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PRINCIPAL INVESTIGATOR: Brad H. Nelson, Ph.D.

CONTRACTING ORGANIZATION: British Columbia Cancer Agency
Victoria BC V8R 6V5
Canada

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14. ABSTRACT We previously demonstrated that hormone therapy (HT) and radiation therapy (RT) induce tumor-specific autoantibody responses in human prostate cancer, and this grant investigates the clinical significance of these findings. In Aim 1, the findings that HT induces autoantibody and T cell responses against PABPN1 in the Shionogi tumor model and that these immune responses are associated with <u>inferior</u> outcomes have recently been submitted for publication. We have also shown that the combination of HT+RT in this model leads to delayed tumor recurrence of a distal untreated tumor. Work is underway to determine whether similar antibody and T cell responses are seen in these mice and whether they too are associated with poor outcomes. In the human setting, we have tested known prostate cancer tumor antigens by ELISPOT and begun cloning our serologically-defined tumor antigens in order to test these against PBMCs collected from prostate cancer patients showing treatment-induced autoantibody responses (Aim 2). We have also continued to assemble cohorts of prostate cancer patients with recurrent versus non-recurrent disease at 5 years post-treatment (Aim 3). In summary, this study is progressing on schedule and is revealing unexpected results that we believe may be highly relevant to prognosis and treatment of prostate cancer.					
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W81XWH-07-1-0259 Annual Report, March 2009

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. Our preliminary results in the androgen-dependent murine Shionogi tumor model indicate that castration (the laboratory equivalent of HT) induces tumor-specific autoantibody responses in approximately 50% of animals. Moreover, parallel studies of human prostate cancer patients undergoing standard treatments at our institution indicate that HT and RT both induce tumor-specific autoantibody responses in up to 30% of patients, depending on the stage of disease and specific treatment. Based on these observations, we hypothesized that treatment-induced autoantibody responses in prostate cancer are accompanied by CD4+ and CD8+ T cell responses that potentially delay or prevent tumor recurrence.

This study has three specific aims:

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

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

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

BODY:

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

As described in the 2008 Annual Report, we identified poly(A) binding protein nuclear 1 (PABPN1) as the treatment-induced ~40 kDa antigen in our Shionogi model and used it to determine that autoantibody and T cell responses were associated with poorer outcomes in castrated mice bearing Shionogi tumors. A manuscript entitled "Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model" describing these results has recently been submitted to the

International Journal of Cancer for publication. A copy of this manuscript can be found in the Appendix.

We have also completed a preliminary CD4 depletion experiment in the castration only setting. A total of 15 mice were used for this experiment. Seven mice served as controls and were injected with PBS x days prior to tumor injection. Eight mice were injected with 400 μ g of anti-CD4 antibody (clone GK1.5) at -3, -1, +3 days relative to castration. An additional 200 μ g of depleting antibody was injected weekly post castration. All mice were castrated when tumors reached 65-100 mm². Mice were re-injected with PBS or anti-CD4 antibody every 1 or 2 weeks. Flow cytometry on PBMCs from a subset of mice was performed to determine the efficacy of anti-CD4 depletion (Figure 1). PBMCs were stained with anti-CD3 to identify the T cell population (first panel, Figure 1 A-D) and with anti-CD4 (second panel, Figure 1 A-D). Results clearly show that the mice that were 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 (Figure 2). We will be repeating these experiments in the near future and expanding them to include depletion of the CD8+ T cells as well as CD19+ B cells. This will help us determine whether the poor outcomes seen in the castration only experiments are caused by the autoantibody or T cell immune response or whether the presence of PABPN1-specific autoantibodies and T cells are merely a marker of early tumor recurrence and poor prognosis.

Several additional mouse experiments were completed in the past year. While the experiments mentioned above focused only on castration, these experiments focused on the combination of castration and radiation therapy (in the form of brachytherapy) in the Shionogi model. These experiments were designed primarily to study the abscopal effect, described for ionizing radiation, where 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.

The first set of castration+RT experiments was initiated to determine whether castration and brachytherapy led to delayed tumor recurrence of an untreated distal tumor. It was established in previous experiments 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 used in the following 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 mm². In the remaining groups, mice were castrated when tumors reached 65-100 mm². 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 3A). Using Kaplan-Meier analysis we then compared the tumor-free survival between those three experimental groups (Figure 1B). 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 3B). 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, 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 Flt3 ligand, a potent stimulator of dendritic cells (DCs) and 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 mm², 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 4). Figure 4A 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 4B 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. 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.

With regard to the Statement of Work for Aim 1, we have completed the majority of the proposed experiments. The work that is left to complete includes performing the immunological assays (Western blots, T cell assays) for both Shionogi experiments described above to determine whether immune responses (autoantibody and/or T cell responses) are associated with poorer outcomes as was seen in the castration only experiments or if the addition of radiation therapy in this setting leads to improved outcomes as is seen in the clinical setting. In addition we plan on repeating the Flt3 ligand experiments with optimized timing of Flt3 ligand administration and repeating the depletion experiments to determine the immunological mechanism responsible for the poor outcomes seen in the castration only experiments. These experiments should be completed in 2009.

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 the 16 prostate cancer patients who showed treatment-induced antibody responses against specific antigens, with emphasis on the four patients in which the treatment-induced antigen was cloned. We were able to collect the large volume of blood from 11 of these patients including the 4 patients from whom antigens were cloned. PBMCs were isolated from these patients and frozen for use in T cell assays. The remaining 5 patients were contacted and agreed to donate a large volume of blood in the future when needed.

To ensure that the ELISPOT methodology was working we decided to test 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 5). 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.

In order to avoid having to map the CD8+ epitopes for each of our antigens, we have established an *in vitro* transcribed mRNA platform (ivt RNA), in which the antigen of interest is expressed in autologous antigen presenting cells (APCs) which process and display peptides regardless of HLA haplotype. We are currently cloning 6 treatment-induced antigens as summarized below (Note that ZNF707/PTMA was a double insert clone, thus each needs to be cloned individually in order to ascertain which one is the treatment-induced antigen):

Antigen Primers	Product Length (bp)	Status
PARP1 F: CAC CAT GGC GGA GTC TTC GGA TAA R: CCA CAG GGA GGT CTT AAA ATT GA	3042	In progress
SDCCAG1 F: CAC CAT GAA GAG CCG CTT TAG CAC R: TTT CCT TTT TAC GTT CAG AAG ATT GG	3228	In progress
ODF2 F: CAC CAT GTC TGC CTC ATC CTC AGG R: TCT TGG TAT GCG GGC C	1915	In progress
ZNF707 F: CAC CAT GGA CAT GGC CCA GGA R: CAC CTC CCC GTG CCT	1114	In progress
PTMA F: CAC CAT GTC AGA CGC AGC CGT A R: GTC ATC CTC GTC GGT CTT CT	331	Cloned successfully
CEP78 F: CAC CAT GAT CGA CTC CGT GAA GCT R: GGA ATG CAG GTC CTT TCC AG	2166	In progress
SWAP70 F: CAC CAT GGG GAG CTT GAA GGA G R: CTC CGT GGT CTT TTT CTC TTT CC	1756	Cloned successfully

Once all the antigens are successfully cloned, they will be transfected into autologous B cells, which will act as APCs to allow *in vitro* transcription and presentation of peptides. ELISPOT assays will then be performed to determine whether antigen-specific T cells are present in these patients.

With regard to the Statement of Work for Aim 2, we will be concentrating our efforts on completing the T cell assays in the human patients. This should be completed by the summer of 2009. We are also planning to construct the yeast display library over the summer of 2009 to facilitate cloning of the remaining treatment-induced antigens identified by Western blot. We have deferred screening of the remaining 79 patients until analysis of the initial cohort is complete.

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=17) or not recurred (n=43). Recruitment of recurrent patients continues to be a challenge thus we have expanded our search to include patients diagnosed in 2003-2004, as this now represents the 5 year time frame. In addition, the subjects in our initial cohort of 174 prostate cancer patients are approaching the 5 year anniversary since treatment. Thus, these patients may provide an additional resource for this Aim, with the added benefit of being accompanied with pre-treatment blood draws. With regard to the Statement of Work, we expect that we will be able to complete recruitment of these two groups within 6 months. During this time, as mentioned above, we plan to construct the yeast display library and then begin analysis of serum samples at the end of 2009.

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. Submitted to Int J Cancer, March 2009).

- The combination of castration and brachytherapy led to a delay in tumor recurrence, evidence of an abscopal response, in the murine Shionogi tumor model.
- An *in vitro* transcribed mRNA (ivt RNA) platform has been established to allow analysis of CD8+ T cell responses regardless of HLA haplotype.

REPORTABLE OUTCOMES:

Manuscripts:

Hahn S, Nesslinger NJ, Drapala RJ, Bowden M, Rennie PS, Pai HH, Ludgate C, Nelson BH. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. Submitted to Int J Cancer, March 2009.

Book chapters:

Nesslinger NJ, Pai HH, Ludgate CM, Nelson BH. Exploring the effects of standard treatments on the immune response to prostate cancer. 2009. *Methods of Cancer Diagnosis, Therapy, and Prognosis, Vol. 2.* Hayat, M.A. (Ed.).

Presentations:

Mapping and Manipulating the Immune Response to Cancer. Brad H. Nelson. City of Hope Medical Center, Duarte CA, March 2008.

Mapping and Manipulating the Immune Response to Cancer. Brad H. Nelson. University of Calgary, Calgary AB, April 2008.

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

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

Degrees Obtained:

Sara Hahn

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

CONCLUSION:

Both the murine and human portions of this study are progressing well and on schedule. The Shionogi experiments have produced some interesting and exciting results to date. We have recently submitted a manuscript detailing the castration only experiments in which we found that mice with autoantibody and T cell responses had poorer outcomes than those mice without an immune response. We have also found that the combination of castration and brachytherapy led to delayed tumor recurrence of a distal, unirradiated tumor in the Shionogi model, but only when the tumors had maximally regressed after castration. We are currently performing the immunological analyses to determine whether immune responses are associated with poor outcomes as was seen in the castration only experiments or whether the addition of brachytherapy leads to improved outcomes.

