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PI: Philip D. Greenberg

Project: Developing Models to facilitate the appropriate selection and effective targeting of candidate antigens for specific cellular immunotherapy of prostate cancer

Award: Exploration-Hypothesis Development Award (9/1/05-8/31/06)

Introduction: Immunologically targeting prostate cancer has received increasing attention, [1-3], in part because collateral normal tissue injury is an acceptable toxicity. However, many questions must be resolved, including the nature of antigens that can be effectively targeted, the requisite T cell response, and the relationship between tumor development and progression and the immune system. Many candidate human prostate cancer antigens have been identified, including PSA, PSMA, PAP, PSCA, and TARP [2, 4-11]. These targets, and others suggested by analysis of differential gene expression [6, 12-15], include cytosolic, transmembrane, and secreted proteins, which interface differently with the immune system. Predicting which one or class of antigens might be most effectively targeted is difficult to resolve in human trials, and thus the use of relevant mouse tumor models might provide essential insights into prostate cancer immunobiology that can then be translated to human clinical trials.

The Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model, developed by our collaborator Norm Greenberg, appears particularly useful as a foundation for addressing these questions. The TRAMP model is focused on a mouse strain genetically engineered to express from a minimal rat probasin promoter the pro-oncogenic SV40 early genes in prostatic epithelium in a developmentally and hormonally regulated fashion [16]. Transgene expression, associated with puberty and increased androgen levels, can be detected in prostate tissue as early as 4 weeks of age [17]. Disease begins as prostatic intraepithelial hyperplasia (PIN), and progresses to well-differentiated adenocarcinoma as early as 12 weeks [17, 18], to moderately differentiated over the next 6-weeks, and to poorly differentiated carcinoma by 24-30 weeks. Distant metastases, by both hematogenous and lymphatic spread, have been detected as early as 12 weeks, and approach 100% by 24-30 weeks of age [18]. Although tumor initiation and progression can be reliably predicted, a difficulty for probing immunologic interventions has been an absence of a panel of defined tumor antigens with distinct expression characteristics. Therefore, we have been designing ovalbumin (Ova) to serve as a model tumor antigen expressed selectively in prostate epithelial cells. Specifically, Ova has been designed as a transgene for expression under control of the probasin promoter in normal prostate tissue after puberty and maintained in progressing tumors. Ova is being targeted to different cellular compartments to model different classes of candidate prostatic tumor antigens: (a) sOva- secreted (full length ova) [19], (b) mOva- transmembrane (fusion of aa 1-218 of the transferrin receptor to aa 139-385 of ova) [20], and (c) cOva- cytosolic (deletion of aa 1-62 of ova containing the cryptic export signal) [19, 21].

Body: Our first goal was to determine if Ova could be efficiently expressed in all 3 contexts in prostate cells. Therefore, plasmids were constructed with each of the 3 Ova formats as a bicistronic gene containing an IRES and a downstream GFP reporter gene, and transfected into TRAMP-C tumor cell lines. The 3 constructs produced equivalent Ova message by RT-PCR (Fig.1), and protein by Western blot.

Fluorescence staining with Ab revealed that the mOva construct produced readily detectable protein at the cell surface, whereas surface protein was undetectable with cOva and intermediate levels were found with sOva. Permeabilization of the cells before staining, which detects total cytosolic and membrane protein, revealed nearly equivalent levels of protein with all 3 constructs (Fig.1). Each of these tumor lines was shown to stimulate *in vitro* OT-I cells, derived from Tg mice expressing a TCR specific for the Class I-restricted epitope (SIINFEKL₂₅₇₋₂₆₄) in CD8 T cells, suggesting adequate processing and

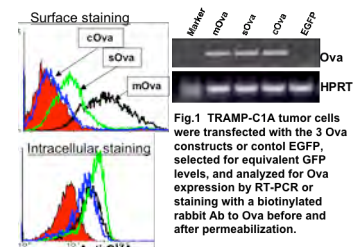


Fig.1 TRAMP-C1A tumor cells were transfected with the 3 Ova constructs or control EGFP, selected for equivalent GFP levels, and analyzed for Ova expression by RT-PCR or staining with a biotinylated rabbit Ab to Ova before and after permeabilization.

presentation of the Class I epitope from Ova in all 3 formats. These constructs were injected into fertilized B6 eggs, and, after 2 sets of injections, candidate founders for each construct have been identified from tail vein DNA. These mice are now being bred for analysis of expression of the genes in prostate tissue *in vivo*.

