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Challenges to Prostate Cancer Immunotherapy

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1. Introduction

Recent approval of Provenge (Sipuleucel-T, Dendreon Corporation, Seattle, WA) by the United States Food and Drug Administration (FDA) has stimulated a new wave of interest in prostate cancer immunotherapy. Sipuleucel-T is an autologous peripheral blood product (APC8015) comprised of partially purified antigen-presenting cells (including monocytes, dendtritic cells, B cells and T cells) loaded *in vitro* with a recombinant fusion protein PA2024, human prostatic acid phosphatase (hPAP) linked to granulocyte-macrophage colony stimulatory factor (GM-CSF) (Small et al., 2000). The conceptual development of this therapy was derived from the fundamental achievements in several different areas of life science in the past several decades. First, the discovery of dendritic cells (DCs) has led to the exploration of these cells as vaccines for cancer immunotherapy (Steinman & Cohn, 1973). Second, the pioneer studies in melanoma indicated that lineage-specific "self" differentiation antigens can be recognized by the immune system, and could be the targets for the immunotherapy (van der Bruggen et al., 2002). Finally, the technological advances in the blood products manufacturing as well as a satisfactory safety record of other cell-based therapies provided an opportunity for the smooth transition of the basic research ideas into the clinical science (ADIS R&D profile, 2006). However, the road to the approval of the first immunological therapy product was not straightforward. Significant difficulties and controversies surrounded all steps from the initial characterization of the immunogenic properties of hPAP to clinical trials and final regulatory approval of Provenge for the use in patients with asymptomatic metastatic castrate-resistant prostate cancer (mCRPC).

1.1 Provenge: Preclinical studies and clinical trials

The Provenge story has started with an early work by Peshwa et al. that demonstrated the induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes (CTL) *in vitro* using antigen-presenting cells (APC) pulsed with the synthetic peptide derived from hPAP (Peshwa et al., 1998). These data indicated that PAP-specific CTL precursors exist and could be potentially recruited to elicit an anti-tumor response. Preclinical studies in animal models demonstrated that DCs loaded with recombinant fusion protein PA2024 could induce strong cellular immune responses *in vivo*, cause tumor delay and improve survival (Valone et al., in press, as cited in (Small et al., 2000). Although very limited information about these

preclinical studies is available, apparently both DCs as well as GM-CSF portion of the fusion protein were essential for the effective anti-tumor immunity, since injections of the fusion protein alone or injections of soulble PAP protein in Freund's adjuvant failed to induce cellular immune responses (Valone et al., in press, as cited in (Small et al., 2000)).

Sequential phase I/phase II trials were performed to determine the safety and efficacy of Provenge and to assess its capacity to break immune tolerance to the normal tissue antigen hPAP (Small et al., 2000). Twelve men were treated in the phase I portion with increasing doses of Provenge, and six patients received all Provenge that could be prepared from a leukapheresis product. The group was heterogeneous in terms of the disease status and previous treatment. Since PA2024 consisted of hPAP fused to the GM-CSF, T-cell and antibody responses to each of these components were examined. All patients developed immune responses to the recombinant fusion protein used to prepare Provenge, and 38% developed immune responses to hPAP. None of the patients had pre-existing T-cell responses to hPAP. Pre-existing T-cell proliferation responses to GM-CSF were observed in 57% patients, which increased after treatment with Provenge to a total 70% of patients. Overall treatment was well tolerated; most patients had no treatment-related adverse events. Three patients had a more than 50% decline in serum prostate-specific antigen (PSA) level, and another three patients had 25% to 49% decreases in PSA levels. However, no improvements in bone scans or soft tissue disease were observed (Small et al., 2000).

In another phase II study (Burch et al., 2004), 21 patients with histologically documented androgen-independent prostate carcinoma were enrolled. Two patients exhibited a transient 25-50% decrease in serum PSA levels. For one patient, serum PSA level dropped to undetectable level, and remained so for more than 4 years. The patient's retro-peritoneal and pelvic adenopathy also resolved. Most of the patients developed antibody and cellular immune responses to the PA2024 fusion protein. However, the responses to purified hPAP or GM-CSF proteins were not observed. Thus, the antigen-specific immuno-reactivity of Provenge observed in earlier studies was not confirmed in later studies.

The safety and efficacy of sipuleucel-T was evaluated in two identically designed, randomised, double-blind, placebo-controlled phase III trials conducted in men with advanced prostate cancer. In one of the studies, 127 patients with radiological evidence of metastasis, evidence of progressive disease, and an expected survival of at least 3 months were enrolled (Small et al., 2006). Time to progression (TTP) was chosen as the primary end point. Patients were randomly assigned in a 2:1 ratio to receive sipuleucel-T therapy or placebo. In the placebo group, APC were isolated from the leukapheresis product and re-infused without being pulsed with PA2024. This phase III study did not demonstrate an improvement in a primary end point (TTP) in patients treated with sipuleucel-T compared to the placebo group. The median TTP for the sipuleucel-T was 11.7 weeks compared with 10.0 weeks for placebo ($p=0.052$, log rank test). The study was not powered to detect survival difference between the two treatment arms, however there was a protocol-specified requirement to follow each patient for survival up to 36 months. This analysis demonstrated that patients treated with sipuleucel-T had significant improvement in overall survival. Median survival time was 25.9 months for sipuleucel-T group, and 21.4 months for placebo ($p=0.01$). The second phase III study revealed no statistically significant benefit in TTP in the overall group (ADIS R&D profile, 2006).

The integrated data from both studies have been recently published (Higano et al., 2009). A total of 225 patients were randomised in both studies to sipuleucel-T (n=147) or placebo (n=78). In the integrated analysis of both studies, patients randomised to sipuleucel-T demonstrated a 33% reduction in the risk of death (hazard ratio, 1.50; 95% confidence interval, 1.10-2.05; P = .011; log-rank test). The treatment effect remained strong after performing adjustments for imbalances in baseline prognostic factors, post-study treatment chemotherapy use, and non-prostate cancer-related deaths. The generally modest toxicity profile, coupled with the survival benefit, suggested a favourable risk-benefit ratio for sipuleucel-T in patients with advanced prostate cancer.

1.2 Provenge: Problems and controversy during regulatory approval

Based on the results of the phase III studies (Small et al., 2006; Higano et al., 2009), Dendreon Corporation submitted the Biologics License Application to the FDA. This decision had raised significant concerns and objections in the scientific community, mostly because the primary endpoint, TTP (for which the study was powered), has not been met, and the claim of the treatment effectiveness (overall survival) was based on the post-hoc analysis. In the opinion of one of the statisticians involved in the initial evaluation of the data, "the trials presented by the company don't provide substantial evidence of efficacy, as required by the FDA regulations. At its best, these trials provided plausibility of efficacy that would justify the conduct of a confirmatory survival trial, and the agent's benefit-to-risk profile remains unknown. The current data are inadequate to make a reliable assessment" (letter from Dr. Flemming, as cited in (Goldberg, 2007a)).

As a part of evaluation process, the application was reviewed by the Cellular, Tissue and Gene Therapies (CTGT) Advisory Committee. However, the process of the approval at the advisory meeting has been conflicting, and required clarification of the approval standards. As the initial discussion at the committee indicated that the data "failed to establish the efficacy" of Provenge, the wording of the question was altered in mid-vote asking whether the sponsor had provided "substantial evidence" of efficacy. This resulted in the vote swing for several members of the advisory panel, and the Committee voted 13 to 4 to recommend Provenge's approval (Goldberg, 2007b). The regulatory terminology for "substantial evidence" and "established efficacy" had led to the vote change by some members, although their position remained the same. For the "established efficacy", the doctor should be able to tell the patient with full confidence that this is established to be an efficacious therapy for the disease. At that time, there were no sufficient grounds for such confidence. However, there was "substantial evidence" that the treatment may work, so the efforts should be continued to evaluate it. Several members of the committee agreed that while the data did not establish efficacy of Provenge, there was substantial evidence that the drug works. There were no significant concerns about the safety of the treatment. Yet, the issue of cerebrovascular events as a potential safety signal was raised. 4.9% of sipuleucel-T-treated patients experienced a cerebrovascular event compared to 1.7% of "placebo" treated group, although the difference was not statistically significant (letter from Dr. Scher, as cited in (Goldberg, 2007b)). The panel voted Provenge was safe by an overwhelming vote of 17 to 0 (Goldberg, 2007b).