REFERENCES:

None

APPENDIX:

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

Please see following page for manuscript.



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

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

SARA HAHN^{1,2}, NANCY J. NESSLIN³, ROBERT J. DRAY⁴, MARY BOWDEN⁴, PAUL S. RENNEL⁴, HOWARD H. PAI³, CHARLES LUDGATE³, BRAD H. NELSON^{1,2}

¹DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY, UNIVERSITY OF VICTORIA, VICTORIA

²TREV AND JOYCE DEELEY RESEARCH CENTRE, BRITISH COLUMBIA CANCER AGENCY, 2410 LEE AVENUE, VICTORIA, BC, CANADA

³RADIATION ONCOLOGY PROGRAM, BC CANCER AGENCY-VANCOUVER ISLAND CENTRE, VICTORIA, BC, CANADA

⁴PROSTATE CENTRE AT VANCOUVER GENERAL HOSPITAL, VANCOUVER, BC, CANADA

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Correspondence: DR. BRAD NELSON, BC CANCER AGENCY, 2410 LEE AVENUE, VICTORIA, BC, V8P 6V5, CANADA. EMAIL: BNELSON@BCCANCER.BC.CA

ABSTRACT

WE RECENTLY REPORTED THAT HORMONE THERAPY INDUCES ANTIGEN-SPECIFIC AUTOANTIBODY RESPONSES IN PROSTATE CANCER PATIENTS. HOWEVER, THE CONTRIBUTION OF AUTOANTIBODIES TO CLINICAL OUTCOMES IS UNKNOWN. WE USED AN ANIMAL MODEL TO TEST THE HYPOTHESIS THAT THERAPY-INDUCED IMMUNE RESPONSES MAY BE ASSOCIATED WITH DELAYED TUMOR RECURRENT. DD/S MICE BEARING ESTABLISHED TUMORS FROM THE ANDROGEN-DEPENDENT SHIONOGI CARCINOMA WERE CASTRATED TO INDUCE TUMOR REGRESSION. TUMOR-SPECIFIC AUTOANTIBODY RESPONSES WERE MEASURED BY IMMUNOBLOT, AND THE UNDERLYING ANTIGEN WAS IDENTIFIED BY SEROLOGICAL ANALYSIS OF A CDNA EXPRESSION LIBRARY. T CELL RESPONSES WERE ASSESSED BY IMMUNOHISTOCHEMISTRY AND ELISPOT. FOLLOWING CASTRATION, 97% OF MICE UNDERWENT COMPLETE TUMOR REGRESSION. 72% EXPERIENCED TUMOR RECURRENCE 18-79 DAYS POST-CASTRATION, WHILE 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 PHOSPHOPROTEIN 1 (PABPN1). CASTRATION ALSO INDUCED PABPN1-SPECIFIC T CELL RESPONSES, WHICH WERE HIGHLY CORRELATED WITH AUTOANTIBODY RESPONSES, AND THIS WAS ACCOMPANIED BY DENSE INFILTRATION OF TUMOR-INFILTRATING LYMPHOCYTES 1-2 WEEKS AFTER CASTRATION. UNEXPECTEDLY, MICE THAT DEVELOPED AUTOANTIBODY RESPONSES TO PABPN1 SHOWED A HIGHER RATE AND SHORTER LATENCY OF TUMOR RECURRENCE. WITH RECURRENT TUMORS, T CELL RESPONSES TO PABPN1 WERE STILL DETECTABLE; HOWEVER, INFILTRATES WERE RESTRICTED TO THE PERIPHERAL STROMA OF TUMORS. IN CONCLUSION, TREATMENT-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.

INTRODUCTION

PROSTATE CANCER IS THE MOST FREQUENTLY DIAGNOSED CANCER IN NORTH AMERICA. DESPITE IMPROVEMENTS IN EARLY DETECTION DUE TO PROSTATE-SPECIFIC ANTIGEN (PSA) SCREENING, IT REMAINS THE SECOND LEADING CAUSE OF CANCER-RELATED DEATHS IN MEN. ANDROGEN DEPRIVATION OR RADIATION THERAPIES (RT) ARE USUALLY SUCCESSFUL IN CONTROLLING ORGAN-CONFINED TUMORS. HOWEVER, IF TUMORS RECUR, THE DISEASE IS TYPICALLY SYSTEMIC AND HORMONE THERAPY (HT) REMAINS THE MAIN TREATMENT OPTION. WHILE HT IS INITIALLY EFFICACIOUS, PATIENTS EVENTUALLY PROGRESS TO ANDROGEN-RESISTANT DISEASE, WHICH IS THE MAIN CAUSE OF DEATH. THERE IS CLEAR NEED FOR IMPROVED TREATMENTS TO PREVENT THE DEVELOPMENT OR OUTGROWTH OF ANDROGEN-INDEPENDENT TUMORS.

WHILE BOTH HT AND RT MEDIATE DIRECT KILLING OF TUMOR CELLS, THERE IS GROWING EVIDENCE THAT THESE TREATMENTS CAN ALSO INDUCE TUMOR-SPECIFIC IMMUNE RESPONSES. RODENRATH *et al.* DEMONSTRATED THAT ANDROGEN DEPRIVATION IN TUMOR-FREE MALE MICE INCREASED THE NUMBER OF T CELLS RESIDING IN PERIPHERAL LYMPHOID TISSUES, AND ALSO LED TO TRANSIENT INCREASED 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 ANTIGENS FOR ANTIGEN PRESENTING CELLS (APCs). INDEED, MERCADER *et al.* DEMONSTRATED THAT HT CAUSED INCREASED LEVELS OF APCs EXPRESSING THE T CELL CO-STIMULATORY MOLECULES B7.1 AND B7.2 IN PROSTATE CANCER PATIENTS, WHICH WAS ACCOMPANIED BY PROFUSE INFILTRATION OF TUMOR-INFILTRATING T CELLS. SIMILARLY, EXTERNAL BEAM RT INDUCES TUMOR CELL NECROSIS AND APOPTOSIS, WHICH IS ACCOMPANIED WITH INFLAMMATORY OR OTHER "DANGER" SIGNALS. THESE SIGNALS PROVIDE BOTH ANTIGEN PRESENTATION AND MATURATION SIGNALS TO DENDRITIC CELLS. AND OTHER FACTORS LEAD TO THE INDUCTION OF CD4+ AND CD8+ T CELL RESPONSES, WHICH CAN ULTIMATELY ELICIT AN ANTI-TUMOR EFFECT. 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. AMOUNTING 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. IN THE SHIONOGI TUMOR MODEL, TUMORS ARE ANDROGEN-DEPENDENT SUCH THAT SURGICAL CASTRATION PRECIPITATES APOPTOSIS AND TUMOR REGRESSION IN A REPRODUCIBLE MANNER, SIMILAR TO THAT SEEN AFTER ANDROGEN WITHDRAWAL IN HUMANS.

PATIENTS. HOWEVER, SIMILAR TO HUMAN PROSTATE CANCER, THE ANDROGEN-DEPLETED EL
RSE TO ANDROGEN-INDEPENDENT RECURRENT TUMORS^{15,17} IN ~~PROSTATE~~ MORE, SHIONOGI
TUMOR CELLS THAT SURVIVE HORMONE WITHDRAWAL, LIKE HUMAN PROSTATE TUMOR CEL
PROTEINS IMPLICATED IN CELL SURVIVAL AND PROGRESSION TO¹⁸ ANDROGEN INDEPENDENCE
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 HUMA
DISCUSSED ABOVE¹² THEUS, THE SHIONOGI MODEL PROVIDES AN EXPERIMENTAL SYSTEM FOR STU
RELATIONSHIP BETWEEN TREATMENT-INDUCED IMMUNE RESPONSES AND OUTCOMES. HERE,
MODEL TO TEST THE HYPOTHESIS THAT CASTRATION-INDUCED IMMUNE RESPONSES MAY BE
DELAYED TUMOR RECURRENCE.

MATERIALS AND METHODS

SEREX screening

SEREX SCREENING OF A PROSTATE CANCER PHAGE CDNA LIBRARY WAS CARRIED OUT
DESCRIBED¹³ USING MOUSE SERA DILUTED 1/400 IN TBS/1% BSA. A DONKEY ANTI-MOUSE IGG AL
PHOSPHATASE-CONJUGATED ANTIBODY WAS USED FOR SECONDARY SCREENING (JACKSON I
LABORATORIES, WEST GROVE, PA).

