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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of the proposed research was to test the following concept: Due to the prostate restricted expression of Pse in normal tissues of mice, Pse is likely to be immunogenic in female mice. T cell responses of male and female mice to Pse and a control antigen Her2/neu were compared. Two different assays were used for this purpose; the ELIspot assay and the Cytotoxicity assay. We found that in the FVB and C57BL/6 strains of mice, immunization with Pse induced specific T cell responses in the female mice, but showed no or weak responses in the male mice. In contrast, the difference in T cell responses of male and female mice to immunization with Her2/neu antigen was relatively small. These results are novel and highly significant and they support our concept that uncharacteristically high difference in responsiveness to Pse of female mice in comparison to male mice is due to the lack of significant expression of Pse as a self-antigen in female mice. Further, these results suggest that PDEF (the human homologue of Pse) is likely to be significantly immunogenic in female breast cancer patients. The observed poor immunogenicity of Pse in the male mice is probably due to some mechanism of tolerance against this protein due to its strong expression in the prostate gland.

15. SUBJECT TERMS
Prostate specific Ets, Female specific immunogenicity, A candidate breast cancer antigen

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INTRODUCTION

Prostate derived Ets factor is a relatively novel member of the Ets family of transcription factors (1). It is also unique since it shows most restricted expression in normal human tissues that is primarily limited to normal prostate with weaker expression in normal bronchus/trachea tissue (2-4). Similarly, Pse (prostate specific Ets, the mouse homologue of PDEF) also shows highly restricted expression in normal mouse tissues that is primarily restricted to normal prostate and colon tissues (5). Based on this understanding, we proposed that Pse is likely to be preferentially immunogenic in female mice and the test of this concept was the focus of our research.

BODY

Our first task was to immunize male and female mice with Pse expression plasmid and enumerate total T cell responses and cytotoxic T cell responses using the ELIspot assay and ^{51}chromium release assay. This experiment was performed and the representative ELIspot images are shown in Figure 1. Our data show that in contrast to female mice, male mice fail to induce appreciable Pse specific IFN-γ secreting T cell responses. The average number (from triplicate wells) of IFN-γ secreting T cells from Pse immunized female (panel A) and male mice (panel C) was 143 and 13 per $5x10^5$ immune splenocytes respectively. The number from male mice is similar to that observed with negative control, i.e., vector immunized mice (panels B and D).

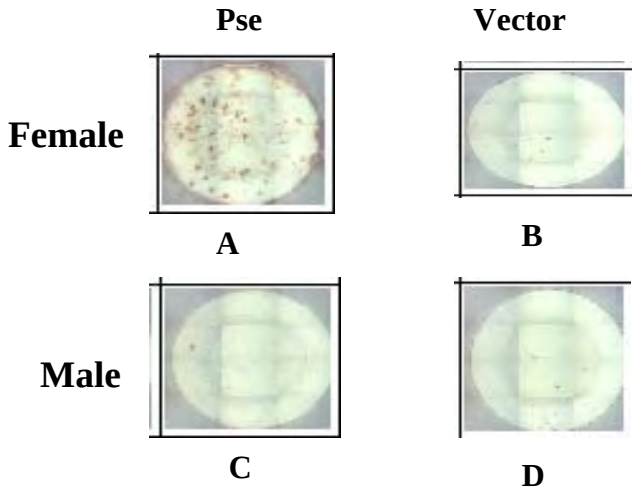

Figure 1. Testing of Pse specific cellular immunity by ELIspot assay for cytokine IFN-γ. Upper panels A and B respectively show representative images of individual wells showing IFN-γ secreting T cells from Pse-transfected dendritic cell (Pse- DC)- immunized (A) and vector-DC-immunized (B) female mice. The lower panels C and D respectively show data for similarly immunized male mice.

