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13. SUPPLEMENTARY NOTES

14. ABSTRACT We had previously demonstrated that hormone therapy (HT) and radiation therapy (RT) induce tumor-specific autoantibody responses in human prostate cancer, and this project investigated the clinical significance of these findings. In Aim 1, we showed that HT induces autoantibody and T cell responses against an antigen called PABPN1 in a mouse tumor model and that these immune responses are associated with inferior outcomes. We also showed that the combination of HT+RT in this model leads to delayed tumor recurrence of a distal untreated tumor. In Aim 2, we developed methods to test prostate cancer tumor antigens for recognition by CD8+ and CD4+ T cells from patients, and we are now in a position to test recognition of serologically-defined tumor antigens associated with treatment-induced autoantibody responses. In Aim 3, we have almost completed assembly of prostate cancer patient cohorts with recurrent versus non-recurrent disease at 5 years post-treatment. In a collaborative project, we showed that prostate cancer vaccines frequently induce seemingly deleterious autoantibody responses in prostate cancer patients. In summary, our results indicate that the immune system has a profound influence on the efficacy of standard treatments, as well as experimental vaccines, in prostate cancer patients. With further understanding, it should be possible to steer the immune response in favor of improved clinical outcomes.

15. SUBJECT TERMS
Tumor immunology, immunotherapy, prostate cancer, antibody, T cell, tumor antigen, hormone therapy, radiation therapy

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Table of Contents

W81XWH-07-1-0259 Final Report, April 2011

PI: Brad H. Nelson, Ph.D.

Title of Project: Exploiting the Immunological Effects of Standard Treatments in Prostate Cancer

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. In our original application, we presented preliminary results in the androgen-dependent murine Shionogi tumor model indicating that castration (the laboratory equivalent of HT) induces tumor-specific autoantibody responses in approximately 50% of animals. Moreover, we showed in parallel studies of human prostate cancer patients undergoing standard treatments at our institution 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 had 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.

Development of autoantibodies to PABPN1 after castration is associated with inferior outcome in the Shionogi mouse model

We had previously shown that castration of mice bearing Shionogi tumors

induce an antibody response against a ~40kd antigen in about 50% of animals (Nesslinger et. al. *Clin. Can. Res.* 13:1493). During the funding period of this grant, SEREX screening was performed and lead to the successful identification of the ~40kd antigen as Poly A Binding Protein (PABPN1). Western blotting revealed that PABPN1 is widely expressed in various normal tissues, and is expressed at slightly higher levels in Shionogi tumors. In this regard, PABPN1 is typical of many serologically defined human tumor antigens, in that it exhibits a fairly widespread expression pattern. Antibodies to PABPN1 arose in 55% of tumor-bearing mice within ~26 days of castration (Figure 1). Interferon-gamma ELISPOT analysis revealed the presence of CD4+ and CD8+ T cell response to PABPN1 in castrated, tumor-bearing mice. This was accompanied by dense infiltration of Shionogi tumors by CD4+ and CD8+ T cells within 1-2 weeks of castration (Figure 2). Thus, it appears that the process of tumor regression after castration induces both T cell and antibody responses against PABPN1 in the majority of mice.

In the Shionogi tumor model, the vast majority of castrated mice experience tumor recurrence within 2-3 weeks of castration. Contrary to our hypothesis, we found that those mice that developed antibody responses to PABPN1 were more likely to experience tumor recurrence (Figure 3). Moreover, tumor recurrence tended to occur more rapidly in mice with antibodies to PABPN1. Mice with recurrent tumors had robust systemic T cell responses to PABPN1, as measured by interferon-gamma ELISPOT analysis of splenocytes (Figure 4). However, histological examination revealed that T cells were invariably restricted to the peripheral stroma of recurrent tumors and failed to penetrate the malignant epithelium (Figure 5). 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 was published in the 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.

In summary, the work performed in the first part of Aim 1 demonstrated that treatment-induced immune responses are ineffective and possibly even detrimental with respect to tumor recurrence in the Shionogi tumor model. We believe these findings may have profound implications for those human prostate cancer patients who mount tumor-associated antibody responses during treatment (Nesslinger et. al. *Clin. Can. Res.* 13:1493). It may prove effective to inhibit rather than enhance such responses. Alternatively, it may be possible to use immunomodulatory cytokines to convert these immune responses to a cytolytic, tumor-destructive phenotype.

Depletion of CD4+ T cells may delay tumor recurrence in the Shionogi mouse model

Given the above findings, we went on to test the revised hypothesis that tumor-associated T cell and B cell responses may promote rather than inhibit tumor recurrence. To test this hypothesis, we attempted to deplete CD4+ T cells prior to castration. A total of 15 mice were used in the first experiment. Seven mice served as controls and were injected with PBS at -3, -1, +1 days relative to castration. Eight mice were injected with 400 μg of anti-CD4 antibody (clone GK1.5) at -3, -1, +3 days relative to castration. An additional 200 μg of depleting antibody or PBS was injected weekly post castration. All mice were castrated when tumors reached 65-100 mm^2. Flow cytometry on PBMCs from a subset of mice was performed to determine the efficacy of anti-CD4 depletion (Figure 6). PBMCs were stained with anti-CD3 to identify the T cell population (first panel, Figure 6 A-D) and with anti-CD4 (second panel, Figure 6 A-D). Results clearly show that the mice injected with PBS had healthy CD4+ T cell populations (~63% of the CD3+ population), whereas the CD4 depleted mice had virtually no CD4+ T cells remaining (<1% of CD3+ T cells). We then compared the tumor growth between the PBS and CD4 depleted mice and found that there was a significant difference in the rate of

recurrence between the two groups of mice in favor of the CD4-depleted mice (Figure 7). These results were confirmed in three additional experiments using small numbers of animals. In order to publish these results, we are performing several more large-scale experiments to increase the sample size. If the results are confirmed, this would provide strong support for our revised hypothesis that tumor-associated T cell and B cell responses promote rather than inhibit tumor recurrence.

The combination of castration and radiation therapy may lead to a protective immune response in the Shionogi model

In parallel studies, we investigated whether the combination of castration and radiation therapy (in the form of brachytherapy) might induce a more beneficial immune response than castration alone in the Shionogi model. More specifically, we sought to investigate the abscopal effect, in which reduced tumour growth is observed outside of the field of radiation. It is thought that an immune-mediated mechanism is responsible for the abscopal effect.

We first established that the number of I-125 radioactive seeds required to give optimal control of the primary tumor was 6, therefore this is the number of seeds was used for all subsequent experiments. Five experimental groups were established as follows:

Group ID	**Description**	**# Mice**
No Tx	No treatment	7
RT	six I-125 seeds only	7
Cx	Castration only	13
Cx+RT	Castration → Max. Regression → six I-125 seeds	13
Cx+RT@1/2	Castration → Partial Regression → six I-125 seeds	13

Cohorts of mice were implanted with two Shionogi tumors (one tumor per flank). No treatment was given in the No Tx group. In the RT group, 6 seeds were implanted in one tumor only once the tumor reached 65-100 mm^2. In the remaining groups, mice were castrated when tumors reached 65-100 mm^2. If the mice went on to receive radiation, seeds were implanted in only one (the primary) tumor at the point the tumor maximally regressed (Group Cx+RT) or when the tumor partially regressed (Group Cx+RT@1/2). All mice were monitored for primary and distal tumor recurrence.

As expected, both primary and distal tumors in the No Tx group grew unchecked, whereas growth of primary tumors in the RT group was controlled. Distal tumors in the RT group grew at a rate similar to that seen in the No Tx group, indicating that no abscopal response was produced by RT alone in this setting. In the remaining groups, treated with castration alone or castration and radiation, radiation of the primary tumor had a clear effect on the rate of primary tumor growth, as expected (Figure 8A). Using Kaplan-Meier analysis we then compared the tumor-free survival between those three experimental groups (Figure 8B). As expected, when comparing primary tumor recurrence, addition of radiation leads to significant delays in tumor recurrence (Cx+RT vs Cx, $p<0.001$; Cx+RT@1/2 vs Cx, $p=0.0009$). When comparing distal tumor recurrence, evidence of an abscopal effect was seen in those mice who received radiation when their tumors regressed maximally after castration versus

those that received only castration (Cx+RT vs Cx, p=0.0096) (Figure 8B). Interestingly, this abscopal effect was not seen in mice radiated when tumors only partially regressed after castration (Cx+RT@1/2 vs Cx, p=0.54), underscoring the importance of maximal tumor regression after castration. This is reminiscent of what is seen in the clinic with high-risk patients treated with HT+RT (Ludgate et al. Intl Journ. Of Rad Onc., Biol., phys. 2005, 62: 1309-15), where the best results are achieved if RT is given only after PSA nadir has been reached with HT.

The second set of castration+RT experiments included the use of the Flt3 ligand, as originally proposed. Flt3 ligand is a potent stimulator of dendritic cells (DCs) and is thought to be an important mediator of anti-tumour immune responses such as those needed for an abscopal effect. The hypothesis with this set of experiments was that administration of Flt3 ligand would enhance the immune-stimulatory effects of combined neo-adjuvant androgen withdrawal and brachytherapy (HT+BT) in mice to prevent or delay re-growth of the not only the primary irradiated tumour, but also the distal, non-irradiated tumour through an abscopal effect. To test the hypothesis, 4 treatment groups were established as follows:

Group ID	**Description**	**# Mice**
Cx	Castration only	13
Cx+RT	Castration → Max. Regression → six I-125 seeds	13
Cx+Flt3L	Castration → Max. Regression → Flt3 ligand *	7
Cx+RT+Flt3L	Castration → Max. Regression → six I-125 seeds → Flt3 ligand **	19

* Flt3 ligand was injected once a day for 10 consecutive days 1 day following maximal regression of tumours
** Flt3 ligand was injected day 1 following implantation of I-125 seeds, for 10 consecutive days

Cohorts of mice were implanted with two Shionogi tumors (one tumor per flank). When tumors reached 65-100 mm^2, mice were castrated to induce regression of both tumors. When tumors had fully regressed, additional treatment was given as described above. In mice given brachytherapy, six seeds were implanted in one flank only at the site of the regressed tumor (primary tumor). All mice were monitored for recurrence of both the primary and distal tumors.

To confirm *in vivo* DC expansion, we performed flow cytometry analysis on blood samples taken from the different groups at time points during and post administration of Flt3 ligand and stained for mAb against dendritic cells (FITC-anti-CD11b and PE-anti-CD11c). As expected, we observed an expansion of dendritic cells in mice treated with Flt3 ligand compared to mice treated with castration only (13% vs. 3% of $CD11c^+$ cells, 59% vs 29% $CD11b^+$ cells).

We then compared the tumor-free survival of both the primary and distal tumor between the different treatment groups (Figure 9). Figure 9A demonstrates a direct anti-tumour response against the primary tumour to brachytherapy, Flt3 ligand and the combined treatment compared to castration alone, however there was no additional survival advantage using the combination treatment compared to brachytherapy alone. Figure 9B also demonstrates a slight anti-tumour effect against the distal tumor using I-125 seeds (graph Cx vs Cx+RT), supporting an abscopal response with therapeutic radiation. However, the combination of I-125 radiation and Flt3 ligand failed to demonstrate an abscopal response or enhancement of abscopal response. This may be due to the timing of administration of Flt3 ligand with respect to I-125 implantation. In future,

additional experiments will be performed which will include a change in timing of Flt3 ligand administration to what we believe would be the time at which maximal DC expansion would occur, followed shortly by exposure to therapeutic radiation using I-125 radioisotopes. These experiments have been delayed due to difficulty obtaining more Flt3 ligand from the manufacturer (Amgen).

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 10A), 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 10B). 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 10C), suggesting that the addition of RT into the treatment regimen may convert a suppressive immune environment into a more productive immune response. This exciting result will be further investigated in future experiments.

With regard to the Statement of Work for Aim 1, we have completed the majority of the proposed experiments. We intend to finish the CD4 depletion experiments and also repeat the same experiments using the CD8 depleting antibody that we have available. We are applying for additional grant funding to complete these experiments. We believe the promising preliminary data obtained with DOD support will help ensure the success of this effort.

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 (Nesslinger et al., *Clinical Cancer Research*, 2007). 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 ensure that the ELISPOT methodology was working we tested five of our HLA-A2+ patients with HLA-A2-restricted peptides from three well-known prostate cancer antigens, including PSA, PSCA and NY-ESO-1. We included 2 positive controls, PHA, which should stimulate T cells non-specifically, and CEF peptide, a pool of peptides from 3 different common viruses that most people have had exposure to. The results showed that of the 5 patients tested, none had T cells specific to the PSA, PSCA or NY-ESO-1 peptides. This is not unexpected as the frequency of T cells against these antigens is low amongst prostate cancer patients in general (Figure 11). Three patients were positive for T cells against the CEF peptide

and both patients stimulated with PHA were also positive, indicating that the assay is working in our hands.

To avoid having to map the CD8+ epitopes for each of our antigens, we 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 successfully cloned three antigens into the ivtRNA vector: 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 ivtRNA 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 12A) 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 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 12B). 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 are concentrating our efforts on completing the T cell assays as described above now that the major technical hurdles appear to have been resolved. We are also planning to perform a 5-year outcome analysis on the entire cohort of patients that was collected beginning in 2004 (Nesslinger et al., *Clinical Cancer Research*, 2007). 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 and 5-year outcome analysis will be completed in 2012.

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 2004-2006, as this now falls within the 5-year time frame. In addition, most of the subjects in our initial cohort of 174 prostate cancer patients have reached 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 a side project that fell within the conceptual theme of this aim, we investigated the relationship between tumor-specific autoantibodies and tumor recurrence in prostate cancer

patients who received a viral vaccine intended to trigger an immune response against their tumor. For this, we teamed up with Drs. Jeffrey Schlom and James Gulley at the US NCI. Their group had previously reported a randomized phase II clinical trial combining a poxvirus-based vaccine encoding prostate-specific antigen (PSA) with radiotherapy in patients with localized prostate cancer. We investigated whether vaccination against PSA induced immune responses to additional tumor-associated antigens and how this influenced clinical outcome. Pretreatment and posttreatment serum samples from patients treated with vaccine + external beam radiation therapy (EBRT) versus EBRT alone were evaluated by Western blot and serologic screening of a prostate cancer cDNA expression library (SEREX) to assess the development of treatment-associated autoantibody responses. Western blotting revealed treatment-associated autoantibody responses in 15 of 33 (45.5%) patients treated with vaccine + EBRT versus 1 of 8 (12.5%) treated with EBRT alone. SEREX screening identified 18 antigens, which were assembled on an antigen array with 16 previously identified antigens. Antigen array screening revealed that 7 of 33 patients (21.2%) treated with vaccine + EBRT showed a vaccine-associated autoantibody response to four ubiquitously expressed self-antigens: DIRC2, NDUFS1, MRFAP1, and MATN2. These responses were not seen in patients treated with EBRT alone, or other control groups. Patients with autoantibody responses to this panel of antigens had a trend toward decreased biochemical-free survival. In conclusion, we found that Vaccine + EBRT induced antigen spreading in a large proportion of patients. A subset of patients developed autoantibodies to a panel of four self-antigens and showed a trend toward inferior outcomes. Thus, cancer vaccines directed against tumor-specific antigens can trigger autoantibody responses to self-proteins, which may influence the efficacy of vaccination. These findings were published last year (Nesslinger et al. Clin Cancer Res 2010, 16:4046).

With regard to the Statement of Work for Aim 3, we did not meet our milestones due to unanticipated difficulty recruiting patients with recurrent disease. However, as described above, we have a solid plan for circumventing this problem over the next 1-2 years. In the meantime, we discovered that cancer vaccines can sometimes induce seemingly deleterious autoantibody responses, which may explain why vaccination is ineffective in many patients.

KEY RESEARCH ACCOMPLISHMENTS

- Treatment-induced autoantibody responses are seen in approximately ~30% of prostate cancer patients undergoing hormone therapy and/or radiation therapy (Nesslinger et al. Clin Cancer Res 2007;13:1493-1502).

- 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).

- The combination of castration and brachytherapy led to a delay in tumor recurrence, evidence of an abscopal response, in the murine Shionogi tumor model. 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.

- Cancer vaccines directed against tumor-specific antigens can trigger autoantibody responses to self-proteins, which may impair the efficacy of vaccination (Nesslinger et al. Clin Cancer Res 2010, 16:4046).

REPORTABLE OUTCOMES

Manuscripts (direct relevance):

1. Nesslinger, N.J., Sahota, R.A., Stone, B., Johnson, K., Chima, N., King, C., Rasmussen, D., Bishop, D., Rennie, P.S., Gleave, M., Blood, P., Pai, H., Ludgate, C., **Nelson, B.H.** 2007. Standard treatments induce antigen-specific immune responses in prostate cancer. *Clin Cancer Res.* Mar 1; 13(5):1493-502. PMID: 17332294.
2. Nesslinger, N.J., Pai, H.H., Ludgate, C.M., **Nelson, B.H.** 2008. Exploring the effects of standard treatments on the immune response to prostate cancer. *Methods of cancer diagnosis, therapy, and prognosis*. *Vol. 2*, Springer.
3. Hahn, S., Nesslinger, N.J., Drapala, R., Bowden, M., Rennie, P., Pai, H.H., Ludgate, C., **Nelson, B.H.** 2009. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. *Int J Cancer. Dec 15; 125(12):2871-8.* PMID: 19554630.
4. Nielsen, J.S., Wick, D.A., Tran E., **Nelson, B.H.** and Webb, J.R. 2010. An in vitro-transcribed-mRNA polyepitope construct encoding 32 distinct HLA class I-restricted epitopes from CMV, EBV, and Influenza for use as a functional control in human immune monitoring studies. *J Immunol Methods*. Aug 31; 360(1-2):149-56. Epub 2010 Jul. PMID: 20637775.
5. Nesslinger, N.J., Ng, A., Tsang, K-Y., Ferrara T., Schlom, J., Gulley, and **Nelson, B.H.** 2010. A viral vaccine encoding prostate-specific antigen induces antigen spreading to a common set of self proteins in prostate cancer patients. *Clin Cancer Res.* Aug 1;16(15):4046-56. Epub 2010 Jun 18. PMID: 20562209.

Manuscripts (conceptually related):

1. Milne, K., Barnes, R.O., Girardin, A., Mawer, M.A., Nesslinger, N.J., Ng, A., Nielsen, J.S., Sahota, R., Tran, E., Webb, J.R., Wong, M.Q., Wick., D.A., Wray, A., McMurtrie, E., Köbel, M., Kalloger, S.E., Gilks, B., Watson, P.H. , **Nelson, B.H.** 2008. Tumor-infiltrating T cells correlate with NY-ESO-1-specific autoantibodies in ovarian cancer. *PLoS One*. Oct 8; 3(10):e3409. PMID: 18923710.
2. Tran, E., Nielsen, J.S., Wick, D.A., Ng, A.V., Nesslinger, N.J., McMurtrie, E., Webb, J.R., **Nelson, B.H**., 2010. Polyfunctional T-cell responses are disrupted by the ovarian cancer ascites environment and only partially restored by clinically relevant cytokines. PLoS ONE 5(12): e15625.
3. **Nelson, B.H.** 2010. CD20+ B cells: the other tumor-infiltrating lymphocytes. *J Immunol.* Nov 1;185(9):4977-82. PMID: 20962266.
4. **Nelson, B.H.** and Webb, J.R. 2011. Tumor-specific mutations as targets for cancer immunotherapy. Chapter 7 (pgs. 151-172) in *Experimental and Applied Immunotherapy,* J. Medin and D. Fowler (eds.). Springer. ISBN: 978-1-60761-979-6.

5. Nielsen, J.S., Sahota, R.A., Nesslinger, N.J., Milne, K., Ng, A.V., Watson, P.H., **Nelson, B.H**. 2011. Tumour-infiltrating B cells promote favourable prognosis in ovarian cancer through cellular rather than humoral mechanisms. *In preparation.*
6. Nesslinger, N., Nielsen, J., Ng, A., Sahota, R. Brandon, J., Chalk, S., Milne, K., Smyth, L., Mulens, V., **Nelson, B.H.** 2011. Standard treatments for EOC induce antigen-specific antibody responses: relationship to tumor-infiltrating B cells. *In preparation*.

Presentations:

1. Standard Treatments Induce Antigen-Specific Immune Responses in Prostate Cancer. **Brad H. Nelson.** Prostate Cancer Research Foundation of Canada Retreat, Orangeville, ON, January 2007.

2. Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. Dept. of Immunology, University of Minnesota, Minneapolis MN, April 2007.

3. Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. UBC Medical Genetics, Vancouver BC, May 2007.

4. Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. Canadian Melanoma Conference, Banff AB, Oct 2007.

5. Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. UBC Immunology Department Seminar, Vancouver BC, Oct. 2007.

6. Tracking and Manipulating the Immune Response to Cancer. **Brad H. Nelson**. City of Hope Medical Center, Duarte CA, Mar. 2008.

7. Mapping and Manipulating the Immune Response to Cancer. **Brad H. Nelson.** City of Hope Medical Center, Duarte CA, March 2008.
8. Mapping and Manipulating the Immune Response to Cancer. **Brad H. Nelson**. University of Calgary, Calgary AB, April 2008.
9. Hormone therapy induces antigen-specific antibody and T cell responses in the Shionogi mouse tumour model. Sara Hahn, Nancy Nesslinger, Rob Drapala, Mary Bowden, Paul Rennie, Howard Pai, Charles Ludgate, **Brad H. Nelson**. Annual Meeting of the American Association for Cancer Research, San Diego, CA, April 2008.

10. Mapping and Manipulating the Immune Response to Cancer. **Brad H. Nelson**. MD Anderson Cancer Center, Houston TX, May 2008.

11. Toward personalized immunotherapy of cancer. **Brad H. Nelson.** Canadian Cancer Immunotherapy Workshop 2008, Toronto ON, Nov 2008.

12. Immunotherapy of cancer. **Brad H. Nelson**. Genome BC Winter Symposium, Jan 27 09, Vancouver BC.
13. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Centre Hospitalier de l'Universite de Montreal, Montreal PQ, July 8 2009.

14. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby BC Sept. 18 2009.
15. Toward effective immunotherapy of ovarian cancer. **Brad H. Nelson**. 1st Translational Cancer Immunology Conference, Havana, Cuba, Nov 2009.
16. Immune response to prostate cancer. **Brad H. Nelson**. Centre for Genetic Engineering and Biotechnology, Camaguey, Cuba, Nov 23 2009.
17. A novel gene signature associated with response to adoptive T cell therapy in a mouse model of breast cancer. **Brad H. Nelson**. Canadian Society for Immunology Annual Conference, Niagara Falls ON April 2010.
18. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Dept. of Biochemistry, University of Victoria, Victoria BC Sept 2010.
19. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Benaroya Research Institute, Seattle WA Oct 27 2010.
20. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Ontario Health Research Institute, Ottawa ON, Nov 4 2010.
21. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Oxford Biomedica, Oxford UK, Nov 29 2010.
22. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy, Dec 6 2010.
23. Anti-Tumour Immunity: Impact of host immunity on cancer outcomes. **Brad H. Nelson**. CIHR Immunomodulation of Cancer Workshop, Vancouver BC, Mar 11 2011.
24. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. Universität Rostock, Rostock, Germany, Mar 21 2011.
25. Toward personalized immunotherapy of cancer. **Brad H. Nelson**. University Medical Centre Groningen, Groningen, The Netherlands, Mar 22 2011.

Degrees Obtained:

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Master of Science, Department of Biochemistry and Microbiology, University of Victoria
Thesis title: “The influence of host immunity on outcomes following hormone therapy for cancer”
Degree awarded: June 2008

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CONCLUSION

This project started with an original clinical observation: standard treatments for prostate cancer (specifically hormone and radiation therapy) induce autoantibody responses to tumor-associated antigens in a significant proportion of patients (25-30%). With support from the DOD, we have now shown in a murine model that these responses, rather than being protective,

appear to promote tumor recurrence. We also showed that this effect can be reversed by depleting CD4+ T cells from the host. Of greater clinical relevance, we also obtained preliminary data indicating that the combination of hormone and radiation therapy may induce protective immune responses. Overall, our results indicate that the immune system has a profound influence on the efficacy of standard treatments for prostate cancer. With further understanding, it should be possible to steer the immune response in favor of improved clinical outcomes. With several peer-reviewed publications now in place, and a unique patient cohort with serial serum specimens and associated clinical data, we are in a strong position to seek additional funding to continue this important work.

REFERENCES

Hahn, S., Nesslinger, N.J., Drapala, R., Bowden, M., Rennie, P., Pai, H.H., Ludgate, C., **Nelson, B.H.** 2009. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. *Int J Cancer. Dec 15; 125(12):2871-8.* PMID: 19554630.

Nesslinger, N.J., Sahota, R.A., Stone, B., Johnson, K., Chima, N., King, C., Rasmussen, D., Bishop, D., Rennie, P.S., Gleave, M., Blood, P., Pai, H., Ludgate, C., **Nelson, B.H.** 2007. Standard treatments induce antigen-specific immune responses in prostate cancer. *Clin Cancer Res.* Mar 1; 13(5):1493-502. PMID: 17332294.

Ludgate CM, Bishop DC, Pai H*, et al.* Neoadjuvant hormone therapy and external-beam radiation for localized high-risk prostate cancer: the importance of PSA nadir before radiation. International journal of radiation oncology, biology, physics 2005;62: 1309-15.

Nesslinger, N.J., Ng, A., Tsang, K-Y., Ferrara T., Schlom, J., Gulley, and **Nelson, B.H.** 2010. A viral vaccine encoding prostate-specific antigen induces antigen spreading to a common set of self proteins in prostate cancer patients. *Clin Cancer Res.* Aug 1;16(15):4046-56. Epub 2010 Jun 18. PMID: 20562209.

APPENDIX

Manuscripts:

1. Nesslinger, N.J., Sahota, R.A., Stone, B., Johnson, K., Chima, N., King, C., Rasmussen, D., Bishop, D., Rennie, P.S., Gleave, M., Blood, P., Pai, H., Ludgate, C., **Nelson, B.H.** 2007. Standard treatments induce antigen-specific immune responses in prostate cancer. *Clin Cancer Res.* Mar 1; 13(5):1493-502. PMID: 17332294.
2. Hahn, S., Nesslinger, N.J., Drapala, R., Bowden, M., Rennie, P., Pai, H.H., Ludgate, C., **Nelson, B.H.** 2009. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. *Int J Cancer. Dec 15; 125(12):2871-8.* PMID: 19554630.
3. Nesslinger, N.J., Ng, A., Tsang, K-Y., Ferrara T., Schlom, J., Gulley, and **Nelson, B.H.** 2010. A viral vaccine encoding prostate-specific antigen induces antigen spreading to a common set of self proteins in prostate cancer patients. *Clin Cancer Res.* Aug 1;16(15):4046-56. Epub 2010 Jun 18. PMID: 20562209.

