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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 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), 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 HPSA-T or human prostate acidic phosphatase (HPAP-T), both of which lack signal peptide sequences and are retained in the cytosol and degraded by the proteasomes following expression. Additionally, we tested in a rat model 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. Spleen cells from the immunized rats are cytotoxic to both the parental (AT3B-1) and transfected cell lines. Inoculation of rats with AT3B-1^{PSA} cells immunize them against human PSA. The immune response could be detected as cytotoxicity against the transfected cell line or as antibodies against the native PSA detected by ELISA.

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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 HPSA-T or human prostate acidic phosphatase (HPAP-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.

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, stimulate cytotoxic T cell response in autologous T cells [1]. 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 T-PSMA H (tPSA)-DCs and S-PSA H (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 S-PSMA. In contrast, T-PSMA or T-PAP transfected DCs primed T cells towards several PSMA 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 have been incorporated in a manuscript which has been submitted for publication in Cancer Gene Therapy [2].

2. Titration of AT3B-1 and AT3B-1PSA for tumor cell development

Preliminary studies using the Copenhagen rat tumor prostate model showed uniform tumor development in rats that were injected subcutaneously with 100 000 AT3B-1 cells. We needed to define the tumorogenicity of the PSA-transfected cell line and compare it to the parental cell line. To do that, healthy, Copenhagen 2331 male retired rat breeders were injected sub-cutaneously in the right flank with different numbers of tumor cells from the AT3B-1 or the AT3-B1^{PSA} rat prostate carcinoma cell line (see below). For the AT3B-1 cell line, cells (passage 85, delivered 9/03) were purchased from the ATCC. Three rats per group were injected. Injections were performed using 25-ga needles. 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. Tumor size was evaluated every other day by measuring two perpendicular diameters by a caliper. Three days after tumor formation or five weeks after tumor inoculation, all rats were sacrificed, tumor when present were excised and measured. Necropsy was performed, tumor and sera was collected and liver and lungs were inspected macroscopically for metastases.

1. Titration of AT3B-1^{PSA} for tumor induction
   - $5x10^6$ – tumor development in 10-12 days
   - $1x10^6$ – tumor development in 15-19 days
   - $0.500x10^6$ – tumor development 27-29 days
   - $0.250x10^6$ – no tumors

- $0.1x10^6$ – no tumors

2. Titration of AT3B-1 cells for tumor induction
   - $1x10^6$ – tumor development day 10-12
   - $0.5x10^6$ – tumor development day10-12
   - $0.25x10^6$ – tumor development day 19-25
   - $0.1x10^6$ – tumor development day 23-25

Conclusion:

1. Injection of the parent cell line (AT-3B1) leads to uniform development of tumors and there is a relationship between number of cell injected and the time of tumor appearance.
2. Injection of 0.25 and 0.1 $x10^6$ of PSA-transfected AT3B-1 cells does not result in tumor development.
3. Injection of a larger dose of the transfected cells leads to tumors, but their development is delayed.

For future experiments all immunized rats will be challenged with $1x10^6$ $AT3B\text{-}1^{PSA}$ cells.

3. In vivo testing in a rat model the safety and efficacy of a combination of gene-based vaccines encoding human PSA, PAP and PSMA (months 10-30)

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

1. **Immunization cocktail A:** 50 ug H PSMA-T plasmid + 50 ug H PSA-T plasmid + 50 ug H PAP-T plasmid
2. **Immunization cocktail G:** 150 ug empty plasmid backbone
3. **Immunization cocktail H:** saline

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 104 animals were injected with the immunization cocktails (35 rats with cocktail A, 39 with cocktail G and 30 with cocktail H).

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. When re-immunizations were performed, mice were re-immunized at weekly intervals. Two weeks after the last

immunization, all mice either received a tumor inoculation or were sacrificed, blood and sera were collected and spleens harvested.

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. 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.

