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BRIEF REPORT

Attenuated *Toxoplasma gondii* therapy of disseminated pancreatic cancer generates long-lasting immunity to pancreatic cancer

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ABSTRACT

We have recently reported that treatment of disseminated pancreatic cancer with an attenuated *Toxoplasma gondii* uracil auxotroph vaccine promoted antitumor $CD8^+$ T cell responses and long-term survival. Here, we optimized the treatment strategy for disseminated pancreatic cancer and show that attenuated *Toxoplasma gondii* therapy stimulated effective long-term immunity to pancreatic cancer through mechanisms involving $CD4^+$ T cells and pancreatic tumor-specific IgG. Our results suggest that cell-mediated immunity in conjunction with humoral antibody immunity may offer greater resistance to recurrence of highly aggressive tumors. Cancer immunotherapeutic strategies using attenuated *Toxoplasma gondii* vaccines merit further investigation as a novel strategy to reawaken immunity to primary pancreatic carcinoma and to generate long-lasting immunity to pancreatic cancer recurrences.

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Introduction

Despite advances made in the understanding of the development and tumor microenvironment and progress in the development of drugs and therapies, pancreatic ductal adenocarcinoma (PDAC) still remains one of the deadliest cancers with an average survival rate of only 4 to 6 mo following initial diagnosis.[1] Thirty percent of patients present with locally advanced disease and 50% of patients present with metastatic disease upon diagnosis.[2] Furthermore, metastases develop in ~70% of patients that have received successful surgical resection of their primary tumor. For the 15 to 20% of patients eligible for and receiving surgery, the 5-year survival rate with additional therapeutic intervention remains around 20%.[3-5] Although some progress has been made with regards to improving patient survival, there is still a strong need for the development of drugs and therapies to enhance the immune response to further combat advanced pancreatic cancer in conjunction with current therapies. In particular, innovative therapies that confer long-term protection against highly aggressive metastatic pancreatic cancer and tumor recurrence will be crucial to improve long-term patient survival.[6]

One recently reported effective immunotherapy for established solid tumors is immunotherapeutic treatment with the non-replicating attenuated *Toxoplasma gondii* uracil auxotroph *CPS*.[7,8] *CPS* treatment of tumor-bearing mice promoted long-lasting tumor-free survival in highly aggressive murine solid tumor models for ovarian cancer, for melanoma, and for pancreatic cancer.[9-11] This remarkably effective protection from the primary tumor is triggered and coordinated by *CPS* manipulation of tumor-associated myeloid cells, which leads to the elimination of tumor-associated immune suppression, thereby promoting the activation of significant antitumor immune responses.[9-13] *CPS* therapy strongly induced the production of IL-12 and IFNγ, and stimulated tumor cell specific effector $CD8^+$ T cell populations. Moreover, mice that survived B16 melanoma after *CPS* treatment exhibited increased survival against B16 melanoma re-challenge 40 d post-primary tumor challenge. However, this resistance to B16 tumor recurrence waned with time and only 15% of surviving mice re-challenged at 120 d post-primary challenge survived.[9] We recently reported that *CPS* therapy of a highly aggressive model of disseminated pancreatic cancer promoted long-term tumor-free survival of mice (>1 year).[11] Here, we report improved *CPS* therapy regimens and investigate *CPS*-triggered immunotherapeutic mechanisms that promote long-term protection against recurrence of pancreatic cancer.

Results and discussion

CPS therapy confers protection against re-challenge with pancreatic tumor

CPS treatment of established Pan02 disseminated pancreatic cancer using a three-dose treatment schedule at 7, 19, and 31 d post-tumor challenge provided a 10 to 15% long-term survival rate.[11] To determine whether additional *CPS* treatments would increase the proportion of mice protected against primary tumor, we tested a five-dose *CPS* treatment schedule at 7, 19, 31, 43, and 55 d post Pan02 tumor challenge (Fig. 1A). The five-dose *CPS* treatment schedule significantly increased long-term survival of pancreatic tumor-bearing mice, with ~35% of mice surviving the primary pancreatic tumor (Fig. 1B). The *CPS*-treated mice that survived for >200 d were then re-challenged with Pan02 tumor cells to determine whether *CPS* treatment had generated detectable long-term protection against