Standard Treatments Induce Antigen-Specific Immune Responses in Prostate Cancer

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Abstract

Purpose: Prostate tumors express antigens that are recognized by the immune system in a significant proportion of patients; however, little is known about the effect of standard treatments on tumor-specific immunity. Radiation therapy induces expression of inflammatory and immune-stimulatory molecules, and neoadjuvant hormone therapy causes prominent T-cell infiltration of prostate tumors. We therefore hypothesized that radiation therapy and hormone therapy may initiate tumor-specific immune responses.

Experimental Design: Pretreatment and posttreatment serum samples from 73 men with nonmetastatic prostate cancer and 50 cancer-free controls were evaluated by Western blotting and SEREX (serological identification of antigens by recombinant cDNA expression cloning) antigen arrays to examine whether autoantibody responses to tumor proteins arose during the course of standard treatment.

Results: Western blotting revealed the development of treatment-associated autoantibody responses in patients undergoing neoadjuvant hormone therapy (7 of 24, 29.2%), external beam radiation therapy (4 of 29, 13.8%), and brachytherapy (5 of 20, 25%), compared with 0 of 14 patients undergoing radical prostatectomy and 2 of 36 (5.6%) controls. Responses were seen within 4 to 9 months of initiation of treatment and were equally prevalent across different disease risk groups. Similarly, in the murine Shionogi tumor model, hormone therapy induced tumor-associated autoantibody responses in 5 of 10 animals. In four patients, SEREX immunoscreening of a prostate cancer cDNA expression library identified several antigens recognized by treatment-associated autoantibodies, including PARP1, ZNF707 + PTMA, CEP78, SDCCAG1, and ODF2.

Conclusion: We show for the first time that standard treatments induce antigen-specific immune responses in prostate cancer patients. Thus, immunologic mechanisms may contribute to clinical outcomes after hormone and radiation therapy, an effect that could potentially be exploited as a practical, personalized form of immunotherapy.

Prostate tumors are recognized by the immune system in a significant proportion of patients, as evidenced by serum antibody responses to prostate-specific antigen (PSA), prostatic acid phosphatase, p53, and HER2/neu (1). In addition, many prostate tumors contain significant numbers of tumor-infiltrating lymphocytes, which are associated with favorable outcomes in prostate cancer and other cancers (2–10). In an attempt to enhance the immune response to prostate cancer, clinical vaccine trials have been conducted targeting such antigens as PSA (11, 12), prostate-specific membrane antigen (13), and prostatic acid phosphatase (14). For example, Gulley et al. (15) conducted a phase II clinical trial combining a poxvirus vaccine encoding PSA with external beam radiation therapy (EBRT) and showed significant increases in PSA-specific T cells in the majority of patients, along with emergent responses to antigens not present in the vaccine, providing evidence of antigen spreading. A second example is Provenge (APC8015), an autologous dendritic cell vaccine loaded with human prostatic acid phosphatase–granulocyte macrophage colony-stimulating factor fusion protein (Dendreon Corporation, Seattle, WA; ref. 16). In a phase III trial, Provenge induced prostatic acid phosphatase–specific T-cell responses and significantly increased 3-year overall survival in patients randomized to APC8015 compared with those randomized to placebo (25.9 versus 21.4 months, $P = 0.01$), representing the first survival advantage attributed to an immunotherapy product in prostate cancer (17, 18).

Despite this progress with immune-based therapies for prostate cancer, there is little information available on whether

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standard treatments, such as hormone therapy and radiation therapy, induce tumor-specific immune responses (19). One might expect the apoptotic cellular material resulting from cytotoxic therapies to be processed by macrophages and dendritic cells and presented to the immune system (20). Furthermore, radiation therapy creates an inflammatory milieu by inducing the expression of inflammatory cytokines, MHC molecules, B7 and other costimulatory molecules, adhesion molecules, death receptors, and heat shock proteins in tumor cells, stroma, and vascular endothelium, which can contribute to effective antigen cross-presentation, leading to a $CD8^+$ cytolytic response (21–23). For its part, hormone therapy can induce prominent T-cell infiltration of human prostate tumors (2) and cause the release of tumor antigens for processing by dendritic cells and presentation to both cytotoxic and helper T cells (24). Finally, in normal tumor-free mice, androgen deprivation transiently increases levels of peripheral T cells, and T cells proliferate more vigorously after antigen stimulation (25). Despite these intriguing findings, there is little information available concerning the effect of standard treatments on tumor immunity in prostate cancer patients and the potential influence this has on clinical outcomes.

To investigate whether standard treatments induce tumor-specific immune responses, we studied a cohort of 73 prostate cancer patients undergoing various forms of standard treatment, including hormone therapy, EBRT, brachytherapy and radical prostatectomy, alone or in combination, as well as nine patients who chose watchful waiting instead of treatment. Pretreatment and posttreatment serum samples were evaluated by Western blot and SEREX (serological identification of antigens by recombinant cDNA expression cloning) antigen arrays for the appearance of treatment-associated autoantibody responses. In parallel, an androgen-dependent mouse model was assessed for treatment-induced serologic changes. We show for the first time that standard treatments induce antigen-specific autoantibody responses in a significant proportion of prostate cancer patients. In the future, it may be possible to enhance the immunologic effects of standard treatments as a practical, personalized form of immunotherapy.

Materials and Methods

Subjects. All blood samples were collected with informed consent and approval from the Research Ethics Board of the BC Cancer Agency. Seventy-three patients with nonmetastatic prostate cancer were recruited at the BC Cancer Agency in Victoria, BC, Canada. Typically, pretreatment serum was collected on the first patient visit and subsequent samples were collected approximately every 3 months during treatment, every 6 months for the first year after the completion of treatment, and yearly thereafter to coincide with regularly scheduled PSA tests. If more that one treatment was initiated, samples were collected during and after each treatment. Of the 73 patients, 9 chose watchful waiting instead of treatment and served as an untreated control group. A second control group consisted of 50 men over the age of 50 years with no personal history of cancer. For 27 of these controls, two to three serial blood draws were collected at 2- to 6-month intervals; for the remainder, only a single blood sample was collected. Serum was aliquoted and stored at −80°C.

Western blot assay. LNCaP or PC3 cells were lysed at $\sim 1 \times 10^6$ cells/mL in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5); 150 mmol/L NaCl; 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS] on ice for 30 min. After centrifugation, 400 μg of protein were separated using NuPAGE Novex 4% to 12% Bis-Tris gels (Invitrogen, Burlington, ON, Canada) and transferred to nitrocellulose using the XCell SureLock Mini-Cell (Invitrogen). Sera were diluted 1/500 in Blotto (5% dry milk powder; 0.1% Tween 20; 50 mmol/L Tris; 150 mmol/L NaCl) and incubated with nitrocellulose membranes for 1 h at room temperature using a multichannel immuoblotting device (Mini Protean II Multiscreen, Bio-Rad, Missassauga, ON, Canada). The membrane was then incubated for 1 h at room temperature with horseradish peroxidase–conjugated goat anti-human IgG (H+L; Jackson ImmunoResearch, West Grove, PA) diluted 1/10,000 in Blotto and visualized by enhanced chemiluminescence.

SEREX screening and antigen arrays. A prostate cancer phage cDNA expression library was constructed with a total of 5.0 μg mRNA from three prostate cancer cell lines (LNCaP, PC3, and DU-145) using the ZAP cDNA library construction kit (Stratagene, La Jolla, CA). The LNCaP and PC3 cell lines were purchased from American Type Culture Collection (Manassas, VA), and the DU-145 cell line was a kind gift from Dr. Ralph deVere White (University of California, Davis Medical Centre, Davis, CA). The library contained 6.8×10^5 clones with a 98.6% recombination frequency. SEREX screening, antigen arrays, and plasmid conversions were done as previously described (26) with the following exception: an additional blocking step was done using TBS [50 mmol/L Tris/150 mmol/L NaCl (pH 8.0)] + 1% bovine serum albumin before secondary antibody was added. SEREX screening of the prostate cancer library was done using serum from a total of 15 patients. A previously described testis library (26) was also screened in an attempt to obtain antigens of the cancer-testis class (27). To make screening more efficient, sera from two to five patients were pooled, each represented at a 1/200 dilution.

Shionogi mouse model. Ten male DD/S mice were inoculated s.c. with 5×10^6 Shionogi tumor cells in one flank. When the tumor reached a diameter of 8 mm in size (approximately day 14), the mice were castrated to simulate androgen deprivation–type hormone therapy, causing the tumor to regress. Blood was collected from each mouse before castration, 2 weeks postcastration, and at euthanization. Cytoplasmic protein lysates were made from intact Shionogi tumor using an NP40 lysis buffer [50 mmol/L Tris (pH 7.5); 150 mmol/L NaCl; 1% NP40]. Western blotting using precastration and postcastration serum samples was done as described above.

Results

Description of the treatment and control groups. To assess the effect of standard treatments on the immune response to prostate cancer, we collected serum samples from patients with various stages of disease before, during, and after standard treatments, which included hormone therapy, EBRT, brachytherapy, radical prostatectomy, or combinations thereof. Patient characteristics are shown in Table 1. Control groups included nine prostate cancer patients who underwent watchful waiting in lieu of treatment, as well as 50 age-matched men with no personal history of cancer. The median ages of the patient group, the watchful waiting control group, and the cancer-free control group were 69 years (range 51-81 years), 70 years (range 55-80 years), and 71 years (range 53-85 years), respectively.

Serologic changes associated with hormone therapy. To broadly assess whether standard treatments for prostate cancer induce immune responses to tumor antigens, patient and control sera were first immunoblotted against lysates from the prostate cancer cell line LNCaP. All positive and most negative results were confirmed by at least two independent immunoblots.

Table 1. Patient characteristics and treatment

	HT	EBRT	HT + EBRT	BT	HT + BT	RP	HT + RP	WW
No. patients	1	1	28	10	10	12	2	9
Age, median (range)	79	76	71 (51-81)	69.5 (55-75)	68.5 (58-76)	62.5 (53-71)	69(67-71)	70 (55-80)
Gleason Score, *n* (%)								
6	1 (100)	0 (0)	6 (21.4)	10 (100)	5 (50)	8 (66.7)	1 (50)	6 (66.7)
7	0 (0)	1 (100)	15 (53.6)	0 (0)	5 (50)	3 (33.3)	1 (50)	3 (33.3)
8-10	0 (0)	0 (0)	7 (25.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Median	6	7	7	6	6.5	6*	6.5	6
Stage, *n* (%)								
T_1	0 (0)	0 (0)	5 (17.9)	6 (60)	3 (30)	8 (66.7)	1 (50)	5 (55.5)
T_2	1 (100)	1 (100)	16 (57.1)	4 (40)	7 (70)	2 (16.7)	0 (0)	3 (33.3)
T_3	0 (0)	0 (0)	7 (25)	0 (0)	0 (0)	1 (8.3)	1 (50)	0 (0)
T_4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (11.1)
PSA at diagnosis (ng/mL)								
Median (range)	4.3	8	9.9 (1.7-24)	5.7 (4.2-9.3)	6.65 (1.2-12.7)	5.05 (1.9-6.6)†	15.8 (10-21.6)	6.2 (4.1-18)
Risk group, *n* (%)								
Low	1 (100)	0 (0)	3 (10.7)	10 (100)	4 (40)	10 (83.3)	1 (50)	4 (44.4)
Intermediate	0 (0)	1 (100)	6 (21.4)	0 (0)	6 (60)	1 (8.3)	0 (0)	4 (44.4)
High	0 (0)	0 (0)	19 (67.9)	0 (0)	0 (0)	1 (8.3)	1 (50)	1 (11.1)

Abbreviations: HT, hormone therapy; BT, brachytherapy; RP, radical prostatectomy; WW, watchful waiting.
*One patient's Gleason score and stage were unknown.
†Two patients' PSA values were unknown.

We evaluated 24 patients undergoing hormone therapy. All but one of these patients was subsequently treated with EBRT ($n = 21$) or radical prostatectomy ($n = 2$); however, analysis of their serum at the completion of hormone therapy and before EBRT or radical prostatectomy allowed assessment of the effects of hormone therapy alone. These 24 patients represented a range of disease risk levels for localized disease, including 4 low-risk (all of PSA ≤10, Gleason ≤6, and stage T_1/T_{2a}), 5 intermediate-risk (any of PSA 11-19, Gleason ≤7, or stage > T_{2b}), and 15 high-risk (any of PSA >20, Gleason ≥8 or stage ≥T_{3a}) patients. By Western blot, 7 of 24 patients (29.2%) showed the emergence of new seroreactivities during hormone therapy, as indicated by the appearance of one or more immunoreactive bands by Western blot (Fig. 1A). Treatment-associated changes arose 4 to 9 months after the initiation of hormone therapy and, with one exception described below, persisted for the duration of study, even in patients who went on to receive a second treatment modality.

We next examined whether the emergence of hormone therapy–associated serologic responses correlated in some way to the extent of tumor regression, as measured by changes in PSA values. All 24 patients experienced a decline in their PSA value, with the median value dropping from 11.0 μg/L (range 2.7-42 μg/L) to 0.15 μg/L (range 0.02-20 μg/L), and for most patients, the PSA nadir occurred within 3 months of the initiation of hormone therapy. Intriguingly, patients exhibiting a hormone therapy–associated immune response achieved a lower median PSA value (0.14 μg/L) at 3 months after hormone therapy than those who did not (median 0.26 μg/L). Moreover, the median time to PSA nadir was less in those patients showing an immune response (7 months, range 6-16 months) than those who did not (9 months, range 2-16 months). Although these differences did not reach statistical significance, possibly owing to small sample sizes, they suggest that tumors undergoing more rapid and extensive regression may be the most likely to trigger a serologic response. As for the

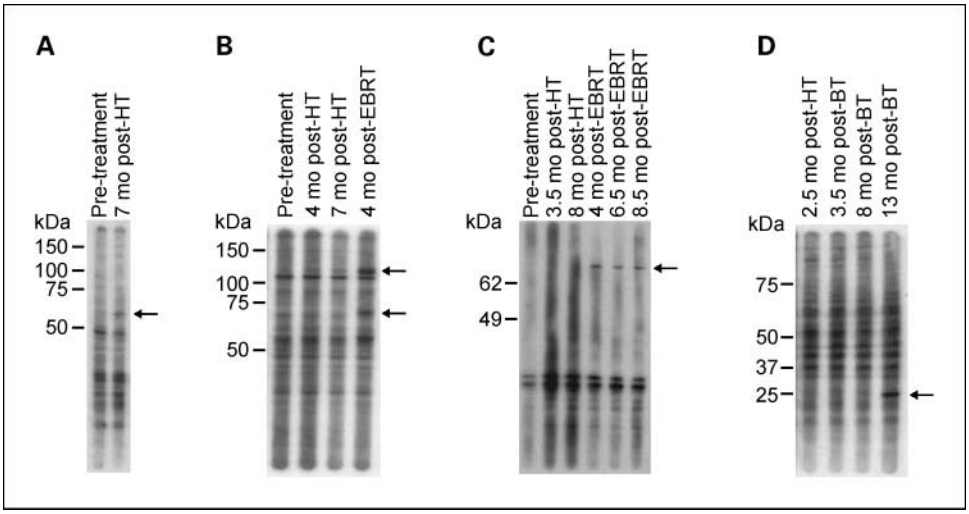

Fig. 1. Standard treatments induce autoantibody responses to tumor proteins in prostate cancer patients. Western blot analysis of serum from five patients probed against LNCaP cell lysates. *A,* patient PC036. Hormone therapy (HT) is associated with the appearance of a ~55 kDa protein (*arrow*). *B,* patient PC015, demonstrating a hormone therapy–associated antibody response to one antigen, which intensifies after EBRT (*top arrow*) and an EBRT-associated response (*bottom arrow*). *C,* patient PC051, showing an EBRT-associated antibody response (*arrow*) of ~100 kDa. *D,* patient PC026, showing a brachytherapy (BT)-associated antibody response (*arrow*).

timing of serologic responses with respect to changes in PSA, in three of seven patients (PC009, PC015, and PC060), the hormone therapy–associated autoantibody response occurred ≥3 months after the PSA nadir (Fig. 2). In one of seven patients (PC036), the immune response occurred during (or possibly before) the drop in PSA. In the remaining three of seven patients, the available time points did not allow us to determine whether the immune responses occurred during or after the drop in PSA.

We considered other factors that might influence the development of hormone therapy–associated immune responses. In general, all patients began hormone therapy with a combination of flutamide and leuprolide acetate or flutamide and goserelin. We found no obvious differences in the hormone therapy drug regimen between those patients who developed a serologic response and those who did not. Similarly, there was no obvious correlation with disease severity, as autoantibody responses were seen in 1 of 4 low-risk, 1 of 5 intermediate-risk, and 5 of 15 high-risk patients.

Although the above results concerned the emergence of new seroreactivities during hormone therapy, we also observed one example (PC001) in which a seroreactive band of ~55 kDa disappeared within 7 months of the initiation of hormone therapy (data not shown). At the same time, a seroreactive band (~62 kDa) appeared, indicating that hormone therapy may both induce and inhibit autoantibody responses to tumor-associated antigens.

Serologic changes associated with radiation therapy. Twenty-nine patients were evaluated by Western blot for the effect of EBRT on the autoantibody profile. The majority (28 of 29) had received prior hormone therapy; therefore, we first considered whether EBRT enhanced or inhibited autoantibody responses that arose during hormone therapy. Five of the patients described above who showed a hormone therapy–associated response went on to receive EBRT. With only one exception, hormone therapy–associated autoantibody responses persisted after EBRT, with follow-up ranging from 7 to 14 months. The one exception was patient PC001, in which a ~62 kDa seroreactive band that appeared during hormone therapy diminished within 3 months of starting EBRT. We do not know whether this change was due to EBRT or would have occurred irrespective of further treatment. One patient (PC015) had a serologic response induced by hormone therapy that intensified after EBRT, as well as a second seroreactivity that emerged after EBRT (Fig. 1B). Thus, in most cases, EBRT is associated with retention or even enhancement of hormone therapy–induced autoantibody responses.

We next considered the emergence of new autoantibody responses associated with EBRT. Four of 29 patients (13.8%) showed new seroreactivities after EBRT (Fig. 1C), none of whom had shown a hormone therapy–associated immune response. One of the four patients underwent EBRT without prior hormone therapy, meaning that the immune response was attributable to EBRT alone. In the other patients that had neoadjuvant hormone therapy, it is not possible to determine whether the response was due to the effects of the EBRT, a combination of the hormone therapy and EBRT, or a delayed response to hormone therapy.

As an alternative to EBRT, 20 patients received brachytherapy, either alone (n = 10) or after hormone therapy (n = 10). Three of 10 hormone therapy + brachytherapy patients showed the emergence of new seroreactivities after brachytherapy that were not seen during hormone therapy. Of the 10 patients receiving brachytherapy without prior hormone therapy, two (20%) showed new immunoreactivities at 4 to 5 months after brachytherapy (Fig. 1D).

The presence or absence of a serologic response during radiation therapy was not attributable to radiation dose, as all patients who underwent EBRT received the same dose of radiation (a total of 7,400 cGy in 37 fractions over 7.5 weeks).

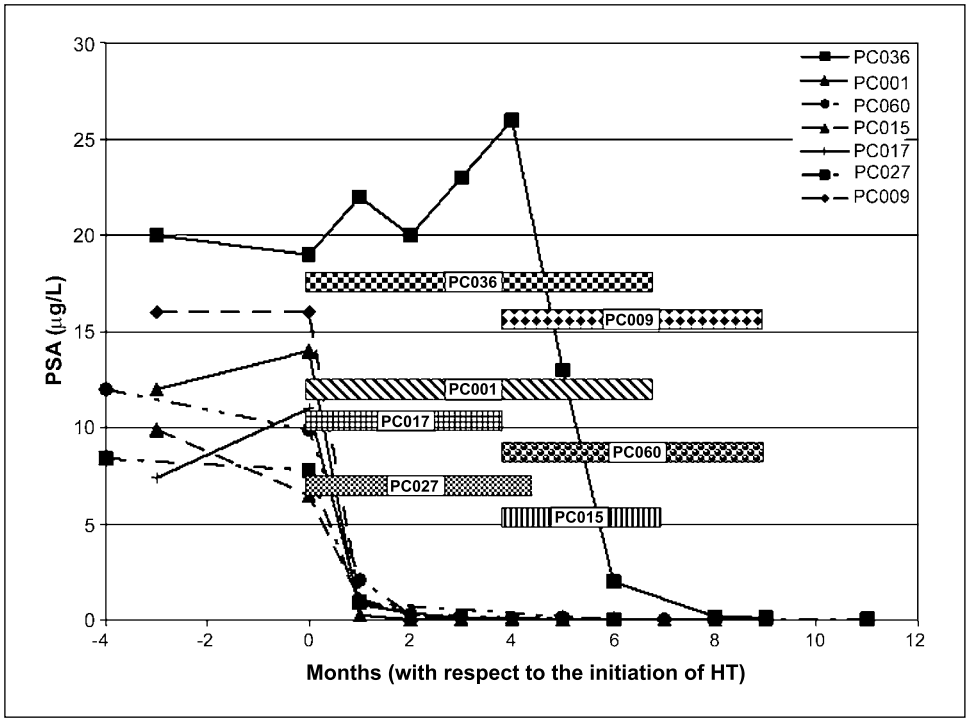

Fig. 2. PSA values over time for the seven patients who showed a hormone therapy – associated seroreactive change by Western blot. PSA values were plotted with respect to the initiation of treatment (t = 0). Shaded bars, time frame in which the hormone therapy – associated seroreactivity emerged according to available blood draws.

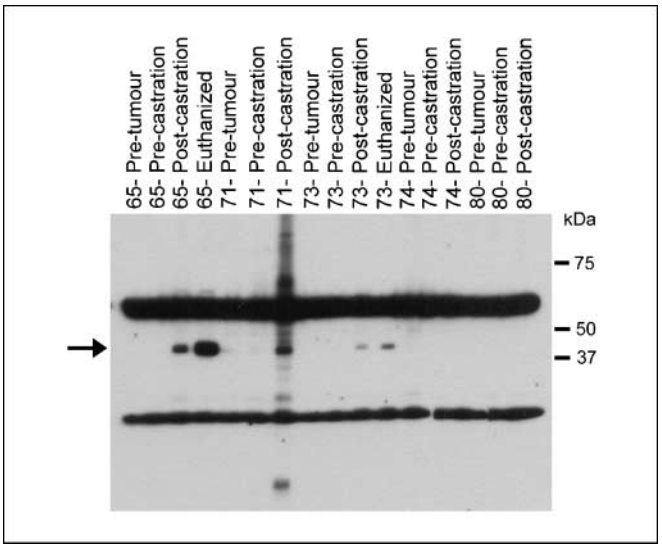

Fig. 3. Androgen withdrawal induces autoantibody responses to a 40 kDa protein in the murine Shionogi tumor model. Western blot analysis of Shionogi mouse sera probed against Shionogi tumor lysate. Serum was collected from five mice at serial time points during treatment, including pretumor, precastration, postcastration, and euthanization. The Western blot shows that three mice (65, 71, 73) have a hormone therapy – induced autoantibody response to a 40 kDa protein (*arrow*) that appears only after castration, whereas two mice (74, 76) do not.

Similarly, all patients who underwent brachytherapy received a peripheral dose of 144 Gy delivered by 120 to 130 ^{125}I seeds (0.334 mCi). Therefore, it appears that patient-specific factors unrelated to radiation dose influenced whether an autoantibody response emerged during treatment.

Serologic analysis of surgical cases and control participants. Fourteen patients in the study had surgery, two of whom received neoadjuvant hormone therapy. No serologic changes were seen by Western blot in any of these patients with a follow-up ranging from 5 to 20 months after radical prostatectomy. Similarly, the nine patients who underwent watchful waiting instead of treatment showed no serologic changes over a 5- to 30-month interval.

We also evaluated 27 cancer-free control subjects from whom two to three serial blood draws were available. Control sera showed a number of immunoreactive bands that varied from individual to individual but did not change over time in 25 of 27 controls. However, 2 of the 27 controls did show a serologic change over a 5- to 10-month interval. One of these individuals displayed a seroreactive band of ~125 kDa at their third time point that was not present at the first two time points. The second individual showed a seroreactive band of ~42 kDa at the second and third time points, as well as a seroreactive band of ~62 kDa at the third time point (data not shown). Thus, the serologic changes seen by Western blot are not exclusive to prostate cancer patients. Nevertheless, the frequency of responses in patients treated with hormone therapy and/or radiation therapy (28.8%; 15 of 52) is significantly higher than that seen in cancer-free controls (2 of 27, 7.4%; $P = 0.028$, χ^2 test) and prostate cancer patients treated surgically (0 of 14, 0%; $P = 0.022$, χ^2 test), and approaches significance in patients undergoing watchful waiting (0 of 9, 0%; $P = 0.064$, χ^2 test), suggesting the majority of responses are indeed induced by the hormone or radiation treatments.