None of the 35 animals immunized with the immunization cocktail A (a mixture of HPSMA-T; HPAP-T and HPSA-T plasmids) developed tumors. In contrast, 37 of the 39 control rats immunized with the empty backbone and all 30 animals immunized with saline developed tumors 14-20 days after AT3B-1^{PSA} cell inoculation. None of the tumor bearing rats showed distant metastases.

There were no macroscopical or histological changes in the proximal kidney tubule, intestines, lungs, liver, prostate or brain tissues of the immunized rats.

Spleen cells from the immunized animals were cytotoxic against the parent (AT3B-1) and the transfected cell line. The spleens were processed individually. A single cell suspension was obtained cutting and filtering the organ through a sterile cell strainer (70 μm; Becton Dickinson, Franklin Lakes, NJ). Thirty million splenocytes were resuspended in 10 ml RPMI 1640 containing 10% heat inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium) and cultured in a T25 flask with 3 x 106 of mitomycin-c-treated of AT3B-1PSA cells in 10 ml culture medium. After 4 days, blasts were isolated on a density gradient, cultured for an additional day in medium supplemented with 20 IU/ml human rIL-2, and tested for cytolytic activity in a 4-hour 3H thymidine labeled DNA fragmentation assay [3] using the respective targets.

Interestingly, spleen cells from 18 of the 39 animals who were immunized with the empty backbone, and from19 of the 30 animals who received saline, were cytotoxic against the transfected but not against the parent cell line. We interpret this as evidence that animals become immunized against PSMA after inoculation with the AT3B-1^{PSA}

cells. Immunization against PSA could also explain the reduced tumorogenicity of the PSA-transfected cell line (see 2. of the present report) but additional experiments are necessary to clarify this hypothesis.

The presence of anti-PSMA or anti-PSA antibodies in serum pre- and post-vaccination was analyzed by Western blot and ELISA. 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 PSMA monoclonal antibodies Y-PSMA1 and Y-PSMA2 were obtained from Yes Biotech Laboratories Ltd (Mississauga, Ontario, Canada). 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 and electro-transfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 min. The membranes were probed with poly- or monoclonal anti-PSMA Abs (see below) for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce) in accordance with the manufacturer's recommendations. Polyvinyl chloride 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were incubated overnight at 4°C with a purified preparation of PSA (Vitro Diagnostics, Littleton, CO), as well as BSA or human serum albumin at 100 ng/well in 50 µl of PBS (pH 7.2). The wells were blocked for 1 h with PBS containing 1% BSA (assay buffer). Tested serum and control pooled rat serum were diluted in assay buffer and added to wells in triplicate in a volume of 50 µl/well. Purified human antimurine PSA-specific IgG antibody (Fitzgerald Industries, Concord, MA) was used as a positive control for PSA binding. Purified murine IgG was used as a negative control. After incubation overnight at room temperature, the wells were washed four times with assay buffer, and 50 µl of a 1:4000 dilution of peroxidase-conjugated goat antihuman IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added to each well. A 1:2000 dilution of peroxidase-conjugated goat antimurine IgG (Kirkegaard & Perry) was used for the PSA antibody control. After incubation at 37°C for 1 h, wells were washed four times with assay buffer, and 100 µl each of the chromogen O-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and hydrogen peroxide was added to each well. After a 10-min incubation in the dark, the reaction was stopped with 25 µl of 4N NH2SO4. The absorbance of each well was measured at 490 nm using an ELISA microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Sera from 17 of the 39 rats animals immunized with cocktail A (HPSMA-T; HPAP-T; HPSA-T) contained very low titer of antibodies against the target antigens that could be detected on Western against a lysate of COS-1 cells transfected with the respected target antigen. No antibodies against recombinant PSMA or PAP could be detected on ELISA. We interpret this as evidence that no antibodies against the native molecule of the target antigen develop when immunization is performed with the "truncated" (HPSMA-T and HPAP-T) vectors whose products are not secreted but proteasomally degraded. In contrast, however, all rats developed antibodies against the native PSA (detectable both on Western blots and ELISA). A possible explanation for