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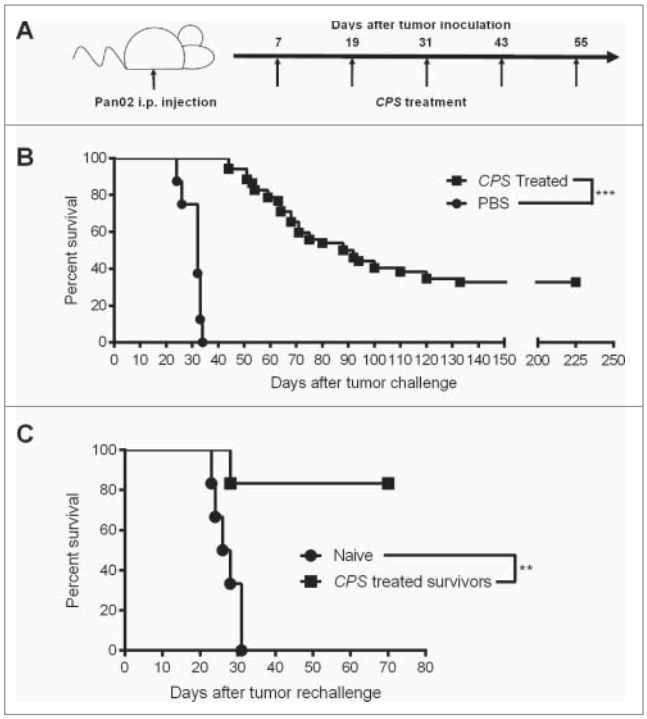

Figure 1. *CPS* treatment confers protection against pancreatic tumor re-challenge. (A) Treatment schematic outlining the treatment used for generating long-term survival. (B) One week after injection of 1.0×10^6 Pan02 cells i.p. mice were treated with *CPS* using a five-dose (n = 52) treatment schedule as outlined in Fig. 1A and survival was monitored. (C) 225 d after initial tumor inoculation, *CPS*-treated survivors (n = 5) or age-matched naive mice (n = 5) were re-challenged with 1.0×10^6 Pan02 cells and survival was monitored. $^{**}= p < 0.01$, $^{***}= p < 0.001$.

recurrence of pancreatic cancer. *CPS*-treated survivors or naive age-matched mice were re-challenged by intraperitoneal injection with 1.0×10^6 Pan02 cells. Following re-challenge, naive age-matched mice succumbed to Pan02 tumor within 35 d, a similar kinetic as seen in 8-week old mice bearing the same tumor type (Fig. 1C). In contrast, *CPS*-treated survivors re-challenged with pancreatic tumor did not succumb to tumor re-challenge. In fact, ~80% percent of these mice survived Pan02 re-challenge (Fig. 1C). Thus, *CPS* treatment of the primary disseminated pancreatic tumor stimulated immune responses that strongly protected against disease recurrence after tumor re-challenge. To our knowledge, this is the first reported therapy against disseminated pancreatic cancer that confers long-lasting protection against tumor recurrence.

CPS therapy increases pancreatic tumor-specific antibodies

Long-lasting protection against Pan02 re-challenge suggested the presence of immune memory to pancreatic tumor. Cancer patients often possess circulating tumor-specific antibody likely generated by the release of antigen during T cell lysis of tumor cells early during tumor progression.[14] To detect the presence of a persistent humoral immune response following *CPS* therapy, we isolated serum from *CPS*-treated survivors and age-matched naive mice and measured bulk IgG antibody levels as well as the IgG populations able to recognize Pan02 cell lysate antigens. Serum obtained from *CPS*-treated survivors contained significantly higher levels of circulating IgG than serum from age-matched naive mice (326 μg/mL compared to ~227 μg/mL) (Fig. 2A). Even more interesting was the presence of increased Pan02-specific IgG in *CPS*-treated survivors (Fig. 2B). While the concentration of circulating Pan02-specific antibody in untreated mice was relatively low (15 μg/mL), *CPS*-treated survivors expressed significantly more circulating Pan02-specific antibody (~120 μg/mL) (Fig. 2B). The accumulation and persistence of pancreatic tumor-specific antibodies following *CPS* therapy highlights the ability of *CPS* to generate a broad antitumor immune response. Moreover, the generation of tumor-specific antibody responses to various solid tumors has been strongly linked as a positive prognostic factor for patient survival.[14-17]