Androgen withdrawal induces autoantibody responses in a murine tumor model. Although the above studies using patient

Table 2. Antigens identified in a SEREX screen of a human prostate cancer cDNA expression library and a human testis cDNA expression library

Name	UniGene no.	Functional significance
RalA binding protein 1 (RALBP1)	Hs.334603	Signaling and drug transport
Synaptonemal complex protein 65 (SC65)	Hs.446459	Cell cycle regulation
RIO kinase 1 (RIOK1)	Hs.437474	Serine protein kinase involved in cell cycle regulation
Kinectin (KTN1)	Hs.509414	A microtubule-associated protein
Peroxisomal D3,D2-eonyl-CoA Isomerase (PECI)	Hs.15250	Metabolic enzyme
Dihydrolipoamide dehydrogenase (DLD)	Hs.131711	Metabolic enzyme
Switch-associated protein 70 (SWAP-70)	Hs.153026	B-cell signaling
C14orf35	Hs.165465	Predicted *N*-acetyltransferase
Son DNA binding protein (SON)	Hs.517262	Possible MYC family oncoprotein
Ubiquitin specific protease 11 (USP11)	Hs.171501	Deubiquinating enzyme exhibiting prosurvival function in response to DNA damage
WD repeat domain 36 (WDR36)	Hs.533237	Signaling
Poly (ADP-ribose) polymerase family, member 1 (PARP1)	Hs.177766	DNA repair
F-box only protein 38 (FBXO38)	Hs.483772	Regulator of gene expression during differentiation
Cellular senescence inhibited gene protein (CSIG)	Hs.592044	Overexpressed in non–small cell lung cancer
Recombining binding protein suppressor of hairless (RBPSUH)	Hs.479396	Notch signaling
Outer dense fiber of sperm tails 2 (ODF2)*	Hs.129055	Maintains the elastic structure and recoil of the sperm tail
Serologically defined colon cancer antigen 1 (SDCCAG1)*	Hs.388584	Nuclear export
PHD finger protein 20 (PHF20)	Hs.517044	Possible transcription factor
ATPase family AAA domain containing 3A (ATAD3A)	Hs.227067	Tumor-associated cell surface antigen
Zinc finger protein 707 (ZNF707)	Hs.521922	Possible regulator of transcription
Centrosomal protein 78 (CEP78)	Hs.374421	Cell cycle regulation

*Isolated from the testis expression library.

sera show a correlation between hormone therapy/radiation therapy and the emergence of serologic responses, they do not establish a causative link. Therefore, to experimentally test whether hormone therapy can induce humoral immunity to tumor antigens, we used the murine Shionogi mammary carcinoma model (SC-115), which has been used extensively to study the conversion from androgen-dependent to -independent neoplasia (28–30). Shionogi tumors are initially androgen dependent and hence are grown in male mice. Castration precipitates apoptosis and tumor regression similar to that seen after androgen withdrawal in prostate cancer patients (31, 32). To evaluate whether tumor regression induces an autoantibody response, Shionogi tumor cells were injected into the flank of 10 male DD/S mice. When tumors reached ~8 × 8 mm, mice were castrated, inducing tumor regression in all cases. Precastration and postcastration sera were subjected to immunoblotting against Shionogi tumor protein lysates. Intriguingly, 5 of 10 mice showed the emergence of an autoantibody response to an ~40 kDa tumor protein within 3 weeks of castration (Fig. 3). Thus, androgen withdrawal can indeed induce autoantibody responses to tumor antigens. However, even in the context of a well-controlled tumor model, there is some degree of variability between individuals, suggesting that other factors influence the initiation of these responses.

Cloning prostate cancer antigens by SEREX. SEREX immunoscreening has been used successfully to identify a large number of antigens from various human cancers, including prostate cancer (33–37). Therefore, to clone the tumor antigens underlying the Western blot results from the above-mentioned prostate cancer patients, a prostate tumor cDNA expression library was constructed using pooled mRNA from the prostate cancer cell lines LNCaP, PC3, and DU-145. Immunoscreening was done using serum from 15 prostate cancer patients, 10 of whom showed a treatment-associated serologic change by Western blot analysis. Each of the 15 patient sera was screened against 1×10^5 to 5×10^5 plaque-forming units of the prostate library, and many were also screened against a previously described testis library (26) in an attempt to identify antigens of the cancer-testis class (27). In total, 21 unique immunoreactive clones were identified (Table 2). The majority of clones contained single cDNA inserts; however, two clones, SWAP-70 + CHYS1 and ZNF707 + PTMA, contained two contiguous cDNA segments, likely representing a double cDNA insertion event during library construction.

To evaluate the frequency of autoantibody responses to these antigens among prostate cancer patients and cancer-free controls, we constructed a SEREX antigen array containing the 21 antigens, as well as three antigens from our previous ovarian cancer study (26). Each array also contained a positive IgG control and a negative control consisting of a nonrecombinant clone. All phage were spotted in duplicate in distinct locations on the array. Replicate arrays were probed with pooled time point sera from 55 patients and 50 cancer-free controls (Table 3). Based on this analysis, antigens could be grouped into three classes. Nine antigens were reactive in approximately equal numbers of patient and control sera, suggesting they represent common autoantigens. Indeed, this group included RBPSUH and SDCCAG1, both of which are known autoantigens (38, 39). By contrast, nine antigens were reactive with serum from cancer patients only. This included two known tumor antigens (MBD2 and p53), one known disease-associated autoantigen (PARP1), and six novel autoantigens (C14orf35, USP11, WDR36, ODF-2, ZNF707+PTMA, and CEP78). A third group encompassed antigens that were recognized by autoantibodies from prostate cancer patients at least twice as frequently as cancer-free controls. This included two known cancer-associated autoantigens (PECI and SEB4), two known cancer- and disease-associated autoantigens (DLD and KTN-1), and two novel autoantigens (RALBP1 and SON).

We evaluated whether autoantibody responses to tumor antigens were more prevalent in the patient versus control groups. For this analysis, we excluded the 15 patients who were used to screen the SEREX libraries, because this would introduce a bias in favor of the patient group. In addition, we excluded the nine antigens that were equivalently reactive with patient and control sera, because these likely represent common autoantigens that are unrelated to prostate cancer. Using these criteria, 13 of 40 (32.5%) of patients were seroreactive to at least one antigen on the array, compared with 7 of 50 (14%) of controls ($P = 0.036$, χ^2 test). It is perhaps not unexpected that some control sera would be seropositive, as the prevalence of autoantibody responses increases with age (40–42) as does the risk of prostate cancer (43). The six antigens that were seroreactive in the 14% of control subjects included PECI, SEB4, DLD, KTN-1, RALBP1, and SON. Of these, only DLD has been previously reported to be immunoreactive in a healthy control subject (44), and PECI, SEB4, DLD, and KTN-1 have all been deemed to be cancer-

Table 3. Summary of the SEREX antigen array results indicating the number of prostate cancer patient and cancer-free control sera that showed autoantibody reactivity to each antigen

	Prostate cancer sera (*n* = 55)	Cancer-free control sera (*n* = 50)
Known common autoantigens		
RBPSUH (38)	4	3
SWAP-70+CHYS1 (77)	6	7
SDCCAG1 (39)	3	4
Known cancer-associated autoantigens		
PECI (45)	3	1
ATAD3A (78)	3	2
RIOK1 (79)	4	3
PHF20 (45, 80)	2	3
MBD2	1	0
p53 (65)	1	0
SEB4	2	1
Known disease-associated autoantigens		
PARP-1 (39, 60, 61)	1	0
DLD (also in cancer; refs. 44, 46)	2	1
SC65 (also in cancer; refs. 44, 76)	3	2
KTN-1 (also in cancer; refs. 81, 82)	3	1
Novel autoantigens		
RALBP1	4	1
C14orf35	1	0
SON	4	2
USP11	1	0
WDR36	2	0
FBXO38	2	2
ODF-2	2	0
CSIG	1	2
ZNF707 + PTMA	1	0
CEP78	1	0

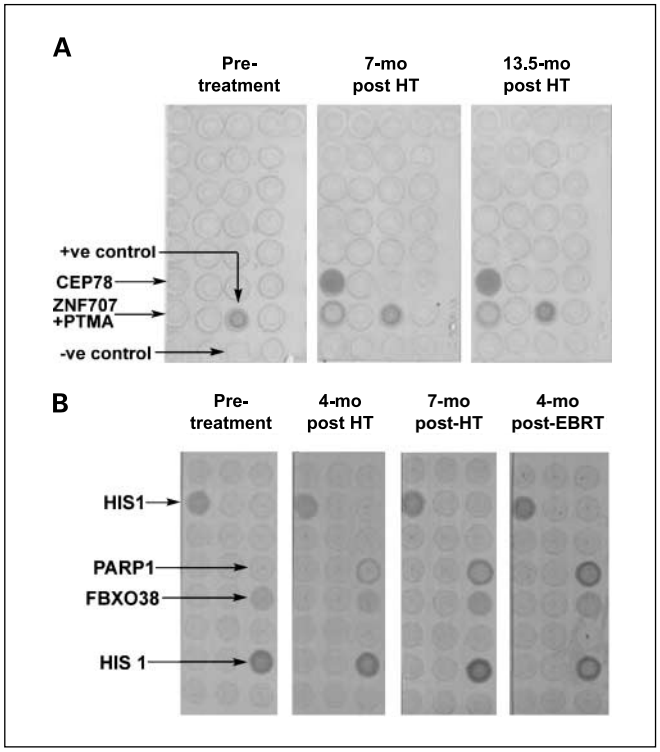

Fig. 4. SEREX antigen array analysis of pretreatment and posttreatment sera in two prostate cancer patients demonstrating treatment-associated changes in seroreactivity. *A,* patient PC036; pretreatment, 7 and 13.5 mo after hormone therapy (HT). Both CEP78 and the double insert clone ZNF707+PTMA are negative in the pretreatment sample and become seroreactive after hormone therapy. +ve control, positive control consisting of an IgG clone; −ve control, consisting of a nonrecombinant λ phage clone. *B,* patient PC015; pretreatment, 4 and 7 mo after hormone therapy and 4 mo after EBRT. Both HIS1 and FBXO38 show an unchanging, positive seroreactivity at every time point. PARP1 is negative in the pretreatment sample, but becomes seroreactive after hormone therapy and intensifies after EBRT.

associated autoantigens in other studies (45–47). In the current study, all six were more frequently immunoreactive in prostate cancer patients than controls. Thus, although not definitive, these results suggest that some or all of these controls may have undiagnosed prostate cancer or another cancer.

An equivalent proportion of patients with high-risk versus low/intermediate-risk disease were seroreactive to one or more antigens on the array [51.7% (15 of 29) versus 54.5% (6 of 11)]. Likewise, 50% (16 of 32) of patients with stage $\leq T_{2b}$ disease were seroreactive versus 62.5% (5 of 8) of patients with stage $\geq T_{2c}$ disease. Thus, similar to the Western blot results, there was no obvious association between disease severity and seroreactivity to antigens on the array.

Treatment-associated immune responses to SEREX-defined antigens. Serial serum samples were analyzed to determine whether autoantibody responses to SEREX-defined antigens developed in association with treatment. In 4 of the 34 patients analyzed, we observed antigen-specific autoantibody responses that were present in posttreatment but not pretreatment sera. In one patient, PC036, autoantibody responses to two antigens (ZNF707+PTMA and CEP78) arose after 7 months of hormone therapy (Fig. 4A). In a second patient, PC011, an autoantibody response to SDCCAG1 arose after EBRT. In a third patient (PC001), three different serologic changes were seen. Specifically, hormone therapy was associated with the disappearance of autoantibodies to two different antigens (KTN1 and RALBP1), whereas EBRT was followed by a significant increase in autoantibodies to a third antigen (ODF2). Finally, patient PC015 had a hormone therapy–associated response to PARP1 that appeared within 4 months of hormone therapy and intensified after EBRT (Fig. 4B). Interestingly, this patient had a ~120 kDa treatment-induced band appear on Western blot after 4 months of hormone therapy (Fig. 1B), making it likely that this band is in fact PARP1, a 113 kDa protein.

For those control subjects who showed seroreactivity to the treatment-associated antigens on the array, serial time point serum samples were analyzed individually. No changes in seroreactivity were seen for any control over a >6-month interval.

One patient, PC011, showed a treatment-associated seroreactivity by antigen array that was not detected by Western blot. Therefore, when the results for the Western blot assay and SEREX antigen array were combined, hormone therapy was associated with a serologic response in 29.2% of patients, EBRT in 17.2% of patients, and brachytherapy in 25.0% of patients.

Discussion

The combined use of adjuvant hormone therapy and radiation therapy in high-risk prostate cancer has led to significantly improved outcomes, with 76% of patients remaining disease-free, without PSA failure at 5 years (48). Our prior results have shown that neoadjuvant hormone therapy given before radiation therapy for >8 months provided prolonged PSA disease-free survival in patients with less well-differentiated tumors (49). Although these clinical results are encouraging, we do not fully understand the basis of synergy between hormone therapy and radiation therapy, which limits our ability to improve upon current treatments. Here, we show for the first time that hormone therapy and radiation therapy induce antigen-specific immune responses in ~29% of prostate cancer patients, as evidenced by the development of autoantibody responses to tumor-associated antigens. Responses typically appeared within a few months of the start of treatment and in most cases persisted for the duration of the study (up to 30 months). A diverse repertoire of antigens was recognized, several of which were successfully cloned by SEREX. In the murine Shionogi tumor model, androgen withdrawal by castration also induced an autoantibody response to the tumor in ~50% of animals. Our findings raise the possibility that the host immune response may influence clinical outcomes after standard treatments. If so, enhancing the frequency, magnitude, and character of these immune responses with immunomodulatory agents could potentially improve outcomes further.

The ~29% prevalence of treatment-associated immune responses shown here may be an underestimate given the limitations of the assays used. First, *Escherichia coli*–based expression systems do not recapitulate the full range of posttranslational modifications characteristic of mammalian proteins, which precludes the expression of many epitopes. We mitigated this in part by performing Western blots of tumor cell lysates, which allows detection of glycosylated or other posttranslationally modified epitopes; however, antigens cannot

be cloned using this method. In the future, use of a yeast cell surface display library (50) may allow cloning of additional posttranslationally modified antigens. Second, we focused exclusively on IgG autoantibodies and therefore would have missed IgM responses, which predominate early in the primary immune response (51). Third, by focusing on autoantibody responses, we may have overlooked treatment-induced responses involving T cells or innate immune effectors such as natural killer or NKT cells. Nevertheless, serologic screening is a useful starting point as it provides recombinant antigens that can then be used to assess antigen-specific T-cell responses, as has been done with SEREX-defined antigens such as NY-ESO-1 (52). In a similar manner, we are currently evaluating whether treatment-associated serologic responses are associated with the emergence of $CD4^+$ and/or $CD8^+$ T-cell responses to the SEREX-defined antigens described here.

Many of the cancer-associated antigens we found have intriguing properties that suggest an involvement in tumor development or progression, including proteins involved in cell cycle regulation (SC65 and CEP78) and DNA damage and repair (USP11 and PARP1). Other antigens are involved in signaling (RALBP1, SWAP-70) or show homology to other oncoproteins (SON). Interestingly, none of the proteins we identified have been previously implicated in prostate cancer (34–37, 53–55). Furthermore, despite extensive screening of both a prostate cancer and testis library, we did not isolate previously reported prostate cancer antigens such as NY-ESO-1, LAGE-1, and XAGE-1 (52, 54). The lack of overlap between different studies suggests that prostate cancer triggers responses to a broad repertoire of autoantigens.

How might tumor antigens become immunogenic during standard treatments? In theory, some may be newly expressed by stressed or dying tumor cells. Others might become immunogenic due to loss of tolerance. Indeed, hormone therapy induces apoptosis of hormone-dependent tumor cells (56), increases tissue levels of dendritic cells and costimulatory molecules, and triggers vigorous T cell–mediated inflammation in the prostate (2). Similarly, radiation therapy induces the apoptosis and necrosis of tumor cells (21) and increases expression of inflammatory and immune mediators, including cytokines, MHC molecules, costimulatory molecules, adhesion molecules, death receptors, and heat shock proteins (57). Although apoptosis was historically considered a nonimmunogenic process, this no longer appears to be the case. In autoimmune disease and cancer, the surface blebs of apoptotic cells contain high concentrations of intracellular antigens that trigger autoantibody responses (58, 59). This process can be enhanced when antigens undergo structural modifications such as cleavage by apoptosis-specific proteases, phosphorylation, or complex formation (58). Dendritic cells can take up apoptotic material and cross-present antigens to $CD8^+$ and $CD4^+$ cells (20), thereby breaking tolerance. In the present study, three of the five treatment-associated antigens we discovered (ZNF707+PTMA, CEP78, and ODF-2) have not been described previously; therefore, we currently have little insight into their mechanism of immunogenicity. On the other hand, PARP1 is a well-known target for cleavage by caspase-3 during apoptosis and necrosis (60) and has been identified as an autoantigen in colorectal cancer (39) and autoimmune conditions such as systemic lupus erythematosus (61). Thus, PARP1 likely becomes immunogenic by virtue of caspase-3–mediated cleavage in apoptotic tumor cells. Intriguingly, anti-PARP1 autoantibodies from patients with systemic lupus erythematosus have been shown to inhibit PARP1 function, even in intact cells (62), which raises the possibility that autoantibodies to antigens such as PARP1 might affect tumor cell biology.

It is difficult to predict whether treatment-induced autoantibody responses will have a positive or negative effect on clinical outcomes. This issue has been most extensively studied with autoantibodies to p53, which are associated with favorable outcomes in lung cancer (63, 64) and poor outcomes in breast, colon, oral, and gastric cancers (65). It remains to be determined whether these disparate results reflect immunologic differences between tumor sites, or an inconsistent biological effect overall. In various autoimmune disorders, autoantibodies are associated with present or future tissue destruction, and may act via complement reactions, antibody-dependent cytotoxicity, immune complex formation, or direct triggering of cell death pathways (66, 67). As mentioned above, autoantibodies can also penetrate target cells and alter the function of their cognate antigen (62). In addition to any potential direct effects on target cells, autoantibodies are usually indicators of an underlying T-cell response, which, in turn, can mediate tissue destruction. For example, coincident autoantibody and $CD8^+$ T-cell responses to antigens such as p53 and NY-ESO-1 have been documented in cancer patients (68, 69). On the other hand, the Th1/Th2 paradigm would suggest that autoantibodies and cytolytic T-cell responses may be mutually exclusive in some cases, as they are promoted by Th2 and Th1 T helper responses, respectively (70).

Given the diversity of autoantigens recognized by cancer patients in the present study and many previous SEREX studies (33), it seems likely that investigation of a single antigen such as p53 is unlikely to fully reveal any relationship between tumor immunity and outcomes. Furthermore, the vast majority of studies to date have measured autoantibody responses at the time of diagnosis rather than posttreatment. As shown here, autoantibody profiles can change significantly during the course of treatment, and one would expect outcomes to be more influenced by the immune status of patients after treatment than before. Indeed, a recent study involving patients undergoing IFN therapy for melanoma showed that the emergence of autoantibody responses to a panel of antigens during treatment correlated with increased relapse-free survival and overall survival (71). Importantly, this latter study focused on common autoimmune indicators such as antinuclear and anti-DNA antibodies. This suggests that even common autoantigens, such as many of the antigens identified in the present study, can potentially serve as clinically relevant target antigens in cancer, or at least represent surrogate markers for effective antitumor responses.

Although much progress has been made with cancer immunotherapy, the clinical effect and feasibility of many immunotherapeutic approaches remain a challenge. For example, peptide-loaded dendritic cells are considered among the most potent of vaccines, yet require specialized expertise and considerable expense to administer (72). By contrast, our data show that standard treatments for cancer may represent a form of *in situ* vaccination, at least at the level of serologic responses. Ongoing studies will address whether standard treatments also induce $CD4^+$ and $CD8^+$ T-cell responses that may contribute

to an antitumor response. The prevalence, magnitude, and nature of such responses could potentially be optimized by administration of immune-stimulatory agents during standard treatments. For example, Flt3 ligand has been shown in preclinical studies to enhance the immune-stimulatory effects of radiation therapy (73). A phase I clinical trial combining conformal radiotherapy and intratumoral injection of dendritic cells was shown to induce tumor-specific and innate immunity (74). Finally, a phase II trial combining chemotherapy and s.c. injection of interleukin 2 and granulocyte macrophage colony-stimulating factor showed strong immunologic and antitumor activity in colorectal cancer patients (75). As shown here, the Shionogi mouse model provides an experimental system to test whether these or other immunomodulatory strategies can enhance the immunologic effects of hormone therapy and radiation therapy to therapeutic levels. The concept of using immunomodulatory agents to maximize the immunologic effects of standard cancer treatments might ultimately provide a cost-effective, clinically feasible alternative to personalized cancer vaccines.

References

1. McNeel DG, Nguyen LD, Storer BE, Vessella R, Lange PH, Disis ML. Antibody immunity to prostate cancer associated antigens can be detected in the serum of patients with prostate cancer. J Urol 2000;164:1825–9.
2. Mercader M, Bodner BK, Moser MT, et al. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A 2001;98:14565–70.
3. Naito Y, Saito K, Shiiba K, et al. $CD8^+$ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 1998;58:3491–4.
4. Schumacher K, Haensch W, Roefzaad C, Schlag PM. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res 2001;61:3932–6.
5. Marrogi AJ, Munshi A, Merogi AJ, et al. Study of tumor infiltrating lymphocytes and transforming growth factor-β as prognostic factors in breast carcinoma. Int J Cancer 1997;74:492–501.
6. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer [comment]. N Engl J Med 2003;348:203–13.
7. Nakano O, Sato M, Naito Y, et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res 2001;61:5132–6.
8. Troy A, Davidson P, Atkinson C, Hart D. Phenotypic characterisation of the dendritic cell infiltrate in prostate cancer [comment]. J Urol 1998;160:214–9.
9. Shimura S, Yang G, Ebara S, Wheeler TM, Frolov A, Thompson TC. Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. Cancer Res 2000;60:5857–61.
10. Vesalainen S, Lipponen P, Talja M, Syrjanen K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 1994;30A:1797–803.
11. Gulley J, Chen AP, Dahut W, et al. Phase I study of a vaccine using recombinant vaccinia virus expressing PSA (rV-PSA) in patients with metastatic androgen-independent prostate cancer. Prostate 2002;53:109–17.
12. Cavacini LA, Duval M, Eder JP, Posner MR. Evidence of determinant spreading in the antibody responses to prostate cell surface antigens in patients immunized with prostate-specific antigen. Clin Cancer Res 2002;8:368–73.
13. Tjoa BA, Lodge PA, Salgaller ML, Boynton AL, Murphy GP. Dendritic cell-based immunotherapy for prostate cancer. CA Cancer J Clin 1999;49:117–28, 65.
14. Valone FH, Small E, MacKenzie M, et al. Dendritic cell-based treatment of cancer: closing in on a cellular therapy. Cancer J 2001;7:S53–61.
15. Gulley JL, Arlen PM, Bastian A, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. Clin Cancer Res 2005;11:3353–62.
16. Small EJ, Fratesi P, Reese DM, et al. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells [comment]. J Clin Oncol 2000;18:3894–903.
17. Lin AM, Hershberg RM, Small EJ. Immunotherapy for prostate cancer using prostatic acid phosphatase loaded antigen presenting cells. Urol Oncol 2006;24:434–41.
18. Small EJ, Schellhammer PF, Higano CS, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. J Clin Oncol 2006;24:3089–94.
19. Webster WS, Small EJ, Rini BI, Kwon ED. Prostate cancer immunology: biology, therapeutics, and challenges. J Clin Oncol 2005;23:8262–9.
20. Leitner WW, Restifo NP. DNA vaccines and apoptosis: to kill or not to kill? J Clin Invest 2003;112:22–4.
21. Friedman EJ. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. Curr Pharm Des 2002;8:1765–80.
22. McBride WH, Chiang CS, Olson JL, et al. A sense of danger from radiation. Radiat Res 2004;162:1–19.
23. Demaria S, Bhardwaj N, McBride WH, Formenti SC. Combining radiotherapy and immunotherapy: a revived partnership. Int J Radiat Oncol Biol Phys 2005;63:655–66.
24. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature 2000;407:784–8.
25. Roden AC, Moser MT, Tri SD, et al. Augmentation of T cell levels and responses induced by androgen deprivation. J Immunol 2004;173:6098–108.
26. Stone B, Schummer M, Paley PJ, et al. Serologic analysis of ovarian tumor antigens reveals a bias toward antigens encoded on 17q. Int J Cancer 2003;104:73–84.
27. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun 2004;4:1.
28. Miyake H, Nelson C, Rennie PS, Gleave ME. Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen independence in prostate cancer. Cancer Res 2000;60:170–6.
29. Akakura K, Bruchovsky N, Rennie PS, et al. Effects of intermittent androgen suppression on the stem cell composition and the expression of the TRPM-2 (clusterin) gene in the Shionogi carcinoma. J Steroid Biochem Mol Biol 1996;59:501–11.
30. Rennie PS, Bruchovsky N, Coldman AJ. Loss of androgen dependence is associated with an increase in tumorigenic stem cells and resistance to cell-death genes. J Steroid Biochem Mol Biol 1990;37:843–7.
31. So AI, Bowden M, Gleave M. Effect of time of castration and tumour volume on time to androgen-independent recurrence in Shionogi tumours. BJU Int 2004;93:845–50.
32. Rennie PS, Bruchovsky N, Buttyan R, Benson M, Cheng H. Gene expression during the early phases of regression of the androgen-dependent Shionogi mouse mammary carcinoma. Cancer Res 1988;48:6309–12.
33. Li G, Miles A, Line A, Rees RC. Identification of tumour antigens by serological analysis of cDNA expression cloning. Cancer Immunol Immunother 2004;53:139–43.
34. Hoeppner LH, Dubovsky JA, Dunphy EJ, McNeel DG. Humoral immune responses to testis antigens in sera from patients with prostate cancer. Cancer Immun 2006;6:1.
35. Jager D, Karbach J, Pauligk C, et al. Humoral and cellular immune responses against the breast cancer antigen NY-BR-1: definition of two HLA-A2 restricted peptide epitopes. Cancer Immun 2005;5:11.
36. Zhou Y, Toth M, Hamman MS, et al. Serological cloning of PARIS-1: a new TBC domain-containing, immunogenic tumor antigen from a prostate cancer cell line. Biochem Biophys Res Commun 2002;290:830–8.
37. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci U S A 1997;94:1914–8.
38. Nickenig G, Wolff M, Stablein A, Pfister H, Bohm M. Enhanced expression and autoimmunity of recombination signal binding protein-jκ in human dilated cardiomyopathy. Biochem Biophys Res Commun 1999;266:432–6.
39. Scanlan MJ, Chen YT, Williamson B, et al. Characterization of human colon cancer antigens recognized by autologous antibodies. Int J Cancer 1998;76:652–8.
40. Manoussakis MN, Tzioufas AG, Silis MP, Pange PJ, Goudevenos J, Moutsopoulos HM. High prevalence of anti-cardiolipin and other autoantibodies in a healthy elderly population. Clin Exp Immunol 1987;69:557–65.
41. Rajczy K, Vargha P, Beregi E. Relationship between immunoglobulin levels and specific antibody titers in the elderly. Z Gerontol 1986;19:158–61.
42. Candore G, Di Lorenzo G, Mansueto P, et al. Prevalence of organ-specific and non organ-specific autoantibodies in healthy centenarians. Mech Ageing Dev 1997;94:183–90.
43. Pienta KJ, Esper PS. Risk factors for prostate cancer. Ann Intern Med 1993;118:793–803.
44. Comtesse N, Zippel A, Walle S, et al. Complex humoral immune response against a benign tumor: frequent antibody response against specific antigens as diagnostic targets. Proc Natl Acad Sci U S A 2005;102:9601–6.
45. Wang Y, Han KJ, Pang XW, et al. Large scale identification of human hepatocellular carcinoma-associated antigens by autoantibodies. J Immunol 2002;169:1102–9.
46. Wu YY, Hsu TC, Chen TY, et al. Proteinase 3 and dihydrolipoamide dehydrogenase (E3) are major autoantigens in hepatitis C virus (HCV) infection. Clin Exp Immunol 2002;128:347–52.
47. Wang HC, Su YR, Han KJ, et al. Multiple variants and a differential splicing pattern of kinectin in human hepatocellular carcinoma. Biochem Cell Biol 2004;82:321–7.
48. Bolla M, Collette L, Blank L, et al. Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate

cancer (an EORTC study): a phase III randomised trial [comment]. Lancet 2002;360:103–6.