The controversy during approval process and continued objections from some members of the CTGT advisory committee as well as from the FDA Oncologic Drug Advisory

Committee (Goldberg, 2007b, 2007c) has led to the delay in the approval of Provenge by the FDA until an ongoing larger Phase III study was completed. The final approval has been granted in 2010 based on the conclusive results of the most recent double-blind, placebo-controlled, multi-center phase III trial in men with mCRPC (Kantoff et al., 2010a). In this study, 512 patients were randomly assigned in a 2:1 ratio to receive either sipuleucel-T (341 patients) or placebo (171 patients). The primary end point was overall survival, analyzed by means of a stratified Cox regression model adjusted for baseline levels of serum PSA and lactate dehydrogenase. In the sipuleucel-T group, there was a relative reduction of 22% in the risk of death as compared with the placebo group (hazard ratio, 0.78; 95% confidence interval [CI], 0.61 to 0.98; P=0.03). This reduction represented a 4.1-month improvement in median survival (25.8 months in the sipuleucel-T group vs. 21.7 months in the placebo group). The 36-month survival probability was 31.7% in the sipuleucel-T group versus 23.0% in the placebo group. While no effect on the time to disease progression was observed, the use of sipuleucel-T prolonged overall survival among men with mCRPC (Kantoff et al., 2010a).

The high level of publicity during of the process of Provenge's approval and significant efforts by public advocates, including advocate patients groups, is understandable since there are not too many options for patients with advanced mCRPC who have a limited life expectancy. Only one drug, docetaxel, has been shown to lengthen overall survival (by approximately 2–3 months) in men with mCRPC (Tannock et al., 2004). The treatment is accompanied by the toxicities so severe that many patients decline to use it at all. Relatively mild and transient toxicity of Provenge compared to the chemotherapy drugs was one of the major factors that have led to its final approval by the FDA.

While Provenge's approval certainly represents a significant achievement in the field of immunotherapy, the controversy surrounding its approval remains unresolved. Recently, the FDA issued a warning letter to Dendreon Corporation for the overstatement of efficacy and omission and minimization of risk information in some promotional materials (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatory Information/ComplianceActivities/Enforcement/UntitledLetters/ucm221635.htm).

The scientific discussion is also still ongoing, as several issues in the Dendreon's studies design remain disputable from the scientific point of view (Longo, 2010). In the Provenge study, the placebo group received autologous untreated APC, while the treatment group APC treated with received a fusion protein consisting of the antigen (hPAP) as well as an immunomodulatory molecule (GM-CSF). APC treated with GM-CSF alone would serve as a more appropriate placebo control. The role of an antigen-specific component (an immune response to hPAP) is completely unclear. While most of the patients developed antibody responses to the fusion protein, there was no convincing evidence of the humoral immune responses to hPAP. Moreover, the cellular immune responses were not thoroughly evaluated. The proliferation assay using a soluble fusion protein could serve as an indicator of CD4 T-cell responses, however antigen-specific CD8 T-cell responses have not been evaluated at all. While the initial scientific rationale for Provenge was based on the promising effects of the DC-based therapy, Provenge is not a DC product in a strict sense, but a partially enriched peripheral blood product that is cultured *in vitro* in the presence of recombinant GM-CSF to initiate DC differentiation. The product is released for the administration based on the increase in the proportion of cells expressing a surface marker

CD54, which is not a specific marker for DCs. CD54+ cells represent only about 10-20% of the final product, the contribution of the other cell populations and cytokines on the potential therapeutic or toxic effects is not known. While the treatment clearly had a survival benefit in men with mCRPC, Provenge group lived only an average of 4.1 months longer compared with men in the control group. No effect on PSA or any other parameter suggesting anti-tumor effect was found. The mechanism for extension of survival with this agent is unknown and may have nothing to do with an immune response. We agree with the opinion of Dr. Longo that "…the prolongation of survival without a measurable anti-tumor effect is surprising. It is hard to understand how the natural history of a cancer can be affected without some apparent measurable change in the tumor… This lack of tumor effect raises concern that the results could have been influenced by an unmeasured prognostic variable that was accidentally imbalanced in study-group assignments" (Longo, 2010).

The controversy surrounding Provenge also highlights the challenges that investigators in the field of cancer vaccines are facing in the attempt to develop an effective vaccine against prostate cancer, including the selection of target antigen and adjuvant for the vaccine development and the choice of best strategy for immunological monitoring during clinical trials. In this chapter, we will review data generated in our laboratory over a period of more than ten years, and describe our attempts to characterize CD8 T-cell responses to prostate tumor-associated antigens (TAA) and understand the role of CD4 T-cells and humoral immunity in the regulation of the anti-tumor immune response and cancer progression.

2. Evaluation of the CD8 T-cell responses to human PSA in patients with prostate cancer

Pioneering studies in melanoma clearly demonstrated that many cancer antigens were not mutated or abnormal proteins that would be logically expected to be expressed by cells with aberrant function but were, in fact, derived from normal proteins expressed by mature cells of the same lineage from which the tumor was derived (van der Bruggen et al., 2002). The first example of this phenomenon was a demonstration that the enzyme tyrosinase, which is expressed by melanocytes and is required for synthesizing melanin, is an antigen for T-cells in melanoma (Coulie et al., 1994). The link between a successful cancer immunotherapy and the induction of autoimmunity was demonstrated by the fact that the appearance of autoimmune vitiligo correlated with anti-tumor response in patients undergoing melanoma immunotherapy (Rosenberg & White, 1996). Based on melanoma studies, it was reasonable to hypothesize that a successful immunotherapy for prostate cancer could be based on the prostate-specific differentiation antigens. The idea to induce an autoimmune response against normal prostatic proteins was particularly attractive since destruction of the prostate during immunotherapy is of no apparent consequence in men after their reproductive years, as well as in prostate cancer patients who underwent a radical prostatectomy.

Several prostate differentiation antigens, including PSA, PAP and others, have been explored as targets for the prostate cancer immunotherapy (reviewed in (McNeel, 2007)). However, the first step in identifying prostate tumor antigens by studying T-cells specific for prostate tumor cells has not occurred to the extent that it did with melanoma. The identification of T-cells that specifically recognize prostate cancer cells has been hampered by two major problems. First, prostate cancer cells are difficult to grow *in vitro* especially for long periods and second, T-cells specifically recognizing prostate tumor cells have been

possible to produce in culture only in rare circumstances (Haas et al., 1990). The T-cell lines can be produced but it is difficult to preserve their function and specificity for an interval long enough to allow for repeated assessments of peptide and whole antigen reactivity that are convincing. These studies have relied upon the *in vitro* cultures of peripheral blood mononuclear cells (PBMC) derived from non-vaccinated cancer patients or normal individuals of known Human Leukocyte Antigen (HLA) types. The responses in such cultures are usually marginal, and may represent fluctuations in the non-specific background or the responses to the impurities in the antigen preparations. Even when peptides show significant immunogenicity *in vitro*, the evidence that CD8 T-cells specific to these peptides could recognize target cells expressing endogenous antigens in Major Histocompatibility Complex (MHC) class I-restricted manner is rarely convincing.

2.1 PSA3 peptide for immunological monitoring and vaccination in patients with prostate cancer

Several computer-based algorithms, either allele-specific, or HLA-unrestricted have also been used to identify immunogenic epitopes (reviewed in (Hattotuwagama et al., 2007)). The major problem of this approach is the low probability that the peptide is made during natural processing and presentation by antigen-presenting cells. This problem is exacerbated by the lack of autologous target cell lines. The data generated using allogeneic partially HLA-matched targets should be interpreted with caution, as this approach is prone to producing false-positive results. In some cases, the epitopes that were selected by this method and initially believed to be naturally processed were later identified as cryptic once lymphocytes from vaccinated patients became available for the analysis.

One of such examples is an HLA-A2.1-restricted peptide $PSA_{154-163}$ (VISNDVCAQV, also known as PSA3). The peptide sequence was selected based on the computer-generated prediction algorithm, and the ability of this peptide to bind HLA-A2 molecule was initially tested in the T2 cell line-based binding assay (Correale et al., 1997). The T2 cell line [(174 x CEM.T2) is deficient in the polymorphic peptide transporter molecule, and expresses very low levels of HLA class I on the surface. An external loading of HLA class I-restricted peptides stabilizes the HLA/peptide complex on the cell surface, which is detected with HLA-specific antibodies by the shift in the immunofluorescence intensity. In the study by Correale at al., the test was performed using a single dose of the peptide (50 μg/ml) at single time point, and demonstrated a reasonably high level of binding (Correale et al., 1997). However, more detailed analysis performed later (Terasawa et al., 2002) using the same assay in a range of peptide doses clearly demonstrated that the PSA3 is a low affinity peptide, as the binding to T2 target cells dramatically decreased at the concentrations lower than 10 μg/ml. Moreover, the analysis of the stability for PSA3 peptide in the T2-based assay also revealed that the PSA3 peptide is unstable, with a half-life of less than 2 hr (Terasawa et al., 2002). PSA3-specific CD8 T-cell lines were established from non-vaccinated HLA-A2.1+ donors but required six or seven *in vitro* stimulation cycles in order to generate a measurable immune response.

