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14. ABSTRACT

In year three of the award period, we tested the effects of pre-immunization of rats against PSMA, PSA or PAP on the development of transplantable tumors. Immunization of rats with a cocktail consisting of plasmid DNA and rat GM-CSF against PAP, PSA or PSMA protected the animals against development of transplantable AT3-B1^{PSA} tumors. None of the animals immunized with H PSA-T or H PSMA-T developed antibodies against the target antigen. In contrast, immunization with the "secreted" vaccines, HPSMA-S or HPSA-S resulted in production of antibodies against the target antigen. The antibodies were of mixed (Th1 and Th2) type (IgG1 and IgG2a). When priming was performed with the "truncated" version of the vaccines (H PSMA-T or H PSA-T), however and boosting with the "secreted" ones, the antibodies were mainly of the Th1 (complement-binding) type (IgG2a and IgG2b). The best protection against PSMA-transfected AT3-B1 tumors was achieved when priming was performed with a plasmid encoding a xenogeneic protein and boosting with a plasmid encoding a syngeneic one.

15. SUBJECT TERMS
Immunotherapy, prostate cancer, dunning rat model, PSMA, PAP, PSA, naked DNA immunization, cytotoxic T cells, tumor prevention

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REPORT

Introduction:

Recent research indicates that loss of expression of certain antigens is a common phenomenon during cancer progression and, therefore, targeting a single, defined tumor antigen will be disadvantageous to inducing an immune response against a broad spectrum of potential targets. Furthermore, the generation of various peptides when the immunization is performed against different tissue-specific antigens will eliminate possible differences in the affinity for peptide binding of different MHC alleles thus increasing the probability of creating a universal tissue-specific vaccine for the highly polymorphic human population. For this reason, we speculated that a cancer vaccine targeting a multitude of tissue-specific antigens is more likely to raise an effective immune response. We have enlarged the target panel to include the prostate-specific membrane antigen (PSMA), the prostate acidic phosphatase (PAP) and the prostate-specific antigen (PSA). During year two of the granting period, we continued to study the efficacy of raising cytotoxic human T cell responses against the auto-antigens PAP and PSA with genetically modified autologous dendritic cells. The ability of genetically modified with one of two types of plasmid DNA vaccines dendritic cells (DCs) to stimulate lymphocytes from normal human donors and to generate antigen-specific responses, is compared. The first type, also called "secreted" vaccine (sVac), encode for the full length of the human prostate-specific antigen (PSA) with a signal peptide sequence so that the expressed product is glycosylated and directed to the secretory pathway. The second type, truncated vaccines (tVacs), encode for either H PSA-T or human prostate acidic phosphatase (H PAP-T), both of which lack signal peptide sequences and are retained in the cytosol and degraded by the proteasomes following expression. Additionally, the safety of a combination of gene-based vaccines against the human PSMA, PAP and PSA, and their efficacy to prevent tumor development after inoculation with the AT3B-1^{PSA} cell line was tested in a rat model. Immunization with both vectors led to generation of cell cytotoxicity providing GM-CSF was administered with the vaccine. Spleen cells from animals immunized with hPSMAt demonstrated stronger cytotoxicity to the target cells. Priming with a vector that encoded a xenogeneic protein (H PSMA-T; "xenogeneic" construct) and boosting with a vector that encoded an autologous protein (R PSMA-T; "autologous" construct) gave the best protection against tumor challenge. Immunization with tVacs did not lead to formation of antibodies to the target protein as detected by Western blot or ELISA, while immunization with sVacs or with the protein did. Antibodies were of mixed Th1-Th2 isotype. Priming with tVacs and boosting with protein also resulted in antibody formation, but in this case the antibodies were from the cytotoxic, Th1 isotype. The best strategy to obtain a strong cellular cytotoxic response, therefore, seems to be gene-based vaccinations with tVacs, priming with the "xenogeneic" and boosting with the "autologous" constructs. When cytotoxic antibody production is the goal, priming should be performed with the tVacs while boosting with the protein.

Body of the report

1. In vitro transfection of human dendritic cells with PAP and PSA

In the last year's annual report we presented evidence that human dendritic cells, genetically engineered to express human prostate-specific membrane antigen (PSMA), PSA and PAP, stimulate cytotoxic T cell response in autologous T cells [1,2]. We next needed to test the efficacy of transfection of human dendritic cells with PSA and PAP and the ability of such transfected cells to stimulate autologous T cells to lyse the target LNCaP cell line. Using a commercially available transfection device from Amaxa, we could transfect differentiated dendritic cells with 20-40% efficiency. Such transfected dendritic cells stimulated in vitro autologous T cells to PAP or PSA. T cells cytotoxicity was then tested against tumor cells or peptide-pulsed T2 target cells. Both H PSA-T (tPSA)-DCs and HPSA-S (sPSA)-DCs generated antigen-specific cytotoxic T cell responses. The immune response was restricted towards one of four PSA derived epitopes when priming and boosting was performed with HPSA-S. In contrast, H PSA-T or H PAP-T transfected DCs primed T cells towards several antigen derived epitopes. Subsequent repeated boosting with transfected DCs restricted the immune response to a single immunodominant epitope. The immunodominance could be alleviated by depletion of CD25+ T cells prior to, or by GITR-L co-expression during, priming. The results were incorporated in a manuscript which was published in Cancer Gene Therapy [2].

Conclusions:

1. Dendritic cells transfected with a construct whose product is retained in the cytosol and degraded in the proteasome, prime to both dominant and subdominant epitopes.
2. Early CD25+ cell depletion during priming in vitro enhances priming to sub-dominant epitopes.
3. Co-expression of GITR-L during priming may alleviate immunodominance.

2. In vivo testing the safety and efficacy of naked DNA immunization when single tissue-specific antigens (PSA, PSMA or PAP) are targeted for immunotherapy (months 18-36)

Immunization cocktails (all cocktails contained 9 μg/m^2 recombinant rat GM-CSF; R&D, Minneapolis, MN):

- Immunization cocktail C: 50 μg H PSA-T plasmid + 100 μg empty plasmid backbone
- Immunization cocktail D: 50 μg H PAP-T plasmid + 100 μg empty plasmid backbone
- Immunization cocktail F: 50 μg H PSMA-T plasmid + 100 μg empty plasmid backbone

The usual dose for DNA immunization of small rodents is 100 μg of plasmid DNA. Safety studies in mice have shown no toxicity in a dose range between 1ug and 100 μg plasmid DNA. After adjusting the dose per body weight, we assumed that injecting of 150 μg plasmid DNA per rat per immunization will have no toxic effect on the animal.

Healthy, Copenhagen 2331 male retired rat breeders were used. All rats were immunized three times at 10-day intervals. The immunizations were intradermal and were performed by a standard intradermal injection technique with a small gauge (25-27 gauge) needle and an intradermal bevel. The needle was advanced into the intradermal region and the material was slowly injected while observing the formation of a "bleb" indicative of a proper injection. Volumes of up to 0.1 ml per site were injected.

A total of 54 animals were injected with the immunization cocktails (18 rats per each cocktail).

The animals were observed once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the study.

Two weeks after the last immunization, all rats were injected sub-cutaneously in the right flank with 1x10^6 tumor cells from the AT3B-1^{PSA} rat prostate carcinoma cell line. Injections were performed using 25-ga needles. The AT3B-1^{PSA} prostate cancer cell line is a non-metastatic cell line. Tumor size was evaluated every other day by measuring two perpendicular diameters by a caliper. The animals were observed twice daily for morbidity and mortality, and once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the whole duration of the study. Two weeks after tumor inoculation all rats were sacrificed, tumor were excised and measured. Necropsy was performed and kidney, especially proximal tubule, intestines, lungs, liver, prostate and brain were examined by histology. Spleen cells and sera were collected and tested for cytotoxicity against AT3B-1^{PSA} cells or for antibody against the target antigen.

All immunizations were tolerated very well and there were no signs of toxicity after immunization. There were no differences in the overall condition of the animals between those who received the gene-based vaccines, the empty plasmid backbone or the saline injections.

Five control animals that were immunized with an empty plasmid + GM-CSF developed tumors 16 days following tumor inoculation. Immunization with any of the vectors that encoded a target antigen led to protection of animals against development of tumors. None of the 18 animals immunized with the immunization cocktail C (H PSA-T plasmid) developed tumors. In

contrast, 4 of the 18 rats immunized with cocktail D and 2 of the 18 rats immunized with cocktail F developed tumor. The difference between group C and D is statistically significant ($\chi^2 = 4.5$; $p<0.05$) while between C and F is insignificant ($\chi^2 = 2.11$; $p<0.2$). None of the tumor bearing rats showed distant metastases. All animals developed antibodies against PSA that were detected by ELISA, probably as a result of the inoculation of the PSA-expressing cell line. From experimentation performed last year (year 2 of the award) we found that AT3B-1^{PSA} cells were immunogenic and injection of low numbers (less than $0.5x10^6$) of cells per rat did not lead to tumor formation, but to immunity against PSA which spread to other tumor antigens so that animals became protected against the non-transfected parental cell line. A no-cost extension of the award to study this phenomenon was requested and granted.

None of the animals immunized with the H PAP-T and H PSMA-T constructs developed antibodies against the target antigen (testing by Western blot and ELISA) – see # 4.

Conclusions:

1. Immunization against the tissue-specific antigens PSMA, PSA and PAP is safe and well tolerated.
2. All 5 control animals immunized with the empty plasmid backbone-rat GM-CSF cocktail develop tumors 16 days after AT3B-1^{PSA} cell inoculation.
3. None of the 18 animals immunized with the immunization cocktail C (H PSA-T plasmid) developed tumors.
4. The difference between the rate of tumor development in rats immunized against PSA, PSMA or PAP was statistically insignificant at the current number of rats tested.
5. All animals developed anti-PSA antibodies as a result of the inoculation of the AT3B-1^{PSA} cells.
6. None of the animals immunized with H PSMA-T or H PAP-T developed antibodies against the target antigen.

3. **Effectiveness of immunotherapy after immunization with plasmids encoding either syngeneic (R-"PSMA"-T) or xenogeneic (H-PSMA-T) proteins (months 24-36)**

Ten healthy, Copenhagen 2331 male retired rat breeders were immunized three times at 10-day intervals with a plasmid encoding the rat equivalent of the extracellular portion of the human PSMA (R PSMA-T plasmid) and rat recombinant GM-CSF. All immunizations were tolerated very well and there were no signs of toxicity after immunization. There were no differences in the overall condition of the animals between those who received the gene-based vaccines, the empty plasmid backbone or the saline injections.

Two weeks after the last immunization, all rats were injected sub-cutaneously in the right flank with $1x10^6$ tumor cells from the AT3B-1^{PSA} rat prostate carcinoma cell line. Injections were performed using 25-ga needles. Tumor size was evaluated every other day by measuring two perpendicular diameters by a caliper. The animals were observed twice daily for morbidity and mortality, and once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the whole duration of the study. Two weeks after tumor inoculation all rats were sacrificed, tumor were excised and measured. Necropsy was performed and kidney, especially proximal tubule, intestines, lungs, liver, prostate and brain were examined by histology. Spleen cells and sera were collected and tested for cytotoxicity against AT3B-1^{PSA} cells or for antibody against the target antigen. The results from this study were compared with the results in which the animals were immunized prior to tumor cell inoculation with the H PSMA-T plasmid (see #2).

None of the animals developed tumors until day 16 after tumor inoculation and were sacrificed. In contrast, 2 of the 18 rats, immunized with H PSMA-T developed tumors, but the difference between the two groups in not statistically significant. Spleen cells from animals from both groups were cytotoxic against the AT3B-1^{PSA} rat prostate carcinoma cell line. No antibodies against PSMA were detected by ELISA or Western blot.

Conclusions:

1. Immunization with the R PSMA-T is safe and well tolerated.
2. Animals, immunized with the R PSMA-T plasmid are protected against development of AT3B-1^{PSA}-inuced tumors.
3. No statistically significant difference in tumor protection is observed when animals immunized with H PSMA-T are compared to animals immunized with the R PSMA-T.
4. Rats immunized with the R PSMA-T do not develop antibodies against the target antigen.

4. **To compare different priming and boosting strategies for tumor prevention when immunizing against PSMA**

Since the AT3B-1 cells that were transfected with human the PSA elicited a tumor response, initially we decided to work for theses experiments with a non-transfected parental cell line. We found, however, that both rat prostate tissue and AT3B-1 cell lysates expressed low level of PSMA when tested by PCR (data not shown). Since PSMA expression is up-regulated in human prostate cancer cells, we decided to transfect the AT3-B1 cells with the HPSMA-S, a vector that contains the murine Ig k-chain leader sequence so that the extracellular portion of the human PSMA is glycosylated and secreted [1]. We did not transfect the cells with the full length PSMA since our goal was to study T cell-mediated and not antibody-mediated tumor protection and we felt that if PSMA was expressed as a membrane protein by the transfected AT3B-1 cells, some of the protection might have been antibody mediated. This would have been true especially when immunization was performed with DNA constructs that led to anti-PSMA antibody development (see below).

3.1 AT3B-1 transfection with HPSMA-S.

Monolayers of AT3B-1 cells (CRL-2375; ATCC, Manassas, VA) were transfected with the HPSMA-S using the FuGENE6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) and assayed for PSMA production by Western blot. The cells were seeded in 6-well tissue culture plates (Nunc, Denmark) at $1.5x10^5$ cells per well and grown to 50-70% confluence in DMEM supplemented with 25 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 10% (v/v) of fetal bovine serum. COS-1 or AT3B-1 cells were transfected with 1.5 μg of plasmid DNA pre-condensed with 4.5 μl of FuGENE 6 reagent in serum-free DMEM for 30 min at room temperature. Cells were then grown for 72 h in complete DMEM and then harvested.

The transfected cells with stable expression of HPSMA-S were selected and cloned after growth in zeocin-containing medium (0.1 mg/ml). We obtained two clones of stably transfected AT3B-1 cells – YM1 and YM2, both of which are high expressors and PSMA can be detected in the culture medium. A significant portion of the expressed product is degraded in the proteasome and could be detected in cell lysates only in the presence of proteasomal inhibitor lactacystin. All further experimentation was performed with the YM2 clone.

All non-immunized rats developed tumors 12 days after inoculation of $1x10^5$ YM2 cells. We found that the protection of the immunized animals from development of tumor, however, depended on the amount of YM2 cells inoculated during tumor challenge. Partial tumor protection could be seen when $2x10^5$ or $3x10^5$ tumor cells were injected. All immunized animals developed tumors when the tumor dose per animal was higher then $4x10^5$ cells. In an attempt to define the best strategy of vaccination, we explored different priming and boosting strategies. A total of 40 rats were included in these experiments.

All immunizations were performed with plasmid DNA cocktails that contained rat GM-CSF. All animals were challenged with $2x10^5$ cells. Rats were sacrificed on the day tumor was detected. All tumor-free animals were sacrificed at day 16 following tumor cell inoculation. Ten rats were included per group. The best protection following immunization involved priming with H PSMA-T and boosting with R PSMA-T and all the animals were tumor free 16 days after

tumor inoculation (figure 4; circles). In contrast, when priming was performed with HPSMA-S and boosting with R PSMA-T, three rats developed tumors on day 12, while the rest of the rats (66%) remained tumor free. The difference between these two groups was not statistically significant ($\chi^2 = 3.52$ at $p<0.1$). Spleen cells from the HPSMA-S-immunized animals, however, gave the strongest proliferative response after stimulation with recombinant PSMA (data not shown). Priming and boosting with R PSMA-T resulted in only partial protection – three rats developed tumor on day 10, and 3 rats on day 11. Four of those rats (34%) remained tumor free until day 16 (figure 4, diamonds). The difference between this group and the control group, though, was statistically significant ($\chi^2 = 5$ at $p<0.05$) since all control rats developed tumors by day 12 after tumor inoculation.