A comparison of the Pse specific cytotoxic T cell response by ^{51} Cr release assay is shown in Figure 2

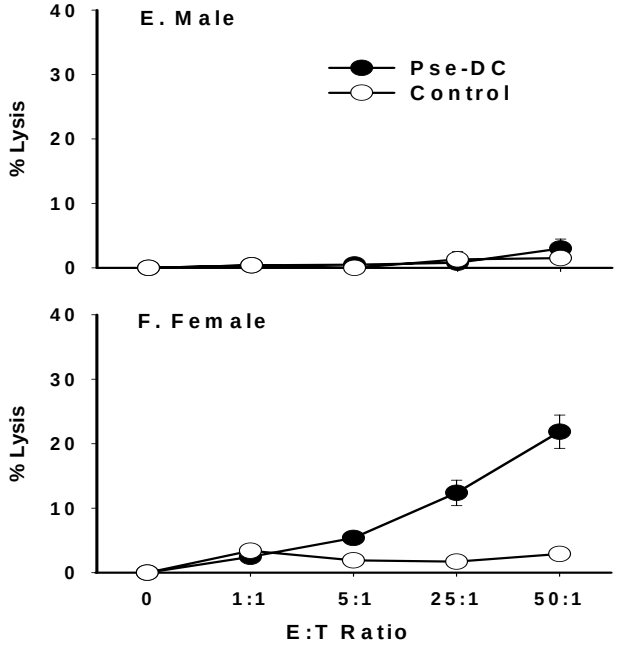

Figure 2. Comparison of Pse specific CTL responses in male and female mice. Specific lysis of Pse-positive targets from a syngeneic mammary tumor cell line was observed with splenocytes from Pse-DC immunized female mice (solid circles in lower panel) but not from male mice (solid circles in upper panel). As control, both male and female mice immunized with vector plasmid failed to elicit Pse-specific CTL responses (open circles in top and bottom panels).

The above experiment was repeated once in the same FVB strain of mice and similar results were obtained (data not shown).

Together, the data shown in figures 1 and 2 support our concept that due to prostate restricted expression of Pse, it is immunogenic in female FVB mice but not in male FVB mice.

In task 2, we proposed to clone the neu oncogene and compare the response of male and female mice to this antigen. To that end, we obtained the Her-2/neu oncogene expression plasmid from colleagues in the field and tested male and female mice for their responses to this antigen. Representative images of the ELIspot assay from male and female FVB mice are shown in Figure 3. The average number of IFN-γ secreting T cells/ $5x10^5$ splenocytes from triplicate wells from Her-2/neu immunized female and male mice was 152 and 98 respectively. These data show that both female and male mice respond to immunization with Her-2/neu antigen, although female mice show a relatively more robust response than male mice.

Female **Male**

Figure 3. Testing of Her2/neu specific T cell response by ELIspot assay for cytokine IFN-γ. The left panel shows representative image of individual wells showing IFN-γ secreting T cells from Her2/neu-transfected dendritic cell (Her2-DC)-immunized female mice. The right panel shows data for similarly immunized male mice.

In task 3, we proposed to compare Pse and neu specific T cell responses in male and female mice in the same experiment. These results are shown below in figure 4.

