Award Number: W81XWH-07-1-0259

TITLE:
Exploiting the Immunological Effects of Standard Treatments in Prostate Cancer

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REPORT DATE:
March 2010

TYPE OF REPORT:
Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT:

   X  Approved for public release; distribution unlimited

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**4. TITLE AND SUBTITLE**

Exploiting the immunological effects of standard treatments in prostate cancer

**5a. CONTRACT NUMBER**

W81XWH-07-1-0259

**5b. GRANT NUMBER**

PC060159

**5c. PROGRAM ELEMENT NUMBER**

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

US Army Medical Research and Material Command
Fort Detrick, Maryland
21702-5012

**10. SPONSOR/MONITOR’S ACRONYM(S)**

**11. SPONSOR/MONITOR’S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

We previously demonstrated that hormone therapy (HT) and radiation therapy (RT) induce tumor-specific autoantibody responses in human prostate cancer, and this grant investigates the clinical significance of these findings. In Aim 1, the findings that HT induces autoantibody and T cell responses against PABPN1 in the Shionogi tumor model and that these immune responses are associated with inferior outcomes have recently been submitted for publication. We have also shown that the combination of HT+RT in this model leads to delayed tumor recurrence of a distal untreated tumor. Work is underway to determine whether similar antibody and T cell responses are seen in these mice and whether they too are associated with poor outcomes. In the human setting, we have tested known prostate cancer tumor antigens by ELISPOT and begun cloning our serologically-defined tumor antigens in order to test these against PBMCs collected from prostate cancer patients showing treatment-induced autoantibody responses (Aim 2). We have also continued to assemble cohorts of prostate cancer patients with recurrent versus non-recurrent disease at 5 years post-treatment (Aim 3). In summary, this study is progressing on schedule and is revealing unexpected results that we believe may be highly relevant to prognosis and treatment of prostate cancer.

**15. SUBJECT TERMS**

Tumor immunology, immunotherapy, prostate cancer, antibody, T cell, tumor antigen, hormone therapy, radiation therapy

**16. SECURITY CLASSIFICATION OF:**

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**17. LIMITATION OF ABSTRACT**

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**18. NUMBER OF PAGES**

19

**19a. NAME OF RESPONSIBLE PERSON**

USAMRMC

**19b. TELEPHONE NUMBER (include area code)**
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INTRODUCTION

While much effort is being made to develop effective immune-based therapies for prostate cancer, there is little information available on whether standard treatments induce tumor-specific immune responses, which could potentially influence clinical outcomes. Radiation therapy causes inflammation associated with the expression of inflammatory cytokines, MHC molecules, B7 and other co-stimulatory molecules. Likewise, neoadjuvant hormone therapy has been shown to cause prominent T-cell infiltration of prostate tumors. Based on such findings, we asked whether radiation therapy (RT) and hormone therapy (HT), by causing tumor cell death in an inflammatory context, might induce tumor-specific immune responses in prostate cancer. Our preliminary results in the androgen-dependent murine Shionogi tumor model indicate that castration (the laboratory equivalent of HT) induces tumor-specific autoantibody responses in approximately 50% of animals. Moreover, parallel studies of human prostate cancer patients undergoing standard treatments at our institution indicate that HT and RT both induce tumor-specific autoantibody responses in up to 30% of patients, depending on the stage of disease and specific treatment. Based on these observations, we hypothesized that treatment-induced autoantibody responses in prostate cancer are accompanied by CD4+ and CD8+ T cell responses that potentially delay or prevent tumor recurrence.

This study has three specific aims:

Aim 1. To determine in the Shionogi mouse tumor model whether castration and brachytherapy induce autoantibody and T-cell responses that prevent or delay tumor recurrence.

Aim 2. To determine in human prostate cancer patients whether treatment-induced autoantibody responses are accompanied by tumor-specific CD4+ and CD8+ T-cell responses.

Aim 3. To determine whether tumor-specific autoantibody profiles differ in prostate cancer patients with recurrent versus non-recurrent disease.

BODY

Aim 1. To determine in the Shionogi mouse tumor model whether castration and brachytherapy induce autoantibody and T-cell responses that prevent or delay tumor recurrence.

As described in the 2009 Annual Report, we identified poly(A) binding protein nuclear 1 (PABPN1) as the treatment-induced ~40 kDa antigen in our Shionogi model and used it to determine that autoantibody and T cell responses were associated with poorer outcomes in castrated mice bearing Shionogi tumors. A manuscript entitled “Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model” describing these results has recently been published in the
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International Journal of Cancer (Hahn et al., Int J Cancer 2009;125:2871-78). A copy of this manuscript can be found in the Appendix.

While the experiments published recently focused only on castration, additional Shionogi experiments have focused on the combination of castration and radiation therapy (in the form of brachytherapy). These experiments were designed primarily to study the abscopal effect, described for ionizing radiation, where reduced tumor growth is observed outside of the field of radiation. It is thought that an immune-mediated mechanism is responsible for the abscopal effect.

We have completed the immunological analysis of the Shionogi castration + RT experiments. These experiments were designed to determine whether castration and brachytherapy led to delayed tumor recurrence of an untreated distant tumor. Although five experimental groups were established, tumor-free survival and immunological analysis focused on three groups:

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<td>Cx</td>
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<tr>
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<tr>
<td>Cx+RT@1/2</td>
<td>Castration → Regression to half tumor volume → six I-125 seeds</td>
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In order to accurately analyze the data, we established a set of criteria that all animals were gauged against. The primary tumor was defined as the first tumor to appear. The subsequent tumor was defined as the distant tumor. Recurrence was defined as the date tumors were first measurable. Tumor-free survival was calculated from date of castration to date of recurrence. In the analysis of the distant tumors, mice were excluded from analysis if castration was given prior to growth of distant tumors, which resulted in 5 mice being eliminated from the analysis. With these criteria, tumor-free survival was re-calculated for the three groups of mice identified above. When the primary tumors were considered, there was a clear survival advantage in those mice that were treated with radiation and castration versus those mice treated with castration alone (Figure 1A), indicating that radiation therapy was effective in controlling primary tumor growth. Although the difference is not statistically significant, those mice that received