this is that the rats become immunized and develop antibodies against the native PSA only after they are inoculated with the transfected tumor cells that secrete human PSA (AT3B-1^{PSA}). Additional support for this hypothesis came from the fact that 22 of the 39 animals immunized with the empty backbone, and 14 of the 30 who received saline, but were all inoculated with AT3B-1^{PSA}, had antibodies against the native PSA. To confirm this observation, the following experiment was performed. Five rats received 3 immunizations with HPSA-T and were then sacrificed and their sera tested against PSA by Western blot and ELISA. None of the rats developed antibodies against native PSA detected on ELISA. Two of them had low titer of antibodies when tested against a lysate of COS-1 cells on Western blot.

Conclusion:

1. Immunization with the mixture of the three plasmids (cocktail A) is safe and well tolerated.
2. Three immunizations with this cocktail provide excellent protection against tumor development induced by inoculation of $1x10^6$ AT3B-1^{PSA} cells.
3. Spleen cells from the immunized rats are cytotoxic to both the parental and transfected cell lines.
4. Spleen cells from some control rats that have been inoculated with AT3B-1^{PSA} cells are cytotoxic to the transfected but no to the parental cell line.
5. Inoculation of rats with AT3B-1^{PSA} cells immunizes them against human PSA. The immune response could be detected as cytotoxicity against the transfected cell line or as antibodies against the native PSA detected by ELISA. This could explain the reduced tumorogenicity of the transfected cell line.
6. Some of the animals immunized with the "truncated" vectors whose products are retained in the cytosol and degraded in the proteasome, contain antibodies to the non-glycosylated forms of the antigen (protein core), but not against the natively folded glycoprotein.

Currently, results from the above experiments are being processed and a manuscript is in preparation.

4. 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:

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

Experiments involving immunizations with the single target encoding vectors have just been initiated. They will involve a total of 54 rats divided in three groups, each comprising of 18 animals.

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 [1].
- Proved in an animal model the safety of naked DNA immunization with a mixture of three "truncated" plasmid vectors that encode for the human PSMA, PSA and PAP that are not glycosylated but are degraded in the proteasome of the cells that express them.
- Proved in rats the efficacy of immunization with the mixture of the gene-based vaccines to prevent AT3B-1^{PSA} induced tumor development.
- Showed that spleen cells derived from rats previously immunized with the mixture of the three "truncated" plasmid vectors are cytotoxic to both the parental (AT3B-1) and the transfected (AT3B-1^{PSA}) cell lines.
- Showed that AT3B-1 cells stably transfected with the human PSA (AT3B-1^{PSA}) have decreased tumorogenicity in rats when compared to the parental cell line.
- Showed that sub-cutaneous inoculation of control (non-immunized) rats with the AT3B-1^{PSA} immunizes them against human PSA. Immune responses can be detected by cytotoxicity against the AT3B-1^{PSA} cells or by ELISA with recombinant PSA.
- Showed that immunization with the "truncated" vectors whose products after expression are degraded in the proteasome lead to development of low titer antibodies against the protein core.

Reportable Outcomes:

1. Mincheff M, Zoubak S, Altankova I, Tchakarov S, Makogonenko Y, Botev C, Ignatova I, Dimitrov R, Madarzhieva K, Hammett M, Pomakov Y, Meryman H, Lissitchkov T. 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 Dec;10(12):907-17

2. Grant application Idea Development Award PC040214, "GENE-BASED VACCINATIONS FOR CANCER IMMUNOTHERAPY", U.S. Army Medical Research and Materiel Command DOD 2004 Prostate Cancer Research Program; M.Mincheff P.I., submitted 2/04