To begin assessing the potential immunogenicity of these constructs, the genes were inserted into a retroviral vector under control of the LTR, and stably expressed in transplantable TRAMP tumor cell lines. Tumor cells were selected at 1 month for equivalent levels of the GFP reporter gene and analyzed for ability to stimulate CFSE labeled OT-I CD8 cells and OT-II CD4 cells, obtained from Tg mice expressing a TCR specific for the Class II-restricted epitope Ova₃₂₃₋₃₃₉. A distinct stimulation hierarchy was observed both *in vitro* and *in vivo*- *mOva* was less efficient than either *sOva* or *cOva* at stimulating OT-I cells, and *cOva* was a very poor stimulator of OT-II cells compared to *mOva* or *sOva* (Fig.2). These results suggest distinct immunogenic patterns may be anticipated in the TRAMP-Ova mice.

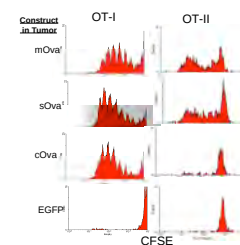


FIG.2 107 CD45.1 OT-I or Thy1.1 OT-II T cells were injected iv into normal CD45.2 Thy1.2 B6 hosts that were inoculated ip 1 day later with 5×10^6 irradiated TRAMP-C1A tumor cells expressing one of the Ova gene constructs. Splens were harvested 4 days later and CFSE dilution of transferred cells assessed.

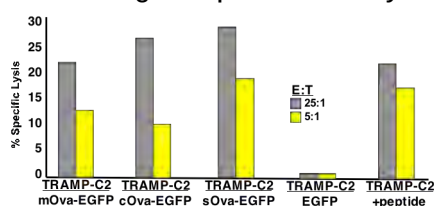


FIG.3 OT-I effector CD8 cells from *in vitro* culture were analyzed for lysis of TRAMP-C2 targets transfected with Ova-GFP constructs or control GFP, or pulsed with 1 μ M SIINFEKL peptide.

We have also performed preliminary studies assessing the ability of T cells to target Ova as a prostatic tumor antigen. We first determined if TRAMP-C2_{Ova} cells could be lysed *in vitro* by effector OTI cells generated by prior *in vitro* activation, and TRAMP-C2_{Ova} cells expressing Ova in each of the formats was recognized. (Fig 3). We then examined if OTI cells could therapeutically target this tumor *in vivo*. Mice were injected sc with 5×10^6 TRAMP-C2 or TRAMP-C2_{mOva}, TRAMP-C2_{sOva}, or TRAMP-C2_{cOva} cells, and

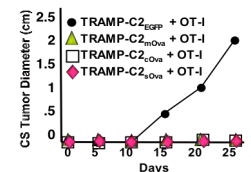


FIG.4 Mice were injected with TRAMP-C2_{GFP} or TRAMP-C2_{mOva}, TRAMP-C2_{sOva}, and TRAMP-C2_{cOva} treated with OT-I cells on day 10 when tumor became detectable

treated on day 10 when the tumor was becoming detectable with 10^7 OT-I cells iv. All untreated mice and mice with TRAMP-C2 tumor transfected with only the GFP gene had to be sacrificed by day 25 due to the progressive large tumor masses, but all mice bearing small TRAMP-C2 tumors expressing either *mOva*, *sOva*, or *cOva* and treated with OTI cells were cured (Fig.4). Thus, OVA in each these formats appears to have the potential to be targeted therapeutically in the spontaneous TRAMP model.

Key Research Accomplishments:

- Developed Ova-transgenic mice that are being mated to TRAMP transgenic mice
- Demonstrated the expression in a prostate tumor of Ova as a model tumor antigen can be immunogenic and induce CD4 and CD8 T cell responses
- Demonstrated that the context of Ova expression in a prostate tumor as a membrane, cytosolic, or secreted protein impacts immunogenicity, and that these contexts of protein expression are differentially recognized by Ova-specific CD4 and CD8 T cells

Reportable Outcomes: None to date (Transgenic mice were developed and are now being bred).

Conclusions: These studies have laid the foundation for having in place a spontaneous prostate tumor model in which the tumor expresses a model antigen, and in which the effect of the nature of expression of that antigen on induction of tolerance, the generation of T cell responses that can recognize the tumor and normal prostate, and the ability to therapeutically target the antigen can be assessed. It is anticipated that the results of such studies will lead to the design of clinical trials by our group as well as other research groups.

Personnel: The personnel who received support for efforts on this project were:

PI: Philip Greenberg

Co-investigator: Norman Greenberg

Res. Tech: Ryan Patrick

Res. Tech: Deborah Kwok

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