Despite its low affinity and stability, PSA3 peptide is still commonly used in PSA-based vaccine clinical trials as a surrogate marker for PSA-specific CD8 T-cell responses. This choice remains highly disputable, and presents significant challenges in the interpretation of the data produced in the immunological assays. A number of recently conducted clinical

trails for prostate cancer-specific vaccines reported the successful achievement of the immunological endpoints (Madan et al., 2009; Lubaroff et al., 2009). However, the detailed examination of the data raises significant concerns. One of the problems in the interpretation of the results is lack of actual "raw" data. For example, when the immune reactivity to PSA peptides is measured by interferon (IFN)-γ Enzyme-linked immunosorbent spot (ELISPOT) assay, the results are usually expressed as precursor frequencies or fold increase in precursor frequencies, which makes the interpretation of these data very difficult. In one of the recently published studies in the series of PSA-based vaccine trials, the best responding patient demonstrated PSA3A-specific precursor frequency of 1/35,294 (Gulley et al., 2010). Although no "raw" ELISPOT data were published in this report, the estimation could be made based on the available data for PBMC plating density (Gulley at al., 2002). Simple calculation shows that precursor frequency of about 1/35,000 for a given plating density of $6x10^5$ cells per well would correspond to about 17 positive spots per well. Since the data for absolute magnitude of the response compared to "no peptide" control were not presented, it is not possible to assess the validity of these measurements. Given a significant variation in the non-specific background responses in human PBMC cultures, this is in general a very low count. In our clinical studies, for example, we use very stringent criteria for the distinguishing "positive" and "negative" responses. The ELISPOT result is considered positive if the number of spots in the wells stimulated with specific peptides is 10% higher than the number of spots in the wells without peptide or irrelevant peptide with a cut-off of 20 spots (regardless of plated cell number) above background provided that the difference was statistically significant by 2-sided Student's t-test (Kouiavskaia et al., 2009b).

So far, a scrutinized analysis of the available published data from most of the clinical trials in prostate cancer does not provide a sufficient evidence that CD8 T-cell responses to the naturally processed tumor antigen were induced by the prostate cancer vaccines. In our opinion, the convincing evidence that the vaccines targeting prostatic differentiation antigens are capable of generating a meaningful T-cell-mediated immunity is currently lacking.

2.2 Vaccination with agonist peptide PSA:154-163 (155L) derived from prostate-specific antigen: A phase 2 study in patients with recurrent prostate cancer

Due to the low affinity and stability of PSA3 peptide, a search for amino acid substitutions has been performed to improve peptide binding to HLA-A2.1. A modified agonist peptide PSA:154–163(155L) (also designated PSA-3A, "A" for agonist), which represents a native PSA3 peptide with substitution of leucine at position 155, has been described (Terasawa et al., 2002). The substitution increased the affinity and stability of binding of PSA3A to HLA-A2.1. In this study, CD8 T-cell lines specific for PSA3A were generated by three cycles of *in vitro* stimulation with the peptide, and were capable of recognizing the native PSA3 peptide and lyse peptide-pulsed HLA-A2.1+ tumor target cells. The immunogenicity of PSA3A peptide was also confirmed using *HLA-A2.1* transgenic (tg) mice (Terasawa et al., 2002). The significantly improved immunogenicity of the agonist peptide in human PBMC cultures and in a "humanized" mouse model generated a sufficient level of enthusiasm for the testing this peptide in a clinical trial in patients with prostate cancer.

We have conducted a clinical trial sponsored by the NCI Cancer Therapy Evaluation Program to evaluate the peptide PSA:154-163(155L) as a vaccination strategy for the

treatment of prostate cancer in HLA-A2 patients with detectable and rising serum PSA after radical prostatectomy (Clinicaltrials.gov identifier NCT00109811). The results of this study have been recently published in the *Journal of Immunotherapy* (Kouiavskaia et al., 2009a) and are discussed below. The trial was a single dose-level, Phase II pilot trial of 1 mg of PSA3A emulsified with adjuvant (Montanide ISA-51). The primary endpoint was determination of immunogenicity of the vaccine; secondary outcomes were determination of toxicity and effect on serum PSA**.** In our study, five patients were enrolled and completed all vaccinations. We were not able to detect PSA-3A peptide-specific responses in primary PBMC in a sensitive IFN-γ ELISPOT assay at any time point during the course of vaccination. However, three of five patients demonstrated strong IFN-γ responses, and one patient demonstrated a marginal response to the PSA3A peptide in advanced CD8 T-cell cultures derived from post-vaccination PBMC (Kouiavskaia et al., 2009a). None of the five cultures derived from the baseline PBMC responded to the peptide. These results can be considered as evidence of a successful vaccination in these patients; however, low frequencies of antigen-specific cells could not be detected by direct ELISPOT assay using unfractioned PBMC.

Functional activity of the PSA3A-specific CD8 T-cell lines was also assessed in IFN-γ and Granzyme B ELISPOT assays using various PSA-expressing HLA-A2+ target cells. While strong IFN-γ responses were detected when the PSA3A peptide was presented by HLA-A*0201-expressing HEK293 cells, none of the peptide-specific T-cell lines could recognize HEK293-A*0201 cells expressing endogenous PSA (Kouiavskaia et al., 2009a). We also failed to detect any response to the PSA-producing HLA-A2.1+ prostate adenocarcinoma cell line LNCaP. This cell line is commonly used as a model cell line in prostate cancer studies. The recognition of LNCaP cells by CD8 T-cells specific to the PSA3 or PSA3A peptides has been previously reported (Correale et al., 1997; Terasawa et al., 2002). However, we could not detect any recognition of the LNCaP cells by the PSA3A-specific CD8 T-cell cultures in a sensitive IFN-γ ELISPOT assay, although the same CD8 T-cell lines strongly responded to the stimulation with the peptide presented by other HLA-A2+ tumor cell lines used in the study. The ability of LNCaP cells to express and up-regulate HLA-A2 is highly disputable in the literature. Some studies describe LNCaP subclones that express low but detectable levels of HLA class I presumably sufficient for antigen presentation (Correale et al., 1997) . On the other hand, several studies indicated that HLA class I is under-expressed on LNCaP, rendering the cells poor at presenting antigens in the context of class I. In addition, the LNCaP cell line is unresponsive to IFN-γ treatment due to the lack of JAK1 gene expression (Dunn et al., 2005; Sanda et al., 1995). In our experiments, HLA-A2 was barely detectable on LNCaP cells by flow cytometric analysis, and could not be up-regulated by IFN-γ treatment.

To test the ability of PSA3A-specific CD8 T-cells to recognize tumor target cells expressing endogenous PSA, we have established a PSA-expressing OVCAR3 tumor cells line, and rigorously tested its ability to up-regulate HLA-A2 and process endogenous antigens. CD8 T-cell lines specific to M1 Influenza peptide demonstrated strong reactivity with influenza A virus-infected OVCAR target cells in the IFN-γ ELISPOT assay. In contrast, CD8 T-cell lines specific to PSA3A peptide demonstrated minimal response to OVCAR cells engineered to express endogenous PSA. The responses were observed only in a minority of cultures, and their magnitude was only about 10-20% of the responses against control OVCAR cells (OVCAR-ev) pulsed with PSA3A peptide (Kouiavskaia et al., 2009a). The responses from the only patient that demonstrated the reactivity (Pr159) are shown in Figure 1.

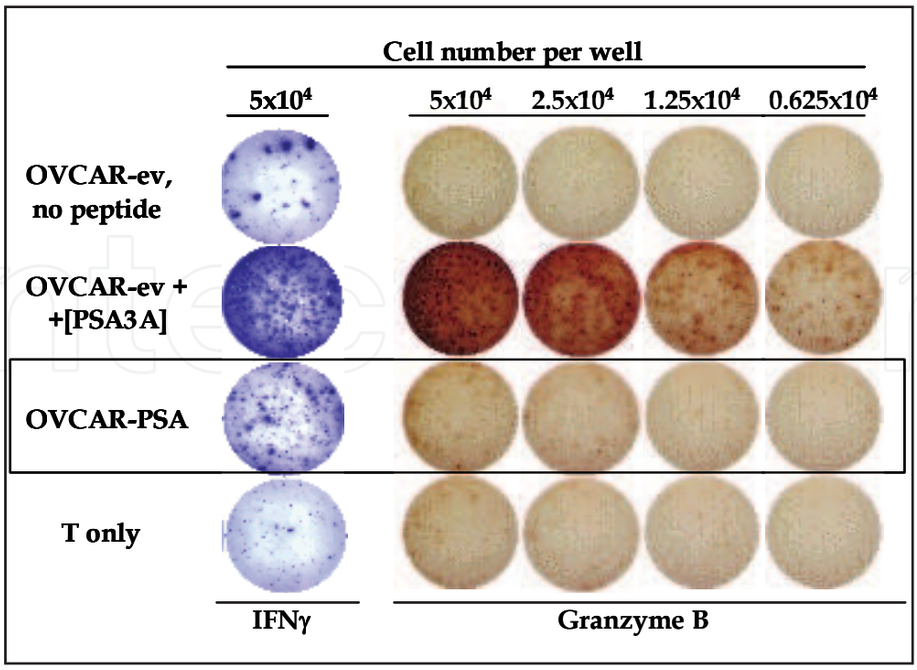

Fig. 1. Recognition of endogenous PSA by PSA3A peptide-specific CD8 T-cells. PSA3A peptide-specific CD8 T-cell line developed from patient's Pr159 PBMC was tested for the reactivity with either specific peptide (presented by OVCAR-ev cells) or PSA-expressing OVCAR tumor cells in IFN-γ (blue dots) or Granzyme B ELISPOT (red dots) assays. Representative wells of triplicates are shown.