3. 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 (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 PSMA expression in the AT3B-1 cell line has been detected only at the mRNA but not protein level. All tumor protection studies will be performed with the AT3B-1^{PSA} cell line.
2. 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.
3. Early CD25+ cell depletion during priming in vitro enhances priming to sub-dominant epitopes.
4. Co-expression of GITR-L during priming may alleviate immunodominance.
5. Injection of the parent cell line (AT-3B1) leads to uniform development of tumors and there is a relationship between number of cell injected and the time of tumor appearance.
6. Injection of 0.25 and 0.1 $x10^6$ of PSA-transfected AT3B-1 cells does not result in tumor development.
7. Injection of a larger dose of the transfected cells leads to tumors, but their development is delayed.
8. Immunization with the mixture of the three plasmids (cocktail A) is safe and well tolerated.
9. Three immunizations with this cocktail provide excellent protection against tumor development induced by inoculation of $1x10^6$ AT3B-1^{PSA} cells.
10. Spleen cells from the immunized rats are cytotoxic to both the parental and transfected cell lines.
11. Spleen cells from some control rats that have been inoculated with AT3B-1^{PSA} cells are cytotoxic to the transfected but no to the parental cell line.
12. Inoculation of rats with AT3B-1^{PSA} cells immunizes them against human PSA. The immune response could be detected as cytotoxicity against the transfected cell line or as antibodies against the native PSA detected by ELISA. This could explain the reduced tumorogenicity of the transfected cell line.
13. Some of the animals immunized with the "truncated" vectors whose products are retained in the cytosol and degraded in the proteasome, contain antibodies to the non-glycosylated forms of the antigen (protein core), but not against the natively folded glycoprotein.

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 application 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**(12): p. 907-17.
2. Mincheff, M., et al., *Depletion of CD25+ cells from human T-cell enriched fraction eliminates immunodominance during priming and boosting with genetically modified dendritic cells.* Cancer Gene Ther, 2004: p. (submitted).
3. Matzinger, P., *The JAM test. A simple assay for DNA fragmentation and cell death.* J Immunol Methods, 1991. **145**(1-2): p. 185-92.

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Human dendritic cells genetically engineered to express cytosolically retained fragment of prostate-specific membrane antigen prime cytotoxic T-cell responses to multiple epitopes

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The ability of two plasmid DNA vaccines to stimulate lymphocytes from normal human donors and to generate antigen-specific responses is demonstrated. The first vaccine (truncated; tPSMA) encodes for only the extracellular domain of prostate-specific membrane antigen (PSMA). The product, expressed following transfection with this vector, is retained in the cytosol and degraded by the proteasomes. For the "secreted" (sPMSA) vaccine, a signal peptide sequence is added to the expression cassette and the expressed protein is glycosylated and directed to the secretory pathway. Monocyte-derived dendritic cells (DCs) are transiently transfected with either sPSMA or tPSMA plasmids. The DCs are then used to activate autologous lymphocytes in an *in vitro* model of DNA vaccination. Lymphocytes are boosted following priming with transfected DCs or with peptide-pulsed monocytes. Their reactivity is tested against tumor cells or peptide-pulsed T2 target cells. Both tPSMA DCs and sPSMA DCs generate antigen-specific cytotoxic T-cell responses. The immune response is restricted toward one of the four PSMA-derived epitopes when priming and boosting is performed with sPSMA. In contrast, tPSMA-transfected DCs prime T cells toward several PSMA-derived epitopes. Subsequent repeated boosting with transfected DCs, however, restricts the immune response to a single epitope due to immunodominance.

Cancer Gene Therapy (2003) **10,** 907–917. doi:10.1038/sj.cgt.7700647

Keywords: PSMA; gene-based vaccine; immunodominance; CTLA-4

Immunotherapy of prostate cancer could be a safe, noninvasive, relatively inexpensive procedure that can avoid bowel and bladder injury and impotence, which often result from surgical, cryosurgical or radiation therapy. Several groups have recently reported on the safety of DNA vaccines for immunizations against tumor antigens.[1–3] A possible target for prostate cancer immunotherapy is the prostate-specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II (GCPII).[4–8] PSMA expression is normally restricted to the prostate gland, brain tissue, jejunum and proximal kidney tubules.[9–11] Its expression is increased nearly 10-fold in prostate cancer cells and is also found in tumor but not normal neovasculature.[7,12]