Role of T cell responses in protection from pancreatic cancer recurrence

The presence of circulating $CD8^+$ T cells is a positive prognostic in pancreatic cancer.[18] In mice bearing disseminated Pan02 tumors, *CPS* treatment significantly increased activated $CD8^+$ T cell infiltration into the tumor microenvironment, and also increased the number of circulating pancreatic tumor-specific T cells.[11] Elimination of $CD8^+$ T cells abrogated the immune protection conferred by *CPS* treatment of mice bearing disseminated Pan02 tumors. To determine whether the $CD8^+$ T cell population was required for long-term protection against pancreatic tumor in *CPS*-treated survivors, we depleted $CD8^+$ T cell populations prior to and after Pan02 tumor re-challenge (Fig. 3A). Following the depletion of $CD8^+$ T cells, age-matched naive mice succumbed to pancreatic cancer with similar kinetics as 8-week old mice (Fig. 3B). Unexpectedly, antibody depletion of $CD8^+$ T cells in *CPS*-treated survivors did not abolish immunity to Pan02 tumor re-challenge compared to *CPS*-treated survivors receiving control isotype antibody (Fig. 3B).

The absence of a strict dependence on $CD8^+$ T cells for immune protection led us to investigate the potential importance of $CD4^+$ T cells in mediating protection against tumor recurrence after Pan02 re-challenge. Following the depletion of $CD4^+$ T cells, age-matched naive mice succumbed to pancreatic cancer with similar kinetics as 8-week old mice (Fig. 3C). Surprisingly, *CPS*-treated survivors depleted of $CD4^+$ T cells were not protected against pancreatic tumor re-challenge (Fig. 3C). Thus, while $CD4^+$ T cells were not required for the treatment efficacy of the primary tumor, the $CD4^+$ T cell population was required for protection against tumor re-challenge. The critical role of $CD4^+$ T cells in re-challenge against Pan02 tumor, in addition to the diminished role of $CD8^+$ T cells in re-challenge, reinforces the evidence that *CPS* therapy of the primary pancreatic tumor induced a robust and dynamic antitumor response to promote long-term survival.

In the B16 melanoma model, immunity to tumor recurrence lasted less than ~120 d in *CPS*-treated survivors.[9] Here, we find that long-term immunity to pancreatic cancer recurrence lasted more than 200 d in mice that survived the primary tumor after treatment with *CPS*. *CPS* therapy triggered a diverse cell-mediated ($CD4^+$ and $CD8^+$ T cells) and a significant humoral antibody response against pancreatic cancer. While $CD8^+$ T cells were essential for survival against the primary Pan02

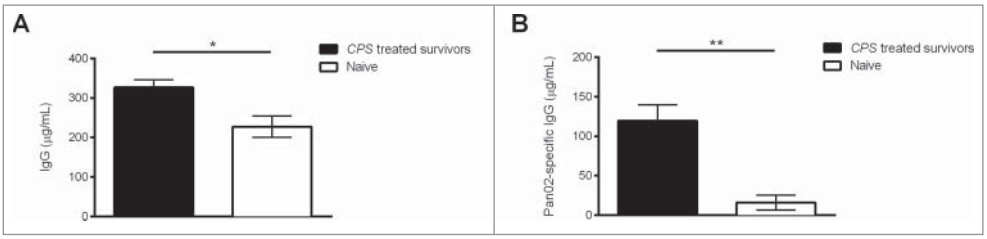

Figure 2. *CPS* therapy increases tumor-specific antibodies in pancreatic tumor-surviving mice. About 200 d after initial Pan02 tumor inoculation, blood serum was isolated from *CPS*-treated survivors or age-matched naive mice. Detection of circulating IgG (A) or Pan02-specific IgG (B) was conducted by ELISA coated with IgG detecting antibodies or with Pan02 cell lysates. $^{*}= p < 0.05$, $^{**}= p < 0.01$.

tumor, immune protection against tumor re-challenge did not rely on the $CD8^+$ T cell population. On the other hand, while $CD4^+$ T cells were not essential for survival against the primary Pan02 tumor, immune protection against tumor re-challenge was dependent on the $CD4^+$ T cell population. In view that the $CD4^+$ T cell population was associated with immune protection, our results do not yet rule out a supporting role for $CD8^+$ T cells in the immunity to tumor recurrence.