Cloning and purification of SEREX-identified antigens

TO SOLATE FULL-LENGTH CDNA CLONES FOR ANTIGENS IDENTIFIED BY SEREX, TOTAL
EXTRACTED FROM⁷ SHIONOGI TUMOR CELLS USING THE RNEASY MINI KIT (QIAGEN, MISSISSA
CANADA) AND THEN 0.08 μ G TOTAL RNA WAS SYNTHESIZED INTO CDNATM USING SUPERS
TRANSCRIPTASE (INVITROGEN, BURLINGTON, CANADA). PCR PRODUCTS WERE PURIFIED USIN
GEL EXTRACTION KIT (INVITROGEN, MISSISSAUGA, CANADA),TM CLONED[®] INTO PENTR
(INVITROGEN BURLINGTON, CANADA) AND TRANSFORMED INTO ONE SHOT[®] TOP10 CHEMICA
E. coli (INVITROGEN, BURLINGTON, CANADA). CLONES WERE VERIFIED BY SEQUENCING BEFO
INTO THE *E. coli* EXPRESSION VECTORTM PDESTTM (INVITROGEN, BURLINGTON, CANADA). CLONES WERE
TRANSFORMED INTO BLTM21TM CELLS (INVITROGEN, BURLINGTON, CANADA), AND PROTEIN PRODUC
INDUCED BY ADDITION OF ARABINOSE. AFTER 2 HOURS, BACTERIAL PELLETS WERE RE-SUSPE
MM TRIS-HCL, PH 7.5, 500 MM NA CL, 20 MM IMIDAZOLE, AND 1 MM DITHIOTHREITOL. AFTER O
FREEZE-THAW CYCLE AT -80 °C, BACTERIA WERE SONICATED AND CENTRIFUGED. SUPERNAT

WERE ANALYZED BY SDS-PAGE, AND FRACTIONS CONTAINING THE MOST PROTEIN WERE LOADED ON TO A HITRAP IMAC FF NICKEL COLUMN (GE HEALTHCARE, PISCATAWAY, NJ) AND PURIFIED BY IMMEDIATELY FOLLOWED BY METAL ION ADSORPTION CHROMATOGRAPHY (IMAC) USING THE ÄKTA FPLC (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 MICE 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 MICE WERE INJECTED SUBCUTANEOUSLY IN THE NECK REGION WITH SHIONOGI CARCINOMA CELLS. WHEN TUMORS REACHED ~8 MM X 10 MM IN SIZE, MICE WERE CASTRATED TO INDUCE ANDROGEN DEPENDENT SUBSEQUENT TUMOR REGRESSION. SERIAL BLOOD SAMPLES WERE COLLECTED FROM THE TAIL VEIN PRIOR TO INOCULATION, BEFORE CASTRATION, AND THEN TWICE WEEKLY FOLLOWING CASTRATION. TUMOR LENGTH (WIDTH) WAS MEASURED USING MICRO CALIPERS. TUMORS WERE CONSIDERED TO HAVE RECURRENTLY BECOME PALPABLE. UNLESS OTHERWISE INDICATED, MICE WERE SACRIFICED WHEN RECURRENT TUMORS WERE APPROXIMATELY 10% OF TOTAL BODY WEIGHT. ON NECROPSY, TERMINAL BLOOD SAMPLES WERE COLLECTED BY CARDIAC PUNCTURE. TUMORS WERE REMOVED AND DIVIDED IN TWO HALVES, WHICH WERE STORED IN LIQUID NITROGEN OR FIXED IN 10% FORMALIN. LYMPH NODES AND SPLEEN WERE PROCESSED TO SINGLE-CELL SUSPENSIONS USING THE BLUNT END OF A 5 MM SYRINGE AND A 40-µM CELL STRAINER. SPLENOCYTES WERE RE-SUSPENDED IN ACK LYSIS BUFFER (1X ACK LYSIS BUFFER, 0.1 MM EDTA, 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 CASTRATED MICE. FROZEN TUMORS WERE PULVERIZED INTO A FINE POWDER IN LIQUID NITROGEN AND SUSPENDED IN LYSIS BUFFER (1X DULBECCO'S PBS, 0.01% TRITON, PROTEASE INHIBITOR COCKTAIL). TUMORS WERE HOMOGENIZED THROUGH 18G AND 21G NEEDLES, AND THEN SONICATED. ALIQUOTS CONTAINING 10 µg OF PROTEIN LYSATE WERE IMMUNOBLOTTED WITH MOUSE SERUM (1/500) FOLLOWED BY HRP-CONJUGATED ANTI-MOUSE IGG (H+L; JACKSON IMMUNORESEARCH, WEST GROVE, PA) AS DESCRIBED PREVIOUSLY.

Flow cytometry

TO ISOLATE TUMOR-INFILTRATING LYMPHOCYTES, TUMOR FRAGMENTS WERE PRESSES μ M MEMBRANE WITH THE BLUNT END OF A 5 MM SYRINGE, AND THE RESULTING CELL SUSPE CENTRIFUGED AND RE-SUSPENDED IN 0.5 ML OF 1.0% BSA/PBS. CELLS WERE STAINED IN VARI COMBINATIONS WITH THE FOLLOWING FLUOROCHROME-CONJUGATED ANTIBODIES AT 1/400 BSA/PBS: CD3-FITC, CD4-PE, CD4-PERCP, CD8-CY-CHROME, AND CD44-PE (BECTON, DICKINSON AND COMPANY, OAKVILLE, ON). ISOTYPE-MATCHED FLUOROCHROME-CONJUGAT SERVED AS NEGATIVE CONTROLS. CELLS WERE ANALYZED ON FACS DIVA EXTENDED BY SYSTEM (BECTON, DICKINSON AND COMPANY, OAKVILLE, CANADA) WITH FLOWJO SOFTWARE INC., ASHLAND, OR).

IFN- γ ELISPOT assay

96-WELL MULTISCREEN 0.45 μ M FILTER PLATES (MILLIPORE, MA) WERE PRE-WET WITH 70% ETHANOL FOLLOWED BY THREE WASHES WITH STERILE PBS. PLATES WERE INCUBA 4°C WITH 50 μ L/WELL ANTI-MOUSE IFN- γ (10 μ G/ML; MABTECH, MARIEMONT, OH). AFTER THREE PBS WASHES, PLATES WERE BLOCKED WITH T CELL MEDIA (RPMI-1640 SUPPLEMENTED WITH 10 MM SODIUM PYRUVATE, 2 MM L-GLUTAMINE, 100 μ G/ML PENICILLIN/STREPTOMYCIN, AND 25 μ M MERCAPTOETHANOL) FOR 2 HOURS AT 37°C. T CELLS WERE ADDED TO EACH WELL. PABPN1 PROTEIN WAS ADDED TO A FINAL CONCENTRATION OF 10 μ G/ML. CONA WAS ADDED TO A FIN OF 2 μ G/ML. T CELL MEDIA WAS USED AS A NEGATIVE CONTROL. SAMPLES WERE RUN IN TRIE WERE INCUBATED FOR AT LEAST 20 HOURS AT 37 °C. AFTER WASHING SIX TIMES WITH PBS/0. 20, 100 μ L OF BIOTINYLATED ANTI-MOUSE IFN- γ (10 μ G/ML IN 0.5% BSA/PBS/0.05% TWEEN-20; MABTECH, R4-6A2, MARIEMONT, OH) WAS ADDED TO EACH WELL. PLATES WERE LI HOURS AT 37 °C AND THEN WASHED 12 TIMES WITH PBS/0.05% TWEEN-20. AVIDIN PEROXIDASE COMPLEX (VECTOR LABORATORIES, BURLINGAME, CA) (100 μ L/WELL) WAS ADDED FOLLOWED INCUBATION AT ROOM TEMPERATURE. PLATES WERE WASHED AS ABOVE AND THEN DEVELO VECTASTAIN AEC SUBSTRATE KIT (VECTOR LABORATORIES, BURLINGAME, CA) FOR APPROXI MINUTES. DEVELOPMENT WAS STOPPED BY RINSING WITH TAP WATER. AIR-DRIED PLATES W ZELNET CONSULTING, INC. (FORT LEE, NJ) FOR ENUMERATION OF SPOTS USING AN AUTOMAT 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 TWO INDIVIDUALS WHO WERE BLIND AS 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 INDUCED IGG AUTOANTIBODY RESPONSES TO AN UNIDENTIFIED ~40 KDA ANTIGEN IN APPROXIMATELY 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 CLONED HUMAN ANTIGEN EXPRESSION LIBRARY DERIVED FROM HUMAN PROSTATE CANCER CELL LINES. WE IMMUNOBLOTTED LYSATE FROM THE HUMAN PROSTATE CANCER CELL LINE LNCAP WITH SERA FROM CASTRATION-BEARING, CASTRATED MICE THAT WERE POSITIVE FOR AUTOANTIBODIES TO THE ~40 KDA ANTIGEN. A SEROREACTIVE BAND WAS SEEN AT ~40 KDA, INDICATING THE ANTIGEN SEEN IN SHIONOGI TUMORS MAY INDEED HAVE A HUMAN HOMOLOG (DATA NOT SHOWN). TO IDENTIFY THE ANTIGEN, WE SCREENED APPROXIMATELY 2,300 CLONES OF THE HUMAN PROSTATE CDNA EXPRESSION LIBRARY WITH MOUSE SERA THAT WERE POSITIVE FOR AUTOANTIBODIES TO THE ~40 KDA ANTIGEN. FOUR SEROLOGICALLY DISTINCT ANTIGENS WERE CLONED. THE CORRESPONDING RECOMBINANT PROTEINS WERE IMMUNOBLOTTED WITH ADDITIONAL MOUSE SERA THAT WERE KNOWN TO BE POSITIVE OR NEGATIVE FOR AUTOANTIBODIES TO THE ~40 KDA ANTIGEN. THE PATTERN OF SEROREACTIVITY TO ONE ANTIGEN, POLY(A) BINDING PROTEIN 1 (PABPN1), WAS IDENTICAL TO THE PATTERN OF SEROREACTIVITY TO THE ~40 KDA ANTIGEN FROM SHIONOGI TUMOR LYSATES (FIGURE 1A), SUGGESTING PABPN1 WAS THE CORRECT ANTIGEN.

TO CONFIRM THAT PABPN1 WAS THE ~40 KDA ANTIGEN IDENTIFIED IN SHIONOGI TUMOR LYSATE, FIVE 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 (FIGURE 1B).

BY WESTERN BLOT, PABPN1 WAS EXPRESSED AT HIGH LEVELS IN SHIONOGI TUMOR LYSATE, BUT NOT IN NORMAL LIVER, LUNG AND UTERINE MOUSE TISSUES. BY CONTRAST, PABPN1 WAS NOT EXPRESSED IN NORMAL KIDNEY, SKELETAL MUSCLE OR HEART (FIGURE 1C). THIS IS IN ACCORD WITH PUBLICLY AVAILABLE DATA ON HUMAN TISSUES, WHERE PABPN1 IS EXPRESSED AT HIGHER LEVELS IN LIVER, LUNG, AND UTERUS THAN TO KIDNEY, MUSCLE, AND HEART.