Conclusions:

1. Priming with a plasmid encoding a xenogeneic construct and boosting with a plasmid encoding a syngeneic protein is safe and well tolerated.
2. Priming with a plasmid encoding a xenogeneic protein followed by re-immunization with a plasmid encoding a syngeneic leads to the best protection against AT3B-1^{PSMA}-induced tumors.
3. Spleen cells from animals immunized with the HPSMA-S plasmid give the strongest proliferative response to PSMA.

5. **To compare the development of anti-PSMA antibodies in a Dunning rat prostate cancer model between groups of animals that are immunized with a truncated R"PSMA"-T plasmid and R-"PSMA"-S plasmid**

Healthy, Copenhagen 2331 male retired rat breeders were used. All rats were immunized three times at 10-day intervals. The immunizations were intradermal and were performed by a standard intradermal injection technique with a small gauge (25-27 gauge) needle and an intradermal bevel. The needle was advanced into the intradermal region and the material was slowly injected while observing the formation of a "bleb" indicative of a proper injection. Volumes of up to 0.1 ml per site were injected.

A total of 20 animals were injected with an immunization cocktail that contained plasmid DNA and 9 $\mu g/m^2$ recombinant rat GM-CSF. Ten rats were immunized with R PSMA-S and ten rats with R PSMA-T. Two weeks following the last immunization, the rats were inoculated with the with $1x10^5$YM2 cells. None of the immunized animals developed tumors.

As observed in earlier studies, rats immunized with "truncated" constructs (H PSMA-T or R PSMA-T) did not develop antibodies to PSMA as detected by ELISA or Western blot, no matter whether GM-CSF was present in the immunization cocktail or not. No antibodies against PSMA (detection by Western blot or ELISA) developed following immunization with plasmid, encoding the secreted PSMA (R PSMA-S), or with a recombinant protein, when GM-CSF was absent from the immunization cocktail. Immunization with R PSMA-S in the presence of GM-CSF led to formation of antibodies against the native protein (detected by ELISA). Antibodies, developing following immunization with R PSMA-S or GCPII, were of mixed Th2 and Th1 type, since both IgG1 and IgG2a subtypes were detected. On the contrary, priming with H PSMA-T or R PSMA-T and boosting with R PSMA-S led to formation of Th1, cytotoxic antibodies (IgG2a and IgG2b) with relatively high titer.

Conclusions:

1. Immunization with R PSMA-T does not lead to development of antibodies against the target antigen.
2. Immunization with R PSMA-S results in the development of antibodies to the target antigen when GM-CSF is used as an adjuvant. The antibodies are of mixed Th2 and Th1 type.
3. Priming with H PSMA-T or R PSMA-T and boosting with R PSMA-S led to formation of Th1, cytotoxic antibodies (IgG2a and IgG2b) with relatively high titer.

6. **To compare the development of anti-PSA antibodies in a Dunning rat prostate cancer model with already established tumors after immunization with full length or human truncated PSA constructs (months 24-36)**

In experiments performed in year two of the award, we found that AT3B-1^{PSA} cells were immunogenic and that their inoculation per se led to development of anti-PSA antibodies. As a result of this, the experiments listed in Task 6 were modified and no tumor cell inoculation prior to immunization was performed. Healthy, Copenhagen 2331 male retired rat breeders were used. All rats were immunized three times at 10-day intervals. The immunizations were intradermal and were performed by a standard intradermal injection technique with a small gauge (25-27 gauge) needle and an intradermal bevel. The needle was advanced into the intradermal region and the material was slowly injected while observing the formation of a "bleb" indicative of a proper injection. Volumes of up to 0.1 ml per site were injected.

A total of 57 animals (19 rats per group) were immunized 3 times at 10-day intervals with either H PSA-T, HPSA-S or empty plasmid cocktails. All immunizations were tolerated very well and there were no signs of toxicity after immunization. There were no differences in the overall condition of the animals between those who received the gene-based vaccines or the empty plasmid backbone. Two weeks after the last immunization, all animals were sacrificed, spleen cells and sera were collected and frozen until further use. The sera were tested for anti-PSA antibodies by Western blot and ELISA. None of the rats immunized with H PSA-T or the empty plasmid developed antibodies against PSA. In contrast, all animals immunized with HPSA-S developed antibodies against the target antigen that were of mixed Th1-Th2 type (IgG1 and IgG2a).

Conclusion:

1. No anti-PSA antibodies are detected in the sera of animals immunized with the H PSA-T plasmid.
2. Immunization with HPSA-S plasmid in the presence of rat GM-CSF leads to development of antibodies against PSA that are of mixed Th1-Th2 type.

Key Research Accomplishments:

During the second year we have:

- Showed the efficacy of human dendritic cells transfected with the H-PSA and human PAP to stimulate autologous T cells in vitro. Tested their ability to lyse target tumor cells or HLA-identical cells that have been pulsed with PSMA plasmids.
- Proved in an animal model the safety of naked DNA immunization with the human PSMA, PSA or PAP.
- Proved in rats the efficacy of immunization with these plasmid vaccines to prevent AT3B-1^{PSA} induced tumor development.
- Showed that spleen cells derived from rats previously immunized with one of the three "truncated" plasmid vectors are cytotoxic to both the parental (AT3B-1) and the transfected (AT3B-1^{PSA}) cell lines.
- Showed that immunization with H PSA-T leads to the better protection against development of AT3B-1^{PSA} tumors when compared to immunization with H PAP-T.
- Confirmed that inoculation of AT3B-1^{PSA} cells in non-immunized rats leads to development of anti-PSA antibodies
- Showed that immunization with the "secreted" versions of the plasmid vaccines (including HPSA-S) leads to antibodies against the target antigen. Contrary to that, immunization with the "truncated" vaccines does not result in development of antibodies against the target antigen. The latter vaccines may be advantageous when targeting secreted products used for tumor markers such as PSA or PAP where development of antibodies my interfere with the measurement of the serum concentration of the marker.
- Showed that priming with a "truncated" plasmid vaccine followed by boosting with a "secreted" plasmid vaccine or soluble protein leads to development of Th1-type (complement-binding) antibodies against the target antigen
- Showed that the best immunization strategy involves priming with a plasmid encoding a xenogeneic antigen and boosting with a plasmid encoding a syngeneic antigen. Such prime-boost regimen gives the best protection against development of AT3B-1^{PSMA} transplantable tumors.

Reportable Outcomes:

1. Mincheff M, Zoubak S, Altankova I, Tchakarov S, Pogribnyy P, Makogonenko Y, Botev C, Meryman, HT. Depletion of CD25+ cells from human T-cell enriched fraction eliminates immunodominance during priming and boosting with genetically modified dendritic cells. Cancer Gene Therapy 12, 185, 2005

2. The AT3B-1^{PSMA} (YM2) cell line which is the AT3B-1 cell line transfected to secrete the extracellular portion of the human prostate specific membrane antigen

3. Mincheff M, Makogonenko Y, Zoubak S. Immune responses against PSMA after gene-based vaccination for immunotherapy: A. Results from immunizations in animals. Cancer Gene Therapy (submitted for publication)

Conclusions

1. The research has progressed according to the approved Statement of Work. There have been no problems so far connected with the experimental design except for the fact that inoculation of AT3B-1^{PSA} cells leads to immunity against PSA and no tumor formation when less than $0.5x10^6$ cells per rat are inoculated. For this particular reason, when testing immunization targeting single tissue-specific antigens, $1x10^6$ cells per rat were inoculated. Since inoculation of AT3B-1^{PSA} cells per se led to development of antibodies against PSA in naive rats, in experiments designed to compare the development of anti-PSA antibodies following immunization with different plasmids, the prior inoculation of AT3B-1^{PSA} cells was omitted. Additionally, since PSMA expression in the AT3B-1 cell line was detected only at the mRNA but not the protein level, tumor protection studies when comparing immunizations with the R PSMA-T to immunizations with the H PSMA-T were performed with the AT3B-1^{PSMA} cell line (YM2).
2. Immunization against the tissue-specific antigens PSMA, PSA and PAP is safe and well tolerated.
3. All 5 control animals immunized with the empty plasmid backbone-rat GM-CSF cocktail develop tumors 16 days after AT3B-1^{PSA} cell inoculation.
4. None of the 18 animals immunized with the immunization cocktail C (H PSA-T plasmid) developed tumors.
5. The difference between the rate of tumor development in rats immunized against PSA, PSMA or PAP was statistically insignificant at the current number of rats tested.
6. All animals developed anti-PSA antibodies as a result of the inoculation of the AT3B-1^{PSA} cells.
7. None of the animals immunized with H PSMA-T or H PAP-T developed antibodies against the target antigen. Immunization with the R PSMA-T is safe and well tolerated.
8. Animals, immunized with the R-PSMA plasmid are protected against development of T3B-1^{PSA}-inuced tumors.
9. No statistically significant difference in tumor protection is observed when animals immunized with H PSMA-T are compared to animals immunized with the R PSMA-T.
10. Rats immunized with the R PSMA-T do not develop antibodies against the target antigen.
11. Priming with a plasmid encoding a xenogeneic construct and boosting with a plasmid encoding a syngeneic protein is safe and well tolerated.
12. Priming with a plasmid encoding a xenogeneic protein followed by re-immunization with a plasmid encoding a syngeneic leads to the best protection against AT3B-1^{PSMA}-induced tumors.
13. Spleen cells from animals immunized with the HPSMA-S plasmid give the strongest proliferative response to PSMA.
14. Immunization with R PSMA-T does not lead to development of antibodies against the target antigen.
15. Immunization with R PSMA-S results in the development of antibodies to the target antigen when GM-CSF is used as an adjuvant. The antibodies are of mixed Th2 and Th1 type.
16. Priming with H PSMA-T or R PSMA-T and boosting with R PSMA-S led to formation of Th1, cytotoxic antibodies (IgG2a and IgG2b) with relatively high titer.
17. No anti-PSA antibodies are detected in the sera of animals immunized with the H PSA-T plasmid. A "truncated" version of gene-based vaccines, therefore, can be safely used when targeting tumor markers that are used for monitoring disease such as PSA and PAP.
18. Immunization with HPSA-S plasmid in the presence of rat GM-CSF leads to development of antibodies against PSA that are of mixed Th1-Th2 type.

The "so what" section: Loss of expression of certain antigens is a common phenomenon during cancer progression and, therefore, targeting a single, defined tumor antigen will be disadvantageous to inducing an immune response against a broad spectrum of potential targets. Our results show that a combination of vaccines that target three different auto-antigens is safe to administer and is very effective in prevention of tumor development. Surprising to us, genetic modification of rat tumor cells that leads to expression of human auto-antigens, makes them immunogenic and this research will be broadened to include experiments identifying possible practical applications for vaccine design. New funding for those studies will be sought. The fact that immunization with "truncated" DNA sequence does not lead to formation of antibodies against the native antigen opens new venues for using DNA vaccines encoding truncated PSA for immunotherapy since no antibodies that will interfere with PSA testing will be developed.

References:

1 Mincheff M *et al.* Human dendritic cells genetically engineered to express cytosolically retained fragment of prostate-specific membrane antigen prime cytotoxic T-cell responses to multiple epitopes. *Cancer Gene Ther* 2003; **10:** 907-917.

2 Mincheff M *et al.* Depletion of CD25+ cells from human T-cell enriched fraction eliminates immunodominance during priming with dendritic cells genetically modified to express a secreted protein. *Cancer Gene Ther* 2005; **12:** 185-197.

ACCOMPANYING DOCUMENTS:

A. Manuscript published in Cancer Gene Therapy, 12, 185, 2005

Depletion of $CD25^+$ Cells from Human T-Cell Enriched Fraction Eliminates Immunodominance during Priming with Dendritic Cells Genetically Modified to Express a Secreted Protein

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Running title: Depletion of $CD25^+$ cells, GITR-L co-transfection and immunodominance

Keywords: PSA, PAP, Gene-based vaccine, immunodominance, CD4+CD25+ cells, T regulatory cells, GITR-L

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ABSTRACT

The ability of dendritic cells (DCs), genetically modified with one of two types of plasmid DNA vaccines to stimulate lymphocytes from normal human donors and to generate antigen-specific responses, is compared. The first type, also called "secreted" vaccine (sVac), encodes for the full length of the human prostate-specific antigen (PSA) with a signal peptide sequence so that the expressed product is glycosylated and directed to the secretory pathway. The second type, truncated vaccines (tVacs), encodes for either hPSA or human prostate acidic phosphatase (hPAP), both of which lack signal peptide sequences and are retained in the cytosol and degraded by the proteasomes following expression. Monocyte-derived dendritic cells are transiently transfected with either sVac or one of two tVacs. The DCs are then used to activate $CD25^+$-depleted or non-depleted autologous lymphocytes in an *in vitro* model of DNA vaccination. Lymphocytes are boosted following priming with transfected DCs, peptide pulsed DCs or monocytes. Their reactivity is tested against tumor cells or peptide-pulsed T2 target cells. Both tVacDCs and sVacDCs generate antigen-specific cytotoxic T cell responses. The immune response is restricted towards one of three antigen-derived epitopes when priming and boosting is performed with sVacDCs. In contrast, tVac transfected DCs prime T cells towards all antigen-derived epitopes. Subsequent repeated boosting with transfected DCs, however, restricts the immune response to a single epitope due to immunodominance. While $CD25^+$ cell depletion prior to priming with sVacDCs alleviates immunodominance, co-transfection of dendritic cells with GITR-L does so in some but not all cases.

INTRODUCTION

In a previous study, [1] we found that dendritic cells transfected with products that were cytosolically retained and degraded in the proteasomes, primed autologous T cells to multiple epitopes. Priming with sVacDCs, however, restricted the immune response to one of the epitopes due to immunodominance and the latter was alleviated if anti-CTLA-4 antibodies were present. We wanted to extend these observations to other tumor-associated antigens such as prostate-specific antigen (PSA) and prostate acidic phosphatase (PAP), as well as to study the effect that the removal of $CD4^+CD25^+$ cells prior to priming may have on immunodominance development. Both PSA and PAP are currently used as targets for immunotherapy of cancer. [2-4] On the other hand, gene-based vaccination in its current mode of application is effective in breaking tolerance to a self-antigen, but the response appears to be narrow and restricted to few of the potential epitopes. For example, the post-vaccination T cell response of some of the HLA A2 patients from the clinical trial performed by us [5] was directed against only two of the potential 4 PSMA peptide motifs that had high affinity for binding [M. Mincheff, unpublished]. Immunodominance is a natural mechanism for control that ensures the tight specificity of the immune reaction and prevents untoward autoimmunity, but it also carries the risk of inefficient immune surveillance in cases such as cancer where mutations of the epitope or downregulation of MHC alleles occur. [6-8] Malignant transformation and tumor progression are frequently associated with loss of HLA class I antigens. For example, a recent review of the literature [9] has reported that ~ 15% and 55% of surgically removed primary and metastatic melanoma lesions, respectively, are not stained in immunohistochemical reactions by monoclonal antibodies to monomorphic determinants of HLA class I antigens. Loss or reduced HLA class I antigen expression enables tumor cells to evade the host's immune response[6,7,10-12] and downregulation of HLA class I antigens in metastases from patients with malignant melanoma has been associated with poorer prognosis. [13] Immunodominance, therefore, presents a problem in vaccinology.[8,14,15] New vaccines and/or new methods of immunizations need to be developed for those instances. These hopefully will raise responses to subdominant determinants so that the selection of tumor escape variants that fail to express immunodominant epitopes will be prevented. [8]

Numerous factors combine to establish an immunodominance hierarchy. [16] Preliminary results from our laboratory suggest that the enhanced priming to sub-dominant epitopes by CTLA-4 inhibition is at least partially mediated through the inhibition of CD4+CD25+ T cell function. These CD4+ T cells are a minor subpopulation (10%) that co-expresses the IL-2 receptor α-chain (CD25) [17] and they can prevent both the induction and effector function of autoreactive T cells. [18-20] Additionally, they suppress polyclonal T cell activation in vitro by inhibiting IL-2 production. [21] Very little is known of the physiologic regulation of CD4+CD25+ T cells *in vivo*. [22] Recent reports suggest that glucocorticoid-induced tumor necrosis factor receptor (GITR), also known as TNFRSF18 – a member of the TNF-nerve growth factor receptor gene superfamily – is predominantly expressed on CD4+CD25+ T cells [22,23] and stimulation of GITR abrogates CD4+CD25+ T cell-mediated suppression. [23] The gene encoding the natural ligand of human GITR has been cloned and characterized. It is called GITR-L, a human activation-inducible TNF receptor (AITR) ligand, or TL6. Expression of the GITR-L is detected in immature and mature splenic dendritic cells. GITR-L binds GITR expressed on HEK 293 cells and triggers NF-kappa B activation. Functional studies reveal that soluble CD8-GITR-L prevents CD4+CD25+ regulatory T-cell-mediated suppressive activities [24]. Would CD25+ T cell depletion prior to priming alleviate immunodominance? If CD25+ cells suppress priming of sub-dominant T cell clone, could immunodominance be restricted by GITR signaling? Could this be achieved by enhanced GITR-L co-expression during re-immunization? The following

experiments were designed to test these hypotheses in an *in vitro* immunization system with human cells.