$CD4^+$ T cells were shown to be required for development of optimal effector $CD8^+$ T cell populations following vaccination with the *CPS* vaccine strain.[19] Moreover, the relationship between $CD4^+$ T cells and B cells plays a critical role in protection against viral infections and in the generation of protection against tumor growth.[20,21] Vaccination of μMT mice (deficient in B cells) with *CPS* failed to provide protective immunity to re-challenge with virulent *T. gondii* infection.[22] While significant levels of antibody are not detected within 10 d after *CPS* therapy of primary Pan02 tumors (data not shown), antibody may play a central role in early identification of recurrent tumor. The persistence of circulating antibody raises interesting questions regarding the role these antibodies and circulating $CD4^+$ T cells play during re-challenge. These data suggest the $CD8^+$ effector T cells recruited during primary treatment with *CPS* may release high levels of pancreatic tumor antigen that stimulate circulating pancreatic tumor-specific IgG as well as potential $CD4^+$ T cell memory.

In conclusion, our data show that *CPS* treatment of mice bearing primary Pan02 tumors generates long-lasting antitumor immunity to disseminated pancreatic cancer. These results reveal early modifications made to the tumor microenvironment mediated by *CPS* invasion of tumor-associated myeloid cells leads to recognition of Pan02 tumor and generates immune memory against future recurrence.[11] Further work is necessary to examine the basis for protective immunity to pancreatic cancer to understand the relationship between memory $CD4^+$ T cells, potential memory B cells, and circulating persistent antibody specific to pancreatic tumor cells. Our results present compelling evidence of the potency of *CPS* therapy for treatment of primary disseminated pancreatic cancer. Mice surviving the primary pancreatic tumor after *CPS* treatment exhibited potent immune protection against the recurrence of pancreatic cancer. Our results suggest that diverse cell mediated and humoral antibody mediated antitumor immune responses generated by primary immunotherapeutic treatment may be critical to the generation of long-lasting protective immunity to highly aggressive cancers such as PDAC.