49. Ludgate CM, Lim JT, Wilson AG, Alexander AS, Wilson KS. Neoadjuvant hormone therapy and external beam radiation for localized prostate cancer: Vancouver Island Cancer Centre experience. Can J Urol 2000;7:937–43.

50. Wadle A, Mischo A, Imig J, et al. Serological identification of breast cancer-related antigens from a *Saccharomyces cerevisiae* surface display library. Int J Cancer 2005;117:104–13.

51. Boes M. Role of natural and immune IgM antibodies in immune responses. Mol Immunol 2000;37:1141–9.

52. Nakada T, Noguchi Y, Satoh S, et al. NY-ESO-1 mRNA expression and immunogenicity in advanced prostate cancer. Cancer Immun 2003;3:10.

53. Dunphy EJ, McNeel DG. Antigen-specific IgG elicited in subjects with prostate cancer treated with flt3 ligand. J Immunother 2005;28:268–75.

54. Fossa A, Alsoe L, Crameri R, Funderud S, Gaudernack G, Smeland EB. Serological cloning of cancer/testis antigens expressed in prostate cancer using cDNA phage surface display. Cancer Immunol Immunother 2004;53:431–8.

55. Wang X, Yu J, Sreekumar A, et al. Autoantibody signatures in prostate cancer. N Engl J Med 2005; 353:1224–35.

56. Isaacs JT, Furuya Y, Berges R. The role of androgen in the regulation of programmed cell death/apoptosis in normal and malignant prostatic tissue. Semin Cancer Biol 1994;5:391–400.

57. Chakraborty M, Abrams SI, Camphausen K, et al. Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy. J Immunol 2003;170:6338–47.

58. Rosen A, Casciola-Rosen L. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. Cell Death Differ 1999;6:6–12.

59. Hansen MH, Nielsen H, Ditzel HJ. The tumor-infiltrating B cell response in medullary breast cancer is oligoclonal and directed against the autoantigen actin exposed on the surface of apoptotic cancer cells. Proc Natl Acad Sci U S A 2001;98:12659–64.

60. Casiano CA, Ochs RL, Tan EM. Distinct cleavage products of nuclear proteins in apoptosis and necrosis revealed by autoantibody probes. Cell Death Differ 1998;5:183–90.

61. Lim Y, Lee DY, Lee S, et al. Identification of autoantibodies associated with systemic lupus erythematosus. Biochem Biophys Res Commun 2002;295: 119–24.

62. Decker P, Isenberg D, Muller S. Inhibition of caspase-3-mediated poly(ADP-ribose) polymerase (PARP) apoptotic cleavage by human PARP autoantibodies and effect on cells undergoing apoptosis. J Biol Chem 2000;275:9043–6.

63. Winter SF, Sekido Y, Minna JD, et al. Antibodies against autologous tumor cell proteins in patients with small-cell lung cancer: association with improved survival. J Natl Cancer Inst 1993;85:2012–8.

64. Bergquist M, Brattstrom D, Larsson A, et al. p53 auto-antibodies in non-small cell lung cancer patients can predict increased life expectancy after radiotherapy. Anticancer Res 1998;18:1999–2002.

65. Soussi T. p53 Antibodies in the sera of patients with various types of cancer: a review. Cancer Res 2000; 60:1777–88.

66. Lim PL, Zouali M. Pathogenic autoantibodies: emerging insights into tissue injury. Immunol Lett 2006;103:17–26.

67. Sisto M, Lisi S, Castellana D, et al. Autoantibodies from Sjogren's syndrome induce activation of both the intrinsic and extrinsic apoptotic pathways in human salivary gland cell line A-253. J Autoimmun 2006;27:38–49.

68. Hoffmann TK, Donnenberg AD, Finkelstein SD, et al. Frequencies of tetramer+ T cells specific for the wild-type sequence p53(264-272) peptide in the circulation of patients with head and neck cancer. Cancer Res 2002;62:3521–9.

69. van Rhee F, Szmania SM, Zhan F, et al. NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and induces spontaneous humoral and cellular immune responses. Blood 2005;105:3939–44.

70. Crane IJ, Forrester JV. Th1 and Th2 lymphocytes in autoimmune disease. Crit Rev Immunol 2005;25: 75–102.

71. Gogas H, Ioannovich J, Dafni U, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. N Engl J Med 2006;354: 709–18.

72. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10:475–80.

73. Demaria S, Ng B, Devitt ML, et al. Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys 2004;58:862–70.

74. Chi KH, Liu SJ, Li CP, et al. Combination of conformal radiotherapy and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. J Immunother 2005;28:129–35.

75. Correale P, Cusi MG, Tsang KY, et al. Chemoimmunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. J Clin Oncol 2005;23:8950–8.

76. Ochs RL, Stein TW, Jr., Chan EK, Ruutu M, Tan EM. cDNA cloning and characterization of a novel nucleolar protein. Mol Biol Cell 1996;7:1015–24.

77. Comtesse N, Heckel D, Maldener E, Glass B, Meese E. Probing the human natural autoantibody repertoire using an immunoscreening approach. Clin Exp Immunol 2000;121:430–6.

78. Geuijen CA, Bijl N, Smit RC, et al. A proteomic approach to tumour target identification using phage display, affinity purification and mass spectrometry. Eur J Cancer 2005;41:178–87.

79. Line A, Slucka Z, Stengrevics A, Silina K, Li G, Rees RC. Characterisation of tumour-associated antigens in colon cancer. Cancer Immunol Immunother 2002;51: 574–82.

80. Struss AK, Romeike BF, Munnia A, et al. PHF3-specific antibody responses in over 60% of patients with glioblastoma multiforme. Oncogene 2001;20: 4107–14.

81. Lu Y, Ye P, Chen SL, Tan EM, Chan EK. Identification of kinectin as a novel Behcet's disease autoantigen. Arthritis Res Ther 2005;7:R1133–9.

82. Hirano N, Butler MO, Von Bergwelt-Baildon MS, et al. Autoantibodies frequently detected in patients with aplastic anemia. Blood 2003;102:4567–75.

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

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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.

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.

Material and methods

SEREX screening

SEREX screening of a prostate cancer phage cDNA library was carried out as previously described[13] 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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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 RNeasy 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 *E. coli* expression vector pDEST™17 (Invitrogen, Burlington, Canada). Clones were transformed into BL21-AI™ 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 Tris-HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole and 1 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) and purified by IMAC using the ÄKTAprime™ plus (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 $\sim 5 \times 10^6$ Shionogi carcinoma cells. When tumors reached $\sim 8 \times 10$ 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 single-cell suspensions using the blunt end of a 5 ml syringe and a 40-μm cell strainer. Splenocytes were resuspended in ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA and pH 7.3). Lymphocytes and splenocytes were combined, counted and frozen in 50%FBS/10%DMSO for long-term storage.

Immunoblotting of tumor lysates

Cytoplasmic protein lysate was made from intact Shionogi tumors pooled from 5 noncastrated mice. Frozen tumors were pulverized into a fine powder in liquid nitrogen, resuspended in lysis buffer (1 × Dulbecco's PBS, 0.01% Triton, protease inhibitor cocktail), homogenized through 18 G and 21 G needles and then sonicated. Aliquots containing 400 μg of protein lysate were immunoblotted with mouse serum (1/500) followed by HRP-conjugated goat antimouse IgG (H+L; Jackson ImmunoResearch, West Grove, PA) as described previously.[13]

Flow cytometry

To isolate tumor-infiltrating lymphocytes, tumor fragments were pressed through a 40-μm membrane with the blunt end of a 5 ml syringe, and the resulting cell suspension was centrifuged and resuspended in 0.5 ml of 1.0% BSA/PBS. Cells were stained in various combinations with the following fluorochrome-conjugated antibodies at 1/400 in 1.0% BSA/PBS: CD3-FITC, CD4-PE, CD4-PerCP, CD8-Cy-Chrome and CD44-PE (Becton, Dickinson and Company, Oakville, Canada). Isotype-matched fluorochrome-conjugated antibodies served as negative controls. Cells were analyzed on a BD FACSCalibur™ flow cytometry system (Becton, Dickinson and Company, Oakville, Canada) with FlowJo software (Tree Star, Ashland, OR).

IFN-γ ELISPOT assay

Ninety-six-well MultiScreen$_{HTS}$ IP, 0.45 μm filter plates (Millipore, 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 antimouse 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 antimouse 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.[13] 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.[13] To this end, we immunoblotted lysate from the human prostate cancer 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 $\sim 2.3 \times 10^4$ 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

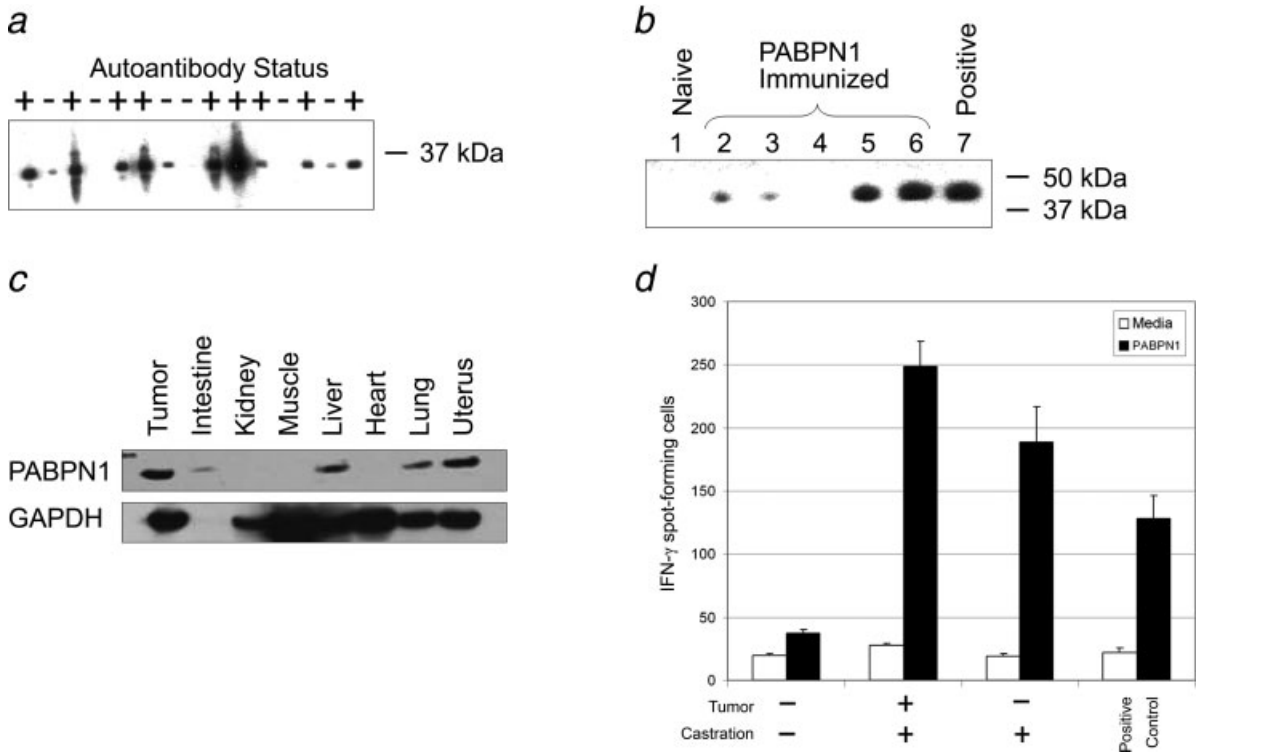

FIGURE 1 – Recognition of PABPN1 by serum antibodies and T cells from castrated mice. (*a*) Recombinant PABPN1 (10 μg) was immunoblotted with serum from mice that were known to be positive (+) or negative (−) for autoantibodies to the ~40 kDa antigen. The correlation between expected positives and negatives was high, although some expected negatives were weakly positive against purified PABPN1, likely reflecting the higher sensitivity of this assay owing to the use of recombinant PABPN1. (*b*) Shionogi tumor lysate was immunoblotted with sera from a naïve DD/S mouse and 5 mice that had been immunized with 100 μg of PABPN1 in incomplete Freund's adjuvant. The presence of an immunoreactive band at ~40 kDa confirms that PABPN1 is the ~40 kDa antigen. (*c*) 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.

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. 1*a*), 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 confirmed that PABPN1 was indeed the 40 kDa antigen (Fig. 1*b*).

By Western blot, PABPN1 was expressed at high levels in Shionogi tumor lysate, as well as normal liver, lung and uterine mouse tissues. By contrast, PABPN1 was not expressed in normal kidney, skeletal muscle or heart (Fig. 1*c*). This is in accord with published data for human tissues, where PABPN1 is expressed at higher levels in liver, lung and uterus compared with kidney, muscle and heart.[19]

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. 1*d*). 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. 1*d*). 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-

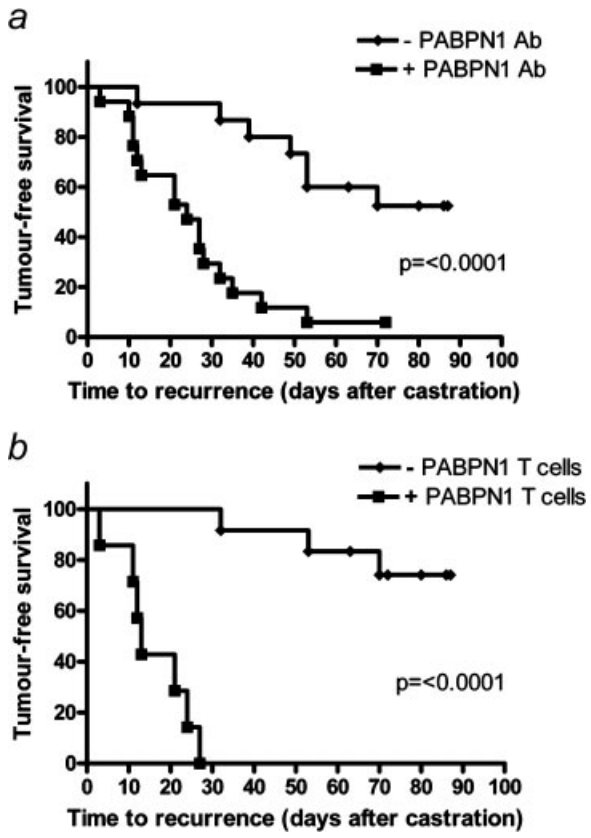

Figure 2 – Castration-induced autoantibody and T cell responses to PABPN1 are associated with tumor recurrence. (*a*) Kaplan–Meier curve comparing the time to recurrence of tumors in mice with (+) or without (−) autoantibodies to PABPN1. (*b*) Kaplan–Meier curve comparing the time to recurrence of tumors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

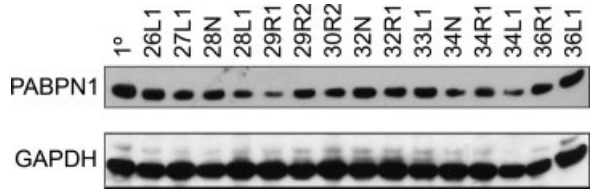

Figure 3 – Recurrent Shionogi tumors retain PABPN1 expression. PABPN1 expression was examined in 15 recurrent Shionogi tumors (labeled with mouse identification numbers such as 26L1) compared with 1 primary tumor (1°). Each lane was loaded with 20 μg tumor lysate and screened with serum from a PABPN1-immunized mouse. GAPDH served as a loading control.

tion, 32/33 mice experienced complete tumor regression. Of these 32 mice, 72% (23/32) experienced tumor recurrence 18–79 days postcastration, whereas the remaining 28% (9/32) remained tumor-free for the duration of the experiment (77–92 days postcastration) after which they were sacrificed for analysis. Seventy percent (16/23) of mice with recurrent tumors had an autoantibody response to PABPN1, compared with only 11% (1/9) of mice that remained tumor-free ($p = 0.005$, Fisher's exact test). Accordingly, the mean tumor-free interval for mice with autoantibodies to PABPN1 was 24.5 days compared with 62.9 days for mice without autoantibodies to PABPN1 ($p < 0.0001$, two-tailed unpaired *t*-test).

T-cell responses to PABPN1 were assessed by IFN-γ ELISPOT using splenocytes harvested at the time of euthanasia. As before, mice with autoantibodies to PABPN1 had stronger T cell responses to PABPN1 (mean = 406 spot-forming cells/10^6 splenocytes, $n = 8$) than mice without autoantibodies to PABPN1 (mean = 38 spot-forming cells/10^6 splenocytes, $n = 11$). Accordingly, mice with recurrent tumors had stronger PABPN1-specific T cell responses (mean = 335 spot-forming cells/10^6 splenocytes, $n = 10$) than mice without recurrent tumors (mean = 36 spot-forming cells/10^6 splenocytes, $n = 9$). Kaplan–Meier analysis revealed a significant difference in time to recurrence between mice with and without PABPN1 antibody and T cell responses (Fig. 2). Thus, contrary to our initial hypothesis, treatment-induced autoantibody and T cell responses to PABPN1 were correlated with inferior outcomes.

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 mm^2 compared with 79.7 mm^2 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 in tumor epithelium or stroma (Fig. 4*a*- noncastrated). In contrast, on Days 7 and 14 postcastration, 12/18 tumors were densely infiltrated by CD3+ T cells (Fig. 4*a*- Day 7 and Day 14). By FACS analysis averaged over 3 experiments, ∼55% of tumor-infiltrating CD3+ T cells were CD8+, and ∼24% were CD4+ T cells. Over 95% of T cells were CD44 high, indicating an activated phenotype (data not shown). By contrast, very few

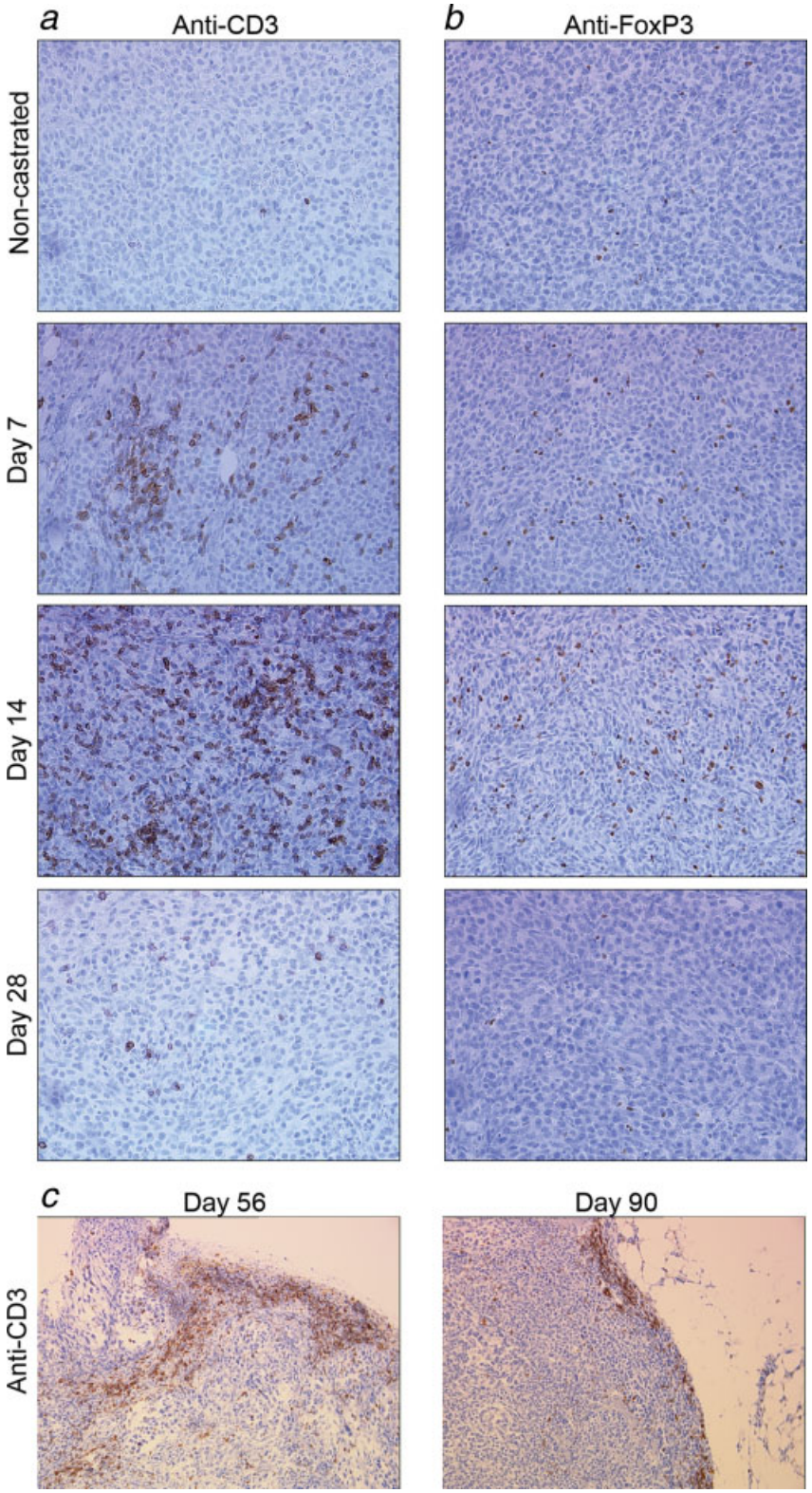

FIGURE 4 – CD3+ T cell infiltration of Shionogi tumors after castration. (*a*) Anti-CD3 staining of Shionogi tumors shows dense infiltration of CD3+ T cells beginning at Day 7 postcastration and reaching maximal levels at Day 14. By Day 28, when most of the tumors had recurred, CD3+ T cells were sparse (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.[20] Tumors were also examined by IHC for cells expressing FoxP3, a transcription factor expressed by regulatory T cells (Tregs).[21] Very few FoxP3+ cells were seen in tumors from noncastrated mice. In castrated mice, the number of tumor-infiltrating FoxP3+ cells was proportional to the number of CD3+ T cells (*i.e.*, about 5–10%) (Fig. 4*b*). 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).[22]

In contrast to the regressing tumors described earlier, recurrent tumors contained relatively sparse CD3+ T cell infiltrates in tumor epithelium (Fig. 4*a*- Day 28). Likewise, very few cells expressing FoxP3, Granzyme B or Pax-5 were seen (Fig. 4*b*- Day 28 and data not shown). However, the peripheral and stromal regions of recurrent tumors showed dense accumulations of CD3+ T cells (Fig. 4*c*). 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,[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. Contrary to 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+ 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[23,24] but not others.[25–29] Autoantibodies to other target antigens have been associated with improved prognosis in melanoma, glioblastoma, gastric cancer and breast cancer.[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.[35] Likewise, in other murine models, B cells were shown to inhibit T cell-mediated rejection of thymoma, melanoma and colon carcinoma cells.[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.[38] Likewise, T cells directed against Helicobacter pylori are thought to promote the development of gastric adenocarcinoma.[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 played 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-γ 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.[40,41] Castration can reverse thymic atrophy, resulting in rapid restoration of peripheral T cell numbers and function.[4,40] 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+ T cells.[42]

Castration-induced T cell responses were accompanied by the rapid and dense infiltration of tumor epithelium by CD4+, CD8+ and FoxP3+ 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.[6] It is noteworthy that in primary prostate tumors collected prior to treatment, the presence of tumor-infiltrating CD4+, CD8+ and CD20+ lymphocytes is associated with inferior outcomes,[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.[44] The role of PABPN1 in transcription may explain its high level of expression in Shionogi

tumor cells.[45] It may seem counter intuitive 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*.[46] 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.[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.[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.[13] 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.[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.