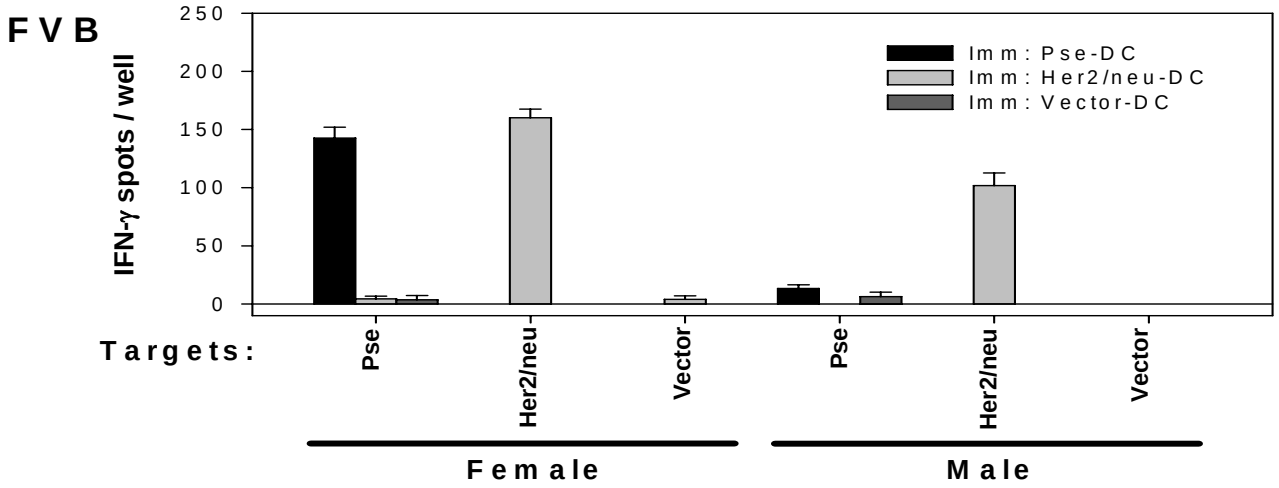

Figure 4. *A comparison of Pse and Her-2/neu specific T cell responses induced in female and male mice of FVB strain. Spleens were harvested from female/male FVB mice immunized three times with Pse-DC, Her2/neu-DC or Vector-DC respectively. Immune splenocytes were cultured for 5 days in the presence of IL-2 and the respective target (stimulator) cells i.e., the Pse-DC, Her2/neu-DC or Vector-DC. The number of Pse-specific or Her2/neu specific IFN-g-secreting T cells were determined by the ELIspot assay, and are shown per* $5X10^5$ *immune splenocytes.*

From the experiment in Figure 4, we find that whereas Pse specific immune response was seen only in female mice, both male and female mice induced specific T cell responses to the control Her-2/neu antigen. These results strongly support our hypothesis that due to the prostate restricted expression, Pse is likely to be more immunogenic in female mice than in male mice. The lack of the Pse specific T cell response in male mice is likely due to some mechanism of tolerance to Pse as a self protein in male mice.

Since the last Annual Report, we have tested the immune responses against Pse and Her2-neu in male and female mice of another mouse strain i.e. the C57BL/6 strain. The data shown in Figure 5 is quite similar to that shown in Figure 4 except that the responses of the C57BL/6 mice are relatively higher than those of the FVB mice. This may be due to the generally stronger antigen specific T cell responses observed in the C57BL strain of mice.