Autoantibody and T cell responses to PABPN1

TO ESTABLISH THE TIME COURSE OF AUTOANTIBODY RESPONSES TO PABPN1, A COHORT OF TUMOR-BEARING MICE BEARING ESTABLISHED SHIONOGI TUMORS WERE CASTRATED, AND SERIAL BLOOD SAMPLES WERE COLLECTED. AUTOANTIBODY RESPONSES TO PABPN1 BY IMMUNOBLOTTING. OVERALL, 18/33 MICE (54.5%) SHOWED AN AUTOANTIBODY RESPONSE TO PABPN1, WHICH APPEARED AN AVERAGE OF 26 DAYS POST-CASTRATION (RANGE 6-47 DAYS POST-CASTRATION). BY CONTRAST, AUTOANTIBODIES TO PABPN1 WERE NOT FOUND IN SERA FROM TUMOR-BEARING, NON-CASTRATED MICE OR CASTRATED NON-TUMOR-BEARING MICE.

THE FACT THAT AUTOANTIBODIES TO PABPN1 WERE OF THE IGG SUBCLASS SUGGESTED THE PRESENCE OF AN UNDERLYING T CELL RESPONSE. THIS WAS INVESTIGATED BY MEANS OF IN VITRO T CELL ASSAYS OF SPLENOCYTES. AS EXPECTED, WILD-TYPE MICE IMMUNIZED WITH RECOMBINANT PABPN1 SHOWED STRONG T CELL RESPONSES TO PABPN1, WHEREAS NON-IMMUNIZED CONTROL MICE SHOWED NO RESPONSE (FIGURE 1D). OF 19 TUMOR-BEARING CASTRATED MICE EXAMINED, 7 (36.8%) SHOWED A STRONG T CELL RESPONSE TO PABPN1. ALL OF THESE MICE ALSO HAD AN AUTOANTIBODY RESPONSE TO PABPN1. CONVERSELY, OF 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 LOW CONCORDANCE BETWEEN AUTOANTIBODY AND T CELL RESPONSES. SPECIFICALLY, NON-TUMOR-BEARING MICE CASTRATED SHOWED MODEST T CELL RESPONSES TO PABPN1 (FIGURE 1D). THUS, CASTRATION 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 AUTOANTIBODIES AND TUMOR RECURRENCE. FOLLOWING CASTRATION, 32/33 MICE EXPERIENCED COMPLETE TUMOR RESECTION. OF THESE 32 MICE, 72% (23/32) EXPERIENCED TUMOR RECURRENCE 18-79 DAYS POST-CASTRATION. THE REMAINING 28% (9/32) REMAINED TUMOR-FREE FOR THE DURATION OF THE EXPERIMENT (28 DAYS POST-CASTRATION) AFTER WHICH THEY WERE SACRIFICED FOR ANALYSIS. SEVENTY PERCENT OF MICE WITH RECURRENT TUMORS HAD AN AUTOANTIBODY RESPONSE TO PABPN1, COMPARED TO 0% OF MICE THAT REMAINED TUMOR-FREE (P=0.005, FISHER'S EXACT TEST). ACCORDINGLY, THE MEDIAN TIME INTERVAL FOR MICE WITH AUTOANTIBODIES TO PABPN1 WAS 24.5 DAYS COMPARED TO 62.9 DAYS WITHOUT AUTOANTIBODIES TO PABPN1 (P<0.0001, TWO-TAILED UNPAIRED T-TEST).

T CELL RESPONSES TO PABPN1 WERE ASSESSED BY SPOT-ASSAY 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/10⁶ SPLENOCYTES, N=8) THAN MICE WITHOUT AUTOANTIBODIES TO PABPN1 (MEAN = 38 SPOT-FORMING CELLS/10⁶ SPLENOCYTES, N=10). ACCORDINGLY, MICE WITH RECURRENT TUMORS HAD STRONGER PABPN1-SPECIFIC T CELL RESPONSES (MEAN = 335 SPOT-FORMING CELLS/10⁶ SPLENOCYTES, N=10) THAN MICE WITHOUT RECURRENT TUMORS (MEAN = 12 SPOT-FORMING CELLS/10⁶ SPLENOCYTES, N=9). KAPLAN-MEIER ANALYSIS REVEALED A SIGNIFICANT DIFFERENCE IN TIME TO RECURRENCE BETWEEN MICE WITH AND WITHOUT PABPN1 ANTIBODIES (FIGURE 2). THUS, CONTRARY TO OUR INITIAL HYPOTHESIS, TREATMENT-INDUCED AUTOANTIBODIES AND T CELL RESPONSES TO PABPN1 WERE CORRELATED WITH INFERIOR OUTCOMES.

TO INVESTIGATE WHETHER AUTOANTIBODIES TO PABPN1 PROMOTE TUMOR RECURRENCE, WE ASSESSED THE TIMING OF AUTOANTIBODY RESPONSES TO PABPN1 PRIOR 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 RESPONSE WAS NOT DETECTED UNTIL 2 OR MORE DAYS AFTER TUMOR RECURRENCE, MAKING IT UNCLEAR IF AUTOANTIBODIES CONTRIBUTED DIRECTLY TO RECURRENCE. IN THE REMAINING 2/16 MICE, THE RESPONSE WAS DETECTED WITHIN ONE 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 MECHANISM OF TUMOR RECURRENCE.

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 DEVELOPED AN AUTOANTIBODY RESPONSE WAS COMPARED TO 79.7 mm³ 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, THE AVERAGE OF 9.7 DAYS FOR TUMORS TO REGRESS AFTER CASTRATION COMPARED TO 6.8 DAYS FOR THOSE 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 EXPRESSED THE TARGET ANTIGEN PABPN1. WE EVALUATED THIS ISSUE BY IMMUNOBLOTTING RECURRENT TUMORS FOR EXPRESSION OF PABPN1. ALL RECURRENT TUMORS EXPRESSED PABPN1, AND IN RECURRENT TUMORS THE LEVEL EXPRESSION WAS SIMILAR TO PRIMARY TUMOR (FIGURE 3).

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

SINCE MICE WITH RECURRENT TUMORS HAD SUBSTANTIAL PABPN1-SPECIFIC T CELL RESPONSES AND RECURRENT TUMORS STILL EXPRESSED ANTIGEN, WE NEXT INVESTIGATED WHETHER T CELLS TRAFFICKED TO 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 NON-CASTRATED MICE HAD VERY FEW CD3+ T CELLS INFILTRATING THE EPITHELIUM OR STROMA (FIGURE 4A- NON-CASTRATED). IN CONTRAST, ON DAYS 7 AND 14 POST-CASTRATION, 12/18 TUMORS WERE DENSELY INFILTRATED BY CD3+ T CELLS (FIGURE 4A- DAY 7 AND DAY 14). FACS ANALYSIS AVERAGED OVER THREE EXPERIMENTS, ~55% OF TUMOR-INFILTRATING CD3+ T CELLS WERE CD8+, AND ~24% WERE CD4+ T CELLS. OVER 95% OF T CELLS WERE HIGHLY ACTIVATED, EXPRESSING AN ACTIVATED PHENOTYPE (DATA NOT SHOWN). BY CONTRAST, VERY FEW TUMOR-INFILTRATING T CELLS EXPRESSED GRANZYME B AS ASSESSED BY IHC, SUGGESTING A PAUCITY OF MATURE CYTOLYTIC EFFECTOR T CELLS. RECURRENT TUMORS WERE ALSO EXAMINED BY IHC FOR CELLS EXPRESSING FOXP3, A TRANSCRIPTION FACTOR ASSOCIATED WITH REGULATORY T CELLS.

BY REGULATORY T CELLS²¹ (VERY FEW FOXP3+ CELLS WERE SEEN IN TUMORS FROM NON-CASTRATED 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%) (FIGURE 4B). FINALLY, TUMORS FROM CASTRATED MICE CONTAINED VERY FEW B CELLS, AS ASSESSED BY IHC WITH AN ANTIBODY AGAINST B CELLS²² (NOT SHOWN).

IN CONTRAST TO THE REGRESSING TUMORS DESCRIBED ABOVE, RECURRENT TUMORS CONTAINED RELATIVELY SPARSE CD3+ T CELL INFILTRATES IN TUMOR EPITHELIUM (FIGURE 4A- DAY 28). FEW CELLS EXPRESSING FOXP3, GRANZYME B OR PAX-5 WERE SEEN (FIGURE 4B- DAY 28 AND 4C- DAY 28 SHOWN). HOWEVER, THE PERIPHERAL AND STROMAL REGIONS OF RECURRENT TUMORS SHOWED ACCUMULATIONS OF CD3+ T CELLS (FIGURE 4C). THUS, IT APPEARS THAT RECURRENT SHIONO 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-DEPENDENT AUTOANTIBODY RESPONSES IN A SIGNIFICANT PROPORTION OF HUMAN¹³ PROSTATE CANCER PATIENTS. 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 SHIONO TUMOR MODEL, WHICH EXHIBITS TREATMENT-INDUCED AUTOANTIBODY RESPONSES SIMILAR TO THOSE OBSERVED IN PROSTATE CANCER PATIENTS. WE HYPOTHESIZED THAT TREATMENT-INDUCED AUTOANTIBODY RESPONSES WOULD BE ASSOCIATED WITH DELAYED TIME TO TUMOR RECURRENCE AND PROLONGED SURVIVAL. TO TEST THIS HYPOTHESIS, WE FIRST CLONED PABPN1, THE ANTIGEN THAT UNDERLIES TREATMENT-INDUCED AUTOANTIBODY RESPONSES IN THIS MODEL. IN GENERAL, MICE THAT DEVELOPED PABPN1-SPECIFIC AUTOANTIBODY RESPONSES AFTER CASTRATION ALSO DEVELOPED PABPN1-SPECIFIC T CELL RESPONSES. CONTRARY TO OUR HYPOTHESIS, HOWEVER, DEVELOPMENT OF PABPN1-SPECIFIC AUTOANTIBODY AND T CELL RESPONSES WAS ASSOCIATED WITH 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 RECURRENT TUMORS. NOTABLY, HOWEVER, RECURRENT TUMORS HAD GREATLY REDUCED LYMPHOCYTIC INFILTRATION, WITH T CELLS BEING RESTRICTED TO PERIPHERAL STROMAL REGIONS. THESE FINDINGS RAISE CONCERNS THAT TREATMENT-INDUCED IMMUNE RESPONSES MAY HAVE A NEGATIVE IMPACT ON CLINICAL OUTCOMES IN PROSTATE CANCER PATIENTS.