![Graph A: Tumor-free survival of primary tumors](image)

![Graph B: Tumor-free survival of distant tumors](image)

Figure 1: Kaplan-Meier analysis of mice with Shionogi tumors that were treated with castration (Cx), castration followed by brachytherapy once the tumors fully regressed (Cx+RT) or castration and brachytherapy once tumors partially regressed (Cx+RT@1/2). A. A significant difference is seen in time to tumor recurrence between those mice treated with brachytherapy compared to those treated only with castration. Although not significant the mice treated after complete tumor regression tended towards increased tumor-free survival. B. The tumor-free survival advantage is lost when distant tumors are considered.
radiation when the tumors fully regressed tended to do better than those mice that received radiation when their tumors were only partially regressed. This is reminiscent of what is seen in human prostate cancer patients with high risk disease who have superior clinical outcomes with hormone and radiation therapy if radiation therapy is delayed until PSA nadir is reached on hormone therapy (1). When considering the distant tumors, there was no difference in tumor-free survival between any of the three treatment regimens (Figure 1B).

Immunological analysis was performed on the serum from all mice in the castration only (Cx) and castration + brachytherapy (Cx+RT) treatment groups. Western blots against purified recombinant PABPN1 protein were performed on serum samples from serial time points including pre-tumor, pre-castration, post-castration, post-radiation (if applicable) and a final sample from the terminal bleed. Positive autoantibody responses against PABPN1 were seen in 7/11 (63.6%) of mice in the Cx only group and 6/10 (60%) of mice in the Cx+RT group. These numbers are in accord with our previous experience in which an average of 55% of mice demonstrated a castration-induced autoantibody response to PABPN1 (2).

We then compared the tumor-free survival of those mice with or without an autoantibody response to PABPN1. In the Cx group, there was a clear survival advantage for the group of mice without an autoantibody response (Figure 2A), replicating the results that were seen previously (2). However, when we compared the tumor-free survival of those mice with or without an autoantibody response in the Cx+RT group, the survival advantage was reversed. That is, the mice with an autoantibody response tended to have a survival advantage over those mice without one (Figure 2B). This is most clearly demonstrated when we compared the tumor-free survival of the distant tumor from mice with autoantibody responses from both treatment groups (Figure 2C), suggesting that the addition of RT into the treatment regimen converts a potentially suppressive immune environment into a more productive immune response.

In addition to the castration + brachytherapy experiments we have continued the CD4 depletion studies in the setting of castration only in order to explore the influence of treatment-associated immune
responses on tumor-free survival more fully. A preliminary CD4 depletion experiment was described in the 2009 Annual Report. In order to improve the logistics of these experiments, we established a breeding colony of DD/S mice at the University of Victoria animal facility, which is affiliated with the Deeley Research Centre. Thus all Shionogi experiments can now occur in Victoria rather than at the Jack Bell Research Centre in Vancouver.

To date, three CD4 depletion experiments have been initiated. In the first experiment, tumors were established in a total of eight mice. Five were designated experimental animals and were injected with 400 µg CD4 depleting antibody (purified from the GK1.5 hybridoma) at -3, -1 and +1 days relative to castration. The remaining three mice were designated controls and were injected on the same schedule with PBS. All mice were castrated once the tumors reached ~8 x 8 mm (length x width). One mouse in the control group died unexpectedly after castration, leaving 2 mice in the control group and the tumor of one CD4 depleted mouse never fully regressed, leaving 4 mice in the depleted group. After castration tumors were measured three times per week and monitored for regression and recurrence. Depleting antibody (200 µg) or PBS was injected weekly post-castration to maintain CD4 depletion. Blood was collected weekly and stored as serum for immunological analysis. PBMCs were also collected weekly to confirm depletion by flow-cytometry. Immunological analysis of these animals was performed by running serum from serial time points against purified PABPN1 by Western blot. Interestingly, none of the animals from either the depleted or control groups had autoantibodies against PABPN1.

A tumor growth curve was plotted for each mouse which demonstrated that, in general, CD4 depleted mice had a delayed time to tumor recurrence with one depleted mouse (#9) showing no signs of recurrence (Figure 3). In fact, mouse #9 also had the most successful depletion with only an average of 0.2% CD4+ T cells remaining (data not shown).

Splenocytes were collected at the time of sacrifice and an ELISPOT assay was performed to determine whether PABPN1-specific T cells were present. A naïve mouse immunized with recombinant PABPN1 protein plus poly I:C as adjuvant was used as a positive control. Results of the ELISPOT assay are presented in Figure 4. Although the assay was very clean with our positive control (splenocytes stimulated with ConA) clearly positive and our negative control (splenocytes stimulated with an irrelevant protein-SH3GLB1) clearly negative, the results of the PABPN1 stimulation are inconclusive. There are several reasons for this. First, CD4 depleting was not complete in all cases (only mouse #9 had virtually complete depletion), therefore the positive result in the depleted mice may stem from the remaining undepleted CD4 T cells. In addition, stimulating the splenocytes with recombinant PABPN1 protein also activates CD8+ T cells, which could account for the positive signal in all mice. To address these issues we plan on repeating the ELISPOT using magnetically sorted CD4+ and CD8+ populations.

Two additional CD4 depletion experiments are currently underway and we expect to have the results of these experiments available in the next 2-3 months. One adjustment we have made in experiment 3 is the timing of CD4 depleting antibody injections in order to improve the efficacy of depletions. We are now giving the first injection of antibody or PBS at the first sign of tumor growth, with additional injections given every 2 days for a total of three before
Figure 4: ELISpot results from the Group 1 CD4 depletion experiment. Splenocytes from CD4 depleted or PBS placebo experimental mice as well as a PABPN1-vaccinated and a naive mouse, were stimulated with whole PABPN1 protein, an irrelevant protein (SH3GLB1) as a negative control or ConA as a positive control. Although we expected the CD4 depleted mice (m2, m5, m9, m16) to have a reduced number of spots (each spot represents a single T cell producing IFN-γ), this was not the case.

castration. We are hopeful that these experiments will help us elucidate the role of autoantibodies and T cells in mice with these immune responses that do poorly after treatment with castration.