MATERIALS AND METHODS

All human cellular material used in these experiments was obtained following informed consent through protocols approved by the local Committee for Bioethics (Bulgaria) or the Investigational Review Board (IRB) at George Washington University Medical Center in Washington, DC. Use of recombinant DNA was approved by the local IRAC committees.

htPAP-, hGITR-L-, hsPSA- and htPSA plasmids construction

hPAP was obtained by RT-PCR of total RNA from LNCaP cells using TriPure RNA/DNA isolation reagent and Titan RT-PCR kit (Roche). The first cDNA strand was synthesized using hPAP-specific reverse primer (5'-GAGATCTCTGTGCACACTAATCTGTA-3'). Amplification was performed using the direct primer 5'-TCCTAACTCCTGCCAGAAACAGCTCT-3' and the same reverse primer. After initial denaturation, step (2 min, 94°C) and the first 10 cycles (30 sec at 94°C, 25 sec at 60°C, 45 sec at 72°C) the extension time was progressively incremented by 15 sec after each of the additional 20 cycles. The gel purified PCR product was cloned into a pCR2.1 vector (Invitrogen) and several clones were sequenced. All of them contained silent or non-silent substitutions. Two clones were selected for further work: 150.16 (no substitution in the 5'-region) and 150.20 (no substitution in the 3'-region).

Truncated hPAP (H PAP-T) was obtained by PCR of the clone 150.16 containing no substitution in the region between the end of signal peptide (position 97 on cds) and a single SfuI site (position 311 on cds). The direct primer (CGGCGGGGTACC***ATG***GAGTTGAAGTTTGTGACTTTGGTG) introduces a KpnI site and a Kozak sequence (underlined). The first amino acid next to the signal peptide (Lys) was replaced by Met (bold italic). The reverse primer (5-GGCTGCCAGAGTAGGATAGGATTC-3') anneals to the region downstream of a single SfuI site. The PCR product was digested with KpnI and SfuI endonucleases, gel purified, and sub-cloned by KpnI-SfuI sites into the clone 150.20 replacing its 5'-region with substitutions and signal peptide sequence. The correctness of the resulting clone was checked by sequencing.

Finally, the *H PAP-T* sequence was transferred by BamHI-XbaI sites from the pCR2.1 vector to the mammalian expression vector p147 (see below).

Human GITR-L was obtained by RT-PCR of total RNA from HuVec cells using specific primers (direct: 5'- GGTACCATGTGTTTGAGCCACTTGGAAAATATGCC-3'; reverse: 5'-CTAGGAGATGAATTGGGGATTTGC-3'). The PCR product (544 bp long) was cloned into a pCR2.1 vector and several clones were sequenced. All of them contained mutations. hGITR-L insert from one of the clones was transferred by HindIII-XhoI sites into the vector p147 and the mutations were corrected using QuickChange Site Directed Mutagenesis kit (Stratagen). The sequence of the final clone is identical to hGITR-L genebank entry AF125303.

The *hPSA* containing plasmid was kindly provided by Jan Geliebter, New York Medical College, Department of Microbiology and Immunology, Valhalla, NY, USA. The clone represents the complete hPSA cDNA sequence included in a pCDNA3 (Invitrogen) vector. The clone contains two non-silent nucleotide substitutions: C to A at cds position 289, and G to A at cds position 406 leading to substitutions Pro97 by Thr and Val136 by Met respectively. These mutations do not affect the generation of PSA-derived 9-mer epitopes with high affinity for binding to HLA A201 as predicted by the computer-based algorithm available at http://bimas.cit.nih.gov/.

To obtain a 5'-truncated form of hPSA (*H PSA-T*) that lacks a signal peptide, a PCR of the hPSA region from the end of signal peptide (cds position 52) to the end of cds was performed. A direct primer (GCGGCCGCCGCCACC***ATG***GCACCCCTCATCCTGTCTCGG) introduced a NotI recognition site and a Kozak sequence (underlined) as well as the start methionine (bold italic). A reverse primer (5'-GTTTAAACTCAGGGGTTGGCCACGATGGTGTC-3') introduced the PmeI site (underlined) just next to stop codon. The PCR product was cloned into a pCR2.1 vector and sequenced. The H PSA-T was then excised from the pCR2.1 vector by cutting with NotI+PmeI, gel purified and ligated with pVAX-1 mammalian expression vector (Invitrogen) by NotI and XhoI sites. The last one was blunted with Klenow enzyme in the presence of excess of dNTP. Consequently, H PSA-T was transferred by BamHI-XbaI sites to the mammalian expression vector p147.

The mammalian expression vector p147 represents a modified pCDNA3 vector (Invitrogen) in which the ampicillin resistance gene was replaced with kanamycin resistance gene. The replacement was performed by ligation of BclI-BspHI fragment from pVAX-1 plasmid with BglII-BspHI fragment of plasmid pCDNA3. The former fragment contains kanamycin resistance gene, the last one includes all elements of pCDNA3 vector except the ampicillin resistance gene.

The plasmid-DNA product specifications include endotoxin content below 0.1 EU per microgram of DNA; >90% of covalently closed circle DNA, lack of detectable amounts of bacterial RNA, genomic DNA or ssDNA as determined by agarose-gel electrophoresis; less than 10 microgram of protein per 1 mg of plasmid DNA as determined by colorimetric assay (Bio-Rad, Hercules, CA).

COS-1 transfection

Expression of PSA and PAP constructs (sVacs or tVacs) was performed in COS-1 cells (ATCC). A stably transfected AT3B-1 (ATCC) cell line which secretes PSA was obtained. Monolayers were transfected with FuGENE 6 transfection reagent (Roche) and assayed for PSA or PAP production by Western blot. COS-1 or AT3B-1 cells were seeded in 6-well tissue culture plates (Nunc, Denmark) at $1.5x10^5$ cells per well and grown to 50-70% confluence in DMEM supplemented with 25 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 10% (v/v) of heat inactivated fetal bovine serum. COS-1 or AT3B-1 cells were transfected with 1.5 μg of plasmid DNA pre-condensed with 4.5 μl of FuGENE 6 reagent in serum-free DMEM for 30 min at room temperature. Cells were then grown for 72 h in complete DMEM and then harvested.

In the proteasome inhibition studies, lactacystin (Sigma) was added to the culture media (final concentration 10 μM) 24 h before harvesting.

Cells were harvested by gentle scraping, washed twice with 2 ml of cold PBS and 0.25 ml cold lysing buffer (0.5 M NaCl, 1% triton X-100, 0.2% Tween 20, 50 mM HEPES, pH 7.0) was added to each well. Lysates were transferred to Eppendorf tubes and homogenized by repeated pipetting on ice.

For detection of secreted PSA, the serum containing DMEM was removed 48 h after transfection, the cells were washed twice with 2 ml of PBS, serum free DMEM (2 ml per well) was added and cells were incubated for additional 24 h in the 6-well plates. After collection of the medium, the cell debris was removed by centrifugation (35,000 g, 20 min) and supernatants were concentrated with Centricom centrifuge filtering device (Millipore) and then stored at -30°C until further use.

Electrophoresis and immunoblotting

Immunoblotting and electrophoretic assays of cell lysates and culture supernatants were performed using NuPage BioTris electrophoretic system (Invitrogen). Protein samples in loading buffer were heated at 70°C for 10 min and loaded on 10% Bis-Tris gels. After electrophoresis, the proteins were electro-transferred onto nitrocellulose membrane and blocked with 1% casein in TBS/T for 40 min. The membranes were probed with poly- or monoclonal anti-PSA or PAP Abs (see below) for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG conjugated with HRP (Sigma) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce) in accordance with the manufacturer's recommendations.

Anti-human PSA monoclonal antibodies sc-7316 and sc-7638 were obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). Purified PSA protein was obtained from International Immuno-Diagnostics, Foster City, CA. Anti-human PAP monoclonal antibody (mouse, IgG1), clone Pase/4LJ, was obtained from Acris Antibodies GmbH (Germany).

Flow cytometry analysis

Antibodies used to phenotype the cells were anti-CD1a, anti-HLA-DR-PE, anti-CD80-PE, anti-CD86-PE, anti-CD83-FITC, anti-CD54-PE, anti-HLA-ABC-FITC, anti-CD14-FITC, anti-CD8-PE, anti-CD4-PE, anti-CD69-FITC (PharMingen, San Diego, CA), and anti-CD3-PerCP (Becton Dickinson, San Jose, CA). Anti-GITR-L antibodies (clones 109114 and 109117) and anti-GITR (clone 110416) were supplied by R&D Systems Inc.; Minneapolis, MN 55413, USA. For HLA-A2 Typing, aliquots of PBMC from buffy coats of healthy donors were tested with the FITC-labeled anti-HLA A2 antibody BB7.2 (Becton Dickinson). For staining, 10^5 cells were suspended in 100 µl of PBS and were incubated with 10 µl of the antibodies for 20 min on ice. Flow cytometric analysis was performed on a FACS Calibur (Becton Dickinson).

Phenotyping of CD25+ regulatory T cells (TR)

Briefly, fresh or frozen PBMC were washed once in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, Mo.) and stained with antibodies for CD3 (peridinin chlorophyll protein; PerCP), CD4 (fluorescein isothiocyanate; FITC), CD8 (allophycocyanin; APC), CD25-FITC or -phycoerythrin (PE), CD38-APC or -PE, and/or HLA-DR—APC (BD BioSciences, San Jose, Calif., and BD BioSciences PharMingen, San Diego, Calif.) for 20 min at 4°C. The cells were then washed twice with PBS containing 1% BSA, fixed in 1% paraformaldehyde, acquired on a flow cytometer (FACSCalibur; BD BioSciences), and analyzed using FlowJo software (Tree Star, San Carlos, Calif.).

Cell cultures

Cell cultures from peripheral blood mononuclear cells were maintained in research grade serum-free AIM-V medium (Invitrogen, Carlsbad, CA). The human prostate cancer cell line LNCaP was purchased from ATCC and was maintained in RPMI supplemented with 10% FCS (Life Technologies Inc., Rockville, MD), 2 mM L-glutamine, 50 units/ml penicillin and 50 mg/ml streptomycin (complete medium; CM). The human T2 cell line is transporter associated with antigen processing (TAP) deficient, resulting in inefficient loading of human leukocyte antigen class I molecules with endogenous peptides [25]. As a consequence, the HLA-A0201 molecules of T2 cells can be efficiently loaded with exogenous peptides. The T2 cell line was purchased from ATCC and maintained in IMDM supplemented with 20% FBS (Life Technologies Inc., Rockville, MD).

For T cell stimulation, the leukocyte fraction was collected by leukapheresis and mononuclear cells were separated on a density gradient. Cells were resuspended in serum-free AIM-V medium at $2x10^7$/ml in culture flasks for 2 hrs in a humidified incubator at 37°C. The

non-adherent T-cell enriched fraction (later in the text referred to as "T cells") and part of the adherent cells were harvested and frozen for future use.

Depletion of $CD25^+$ cells

$CD25^+$ cells were purified with MACS CD25 MicroBeads (Miltenyi Biotec, Auburn, Calif.). Briefly, the non-adherent T-cell enriched fraction (T cells) was washed twice in PBS containing 0.5% BSA and 2 mM EDTA, resuspended in 80 µl of PBS containing 0.5% BSA-2 mM EDTA and 20 µl of MACS CD25 MicroBeads per 10^7 total PBMC, and incubated for 15 min at 6 to 12°C. The cells were washed twice in PBS containing 0.5% BSA and 2 mM EDTA and applied to a magnetic column on a MidiMACS separation unit (Miltenyi Biotec). CD25+ and CD25- T-cell fractions were collected. The CD25+ cell fraction contained >90% CD4+ T cells. In some experiments the CD25+ cell fraction was purified to >99% CD4+ T cells by cell sorting after staining with monoclonal antibodies to CD3 and CD4 (FACSvantage; BD Biosciences) and frozen for add-back experiments. In other experiments, CD4+CD25+ T cells were stimulated for 48 hours with plate-bound anti-CD3 monoclonal antibody and IL-2 and used for staining with anti-GITR antibody.

Generation of monocytes-derived dendritic cells

The rest of the adherent cells were differentiated into DC by culture in serum-free AIM-V medium with IL-4 (PeproTech, Rocky Hill, NJ) and GM-CSF (Oncology Supplies Inc. (Dothan, AL) for 6 days. The non-adherent cells were harvested at that time and used for transfection. Transfection was performed using the Nucleofector™ device and transfection kit (Amaxa GmbH, Cologne, Germany). The green fluorescence protein (GFP) transfection efficiency of human DCs after Nucleofector™ transfection was 32+/- 8.8 % (n=5). Following transfection, dendritic cells were resuspended in serum-free AIM-V medium and matured with TNF-α (Becton Dickinson Inc., Bedford, MA) at 37°C for 24 hours. Following maturation, the DCs were resuspended in AIM-V medium at $1x10^5$ cells/ml. Treatment of tPSMA- and sPSMA-transfected DCs with TNF alpha triggers a coordinate series of phenotypic changes, resulting in an up-regulation of co-stimulatory molecules (CD80, CD86, CD40) and HLA class II antigens [1].

In Vitro Generation of CTL Responses

For T cell stimulation, the T cells with or without prior $CD25^+$-depletion, were thawed, washed, resuspended in AIM-V medium and added to the DC suspension so that the final concentration of the T cells was $1x10^6$ cells/ml (T cell/DC ratio = 10:1). The cell suspension was then distributed into 24-wells plates (1ml/well; Costar plates) and cultured at 37°C.

In some experiments $CD4^+CD25^+$ T cells were added back to the stimulated T cells. In those, $0.25x10^6$ $CD4^+CD25^+$ T cells were added to each well (responder T cells/$CD4^+CD25^+$ T cell ratio = 4:1) at different time points following priming.

Three days later, the medium was removed and the T cells were cultured from that moment in serum free AIV-M medium supplemented with human IL-2 (20 U/ml) and human IL-7 (10 U/ml) (PeproTech, Rocky Hill, NJ). Cells were additionally stimulated with autologous PSMA-transfected DCs (stimulator:effector ratio of 1:10) or with peptide-pulsed autologous monocytes (stimulator:effector ratio of 1:1) twice, 8 days apart.

For pulsing with peptides, monocytes were resuspended at 10^6/ml in serum-free RPMI-1640 with L-glutamine and penicillin/streptomycin. Peptide was added to a final concentration of 0.05 mg/ml and the cells were incubated for 4 hours at 37°C in a controlled CO_2 humidified incubator. The cells were then washed twice with serum-free medium, irradiated (1500 rads) and used for boosting in IL-2 and IL-7 containing medium.