BASED ON OUR RESULTS, WE CONSIDERED THE POSSIBILITY THAT TREATMENT-INDUCED B AND T CELL RESPONSES MIGHT SOMEHOW PROMOTE TUMOR RECURRENCE. FOR EXAMPLE, B CELLS HAVE BEEN SHOWN TO PLAY AN ESSENTIAL ROLE IN INFLAMMATION-INDUCED TUMORIGENESIS IN A MURINE COLON CANCER MODEL.³³ FURTHERMORE, T CELLS CAN PROMOTE CARCINOGENESIS IN THE SETTING OF CHRONIC INFLAMMATION. FOR EXAMPLE, IN CHRONIC HEPATITIS B AND C INFECTION, CYTOTOXIC LYMPHOCYTES PROMOTE HEPATOCYTE DAMAGE AND FIBROSIS THROUGH DIRECT CELLULAR TOXICITY AND RELEASE OF INFLAMMATORY CYTOKINES.²⁴ HOWEVER, T CELLS DIRECTED AGAINST *HELICOBACTER PYLORI* ARE THOUGHT TO PROMOTE THE DEVELOPMENT OF GASTRIC ADENOCARCINOMA.²⁵ IN OUR CAHONOGI MODEL DOES NOT INVOLVE CHRONIC INFLAMMATION NOR PRIMARY CARCINOGENESIS, THEREFORE THE RELEVANCE OF THESE EXAMPLES IS UNCLEAR. IN 6/16 CASES FROM OUR STUDY, AUTOANTIBODY RESPONSES TO PABPN1 WERE DETECTED AFTER RECURRENT TUMORS WERE DETECTED, WHICH SEEMS INCOMPATIBLE WITH THE NOTION THAT AUTOANTIBODIES PLAY A CAUSATIVE ROLE IN TUMOR RECURRENCE. AN ALTERNATIVE POSSIBILITY IS THAT AUTOANTIBODIES ARE A MARKER RATHER THAN MEDIATOR OF TUMOR RECURRENCE. INDEED, PRESENCE OF AUTOANTIBODIES TO PABPN1 WAS ASSOCIATED WITH TUMORS THAT REGRESSED MORE RAPIDLY AFTER CASTRATION. IT MAY BE THAT A SLOWER RATE OF TUMOR REGRESSION ALLOWS SUFFICIENT PABPN1 PRESENTATION TO THE IMMUNE SYSTEM, RESULTING IN AUTOANTIBODY AND T CELL RESPONSES. IN REGRESSING TUMORS MAY ALSO BE LESS ANDROGEN-DEPENDENT AND HENCE MORE LIKELY TO BE DETECTED. OUR 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 AND ASSESSING THE EFFECT ON TUMOR RECURRENCE.

IN ADDITION TO AUTOANTIBODIES, WE ALSO DETECTED ROBUST T CELL RESPONSES TO PABPN1 BY IFN- γ ELISPOT. INTRIGUINGLY, MODEST T CELL RESPONSES TO PABPN1 WERE ALSO SEEN IN TUMOR-BEARING MICE THAT UNDERWENT CASTRATION. CASTRATION HAS BEEN SHOWN TO INCREASE LEVELS OF CD4+ T CELLS IN PERIPHERAL LYMPHOID TISSUES OF MICE DUE TO A LOSS OF THE IMMUNE SUPPRESSIVE EFFECT OF CD4+ T CELLS.⁴ THUS, CASTRATION-INDUCED T CELL RESPONSES TO PABPN1 MAY IN PART REFLECT THE GENERAL HOMEOSTATIC EXPANSION OF LYMPHOCYTES IN RESPONSE TO ANDROGEN DEPRIVATION. THE STRONGER T CELL RESPONSES TO PABPN1 DEVELOP IN TUMOR-BEARING MICE SUGGESTS THAT TUMOR-INDUCED APOPTOSIS FURTHER STIMULATES PABPN1-SPECIFIC T CELLS THROUGH THE RELEASE OF SOLUBLE ANTIGENS AND/OR INFLAMMATORY FACTORS. CASTRATION-INDUCED T CELL RESPONSES WERE ACCOMPANIED BY AND DENSE INFILTRATION OF TUMOR EPITHELIUM BY CD3+ T CELLS BY DAY 7. THE MAJORITY OF THESE T CELLS EXPRESSED CD8, A SMALLER PROPORTION (24%) EXPRESSED CD4+, AND A MINORITY EXPRESSED CD4+.

POPULATION (5-10%) EXPRESSED FOXP3. INTRIGUINGLY, HORMONE THERAPY OF HUMAN PROSTATE
ALSO PROMOTES T CELL INFILTRATION OF TUMORS, ALTHOUGH THE CLINICAL SIGNIFICANCE
IS NOT KNOWN. LIKEWISE, IN THE SHIONOGI MODEL, WE WERE UNABLE TO ASSESS THE RELATIONSHIP
BETWEEN T CELL INFILTRATION OF TUMORS AND SUBSEQUENT OUTCOMES, SINCE MICE HAD TO BE
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
PERIPHERAL STROMAL REGIONS. COLLECTIVELY, OUR RESULTS INDICATE THAT SHIONOGI TUMORS ARE
PERMISSIVE TO T CELL INFILTRATION, WHEREAS RECURRENT TUMORS DEVELOP LYMPHOCYTIC
BARRIERS. THIS SUGGESTS THAT THE T CELL RESPONSE TRIGGERED BY CASTRATION MAY CREATE
PRESSURE FOR THE DEVELOPMENT OF IMMUNOLOGICALLY RESISTANT TUMORS UPON RECURRENT
DISEASE. IT IS INTERESTING TO ASSESS WHETHER RECURRENT HUMAN PROSTATE TUMORS ARE SIMILARLY

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
DEVELOPMENT, EXPANSION OF THE TRINUCLEOTIDE REPEAT IN A POLYALANINE TRACT OF THE PABPN1 GENE
CAUSES OCULOPHARYNGEAL MUSCULAR DYSTROPHY, AN AUTOSOMAL DOMINANT INHERITED DISORDER.
THE ROLE OF PABPN1 IN TRANSCRIPTION MAY EXPLAIN ITS HIGH LEVEL OF EXPRESSION IN SOME
CELLS. ON THE ONE HAND, IT MAY SEEM COUNTER INTUITIVE THAT A WIDELY EXPRESSED PROTEIN
PABPN1 WOULD BE A TARGET ANTIGEN OF CASTRATION-INDUCED IMMUNE RESPONSES IN THE
SHIONOGI MODEL. HOWEVER, SAVAGE ET AL. DESCRIBED A NATURALLY ARISING CD8+ T CELL RESPONSE AGAINST A
PEPTIDE DERIVED FROM HISTONE H4, A UBIQUITOUSLY EXPRESSED PROTEIN, IN THE MURINE
PROSTATE CANCER MODEL. MOREOVER, IN OUR STUDY OF TREATMENT-INDUCED AUTOANTIBODIES IN
HUMAN PROSTATE CANCER PATIENTS, MANY OF THE UNDERLYING ANTIGENS WERE WIDELY
EXPRESSED. ¹³ THUS, TREATMENT-INDUCED IMMUNE RESPONSES ARE NOT NECESSARILY DIRECTED AGAINST
TUMOR-ASSOCIATED PROTEINS, BUT MIGHT INSTEAD INVOLVE A BREAKDOWN OF PERIPHERAL TOLERANCE TO WIDELY
EXPRESSED PROTEINS, AS OCCURS IN MANY AUTOIMMUNE CONDITIONS.

OUR FINDINGS IN THE SHIONOGI MODEL RAISE CONCERNS ABOUT HUMAN PROSTATE CANCER
TREATMENT-INDUCED AUTOANTIBODY RESPONSES ARE SEEN IN 20-30% OF PATIENTS TREATED WITH
HORMONE THERAPY AND/OR RADIATION THERAPY. THE RELATIONSHIP BETWEEN TUMOR-ASSOCIATED
ANTIGENS AND CLINICAL OUTCOMES IN HUMANS IS CONTROVERSIAL. AUTOANTIBODIES TO P53 HAVE BEEN ASSOCIATED WITH
FAVORABLE OUTCOMES IN SOME STUDIES, ^{29, 30} WHILE OTHERS ³¹⁻³⁵ HAVE ASSOCIATED AUTOANTIBODIES TO OTHER TARGET
ANTIGENS HAVE BEEN ASSOCIATED WITH IMPROVED PROGNOSIS IN MELANOMA, GLIOBLASTOMA, AND

CANCER AND BREAST CANCER³⁶⁻⁴⁰ HOWEVER, MOST OF THE ABOVE STUDIES HAVE EXAMINED AUTOANTIBODIES PRESENT AT THE TIME OF DIAGNOSIS, WHEREAS OUR WORK IN HUMANS AND THE SHIONOGI MICE WAS FOCUSED ON AUTOANTIBODY RESPONSES THAT ARISE DURING TREATMENT. IF THE PRESENT FINDINGS IN HUMANS, THEN TREATMENT-INDUCED AUTOANTIBODY AND T CELL RESPONSES MAY PORTEND A BENEFICIAL EFFECTOR PHENOTYPE.