Since we detected a weak response to PSA-expressing OVCAR tumor cells in some cultures, we established PSA3A-specific CD8 T-cell clones by limiting dilution from post-vaccination CD8 T-cell cultures of all four responding patients. However, none of the T-cell clones could recognize both the specific peptide and PSA-expressing tumor cells in the same experiment. Interestingly, on a rare occasion we observed clones that reacted with the PSA-expressing tumor cells, but were not reactive with the PSA3A peptide (Figure 2). These data suggest that low levels of reactivity to target cells expressing endogenous PSA that could be detected in some of the cultures were probably due to the non-specific reaction with allogeneic target cells and were not related to the specific anti-PSA immune response. These findings provided evidence that the native peptide, PSA3, is not effectively processed and presented by PSA-expressing tumor cells.

Recent studies in *HLA-A2 tg* mice also confirmed that the PSA3 peptide is poorly immunogenic and is not naturally processed efficiently (Lundberg et al., 2009). In this study, *HLA-A2 tg* mice were immunized with recombinant vaccinia virus encoding whole human PSA, and the CD8 T-cell reactivity with a panel of HLA-A2 restricted peptides was tested by intracellular cytokine staining for IFN-γ. Only a small proportion of CD8 T-cells were reactive against the PSA3 peptide, and the signal was detected only after extensive course of immunization. These data, as well as the findings in our clinical trial, contradict the findings by Terasawa et al. discussed above (Terasawa et al., 2002). One possible explanation of the discrepancy between two studies in *HLA-A2 tg* mice could be the difference in the mouse strains used in both studies. Lundberg et al. used HHD homozygous mice that express a

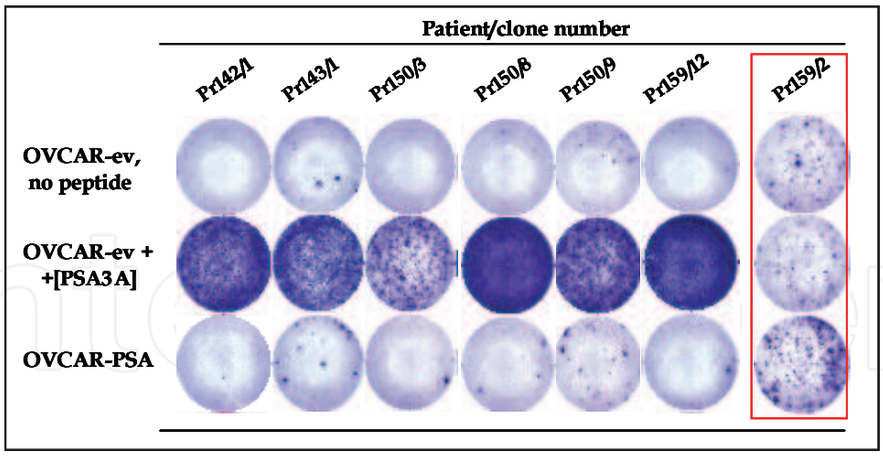

Fig. 2. Lack of recognition of endogenously-expressed PSA by PSA3A peptide-specific CD8 T-cell clones. PSA3A peptide-specific CD8 T-cell clones were developed by limiting dilution (patients Pr142, Pr143, Pr150, Pr159) and tested for reactivity with either specific peptide or PSA-expressing tumor cells in IFN-γ ELISPOT assay. Clone that responded to OVCAR-PSA but lacked peptide-specific reactivity is enclosed into red rectangle (reproduced from Kouiavskaia et al., 2009a).

*HLA-A*0201* chimeric monochain (α1 and α2 domains of *HLA-A*0201* allele and α3 intracellular domain of *H-2D*^{b} allele). The genes for both the *H-2D*^{b} and mouse *β2m* have been disrupted and therefore the HHD mice are deprived of cell surface H-2 molecules (Lundberg et al., 2009). In contrast, Terasawa et al. used different strain of mice that expressed the product of the *HLA-A2.1/K*^{b} chimeric gene as well as native H-2^b molecules (Terasawa et al., 2002). In addition, Lindberg et al. vaccinated mice with a vector encoding whole PSA, hence the responses were detected against naturally processed epitopes. In contrast, Terasawa et al. used a "reversed" approach (immunization with the synthetic peptide in adjuvant), which was appropriate for the evaluation of the peptide immunogenicity but did not address the issues of natural processing. In both studies, mice did not express human PSA as a "self" antigen, hence questions of tolerance could not be addressed in these models. Given a low affinity, low stability, poor natural processing and poor immunogenicity of the PSA3 peptide even in a "foreign" antigen setting, it is not clear why the responses to this peptide are frequently detected in human PBMC cultures.

Significant variations in the methods for the immunological monitoring, and the difficulties in the interpretation of the data generated in different laboratories clearly indicate that there is an overwhelming need for a standardized approach for the data collection and interpretation. Significant attempts have been made recently to unify and integrate immunological monitoring in cancer immunotherapy. The examples of these efforts could be the NCI-supported new initiative, Cancer Immunotherapy Trials Network (http://grants.nih.gov/grants/guide/rfa-files/RFA-CA-10-007.html), or Minimal Information About T-cell Assays ("MIATA") initiative (http://www. miataproject.org). However, these initiatives still do not address all challenges in the interpretation of the immunological data. In many cases, the responses registered in the immunological assays are objectively positive, yet their interpretation could be misleading unless other supportive assays confirm or disprove the original findings. In our clinical trial with the PSA3A peptide, for example, the

bulk peptide-specific CD8 T-cell cultures from some patients did display the reactivity against PSA-expressing tumor targets, which could have been interpreted as an indicator that the PSA3 epitope is naturally processed and presented. Only through labour- and time-consuming limiting dilution analysis were we able to demonstrate that the reactivities against the peptide and against the tumor target expressing endogenous protein were clearly separated. Cloning by limiting dilution is one of the examples of the assay that currently cannot be formally standardized. However, utilizing this method in our clinical trial was critical for avoiding a misleading conclusion about potential utility of the PSA3A peptide for the prostate cancer vaccine. Careful selection of target tumor cells tested for the ability to express HLA class I molecules and process endogenous antigens was also critical.

Overall, the immunization with PSA3A peptide in Montanide ISA-51 adjuvant induced a peptide-specific CD8 T-cell response in 4 out of 5 patients. Since the responses can only be detected after CD8 T-cell enrichment and 1–2 rounds of amplification *in vitro*, the vaccine was not very immunogenic, at least in the combination with Montanide ISA-51 adjuvant. We confirmed that CD8 T-cell lines specific to the agonist peptide were capable of recognizing a native PSA3 peptide. However, peptide-specific T-cells failed to recognize HLA-A2 targets expressing endogenous PSA. We concluded that the PSA3 peptide is poorly processed from endogenous PSA and therefore represents a cryptic epitope of PSA in HLA-A2 antigen-presenting cells. There were no significant changes in serum PSA level in any subject. Based on our findings, the trial was terminated.

3. Evaluation of CD4 T-cell-mediated immune responses to prostatic antigens

Given the importance of CD4 T-cells in sustaining effective anti-tumor CTL responses, and their role as effectors mediating autoimmune responses, we studied MHC class II-restricted immune responses to prostatic antigens in patients with prostatic diseases. The identification of the antigens involved in the autoimmune processes in the prostate remains a difficult task since the proliferative responses detected in the peripheral blood of the patients in response to prostatic proteins are weak and difficult to interpret. In our previous work, we were able to establish CD4 and CD8 T-cell clones reactive with human PSA, however we were not able to convincingly establish their epitope specificity using overlapping PSA peptide library (Klyushnenkova et al., 2004). Characterization of immunogenic and naturally processed epitopes derived from human antigens can be significantly facilitated by the use of *HLA tg* mice in which mouse molecules are deleted so that immune-mediated processes are restricted exclusively by human molecules (Taneja & David, 1998).

3.1 Strain-specific differences in the immune response to human PSA in *DR2b tg* mice

The association of multiple sclerosis (MS) with *HLA-DRB1*1501* has led to the development of transgenic mice expressing this allele for the study of experimental allergic encephalomyelitis (EAE) (Madsen et al., 1999). The mice are termed *DR2b tg* in deference to the older designation of *HLA-DRB1*1501* allele. The mice express a hybrid of the α1 and β1 sequences of human alleles *DRA1*0101* and *DRB1*1501* and the α2 and β2 domains of mouse *IEα* and *IEβ*, respectively to ensure efficient interaction with mouse CD4. All normal mouse Class II molecules in *DR2b tg* mice have been deleted, hence all CD4 T-cells in these

mice are restricted by HLA-DRB1*1501, allowing the CD4 T-cell responses to vaccinated antigens to be studied in detail. Using these mice, we have recently identified two immunogenic epitopes derived from human PSA, $PSA_{171-190}$ and $PSA_{221-240}$. The results of this study have been published in *Clinical Cancer Research* (Klyushnenkova et al., 2005) and are briefly described in Figure 3. In these studies, we immunized *DR2b tg* mice with highly purified human PSA derived from seminal plasma in Complete Freund's Adjuvant (CFA), and screened a library of 20-mer peptides that overlapped by 10 amino acids and spanned the entire protein sequence. Using the "reverse" approach, we also confirmed that immunization with either $PSA_{171-190}$ or $PSA_{221-240}$ induced CD4 T-cell-mediated immunity against whole, naturally processed antigen (Klyushnenkova et al., 2005).