For peptide pulsing of T2 cells, we initially performed peptide-binding assays. For that purpose, T2 cells were incubated (10^5 cells/well) overnight in 96-well plates with serial dilutions of peptides in RPMI 1640/10% boiled fetal calf serum (to prevent protease activity), and then analyzed by FACS analysis for surface expression of HLA-A0201. Mean fluorescence intensities at varying concentrations of peptide were compared (data not shown). In subsequent experiments, T2 cells were pulsed for 6 hours with 0.01mg/ml peptide and 1mCi/well ^{3}H thymidine (ICN Biomedical Inc., Irvine, CA).

After 20 days of culture, effector cells were harvested without further separation for micro-cytotoxicity assays. The cells were analyzed by flow cytometry and 83% +/- 10% of them were $CD3^+$ and ~ 45% of them (when primed with the sPSA DCs) and ~60% of them (when primed with the tPSA DCs) were $CD3^+CD8^+$ (data not shown).

Selection of HLA-A2-binding PSA- and PAP- peptides

For the present study, the aminoacid sequence of the truncated (no-leader sequence) PSA (AA 25-161) and PAP (AA 33-386) were analyzed for the existence of 9-aminoacid peptides predicted to bind to HLA-A201, the most common human MHC class I allele, using the computer-based algorithm (http://bimas.cit.nih.gov/) (table1). Three 9-mer peptides from each sequence that contained peptide-binding motifs for the HLA A201 class I molecule were identified:

Cytotoxicity testing

Cytotoxicity against LNCaP cells or against peptide pulsed T2 cells (both HLA A2-positive) was tested after 20 days of culture and compared to a control cell line that did not express PSA or PAP. Cytotoxicity was tested using the JAM test [26]. Briefly, target or control cells were grown overnight with ^{3}H-thymidine, then washed, resuspended in CM and used in 4-hour cytotoxicity test. The killing was detected as a fall in counts per minute due to DNA fragmentation in cell samples undergoing apoptosis. All of the E:T ratios were tested in triplicate. Spontaneous cytotoxicity was determined in medium alone without effector cells.

Unlabelled K562 cells (no MHC expression and sensitive to natural killer cell-mediated lysis) were included at 50 x the target cell number to inhibit nonspecific lysis. Control experiments involved the Malme M3 melanoma cell line, which is also HLA A2 positive.

Cell Lines

The human LNCaP (CRL-1740), T2 (CRL-1992), Malme 3M (HTB-64), COS-1 (CRL-1650) and AT3B-1 (CRL-2375) cell lines were purchased from ATCC and were maintained according to ATCC instructions.

Cytokines and 3H-thymidine

GM-CSF was purchased from Oncology Supplies Inc. (Dothan, AL), IL-4 and IL-7 from PeproTech Inc. (Rocky Hill, NJ), IL-2 and TNF-α from Becton Dickinson Inc. (Bedford, MA). 3H-thymidine was purchased from ICN Biomedical Inc (Irvine, CA).

Statistics and Epitope Binding Predictions

Analysis of cytotoxicity data were performed using two-tailed Student's t tests assuming equal variance. We used the predictive algorithm from the Bioinformatics and Molecular Analysis Section of the NIH ("BIMAS") that was developed by Parker et al [27], ranking potential MHC binders according to the predictive one-half-time disassociation of peptide/MHC complexes for epitope binding prediction.

Peptide synthesis and purification

Peptides were custom synthesized and purified by Sigma Genosys (The Woodlands, Texas).

RESULTS

COS-1 cells transfected with tPSA, sPSA or GITR-L plasmids express the encoded product

Following transfection with sPSA plasmid, the encoded product is N-glycosylated and could be detected intra- and extracellularly – i.e. is secreted (fig.1 A and B). The product expressed following transfection with the tPSA plasmid is not glycosylated, but is retained in the cytosol and rapidly degraded in the proteasome. Similarly to transfection with another "truncated" gene-based vaccine [1], the product could be detected following proteasomal inhibition with lactacystin. COS-1 cells transfected with GITR-L stain positively with antibodies specific for the GITR-L (fig.2).

tVacDCs (tPSA DCs, tPAPDCs) and sVac DCs (sPSA DCs) prime and support development of T cells that are cytotoxic against LNCaP cells

The T cell enriched fraction from each leukapheresis was primed and then boosted twice, at 8-day intervals with autologous PSA-transfected DCs. Their cytotoxicity was then tested against LNCaP cells or control Malme M3 melanoma cells. Both tVacDCs and sVacDCs primed and supported development of T cells that are cytotoxic against LNCaP cells (fig. 3).

tVacDCs but not sVacDCs prime T cells that are reactive to sub-dominant PSA or PAP epitopes

Recently, we found that dendritic cells transfected with a fragment of the human prostate-specific membrane antigen that was cytosolically retained and degraded in the proteasome (tPSMA DCs), primed autologous T cells to multiple epitopes [1]. To extend these observations to other prostate antigens such as PSA and PAP, we primed the T cell enriched fraction from leukapheresis with either tPSA DCs, sPSA DCs or tPAP DCs and boosted them twice, at 8-day intervals, with autologous monocytes pulsed with one of several PSA or PAP derived peptides (table 1). Twenty days after priming, cytotoxicity was tested against T2 cells pulsed with the same peptide used for boosting. Unlike dendritic cells pulsed with the sPSA plasmid, tVac DCs prime T cells to all PSA- or PAP-derived peptides in all five donors tested (fig. 4). DCs or monocytes, loaded with PSA peptides, support development of T cell effectors with similar efficacy (fig. 4).

Repeated boosting with transfected DCs restricts the response towards one immunodominant epitope

Previously, we found that boosting with polyepitope expressing DCs restricts the immune response towards a single immunodominant epitope. To determine the effect of a prime/boost vaccination strategy on the clonality of the T cell response, tVac DCs-primed cultures, known to contain CTLs to sub-dominant epitopes (fig. 4), were boosted with transfected or peptide pulsed dendritic cells or monocytes, and their cytotoxicity was tested against PSA- or PAP-peptide pulsed T2 targets. Boosting with antigen presenting cells that express multiple PSA- or PAP-derived epitopes (transfected DCs, or DCs or monocytes pulsed with multiple peptides) restricts the immune response towards one immunodominant epitope (table 2), a finding that has already been observed by others. [28] A subdominant T cell response could only be preserved if boosting is performed with an APC (DC or monocyte) pulsed with the particular sub-dominant epitope (table 2; fig.4).

Depletion of CD25+ cells prior to initial exposure to antigen leads to generation of T cells reactive to both dominant and sub-dominant epitopes

Subdominant epitopes are generated when dendritic cells are transfected with sVacDCS, but factors other than TCR signaling such as the CTLA-4/B7 pathway, are contributing to the ineffective proliferation of T cell to subdominant epitopes [1]. Since non-activating anti-CTLA4 antibodies block the suppressor activity of regulatory cells in vitro [29], we decided to explore the effect of CD25+ cell depletion prior to priming of peripheral blood T cells with genetically modified autologous dendritic cells. Similarly to CTLA-4 inhibition [1], CD25+ cell depletion prior to priming with sPSA DCs led to stimulation of T cells reactive to sub-dominant PSA-derived epitopes. We interpret this as evidence that T cell responses to sub-dominant epitopes are generated following priming with sPSA DCs but are inhibited by CD25+ cells present in the T cell-enriched fraction (fig.4). Both peptide pulsed DCs and peptide-pulsed MCs support development of T cells effectors in cultures depleted of CD25+ cells prior to priming with sPSA DCs (fig.5).

Addition of CD25+ cells back to T cells within the first hour of priming with sPSA DCs reverses immunodominance.

Suppression of T cells reactive to sub-dominant epitopes by CD25+ cells occurs early during T cell priming. Addition of CD25+ T cell to T cells that are primed by sPSA DCs is suppressive only if CD4+CD25+ T cells are added within the first hour after initiation of culture (figgs.6 and 7). No suppression is seen if CD4+CD25+ T cells are added 8 hours after initiation of priming.

sPSA DCs co-transfected with the human GITR-L may support priming and development of T cells reactive to sub-dominant epitopes

Murine CD25+ T regulatory cells expressed high levels of GITR. We find that human CD4+CD25+ cells also express high levels of GITR in an activation-dependent manner (fig.8), similarly to human cytotoxic T cells isolated from tumor lesions [30]. Since signaling through GITR has been found to downregulate the function of T regulatory cells and enhance the development of autoimmunity, [22,23,31,32] we decided to explore the effect of co-transfection of DCs with sPSA and GITR-Ligand. In two out of five separate experiments (donor B and C), such DCs primed T cells to the sub-dominant epitopes (fig.9).

DISCUSSION

T cells that are specific for PSA- or PAP-derived peptides exist in the adult male since both sVac DCs and tVacDCs prime T cells that are cytotoxic to LNCaP cells in vitro (fig.3). Gene-based vaccination in its current mode of application is effective in breaking tolerance to a self-antigen, but the response is narrow and is restricted to few of the potential epitopes. This presents a problem in vaccinology since loss of an MHC haplotype that participates in the conformation of the T cell antigen, or point mutation in the recognized sequence would result in ineffective immune surveillance.[8,14,15,33]

Unlike sVacDCs, tVacDCs prime cytotoxic T cells that are specific for both dominant and sub-dominant epitopes (fig.4). Numerous factors combine to establish an immunodominance hierarchy, [16] among them the ineffective generation and transport of sub-dominant epitopes by antigen-presenting cells (APCs). Since proteasomal degradation is the main source of antigenic fragments destined for MHC presentation,[34] purposeful cytosolic retention of newly synthesized tumor-associated antigens in genetically manipulated antigen presenting cells increases both the quantity and the diversity of such fragments. Dendritic cells, transfected to synthesize such

products, clearly have the advantage to prime to both dominant and sub-dominant epitopes (fig.4). [1]

Similarly to boosting with tPSMA transfected DCs,[1] boosting with polyepitope expressing DCs or monocytes, restricts the immune response to the dominant epitope (table 2; fig.4). New vaccines and/or new methods of immunizations need to be developed for those instances. These hopefully will preserve responses to subdominant determinants during re-immunization so that the selection of tumor escape variants that fail to express immunodominant epitopes will be prevented. [8]

In a previous study we found that, under conditions that favor priming of T cells to dominant epitopes, CTLA-4 inhibition alleviates immunodominance. Possible operational mechanisms involved CTLA-4 acting as a non-signaling"decoy" receptor reducing the available ligand for CD28 costimulation [35,36]or creating opportunities for weak signals coming from sub-dominant epitopes otherwise prompt to inhibition by CTLA-4. [37]

A third possibility also existed that needed to be explored.[1] Since non-activating anti-CTLA4 antibodies have been found to block the suppressor activity of regulatory cells *in vitro* [29], a possibility existed [38,39] that a small number of CTLA-4-expressing and -stimulated T cells exerted a suppressive or regulatory effect on other T cells. These cells appeared to be similar if not identical to T regulatory cells.[40-42] In murine models, suppression of auto-reactive T cells has been attributed to a population of spontaneously occurring CD4+CD25+ T cells.[43] Cells with similar phenotype and function have been found in healthy humans.[44-47] Of interest for cancer immunotherapy is the fact that depleting these cells results in the induction of anti-tumor immune responses, particularly after tumor specific vaccination. [48,49] One hypothesis is that depleting these CD4+CD25+ regulatory T cells in humans enhances a polyclonal T cell response.[21,50]

Removal of CD25+ T cells from the T cell reactive pool prior to priming does result in elimination of immunodominance so that T cells are primed by sVac DCs to both the dominant and the sub-dominant epitopes (fig.5). Similarly to CTLA-4 inhibition,[1] CD25+ cell depletion is effective if performed early during priming – actually within the first hour after initiation of culture (fig.6). Whether alleviation of immunodominance through CTLA-4 inhibition acts through T regulatory cell suppression needs additional experimentation. One way would be to look whether CD25+ cell removal and CTLA-4 inhibition have a synergistic effect. A synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy has already been described [51] and this suggests that these interventions may act through different pathways. Unfortunately, our current experimentation system does not permit quantitative analysis of responses to sub-dominant epitopes under different conditions.

Finally, the glucocorticoid-induced TNFR (GITR) is expressed at high levels on resting CD4 +CD25+ T regulatory cells and regulates their suppressive phenotype.[32] Antibodies to GITR abrogate suppression, demonstrating a functional role for this receptor in regulating the CD4+CD25+ T cell subset. [22] In our hands, co-transfection of DCs with the natural ligand for GITR leads to T cell priming to sub-dominant epitopes in 2 out of 5 experiments (fig.7). This could be the result of T regulatory cell suppression by GITR signaling. [22,23,31,32] On the other hand, GITR-GITR-L interaction could also provide a costimulatory signal for the antigen-driven proliferation of naive T cells.[52,53] No matter what the mechanism is, GITR-L co-expression during gene-based vaccination may lead to enhancement of the immune response and alleviation of immunodominance. All of these results, however, have been obtained in an in vitro experimentation system. Additional in vivo experimentation is necessary to validate their significance in immunocompetent hosts.

In conclusion, we have shown that:

1. Dendritic cells transfected with a construct whose product is retained in the cytosol and degraded in the proteasome, prime to both dominant and subdominant epitopes.
2. Early CD25+ cell depletion during priming in vitro enhances priming to sub-dominant epitopes.
3. Co-expression of GITR-L during priming may alleviate immunodominance.

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Table 1. HLA A201-restricted PSA- and PAP-derived peptides

Rank	Start position	Subsequence residue listing	BIMAS Score (estimate of half-time of disassociation of a molecule containing this sequence)
1 PSA	170	KLQCVDLHV	243
2 PSA	52	GVLVHPQWV	124
3 PSA	53	VLVHPQWVL	123
1 PAP	135	ILLWQPIPV	437
2 PAP	112	TLMSAMTNL	182
3 PAP	33	KELKFVTLV	153

Table 2. Priming with tVac DCs stimulates T cells to all 3 PSA- or PAP-derived epitopes. Boosting with dendritic cells and monocytes that express multiple antigenic epitopes restricts the response to an immunodominant epitope

Prime/Boost Strategy*	Number of Patients Developing CTL Activity against PSA or PAP Peptide-Pulsed T2 cells					
	PSA_{170}	PSA_{52}	PSA_{53}	PAP_{135}	PAP_{112}	PAP_{33}
Prime: tVac (PSA or PAP) DCs 2 Boosts tVac(PSA or PAP) DCs	5 of 5	1 of 5	0 of 5	5 of 5	0 of 5	1 of 5
Prime: tVac (PSA or PAP) DCs 2 Boosts 1P (PSA- or PAP-derived) MCs	5 of 5	4 of 5	4 of 5	5 of 5	3 of 5	5 of 5
Prime: tVac (PSA or PAP) DCs 2 Boosts 3P (PSA- or PAP-derived) MCs	5 of 5	0 of 5	0 of 5	5 of 5	0 of 5	0 of 5
Prime: sVac (PSA) DCs 2 Boosts sVac (PSA) DCs	5 of 5	0 of 5	0 of 5	5 of 5	0 of 5	0 of 5
Prime: s Vac (PSA) DCs. 2 Boosts 1P (PSA-derived) MCs	5 of 5	0 of 5	0 of 5	5 of 5	0 of 5	1 of 5

** tVac – "truncated" (no signal sequence) plasmid DNA vaccine; sVac – plasmid vaccine that encodes for the mature form of human PSA with a signal sequence; 1P – pulsed with single either PSA-, or PAP-derived peptide; 3P – pulsed with all three, either PSA- or PAP-derived, peptides,; MCs – monocytes*

FIGURES AND LEGENDS TO FIGURES

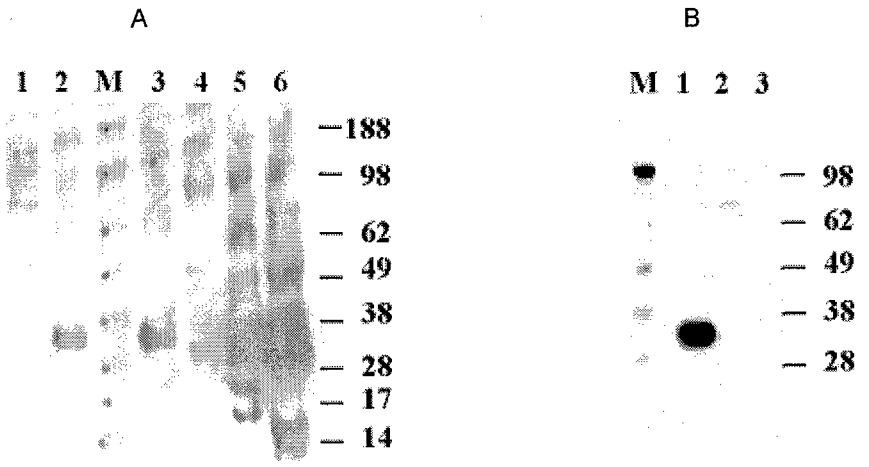

Fig. 1. A. Detection of human PSA in cell lysates. Line 1: The parent AT3B-1; Line 2: AT3B-1 transfected with hPSA plasmid, mass culture; Line M: molecular weight marker; Line 3: D12/1, a PSA producing AT3B-1 clone (reduced conditions); Line 4: The D12/1 clone (non-reduced conditions); Lines 5 and 6: Recombinant PSA protein, reduced and non-reduced conditions respectively, loading 200 ng per line.