With regard to the Statement of Work for Aim 1, we have completed the majority of the proposed experiments. We will finish the CD4 depletion experiments and also repeat the same experiments using the CD8 depleting antibody that we have available. These experiments should be completed by August 2010 and we hope to submit a manuscript describing this work by the end of the year.

Aim 2. To determine in human prostate cancer patients whether treatment-induced autoantibody responses are accompanied by tumor-specific CD4+ and CD8+ T-cell responses.

In order to investigate tumor-specific T cell responses in human prostate cancer, we collected large (200 ml) blood samples from 11 of 16 prostate cancer patients who showed treatment-induced antibody responses against specific antigens, including the 4 patients from whom treatment-induced antigens were cloned. PBMCs were isolated from these patients and frozen for use in T cell assays. The remaining 5 patients agreed to donate a large volume of blood in the future when needed.

To avoid having to map the CD8+ epitopes for each of our antigens, we have established an in vitro transcribed mRNA platform (ivt RNA), in which the antigen of interest is expressed in autologous antigen presenting cells (APCs) which process and display peptides regardless of HLA haplotype. We have successfully cloned three antigens: PARP1 (treatment-induced in patient PC015); PTMA (treatment-induced in patient PC036) and SWAP-70 (treatment-induced in patient PC047). These antigens were transfected into autologous B cells, which acted as APCs to allow in vitro transcription and presentation of peptides. ELISpot assays were then performed to determine whether antigen-specific T cells are present in these patients. The first ELISpot analysis we attempted using the ivt method utilized ex vivo PBMCs from the three patients that demonstrated treatment-induced autoantibody responses against their respective antigen of interest. Although we were hopeful that we would be able to detect antigen-specific T cells ex vivo we realized that these T cells might be present in numbers that would be below the detection limits of the assay. The results of this assay (shown in Figure 5A) confirmed that although the assay was successful (note the high number of CEF-specific T cells in 2 patients) the results of the antigen-specific stimulation were negative, likely due to the low number of these circulating T cells in the periphery. To increase the number of antigen-specific
Figure 5: ELISPOT analysis of patients with treatment-induced autoantibody responses. IFN-γ ELISPOT was used to determine whether patients with an autoantibody response to a particular antigen also had T cell response to the same antigen. Patient PBMCs were stimulated with ivt mRNA of the antigen of interest.

Panel A: ex vivo analysis demonstrates circulating T cells are present at the same level as the negative controls. Panel B: in vitro transfected B cells were used to stimulate PBMCs prior to ELISPOT analysis, resulting in high levels of background.

T cells we performed 2 cycles of in vitro stimulations using autologous B cells transfected with the antigen of interest. Unfortunately, the use of transfected B cells led to a high degree of background, which obscured the results of the assay (Figure 5B). A post-doctoral fellow in the Nelson lab has spent a considerable amount of time trouble-shooting this problem and has learned that using serum-free media helps decrease the background as does the use of T2 cells as APC instead of autologous B cells in HLA-A2+ patients. We will therefore repeat these experiments using the new protocol. In addition we will clone the remaining antigens and perform ELISPOT assays with the remaining patients that demonstrated a treatment-induced autoantibody response.

With regard to the Statement of Work for Aim 2, we will be concentrating our efforts on completing the T cell assays in the human patients. This should be completed by the summer of 2010. We are also planning to perform a 5-year outcome analysis on the entire cohort of patients that was collected beginning in 2004. This will require performing serological analysis including Western blots and SEREX screening and antigen arrays on a total of 143 patients that have pre- and post-treatment serum samples available. We anticipate that the serological analysis will be completed by early 2011 and that the 5 year outcome analysis can be performed in mid 2011, once the majority of patients reach their 5 year post-treatment time point.

**Aim 3. To determine whether tumor-specific autoantibody profiles differ in prostate cancer patients with recurrent versus non-recurrent disease.**

We have continued to collect blood from prostate cancer patients treated ~5 years ago and who have since recurred (n=22) or not recurred (n=50). Recruitment of recurrent patients continues to be a challenge thus we have expanded our search to include patients diagnosed in 2004-2005, as this now represents the 5 year time frame. In addition, the subjects in our initial cohort of 174 prostate cancer patients are approaching the 5 year anniversary since treatment. Thus, these patients may provide an additional resource for this Aim, with the added benefit of being accompanied with pre-treatment blood draws. In fact, we have 6 such patients who
completed their treatment in 2005 and have recurred, increasing the number of recurrent patients available to 28.

**KEY RESEARCH ACCOMPLISHMENTS**

- Castration-induced autoantibody and T cell responses are associated with poor outcomes in the murine Shionogi tumor model (Hahn *et al.*, Int J Cancer 2009; 125:2871-78).

- Castration and brachytherapy-induced autoantibody responses may be associated with superior outcomes in the murine Shionogi tumor model, suggesting that the addition of radiation converts a suppressive immune environment into a beneficial one.

- Troubleshooting of the *in vitro* transcribed mRNA methodology has been completed which will reduce the background following *in vitro* stimulations of PBMCs for patients with treatment-induced immune responses, allowing us to detect low numbers of peripheral antigen-specific T cells using ELISPOT assays.

**REPORTABLE OUTCOMES**

**Manuscripts:**

**Presentations:**
1. Immunotherapy of cancer. Brad H. Nelson. Genome BC Winter Symposium, Jan 27 09, Vancouver BC.

**Funding Applications:**
1. Canadian Institutes of Health Research (CIHR) Operating Grant- Sepember 2009
   **Title:** Standard treatments induce immune responses in prostate cancer: understanding the mechanisms and implications for clinical outcomes
   **Amount Requested:** $155,116/year for 5 years
   **Status:** Unsuccessful

2. Canadian Cancer Society Research Foundation (CCSRI) Operating Grant- October 2009
   **Title:** Standard treatments induce immune responses in prostate cancer: understanding the mechanisms and implications for clinical outcomes
   **Amount Requested:** $149,116/year for 5 years
   **Status:** Unsuccessful

3. Prostate Cancer Canada (PCC) Research Grant- March 2010
   **Title:** Understanding the role of treatment-associated immune responses in prostate cancer
CONCLUSION

Both the murine and human portions of this study are progressing. The Shionogi experiments continue to produce interesting results and we hope to have sufficient data from the CD4 depletion experiments to publish another paper in 2011. The castration + brachytherapy results are provocative and justify additional experiments to determine whether addition of radiation therapy can change the immunologic milieu such that the tumor promoting environment that was created by castration can be converted to a tumor suppressive environment upon the addition of radiation therapy. We hope to recruit a post-doctoral fellow or graduate student to address this question more fully. After considerable troubleshooting to resolve the background problems associated with the ivt mRNA platform, we are confident that the human T cell studies can continue. In addition, we plan to serologically analyze the entire 2004 cohort in order to perform a 5-year outcome analysis and determine in our human prostate cancer patients who underwent standard therapy whether treatment-induced immune responses are associated with clinical outcome.