Since *DR2b tg* mice were engineered on C57BL6/J (B6) background, we also compared the immune responses to PSA protein in *DR2b tg* mice and wild type (wt) B6 mice. While *DR2b tg* mice developed a rigorous IFN-γ response to PSA, wt B6 mice developed only marginal response to whole PSA protein, and no significant reactivity was found to any of the 20-mer PSA peptides in the library (Figure 3). These data served as an initial indication that native murine $I\text{-}A^b$ allele cannot support a CD4 T-cell-mediated response to human PSA. The MHC class II-dependent strain-specific disparity in the response to PSA had significant consequences for the outcome of tumor growth in our mouse tumor model that will be discussed below in the Section 4.

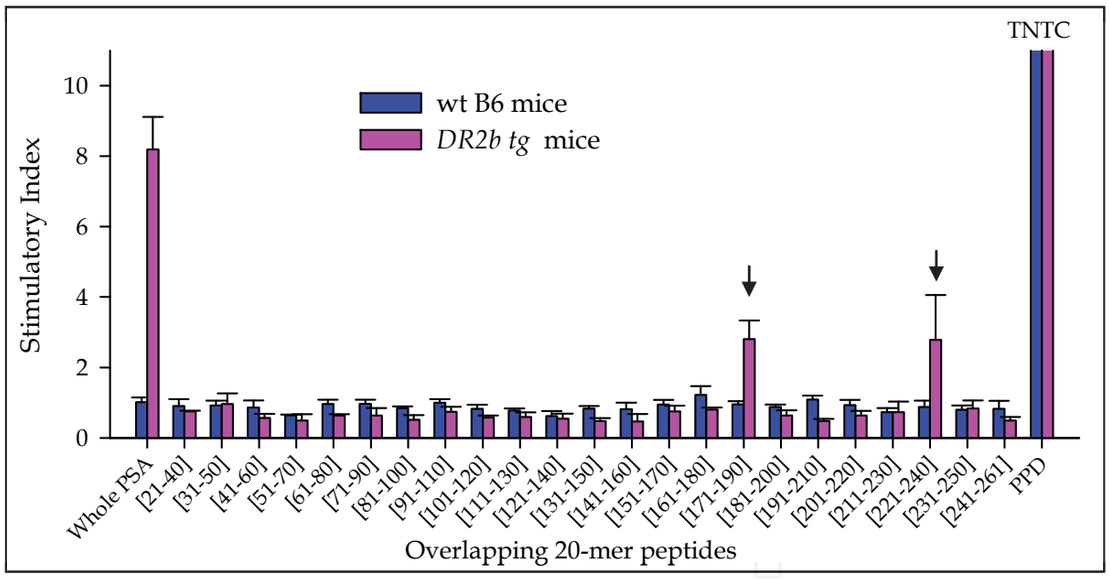

Fig. 3. CD4 T-cell-mediated responses to human PSA in mouse models. *DR2b tg* (pink bars) or wt B6 (blue bars) mice were immunized subcutaneously (s.c.) with human PSA in CFA. Spleens were harvested two weeks later, and lymphocytes were cultured in the presence of PSA protein or a series of overlapping 20-mer peptides derived from the primary amino acid sequence of human PSA. The responses to the purified protein derivate (PPD) of tuberculin served as positive controls. IFN-γ secretion was measured by ELISPOT assay. Stimulatory index was calculated by equation: [Spot number (sample)] / [Spot number (no Ag)]. Data are means ± SD of triplicates. TNTC: "too numerous to count". The 20-mer peptide sequences are published in (Klyushnenkova et al., 2005).

3.2 CD4 T-cell responses to human PAP in mouse models

While our initial efforts were focused on the characterization of the immunogenic properties of PSA, we still do not know whether PSA is an immunodominant antigen or even a particularly strong antigen. Almost exclusive expression of human PAP in normal prostate and prostate tumors makes this protein a promising candidate target for prostate cancer immunotherapy (Fong & Small, 2006). Using *DR2b tg* mice, we studied the immune reactivity to hPAP and identified two immunogenic and naturally processed epitopes within hPAP amino acid sequence. The results of this study have been published in the *Prostate* (Klyushnenkova et al., 2007) and are briefly described in Figure 4.

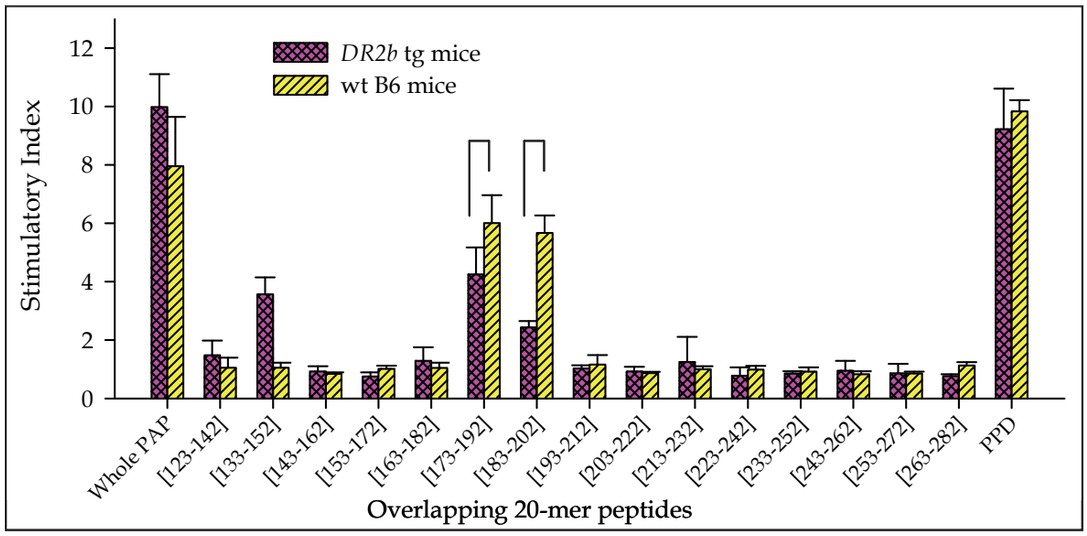

Fig. 4. CD4 T-cell-mediated responses to hPAP in mouse models. *DR2b tg* or wt B6 mice were immunized s.c. with hPAP in CFA. IFN-γ secretion was measured as described in the legend to Figure 3. The peptides from the regions PAP(33-132) and PAP(272-386) showed no significant reactivity and are omitted on the graph. The 20-mer hPAP peptide sequences were published in (Klyushnenkova et al., 2007). Brackets indicate 20-mer peptides common for *DR2b tg* and wt B6 mice.

One of the epitopes was defined by overlapping 20-mer peptides $hPAP_{123-142}$ and $hPAP_{133-152}$, and the other was defined by overlapping peptides $hPAP_{173-192}$ and $hPAP_{183-202}$. These peptides were immunogenic and naturally processed from whole PAP in *DR2b tg* mice and human CD4 T-lymphocyte cultures (Klyushnenkova et al., 2007). Since PSA-specific immunoreactivity demonstrated clear allele-specific differences, we also analyzed hPAP-specific immune responses in wt B6 mice. In contrast to PSA, which showed striking strain-specific differences in CD4 T-cell-mediated reactivity (Figure 3), both *DR2b tg* mice and wt B6 mice developed rigorous immune responses to hPAP protein (Figure 4). Screening of overlapping 20-mer hPAP peptides in wt B6 mice identified an immunodominant *I-A^b*-restricted epitope defined by overlapping 20-mer peptides $hPAP_{173-192}$ and $hPAP_{183-202}$. This region was identical to the one defined in *DR2b tg* mice (Figure 4) although more detailed analysis would be required to demonstrate whether the anchor residues involved in binding to the I-A^b and DR2b molecule are the same.