The main effectors in antitumor immunity are $CD8^+$ cytotoxic T cells that recognize tumor-associated antigen-derived peptides in association with major histocompatibility complex (MHC) class I molecules.[13–15] 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.[16–18] 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.[16]

Numerous factors combine to establish an immunodominance hierarchy,[19] among which are the ineffective generation and transport of subdominant epitopes by antigen-presenting cells (APCs). Since proteasomal degradation is the main source of antigenic fragments destined for MHC presentation,[14] we speculated that purposeful cytosolic retention of newly synthesized

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tumor-associated antigens in genetically manipulated APCs would increase both the quantity and the diversity of such fragments. PSMA is a type II transmembrane glycoprotein which is comprised of 750 amino acids.[20,21] It lacks a signal peptide sequence, and we speculated that elimination of sequences for its transmembrane region might impede the translocation of the encoded product to the endoplasmic reticulum. Such product should not be N-glycosylated, should be retained in the cytosol and rapidly degraded in the proteasome. In theory, dendritic cells (DC) transfected with such "truncated" sequences may have an advantage of presenting "subdominant" antigenic determinants that otherwise may not be generated at sufficient density to prime antigen-specific cytotoxic T-cell responses. The following experiments were designed to test this hypothesis 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 Institutional Review Board (IRB) at George Washington University Medical Center in Washington, DC.

tPSMA and sPSMA plasmids construction

The cDNA encoding the extracellular portion (AA 44–750) of the human PSMA (XC-PSMA) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) after RT-PCR of total mRNA from the human prostate cancer cell line LNCaP (CRL 1740, ATCC). The forward primer introduced a *Not*I cloning site and a Kozak sequence with a Met codon (GCCACCATG) into the 5′-end of XC-PSMA. The tPSMA plasmid for the transfection experiments was obtained by *Not*I–*Xho*I subcloning of XC-PSMA into a pVAX1 mammalian expression vector (Invitrogen, Carlsbad, CA). A secretable variant of the tPSMA plasmid — the sPSMA plasmid — was obtained by fusion of the XC-PSMA with a murine Ig k-chain leader sequence. The insert from tPSMA plasmid was subcloned by *Bam*HI-*Xho*I into the mammalian expression vector pSecTag2A (Invitrogen, Carlsbad, CA), providing the murine Igk-chain leader sequence (clone 96). The single *Sfi*I cloning site of the vector was used to fuse the XC-PSMA in-frame with the leader. The 5′-portion of the XC-PSMA between start Met and a single *Hpa*I site was reamplified in order to introduce *Sfi*I site (Met codon was not included). The *Sfi*I–*Hpa*I fragment of clone 96 was replaced with the PCR product predigested with the same restriction endonucleases, and the *Nru*I–*Xho*I fragment from obtained construct was moved to the pVAX1 vector. The inserts in both constructs are under the regulation of a human cytomegalovirus (CMV) immediate-early promoter/enhancer and a bovine growth hormone polyadenylation signal. The plasmid DNA specifications include: endotoxin content below 0.1 EU per microgram of DNA; lack of detectable amounts of bacterial RNA, genomic DNA or ssDNA as determined by agarose-gel electrophoresis; less than 10 μg of protein per 1 mg of plasmid DNA as determined by a colorimetric assay (Bio-Rad, Hercules, CA).

COS-1 transfection

Expression of PSMA constructs was performed in Cos-1 cells (ATCC). Monolayers were transfected with FuGENE 6 transfection reagent (Roche) and assayed for PSMA production by Western blot. Cos-1 cells were seeded in six-well tissue culture plates (Nunc, Denmark) at 1.5×10^5 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 cells were transfected with 1.5 μg of plasmid DNA precondensed with 4.5 μl of FuGENE 6 reagent in serum-free DMEM for 30 minutes at room temperature. Cells were then grown for 72 hours in supplemented DMEM and then harvested.

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

Cells were harvested by gentle scraping after double wash with 2 ml of cold PBS and by adding 0.25 ml of cold lysis buffer (0.5 M NaCl, 1% Triton X-100, 0.2% Tween 20, 50 mM HEPES, pH 7.0) to each well. Lysates were transferred to Eppendorf tubes and homogenized by repeated pipetting on ice.