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FIGURE LEGENDS

Figure 1. RECOGNITION OF PABPN1 BY SERUM ANTIBODIES AND T CELLS FROM CASTRATED MICE. **A.** RECOMBINANT PABPN1 (40 KDA) 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 POSITIVE AGAINST PURIFIED PABPN1, LIKELY REFLECTING THE HIGHER SENSITIVITY OF THIS ASSAY. **B.** THE USE OF RECOMBINANT PABPN1. **B.** SHIONOGI TUMOR LYSATE WAS IMMUNOBLOTTED WITH SERUM FROM A NAÏVE DD/S MOUSE AND 5 MICE THAT HAD BEEN IMMUNIZED WITH PABPN1 IN INCOMPLETE FREUND'S ADJUVANT. THE PRESENCE OF AN IMMUNOREACTIVE BAND AT ~40 KDA CONFIRMS THAT THIS 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 PABPN1 WERE LOADED DEPENDING ON SAMPLE AVAILABILITY AS SHOWN. **D.** ELISPOT ASSAY TO MEASURE PABPN1-SPECIFIC T CELL RESPONSE, AS MEASURED BY 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 NON TUMOR-BEARING MICE. PABPN1-IMMUNIZED MICE SERVED AS A POSITIVE CONTROL. NON TUMOR-BEARING, NON-CASTRATED DD/S MICE SERVED AS A NEGATIVE CONTROL. FOR EACH SAMPLE WAS RUN IN TRIPLICATE TO PRODUCE AN AVERAGE AND STANDARD DEVIATION.

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 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 COMPARED TO ONE PRIMARY TUMOR (1 TUMOR) LOADED WITH PABPN1. TUMOR LYSATE AND SCREENED WITH SERUM FROM A PABPN1-IMMUNIZED MOUSE. GAPDH SERVED AS A LOADING CONTROL.

Figure 4. CD3+ T CELL INFILTRATION OF SHIONOGI TUMORS AFTER CASTRATION. **A.** ANTI-CD3 STAINING OF SHIONOGI TUMORS SHOWS DENSE INFILTRATION OF CD3+ T CELLS BEGINNING AT DAY 7 POST CASTRATION.

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 SMALL SUBPOPULATION OF CD3+ T CELLS EXPRESSES FOXP3 (400X). **C.** REPRESENTATIVE RECURRENT TUMORS FROM MICE SACRIFICED ON DAY 56 AND DAY 90 POST-CASTRATION. NOTE THAT CD3+ T CELLS WERE RESTRICTED AND CONFINED TO THE PERIPHERAL STROMA OF TUMORS (200X).

For Peer Review

Hahn et al. Figure 1.

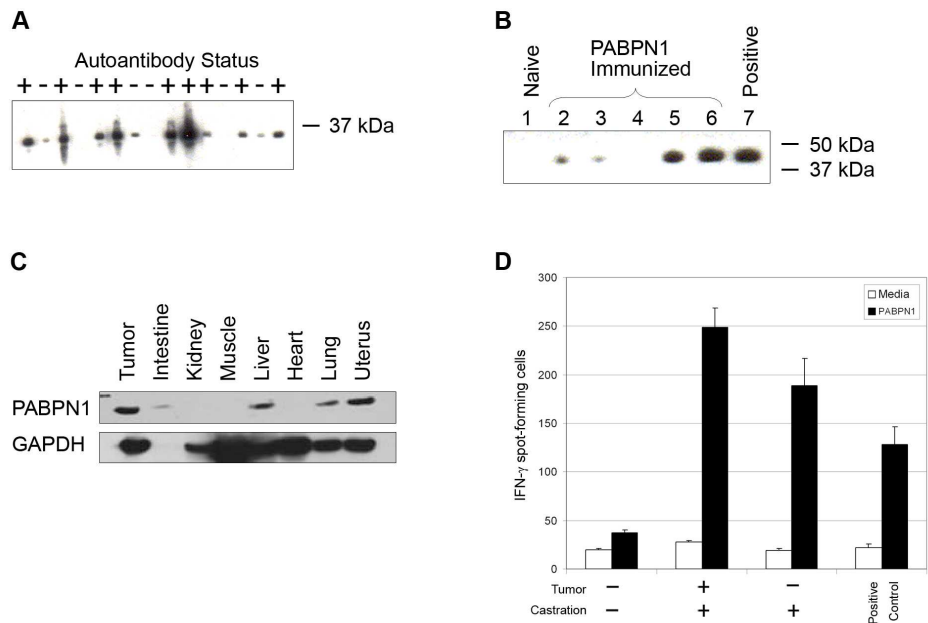


Figure 1
171x127mm (300 x 300 DPI)

Hahn et al. Figure 2.

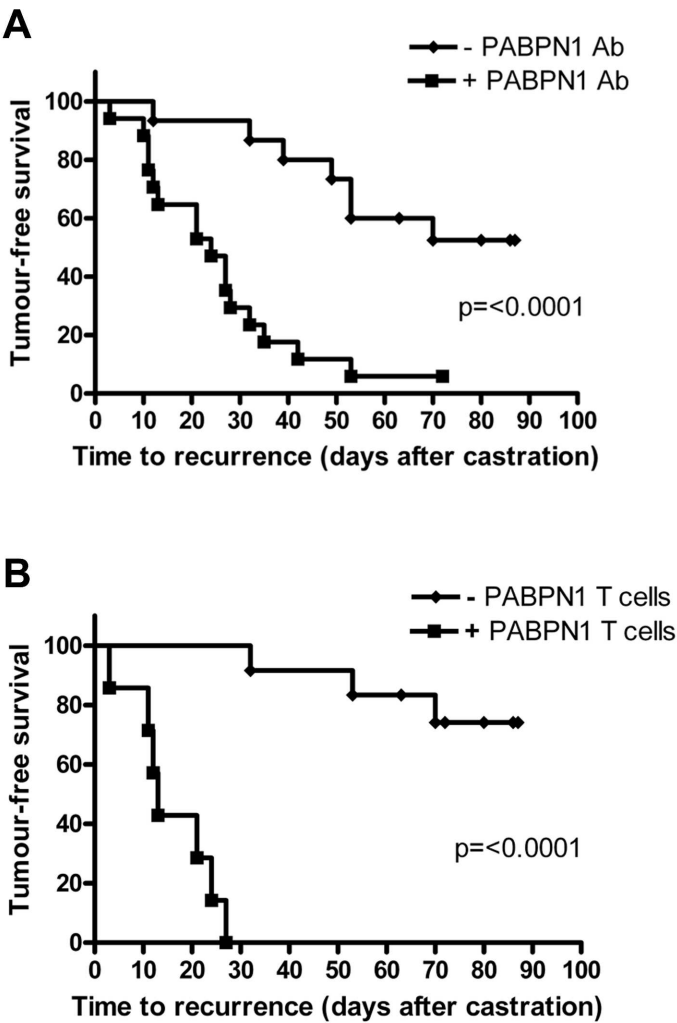


Figure 2
127x211mm (600 x 600 DPI)

Hahn et al. Figure 3.

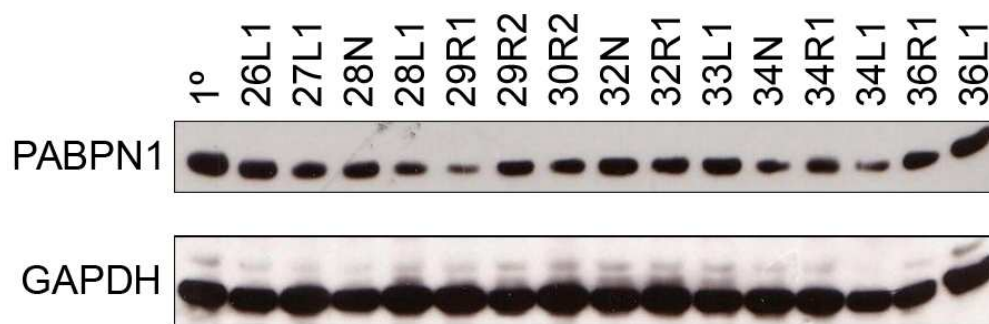


Figure 3
76x43mm (300 x 300 DPI)

Hahn et al. Figure 4.