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References

1. Hoeppner LH, Dubovsky JA, Dunphy EJ, McNeel DG. Humoral immune responses to testis antigens in sera from patients with prostate cancer. Cancer Immun 2006;6:1.
2. Garcia-Hernandez Mde L, Gray A, Hubby B, Kast WM. In vivo effects of vaccination with six-transmembrane epithelial antigen of the prostate: a candidate antigen for treating prostate cancer. Cancer Res 2007;67:1344–51.
3. Eigl BJ, Eggener SE, Baybik J, Ettinger S, Chi KN, Nelson C, Wang Z, Gleave ME. Timing is everything: preclinical evidence supporting simultaneous rather than sequential chemohormonal therapy for prostate cancer. Clin Cancer Res 2005;11:4905–11.
4. Roden AC, Moser MT, Tri SD, Mercader M, Kuntz SM, Dong H, Hurwitz AA, McKean DJ, Celis E, Leibovich BC, Allison JP, Kwon ED. Augmentation of T cell levels and responses induced by androgen deprivation. J Immunol 2004;173:6098–108.
5. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature 2000;407:784–8.
6. Mercader M, Bodner BK, Moser MT, Kwon PS, Park ES, Manecke RG, Ellis TM, Wojcik EM, Yang D, Flanigan RC, Waters WB, Kast WM, et al. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci USA 2001;98:14565–70.
7. Matzinger P. Tolerance, danger, and the extended family. Ann Rev Immunol 1994;12:991–1045.
8. McBride WH, Chiang CS, Olson JL, Wang CC, Hong JH, Pajonk F, Dougherty GJ, Iwamoto KS, Pervan M, Liao YP. A sense of danger from radiation. Radiat Res 2004;162:1–19.
9. Garnett CT, Palena C, Chakraborty M, Tsang KY, Schlom J, Hodge JW. Sublethal irradiation of human tumor cells modulates phenotype resulting in enhanced killing by cytotoxic T lymphocytes. Cancer Res 2004;64:7985–94.
10. Chakraborty M, Abrams SI, Camphausen K, Liu K, Scott T, Coleman CN, Hodge JW. Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy. J Immunol 2003;170:6338–47.
11. Friedman EJ. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. Curr Pharm Des 2002;8:1765–80.
12. Demaria S, Bhardwaj N, McBride WH, Formenti SC. Combining radiotherapy and immunotherapy: a revived partnership. Int J Radiat Oncol Biol Phys 2005;63:655–66.
13. Nesslinger NJ, Sahota RA, Stone B, Johnson K, Chima N, King C, Rasmussen D, Bishop D, Rennie PS, Gleave M, Blood P, Pai H, et al. Standard treatments induce antigen-specific immune responses in prostate cancer. Clin Cancer Res 2007;13:1493–502.
14. Miyake H, Nelson C, Rennie PS, Gleave ME. Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen independence in prostate cancer. Cancer Res 2000;60:170–6.
15. Minesita T, Yamaguchi K. An androgen-dependent mouse mammary tumor. Cancer Res 1965;25:1168–75.
16. Rennie PS, Bruchovsky N, Akakura K, Goldenberg SL, Otal N, Akakura S, Wong P, Tenniswood M. Effect of tumour progression on the androgenic regulation of the androgen receptor. TRPM-2 and YPT1 genes in the. Shionogi carcinoma. J Steroid Biochem Mol Biol 1994;50:31–40.
17. Bruchovsky N, Rennie PS, Coldman AJ, Goldenberg SL, To M, Lawson D. Effects of androgen withdrawal on the stem cell composition of the Shionogi carcinoma. Cancer Res 1990;50:2275–82.
18. Skov K, Adomat H, Bowden M, Dragowska W, Gleave M, Koch CJ, Woo J, Yapp DT. Hypoxia in the androgen-dependent Shionogi model for prostate cancer at three stages. Radiat Res 2004;162:547–53.
19. Shmueli O, Horn-Saban S, Chalifa-Caspi V, Shmoish M, Ophir R, Benjamin-Rodrig H, Safran M, Domany E, Lancet D. GeneNote: whole genome expression profiles in normal human tissues. C R Biol 2003;326:1067–72.
20. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 2003;3:361–70.
21. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942–9.
22. Torlakovic E, Slipicevic A, Robinson C, DeCoteau JF, Alfsen GC, Vyberg M, Chibbar R, Florenes VA. Pax-5 expression in nonhematopoietic tissues. Am J Clin Pathol 2006;126:798–804.
23. Winter SF, Sekido Y, Minna JD, McIntire D, Johnson BE, Gazdar AF, Carbone DP. Antibodies against autologous tumor cell proteins in patients with small-cell lung cancer: association with improved survival. J Natl Cancer Inst 1993;85:2012–8.
24. Bergquist M, Brattstrom D, Larsson A, Holmertz J, Hesselius P, Rosenberg L, Wagenius G, Brodin O. p53 auto-antibodies in non-small cell lung cancer patients can predict increased life expectancy after radiotherapy. Anticancer Res 1998;18:1999–2002.
25. Vogl FD, Frey M, Kreienberg R, Runnebaum IB. Autoimmunity against p53 predicts invasive cancer with poor survival in patients with an ovarian mass. Br J Cancer 2000;83:1338–43.
26. Yamazaki Y, Chiba I, Ishikawa M, Satoh C, Notani K, Ohiro Y, Totsuka Y, Mizuno S, Kitagawa Y. Serum p53 antibodies as a prognostic indicator in oral squamous cell carcinoma. Odontology 2008;96:32–7.
27. Bergqvist AS, Bergqvist M, Brattstrom D, Hesselius P, Larsson A, Brodin O, Wagenius G. Serum p53 autoantibodies as prognostic marker in patients with oesophageal carcinoma. Anticancer Res 2001;21:4141–5.
28. Gumus E, Erdamar S, Demirel G, Horasanli K, Kendirci M, Miroglu C. Association of positive serum anti-p53 antibodies with poor prognosis in bladder cancer patients. Int J Urol 2004;11:1070–7.
29. Mack U, Ukena D, Montenarh M, Sybrecht GW. Serum anti-p53 antibodies in patients with lung cancer. Oncol Rep 2000;7:669–74.
30. Pallasch CP, Struss AK, Munnia A, Konig J, Steudel WI, Fischer U, Meese E. Autoantibodies against GLEA2 and PHF3 in glioblastoma: tumor-associated autoantibodies correlated with prolonged survival. Int J Cancer 2005;117:456–9.

31. Bachelot T, Ratel D, Menetrier-Caux C, Wion D, Blay JY, Berger F. Autoantibodies to endostatin in patients with breast cancer: correlation to endostatin levels and clinical outcome. Br J Cancer 2006; 94:1066–70.
32. Gogas H, Ioannovich J, Dafni U, Stavropoulou-Giokas C, Frangia K, Tsoutsos D, Panagiotou P, Polyzos A, Papadopoulos O, Stratigos A, Markopoulos C, Bafaloukos D, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. N Engl J Med 2006;354:709–18.
33. Satzger I, Meier A, Schenck F, Kapp A, Hauschild A, Gutzmer R. Autoimmunity as a prognostic factor in melanoma patients treated with adjuvant low-dose interferon alpha. Int J Cancer 2007;121: 2562–6.
34. Xue LJ, Su QS, Yang JH, Lin Y. Autoimmune responses induced by Helicobacter pylori improve the prognosis of gastric carcinoma. Med Hypotheses 2008;70:273–6.
35. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006; 6:24–37.
36. Shah S, Divekar AA, Hilchey SP, Cho HM, Newman CL, Shin SU, Nechustan H, Challita-Eid PM, Segal BM, Yi KH, Rosenblatt JD. Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells. Int J Cancer 2005;117:574–86.
37. Inoue S, Leitner WW, Golding B, Scott D. Inhibitory effects of B cells on antitumor immunity. Cancer Res 2006;66:7741–7.
38. Nakamoto Y, Kaneko S. Mechanisms of viral hepatitis induced liver injury. Curr Mol Med 2003;3:537–44.
39. Lundin BS, Enarsson K, Kindlund B, Lundgren A, Johnsson E, Quiding-Jarbrink M, Svennerholm AM. The local and systemic T-cell response to Helicobacter pylori in gastric cancer patients is characterised by production of interleukin-10. Clin Immunol 2007;125:205–13.
40. Sutherland JS, Goldberg GL, Hammett MV, Uldrich AP, Berzins SP, Heng TS, Blazar BR, Millar JL, Malin MA, Chidgey AP, Boyd RL. Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol 2005;175:2741–53.
41. Aragon-Ching JB, Williams KM, Gulley JL. Impact of androgen-deprivation therapy on the immune system: implications for combination therapy of prostate cancer. Front Biosci 2007;12:4957–71.
42. Drake CG, Doody AD, Mihalyo MA, Huang CT, Kelleher E, Ravi S, Hipkiss EL, Flies DB, Kennedy EP, Long M, McGary PW, Coryell L, et al. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. Cancer Cell 2005;7:239–49.
43. Karja V, Aaltomaa S, Lipponen P, Isotalo T, Talja M, Mokka R. Tumour-infiltrating lymphocytes: a prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy. Anticancer Res 2005;25:4435–8.
44. Kuhn U, Wahle E. Structure and function of poly(A) binding proteins. Biochem Biophys Acta 2004;1678:67–84.
45. Hosoda N, Lejeune F, Maquat LE. Evidence that poly(A) binding protein C1 binds nuclear pre-mRNA poly(A) tails. Mol Cell Biol 2006;26:3085–97.
46. Savage PA, Vosseller K, Kang C, Larimore K, Riedel E, Wojnoonski K, Jungbluth AA, Allison JP. Recognition of a ubiquitous self antigen by prostate cancer-infiltrating CD8+ T lymphocytes. Science 2008; 319:215–20.

Cancer Therapy: Clinical

Clinical Cancer Research

A Viral Vaccine Encoding Prostate-Specific Antigen Induces Antigen Spreading to a Common Set of Self-Proteins in Prostate Cancer Patients

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Abstract

Purpose: We previously reported a randomized phase II clinical trial combining a poxvirus-based vaccine encoding prostate-specific antigen (PSA) with radiotherapy in patients with localized prostate cancer. Here, we investigate whether vaccination against PSA induced immune responses to additional tumor-associated antigens and how this influenced clinical outcome.

Experimental Design: Pretreatment and posttreatment serum samples from patients treated with vaccine + external beam radiation therapy (EBRT) versus EBRT alone were evaluated by Western blot and serologic screening of a prostate cancer cDNA expression library (SEREX) to assess the development of treatment-associated autoantibody responses.

Results: Western blotting revealed treatment-associated autoantibody responses in 15 of 33 (45.5%) patients treated with vaccine + EBRT versus 1 of 8 (12.5%) treated with EBRT alone. SEREX screening identified 18 antigens, which were assembled on an antigen array with 16 previously identified antigens. Antigen array screening revealed that 7 of 33 patients (21.2%) treated with vaccine + EBRT showed a vaccine-associated autoantibody response to four ubiquitously expressed self-antigens: DIRC2, NDUFS1, MRFAP1, and MATN2. These responses were not seen in patients treated with EBRT alone, or other control groups. Patients with autoantibody responses to this panel of antigens had a trend toward decreased biochemical-free survival.

Conclusions: Vaccine + EBRT induced antigen spreading in a large proportion of patients. A subset of patients developed autoantibodies to a panel of four self-antigens and showed a trend toward inferior outcomes. Thus, cancer vaccines directed against tumor-specific antigens can trigger autoantibody responses to self-proteins, which may influence the efficacy of vaccination. *Clin Cancer Res; 16(15); 4046–56. ©2010 AACR.*

Prostate cancer is the most frequently diagnosed cancer in North American men and remains the second leading cause of cancer-related death in men despite the improvement of early detection using prostate-specific antigen (PSA) screening. Choice of curative treatment for localized disease is challenging and is based on several factors, including stage and grade of disease, PSA value, comorbidities, and patient preference. In general, localized disease is treated with either surgery or radiation therapy (external beam or brachytherapy), with the addition of androgen deprivation therapy (ADT) for higher risk patients. Unfortunately, ~35% of patients fail definitive local therapy within 10 years as evidenced by an increase in PSA, often due to occult metastatic disease (1). Most recurrences are treated with ADT, which, while initially efficacious, inevitably develops into castration-resistant disease. Although chemotherapy can provide modest benefit at this stage, recurrent cancers are incurable. Thus, there is a clear need for more effective therapies for both primary and recurrent disease.

There is considerable evidence that the immune system recognizes prostate tumors in a significant proportion of patients. Serum antibody responses to several different antigens have been detected in patients with prostate cancer (2–9). The presence of tumor-infiltrating lymphocytes provides additional evidence that prostate tumors are recognized by the immune system. Vesalainen et al. (10) found that tumors with lower densities of tumor-infiltrating

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Translational Relevance

Although cancer vaccines offer promise as a new form of treatment, the results of most cancer vaccine trials to date have been disappointing. To improve on these results, we require a better understanding of the effect of vaccination on the patient's immune system. This report is focused on patients with localized prostate cancer who were treated as part of a phase II clinical trial with a recombinant poxvirus-based vaccine targeting prostate-specific antigen (PSA). In addition to inducing immunity to PSA, as intended, vaccination induced autoantibody responses to a panel of widely expressed self-antigens in a significant proportion of patients. Moreover, these vaccine-induced autoantibody responses seemed to be associated with inferior outcomes. Therefore, tumor-specific vaccines can induce immune responses to self-proteins in patients, which we hypothesize may compromise therapeutic efficacy by provoking regulatory responses by the host to minimize autoimmune damage.

lymphocyte were associated with higher risk of tumor progression and poor prognosis; however, in another study, the presence of CD4+, CD8+, and CD20+ tumor-infiltrating lymphocytes in primary prostate tumors was associated with inferior outcomes (11). Thus, the immune system responds to prostate tumors, but it is unclear whether these responses are beneficial or detrimental to patients.

Although much is known about immune responses at the time of diagnosis, far less is known about how tumor immunity changes when patients undergo treatment. We have recently shown that ADT and radiation therapy induce autoantibody responses to a variety of tumor-associated antigens in 17% to 30% of prostate cancer patients (12). Although we do not yet know the influence of these treatment-associated immune responses on clinical outcomes, we examined this issue in a mouse model. Using the androgen-dependent Shionogi carcinoma model, we found that castration (the experimental equivalent of ADT) induced autoantibody and T-cell responses to the self-antigen PABPN1 in ~50% of animals. Remarkably, mice that developed autoantibody and T-cell responses to PABPN1 showed a higher rate and shorter latency of tumor recurrence (13). Thus, castration-induced autoantibody responses are associated with inferior outcomes in the Shionogi tumor model, consistent with a growing number of studies indicating that antitumor immunity can sometimes promote tumor growth (14–17).

Although the foregoing discussion concerns the effects of standard treatments on tumor immunity, many groups are attempting to use vaccines to deliberately induce an immune response to prostate cancer. We conducted a clinical trial in which patients with localized prostate cancer were treated with a combination of external beam radiation therapy (EBRT; with or without neoadjuvant ADT) and a poxvirus-based vaccine encoding PSA (18, 19). Initially, 30 patients were randomized to receive EBRT with PSA vaccine (vaccine + EBRT; n = 19) or EBRT alone (n = 11; ref. 18). Patients in the vaccine arm also received granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose interleukin 2 (IL-2) as immunologic adjuvants. The primary end point was immunologic response specific to PSA. We found that 13 of 17 patients treated with vaccine + EBRT had significantly increased PSA-specific T-cell responses compared with 0 of 8 patients treated with EBRT alone. However, most patients on the vaccine arm were not able to receive the full dose of IL-2 due to significant adverse events. Therefore, we treated an additional 18 patients with vaccine + EBRT and lowered the dose of IL-2 from 4 MIU/M^2/d for 5 days to 0.6 MIU/M^2/d for 14 days (19). Use of very low-dose IL-2 significantly reduced the number of adverse events while still promoting vaccine-induced T-cell responses.

In addition to the significant increases in PSA-specific CD8+ T cells seen in the majority of vaccinated patients, there was also evidence of antigen spreading in 9 of 13 vaccinated patients, as manifested by the development of T-cell responses to antigens not contained in the vaccine, including PSMA, PAP, PSCA, MUC-1, XAGE-1, and PAGE-4 (18, 19). Such responses were also seen in one of six evaluated patients receiving EBRT alone. These positive results with a limited set of known prostate cancer antigens led us to hypothesize that antigen spreading may have extended to a broader repertoire of prostate cancer antigens. To test this hypothesis, we used Western blotting and SEREX immunoscreening to broadly assess the development of autoantibody responses to prostate antigens in patients from this trial.

Materials and Methods

Subjects and treatment schedule

This study included 41 prostate cancer patients from a clinical trial (00-C-0154) conducted at the National Cancer Institute (18, 19). The clinical trial protocol was approved by the Institutional Review Board of the National Cancer Institute, and the current immunologic study was approved by the University of British Columbia/British Columbia Cancer Agency Research Ethics Board. On this trial, men with previously nontreated, biopsy-confirmed prostate cancer were treated with EBRT or vaccine + EBRT. ADT was also given to most patients (n = 34) at the discretion of the attending radiation oncologist. The first vaccination cycle consisted of a priming dose of vaccinia PSA admixed with vaccinia B7.1, given on day 2. Patients then received seven boosts with fowlpox PSA on a 28-day cycle. GM-CSF was given at 100 μg/d at the vaccination site on days 1 to 4 of each cycle. IL-2 was given s.c. in the abdomen either at 4 MIU/M^2 on days 8 to 12 (n = 17) or at 0.6 MIU/M^2 on days 8 to 21 (n = 16). EBRT was administered after the third cycle of vaccine and consisted of a total dose of 70 Gy, with 1.8 to 2.0 Gy fractions given over 2 months (over cycles 4 and 5 of vaccine). In the vaccine

arm, blood samples were collected before vaccine treatment was initiated, after every vaccine cycle and after all treatment was completed. In the EBRT arm, blood samples were collected before EBRT, immediately after, and 3 months after completion of EBRT.

An additional 24 prostate cancer patients receiving standard treatment at the BC Cancer Agency in Victoria, British Columbia, Canada served as controls. Of these, 15 patients were treated with ADT + EBRT, whereas 9 patients chose active surveillance. Fifteen age-matched men with no personal history of prostate cancer served as an additional control group. Blood samples were collected with informed consent and approval from the University of British Columbia/British Columbia Cancer Agency Research Ethics Board. Patient characteristics and treatment categories are summarized in Table 1.

Western blot assay

Western blots were done as previously described (12). Briefly, 400 μg of protein isolated from LNCaP and PC3 cells was separated by standard PAGE and transferred to nitrocellulose. Sera were diluted 1:500 in Blotto (5% dry milk powder, 0.1% Tween 20, 50 mmol/L Tris, 150 mmol/L NaCl) and incubated for 1 hour at room temperature using the Mini Protean II MultiScreen multichannel immunoblotting device (Bio-Rad). The membrane was then incubated for 1 hour at room temperature with horseradish peroxidase–conjugated goat anti-human IgG secondary antibody (H+L; Jackson ImmunoResearch), diluted to 1:10,000, and visualized by enhanced chemiluminescence. Pretreatment and posttreatment sera were run against both LNCaP and PC3 protein lysate a minimum of two times each.

SEREX screening and antigen arrays

SEREX screening of a previously described prostate cancer phage cDNA expression library (12) was done using posttreatment serum samples from six patients who showed treatment-associated serologic changes by Western blot. To make screening more efficient, sera were pooled in pairs, with each serum represented at a 1:200 dilution. Approximately 3.0 to 4.8 × 10^4 clones were screened with each patient serum. Screening of the prostate cancer library and antigen arrays was carried out as previously described (12, 20). Antigen arrays were screened with a 1:200 dilution of pretreatment and posttreatment serum from the 33 vaccine + EBRT, 8 EBRT without vaccine, 15 ADT+EBRT, and 9 active surveillance prostate cancer patients as well as 2 serial serum samples from 15 cancer-free controls.

Results

Serologic changes associated with vaccination against PSA

To broadly assess vaccine-induced immune responses, patient sera were first analyzed by Western blot against

Table 1. Patient characteristics and treatment categories

	Vaccine + EBRT*	EBRT (no vaccine)	ADT + EBRT	WW
No. patients	33	8	15	9
Age, median (range)	61 (50-76)	70 (61-80)	69 (57-79)	67 (60-80)
Gleason score, *n* (%)				
5	2 (6.1)	0 (0)	0 (0)	1 (11.1)
6	8 (24.2)	2 (25)	5 (33.3)	7 (77.8)
7	9 (27.3)	5 (62.5)	10 (66.7)	1 (11.1)
8	7 (21.2)	1 (12.5)	0 (0)	0 (0)
9	7 (21.2)	0 (0)	0 (0)	0 (0)
Median	7	7	7	6
Stage, *n* (%)				
T_1	12 (36.4)	4 (50)	2 (13.3)	7 (77.8)
T_2	15 (45.5)	1 (12.5)	7 (46.7)	2 (22.2)
T_3	6 (18.2)	3 (37.5)	6 (40)	0 (0)
PSA at diagnosis (ng/mL)				
Median (range)	14.7 (3.84-206)	9.66 (5.5-23)	11.3 (3.1-100)	6.2 (3.1-18)
Risk group, *n* (%)				
Low	5 (15.2)	1 (12.5)	3 (20)	7 (77.8)
Intermediate	7 (21.2)	2 (25)	3 (20)	2 (22.2)
High	21 (63.6)	5 (62.5)	9 (60)	0 (0)

Abbreviation: WW, watchful waiting = active surveillance.

*The vaccine + EBRT and EBRT (no vaccine) patients were part of the NIH 00-C-0154 clinical trial. The ADT + EBRT and WW patients were treated by standard therapy at the BC Cancer Agency Vancouver Island Centre.

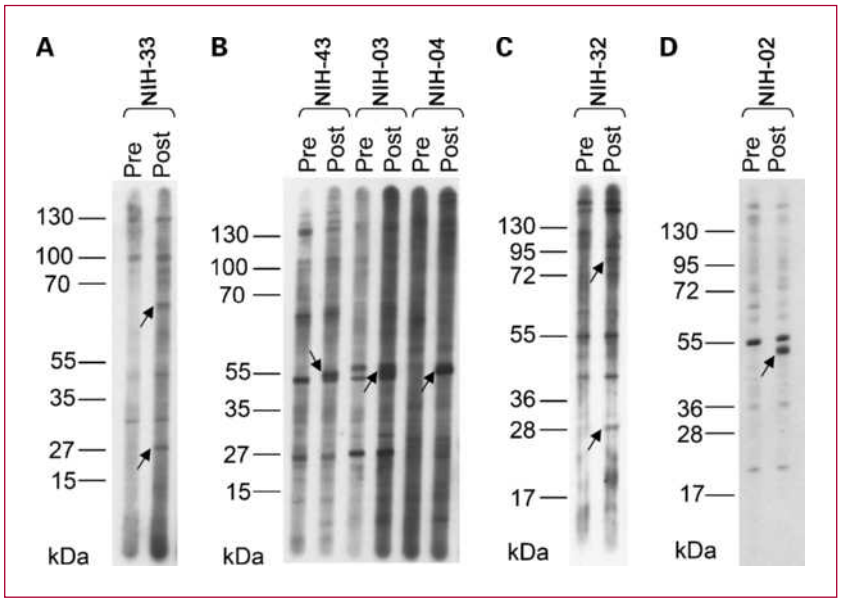

Fig. 1. Treatment with vaccine + EBRT or EBRT without vaccine induces autoantibody responses to a variety of tumor antigens. Western blot analysis of serum from six patients probed against LNCaP (A-C) or PC3 (D) cell lysates. A, treatment with vaccine + EBRT in patient NIH-33 is associated with the development of autoantibody responses against antigens of approximately 65 and 28 kDa. B, vaccine + EBRT treatment in patients NIH-43, NIH-03, and NIH-04 is associated with the development of autoantibody responses against a ~55-kDa antigen in all three patients (arrows). C, vaccine + EBRT treatment in patient NIH-32 is associated with development of autoantibody responses to two antigens of approximately 90 and 28 kDa. D, patient NIH-02, treated with EBRT without vaccine, developed an autoantibody response to a ~53-kDa antigen.

lysates from the prostate cancer cell lines LNCaP and PC3, as per Nesslinger et al. (12, 21). Two groups of patients were assessed as follows: those who received vaccine + EBRT (n = 33) and those who received EBRT without vaccine (n = 8). Of the patients receiving vaccine + EBRT, 15 of 33 (45.5%) showed a serologic change when comparing the prevaccine and postvaccine serum samples (Fig. 1). In contrast, only 1 of 8 (12.5%) patients who received EBRT without vaccine showed a serologic change during EBRT, in accord with our previous study of an independent cohort of patients receiving EBRT (12). The difference between the vaccine + EBRT versus EBRT without vaccine groups was not statistically significant (P = 0.12, Fisher's exact test), likely due to the low number of patients in the EBRT without vaccine group. Indeed, if we increase the sample size of the EBRT without vaccine group by including patients from our prior study (12), the trend reaches significance (P = 0.003, Fisher's exact test; 15 of 33 vaccine + EBRT versus 5 of 40 EBRT without vaccine). Thus, vaccination seems to induce serologic changes beyond those induced by EBRT.

A wide variety of antigens seemed to underlie the vaccine-associated serologic changes, as evidenced by the diversity of protein sizes seen on Western blot (Fig. 1). Although the majority of serologic changes were seen with both LNCaP and PC3 protein lysates, four serologic changes were seen with one protein lysate but not the other, indicating these two cell lines have somewhat distinct antigen repertoires.

Identification of antigens underlying vaccine-induced autoantibody responses

To identify the antigens underlying the above serologic changes, we performed SEREX immunoscreening of a cDNA expression library derived from the prostate cancer cell lines LNCaP, PC3, and DU-145 (12). The library was screened with posttreatment serum samples from six patients who had shown vaccine-associated serologic changes by Western blot (patients NIH-04, NIH-32, NIH-33, NIH-37, NIH-43, and NIH-10). Eighteen antigens were identified, representing a wide range of structural and functional protein classes (Supplementary Table S1). To assess serologic responses to these antigens across the entire patient cohort, SEREX antigen arrays were constructed containing these 18 antigens plus 16 antigens derived from previous SEREX screens of the same prostate cancer library, a cancer-testis library, and an ovarian cancer library (ref. 12; Supplementary Table S1). Antigen arrays were screened with pretreatment serum (serum collected before initiation of vaccine or EBRT) and posttreatment serum (i.e., serum collected after completion of all eight cycles of vaccine and/or EBRT) from all patients from the clinical trial (33 vaccine + EBRT patients and 8 EBRT without vaccine patients). Arrays were also screened with pretreatment and posttreatment sera from an additional group of patients treated with standard ADT + EBRT. Finally, as additional controls, antigen arrays were screened with serial serum samples from prostate cancer patients undergoing active surveillance (n = 9), as well as age-matched men with no personal history of prostate cancer (n = 15).

This analysis revealed several patterns of autoantibody response. First, some antigens were recognized at baseline (i.e., pretreatment) by multiple cancer patients and cancer-free controls, suggesting they represent normal autoantigens (Supplementary Table S2). Second, some antigens showed baseline responses in prostate cancer patients only (Supplementary Table S2). Third, and of greatest interest, some antigens were associated with treatment-induced autoantibody responses. Specifically, 7 of 33 (21.2%)

patients in the vaccine + EBRT group (compared with 0 of 8 patients in the EBRT without vaccine group) showed treatment-induced autoantibody responses by antigen array (Table 2). Of these seven patients, five had shown a response by Western blot in the preceding section, whereas two had not. Thus, when the results of the Western blot and the antigen array were combined, the total number of treatment-induced autoantibody responses was 17 of 33 (51.5%) in the vaccine + EBRT group and 1 of 8 (12.5%) in the EBRT without vaccine group ($P = 0.059$, Fisher's exact test; Table 2). Again, this reached statistical significance when we included additional EBRT without vaccine patients from our previous study; 17 of 33 vaccine + EBRT versus 6 of 40 EBRT without vaccine; $P = 0.0009$, Fisher's exact test; ref. 12).