Fig. 1. B. Detection of secreted human PSA in the culture medium. M molecular weight marker; Line 1: culture medium from the clone D12/1; Line 2: culture medium from the parent AT3B-1 cell line; Line 3: culture medium from a sham transfected AT3B-1 cell line. Cells were crown during 60h in serum free medium, then media were collected, concentrated by filtration and normalized by protein mass.

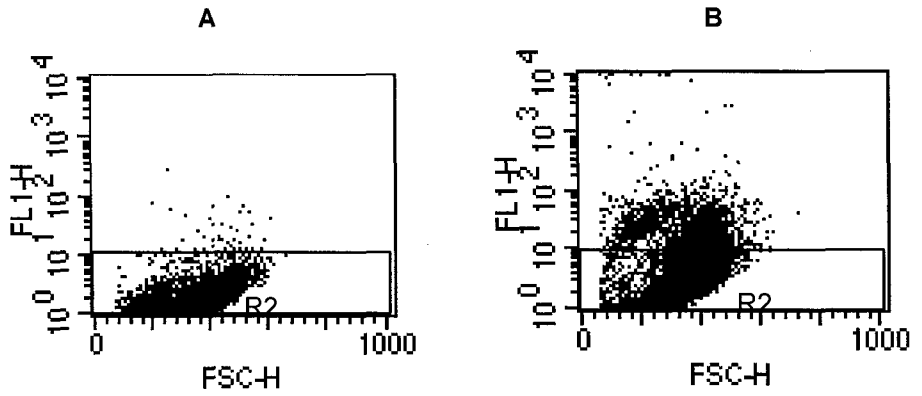

Fig.2. Staining COS-1 cells with FITC-labeled monoclonal antibody (clone 109114) against the GITR-L. A – prior to transfection; B – following transfection with plasmid encoding for the human GITR-L.

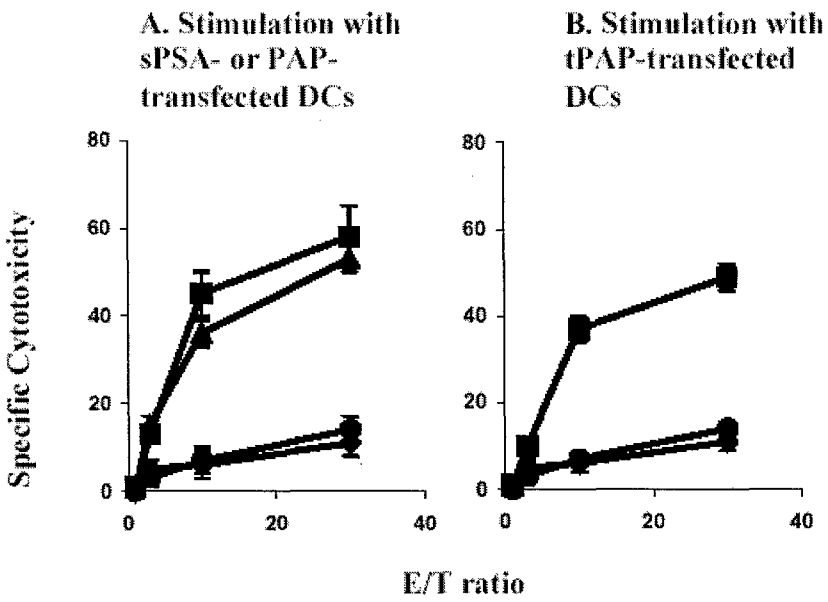

Fig.3. Generation of CTLs by:

A. PSA DCs (tPSA – squares; sPSA - triangles)
B. tPAP DCs (squares).

Non-adherent (T cell-enriched) HLA A2(+) peripheral blood mononuclear cells were primed and then boosted twice at 8-day intervals with autologous monocytes-derived dendritic cells that were transiently transfected with either sPSA, tPSA or tPAP plasmid. The medium was changed, initially 72 hours following priming, and then during boosting. The cells were grown in IL-2 and IL-7 medium for 20 days and the specific cytotoxicity was tested against LNCaP cells. Each point represents the mean and SD of triplicate experiments. Control experiments involved priming and boosting of the T-cell enriched fraction with DCs transfected with empty plasmid (diamonds) or testing of cytotoxicity against the Malme-3M melanoma cell line (circles).

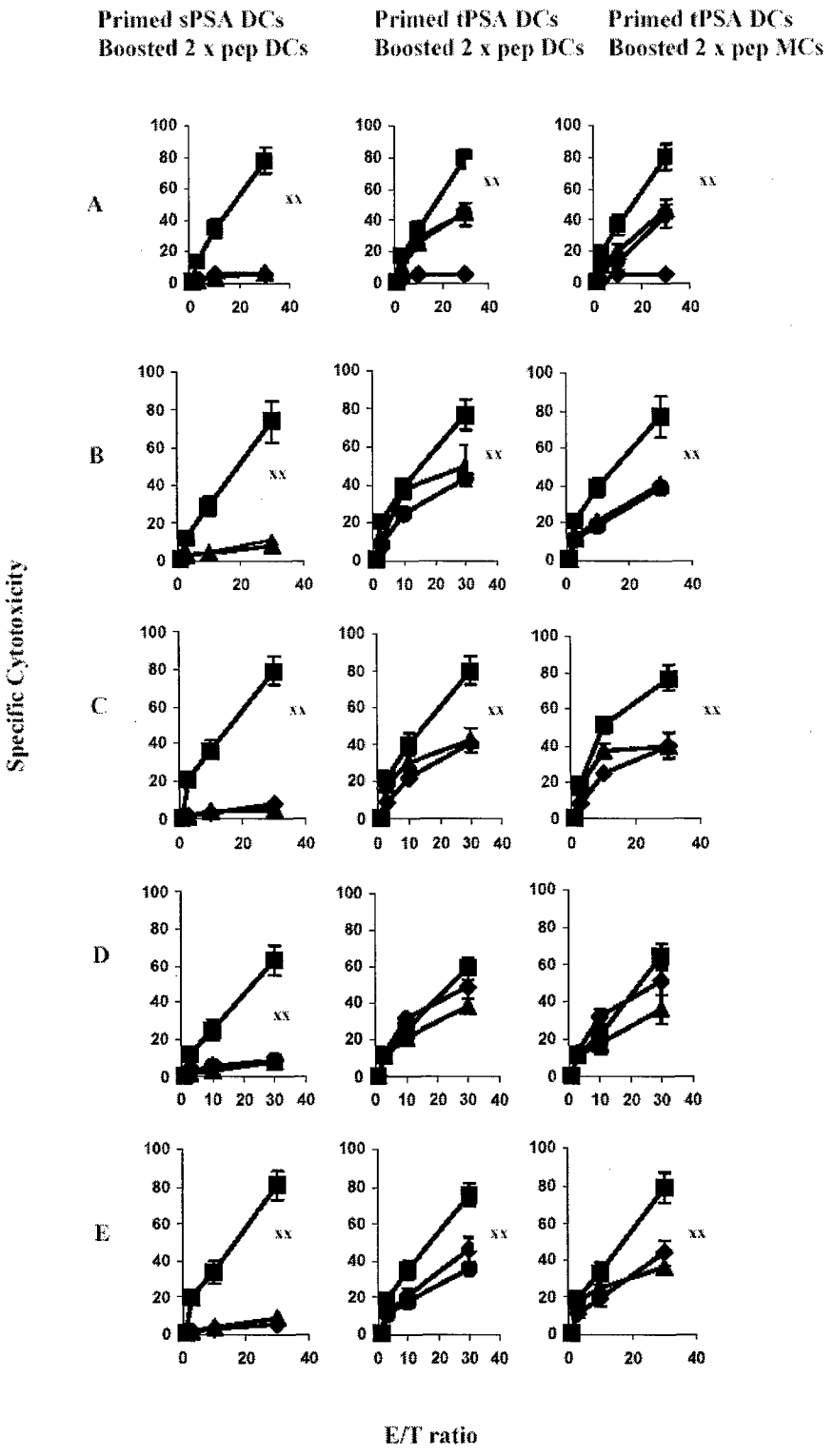

Fig. 4. tVacDCs but not sVacDCs prime T cells that are reactive to sub-dominant PSA or PAP epitopes. Induction of CTL responses with autologous dendritic cells that have been transfected with either the secreted or truncated version of the human PSA. Peripheral blood mononuclear cells that had been depleted of monocytes were primed with autologous DCs transfected with either sPSA (first column) or tPSA (second and third column). Responding cultures were then boosted with monocytes pulsed with one of three HLA-A2 restricted PSA-derived peptides

(table 1) and their cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during boosting (PSA_{170} – squares; PSA_{51} – triangles and PSA_{53} – circles). Data points for the control (influenza) peptide are not shown but are identical to those obtained with empty plasmid-transfected DCs (donor A) in all donors tested. Each point represents the mean and SD from three different experiments. Both tPSA DCs- and sPSA DCs-primed T cells are cytotoxic against T2 cells pulsed with PSA_{170} peptide. Values for cytotoxicity of either tPSA DCs- or sPSA DCs-primed T cells against T2 targets pulsed with PSA_{170} and PSA_{51} or PSA_{53} peptides were compared. Significant differences at the 30:1 E/T ratio are indicated with xx ($p<0.01$). Level of cytotoxicity for sPSA DCs primed T cells against T2 cells pulsed with either PSA_{51} or $PSMA_{53}$ for all 5 donors are identical to controls (T cells primed with empty plasmid transfected DCs).

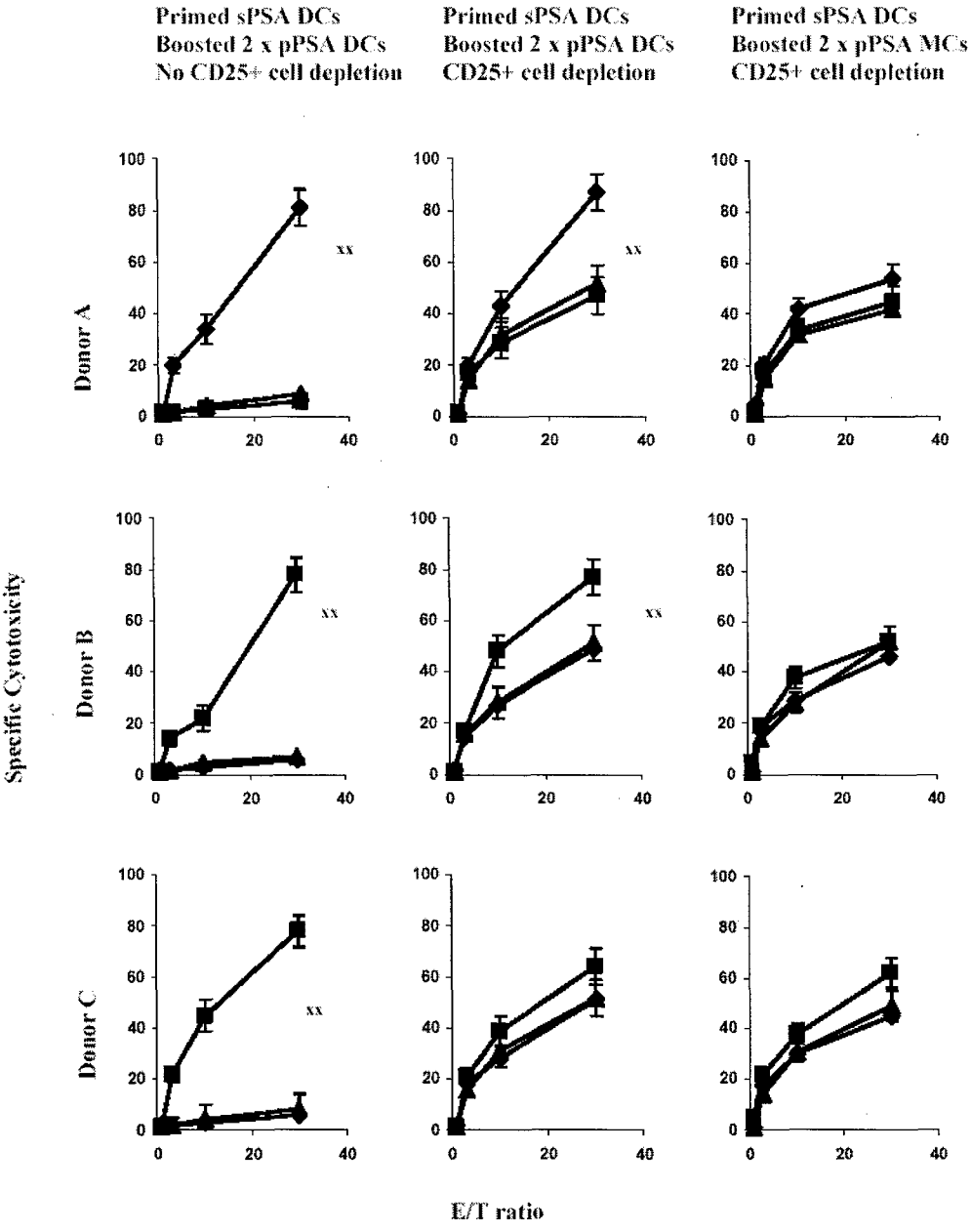

Fig. 5. Depletion of $CD25^+$ cells prior to initial exposure to antigen leads to generation of T cells reactive to both dominant and sub-dominant epitopes. HLA A2(+) peripheral blood mononuclear cells that had been depleted of monocytes, with or without additional removal of CD25+ cells, were primed with autologous dendritic cells transfected with the sPSA plasmid. Responding cultures were then boosted twice with monocytes pulsed with one of three HLA-A2 restricted PSA-derived peptides (PSA_{170} – squares; PSA_{51} – triangles or PSA_{53} – diamonds) (table 1) and their cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during boosting. Each point represents the mean and SD from three different experiments. Values for cytotoxicity of sPSA DCs-primed T cells against T2 targets pulsed with PSA_{170}, PSA_{51} or PSA_{53} peptides were compared. Significant differences at the 30:1 E/T ratio are indicated with xx ($p<0.01$).