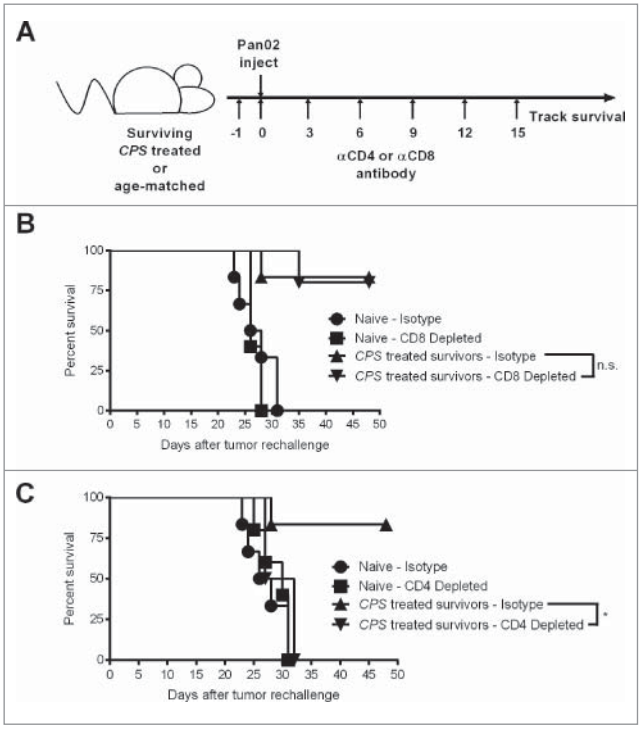

Figure 3. Protection against Pan02 re-challenge depends on $CD4^+$ T cells. (A) *CPS*-treated survivors or age-matched naive mice were re-challenged by i.p. injection with 1.0×10^6 Pan02 cells at 225 d after initial tumor inoculation and survival was monitored. (B) αCD8 antibody was injected i.p. on days indicated in the schedule shown in panel (A) (n = 5 per group). Depletion of $CD8^+$ T cells was verified to be >99% by flow cytometry 16 d after tumor re-challenge. (C) αCD4 antibody was injected i.p. on days indicated in the schedule shown in panel A (n = 5 for $CD4^+$-depleted age-matched naive mice and n = 4 for $CD4^+$-depleted *CPS*-treated survivors). Depletion of $CD4^+$ T cells was verified to be >99% by flow cytometry 16 d after tumor re-challenge. ns = not significant, $^{*}= p < 0.05$.

Materials and methods

Mice and cell lines

Six–eight week old female C57BL/6 (000664) were purchased from Jackson Laboratory. All animal work was performed at the Dartmouth Hitchcock Medical Center animal facility with Dartmouth IACUC approval. The murine pancreatic adenocarcinoma Pan02 cell line, also known as Panc02, was acquired from the Division of Cancer Treatment Tumor Repository (NCI).[23] Pan02 cells were maintained in high glucose Roswell Park Memorial Institute (RPMI) 1640 media. Cell culture media was supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin.

Parasites and tachyzoite isolation

CPS tachyzoites were grown in HFF cells supplemented with 300 μM of uracil.[7,8] Tachyzoites were purified through a 3.0 μM nuclepore membrane and washed twice with phosphate buffer saline (PBS) prior to treatment of tumor-bearing mice.

Tumor inoculation and treatment

10^6 Pan02 cells were injected intraperitoneally (i.p.) in 200 μL of PBS. All CPS treatments used 2.0×10^6 tachyzoites injected i.p. For survival studies, mice were treated with CPS using a five-dose schedule (7, 19, 31, 43, and 55 d). Surviving mice were re-challenged 225 d after primary tumor inoculation.

Circulating IgG and Pan02-specific antibody ELISA

Blood sera were collected via cheek bleeds from animals ~200 d after primary tumor inoculation. Blood was allowed to clot for one hour at room temperature and spun at 1500 rcf for 10 min without break. Collected sera were stored at −80°C until needed.

Pan02 cell lysates were generated by isolating cells from confluent cell cultures. Cells were incubated with protease inhibitor and RIPA buffer (Sigma). Detached cells were isolated utilizing the protocol provided by Sigma. Protein levels were measured utilizing BCA Assay (Life Technologies). For IgG ELISA, purified mouse IgG was diluted in coating buffer (BD Bioscience) at 500 ng/mL. Following overnight incubation, cells were blocked with animal serum in a buffered solution with ProClin®-150 (assay diluent) (BD Bioscience), followed by three washes with 1X wash buffer (concentrated detergent solution with ProClin®-150 as a preservative). Blood sera were serially diluted in assay diluent and incubated for 2 h at room temperature. Bound IgG was detected by incubating goat anti-mouse IgG (Life Technologies) for 1 h at room temperature. Following incubation, samples were incubated with HRP-streptavidin (Biolegend). Samples were then incubated in substrate solution (BD Bioscience) and the reaction was stopped with Stop Solution (BD Bioscience). For Pan02-specific ELISA, Pan02 lysates (0.2 mg/mL) were incubated in coating buffer. Subsequent steps were conducted as stated above.

$CD4^+$ and $CD8^+$ T cell depletions

Purified anti-CD4 (GK1.5), anti-CD8 (2.43), and isotype control (rat IgG2a) antibodies were purchased from BioXCell. 500 μg of antibody was administered i.p. by the schedule outlined in Fig. 3A. Target cell populations were depleted by greater than 99%.

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 unpaired student t-test. Error bars show the SEM. p values of less than 0.05, 0.01, or 0.001 are indicated by *, **, ***, respectively, and non-significant differences are indicated by "n.s.".

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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