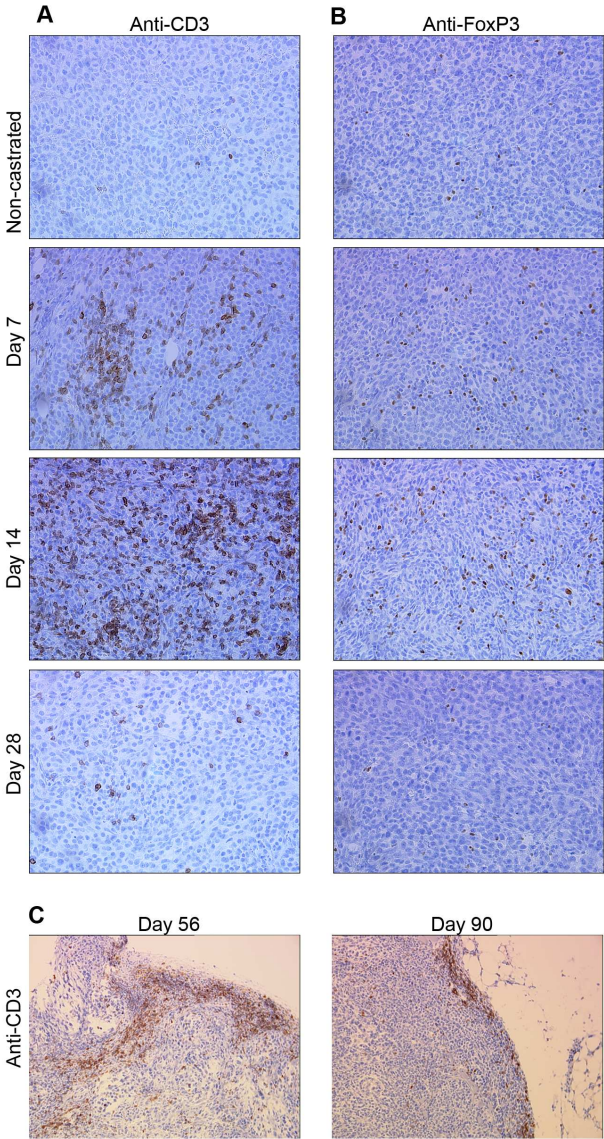
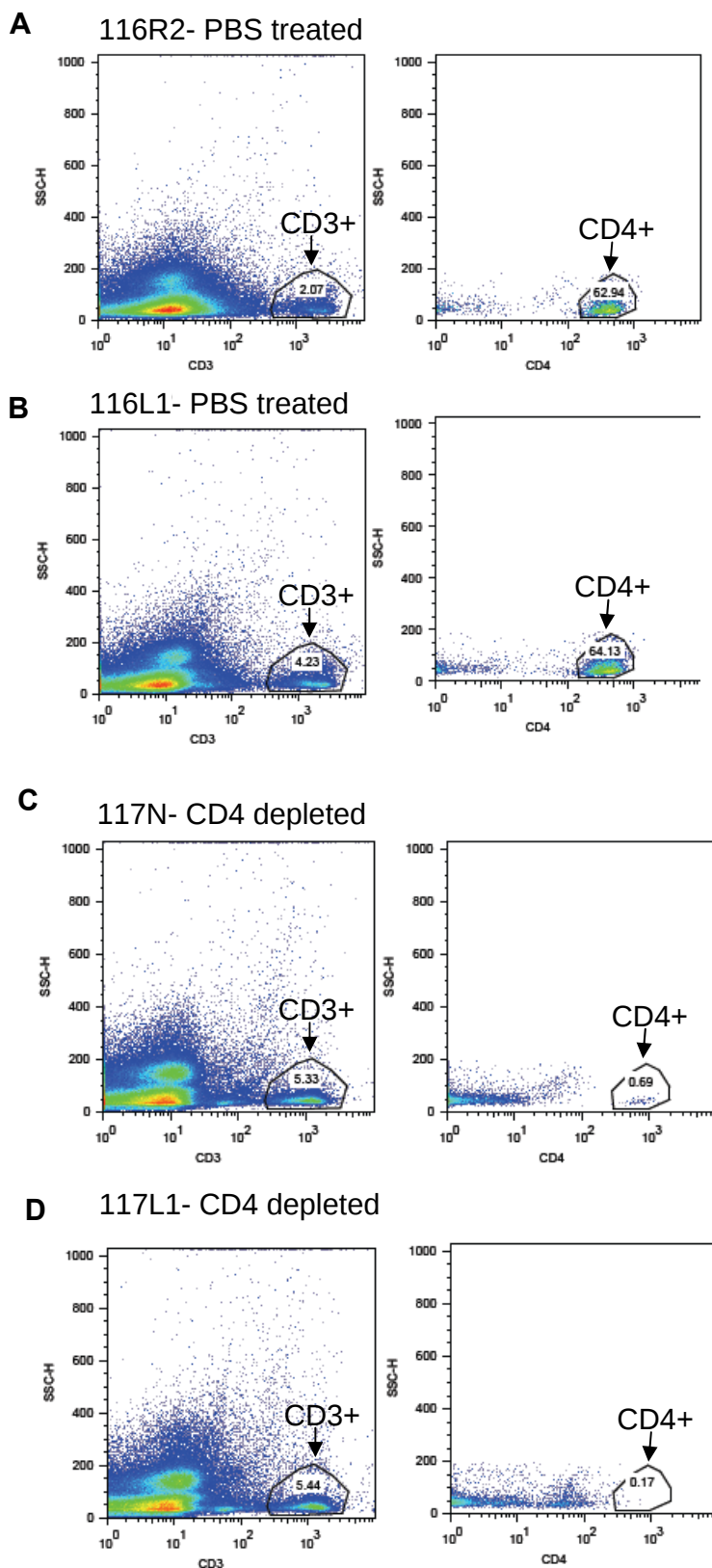


Figure 4
116x215mm (300 x 300 DPI)

SUPPORTING DATA:**Figure 1:****Figure 1:**

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 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.

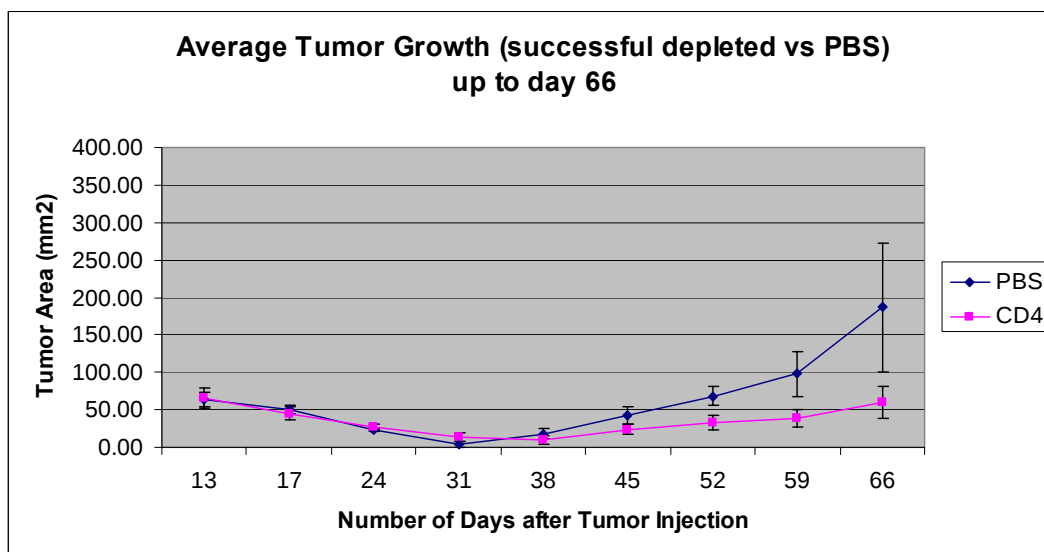
Figure 2

Figure 2: 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.

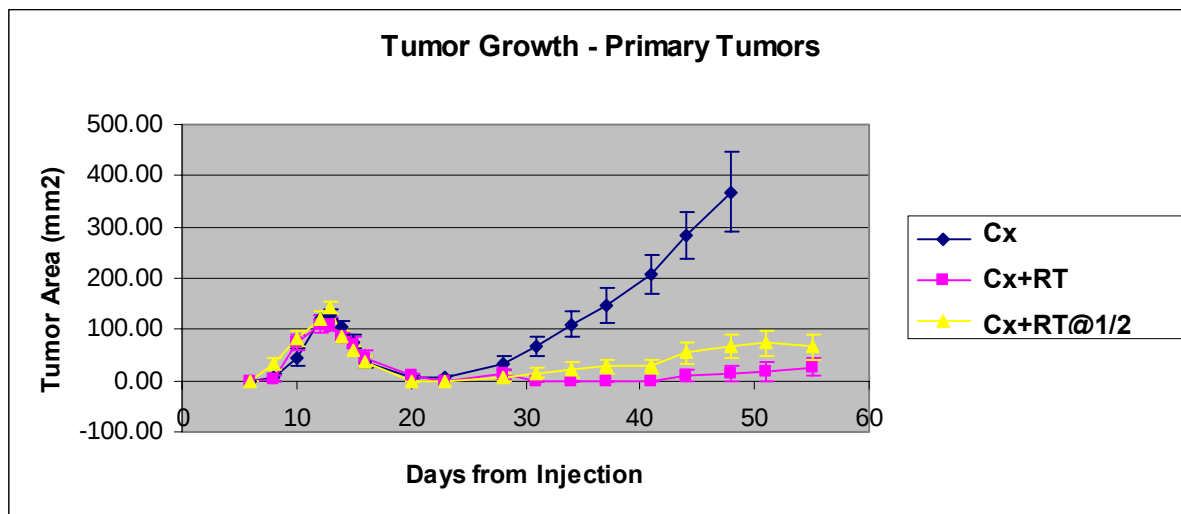
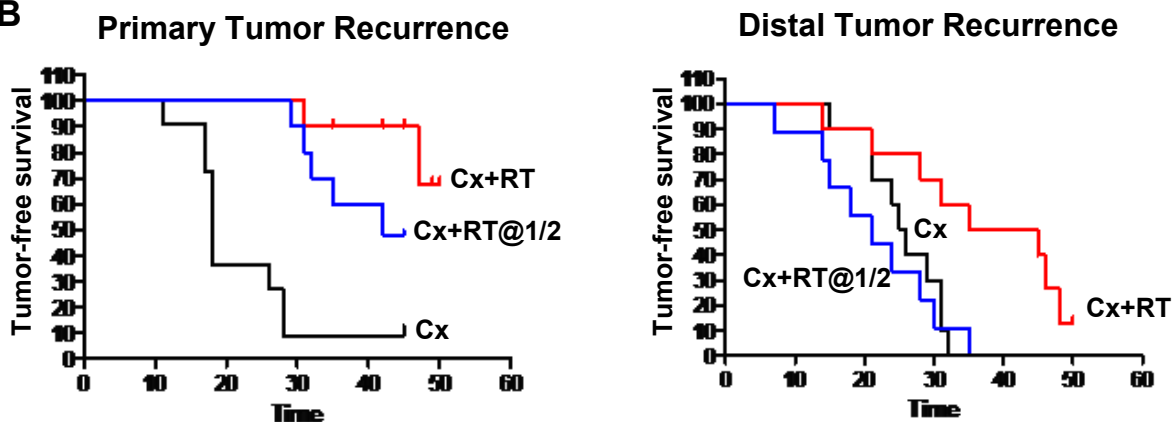
Figure 3**A****B**

Figure 3: 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 mm² 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.