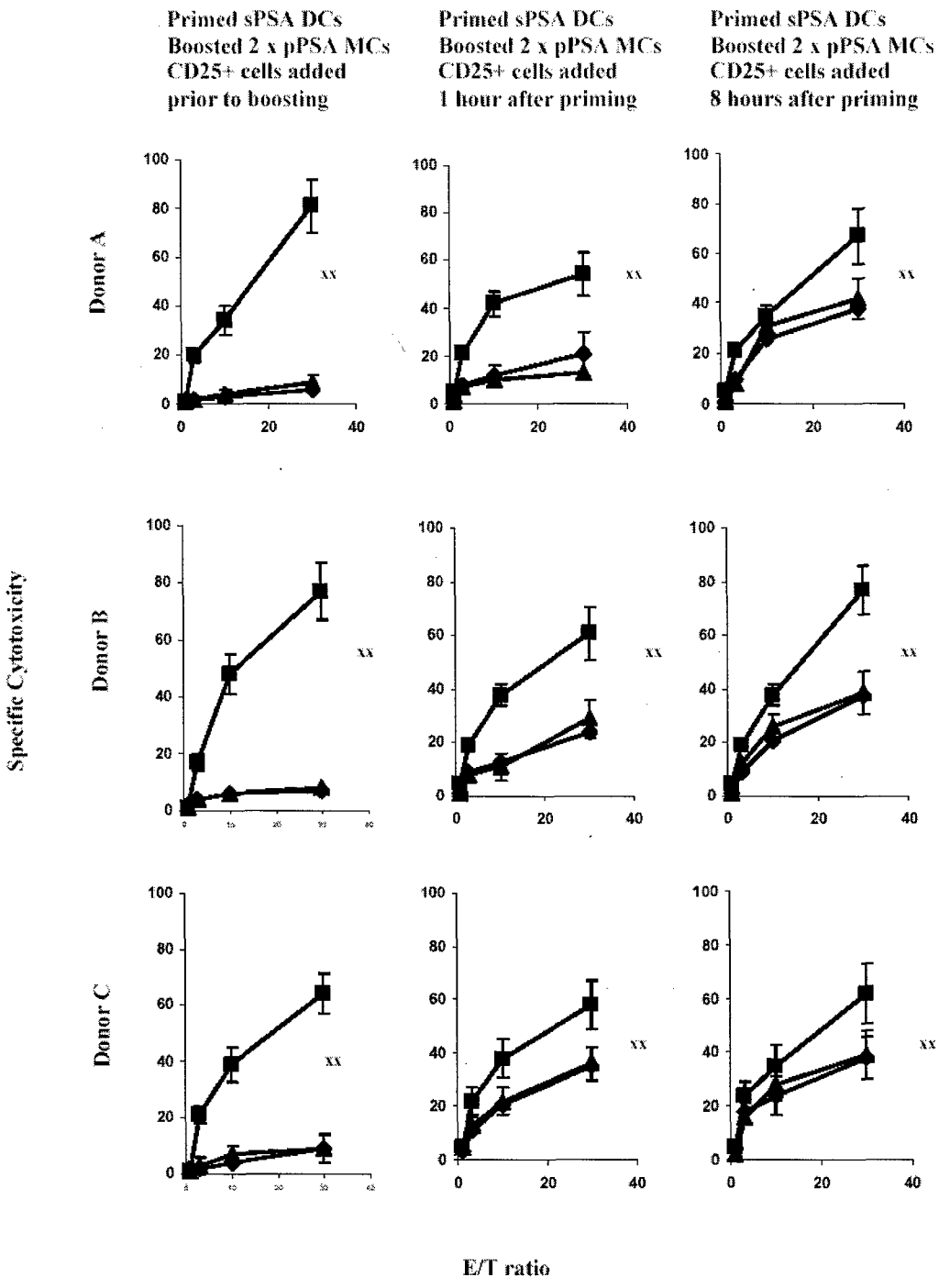

Fig.6. Addition of $CD4^+CD25^+$ cells back to T cells within the first hour of priming with sPSA DCs reverses immunodominance. HLA A2(+) peripheral blood mononuclear cells that had been depleted of monocytes and $CD25^+$ cells were primed with autologous dendritic cells transfected with the sPSA plasmid. Purified $CD4^+CD25^+$ T cells were added back at different time points following the initiation of the cultures (responder T cells/$CD4^+CD25^+$ T cell ratio = 4:1). Responding cultures were then boosted twice with monocytes pulsed with one of three HLA-A2 restricted PSA-derived peptides (PSA_{170} – squares; PSA_{51} – triangles or PSA_{53} – diamonds) and their cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during

boosting. Each point represents the mean and SD from three different experiments. Values for cytotoxicity of sPSA DCs-primed T cells against T2 targets pulsed with PSA_{170}, PSA_{51} or PSA_{53} peptides were compared. Significant differences at the 30:1 E/T ratio are indicated with xx ($p<0.01$).

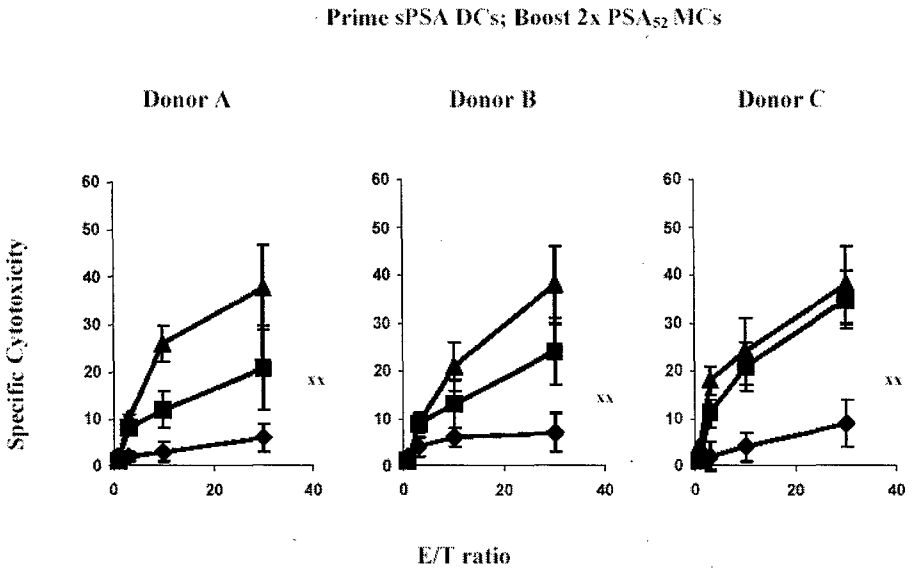

Fig.7. $CD4^+CD25^+$ T cells act early during priming to establish immunodominance to PSA_{52}. HLA A2(+) peripheral blood mononuclear cells that had been depleted of monocytes and $CD25^+$ cells were primed with autologous dendritic cells transfected with the sPSA plasmid. Purified $CD4^+CD25^+$ T cells were added back at different time points (triangles – before; squares – 1 hour after; diamonds – 8 hours after)onset of the (responder T cells/$CD4^+CD25^+$ T cell ratio = 4:1). Responding cultures were then boosted twice with PSA52-pulsed monocytes and their cytotoxicity was tested against T2 cells pulsed with the same peptide.

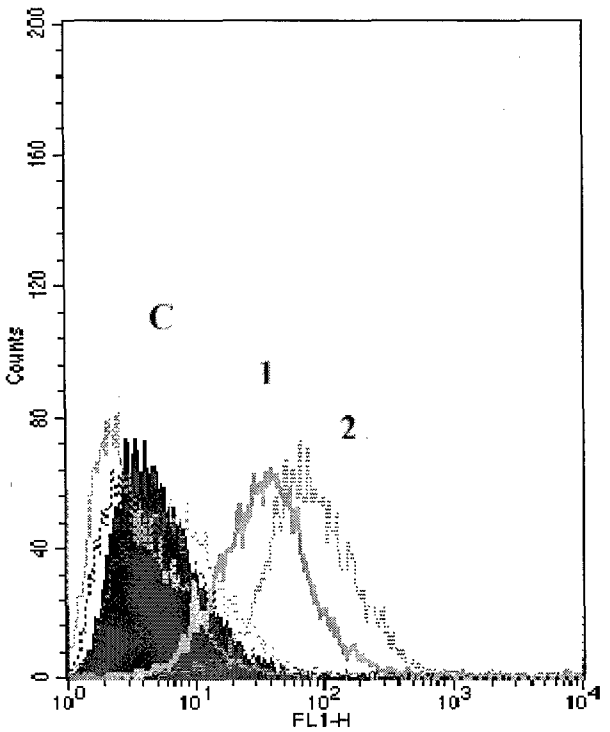

Fig. 8. Activation of human $CD4^+CD25^+$ T cells increases the membrane GITR expression. Freshly isolated (1) or stimulated human $CD4^+CD25^+$ T cells stained with anti-GITR antibody. FITC conjugated mouse IgG1 was used as a control.

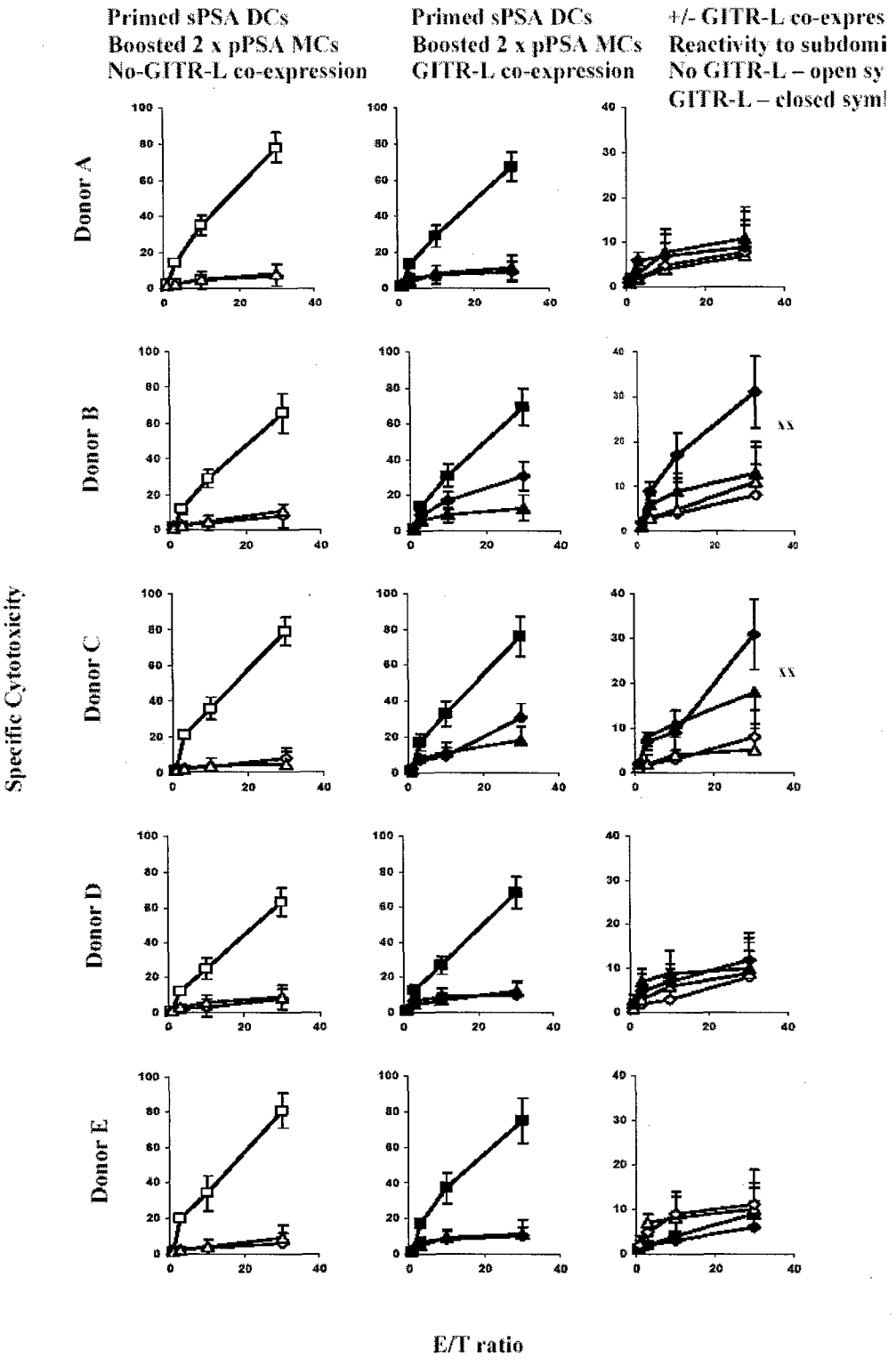

Fig.9. sPSA DCs co-transfected with the human GITR-L may support priming and development of T cells reactive to sub-dominant epitopes. HLA A2(+) peripheral blood mononuclear cells that had been depleted of monocytes were primed with sPSA plasmid-transfected autologous dendritic cells that had (filled in symbols) or had not been (open symbols) transfected with the human GITR-L. Responding cultures were then boosted twice with monocytes pulsed with one of three HLA-A2 restricted PSA-derived peptides (PSA_{170} – squares; PSA_{51} – triangles or PSA_{53}

– diamonds) and their cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during boosting. Each point represents the mean and SD from three different experiments. Values for cytotoxicity of sPSA DCs-primed T cells against T2 targets pulsed with PSA_{51} or PSA_{53} peptides were compared (third column). Significant differences at the 30:1 E/T ratio are indicated with xx ($p<0.01$; reactivity against PSA_{53} for donor B and PSA_{51} or PSA_{53} for donor C).

B. Manuscript submitted in Cancer Gene Therapy

Immune responses against PSMA after gene-based vaccination for immunotherapy: A. Results from immunizations in animals

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Running title: Anti-PSMA gene-based vaccination in animals

Keywords: PSMA, Gene-based vaccine, prostate cancer, cytotoxicity, antibody production

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ABSTRACT

Two plasmid DNA vaccines, encoding either products that are retained in the cytosol and degraded in the proteasome (tVacs; hPSMAt), or secreted proteins (sVacs; hPSMAs) were evaluated for stimulation of cytotoxic cell or antibody responses. Immunization with both vectors led to generation of cell cytotoxicity providing GM-CSF was administered with the vaccine. Spleen cells from animals immunized with hPSMAt demonstrated stronger cytotoxicity to the target cells. Priming with a vector that encoded a xenogeneic protein (hPSMAt; "xenogeneic" construct) and boosting with a vector that encoded an autologous protein (rPSMAt; "autologous" construct) gave the best protection against tumor challenge. Immunization with tVacs did not lead to formation of antibodies to the target protein as detected by Western blot or ELISA, while immunization with sVacs or with the protein did. Antibodies were of mixed Th1-Th2 isotype. Priming with tVacs and boosting with protein also resulted in antibody formation, but in this case the antibodies were from the cytotoxic, Th1 isotype. The best strategy to obtain a strong cellular cytotoxic response, therefore, seems to be gene-based vaccinations with tVacs, priming with the "xenogeneic" and boosting with the "autologous" constructs. When cytotoxic antibody production is the goal, priming should be performed with the tVacs while boosting with the protein.

INTRODUCTION

Several groups have recently reported on the safety and biologic effectiveness of DNA vaccines for immunization against pathogen or tumor antigens.[1-4] The use of PSA-recombinant vaccinia virus has also been found to be safe and to lead to an immune response.[5,6] The main effectors in anti-tumor immunity after DNA immunization are CD8+ cytotoxic T cells that recognize tumor or tumor-associated antigen-derived peptides expressed in association with MHC class I molecules.[7] A rate limiting step in loading the latter with peptides seem to be the antigen supply.[8] We have found that newly synthesized proteins that are purposefully retained in the cytosol are targeted for proteasomal degradation.[9] Transfection with truncated DNA constructs (tVacs) that lack sequences encoding the leader peptide of a secreted protein, or the membrane domain of type II protein, lead to expression of such products. In our immunotherapeutic studies, we have targeted the prostate-specific membrane antigen (PSMA; gamma carboxyl peptidase II, GCPII). PSMA expression is normally restricted to the prostate gland, brain tissue, jeunum and proximal kidney tubules.[10-12] Its expression is increased nearly 10-fold in prostate cancer cells and is also found in tumor but not normal neovasculature.[13,14] Elevated PSMA serum levels have been observed in healthy males and females, as well as in patients with benign prostate hypertrophy, prostate and breast cancer.[15-17] It is a type II integral membrane protein[18,19] whose main portion is extracellular. We have cloned the cDNA encoding the extracellular portion (AA 44-750) of the human PSMA (XC-PSMA) and included it in a mammalian expression vector (hPSMAt). Dendritic cells, transfected with hPSMAt, stimulate in vitro autologous T cell proliferation and cytotoxicity against the target antigen.[9] We speculate that, similarly to results from in vitro experiments, in vivo immunization with this vector will result in enhanced cytotoxicity and protection from tumor development. [9,20]

Additionally, gene-based vaccines encoding for a xenogeneic rather than autologous protein have been shown to more likely break existing immunological tolerance.[3,21-24] We tested this approach and compared immunizations in rats with tVacs encoding the human PSMA or its rat equivalent. Additionally, since transfection with hPSMAt does not result in the expression of a full length glycosylated and folded protein,[9] it is of academic interest whether immunization with hPSMAs will lead to formation of antibody against the native antigen, against carbohydrate residues or against linear epitopes. To study this, we have developed a transplantable tumor rat model in which animals are immunized against PSMA with different DNA vectors with or without recombinant rat GM-CSF, and then inoculated with syngeneic tumor cells (AT3B-1 cells) transfected to express the target protein.