REFERENCES


APPENDIX

Manuscript: “Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model”

Please see following page for manuscript.
Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model

Sara Hahn1,2, Nancy J. Nesslinger2, Robert J. Drapala3, Mary Bowden3, Paul S. Rennie3, Howard H. Pai4, Charles Ludgate4 and Brad H. Nelson1,2

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Key words: prostate cancer; immune response; tumor antigens; prognosis; autoantibody; T lymphocyte; hormone therapy; tumor recurrence

Prostate cancer is the most frequently diagnosed cancer in North American men and, despite improvements in early detection because of prostate-specific antigen (PSA) screening, it remains the second leading cause of cancer-related death among men.1,2 Standard surgical or radiation therapies (RT) are usually successful in controlling organ confined disease, however if tumors recur, the disease is typically systemic and hormone therapy (HT) remains the only treatment option. Although HT is initially efficacious, patients eventually progress to castration-resistant disease, which is incurable.3 Thus, there is clear need for improved treatments to prevent the development or outgrowth of androgen-independent tumors.

Although both HT and RT mediate direct killing of tumor cells, there is growing evidence that these treatments can also induce tumor-specific immune responses. Roden et al.4 demonstrated that androgen deprivation in tumor-free male mice increased the absolute number of T cells residing in peripheral lymphoid tissues, and also led to transient increases in T cell proliferation in response to T cell receptor stimulation. Moreover, HT induces apoptosis of tumor cells, and the resulting apoptotic bodies can serve as an efficient source of antigen to prime antigen presenting cells (APCs).5 Indeed, Mercader et al.6 demonstrated that HT caused increased levels of APCs expressing the T cell costimulatory molecules B7.1 and B7.2 in human prostate cancer patients, which was accompanied by profuse infiltration of tumor tissue by CD3+ T cells. Similarly, external beam RT induces tumor cell necrosis and apoptosis, which when accompanied with inflammatory or other “danger” signals,7 can potentially provide both antigen and maturation signals to dendritic cells and other APCs.8–12 This in turn leads to the induction of CD4+ and CD8+ T cell responses, which can ultimately elicit an antitumor effect.12 Finally, we have recently shown that HT and RT induce autoantibody responses to a variety of tumor-associated antigens in 25–30% of prostate cancer patients.13 Despite the mounting evidence that HT and RT induce T and B cell responses, it is not yet known whether these immune responses influence clinical outcomes.

The murine Shionogi carcinoma model (SC-115) is a transplantable androgen-dependent tumor that despite being of mammary origin, is used to study the conversion from androgen-dependent to androgen-independent neoplasia.14 Initially, Shionogi tumors are androgen-dependent such that surgical castration precipitates apoptosis and tumor regression in a highly reproducible manner, similar to that seen after androgen withdrawal in human prostate cancer patients. However, similar to human prostate cancer, the androgen-depleted environment gives rise to androgen-independent recurrent tumors in >80% of mice.15–17 Furthermore, Shionogi tumor cells that survive hormone withdrawal, like human prostate tumor cells, up-regulate proteins implicated in cell survival and progression to androgen independence.18 We recently showed that castration induces autoantibody responses to a ~40 kDa antigen in ~50% of Shionogi tumor-bearing mice, which is reminiscent of our findings in human patients, discussed earlier.13 Thus, the Shionogi model provides an experimental system for studying the relationship between treatment-induced immune responses and outcomes. Here, we use this model to test the hypothesis that castration-induced immune responses may be associated with delayed tumor recurrence.

We recently reported that hormone therapy induces antigen-specific autoantibody responses in prostate cancer patients. However, the contribution of autoantibody responses to clinical outcomes is unknown. We used an animal model to test the hypothesis that hormone therapy-induced immune responses may be associated with delayed tumor recurrence. Male DD/S mice bearing established tumors from the androgen-dependent Shionogi carcinoma line were castrated to induce tumor regression. Tumor-specific autoantibody responses were measured by immunoblot, and the underlying antigen was identified by serological screening of a cDNA expression library. T cell responses were assessed by immunohistochemistry and IFN-γ ELISPOT. Following castration, 97% of mice underwent complete tumor regression. Of these, 72% experienced tumor recurrence 18–79 days postcastration, whereas the remaining 28% remained tumor-free for the duration of the experiment. In 55% of mice, castration induced autoantibody responses to an antigen identified as poly(A) binding protein nuclear 1 (PABPN1). Castration also induced PABPN1-specific T cell responses, which were highly correlated to autoantibody responses, and this was accompanied by dense infiltration of tumors by CD3+ T cells 1–2 weeks after castration. Unexpectedly, mice that developed autoantibody and T cell responses to PABPN1 showed a higher rate and shorter latency of tumor recurrence. In mice with recurrent tumors, T cell responses to PABPN1 were still detectable; however, T cell infiltrates were restricted to the peripheral stroma of tumors. In conclusion, castration-induced immune responses are associated with inferior outcomes in the Shionogi carcinoma model, raising concerns about the influence of treatment-induced immune responses on clinical outcomes in humans.

Material and methods

SEREX screening

SEREX screening of a prostate cancer phage cDNA library was carried out as previously described13 using mouse sera diluted 1/400 in TBS/1% BSA. A donkey antimouse IgG alkaline phosphatase-conjugated antibody was used for secondary screening (Jackson ImmunoResearch Laboratories, West Grove, PA).


