded myeloid cells, upregulation of CD80 and CD86 expression also detectably occurred on DCs and macrophages not invaded by CPS. Although toll-like receptors (TLR) may trigger upregulation of CD80 and CD86, recent work has revealed an alternative mechanism for cellular activation based on the injection of specialized parasite-secreted molecules directly into host cells contacted by *T. gondii* (48). More significantly, our results demonstrate active invasion by CPS is essential for the therapeutic benefit in view that noninvasive heat-killed parasites failed to stimulate any therapeutic benefit, even though parasite molecules recognized through TLRs are present. Thus, our results reinforce the emerging view that *Toxoplasma* gains preferential access to myeloid cell populations and actively manipulates myeloid cell types from “within” via specialized parasite-secreted molecules (49).

Concurrent with high-level induction of CD80 and CD86, high-level expression of IL12 was observed in CPS-invaded DCs and macrophages. CPS invasion of tumor-associated myeloid cells activated these cell types in the TME, and could represent the predominant source of costimulation and IL12 production within the pancreatic TME. Our results in pancreatic tumor-bearing mice parallel recent results observed in naïve mice and ovarian cancer-bearing mice, highlighting the critical importance of myeloid cell invasion by *Toxoplasma* in stimulating upregulation of costimulatory molecules (20, 23, 50), and activation of T-cell immunity (20).

Although systemic administration of IL12 is beneficial to cancer patients, the treatment is highly toxic (33). CPS treatment selectively increased production of IL12 by macrophages and DCs enhanced in comparison with the response to ID8-GFP cells or to HFF cells (Fig. 7), suggesting CPS treatment initiated an antigen-specific antitumor CD8⁺ T-cell response.

Figure 6.

CD8⁺ T cells are required for the therapeutic benefit of CPS treatment. A, mice were injected i.p. with 1.0 × 10⁶ Pan02 cells, and tumor-bearing mice were treated with CPS 7 days later as indicated. B, CD4 or isotype control antibody was injected on days indicated in the schedule shown in A. Depletion of CD4⁺ T cells was verified >99.9%. C, CD8b or isotype control antibody was injected on days indicated in the schedule shown in A. Depletion of CD8⁺ T cells was verified >99.9%. D, Pan02 tumor-bearing CD8a⁻/⁻ mice were treated with CPS (n = 4) or were treated with PBS (n = 4) using the 3-dose treatment schedule. Survival experiments represent cumulative percentages of two independent experiments. “***”, P < 0.001. n.s., not statistically significant.
CPS provided by as NK cells were not required for the therapeutic bene-

In models of melanoma and ovarian cancer, CPS treatment has provided a significant survival benefit to tumor-bearing mice (16, 23–25). Interestingly, there are variations in the immune cells and mechanisms required for CPS-induced therapeutic benefit in different tumor models. In the B16 melanoma tumor model, NK-cell production of IFNγ was essential for the therapeutic benefit provided by CPS treatment (16), whereas NK cells were not required for the therapeutic benefit provided by CPS treatment of the disseminated pancreatic tumor model. In the aggressive ovarian cancer CD8 cell tumor model as well as the B16 melanoma model, MyD88 expression was not required for the therapeutic benefit provided by CPS treatment (16, 23–25). Here, we identified a key role for MyD88 signaling in the antitumor response to pancreatic cancer. In all solid tumor models examined to date, effective CPS therapy depends on activation of myeloid cell populations that stimulate tumor antigen–specific CD8+ T-cell populations (16, 23–25). In the absence of engineered exogenous antigen expression, CPS treatment induced host recognition of aberrant tissue that leads to the amplification of antigen-specific antitumor CD8+ T-cell responses.

Generation of tumor-specific responses that extend survival is a prime goal of immunotherapeutic treatment. CPS treatment stimulates the removal of immunosuppressive barriers and generates tumor-specific CD8+ T cells. CPS activated proinflammatory myeloid cell populations, reduced regulatory T-cell populations, and promoted the infiltration of T cells into the TME. The increase in infiltrating T cells and the generation of antitumor-specific responses are all key elements known to be important in tumor immunotherapy. CPS accomplishes all of these goals without the need for secondary chemotherapeutic or small-molecule treatments, although coadministration of these or other treatments may synergize with CPS treatment to provide even more effective antitumor therapy or increased immunity to tumor recurrences. The effect of CPS treatment on disseminated pancreatic tumors reveals the high potency and potential of CPS as an immunotherapeutic treatment for metastatic/advanced pancreatic cancer. In conclusion, this study shows that selective targeting and immune activation of tumor-associated myeloid cells by CPS provide an effective immunotherapeutic mechanism to trigger immunity to pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.L. Sanders, B.A. Fox, D.J. Bzik

Development of methodology: K.L. Sanders, B.A. Fox

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Sanders

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.L. Sanders

Writing, review, and/or revision of the manuscript: K.L. Sanders, B.A. Fox, D.J. Bzik

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.L. Sanders

Study supervision: K.L. Sanders, D.J. Bzik

Acknowledgments

The authors thank DartLab for flow cytometry instrument resources and Charles Sentsman and Edward Usherwood for advice throughout the project.

Grant Support

This work was supported by grants from the NIH (NIH AI041930 to D.J. Bzik), and K.L. Sanders was a trainee on NIH training grants T32AI070363-23 and T32AI070751.

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 December 12, 2014; revised February 23, 2015; accepted March 16, 2015; published OnlineFirst March 24, 2015.
References


43. Stromnes IM, Brockenbrough JS, Izeradjene K, Carlson MA, Cuevas C, Simmons RM, et al. Targeted depletion of an MDSC subset unmasks...


Cancer Immunology Research

Attenuated *Toxoplasma gondii* Stimulates Immunity to Pancreatic Cancer by Manipulation of Myeloid Cell Populations

Kiah L. Sanders, Barbara A. Fox and David J. Bzik


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-14-0235

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2015/03/24/2326-6066.CIR-14-0235.DC1

Cited articles
This article cites 50 articles, 19 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/3/8/891.full#ref-list-1

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.

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