3.3 Cross-reactivity between human and mouse PAP: Application to autoimmunity

Given a high level of homology between mouse (mPAP) and human PAP (81% on an amino acid level (Fong et al., 1997)), we examined whether immunization with peptides derived from hPAP could induce cross-reactivity to the corresponding mouse epitope. We chose a 20-mer peptide $hPAP_{173-192}$, which was an immunodominant epitope in both *DR2b tg* and wt B6 mice. As shown in Figure 5, *DR2b tg* mice immunized with $hPAP_{173-192}$ developed strong IFN-γ response to the specific human peptide as well as whole naturally processed hPAP protein, but completely lacked reactivity with a mouse homolog peptide. Similar data were obtained in wt B6 mice (data not shown). Since both $hPAP_{173-192}$ and $hPAP_{183-202}$ overlapping 20-mers showed strong reactivity during peptide library screening in both *DR2b tg* and wt B6 mice (Figure 4), the minimal reactive epitope was most likely located in the region common for these two peptides. As shown in Figure 5, mouse and human PAP differed in 4 amino acids within this overlapping region. These substitutions were sufficient to completely abrogate the response to mPAP in mice immunized with $hPAP_{173-192}$. We also could not detect an antigen-specific inflammation in the prostates of these mice. CD45+ leukocyte infiltrates were present in the prostate stroma, but were attributed to the inflammation induced by the CFA (data not shown).

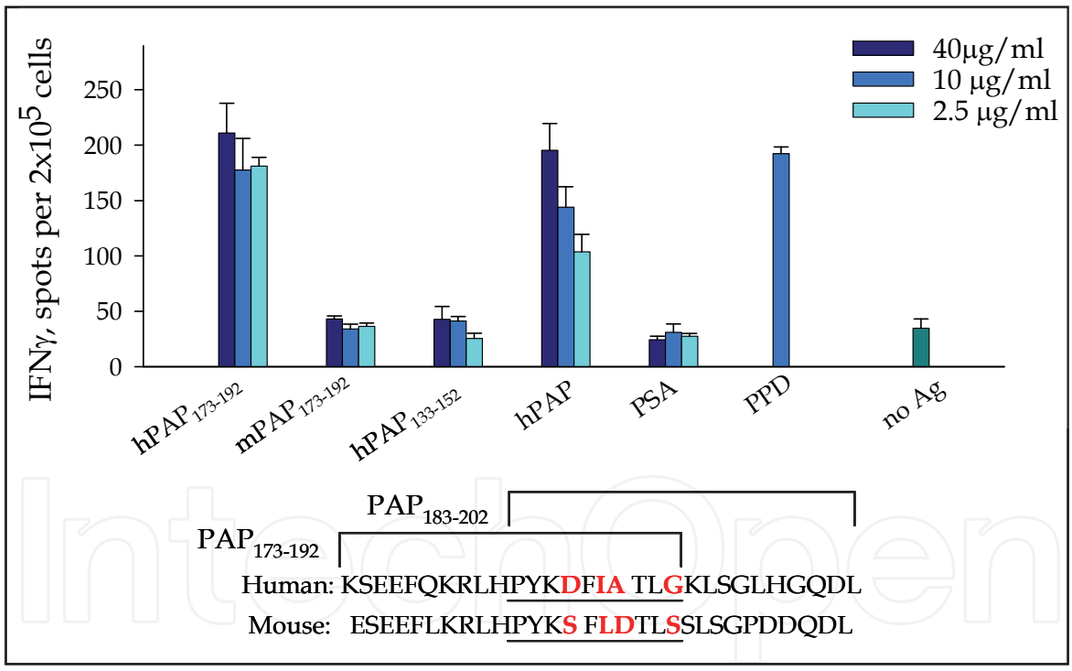

Fig. 5. Lack of cross-reactivity between human and mouse $PAP_{173-192}$ in *DR2b tg* mice. *Top: DR2b tg* mice were immunized s.c. with $hPAP_{173-192}$ peptide in CFA. Spleens were harvested two weeks later and tested for the reactivity with $hPAP_{173-192}$, $mPAP_{173-192}$, or whole soluble hPAP at indicated concentrations. The response to the $hPAP_{133-152}$ and soluble whole PSA served as negative controls; PPD served as a positive control. IFN-γ secretion was measured by ELISPOT assay. Data are means ± SD of triplicates. *Bottom:* amino acid sequences corresponding to human and mouse overlapping peptides $PAP_{171-192}$ and $PAP_{183-202}$ are shown. Overlapping 10-mer regions are underlined. Amino acids that are different between two species are shown in bold red colour.

The cross-reactivity between mouse and human PAP has been previously reported, and served as a rationale for the development of the xenoantigen-based cancer vaccine (Fong et al., 2001; Castelo-Branco et al., 2010). In these studies, wt B6 mice immunized with hPAP developed the immune response to mPAP, which was implied as an underlying mechanism for autoimmune destruction of the prostate gland epithelium in immunized mice. In our experiments, we were not able to establish a molecular basis for the cross-reactivity between human and mouse PAP for the *DR2b* chimeric transgene or the native *I-A^b* allele. Cross-reactivity between human and rodent PAP has been also demonstrated in other models. For example, Copenhagen rats (RT1^{av1} MHC haplotype) immunized with naked DNA vector encoding hPAP developed cross-reactive proliferative and CTL responses to rat PAP that were accompanied by the destructive autoimmune prostatitis (Fong et al., 1997). Similar results were obtained in Lewis rats (RT1^l MHC haplotype) (Johnson et al., 2007). Unfortunately, the molecular mechanism of such cross-reactivity has not been established in these studies. The MHC-restricted epitopes involved into the pathogenic responses in the prostate have not been identified, and the induction of autoimmunity by either immunization with such epitopes or an adoptive transfer of epitope-specific T-cells has not been demonstrated. The pathogenic activity in these models appeared to be associated with CD8 but not CD4 T-cells (Fong et al., 1997; Johnson et al., 2007). These results imply the existence of the H-2^b- and RT allele-restricted epitopes within hPAP amino acid sequence that are identical to or agonistic for the corresponding rodent PAP peptides. Once such epitopes are identified, it would serve as a definitive proof of cross-reactivity between mouse (rat) and human PAP.

3.4 Immune responses to prostatic antigens in patients with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS)

Several other rodent models of autoimmune prostatitis have been described in the literature (reviewed in (Motrich et al., 2007)), however, it is still unclear how adequate these animal models are for the study of the human disease. Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a common but poorly understood condition, and is defined only by non-specific symptoms such as pelvic pain, void dysfunction, and lack of bacterial infection (Alexander & Trissel, 1996; Schaeffer et al., 2002). The presence of autoimmune inflammation in CP/CPPS has been suggested based on the cellular and humoral reactivity to the prostatic antigens detected in the peripheral blood (Alexander et al., 1997; Fong et al., 1997; Dunphy et al., 2004; Motrich et al., 2005; Kouiavskaia et al., 2009b), while the histopathological evidence of the autoimmune infiltration in the human prostate is very limited. In contrast, autoimmune prostatitis in animal models is defined based on the presence of inflammatory infiltration accompanied by pathological changes in the prostate epithelium, while the neuro-physiological mechanisms are rarely, if ever, studied.

The assumptions for the autoimmune nature of the CP/CPPS are mostly based on the similarities with other autoimmune diseases and their corresponding animal models (for example, MS and EAE, diabetes etc.) One of the important lessons learned from the rodent models of autoimmunity is the existence of "susceptible" and "resistant" MHC haplotypes. In the"classical" models of autoimmunity, it is a unique combination of an immunogenic epitope and a "susceptible" MHC (usually MHC class II) allele that triggers and governs the process of autoimmune inflammation. In a polymorphic human population, it remains unclear whether T-cells activated by a given prostatic antigen in a context of a given HLA

allele are directly involved in the pathogenesis of the disease. We have previously demonstrated that men with a destructive inflammatory process in the prostate gland called granulomatous prostatitis showed the strongest recognition of PSA by the peripheral CD4 and CD8 T-cells, and that CD4 T-cell-mediated recognition occurred in the context of *HLA-DRB1*1501* (Klyushnenkova et al., 2004). In addition, an association of this disease with *HLA-DRB1*1501* in Caucasian men provided the evidence that an autoimmune recognition of a normal self prostatic protein is occurring in a human disease (Alexander et al., 2004). However, we could not establish an association with any common HLA-DR/DQ allele for patients with CP/CPPS (Kouiavskaia et al., 2009b). Some indirect evidence, for example, the presence of autoreactivity with prostatic proteins in the peripheral blood, or the presence of autoantibody response implies the existence of underlying autoimmune process, but the definitive proof is yet to be obtained. In our recent study, we have demonstrated that CD4 T-cells from patients with CP/CPPS have a higher frequency of recognition of the self prostatic proteins PAP and PSA compared to normal male blood donors (Kouiavskaia et al., 2009b). Particularly striking was the difference in the response to $hPAP_{173-192}$ peptide. This peptide has been previously identified in the context of *HLA-DRB1*1501* allele (Klyushnenkova et al., 2007), but showed a significant degree of promiscuity based on the HLA binding assay (Kouiavskaia et al., 2009b). Interestingly, there were no differences between *DR15*+ patients and normal controls in the CD4 T-cell responses to this peptide, while the responses of non-*DR15* patients differed dramatically (Kouiavskaia et al., 2009b). We can speculate that a limited response to $PAP_{173-192}$ in healthy men may be due to the higher threshold of activation for the low affinity/avidity autoreactive T-cells found in the peripheral circulation. Under some conditions, such as inflammatory processes, the lower affinity (or functional avidity) epitopes may become "visible" to the immune cells and facilitate autoimmune reactions. It is possible that the pro-inflammatory environment in CP/CPPS may be contributing to the fact that $PAP_{173-192}$ was the most discriminating peptide between cases and normal male donors. This may also explain broader HLA usage repertoire in response to $PAP_{173-192}$ in CP/CPPS patients compared to the control group. Further experiments using humanized animal models are needed to prove directly that CD4 T-cells specific to $hPAP_{173-192}$ or other antigenic peptides are essential and sufficient to induce a destructive autoimmune response in the prostate.