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Received 30 March 2009; Accepted after revision 16 June 2009 DOI 10.1002/ijc.24673

Published online 24 June 2009 in Wiley InterScience (www.interscience.wiley.com).
Cloning and purification of SEREX-identified antigens

To isolate full-length cDNA clones for antigens identified by SEREX, total RNA was extracted from 1 × 10^7 Shionogi tumor cells using the RNAeasy Mini kit (Qiagen, Mississauga, Canada) and then 0.08 μg total RNA was synthesized into cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen, Burlington, Canada). PCR products were purified using the QiAquick Gel Extraction kit (Invitrogen, Mississauga, Canada), cloned into pENTR™ D-TOPO® (Invitrogen, Burlington, Canada) and transformed into One Shot® TOP10 chemically competent E. coli (Invitrogen, Burlington, Canada). Clones were verified by sequencing before subcloning into the pDEST™ 17 (Invitrogen, Burlington, Canada). Clones were verified by expression vector pDEST™ 17 (Invitrogen, Burlington, Canada). Clones were transformed into BL21-AIT™ cells (Invitrogen, Burlington, Canada), and protein production was induced by addition of arabinose. After 2 hr, bacterial pellets were resuspended in 5 ml of 30 mM dithiothreitol. After 1 freeze-thaw cycle at −80°C, bacteria were sonicated and centrifuged. Supernatants and pellets were analyzed by SDS-PAGE, and fractions containing the most protein were loaded onto a HiTrap immobilized metal ion adsorption chromatography (IMAC) FF nickel column (GE Healthcare, Piscataway, NJ). Proteins were eluted by imidazole gradient. Fractions were pooled and dialyzed against 2 l of phosphate buffered saline (PBS) overnight at 4°C.

Shionogi mouse model

Mice were maintained at the Animal Care Unit of the Jack Bell Centre. All protocols followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Advisory Committee of the University of British Columbia. Adult male DD/S mice were injected subcutaneously in the neck region with 5 × 10^6 Shionogi carcinoma cells. When tumors reached ~8 × 10 mm in size, mice were castrated to induce androgen deprivation and subsequent tumor regression. Serial blood samples were collected from the tail vein before tumor inoculation, before castration and then twice weekly following castration. Tumor size (length × width) was measured using micro calipers. Tumors were considered to have recurred once palpable. Unless otherwise indicated, mice were sacrificed when recurrent tumors reached ~10% of total body weight. On necropsy, terminal blood samples were collected by cardiac puncture. Tumors were removed and divided in 2, which were flash frozen in liquid nitrogen or fixed in 10% formalin. Lymph nodes and spleen were processed into cytoplasmic protein lysate was made from intact Shionogi carcinoma cells. When tumors reached ~0.3 × 10^4, tumors were pressed through a 40-μm pore, Billerica, MA) were prewet with 70% ethanol followed by 3 washes with sterile PBS. Plates were incubated overnight at 4°C with 50 μl/well anti-mouse IFN-γ AN18 (10 μg/ml; Mabtech, Mariemont, OH). After 3 PBS washes, plates were blocked with T cell media (RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin and 25 μM 2-mercaptoethanol) for 2 hr at 37°C. About 3 × 10^5 splenocytes were added to each well. Poly(A) binding protein nuclear 1 (PABPN1) protein was added to a final concentration of 10 μg/ml. ConA was added to a final concentration of 2 μg/ml. T cell media was used as a negative control. Samples were run in triplicate. Plates were incubated for at least 20 hr at 37°C. After washing 6 times with PBS/0.05% Tween-20, 100 μl of biotinylated anti-mouse IFN-γ (diluted to 1 μg/ml in 0.5% BSA/PBS/0.05% Tween-20; Mabtech, R4-6A2, Mariemont, OH) was added to each well. Plates were left for 2 hr at 37°C and then washed 12 times with PBS/0.05% Tween-20. Avidin peroxidase complex (Vector Laboratories, Burlingame, CA) (100 μl/well) was added followed by a 1 hr incubation at room temperature. Plates were washed as above and then developed using the Vectastain AEC substrate kit (Vector Laboratories, Burlingame, CA) for ~5–10 minutes. Development was stopped by rinsing with tap water. Air-dried plates were sent to ZellNet Consulting (Fort Lee, NJ) for enumeration of spots using an automated ELISPOT reader with KS ELISPOT Software 4.9 (Carl Zeiss, Thornwood, NY).

Immunohistochemistry

Formalin-fixed tumors were processed following standard methods and stained with hematoxylin and eosin (H&E). A tissue microarray (TMA) containing all experimental tumors was constructed using duplicate 1 mm cores and stained with mouse monoclonal antibodies against CD3 (Lab Vision, RM9107, Fremont, CA), FoxP3 (eBioscience, 14-5773, San Diego, CA), PAX-5 (Lab Vision, Rb9406, Fremont, CA) and Granzyme B (Abcam, ab4059, Cambridge, MA). Scoring of the TMA was performed independently by 2 individuals who were blinded to the experimental status of tumors. A score of 0 (no infiltration) to 3 (dense infiltration) was assigned to each tumor, and scores were averaged. A score of greater than 1 was considered positive for lymphocyte infiltration.

Results

Castration induces autoantibody responses to PABPN1 in the Shionogi carcinoma model

We previously reported that castration of DD/S mice bearing Shionogi tumors induces IgG autoantibody responses to an unidentified ~40 kDa antigen in ~50% of animals. To facilitate cloning of this antigen by SEREX, we first determined whether the ~40 kDa antigen might have a human homolog, as this would allow use of a previously constructed cDNA expression library derived from human prostate cancer cell lines. To this end, we immunoblotted lysate from the human prostate carcinoma cell line LNCaP with serum from tumor-bearing, castrated mice that were positive for autoantibodies to the ~40 kDa antigen. A strong seroreactive band was seen at ~40 kDa, indicating the antigen seen in Shionogi tumor cells does indeed have a human homolog (data not shown). To identify the antigen, we screened ~2.3 × 10^6 clones of the human prostate cDNA expression library with mouse sera that were positive for autoantibodies to the ~40 kDa antigen. Four serologically reactive antigens were cloned. The corresponding recombinant proteins were immunoblotted with
additional mouse sera that were known to be positive or negative for autoantibodies to the ~40 kDa antigen. The pattern of seroreactivity to 1 antigen, PABPN1, was identical to the pattern of seroreactivity to the ~40 kDa antigen from Shionogi tumor lysates (Fig. 1a), suggesting PABPN1 was the correct antigen.