Remarkably, all seven patients from the vaccine + EBRT group who showed a treatment-induced autoantibody response recognized the same set of four antigens: DIRC2, NDUFS1, MRFAP1 and MATN2 (Figs. 2 and 3). These serologic responses were unique to vaccinated patients, as they were not seen in any other treatment or control group. This is the first time in our experience that multiple individuals showed treatment-induced responses to a common set of antigens; therefore, we hypothesized that these responses may have been induced by vaccination. Indeed, analysis of serial serum samples revealed that, in all seven patients, autoantibody responses to DIRC2, NDUFS1, MRFAP1, and MATN2 arose after three cycles of vaccination but before EBRT (Fig. 2B). Thus, this vaccine regimen induces autoantibody responses to a common set of autoantigens in a significant proportion of patients.

In an attempt to explain why patients might show autoantibody responses to a common set of antigens, we considered the possibility that the observed autoantibodies might be cross-reactive. To assess this, we performed sequence homology comparisons between the four antigens, as well as the viral vectors contained in the priming and boosting vaccines. Messenger RNA and protein sequences for DIRC2, NDUFS1, MRFAP1, MATN2, PSA, B7.1, GM-CSF, and IL-2 were obtained from the Genbank database, as were the complete nucleotide sequences of the vaccinia and fowlpox virus genomes. The sequence alignment program ClustalW2 was used to cross-compare all of these sequences (22). There were stretches of homology between the viral vectors and each of the antigens; however, these were short (less than eight nucleotides) and were not shared between the antigens. Similarly, there was little homology between the four antigens and the vaccine components at either the nucleotide or amino acid level (less than eight nucleotides or three amino acids). Alignment between the four antigens themselves revealed no homology at the nucleotide or amino acid level, making it unlikely that the observed autoantibody responses resulted from cross-reactivity.

In our prior study, we reported that 9 of 13 vaccinated patients from this clinical trial had shown evidence of antigen spreading, as manifested by the development of T-cell responses to prostate-associated antigens not contained in the vaccine, including PSMA, PAP, PSCA, MUC-1, XAGE-1, and PAGE-4 (18, 19). Of these nine patients, three also showed treatment-associated autoantibody responses by either Western blot or antigen array in the present study. Conversely, of the 17 patients who showed autoantibody responses, 3 also showed antigen spreading by T-cell assays. Thus, although there was some overlap between autoantibody and T-cell responses, these seemed to be largely independent phenomena (Supplementary Table S3).

We considered whether the seven patients who showed treatment-induced responses against the four antigens had any clinical characteristics in common. Their ages (range, 51-73 y) and ADT use (five of seven patients) were typical of the entire cohort. Five of the patients were treated with IL-2 at the 4 MIU/M^2 dose, whereas the remaining two received the very low 0.6 MIU/M^2 dose. Finally, the seven patients showed no unusual features with respect to the severity of their prostate cancer: Gleason scores ranged from 6 to 8 (median 6.5); stages ranged from T1c to T3b; risk stratification ranged from low to high; and PSA values at diagnosis ranged from 5.7 to 206 (Supplementary Table S4). Thus, these patients had no obvious clinical characteristics that distinguished them from the rest of the cohort.

Kaplan-Meier analysis was done to compare the biochemical-free survival of patients in the vaccine + EBRT group who did or did not show treatment-induced autoantibody responses by Western blot or antigen array. There was no trend or significant difference between the two

Table 2. Frequency of treatment-induced autoantibody responses observed by Western blot, antigen array, or both

	Vaccine +EBRT (n = 33)	EBRT (no vaccine; n = 8)	ADT+EBRT (n = 15)	WW (n = 9)	Cancer-free controls (n = 15)
Western blot	15 (45.5%)	1 (12.5%)	3* (20.0%)	1 (11.1%)	0 (0%)
Antigen array	7 (21.2%)	0 (0%)	2* (13.3%)	1 (11.1%)	0 (0%)
Overall	17 (51.5%)	1 (12.5%)	3 (20.0%)	1 (11.1%)	0 (0%)

*The treatment-induced responses observed in the ADT + EBRT patients by Western blot and antigen array confirms our previously published results (Nesslinger et al. 2007).

A

	1	2	3	4	5
a	SWAP-70	MATN2	CFL1	ZNF707/ PTMA	RALBP1
b	KIF11	NY-ESO-1	GRB2	PARP1	His1
c	SMARCA4	MLCYD	REXO4	NDUFS1	SC65
d	DIRC2	MKRN1	BRD9	MRFAP1	WDR36
e	NDUFS1	UTP15	CEP78	ODF-2	DLD
f	HMBS/ SC65	BRD9	MATN2	SDCCAG1	SON
g	CLNS1A	TBC1D4	DIRC2	p53	RBPSUH
h	MRFAP1	HCFC2	IgG +ve	-ve	FBX038

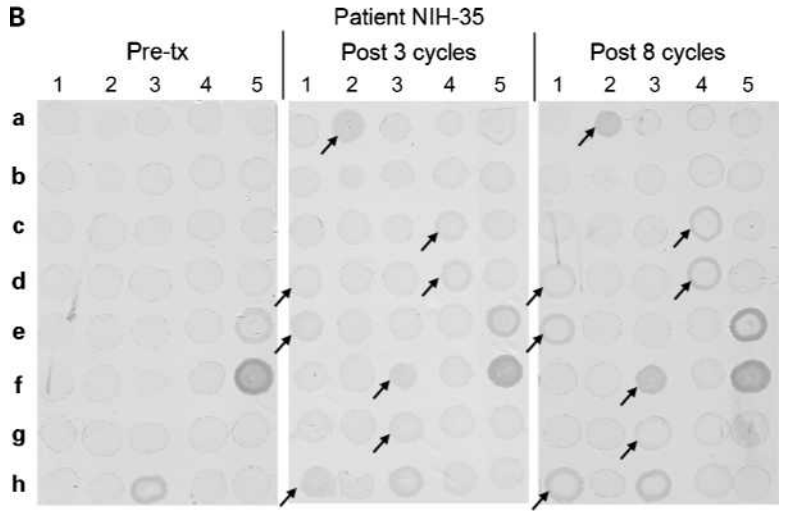

Fig. 2. An example of SEREX antigen array analysis of pretreatment and posttreatment serum from patient NIH-35, treated with vaccine + ERBT, reveals treatment-associated autoantibody responses to a common set of tumor antigens. A, map of the antigen array containing the 18 antigens identified by SEREX screening (using serum from six patients with vaccine-associated autoantibody responses) plus 16 antigens derived from previous screens of prostate or ovarian cancer cDNA expression libraries. Note that several antigens are duplicated within the array. B, Sera collected from patient NIH-35 at three serial time points were used to screen the antigen array: pretreatment (pre-tx), after three cycles of vaccine (post 3 cycles; before EBRT), and after eight cycles of vaccine (post 8 cycles; after completion of EBRT). This revealed baseline autoantibody responses to two antigens (DLD and SON). In addition, autoantibody responses to four antigens (DIRC2, NDUFS1, MRFAP1, and MATN2) appeared only after treatment was initiated (arrows in post 3 and post 8 panels). These responses can be attributed to the vaccine as they are present in the post 3 serum sample, which was taken before the initiation of EBRT.

groups when autoantibody responses to all antigens were considered (P = 0.9537, log-rank test; Fig. 4A). Similarly, there was no significant difference between those patients that had a positive or negative T-cell response to PSA and biochemical-free survival (P = 0.5339, log-rank test). However, the seven patients who showed autoantibody responses to the panel of four common antigens (DIRC2, NDUFS1, MRFAP1, and MATN2) showed a trend toward decreased survival (P = 0.1136, log-rank test; Fig. 4B). Indeed, four of seven of these patients showed biochemical failure, compared with only one of eight nonvaccinated patients. Although not statistically significant, these results suggest that vaccination might not only be ineffective but actually detrimental to patients in those cases in which autoantibody responses to ubiquitous antigens are induced.

Discussion

The use of vaccines to augment the immune response to cancer has been the focus of intense investigation for several decades. Although there have been occasional clinical successes, overall results have been disappointing. The reason for vaccine failure is not known in most cases; however, known barriers to success include tolerance to self-antigens, generation of antigen-loss variants, inadequate immunologic danger signals, and expression of immunosuppressive factors in the tumor microenvironment (23, 24). This study focused on patients with localized prostate cancer who were treated with a recombinant poxvirus-based vaccine containing PSA in combination with radiation therapy (18, 19). By serologic analyses, we found that 17 of 33 (51.5%) of patients developed autoantibody responses after vaccination. Remarkably, seven of these patients developed autoantibody responses to a common set of four antigens. This phenomenon seemed to be unique to vaccination, as it was not observed in patients receiving EBRT without vaccine in this study or our previous study (12). Patients who developed an autoantibody response to the panel of four antigens showed a trend toward reduced biochemical-free survival, suggesting the vaccine could

potentially have induced a detrimental immune response in these patients.

A priori, autoantibodies could play either a beneficial or detrimental role in cancer depending on several factors, including the particular antigen. For example, one could imagine that autoantibody responses to tumor-specific antigens might be beneficial, as they could specifically target tumor cells for destruction by complement or

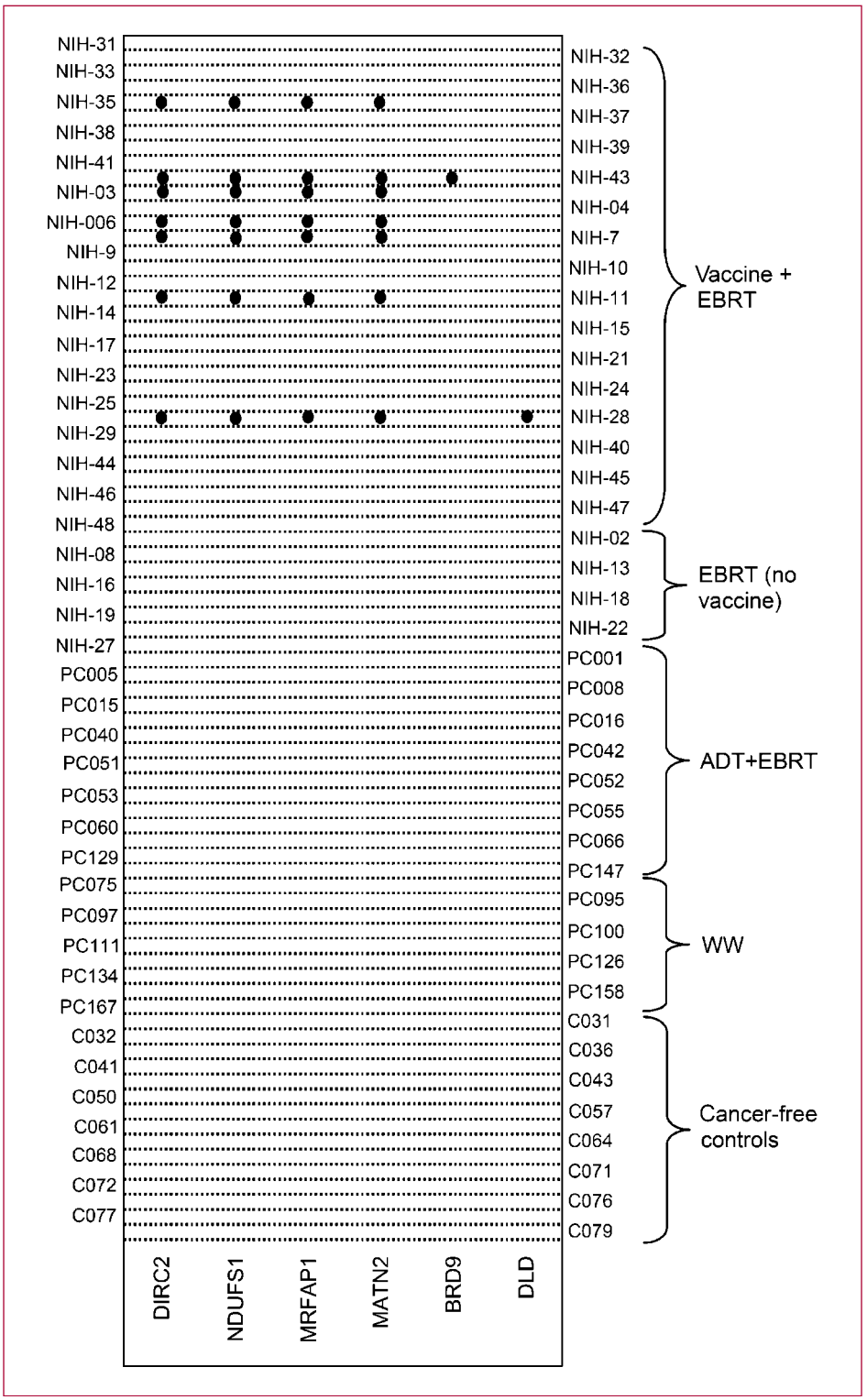

Fig. 3. Summary of all treatment-associated autoantibody responses detected by antigen array. ●, positive autoantibody responses against the indicated antigen. Of 33 patients treated with vaccine + EBRT, seven patients (NIH-35, NIH-03, NIH-43, NIH-006, NIH-7, NIH-11, and NIH-28) developed autoantibody responses to a common set of four antigens (DIRC2, NDUFS1, MRFAP1, and MATN2). Patients NIH-43 and NIH-28 showed an additional treatment-induced response to BRD9 and DLD, respectively. Responses to these antigens can be attributed to the vaccine, as they appeared after three cycles of vaccine but before initiation of EBRT. Additionally, they are not seen in patients treated with ERBT without vaccine (n = 8), ADT + EBRT (n = 15), watchful waiting (WW, n = 9), nor in cancer-free controls (n = 15).

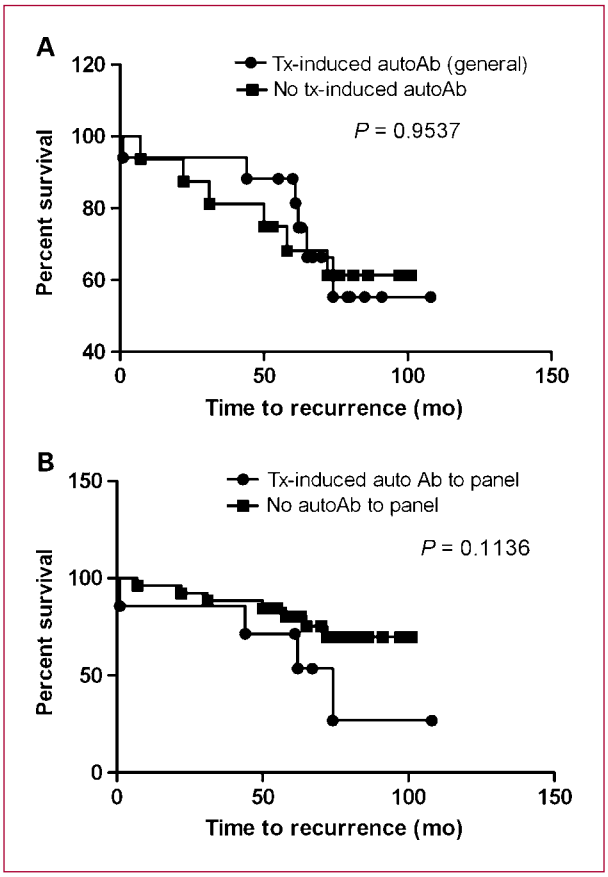

Fig. 4. Kaplan-Meier analysis of biochemical-free survival in relation to the development of autoantiobdy responses. A, patients were stratified according to the presence or absence of treatment-associated autoantibody responses to any antigen seen by Western blot or antigen array. The outcomes of the two groups were similar ($P = 0.9537$). B, patients were stratified according to the presence or absence of treatment-associated autoantibody responses to the panel of four antigens (DIRC2, NDUFS1, MRFAP1, and MATN2) as assessed by antigen arrays. There is a trend toward decreased survival in autoantibody-positive patients ($P = 0.1136$).

antibody-dependent cellular cytotoxicity. By contrast, autoantibody responses to broadly expressed self-antigens (as reported here) could potentially be detrimental, as they might provoke or reflect tolerizing immune responses that serve to minimize autoimmune damage. In reality, many tumor antigens lie somewhere between "tumor specific" and "self"-antigens, and there is no clear consensus on their association with clinical outcomes. The significance of autoantibodies against p53 has been most extensively studied, with some studies finding a positive correlation with clinical outcome (25, 26) and others a negative correlation with outcome (27–31). Autoantibodies to other antigens have been associated with improved prognosis in glioblastoma (32), melanoma (33), gastric cancer (34), and breast cancer (35). Although most of these studies examined the levels of autoantibodies at the time of diagnosis, some examined the development of autoantibodies during treatment. For example, in a clinical trial of melanoma patients treated with IFN-α2B, the development of autoantibodies and clinical signs of autoimmunity were associated with prolonged survival (36).

In the setting of prostate cancer, several groups have conducted phase II clinical trials using GVAX, an allogeneic vaccine composed of the LNCaP and PC3 prostate cancer cell lines engineered to secrete GM-CSF (37–39). By immunoblot analysis, vaccination induced the development of autoantibody responses to LNCaP or PC3 antigens in 79% of patients in one study (38) and 43% to 89% of patients (depending on dose) in a second study (39). In general, these responses were directed against a diverse set of antigens. However, a subset of patients exhibited new or enhanced autoantibody responses to the self-protein filamin-B, which was associated with favorable changes in PSA kinetics (37–39). By contrast, in the present study, the development of autoantibodies during vaccination seemed to be associated with inferior outcomes. Although this trend did not reach statistical significance, possibly due to insufficient sample size, it supports the idea that vaccine-induced autoantibody responses might be detrimental under some conditions.

There are several differences between the present study and the GVAX studies that could account for the different associations between autoantibodies and outcome. With the GVAX approach, autoantibodies are likely induced by a classic, direct immunization mechanism because the cell-based vaccine presented a full complement of human proteins in the context of a strong adjuvant. Thus, the development of autoantibody responses may reflect a successful vaccination overall, hence the correlation to favorable outcomes. By contrast, with the viral vectors used in our trials, autoantibody responses likely developed by an indirect mechanism because the target antigens were not contained within the vaccine. In this scenario, the autoantibody response might arise as a consequence of inflammation at the tumor site, secondary to T-cell recognition of tumor cells expressing the target antigen PSA. One could further speculate that this indirect form of autoantibody induction might be associated with ineffective T-cell responses, such as Th2 responses, rather than cytolytic Th1 responses, which are generally more effective against tumors.

An alternative explanation for the observed trend between autoantibody responses and inferior outcome is that tumors of higher malignant potential could potentially be more immunogenic. However, we saw no obvious differences between the relevant characteristics of patients who developed autoantibody responses to broadly expressed self-antigens and the remainder of the patients (Supplementary Table S4).

The present results are reminiscent of our recent findings in the Shionogi mouse model (12, 13). The Shionogi tumor line is highly androgen-dependent and forms adenocarcinomas when implanted in male mice. Castration of host mice results in rapid regression of established tumors, similar to hormone therapy in human prostate cancer. In 50% to 75% of mice, castration-induced tumor regression is accompanied by the development of autoantibody and

T-cell responses to a self-antigen we identified as poly(A) binding protein N1 (PABPN1). Remarkably, mice that developed autoantibody and T-cell responses to PABPN1 showed inferior outcomes, as manifested by an increased rate of tumor recurrence (13). We are currently investigating whether B and/or T-cell responses to PABPN1 actively promote tumor recurrence in this model or simply serve as markers of other biological processes associated with tumor recurrence. In support of the former possibility, it has recently been shown in a mouse model of prostate cancer that androgen ablation results in infiltration of regressing tumors with leukocytes, including B cells (40). Importantly, tumor-infiltrating B cells were shown to produce cytokines such as lymphotoxin that activate IKK-α and signal transducers and activators of transcription 3 in prostate tumor cells, thereby enhancing hormone-free survival and the transition to androgen independence (40). Other studies have also shown a protumorigenic role for B-cell responses. For example, B cells (or serum antibodies) were shown to promote primary tumor formation in a transgenic mouse model of inflammation-associated carcinogenesis (17). Furthermore, B cells were shown to inhibit T cell–mediated rejection of thymoma, melanoma, and colon carcinoma cells in other murine studies (41, 42). Thus, there is abundant evidence that B-cell responses can promote the development or progression of cancer.

It remains unclear why the antigens DIRC2, NDUFS1, MRFAP1, and MATN2 were common targets of autoantibody responses. According to public databases, all four proteins are ubiquitously expressed in most normal and tumor tissues, and only DIRC2 seems to be overexpressed in prostate cancer tissue compared with normal prostate tissue. As mentioned, the four antigens do not share significant sequence homology with each other or with other coding sequences in the viral vectors. Nonetheless, it is formally possible that these antigens share conformational similarities that are not evident from their primary sequence. In our prior study of ovarian cancer, we found that autoantibody responses were biased toward gene products on chromosome 17 (20); however, the four antigens identified here are located on chromosomes 3, 2, 4, and 8, respectively. In addition, the four antigens have unique cellular functions. Disrupted in renal carcinoma protein 2 (DIRC2) is a membrane-bound protein with homology to members of the major facilitator superfamily of transporters. Intriguingly, it was identified as a breakpoint spanning gene on chromosome 3 in a constitutional familial case of a t(2;3)(q35;q21) translocation resulting in hereditary renal cell carcinoma (43). Mof4 family associated protein 1 (MRFAP1) is an intracellular protein that associates with members of the mortality factor on chromosome 4/MORF4-related gene (MORF4/MRG) family as well as the tumor suppressor protein Rb and thus may play a role in cell growth, immortalization, and/or senescence (44). Matrilin-2 (MATN2) is a secreted adaptor protein of the extracellular matrix that plays a role in cell growth and tissue remodeling (45). Interestingly, we initially cloned MATN2 from an ovarian cancer library using serum from an ovarian cancer patient, suggesting that autoantibodiesto this antigen are not unique to prostate cancer. Finally, NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NADH-coenzyme Q reductase; NDUFS1) is a subunit of respiratory complex I located at the mitochondrial inner membrane. Intriguingly, caspase-mediated cleavage of NDUFS1 is required for several mitochondrial changes during apoptosis (46), which fits with the general notion that caspase-mediated cleavage of proteins can generate neoepitopes that trigger autoantibody responses (47–49). We are currently investigating whether these four proteins serve as autoantigens in the context of other cancer vaccines.