Since it appears that the induction of an anti-tumor immune response is, in fact, the induction of autoimmunity, many of the principles and models in the study of autoimmunity may be applicable to the field of tumor immunology, and could guide the design of immunotherapy for prostate cancer. Mice co-expressing clinically-relevant or model antigens as "self" antigens in the prostate and HLA class I and/or class II molecules on the genetic background that recapitulate features of prostate carcinogenesis in humans can serve as valuable tools to study the connection between inflammation, autoimmunity, and prostate cancer (see, for example review (Abate-Shen & Shen, 2002)).

4. Autoantibody and regulation of anti-tumor immune responses in prostate cancer

Inflammation is one of the critical factors that may contribute to prostatic carcinogenesis, and there is a considerable evidence for an association between prostate cancer development, inflammation and autoantibody generation against tumor proteins (De Marzo

et al., 2007). Various genetic and environmental factors can contribute to the prostate injury, and any of these factors or their combination could lead to a break in immune tolerance and the development of an autoimmune reaction in the prostate. However the exact mechanism that would link autoimmunity and prostate cancer has not been established yet. Since the induction of anti-tumor immunity with prostate differentiation antigens (such as PSA, PAP, PSMA etc), which were logically deduced based on the analogy with melanoma-associated antigens, did not show significant clinical effect, the efforts of the research community have turned to the characterization of naturally occurring anti-tumor immune response in patients with prostate cancer and its role in tumor progression.

The humoral immune responses that occur naturally during the course of tumor development have been extensively characterized during the past decade in an attempt to develop novel, more specific and sensitive assays for early cancer diagnostics, and to identify novel target antigens for the cancer immunotherapy. For example, cancer/testis antigens, a family of molecules with normal expression restricted to male germ cells in the testis but not in adult somatic tissues, are frequently expressed in prostate cancer tumors, and antibodies to these antigens are frequently found in the peripheral circulation in patients with other epithelial cancers (Scanlan et al., 2002; Parmigiani at al., 2006). Other examples include various intracellular antigens over-expressed in prostate cancer (Sreekumar et al., 2004; Bradley et al., 2005; Wang et al., 2005; Arredouani et al., 2009). Because of their very restricted expression pattern in normal tissues and immunogenicity in different types of tumors, these molecules are also considered promising candidates for the prostate cancer immunotherapy.

The tremendous diversity of the target antigens recognized by autoantibodies in different patients represents a significant obstacle for the diagnostics and therapies based on this approach. The development of modern molecular methods including a high throughput methods for identifying personalized tumor-associated antigens would be a significant step in the future development of personalized medicine and can potentially lead to the development of biomarkers for early cancer detection or to distinguish aggressive and indolent forms of prostate cancer. Interpretation of these data in the context of patient's HLA haplotype can provide new insights in the mechanisms that govern the outcome of the anti-tumor immune response.

The list of potential candidate antigens identified by the autoantibody profiling is growing, however the physiological significance of such responses is not completely understood. Many studies in different cancer types, including prostate cancer, have demonstrated that the clinical course of cancer development can be modulated by the naturally occurring anti-tumor immune responses (Taylor et al., 2008). The most intriguing question remains whether or not the autoantibody patterns could serve as a prognostic factor to distinguish aggressive and indolent disease, or to predict the response to the therapy. Our work in *HLA tg* mouse model demonstrated the association between tumor-specific humoral immune responses and tumor progression, and clearly identified a role of "permissive" and "non-permissive" MHC class II alleles in the regulation of anti-tumor immune responses (Klyushnenkova et al., 2009).

In order to understand the role of MHC class II alleles in the development of the anti-tumor immune response we studied the effect of the *HLA-DRB1*1501* expression on the growth of

the transgenic adenocarcinoma of the mouse prostate (TRAMP) -C1 tumor line engineered to express human PSA (TRAMP-PSA). The experiments were carried out in the *DR2b* x C57BL/6J (*DR2bxB6*) *F1* mice that accommodate transplantable tumors of B6 origin. In the F1 mice we found that the chimeric *DR2b* transgene was not always expressed in offspring; this allowed us to examine the influence of the MHC class II gene on the growth of the tumor in transgene positive and negative littermates. We observed unexpected and striking differences in the pattern of immunological response and the overall outcome of tumor growth in DR2b+ and DR2b- F1 mice. These results have been recently published in the *Journal Immunology*, Cutting Edge (Klyushnenkova et al., 2009) and are briefly described in Figure 6.

In our *DR2bxB6 F1* model we found that TRAMP-PSA tumors were frequently rejected by DR2b- F1 mice but grew in DR2b+ F1 littermates (Figure 6A). We hypothesized that these differences in tumorigenicity of TRAMP-PSA may be due to the differences in the CTL response to the tumor antigen (PSA). CD8 T-cells-mediated immunity was tested by IFN-γ ELISPOT assay using an immunodominant CD8 T-cell epitope PSA_{65-73}. As shown in Figure 6B, the frequencies of IFN-γ secreting cells in response to PSA_{65-73} peptide or TRAMP-PSA tumor cells were significantly higher in splenocytes from DR2b- F1 mice that rejected TRAMP-PSA cells compared to DR2b+ tumor-bearing F1 littermates. These results were confirmed using an *in vivo* CTL assay based on the fluorescent dye 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Figure 6C). This suggested that the expression of the *DR2b* transgene in the mice somehow led to inhibition of the CTL responses to PSA expressed by the tumor cells. In contrast to the CD8 T-cell responses, DR2b+ F1 mice developed strong humoral immune responses to TRAMP-PSA tumors, while PSA-specific antibodies were practically absent in DR2b- F1 mice inoculated with TRAMP-PSA tumors (Figure 6D). The antibody responses in DR2b+ mice were predominantly of IgG1 sub-isotype, which immediately implied the differential CD4 T-cell responses to PSA. Indeed, immunization with soluble PSA protein in CFA, which favors CD4 T-cell-mediated immunity, demonstrated significant strain-specific differences (see Section 3, Figure 3).

Mice expressing chimeric *DR2b* transgene (parental *DR2b tg* and *DR2xB6 F1* mice with DR2b+ phenotype) developed strong immune responses to soluble PSA, and multiple CD4 T-cell epitopes were readily identified by overlapping 20-mer library screening (Figure 3). In contrast, mice lacking *DR2b* transgene (wt B6 and *DR2bxB6 F1* mice with DR2b- phenotype) failed to mount CD4 T-cell responses to PSA, and no epitopes restricted by the native $I\text{-}A^b$ allele were identified during library screening. The simple presence of *DR2b* allele was all that was required in our tumor model for the development of strong humoral immune responses to the tumor antigen (PSA), reciprocal suppression of CD8 T-cell responses and enhanced growth of the tumor.

One of the major mechanisms by which the presence of CD4 T-cell epitopes in PSA can negatively affect anti-tumor immunity may involve a classical pathway of humoral immunity. The mechanisms by which humoral immune response may affect tumor growth have been extensively investigated (reviewed in (Tan & Coussens, 2007)), and may involve antibodies/immune complexes, B cells and Th2 type cytokines. Persistent humoral immune responses can exacerbate recruitment and activation of innate immune cells in neoplastic microenvironments where they regulate tissue remodelling, pro-angiogenic and pro-survival pathways that together potentiate cancer development.