To confirm that PABPN1 was the ~40 kDa antigen identified in Shionogi tumor lysates, 5 male mice were immunized with recombinant PABPN1, and sera from immunized mice were used to probe Shionogi tumor lysate. The presence of a strong immunoreactive band at ~40 kDa confirms that PABPN1 is the ~40 kDa antigen (Fig. 1b). Western blot showing expression of PABPN1 in Shionogi tumor lysate as well as normal liver, lung and uterine tissues. Varying amounts of protein were loaded, depending on sample availability as follows: 20 µg Shionogi tumor lysate; 100 µg intestine, liver, muscle, liver; 50 µg heart, lung; 33 µg uterus. Serum from a PABPN1-immunized mouse was used as primary antibody. GAPDH served as a loading control. (d) Castration induces a PABPN1-specific T cell response, as measured by IFN-γ ELISPOT. Fresh splenocytes were used in all ELISPOT experiments. Representative data from a single mouse per treatment group is shown. PABPN1-specific T cells were highest in castrated tumor-bearing mice, followed by castrated nontumor-bearing mice. PABPN1-immunized mice served as a positive control, and nontumor-bearing, noncastrated DD/S mice served as a negative control. For each mouse, the sample was run in triplicate to produce an average and standard deviation.

Autoantibody and T cell responses to PABPN1

To establish the time course of autoantibody responses to PABPN1, a cohort of 33 mice bearing established Shionogi tumors were castrated, and serial blood samples were assessed for autoantibody responses to PABPN1 by immunoblotting. Overall, 18/33 mice (54.5%) had an autoantibody response to PABPN1, which appeared an average of 26 days postcastration (range, 6–47 days postcastration). By contrast, autoantibodies to PABPN1 were not found in serum from tumor-bearing, noncastrated mice or castrated nontumor-bearing mice (data not shown).

The fact that autoantibodies to PABPN1 were of the IgG subclass suggested the presence of an underlying T cell response. This was investigated by IFN-γ ELISPOT analysis of splenocytes. As expected, wild-type mice immunized with recombinant PABPN1 showed robust T cell responses to PABPN1, whereas nonimmunized control mice showed no response (Fig. 1d). Of 19 tumor-bearing castrated mice examined, 7 (36.8%) showed a strong T cell response to PABPN1. All of these mice also had an autoantibody response to PABPN1. Conversely, of the 12 mice that were negative for T cell responses to PABPN1, 11 were also negative for autoantibodies to PABPN1. Thus, there was high concordance between autoantibody and T cell responses in tumor-bearing, castrated mice. However, control mice showed a lower concordance between autoantibody and T cell responses. Specifically, nontumor-bearing mice that underwent castration showed modest T cell responses to PABPN1 (Fig. 1d). Thus, castration alone can induce T cell responses to PABPN1, but the effect is enhanced in tumor-bearing mice.

PABPN1 antibody and T cell responses are associated with early tumor recurrence

We next examined the relationship between castration-induced autoantibody responses and tumor recurrence. Following castra-
To investigate whether autoantibodies to PABPN1 promote tumor recurrence or merely serve as a marker of recurrence, we assessed the timing of autoantibody responses relative to tumor recurrence. In 8 of 16 mice whose tumors recurred, the autoantibody response was detected 5 or more days prior to tumor recurrence. However, in 6 of 16 mice, the autoantibody response was not detected until 2 or more days after tumor recurrence, making it unlikely that autoantibodies contributed directly to recurrence. In the remaining 2/16 mice, the autoantibody response was detected within 1 day of tumor recurrence, such that the temporal relationship could not be reliably discerned. Overall, these data are consistent with the notion that autoantibodies are a marker of tumor recurrence rather than an essential mediator of this process.

This finding led us to consider what other features of tumors might correlate with autoantibody status. The average tumor size at castration for those mice that went on to develop an autoantibody response was 82.4 mm² compared with 79.7 mm² for those with no response, suggesting that the size of the primary tumor did not influence subsequent autoantibody development (p = 0.4963, two-tailed unpaired t-test). We then considered the rate of tumor regression after castration. In those mice that developed an autoantibody response, it took an average of 9.7 days for tumors to regress after castration compared with 6.8 days in those mice that did not develop an autoantibody response (p = 0.049, two-tailed unpaired t-test). Although the temporal difference was only 3 days, this nonetheless suggests that slowly regressing tumors may be more likely to trigger autoantibody responses and, ultimately, to recur.

The unexpected finding that PABPN1-specific autoantibody and T cell responses were associated with inferior outcomes raised the issue of whether recurrent Shionogi tumors still expressed the target antigen PABPN1. We evaluated this issue by immunoblotting 15 recurrent tumors for expression of PABPN1. All recurrent tumors expressed PABPN1, and in most cases the level expression was similar to primary tumor (Fig. 3).

**T cell infiltration of Shionogi tumors after castration and upon recurrence**

Because mice with recurrent tumors had substantial PABPN1-specific T cell responses, and tumors still expressed antigen, we next investigated whether T cells trafficked to and infiltrated the tumor site. To this end, a time course experiment was performed in which 50 tumor-bearing mice were castrated and then groups of 10 mice were euthanized on Day 7 or 14 (while tumors were regressing); Day 28 or 35 (while tumors were recurring); or at later time points when recurrent tumors reached ~10% of the body weight. An additional 10 tumor-bearing mice were not castrated. By IHC, tumors from noncastrated mice had very few CD3+ T cells (Fig. 4a). By contrast, tumors from castrated mice had substantial infiltration of CD3+ T cells in tumor epithelium or stroma (Fig. 4a). In contrast, on Days 7 and 14 postcastration, 12/18 tumors were densely infiltrated by CD3+ T cells (Fig. 4a).