For detection of secreted PSMA, the serum containing DMEM was removed 48 hours after transfection, the cells were washed twice with 2 ml of PBS, then serum-free DMEM (2 ml per well) was added and the cells were incubated for additional 24 hours in the six-well plates. After collection of the medium, the cell debris was removed by centrifugation (35,000 *g*, 20 minutes) and supernatants were concentrated with Centricon-50 centrifuge filtering device (Millipore) and stored at −30°C until further use.

Deglycosylation of PSMA

The deglycosylation of PSMA proteins was carried out by treatment of 25 μg protein samples with 5 U of glycopeptidase F from Chryseobacterium Meningosepticum (Sigma) in 50 mM phosphate buffer (pH 7.5) containing 0.1% (w/v) SDS, 50 mM β-mercaptoethanol, 0.75% (v/v) Triton X-100 for 4 hours at 37°C.[22]

An extracellular portion of the human glutamate carboxypeptidase II (AA 44–750) in Drosophila Schneider's cells, purified to homogeneity,[23] was provided by Dr Jan Konvalinka, Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Prague, The Czech Republic.

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 minutes and loaded on 10% Bis-Tris gels. After electrophoresis and electrotransfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 minutes. The membranes were probed with poly- or monoclonal anti-PSMA Abs (see below) for 1 hour at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma) and visualized with WestPico Super Signal Chemo Luminescent Substrate (Pierce) in accordance with the manufacturer's recommendations.

Anti-human PSMA monoclonal antibodies Y-PSMA1 and Y-PSMA2 were obtained from Yes Biotech Laboratories Ltd (Mississauga, Ontario, Canada).

Cell culture and generation of monocytes-derived DCs

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 U/ml penicillin and 50 μg/ml streptomycin. The human T2 cell line is transporter associated with antigen processing (TAP) deficient, resulting in inefficient loading of leukocyte antigen class I molecules with endogenous peptides.[24] 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 2×10^7/ml and incubated for 2 hours in a humidified incubator at 37°C. The nonadherent T-cell-enriched fraction and part of the adherent cells were harvested and frozen for future use. For convenience, these cells are referred to as "T cells" throughout the text despite the fact that this nonadherent fraction contains B cells and NK cells also.

The rest of the adherent cells were differentiated into DCs by culture in serum-free AIM-V medium supplemented with IL-4 (PeproTech, Rocky Hill, NJ) and GM-CSF (Oncology Supplies Inc., Dothan, AL) for 6 days. The nonadherent cells were harvested and used for transfection. Transfection was performed using the Nucleofector™ device and the corresponding transfection kit (Amaxa GmbH, Cologne, Germany). The efficiency of Nucleofector™ transfection of human DCs was $32 \pm 8.8\%$ ($n = 5$), as estimated by control transfection with green fluorescence protein (GFP). Following transfection, DCs 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 1×10^5 cells/ml.

In vitro *genertion of CTL responses*

For T-cell stimulation, the T-cell-enriched fraction was thawed, washed, resuspended in AIM-V medium and added to the DC suspension, so that the final concentration of the T-cell-enriched fraction was 1×10^6 cells/ml. The cell suspension was then distributed into 24-well plates (1 ml/well) and cultured at 37°C. After 3 days, the medium was replaced with serum-free AIV-M medium supplemented with human IL-2 (20 U/ml) and human IL-7 (10 U/ml) (PeproTech, Rocky Hill, NJ). The 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 medium supplemented 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 rad) and resuspended in IL-2- and IL-7-containing medium.

For cytotoxicity assays, target T2 cells were pulsed for 6 hours with 0.05 mg/ml peptide and 1 μCi/well ^{3}H-thymidine (ICN Biomedical Inc., Irvine, CA).