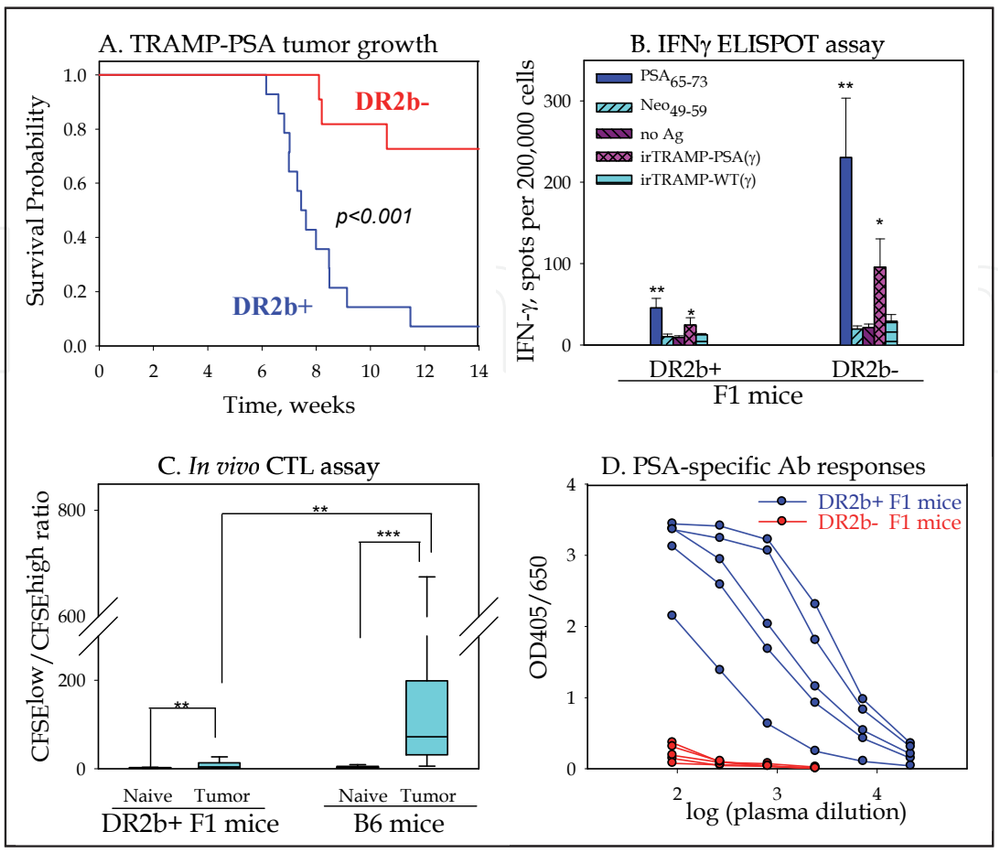

Fig. 6. Strain-specific differences in the pattern of anti-tumor immune responses in the *DR2bxB6 F1* mouse model. *DR2bxB6 F1* male mice (DR2b+ or DR2b- phenotype) were inoculated s.c. with TRAMP-PSA tumor cells. ***A.*** *TRAMP-PSA tumor growth* was monitored for up to 14 weeks. Time-to-event analysis was performed by log rank test using MedCalc software (p value is shown on the graph). Blue line: DR2b+ F1 mice; red line: DR2b- F1 mice. ***B****. CD8 T-cell responses to TRAM-PSA tumor cells* were measured by IFN-γ by ELISPOT assay. Splenocytes were cultured with $PSA_{65\text{-}73}$ peptide, irrelevant $NEO_{49\text{-}59}$ peptide, IFN-γ-treated irradiated TRAMP-PSA or parental TRAMP-C1 tumor cells (irTRAMP(γ)). Spots were counted using ImmunoSpot reader. Mice were tested individually, then means ± SE were calculated for each group (n=5). Data were analyzed by Mann-Whitney U-test (* $p<0.05$; ** $p<0.01$). ***C.*** *In vivo CTL assay*. Syngeneic splenocytes were used as target cells, and were labeled with two different doses of CFSE. $CFSE^{high}$ cells were pulsed with peptide $PSA_{65\text{-}73}$, and $CFSE^{low}$ cells were left untreated. An equal number of target cells were injected i.v. into either tumor-bearing or naïve mice. B6 mice were used because number of DR2b- F1 mice was not sufficient for the assay. Spleens were analyzed by flow cytometry 16 hr later for the loss of $CFSE^{high}$ population, which served as an indicator of the immunity. $CFSE^{low}/CFSE^{high}$ ratios are presented as box plots, the boundaries of the box show the 5th/95th percentile, a line within the box marks the median. Data were analyzed by Mann-Whitney U-test (** $p<0.01$; *** $p<0.001$). D. *PSA-specific antibody responses to TRAMP-PSA tumor cells* were measured by ELISA two weeks after tumor inoculation. The titration curves are shown for the individual mice (same animals as in Figure 6B). Blue circles: DR2b+ F1 mice; red circles: DR2b- F1 mice. Reproduced from (Klyushnenkova et al., 2009).

The association of the autoantibody responses and cancer outcome may not be straightforward. For example, Fossa et al. demonstrated in a small study that spontaneous serological responses against NY-ESO-1 were correlated with poor survival in mCRPC patients (Fossa et al., 2004). In contrast, several other studies demonstrated that treatment with ipilimumab, a fully human antibody against Cytotoxic T-lymphocyte-Associated protein (CTLA)-4, can induce antibody responses to NY-ESO-1, which was associated with positive clinical effect of the CTLA-4 blockade therapy (Yuan et al., 2008; Fong et al., 2009). Other studies could not find such correlation (Goff et al., 2009). It is clear that not just the presence or absence of autoantibody responses to a particular antigen, but the whole pattern of the response in the context of the cancer type, stage, treatment, as well as patients' HLA and non-HLA genes will dictate the outcome of the anti-tumor immune responses.

The other mechanism by which the presence of a CD4 T-cell epitope(s) in PSA could negatively affect anti-tumor immunity is by induction of TAA (PSA)-specific CD4+CD25+ foxp3+ regulatory T-cells (Treg) that could be selectively activated systemically or at the tumor site in an MHC class II-specific manner in DR2b$^+$ mice. Recent studies are revealing a consistent association of Treg infiltration with tumor formation and progression for many different cancers, including prostate cancer (Sfanos et al., 2008). Regardless of the potential effector mechanisms, the presence of IgG antibody is an excellent indicator of CD4 T-cell specific immune response, which occurs in the context of particular HLA class II alleles. The quality of such response (Th1, Th2, Th17, Treg etc.) depends on many factors including epitope structure of the particular antigen, the HLA haplotype and expression of other genes associated with immune response, the origin of the tumor, and the microenvironment.

Based on our observations in the *DR2b* transgenic mouse model of prostate cancer, we hypothesize that the presence of naturally occurring antibody responses to tumor antigen(s) in advanced prostate cancer is an indicator of an immune response that is ineffective at rejecting the tumor. We hypothesize that, in human malignancies, the diversity of available HLA class II haplotypes in the human population may govern the polarization of anti-tumor immune response. In the polymorphic human population, many tumors may contain antigens that are presented by the patient's HLA class II alleles in this fashion and affect the ability of the host to mount an effective response against the tumor. Our current goal is to identify tumor antigens specific to prostate cancer that can differentially induce CD4 T-cell response in HLA class II allele-specific manner, and to demonstrate the correlation between the expression of "permissive" and "non-permissive" HLA class II alleles, and the magnitude of tumor-specific humoral and cellular immune responses in patients with prostate cancer.

5. Conclusions

An effective immunotherapy against prostate cancer remains a dream. The most successful vaccines against bacterial and viral pathogens are based on the ability to induce strong CTL responses as well as neutralizing antibody responses. For certain categories of diseases, including cancer, this conventional approach does not seem to be effective. The role of humoral responses in anti-tumor immunity is a particularly controversial and highly debated topic. The development of a polarized immune response to the tumor during a natural course of the disease represents a unique challenge for the therapeutic vaccines (such as cancer vaccines) compared to the preventive vaccines against infectious diseases.

The major challenge is not only to induce a strong effector immune response against the tumor, but to simultaneously re-direct already ongoing immune response, which was mounted as a part of the natural course of the disease and already went in the "wrong" direction. Learning to harness the cellular arm of the immune response of proper specificity, diversity, magnitude, function and homing properties is a challenging task that requires significant efforts. Rational antigen design, advanced antigen delivery and adjuvant platforms in combination with the strategies directed toward neutralizing immune suppression hold a new promise for prostate cancer immunotherapy. As new potential target antigens emerge as a result of a next generation screening, their careful validation by "direct" and "reverse" immunology in human lymphocyte cultures and HLA transgenic mice remains an important task. Clinical samples from the patients that underwent treatment with Provenge and other experimental immunotherapies may provide critical clues in the efforts to create an effective treatment for prostate cancer and other epithelial cancers.

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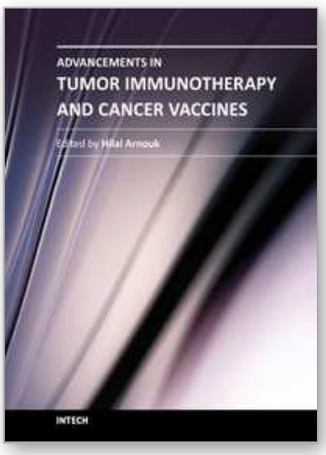

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Harnessing the potential of the human body's own immune system to attack malignant tumor cells has been the goal of many scientific investigators in recent years, with advances in cancer biology and immunology enabling cancer immunotherapy to become a reality. World-class bench and clinical researchers have joined forces to collaborate and review current developments and trends in cancer immunology for the purposes of this book, and the result is a promising review of contemporary clinical treatments. In each chapter the authors present the scientific basis behind such therapeutic approaches, including cancer vaccines with special focus on prostate cancer, melanoma and novel approaches utilizing both innate and adaptive immune responses.

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