**Figure 2** – Castration-induced autoantibody and T cell responses to PABPN1 are associated with tumor recurrence. (a) Kaplan–Meier curve comparing the time to recurrence of tumors in mice with (+) or without (−) autoantibodies to PABPN1. (b) Kaplan–Meier curve comparing the time to recurrence of tumors in mice with (+) or without (−) T cell responses to PABPN1.

**Figure 3** – Recurrent Shionogi tumors retain PABPN1 expression. PABPN1 expression was examined in 15 recurrent Shionogi tumors (labeled with mouse identification numbers such as 26L1) compared with 1 primary tumor (1'). Each lane was loaded with 20 μg tumor lysate and screened with serum from a PABPN1-immunized mouse. GAPDH served as a loading control.
FIGURE 4 – CD3+ T cell infiltration of Shionogi tumors after castration. (a) Anti-CD3 staining of Shionogi tumors shows dense infiltration of CD3+ T cells beginning at Day 7 postcastration and reaching maximal levels at Day 14. By Day 28, when most of the tumors had recurred, CD3+ T cells were sparse (400x). (b) Anti-FoxP3 staining of Shionogi tumors reveals that a minor subpopulation of CD3+ T cells expresses FoxP3 (400x). (c) Representative recurrent tumors from mice sacrificed on Day 56 and Day 90 postcastration. Note that CD3+ T cells are largely restricted to the peripheral stroma of tumors (200x).
tumor-infiltrating cells expressed Granzyme B as assessed by IHC, suggesting a paucity of mature cytolytic effector cells.\textsuperscript{20} Tumors were also examined by IHC for cells expressing FoxP3, a transcription factor expressed by regulatory T (Tregs).\textsuperscript{21} Very few FoxP3\textsuperscript{+} cells were seen in tumors from noncastrated mice. In castrated mice, the number of tumor-infiltrating FoxP3\textsuperscript{+} cells was proportional to the number of CD3\textsuperscript{+} T cells (i.e., about 5–10\%) (Fig. 4b). Finally, tumors from castrated or noncastrated mice contained very few B cells, as assessed by IHC with an antibody to Pax-5 (data not shown).\textsuperscript{22}

In contrast to the regressing tumors described earlier, recurrent tumors contained relatively sparse CD3\textsuperscript{+} T cell infiltrates in tumor epithelium (Fig. 4a–Day 28). Likewise, very few cells expressing FoxP3, Granzyme B or Pax-5 were seen (Fig. 4b–Day 28 and data not shown). However, the peripheral and stromal regions of recurrent tumors showed dense accumulations of CD3\textsuperscript{+} T cells (Fig. 4c). Thus, it appears that recurrent Shionogi tumors may avoid immune rejection by preventing the infiltration of T cells into tumor epithelium.

Discussion

We recently showed that hormone and radiation therapy induce antigen-specific autoantibody responses in a significant proportion of human prostate cancer patients,\textsuperscript{13} however, it is not yet known how these treatment-associated immune responses correlate with clinical outcomes. To address this question experimentally, we utilized the murine Shionogi carcinoma model, which exhibits treatment-induced autoantibody responses similar to those seen in human prostate cancer patients. We hypothesized that treatment-induced autoantibody responses would be associated with delayed time to tumor recurrence and prolonged survival. To investigate this hypothesis, we first cloned PABPN1, the antigen that underlies treatment-induced autoantibody responses in this model. In general, mice that developed PABPN1-specific autoantibodies after castration also developed PABPN1-specific T cell responses. Consistent with our hypothesis, the development of PABPN1-specific autoantibody and T cell responses was associated with more rapid and frequent tumor recurrences. Mice with recurrent tumors retained robust autoantibody and T cell responses to PABPN1, and PABPN1 was still expressed at high levels by tumor cells. Notably, however, recurrent tumors had greatly reduced lymphocytic infiltrates, with CD3\textsuperscript{+} T cells being restricted to peripheral stromal regions. These findings raise concern that treatment-induced immune responses may have a negative impact on clinical outcomes in prostate cancer patients.

In human cancer, the relationship between tumor-associated autoantibodies and clinical outcomes is controversial. Autoantibodies to p53 have been associated with favorable outcomes in some studies\textsuperscript{23,24} but not others.\textsuperscript{25–29} Autoantibodies to other target antigens have been associated with improved prognosis in melanoma, glioblastoma, gastric cancer and breast cancer.\textsuperscript{30–34} Importantly, however, the above studies have examined autoantibodies present at the time of diagnosis, whereas our work in humans and the Shionogi model has focused on autoantibody responses that arise during treatment, an immunological process that remains poorly understood.

Our findings raise the question of whether treatment-induced immune responses might somehow promote tumor recurrence. Indeed, there are several precedents for B cells and T cells promoting tumor formation. For example, B cells were shown to play an essential role in inflammation-induced tumorigenesis in a murine epithelial cancer model.\textsuperscript{35} Likewise, in other murine models, B cells were shown to inhibit T cell-mediated rejection of thymoma, melanoma and colon carcinoma cells.\textsuperscript{36,37} T cells too can have pro-tumorigenic effects. For example, in chronic hepatitis B and C infection, cytotoxic lymphocytes promote hepatocyte damage and fibrosis, which can lead to the development of hepatocellular carcinoma.\textsuperscript{38} Likewise, T cells directed against Helicobacter pylori are thought to promote the development of gastric adenocarcinoma.\textsuperscript{39}

Although the above studies firmly establish a role for B and T cells in promoting primary tumor formation, it remains unclear whether immune responses can promote tumor recurrence after standard treatments. In 6/16 cases from our study, autoantibody responses to PABPN1 appeared after recurrent tumors were detected, which seems incompatible with the notion that autoantibodies to PABPN1 play a causative role in tumor recurrence. An alternative possibility is that autoantibodies are a marker rather than mediator of tumor recurrence. Indeed, the development of autoantibodies to PABPN1 was associated with tumors that regressed more slowly after castration. It may be that a slower rate of tumor regression allows sufficient time for antigen presentation to the immune system, resulting in autoantibody and T cell responses. Slowly regressing tumors may also be less androgen-dependent and hence more likely to recur. Such a model provides a plausible, indirect mechanism linking autoantibodies to tumor recurrence. Future studies will directly test these possibilities by depleting B and T cell subsets from mice and assessing the affect on tumor recurrence.