MATERIALS AND METHODS

Rats and Immunizations and Tumor Cell Inoculation

All animal experiments were approved by the George Washington University Medical Center (GWUMC) Institutional Animal Care and Use Committee. Male retired breeders (Copenhagen rats) were purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN), maintained at the GWUMC Animal Research Facility (ARF), and fed a standard diet. The ARF has maintained continuous accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care since 1974, and has an assurance statement on file with the Office Laboratory Animal Welfare, thus meeting all Public Health Service guidelines for animal use and care.

For immunizations, 100 μg plasmid DNA with or without rat recombinant GM-CSF (R&D Systems, Minneapolis, MN), were brought to100 μl with sterile saline for injection. The immunizations were performed at 10 day-intervals at the right rear flank using insulin syringes.

All injections were intradermal, taking caution that a blister was formed and the infusate was retained at the epidermal-dermal junction.

For tumor cell inoculation, 10^5 of AT3B-1^{PSMA} cells (the YM2 clone, see "*AT3B-1 transfection with hPSMAs*") were resuspended in 100 μl sterile saline and injected sub-cutaneously in the opposite rear flank.

Plasmid DNA Vaccines

The H PSMA-K plasmid (hPSMAt) was constructed on the basis of pVAX1 vector (Invitrogen, Carlsbad, CA) and contained a pUC derived pMB1 origin of replication, a kanamycin resistance gene and the mammalian transcriptional unit with DNA encoding for the extracellular portion of the human PSMA under the regulation of the immediate-early CMV promoter/enhancer and a bovine growth hormone polyadenylation signal. The plasmid size is 5.1 kb.

For the R (rat) "PSMA"–K plasmid (rPSMAt), cDNA encoding for a rat analogue of the human extracellular PSMA was cloned from rat brain tissue and included in a pVAX1 backbone. The cDNA had 85.66% homology with the human PSMA at the nucleotide level. The plasmid size was 5.1 kb. Products expressed from hPSMAt or rPSMAt plasmids were retained in the cytosol and degraded in the proteasome [9].

For the S-PSMA plasmid (hPSMAs), the extracellular portion of the human PSMA was cloned into a pSecTag2 vector (Invitrogen), which provided the murine Ig k-chain leader sequence. The insert from the obtained clone, which represents human XC-PSMA fused with leader sequence, was then sub-cloned into a pVAX1 expression vector (Invitrogen, Carlsbad, CA). The plasmid size was 5.1 kb. Following transfection with this vector, the expressed product was translocated to the endoplasmic reticulum (ER), glycosylated and secreted [9]. Cells, transfected with hPSMAt or hPSMAs synthesize proteins that have identical aminoacid sequences. Due to post-translational modification in the ER, however, the secreted protein is N-glycosylated and secreted, while the other is not transported to the ER and not glycosylated, but remains in the cytosol and is degraded in the proteasome. [9]

For transfection of the AT3B-1 cells with the human PSMA, the extracellular portion of the human PSMA was cloned into a pSecTag2 vector containing zeocin resistance gene.

AT3B-1 transfection with hPSMAs

Monolayers of AT3B-1 cells (CRL-2375; ATCC, Manassas, VA) were transfected with the hPSMAs using the FuGENE6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) and assayed for PSMA production by Western blot. The cells were seeded in 6-well tissue culture plates (Nunc, Denmark) at $1.5x10^5$ cells per well and grown to 50-70% confluence in DMEM supplemented with 25 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 10% (v/v) of fetal bovine serum. COS-1 or AT3B-1 cells were transfected with 1.5 μg of plasmid DNA pre-condensed with 4.5 μl of FuGENE 6 reagent in serum-free DMEM for 30 min at room temperature. Cells were then grown for 72 h in complete DMEM and then harvested.

The transfected cells with stable expression of hPSMAs were selected and cloned after growth in zeocin-containing medium (0.1 mg/ml).

Electrophoresis and immunoblotting

For electrophoresis and Western blotting, cells were harvested by gentle scraping, washed twice with 2 ml of cold PBS and lysed with 0.25 ml cold lysing buffer (0.5 M NaCl, 1% triton X-100, 0.2% Tween 20, 50 mM HEPES, pH 7.0). Lysates were transferred to Eppendorf tubes and homogenized by repeated pipetting on ice. Immunoblotting and electrophoretic assays of cell lysates and culture supernatants were performed using NuPage BisTris electrophoretic system (Invitrogen, Carlsbad, CA). Protein samples in loading buffer were heated at 70°C for 10 min and loaded on 10% BisTris gels. After electrophoresis and electro-transfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 min. The following primary antibodies were used: Y-PSMA1 and Y-PSMA-2 (Yes Biotech Laboratories Limited, Mississauga, Ontario, Canada), rabbit polyclonal anti-GCPII antibodies, anti-GCPII monoclonal antibodies A5, E1, E7, E12 and H2 (all developed in the Institute of Organic Chemistry and Biochemistry, Prague, the Czech Republic). The membranes were probed with poly- or monoclonal anti-PSMA Abs for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma, St. Louis, MO) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce, Rockford, IL) in accordance with the manufacturer's recommendations.

For detection of secreted PSMA, the serum containing DMEM was removed 48 h after transfection, the cells were washed twice with 2 ml of PBS, serum free DMEM (2 ml per well) was added and the cells were incubated for additional 24 h in the 6-well plates. After collection of the medium, the cell debris was removed by centrifugation (35,000 g, 20 min) and supernatants were concentrated with Centricom centrifuge filtering device (Millipore, Bedford, MA) and then stored at -30°C until further use.

For a control, an extracellular portion of the human glutamate carboxypeptidase II (amino acids 44-750) expressed in Drosophila Schneider's cells, purified to homogeneity (GCP II) was used.[25] This material, together with the rabbit anti-PSMA serum and some of the monoclonal antibodies were provided to us by Dr. Jan Konvalinka, Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Prague, The Czech Republic.

Cell Proliferation

Standard [^{3}H]thymidine incorporation assays were performed to assess spleen cell proliferative responses. Splenocytes from immunized rats were resuspended at 5 x 10^6 cells/ml in RPMI 1640 containing 10% FBS. One hundred microliters of cell suspension were added to each well in 96-well plates with 20, 10, 5, or 0 μg/ml of recombinant PSMA. After 4 days of culture, 1 μCi [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) was added to each well. Following a16-h incubation, cells were harvested on glass filter paper and radioactivity was measured in a Beckman LS 2501 liquid scintillation counter. The stimulation index (SI) was calculated as: counts per minute (cpm) with Ag stimulation/background cpm without Ag.

Cell Cytotoxicity

Cell Cytotoxicity was determined as previously described.[26] Briefly, target cells (AT3B-1 or YM2 cells) were grown overnight in complete medium supplemented with 1 with μCi [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) then washed and resuspended in complete medium. Cells in different target to effector cell ratio were plated in 96 round bottom well plates for 6 hours at 37°C in a humidified incubator. Cells were then harvested on glass filter paper, and radioactivity was measured in a liquid scintillation counter. The killing was detected as a fall in counts per minute due to DNA fragmentation in cell samples

undergoing apoptosis. All of the E:T ratios were tested in triplicate. Cell cytotoxicity was normalized to control samples that contained target cells only and expressed as specific lysis. Spontaneous cytotoxicity was determined in medium alone without effector cells.

Tumor Development

Animal weight and tumor development were monitored daily. Tumor size was measured after the animals were euthanized and the tumors were excised.

Antibody determination

For Western blot, SDS gel electrophoresis of cell lysates, cell supernatants or GCP II were performed as described above. After electrotransfer and blocking, the nitrocellulose membranes were stained with sera from immunized rats or anti-PSMA antibodies for an hour. Secondary staining with antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, MO; dilution 1:10,000; for one hour) was performed after three washes with TBS, 0.05% Tween20. Finally, the blots were developed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to BioMax MR film (Kodak, New Haven, CT).

In some cases, serum anti-PSMA antibody titers from immunized rats were measured by a direct ELISA. Ninety-six-well plates coated overnight with 100 μl/well of 10 μg/ml recombinant GCPII in PBS were blocked for 2 hours with PBS containing 2% BSA and 0.05% Tween20. Sera were then added in serial dilutions and incubated for 1 hour. The plates were washed three times with PBS containing 0.05% Tween20 and incubated for 1 hour with 1/5000 dilution of a peroxidase conjugated anti-rat kappa-lambda chain secondary antibody (Jackson Laboratories, Bar Harbor, ME). The plates were then washed three times, developed with tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD), stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader (Dynatech Laboratories, Alexandria, VA).

Statistical Analyses

Significant differences in cytotoxicity of spleen cells or antibody titers were assessed by Student's *t* test. The difference between groups was considered statistically significant when the *P* was lower than 0.05. A non-parametric test of statistical significance for bivariate tabular analysis (chi-square) was performed for data accumulated when tumor development was assayed.

RESULTS

Transfection of AT3B-1 cells with hPSMAs

We find that both rat prostate tissue and AT3B-1 cell lysates express low level of PSMA when tested by PCR (data not shown). Since PSMA expression is up-regulated in human

prostate cancer cells, we decided to transfect the AT3-B1 cells with the hPSMAs, a vector that contains the murine Ig k-chain leader sequence so that the extracellular portion of the human PSMA is glycosylated and secreted [9]. We did not transfect the cells with the full length PSMA since our goal was to study T cell-mediated and not antibody-mediated tumor protection and we felt that if PSMA was expressed as a membrane protein by the transfected AT3B-1 cells, some of the protection might have been antibody mediated. This would have been true especially when immunization was performed with DNA constructs that led to anti-PSMA antibody development (see below). We obtained two clones of stably transfected AT3B-1 cells – YM1 and YM2, both of which are high expressors and PSMA can be detected in the culture medium (figure 1). A significant portion of the expressed product is degraded in the proteasome and could be detected in cell lysates only in the presence of proteasomal inhibitor lactacystin (figure 1). All further experimentation was performed with the YM2 clone.

Addition of rat GM-CSF to the immunization cocktail increases the efficacy of immunization

Male Copenhagen rats were immunized with the plasmid vaccines alone (figure 2A), or with a cocktail that, in addition to the plasmid vaccine, contained 9 $\mu g/m^2$ recombinant rat GM-CSF (R&D, Minneapolis, MN) (figure 2B and C). In separate experiments, rats were primed with the hPSMAt GM-CSF, but boosted with the rPSMAt GM-CSF cocktail (figure 2D). Addition of GM-CSF to the immunization cocktail increased the efficacy of the immunization (figure 2B and C). Rats immunized with hPSMAt showed stronger spleen cell cytotoxicity toYM2 compared to those immunized with rPSMAt, but the best results were obtained when priming was performed with hPSMAt, while boosting with rPSMAt (figure 2D).

Slightly stronger cytotoxicity to YM2 cells was observed in spleen cells from animals immunized with hPSMAt when compared to spleen cells from animals immunized with hPSMAs (figure 3).

In the absence of GM-CSF, immunization with a construct encoding either human or rat PSMA partially protects the rats from developing tumors

Copenhagen rats, inoculated sub-cutaneously with 10^5 syngeneic AT3B-1^{PSMA} YM2 tumor cells, develop detectable tumors by day 10-12 following injection. To explore the role of pre-immunization on tumor development, rats were immunized three times at 10-day intervals with either saline, empty plasmid vector, hPSMAt or rPSMAt (table 1). No rat GM-CSF was included in the immunization cocktail. Fourteen days after the last immunization, all animals received 10^5 of YM2 tumor cells in the right rear flank. Tumors, when developing, were palpable at day 12 following tumor cell inoculations. All animals were sacrificed 15 days following tumor inoculations and tumors, when present, excised and measured. Only two of the ten animals immunized against human PSMA and five of the 10 animals immunized against the rat analogue developed tumors. In contrast, 17 of the 20 animals in the control groups developed tumors. The tumors, developing in the group, immunized with the human or the rat construct, were smaller than the ones in the control group (table 1).

Addition of rat GM-CSF to the immunization cocktail enhances gene-based vaccination-induced tumor protection

From an earlier clinical trial,[4] we found that the magnitude of the response could be increased if the plasmid vaccine is co-injected with GM-CSF, a cytokine that has been known for its adjuvant properties.[27] The above experiment was repeated, but this time recombinant rat GM-CSF (9 $\mu g/m^2$) was added to the immunization cocktail. In this study, full protection was observed when recombinant rat GM-CSF was used as an ingredient of the immunization cocktail (table 2), no matter whether animals were vaccinated with the vaccine encoding the human or the rat protein.

In the presence of GM-CSF, priming with a construct encoding a xenogeneic protein and boosting with a construct encoding an autologous protein lead to the best protection

The protection of the immunized animals from development of tumor depends on the amount of cells inoculated during tumor challenge. Partial tumor protection could be seen when $2x10^5$ or $3x10^5$ tumor cells are injected. All immunized animals develop tumors when the tumor dose per animal is higher then $4x10^5$ cells. In an attempt to define the best strategy of vaccination, we explored different priming and boosting strategies. All immunizations were performed with plasmid DNA cocktails that contained rat GM-CSF. All animals were challenged with $2x10^5$ cells. Rats were sacrificed on the day tumor was detected. All tumor-free animals were sacrificed at day 16 following tumor cell inoculation. Ten rats were included per group. The best protection following immunization involved priming with hPSMAt and boosting with rPSMAt and all the animals were tumor free 16 days after tumor inoculation (figure 4; circles). In contrast, when priming was performed with hPSMAs and boosting with rPSMAt, three rats developed tumors on day 12, while the rest of the rats (66%) remained tumor free. The difference between these two groups was not statistically significant ($\chi^2 = 3.52$ at $p<0.1$). Spleen cells from the hPSMAs-immunized animals, however, gave the strongest proliferative response after stimulation with recombinant PSMA (data not shown). Priming and boosting with rPSMAt resulted in only partial protection – three rats developed tumor on day 10, and 3 rats on day 11. Four of those rats (34%) remained tumor free until day 16 (figure 4, diamonds). The difference between this group and the control group, though, was statistically significant ($\chi^2 = 5$ at $p<0.05$) since all control rats developed tumors by day 12 after tumor inoculation.

Antibodies against PSMA in animals following immunization with different gene-based vaccines

Rats immunized with "truncated" constructs (hPSMAt or rPSMAt) did not develop antibodies to PSMA as detected by ELISA (figure 5) or Western blot (data not shown), no matter whether GM-CSF was present or not. No antibodies against PSMA (detection by Western blot or ELISA) developed following immunization with plasmid, encoding the secreted PSMA (hPSMAs), or with GCP II, when GM-CSF was absent from the immunization cocktail (figure 5). Immunization with hPSMAs in the presence of GM-CSF led to formation of antibodies against the native protein (detected by ELISA; figure 5). The highest titer of antibodies against PSMA was observed when priming was performed with GCPII or tVacs, and boosting was performed with the recombinant protein and GM-CSF was added as an adjuvant (figures 5 and 6). Antibodies, developing following immunization with hPSMAs or GCPII, were of mixed Th2 and Th1 type, since both IgG1 and IgG2a subtypes were detected. On the contrary, priming with tVacs (hPSMAt or rPSMAt) and boosting with hPSMAs or GCP II led to formation of Th1, cytotoxic antibodies (IgG2a and IgG2b) with relatively high titer.