After 20 days of culture, effector T cells were harvested without further separation for microcytotoxicity assays. The cells were analyzed by flow cytometry and $83 \pm 10\%$ of them were $CD3^+$ and ~45 or ~60% of them were $CD3^+CD8^+$ when primed with sPSMA DCs, or tPSMA DCs respectively (data not shown).

For CTLA-4 inhibition experiments, Fab-fragments, prepared (see below) from anti-CD152 (HB-12319, ATCC) monoclonal antibody, were added (0.02 mg/ml final concentration) to the T cells at the initiation of culture or 6, 10 or 18 hours after that. The cultures were incubated for three additional days and the cells were washed and resuspended in IL-2- and IL-7-containing AIV-M medium.

Fab-fragment preparation of anti-CD152 monoclonal antibodies

Purified IgG (2 mg/ml in PBS) was cleaved with papain (Sigma) at a ratio 1:25 (w/w) of papain to IgG in the presence of 0.01 mM L-cystein at 37°C for 5.5 hours. The reaction was stopped by the addition of *N*-ethylmaleimide to a final concentration of 30 mM. The Fab fragment was isolated by gel filtration with a Superdex 75 column (Pharmacia) followed by ion exchange column (ResourceQ, Pharmacia) chromatography. The buffer for the eluted protein was changed to 10 mM Tris-HCl (pH 7.4) by gel filtration, and the pooled protein was concentrated to 15 mg/ml with centricon-10 (Millipore). The protein was tested for purity at each step by SDS-PAGE under reduced and nonreduced conditions.

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 PSMA. Cytotoxicity was tested using the JAM test.[25] Briefly, target or control cells were grown overnight with ^{3}H-thymidine, then washed, resuspended in complete RPMI-1640 medium and used in 4-hour cytotoxicity test. The killing was detected as a fall in counts per minute in cell samples undergoing apoptosis due to DNA fragmentation. All of the E:T ratios were tested in triplicate. Spontaneous cytotoxicity was determined in medium alone without effector cells.

Unlabeled K562 cells (no MHC expression, sensitive to natural killer cell-mediated lysis) were included at 50 × 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), K562 and Malme 3M (HTB-64) cell lines were purchased from ATCC and were maintained according to ATCC instructions.

Flow cytometry analysis and antibodies used

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). For HLA-A2 typing, aliquots of peripheral blood mononuclear cells (PBMC) 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 1% BSA and were incubated with 10 μl of the antibodies for 20 minutes on ice. Flow cytometry analysis was performed on a FACS-Calibur (Becton Dickinson).

Cytokines and ^{3}H-thymidine

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

Statistics and epitope binding predictions

Analysis of cytotoxicity data was 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") which was developed by Parker et al,[26] ranking potential MHC binders according to the predictive one-half-time disassociation of peptide/MHC complexes.

Peptide synthesis and purification

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

Results

Selection of HLA-A2-binding PSMA peptides

The amino-acid sequence of the extracellular domain of PSMA was analyzed for the existence of 9-amino-acid peptides predicted to bind to HLA-A0201, the most common human MHC class I allele. Using the computer-based algorithm (http://bimas.cit.nih.gov/), four 9-mer peptides that contain binding motifs for the HLA-A0201 class I molecule were identified (Table 1).

COS-1 cells transfected with tPSMA or sPSMA plasmids express the encoded product

Following transfection with sPSMA plasmid, the encoded product is N-glycosylated (Fig 1) and could be detected intra- and extracellularly (i.e. is secreted) (data not shown). The product expressed following transfection with the tPSMA plasmid is not glycosylated, but is retained in the cytosol and rapidly degraded in the proteasome. It could be detected following proteasomal inhibition with lactacystin (Fig 2).

Table 1 HLA-A0201-restricted PSMA-derived peptides

Rank	Position at PSMA molecule[a]	Peptide sequence	Score[b]
1	663	MMNDQLMFL	1360
2	711	ALFDIESKV	1055
3	668	LMFLERAFI	261
4	707	GIYDALFDI	251

[a]Positions determined using the numbering from Israeli et al.[4]
[b]Score corresponds to the estimated half-time of dissociation of complexes containing the peptide at 37°C at pH 6.5.