In summary, our results provide further evidence that cancer vaccines targeting a single antigen such as PSA can induce broader immune responses involving multiple host antigens. Although this process of antigen spreading is generally considered a desirable outcome of vaccination, our findings suggest that this assumption may not be universally true. We propose that further studies involving other vaccine strategies are warranted. For example, we vaccinated patients in conjunction with EBRT and, in some cases ADT, both of which can contribute to the development of autoantibody responses, albeit not to the four antigens described here (12). Although the sample size is small, it is interesting to note that of the patients with treatment-induced immune response to the panel of antigens, all four patients who recurred had received prior ADT (Supplementary Table S4). We also included GM-CSF in the vaccine formulation, which several recent studies suggest might be a suboptimal adjuvant for cancer vaccines (50, 51). Finally, the use of IL-2 in the vaccine formulation may have played a role; notably, all four autoantibody-positive patients who recurred received the higher dose of IL-2 (Supplementary Table S4). To better understand the contribution of GM-CSF and IL-2 to the development of autoantibody responses, we intend to analyze samples from patients who received poxvirus-based vaccines without these adjuvants (52, 53). Going forward, if autoantibody responses are indeed detrimental to the efficacy of cancer vaccines, it may be possible to dampen or redirect these responses using other immunomodulatory agents that promote cytolytic over humoral immune responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Freedland SJ, Moul JW. Prostate specific antigen recurrence after definitive therapy. J Urol 2007;177:1985–91.
2. McNeel DG, Nguyen LD, Storer BE, Vessella R, Lange PH, Disis ML. Antibody immunity to prostate cancer associated antigens can be detected in the serum of patients with prostate cancer. J Urol 2000;164:1825–9.
3. Miles AK, Rogers A, Li G, et al. Identification of a novel prostate cancer-associated tumor antigen. Prostate 2007;67:274–87.
4. Hoeppner LH, Dubovsky JA, Dunphy EJ, McNeel DG. Humoral immune responses to testis antigens in sera from patients with prostate cancer. Cancer Immun 2006;6:1.
5. Okada T, Akada M, Fujita T, et al. A novel cancer testis antigen that is frequently expressed in pancreatic, lung, and endometrial cancers. Clin Cancer Res 2006;12:191–7.
6. Jager D, Karbach J, Pauligk C, et al. Humoral and cellular immune responses against the breast cancer antigen NY-BR-1: definition of two HLA-A2 restricted peptide epitopes. Cancer Immun 2005;5:11.
7. Fossa A, Alsoe L, Crameri R, Funderud S, Gaudernack G, Smeland EB. Serological cloning of cancer/testis antigens expressed in prostate cancer using cDNA phage surface display. Cancer Immunol Immunother 2004;53:431–8.
8. Zhou Y, Toth M, Hamman MS, et al. Serological cloning of PARIS-1: a new TBC domain-containing, immunogenic tumor antigen from a prostate cancer cell line. Biochem Biophys Res Comm 2002;290: 830–8.
9. Wang X, Yu J, Sreekumar A, et al. Autoantibody signatures in prostate cancer. N Engl J Med 2005;353:1224–35.
10. Vesalainen S, Lipponen P, Talja M, Syrjanen K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 1994;30A:1797–803.
11. Karja V, Aaltomaa S, Lipponen P, Isotalo T, Talja M, Mokka R. Tumour-infiltrating lymphocytes: a prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy. Anticancer Res 2005;25:4435–8.
12. Nesslinger NJ, Sahota RA, Stone B, et al. Standard treatments induce antigen-specific immune responses in prostate cancer. Clin Cancer Res 2007;13:1493–502.
13. Hahn S, Nesslinger NJ, Drapala RJ, et al. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. Int J Cancer 2009;125: 2871–8.
14. Sharma S, Stolina M, Lin Y, et al. T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function. J Immunol 1999;163:5020–8.
15. Barbera-Guillem E, May KF, Jr., Nyhus JK, Nelson MB. Promotion of tumor invasion by cooperation of granulocytes and macrophages activated by anti-tumor antibodies. Neoplasia 1999;1:453–60.
16. Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. Curr Opin Immunol 2007;19:209–16.
17. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006;6: 24–37.
18. Gulley JL, Arlen PM, Bastian A, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. Clin Cancer Res 2005;11:3353–62.
19. Lechleider RJ, Arlen PM, Tsang KY, et al. Safety and immunologic response of a viral vaccine to prostate-specific antigen in combination with radiation therapy when metronomic-dose interleukin 2 is used as an adjuvant. Clin Cancer Res 2008;14:5284–91.
20. Stone B, Schummer M, Paley PJ, et al. Serologic analysis of ovarian tumor antigens reveals a bias toward antigens encoded on 17q. Int J Cancer 2003;104:73–84.
21. Nesslinger NJ, Pai HH, Ludgate CM, Nelson BH. Effects of standard treatments on the immune response to prostate cancer. In: Hayat MA, editor. Methods of Cancer Diagnosis, Therapy and Prognosis: General Methods and Overviews, Lung Carcinoma and Prostate Carcinoma. New York: Springer; 2009, p. 531–55.
22. Larkin MA, Blackshields G, Brown NP, et al. Clustal W, Clustal X. version 2.0. Bioinformatics 2007;23:2947–8.
23. Attia P, Phan GQ, Maker AV, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. J Clin Oncol 2005; 23:6043–53.
24. Lopez MN, Pereda C, Segal G, et al. Prolonged survival of dendritic cell-vaccinated melanoma patients correlates with tumor-specific delayed type IV hypersensitivity response and reduction of tumor growth factor β-expressing T cells. J Clin Oncol 2009;27:945–52.
25. Winter SF, Sekido Y, Minna JD, et al. Antibodies against autologous tumor cell proteins in patients with small-cell lung cancer: association with improved survival. J Natl Cancer Inst 1993;85:2012–8.
26. Bergquist M, Brattstrom D, Larsson A, et al. p53 auto-antibodies in non-small cell lung cancer patients can predict increased life expectancy after radiotherapy. Anticancer Res 1998;18:1999–2002.
27. Vogl FD, Frey M, Kreienberg R, Runnebaum IB. Autoimmunity against p53 predicts invasive cancer with poor survival in patients with an ovarian mass. Br J Cancer 2000;83:1338–43.
28. Yamazaki Y, Chiba I, Ishikawa M, et al. Serum p53 antibodies as a prognostic indicator in oral squamous cell carcinoma. Odontology 2008;96:32–7.
29. Bergqvist AS, Bergqvist M, Brattstrom D, et al. Serum p53 autoantibodies as prognostic marker in patients with oesophageal carcinoma. Anticancer Res 2001;21:4141–5.
30. Gumus E, Erdamar S, Demirel G, Horasanli K, Kendirci M, Miroglu C. Association of positive serum anti-p53 antibodies with poor prognosis in bladder cancer patients. Int J Urol 2004;11:1070–7.
31. Mack U, Ukena D, Montenarh M, Sybrecht GW. Serum anti-p53 antibodies in patients with lung cancer. Oncol Rep 2000;7:669–74.
32. Pallasch CP, Struss AK, Munnia A, et al. Autoantibodies against GLEA2 and PHF3 in glioblastoma: tumor-associated autoantibodies correlated with prolonged survival. Int J Cancer 2005;117:456–9.
33. Satzger I, Meier A, Schenck F, Kapp A, Hauschild A, Gutzmer R. Autoimmunity as a prognostic factor in melanoma patients treated with adjuvant low-dose interferon α. Int J Cancer 2007;121:2562–6.
34. Xue LJ, Su QS, Yang JH, Lin Y. Autoimmune responses induced by Helicobacter pylori improve the prognosis of gastric carcinoma. Med Hypotheses 2008;70:273–6.
35. Bachelot T, Ratel D, Menetrier-Caux C, Wion D, Blay JY, Berger F. Autoantibodies to endostatin in patients with breast cancer: correlation to endostatin levels and clinical outcome. Br J Cancer 2006;94: 1066–70.
36. Gogas H, Ioannovich J, Dafni U, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. N Engl J Med 2006;354:709–18.
37. Simons JW, Carducci MA, Mikhak B, et al. Phase I/II trial of an allogeneic cellular immunotherapy in hormone-naive prostate cancer. Clin Cancer Res 2006;12:3394–401.
38. Urba WJ, Nemunaitis J, Marshall F, et al. Treatment of biochemical recurrence of prostate cancer with granulocyte-macrophage colony-stimulating factor secreting, allogeneic, cellular immunotherapy. J Urol 2008;180:2011–7, discussion 7–8.
39. Higano CS, Corman JM, Smith DC, et al. Phase 1/2 dose-escalation study of a GM-CSF-secreting, allogeneic, cellular immunotherapy for metastatic hormone-refractory prostate cancer. Cancer 2008;113: 975–84.
40. Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature 2010;464:302–5.
41. Shah S, Divekar AA, Hilchey SP, et al. Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells. Int J Cancer 2005;117:574–86.
42. Inoue S, Leitner WW, Golding B, Scott D. Inhibitory effects of B cells on antitumor immunity. Cancer Res 2006;66:7741–7.
43. Bodmer D, Schepens M, Eleveld MJ, Schoenmakers EF, Geurts van Kessel A. Disruption of a novel gene, DIRC3, and expression of DIRC3-1 fusion transcripts in a case of familial renal cell cancer and t(2;3)(q35;q21). Genes Chromosomes Cancer 2003;38:107–16.

44. Tominaga K, Magee DM, Matzuk MM, Pereira-Smith OM. PAM14, a novel MRG- and Rb-associated protein, is not required for development and T-cell function in mice. Mol Cell Biol 2004;24:8366–73.
45. Malin D, Sonnenberg-Riethmacher E, Guseva D, et al. The extracellular-matrix protein matrilin 2 participates in peripheral nerve regeneration. J Cell Sci 2009;122:995–1004.
46. Ricci JE, Munoz-Pinedo C, Fitzgerald P, et al. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. Cell 2004;117:773–86.
47. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 1994; 179:1317–30.
48. Catera R, Silverman GJ, Hatzi K, et al. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol Med 2008;14:665–74.
49. Radic M, Marion T, Monestier M. Nucleosomes are exposed at the cell surface in apoptosis. J Immunol 2004;172:6692–700.
50. Faries MB, Hsueh EC, Ye X, Hoban M, Morton DL. Effect of granulocyte/macrophage colony-stimulating factor on vaccination with an allogeneic whole-cell melanoma vaccine. Clin Cancer Res 2009;15: 7029–35.
51. Slingluff CL, Jr., Petroni GR, Olson WC, et al. Effect of granulocyte/macrophage colony-stimulating factor on circulating CD8+ and CD4+ T-cell responses to a multipeptide melanoma vaccine: outcome of a multicenter randomized trial. Clin Cancer Res 2009; 15:7036–44.
52. Arlen PM, Skarupa L, Pazdur M, et al. Clinical safety of a viral vector based prostate cancer vaccine strategy. J Urol 2007;178:1515–20.
53. Gulley JL, Arlen PM, Madan RA, et al. Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. Cancer Immunol Immunother 2009;59:663–74.

SUPPORTING DATA:

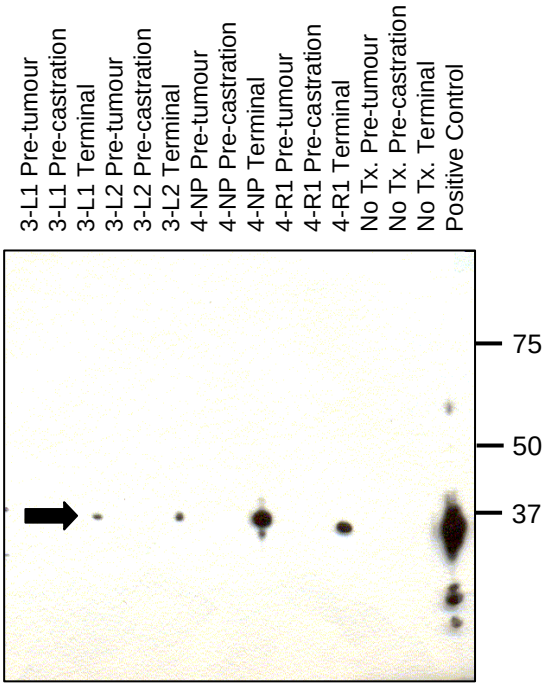


Figure 1. Castration induced an antibody response against PABPN1 in a large proportion of mice. Purified, soluble, *E. coli* recombinant PABPN1 (10 µg) was probed with pre-tumour, pre-castration, and terminal serum samples obtained from castrated mice 3-L1, 3-L2, 4-NP, 4-R1, and a tumour-bearing, non-castrated (No Tx.) mouse. 3-L1 and 3-L2 were sacrificed on day 28 post-castration and 4-NP and 4-R1 were sacrificed on day 33 post-castration. The arrow shows the PABPN1 seroreactive band.

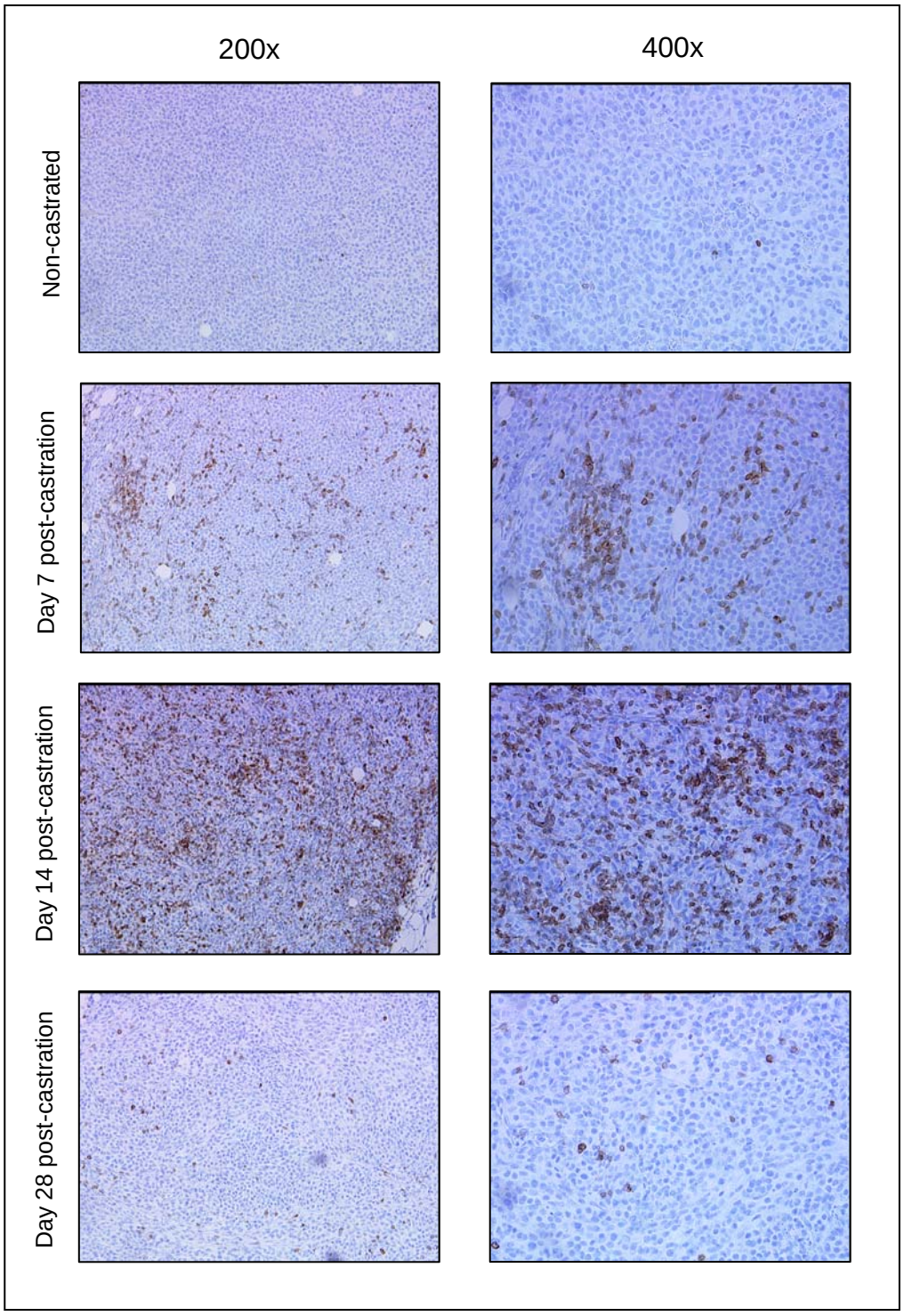


Figure 2. Anti-CD3 staining of Shionogi tumours at specific time points following castration showed dense infiltration of CD3+ T cells between 7-14 days post-castration. By 28 days post-castration, when most of the tumours had recurred, CD3+ T cells were sparse. The tumours from non-castrated mice were essentially void of CD3+ T cells.

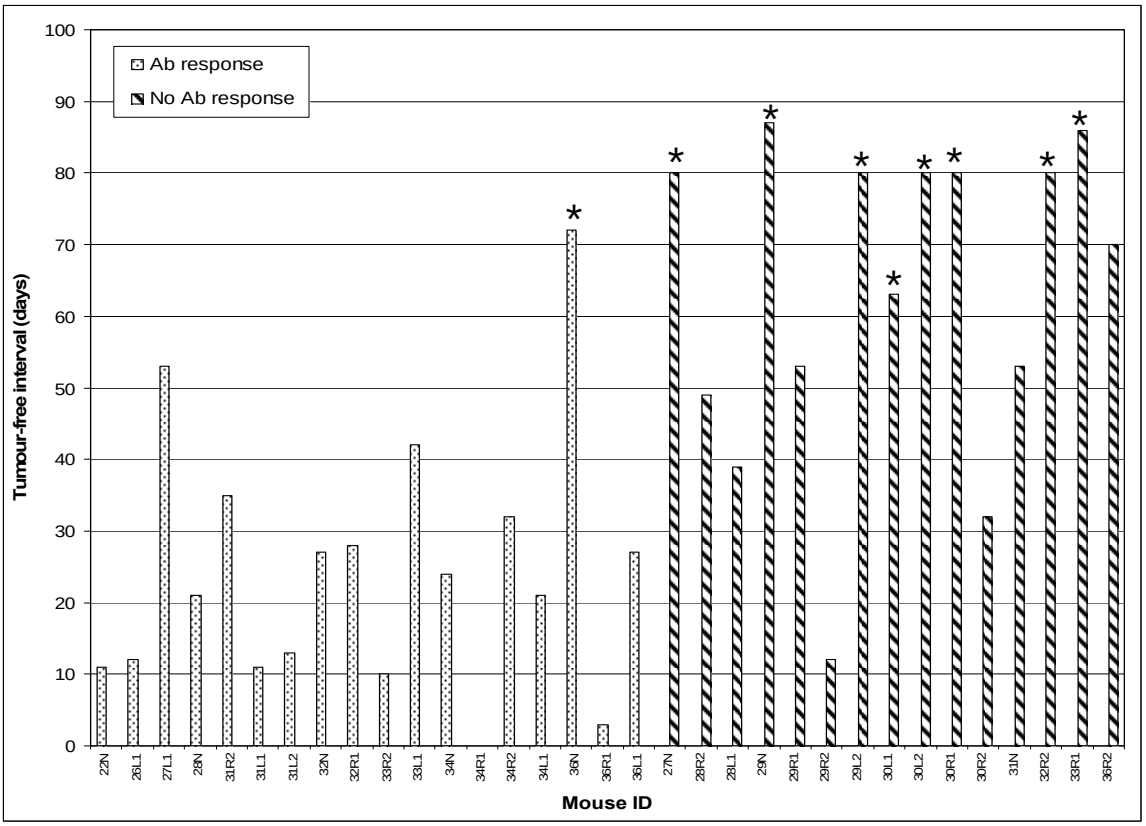


Figure 3. The majority of mice that did not have a PABP1 antibody (Ab) response had a longer tumour-free interval compared to those mice that did have a PABP1 antibody response. The * indicates that for these mice, the tumour-free interval was not determined, as the mice were sacrificed on the indicated day for the purpose of immunological analysis.

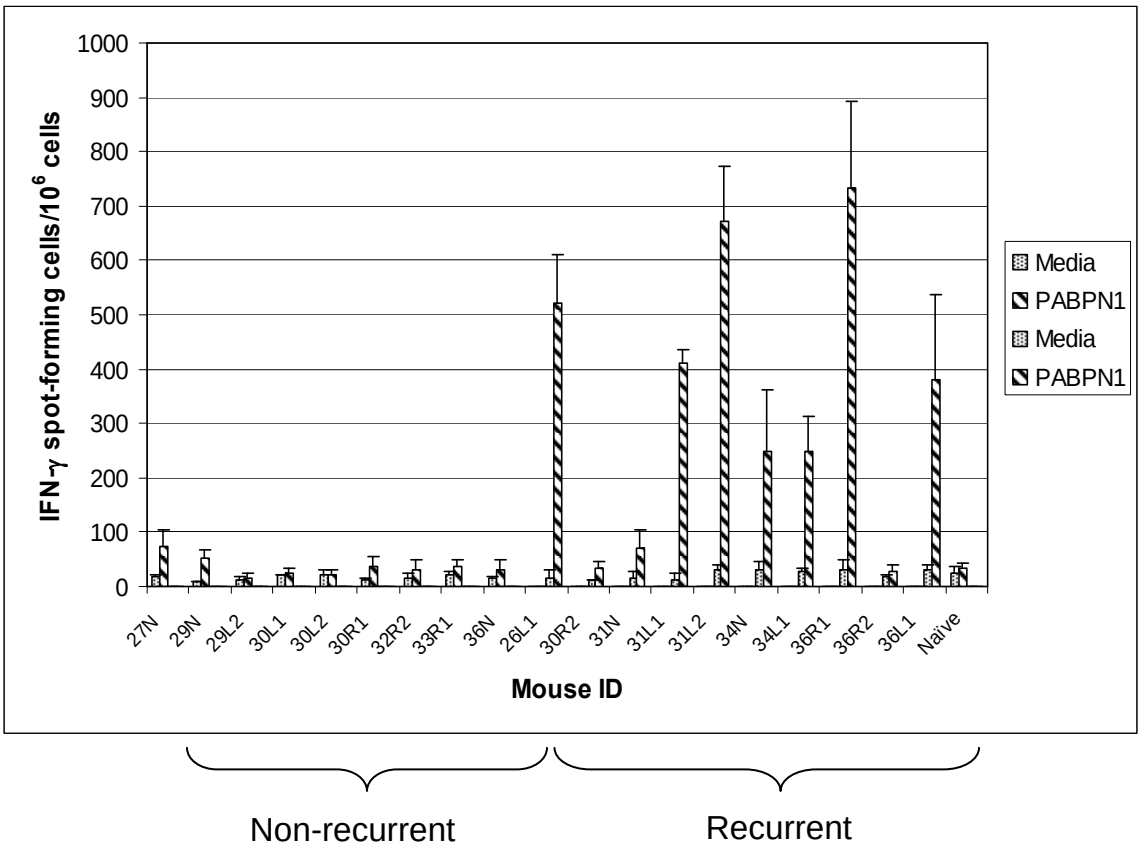


Figure 4. Castrated mice with recurrent tumours generally had a larger PABPN1-specific T cell response compared to those mice that remained tumour-free for the duration of the experiment. For each mouse shown on the graph, fresh splenocytes were used in the ELISPOT assay, which was run in triplicate, with the values being averaged and the standard deviation calculated. The splenocytes were stimulated with media as a negative control.

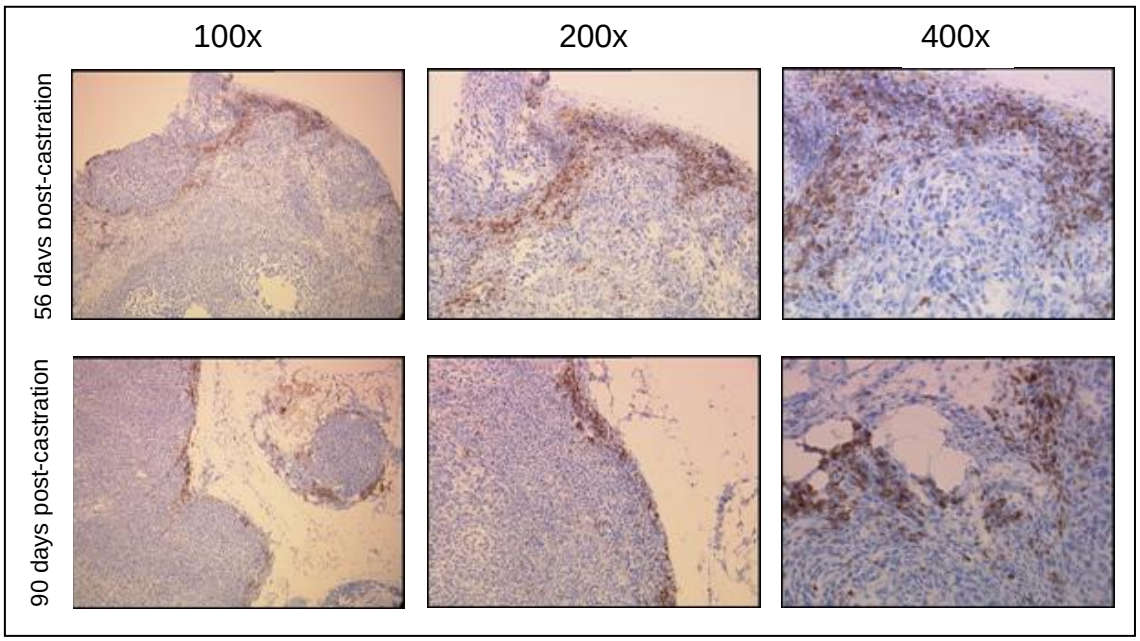


Figure 5. Representative recurrent tumours stained with anti-CD3 from one mouse sacrificed on day 56 post-castration and one mouse sacrificed on day 90 post-castration show that the CD3+ T cells are mainly confined to the periphery of the tumours and surrounding stroma.

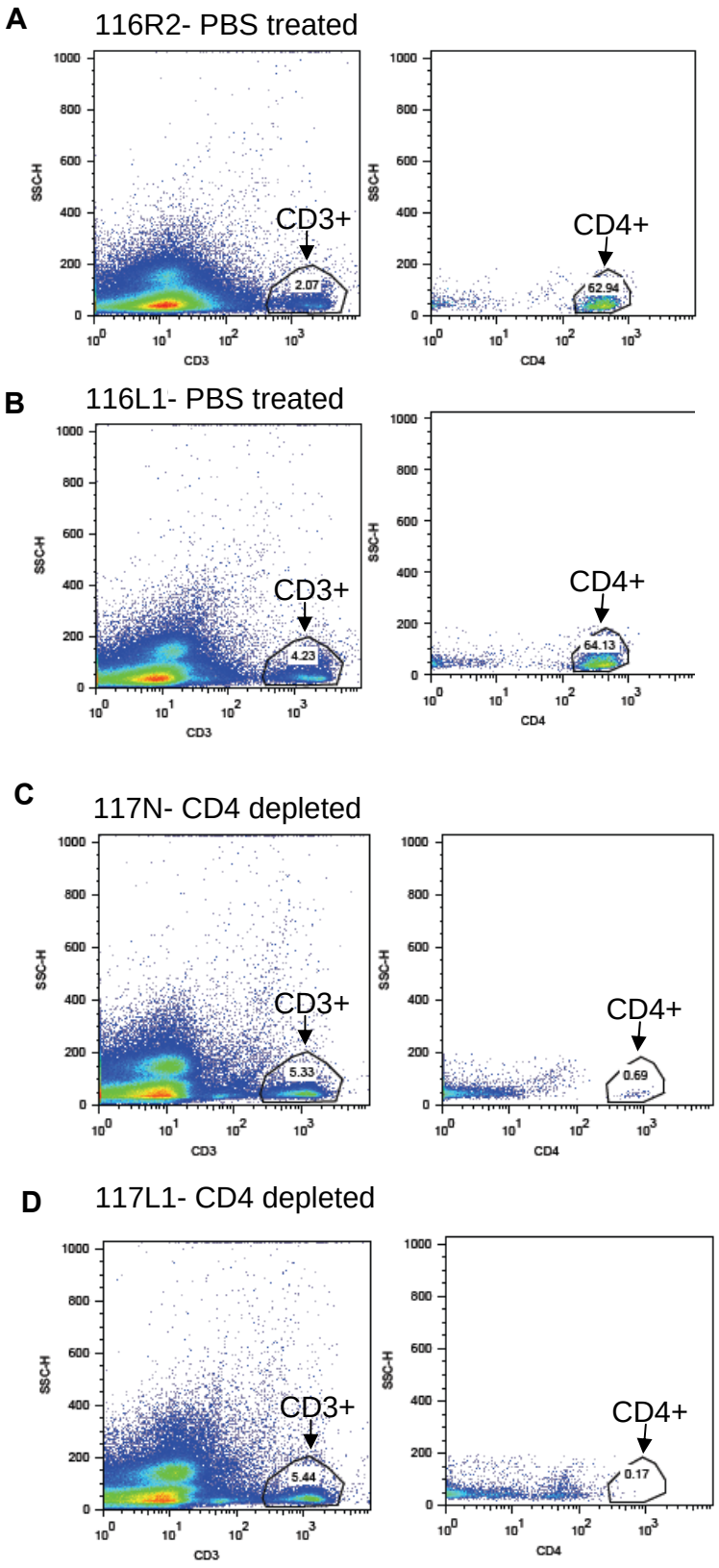


Figure 6:
Flow cytometry data from CD4 depletion experiments. Mice 116R2 (A) and 116L1 (B) were injected with PBS only while 117N (C) and 117L1 (D) were treated with anti-CD4 antibody. PBMCs were isolated from whole blood at Day 66 post-tumor injection. Cells were stained with anti-CD3 and anti-CD4 antibodies for flow cytometric analysis. The first panel shows the total percent of CD3+ T cells and the second panel shows the percent of CD4+ T cells in the CD3+ population. Those mice mice receiving the CD4 depleting antibody have no CD4+ T cells remaining in the CD3+ T cell population whereas the control mice have a healthy CD4+ population, indicating that the depletion was successful.