DISCUSSION

Immunotherapy holds a great promise for controlling and treatment of cancer, but effective immunization against tissue-specific, self-antigens is necessary in order to increase the likelihood of success. In order to increase the effectiveness of immunization, we decided to use plasmid vectors that will provide:

a) effective generation of antigen derived peptide in the transfected cell cytoplasm for MHC class I presentation

b) presence of antigenic determinants that would more-likely break existing tolerance to autologous antigens targeted for cancer immunotherapy.

For this reason we compared vectors that encode for:

a) proteins that do not undergo posttranslational translocation to the endoplasmic reticulum but are degraded in the proteasome[9,28] – i.e. tVacs

b) xenogeneic rather than autologous (syngeneic) proteins.[21,22,24]

Similarly to the in vitro experiments, performed by us, [9] in vivo administration of tVacs or sVacs stimulates cytotoxic T cell responses (figure 3). The stronger cytotoxicity seen after immunization with hPSMAt may reflect stimulation of T cell clones that have high affinity receptors for the antigen. Alternatively, the stronger spleen cell cytotoxicity to the YM2 cells may be due to priming to sub-dominant epitopes which, unlike results in vitro, is maintained after boosting.[9] In such a case the stronger cytotoxicity may originate in the greater number of T cell clones recognizing the target cells. Some of this may have receptors with low affinity for the antigen and greater numbers of responders (respectively responder to target ratio) may be required for induction of cell death in target cells. Whether this is the case, however, is impossible to conclude from the current animal model. Additional experimentation, for example with a transgenic animal model (HLA-A*0201/H-2 Kb mice) to evaluate responses to individual PSMA-derived epitopes, is required before any hypothesis can be put forward.

An important issue for effective vaccines is the development of potent adjuvants that can facilitate induction or augmentation of immunity. Despite the presence of non-methylated CpG motifs in plasmid DNA vaccines,[29-35] naked DNA immunization of rats obviously requires an additional adjuvant, and GM-CSF could act as such (figure 2; table 2). Granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances immune responses by inducing the proliferation, maturation, and migration of dendritic cells.[36] Animals, immunized with a GM-CSF-plasmid DNA cocktail, are fully protected against transplantable tumors. This result is in agreement with earlier observations both in animals and in humans.[4,37-39] The expansion and differentiation of both T and B lymphocytes is affected[36] since the adjuvant effect of GM-CSF influences both cytotoxic T cell generation (figure 2) and antibody development (figure 5).

It has been shown that immunization with xenogeneic rather than autologous proteins is more likely to break existing tolerance[21] and we, as have others,[24] speculated that immunizations with

plasmid DNA that encodes xenogeneic proteins will have the same advantage. This clearly is the case as far as priming is concerned (figure 4). Addition of GM-CSF to the immunization cocktail, however, leads to easier break of immune tolerance to PSMA since good protection against tumor development is observed even in those animals that were immunized with rPSMAt (table 2), a construct encoding the autologous protein.

Rats, primed with a construct encoding a human, but boosted with a construct encoding a rat protein, demonstrate the best protection against a transplantable tumor (figure 5). This may be due to the fact that priming with the construct encoding the xenogeneic protein successfully breaks the tolerant state. Boosting with an autologous protein or construct that encodes it, however, is necessary for fine tuning of the immune response.

Finally, release of the expressed product at the immunization site after gene-based vaccination seems to be necessary for development of antibody (figure 5). Priming and boosting with tVacs, whose product is not released extracellularly, but degraded in the proteasome, leads to no antibody production no matter whether an adjuvant is administered with the immunization cocktail. Others have reported similar findings following immunization with cytosolically retained or secreted viral proteins.[40-47] This is an important observation because, in cases where a tumor marker serves as a target antigen, development of high titer antibodies in patients' sera may interfere with marker testing and the use of the marker in disease monitoring. This is especially true when targeting secreted proteins such as PSA or PAP, where development of antibodies is of no therapeutic value. In those cases using tVacs instead of sVacs is clearly advantageous.

Production of antibodies after immunization for immunotherapy of cancer, however, can be beneficial in those instances where the target antigen is a membrane protein, such as PSMA.[48-51] Recently, Haas et al[52]showed, that immunization with a plasmid vector encoding a PSMA residue that is expressed on the membrane following expression, also leads to development anti-PSMA antibodies that bind to the native antigen. One would expect that the type of the immune response would impact the outcome of the treatment with Th1-type, cytotoxic antibodies that bind complement having the advantage of causing direct damage to the tumor cells. Were such the case, priming with tVacs and boosting with the native protein seem to represent the best immunization strategy (figure 6).

In conclusion we have shown that in vivo immunization with both tVacs and sVacs leads to generation of cytotoxic spleen cells. This shows that both tVacs and sVacs are immunogenic in rodents, and spleen cells from animals immunized with tVacs demonstrate stronger cytotoxicity. Co-administration of GM-CSF during immunization enhances the immune response and immune animals are protected against development of tumor when tumor cells that express the target antigen are inoculated. Priming with a construct encoding a xenogeneic protein and boosting with a construct encoding a syngeneic (autologous) protein confer the best protection. Immunization with gene-based vaccines whose products are retained in the cytoplasm and proteasomally degraded (tVacs) does not lead to antibody formation to the target antigen. Priming with tVacs and boosting with the native protein antigen results in production of cytotoxic antibodies.

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Table 1. Gene-based vaccination induced tumor protection

Injection with:	*Number of animals with tumor (Total number of animals per group)*	*Mean size of tumor*
A. 3 x hPSMAt	2(10)	0.5 +/- 0.1 cm3
B. 3 x rPSMAt	5(10)	1.2 +/- 0.23 cm3
C. 3 xhPSMAs	7(10)	1.6 +/- 0.48 cm3
D. 3 x Empty vector	8(10)	>2.3 +/- 0.3 cm3
E. 3 x Saline	9(10)	>2.44 +/- 0.48 cm3

Differences in the number of tumor developing rats. Difference between groups A and B is not statistically significant (χ^2 = 1,978; $p<0.2$). Differences between groups A and E is statistically significant (χ^2 = 5.05; $p<0.025$), Differences between groups D and E (combined) and A or B are statistically significant (χ^2 = 3.8; $p<0.01$ for A and χ^2 = 4.17; $p<0.05$ for B).

Table 2. Addition of GM-CSF to the immunization cocktail enhances gene-based vaccination induced tumor protection

Injection with:	*Number of animals with tumor (Total number of animals per group)*	*Mean size of tumor*
F. 3 x hPSMAt + rGM-CSF	0(10)	-
G. 3 x rPSMAt + rGM-CSF	0(10)	-
H. 3 x hPSMAs + rGM-CSF	2(10)	1.1 +/- 0.1 cm3
I. 3 x Empty vector + rGM-CSF	9(10)	>1.89 +/- 0.34 cm3

Differences in the number of tumor developing rats. Difference between A and F is not statistically significant (χ^2 = 2.22; $p<0.2$). Difference between B and G is statistically significant (χ^2 = 6.66; $p<0.01$). Difference between C and H is statistically significant ((χ^2 = 5.05; $p<0.2$).

FIGURES AND LEGENDS TO FIGURES

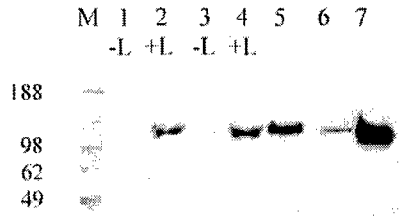

Figure 1. Detection of PSMA in AT3-B1 cells transfected with hPSMAs

+/-L – presence or absence of lactacystin
1 and 2 – AT3B-1 cell clone YM1, stably transfected with hPSMAs
3 and 4 – AT3B-1 cell clone YM2, stably transfected with hPSMAs
5 – supernatant from YM2
6 – supernatant from YM1
7 – recombinant GCPII

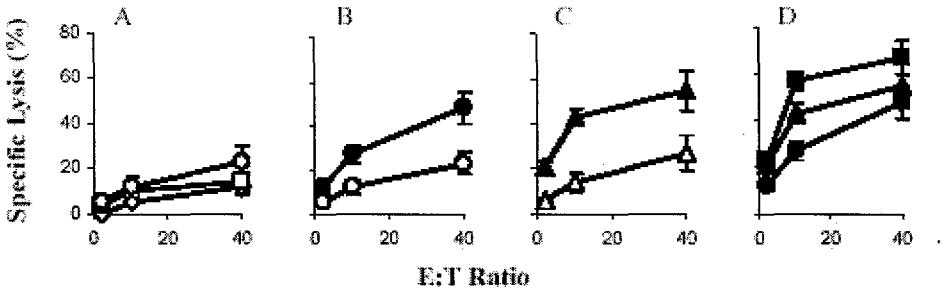

Fig. 2. Cytotoxicity of rat spleen cell towards the YM2 clone. Copenhagen rats were immunized three times at 10 day-intervals, euthanized 14 days later and their spleen cells tested for cytotoxicity against the YM2 clone. A – rats were injected with either rPSMAt (circles), empty vector (squares) or saline (diamonds). B – rats were injected with rPSMAt with (filled in circles) or without rat recombinant GM-CSF. C – rats were injected with hPSMAt with (filled in triangles) or without rat recombinant GM-CSF. D – rats were primed and boosted with cocktails containing rat GM-CSF and either hPSMAt (triangles) or rPSMAt (circles), or primed with hPSMAt and boosted twice with rPSMAt (squares).

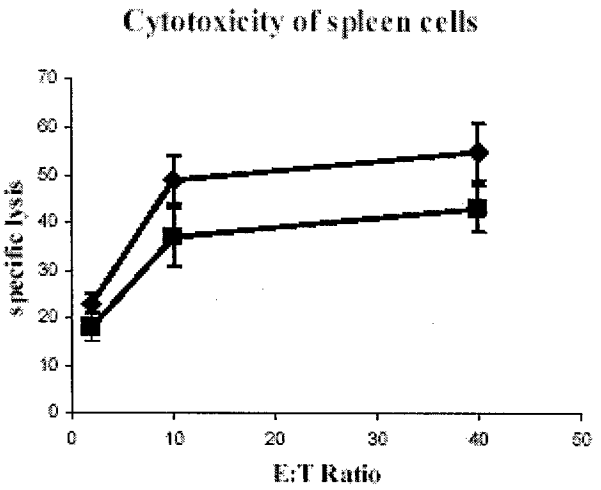

Figure 3. Cytotoxicity of spleen cells from Copenhagen rats that were immunized against the YM2 clone. Rats were immunized three times at 10 day-intervals with a cocktail that contained rat GM-CSF and either hPSMAt (diamonds) or hPSMAs (squares).

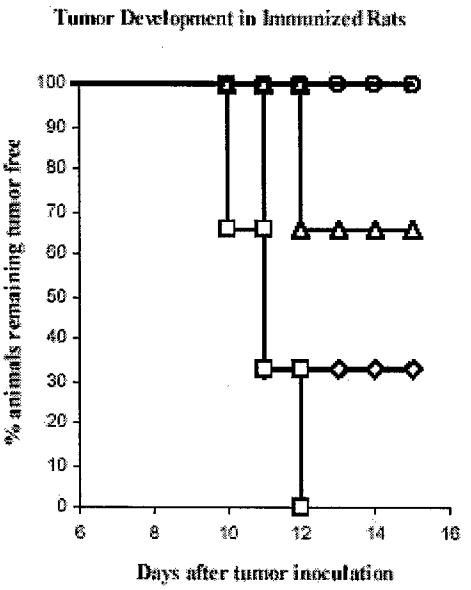

Figure 4. Tumor development in rats following different prime-boosting regimens. All rats were immunized with a cocktail containing 100 μg plasmid DNA and 9μg/m^2 body surface area rat GM-CSF. Circles: Rats were primed with hPSMAt and boosted with rPSMAt; triangles – primed with hPSMAs and boosted with rPSMAt; diamonds – primed and boosted with rPSMAt; squares – primed and boosted with empty vector.

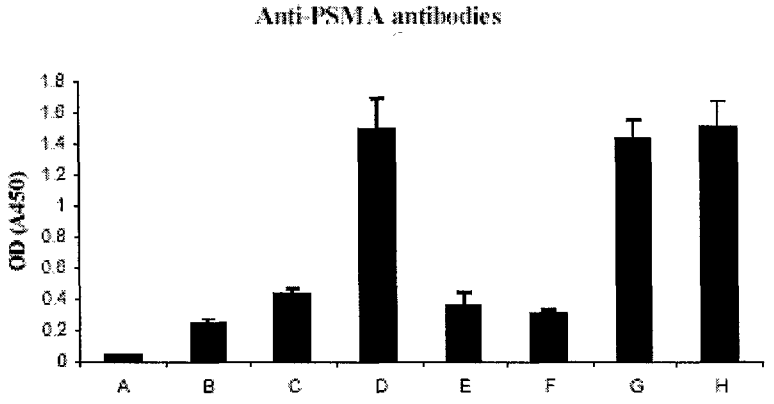

Figure 5. Immunization with the native protein leads to development of antibodies against its native conformation (data from ELISA, 1: 30 serum dilution) ***A*** – priming with hPSMAt, boosting with rPSMAt (with GM-CSF); ***B*** – 3 immunizations with hPSMAs (no GM-CSF); ***C*** – 3 immunizations with hPSMAs (with GM-CSF); ***D*** – 3 immunizations with GCPII (with GM-CSF,); ***E*** – prime with GCPII, boost with hPSMAt and rPSMAt (with GM-CSF); ***F*** – prime with GCPII, boost with rPSMAt (with GM-CSF,); ***G*** – prime with hPSMAt, boost with GCPII (with GM-CSF); ***H*** – prime with rPSMAt, boost with GCPII (with GM-CSF).

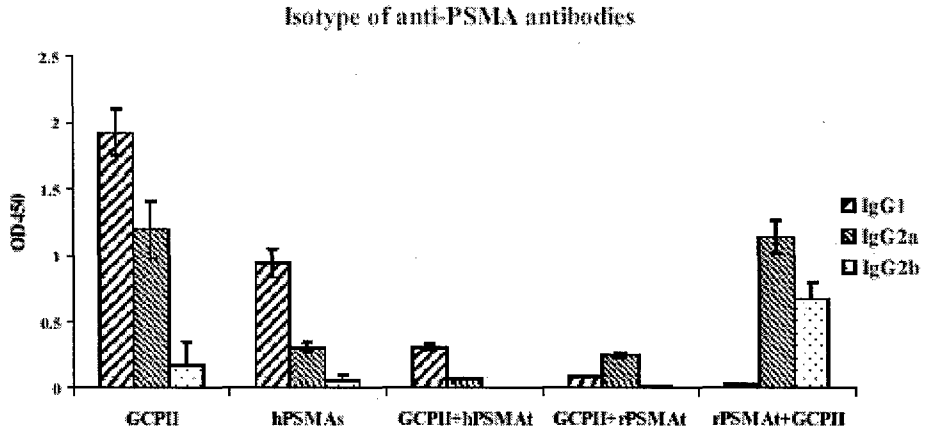

Figure 6. Immunization with tVacs primes for development of cytotoxic antibodies but boosting with the native protein is necessary for development of antibodies (data from ELISA, 1:30 dilution). Rats were primed and boosted twice with either GCPII or hPSMAs, or primed with GCPII and boosted with hPSMAt or rPSMAt; or primed with hPSMAt and boosted with GCPII.