In addition to autoantibodies, we detected robust T cell responses to PABPN1 by IFN-\gamma ELISPOT. Intriguingly, modest T cell responses to PABPN1 were also seen in tumor-free mice that underwent castration. This might be related to a direct effect of castration on the thymus. In normal individuals, the thymus undergoes age-related atrophy, which results in diminished T cell-dependent antibody formation, generation of cytolytic T cells and T cell responses to antigen stimulation.\textsuperscript{40,41} Castration can reverse thymic atrophy, resulting in rapid restoration of peripheral T cell numbers and function.\textsuperscript{42–44} Thus, castration-induced T cell responses to PABPN1 may in part reflect the generalized homeostatic expansion of lymphocytes in response to androgen deprivation. The fact that even stronger T cell responses to PABPN1 develop in tumor-bearing mice suggests that tumor cell apoptosis further stimulates PABPN1-specific T cells through the release of soluble antigen and/or inflammatory factors. Indeed, in the murine TRAMP model of prostate cancer, castration has been shown to enhance the expansion and function of adoptively transferred tumor-specific CD4\textsuperscript{+} T cells.\textsuperscript{45} Castration-induced T cell responses were accompanied by the rapid and dense infiltration of tumor epithelium by CD4\textsuperscript{+}, CD8\textsuperscript{+} and FoxP3\textsuperscript{+} T cells. Intriguingly, HT of human prostate cancer also promotes T cell infiltration of tumors, although the clinical significance of this observation is not known.\textsuperscript{6} It is noteworthy that in primary prostate tumors collected prior to treatment, the presence of tumor-infiltrating CD4\textsuperscript{+}, CD8\textsuperscript{+} and CD20\textsuperscript{+} lymphocytes is associated with inferior outcomes,\textsuperscript{43} suggesting that, in contrast to many other epithelial cancers, tumor-infiltrating lymphocytes may be a negative prognostic factor in prostate cancer. In this study, we were unable to assess the relationship between T cell infiltration of tumors and subsequent outcomes, because mice had to be euthanized on Days 7–14 in order for T cell infiltration to be assessed. However, when recurrent tumors were analyzed, T cells no longer infiltrated tumor epithelium, but rather were restricted to peripheral stromal regions. Thus, while Shionogi tumors are initially permissive to T cell infiltration, recurrent tumors appear to develop lymphocyte infiltration barriers. This suggests that the T cell response triggered by castration may create selective pressure for the development of immunologically resistant tumors upon recurrence. It will be interesting to assess whether recurrent human prostate tumors are similarly devoid of T cells.

PABPN1 is a ubiquitously expressed protein that is involved in the polyadenylation of mRNA in eukaryotes. Although PABPN1 has not been directly implicated in cancer, aberrant expansion of the trinucleotide repeat in a polyalanine tract of the PABPN1 gene causes oculopharyngeal muscular dystrophy, an autosomal dominant inherited disorder in humans.\textsuperscript{46} The role of PABPN1 in transcription may explain its high level of expression in Shionogi
tumor cells.\textsuperscript{15} It may seem counterintuitive that a widely expressed protein such as PABPN1 would be a target antigen of castration-induced immune responses in the Shionogi model. However, Savage et al.\textsuperscript{26} described a naturally arising CD8+ T cell response against a peptide derived from histone H4, a ubiquitously expressed protein, in the murine TRAMP prostate cancer model. Moreover, in our study of treatment-induced autoantibody responses in human prostate cancer patients, many of the underlying antigens were widely expressed proteins.\textsuperscript{13} Thus, treatment-induced immune responses are not necessarily directed against tumor-specific proteins, but might instead involve a breakdown of peripheral tolerance to widely expressed self proteins, as occurs in many autoimmune conditions.

How relevant is this study to human prostate cancer? A priori, there is no reason to believe that PABPN1 itself is an important target antigen in human prostate cancer. Indeed, we tested serum samples from 14 patients by Western blot for the presence of autoantibodies to recombinant PABPN1; only 1 of 14 patients demonstrated autoantibodies to PABPN1, and the response was detected in both pre- and post-HT serum samples (data not shown). This fits with the general finding that human prostate cancer patients recognize a large repertoire of tumor antigens that differ widely between patients.\textsuperscript{13} Rather than the specific target antigen being relevant, we believe this study raises important, fundamental questions about the impact of treatment-induced immune responses on clinical outcomes. It is a clinical reality that hormone and radiation therapy induce autoantibody responses in 20–30% of prostate cancer patients.\textsuperscript{12} If the results from the Shionogi tumor model translate to humans, then treatment-induced autoantibody responses may portend early recurrence. We are investigating this issue in our human prostate cancer cohort as long-term outcomes data becomes available.\textsuperscript{13} If treatment-induced immune responses do indeed show an association with inferior outcomes in humans, this may present an opportunity to improve standard clinical practice. For example, it may prove beneficial to transiently suppress the immune system during hormone and radiation therapy to avoid generating maladaptive immune responses. Alternatively, it may be possible to skew immune responses toward a beneficial effector phenotype by treating patients with immune stimulatory cytokines or other agents as they undergo standard treatments.

Acknowledgements

The authors thank Mr. Alvin Ng and Dr. John Webb for technical assistance and committee members Dr. Terry Pearson, Dr. Perry Howard and Dr. Rob Ingham as well as members of the Deely Research Centre for helpful discussions.

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26. Nelson C, Akakura K, Goldberg SL, Otal N, Akakura S, Wong P, Tenniswood M. Effect of tumour progression on the correlations data becomes available. If treatment-induced immune responses may indeed show an association with inferior outcomes in humans, this may present an opportunity to improve standard clinical practice. For example, it may prove beneficial to transiently suppress the immune system during hormone and radiation therapy to avoid generating maladaptive immune responses. Alternatively, it may be possible to skew immune responses toward a beneficial effector phenotype by treating patients with immune stimulatory cytokines or other agents as they undergo standard treatments.