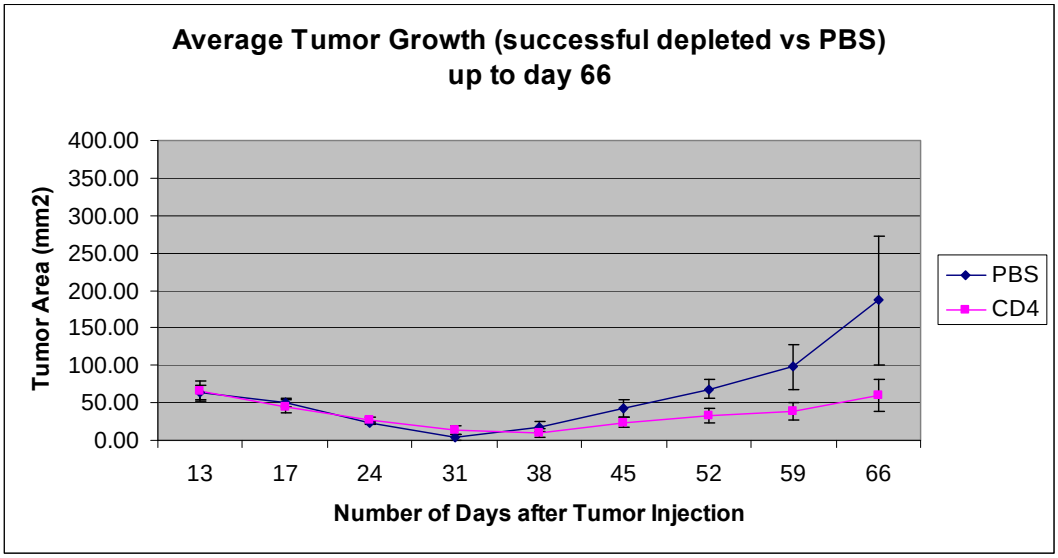


Figure 7: Tumor measurements from the CD4 depletion experiment comparing the average tumor area of the PBS control mice and the CD4 depleted mice. A significant difference between the two groups is demonstrated.

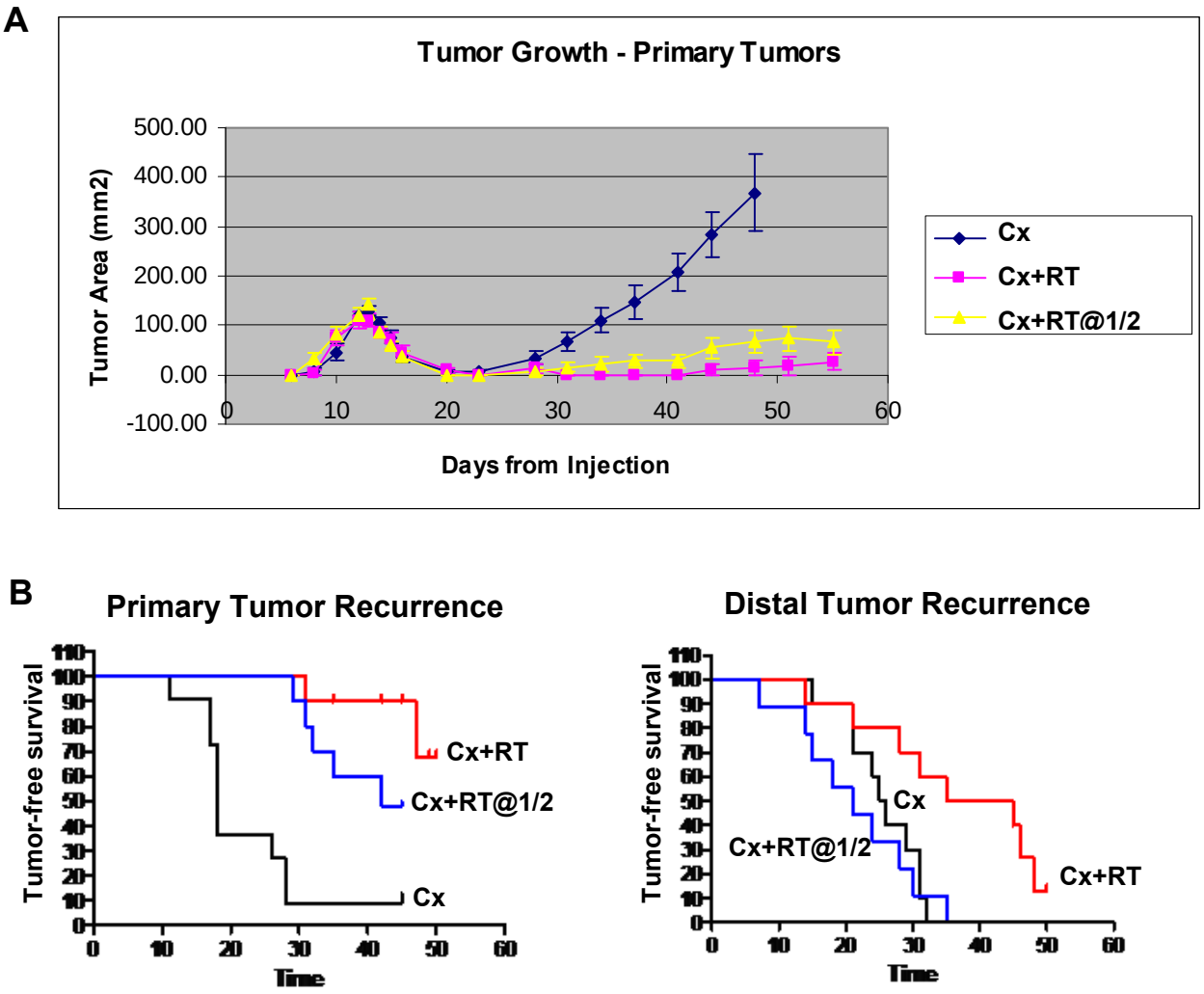
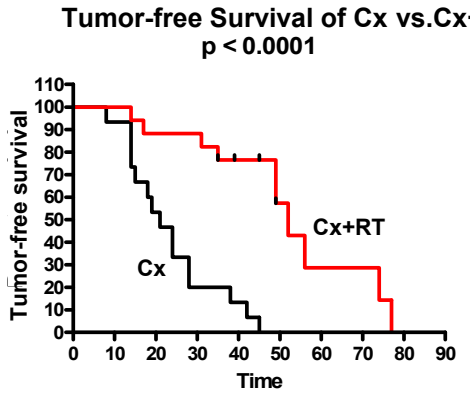
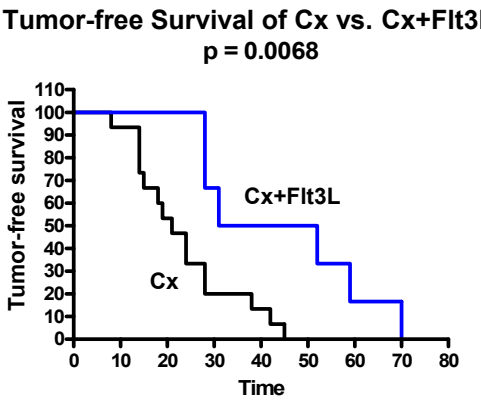
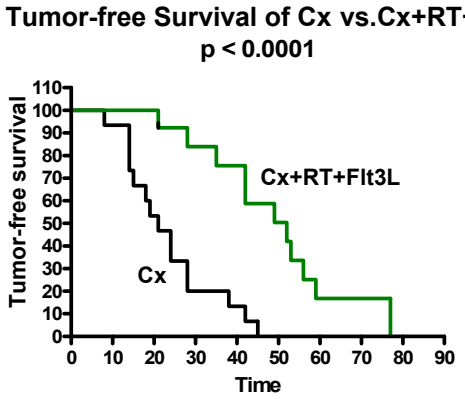


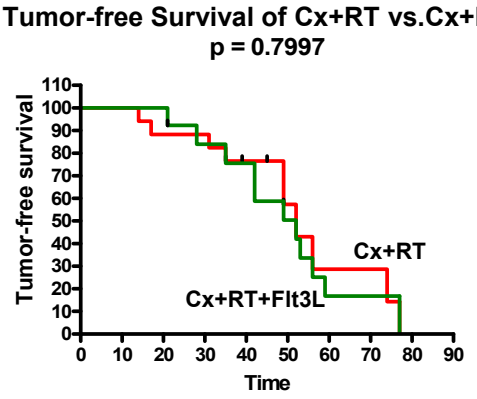
Figure 8: Demonstrating an abscopal response in the Shionogi tumor model. Mice were injected with two Shionogi tumors, one per flank. Tumors were allowed to reach 65-100 mm^2 before all mice were castrated. Some mice received no additional treatment (Cx). A second group of mice received 6 I-125 pellet at the point of maximal tumor regression in the primary tumor only (Cx+RT) while the third group received 6 I-125 pellets when the tumors had only partially regressed (Cx+RT@1/2). Figure 3A shows the rate of tumor growth of the primary tumor and clearly demonstrates the efficacy of radiation on primary tumor growth. Figure 2B shows a Kaplan-Meier curve comparing the rate of tumor recurrence of both the primary and distal tumors. The clear advantage of adding radiation is seen in the primary tumors. In the distal tumors a significant survival advantage is seen in mice treated with Cx+RT, evidence of an abscopal effect. Note this advantage is lost if mice are radiated before the tumors have fully regressed.

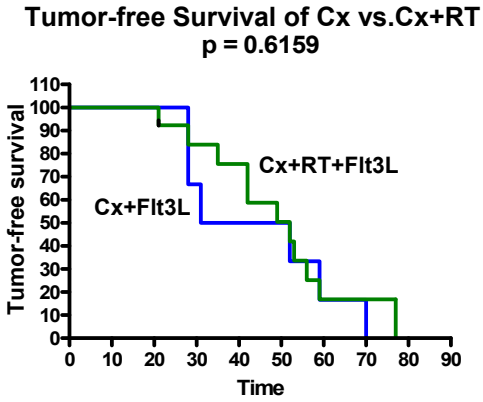
A


Tumor-free Survival of Cx vs.Cx+RT
p < 0.0001
Tumor-free survival
Time
Cx+RT
Cx


Tumor-free Survival of Cx vs. Cx+Flt3L
p = 0.0068
Tumor-free survival
Time
Cx+Flt3L
Cx


Tumor-free Survival of Cx vs.Cx+RT+Flt3L
p < 0.0001
Tumor-free survival
Time
Cx+RT+Flt3L
Cx


Tumor-free Survival of Cx+RT vs.Cx+RT+Flt3L
p = 0.7997
Tumor-free survival
Time
Cx+RT
Cx+RT+Flt3L


Tumor-free Survival of Cx vs.Cx+RT
p = 0.6159
Tumor-free survival
Time
Cx+RT+Flt3L
Cx+Flt3L

B

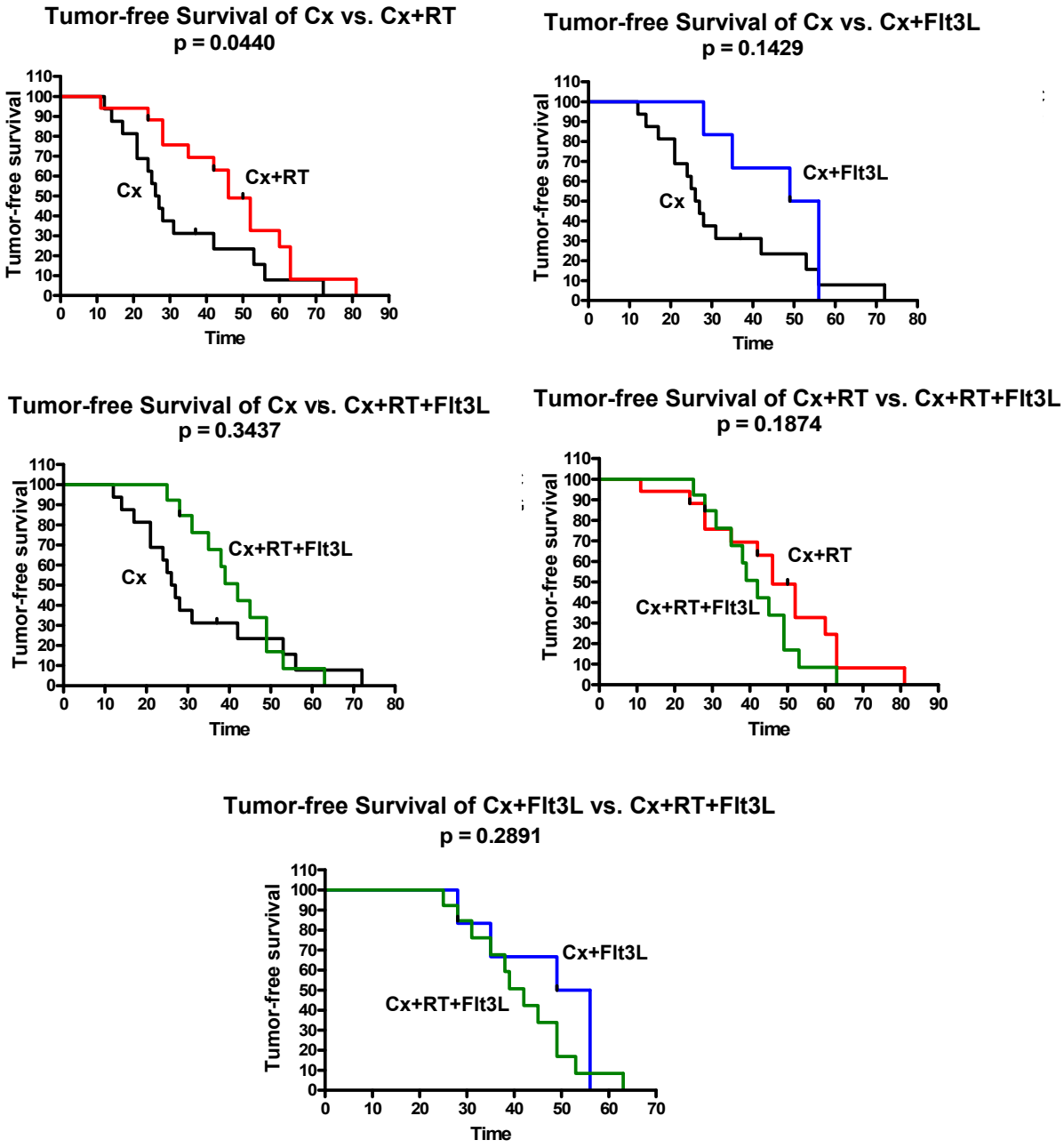


Figure 9: Kaplan-Meier analysis of tumor-free survival in mice treated with a combination of castration, brachytherapy and Flt3 ligand. A survival advantage is seen in the primary tumors of mice treated with a combination of Cx+RT, Cx+Flt3L and Cx+RT+Flt3L compared to Cx alone (Figure 9A). When examining the tumor-free survival of the distal tumors a slight, but significant advantage is seen in those mice treated with Cx+RT, as was noted in Figure 8. However, no additional survival advantage was seen with addition of Flt3L.

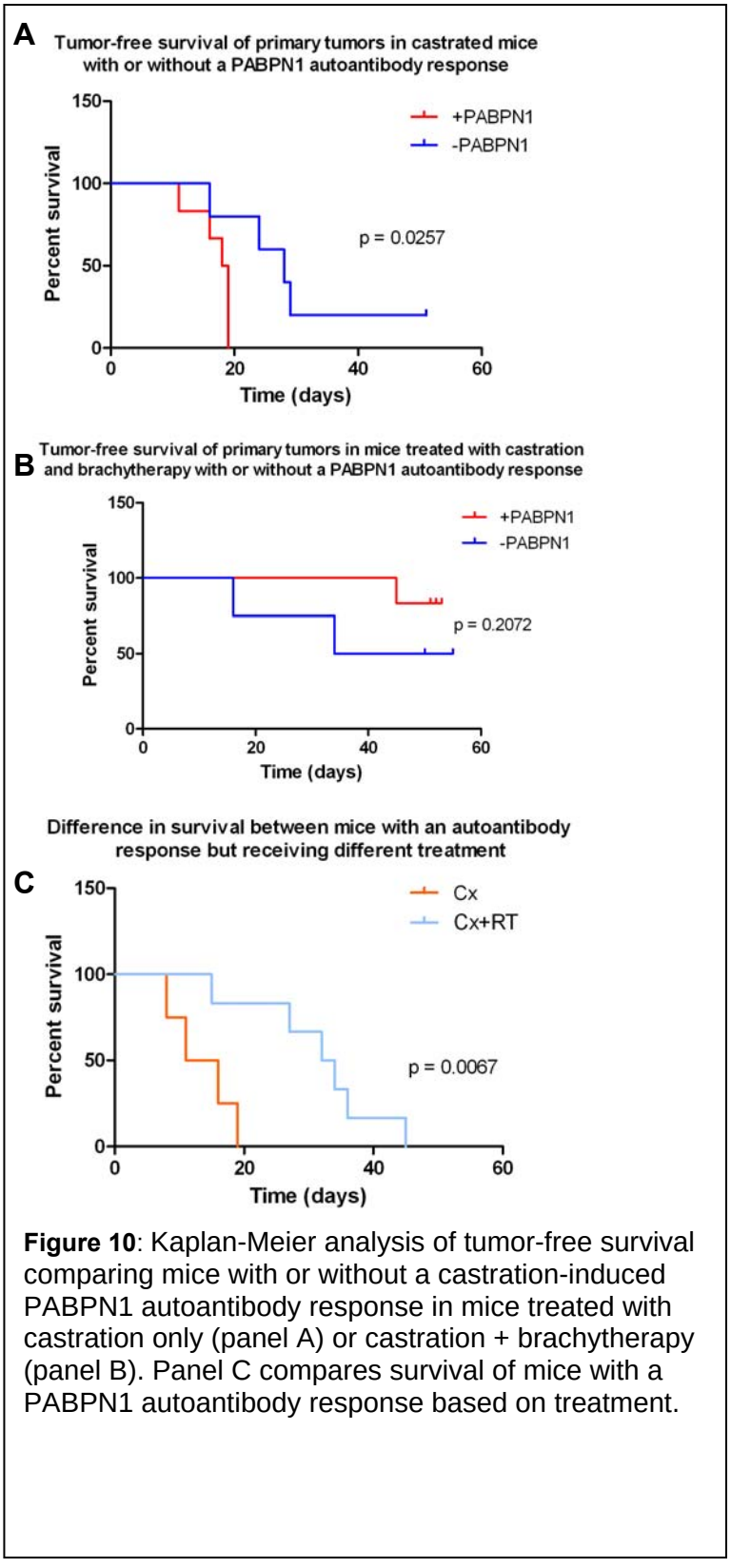


Figure 10: Kaplan-Meier analysis of tumor-free survival comparing mice with or without a castration-induced PABPN1 autoantibody response in mice treated with castration only (panel A) or castration + brachytherapy (panel B). Panel C compares survival of mice with a PABPN1 autoantibody response based on treatment.

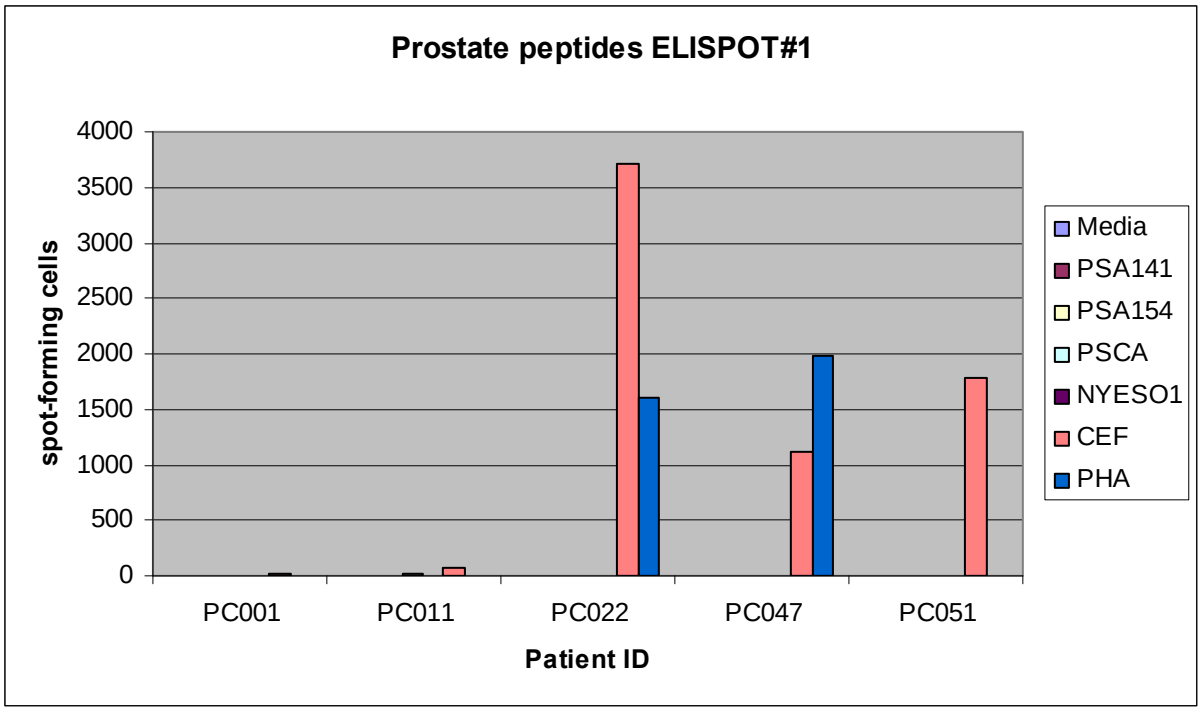


Figure 11: ELISPOT analysis of human prostate cancer patients stimulated with different prostate cancer antigen-specific peptides. Results show that none of the 5 patients tested had antigen-specific T cells against any of the 4 peptides tested. PHA was used as a non-specific T cell stimulant and shows a clear positive result in those 2 patients. In addition, three patients had T cells against the CEF peptide, demonstrating that the ELISPOT methodology worked.

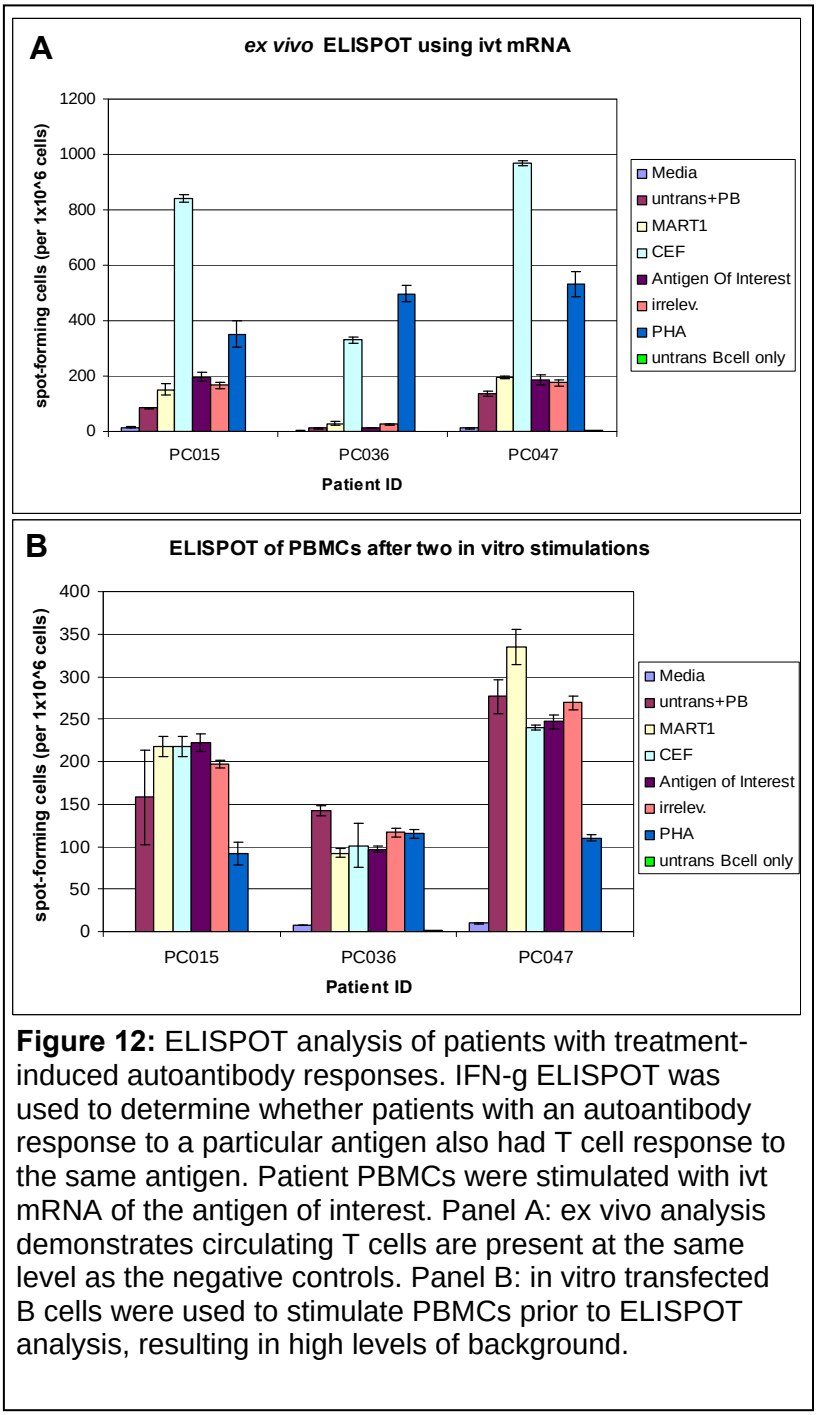


Figure 12: ELISPOT analysis of patients with treatment-induced autoantibody responses. IFN-g ELISPOT was used to determine whether patients with an autoantibody response to a particular antigen also had T cell response to the same antigen. Patient PBMCs were stimulated with ivt mRNA of the antigen of 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.