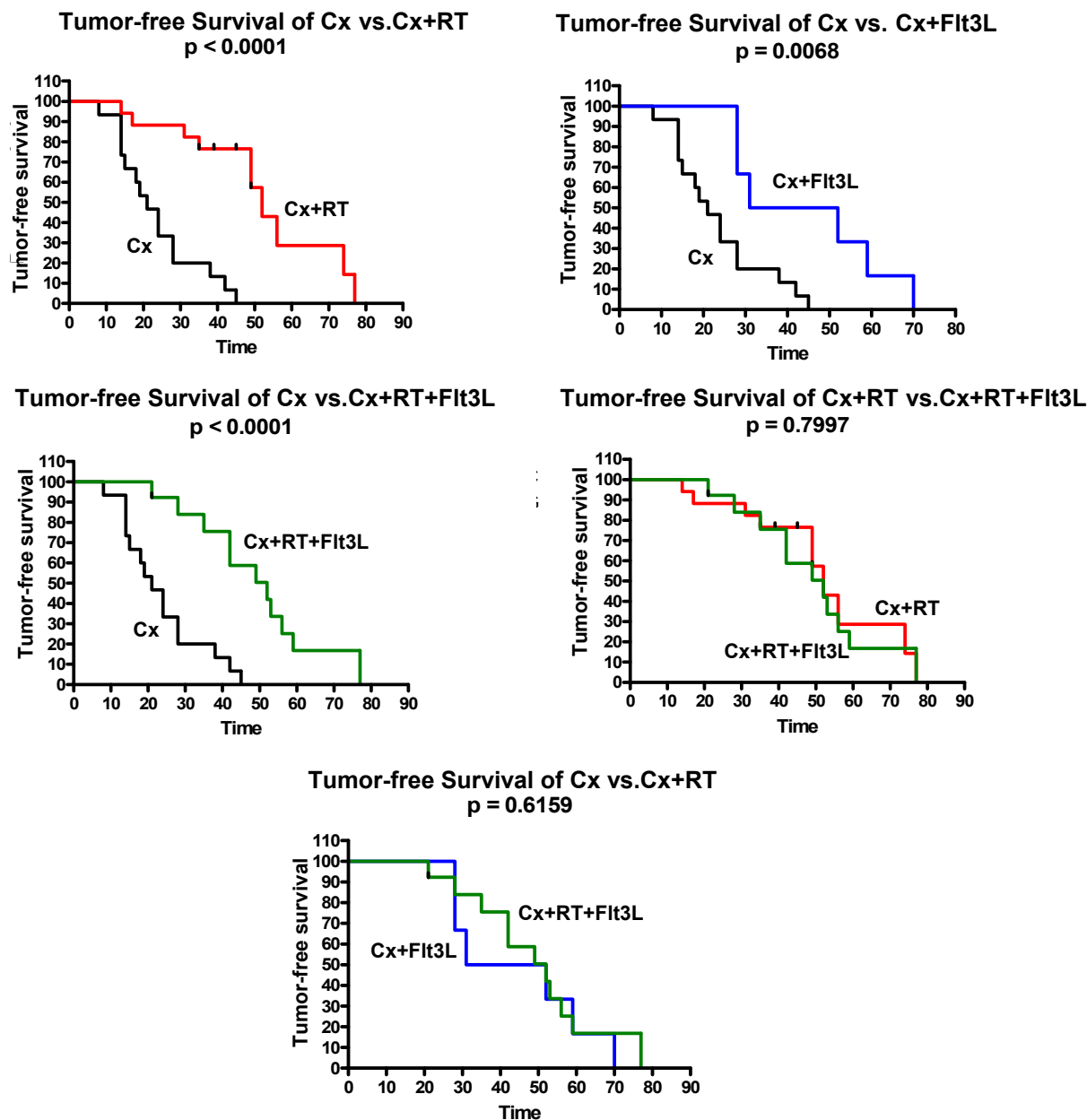
Figure 4A

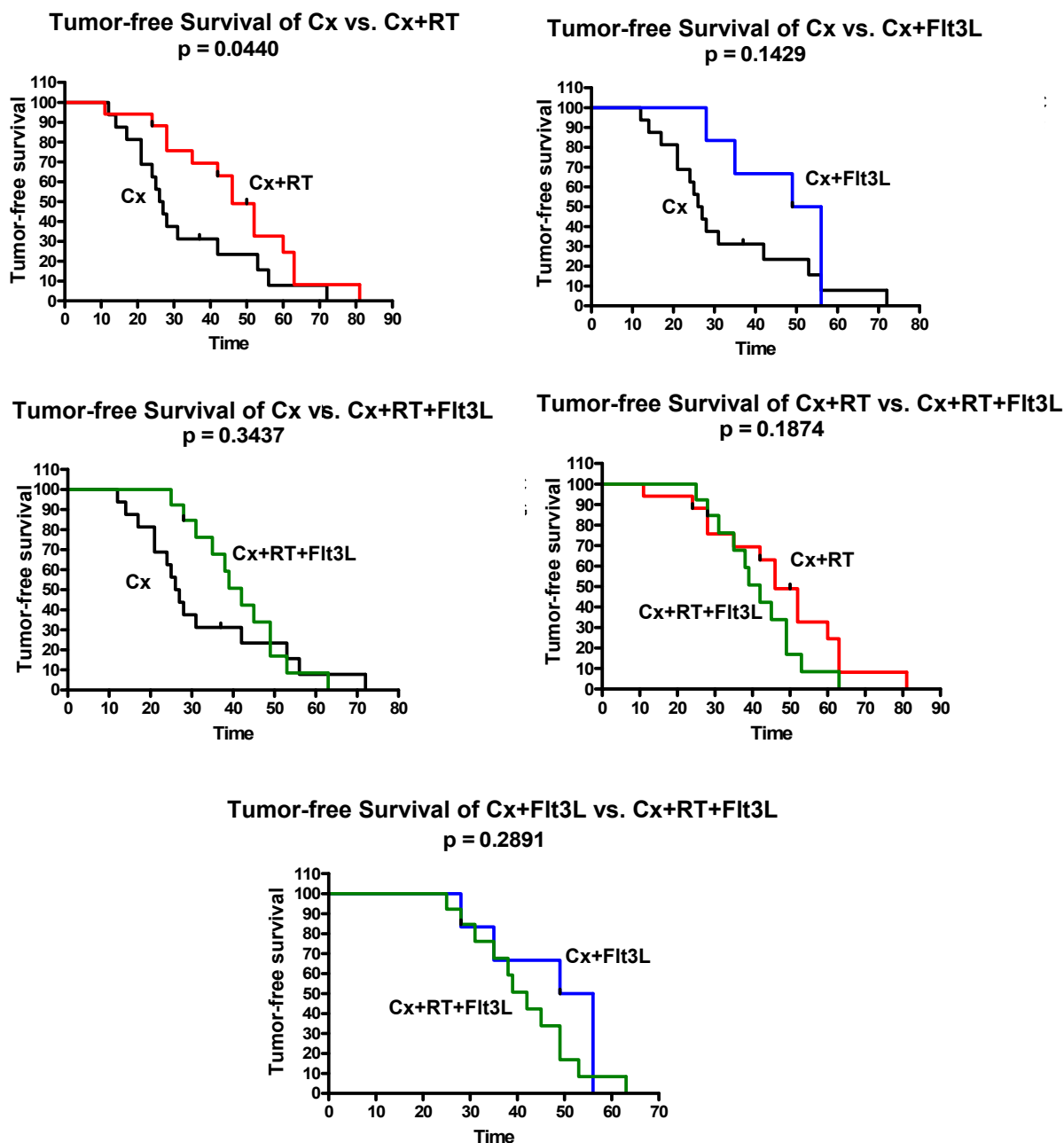
Figure 4B

Figure 4: 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 4A). 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 3. However, no additional survival advantage was seen with addition of Flt3L.

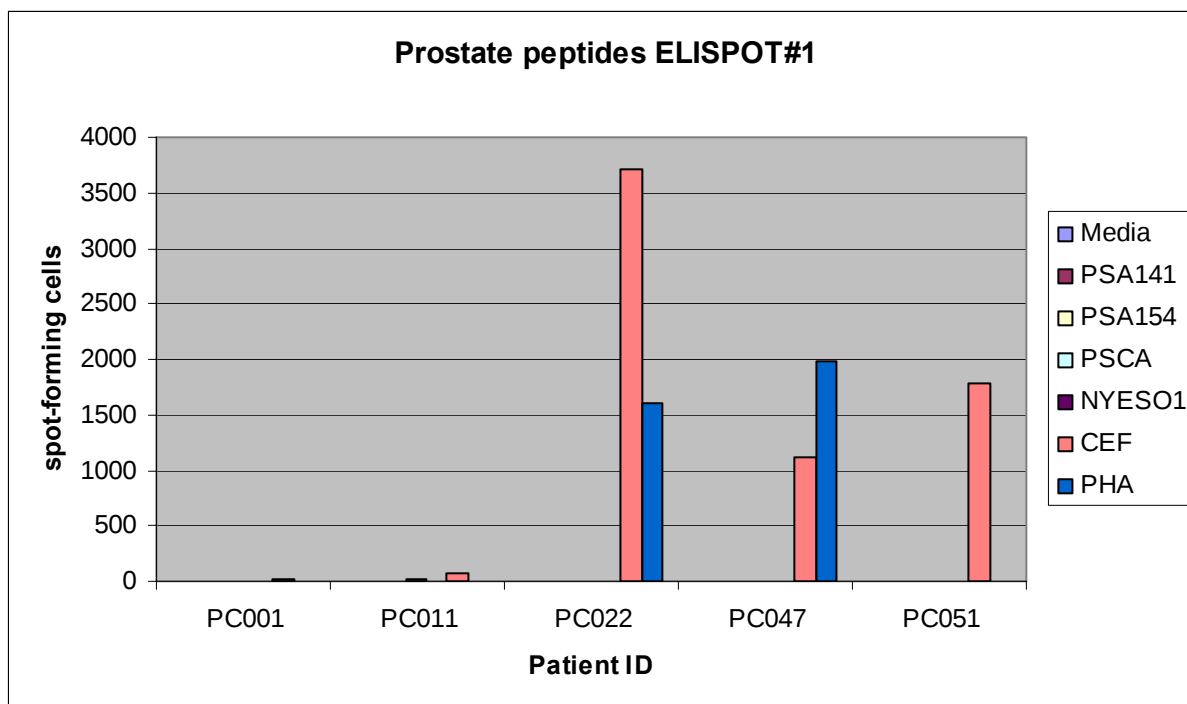
Figure 5

Figure 5: 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.