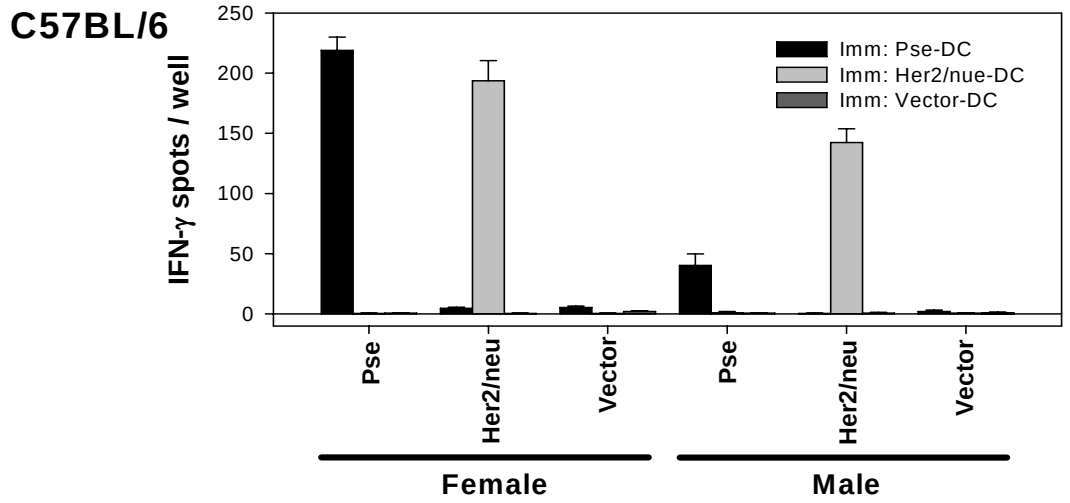

Figure 5. *A comparison of Pse and Her2/neu specific T cell responses induced in male and female mice of C57BL/6 strain. Spleens were harvested from female and male mice following immunization (3 times at two-week interval) with Pse-DC or with Her2/neu-DC or Vector-DC respectively. The splenocytes were cultured for 5 days in the presence of IL-2 and the respective target stimulators i.e., Pse-DC, Her2/neu-DC or vector-DC respectively. The number of IFN-γ secreting T cells was determined by ELIspot assay and are shown per* $5X10^5$ *immune splenocytes.*

Overall, the responses of male C57BL/6 mice remain much lower in comparison to those of the female mice from this strain as well.

Statistical evaluation. The data presented in Figures 4 and 5 was evaluated for statistical significance for response difference of the male and female mice to Pse. The normality of the data was checked using skewness and kurtosis. The data showed a slightly light tail which may result in conservative test results. A linear model with gender and antigen was constructed. The Dunnett's test was used individual pair-wise tests using the least square means. Compared with male FVB mice, female FVB mice showed significantly higher responses for both Pse and Her2/neu treatments ($p < 0.0001$ and $p=0.0003$, respectively). However, the estimated differences between male and female were 129.0 (95% CI: 101.2-157.5) for Pse and 58.3 (95% CI: 30.2-86.5) for Her2/neu. The un-overlapped confidence intervals showed that the difference between gender for Pse was much greater than for Her2/neu. With C57BL/6 mice, female again showed higher responses than male for both Pse and Her2/neu ($p=0.0001$ and $p=0.0253$, respectively). Again, similarly to FVB mice, the difference between gender was much greater for Pse (165, 95% CI: 119.7-210.3) than for Her2/neu (51.3, 95% CI: 6.0-96.6). These data show that the uncharacteristically higher difference in the immune responsiveness of female and male mice to Pse is significantly above that observed for the control Her2/neu antigen which, in contrast to Pse, is not known to show gender specific differences in its expression in normal tissues.

In summary, using the two different strains of mice i.e. FVB and C57BL/6, and using the ELIspot and cytotoxicity assays, we have shown that the female mice induce much stronger T cell responses to Pse than male mice. These data provide a proof for our concept and predict that immunization with PDEF is likely to induce strong T cell responses in female breast cancer patients. Together, our results support the testing of PDEF as a novel antigen in breast cancer patients.

Un-met objectives

As shown in Figure 2, we were able to compare the specific cytolytic activity of the Pse specific immune splenocytes from female and male mice *in vitro,* using a syngeneic mammary tumor cell line as target cells. However, we failed to show similar cytolytic activity of the Her2/neu specific immune splenocytes against the same mammary tumor cell line. We expected to observe such lysis by the Her2/neu specific immune splenocytes from FVB mice since the cell line target is derived from a mammary tumor from neu-transgenic mice of the FVB strain background. One possibility for our failure to observe this lysis is that the target cell line used in our work has lost the expression of the neu oncogene during the long-term culture *in vitro*. This possibility will be examined in future studies.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that Pse is strongly immunogenic in female mice but not in male mice.
- In contrast, Her-2/neu is immunogenic in both female and male mice.
- Our data show preferential immunogenicity of Pse in female mice and support our concept. The data also support the testing of PDEF as a novel breast tumor antigen in female breast cancer patients.

REPORTABLE OUTCOMES

The research results shown in this report are quite novel and therefore are reportable outcomes. We have presented this work at the Annual Retreat Meeting of the Department of Immunology held early this year. Also, this work was presented at the Department of Genetics seminar series at our institute. However, in order to publish this work, we need to determine why Her2/neu specific cytotoxic T cells did not lyse the syngeneic mammary tumor cell line. Once we sort out this problem, we expect to present these findings at the national and international conferences and publish them in a peer-reviewed journal.

CONCLUSIOS

The above results are highly novel and significant and they support our concept and suggest that PDEF (the human homologue of Pse) is likely to be similarly immunogenic in female breast cancer patients. Our results also suggest that the lack of immunogenicity of Pse in male mice is likely due to some mechanism of tolerance to Pse as a self protein expressed in the normal prostate tissue of males. Understanding the mechanism of tolerance to Pse and developing approaches to overcome it will be useful for efforts at breaking tolerance against PDEF in male breast cancer patients.

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APPENDICES

Abstract of the presentation at the Department of Immunology Retreat Meeting, Holiday Valley, N.Y. January, 2007 (Abstract shown on the next page).

SUPPORTING DATA

None

PERSONNEL RECEIVING PAY ON THIS RESEARCH EFFORT

Ashwani K. Sood, Ph.D.	Principal Investigator
Liaomin Peng, M.D.	Postdoctoral Fellow
Jihnhee Yu, Ph.D.	Statistician Consultant

Appendices
Publications and Meeting Abstracts
Meeting Abstract
Title: PDEF in breast and prostate cancer progression and a novel antigen in breast cancer
Oral presentation by Ashwani Sood at the Annual Retreat meeting of Department of Immunology, Roswell Park Cancer Institute

Background: PDEF (prostate derived Ets factor) mRNA was previously reported to be over expressed in human breast tumors and shows highly restricted expression in normal human tissues. However, there is limited knowledge about the expression of PDEF protein in human tumors, and its potential as an antigen. The purpose of this study was to determine PDEF protein expression in various stages of breast and prostate neoplasias and to evaluate its immunogenicity and its potential as a target antigen.

Materials and Methods: A new rabbit polyclonal antibody to PDEF was prepared and reacted with tissue microarrays (TMAs) consisting of 1 mm cores of 62 benign breast tissues (from cancer cases), 46 *in situ* carcinomas, 65 invasive ductal carcinomas and 39 invasive lobular carcinomas. The antibody was also similarly reacted with TMAs from 290 benign prostate tissues, 109 PIN (prostate intraepithelial neoplasia) samples and 230 prostate carcinomas from the same cohort of prostate cancer patients. The average nuclear staining intensity and the percentage of stained epithelial cells were evaluated, a combined score was calculated and a threshold for over expression was set. In addition, we tested the immunogenicity of Pse (prostate specific Ets, the mouse homologue of PDEF) in female and male mice of FVB and C57BL/6 strains.

Results: Relative over expression of PDEF was identified in 11 of 62 (18%) benign breast tissues, 23 of 46 (50%) DCIS lesions, 30 of 65 (46%) invasive ductal carcinomas and 20 of 39 (51%) invasive lobular carcinomas. Further, of the 9 matched samples of benign breast and tumor tissues from same patients, 8 showed an increase in the number and/or intensity of PDEF expressing epithelial cells in tumors. Relative over expression of PDEF was also identified in 79 of 290 (27%) benign prostate tissues, 36 of 109 (33%) PIN samples, 92 of 230 (40%) prostate carcinomas. Importantly, comparison of the matching samples of cancer versus benign and cancer versus PIN showed that in 68% and 70% cases respectively, increased expression of PDEF was seen in cancer in comparison to the matching benign or PIN tissue.

The test of the immunogenicity of Pse showed that in both FVB and C57BL/6 strains of mice, female mice induced much stronger T cell responses to Pse than male mice. In contrast, T cell responses to the control Her2/neu antigen were less different. These data suggest that immunization with PDEF is likely to induce strong T cell responses in female breast cancer patients.

Conclusions: This is the first report of the characteristics of PDEF protein expression in various stages of breast and prostate neoplasias. The data show frequent increase in the number and/or intensity of PDEF expressing epithelial cells in progression from benign breast or prostate tissue to carcinoma. These results together with: i) limited expression of PDEF in normal tissues; ii) its promotion of epithelial cell motility and invasiveness *in vitro* and tumorigenicity *in vivo*; and iii) immunogenicity of its mouse homologue support PDEF as a novel target and antigen against breast and prostate cancers.