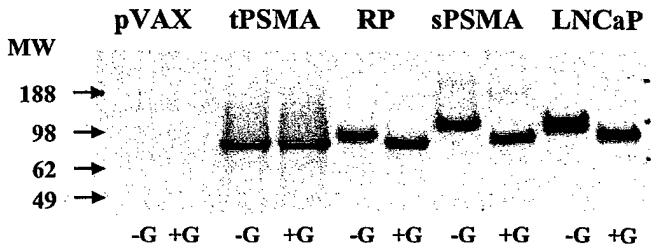

Figure 1 Proteins that lack sequences for translocation to the ER are not N-glycosylated. COS-1 cells were transfected with empty (pVAX), tPSMA or sPSMA plasmids, grown for 72 hours and the cell lysates were analyzed on Western blot with (+G) or without (–G) prior treatment with glycopeptidase F. Unlike the product obtained following transfection with tPSMA, the product obtained following transfection with sPSMA is N-glycosylated. Bands from recombinant PSMA or from cell lysates from LNCaP cells are shown for comparison. Legend: pVAX, tPSMA and sPSMA — lysates from COS-1 cells transfected with the respective plasmids; RP— recombinant PSMA expressed in insect cells; LNCaP — lysate from LNCaP cells.

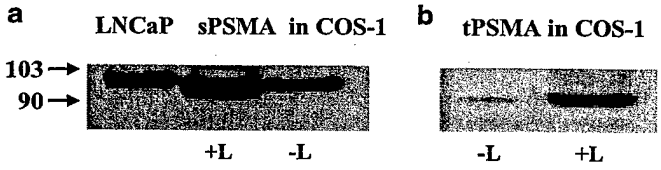

Figure 2 Proteins that are purposefully retained in the cytosol are degraded in the proteasome. COS-1 cells were transfected with the tPSMA plasmids and the cells were grown for 72 hours, the last 24 hours in the presence of lactacystin (10 μM). Unlike the product expressed following transfection with sPSMA (**a**), most of the product expressed following transfection with tPSMA (**b**) is degraded in the proteasome but could be detected following proteasomal inhibition. Legend: LNCaP—LNCaP cell lysate; +/− L—presence (+) or absence (−) of the proteasomal inhibitor lactacystin.

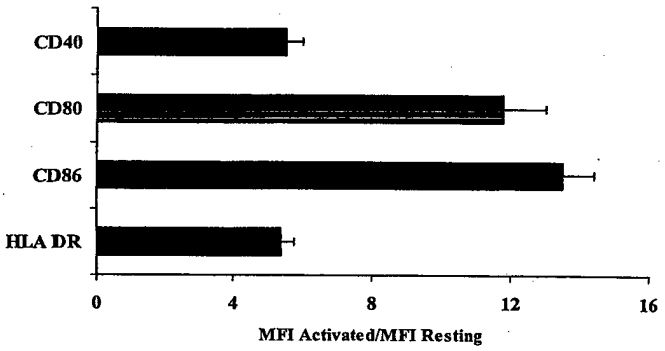

Figure 3 Change in cell surface antigen expression in PSMA-transfected DCs following maturation with TNF-α. Since surface antigens show a heterogeneous baseline expression, the upregulation of those molecules is presented as a ratio of mean fluorescent intensities (MFI activated/MFI resting). Data from five experiments with different donors are shown.

Generation of mature DCs from peripheral blood monocytes

Immature DCs were generated from PBMC after 6 days of culture in GM-CSF- and IL-4-conditioned medium. DC appeared as nonadherent cells with the typical DC morphology and high expression of CD1a (80–90%). Treatment of tPSMA- and sPSMA-transfected DCs with TNF-α triggers a coordinate series of phenotypic changes, resulting in an upregulation of costimulatory molecules (CD80, CD86, CD40) and HLA class II antigens (Fig 3).

Both tPSMA DCs and sPSMA 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 PSMA-transfected DCs. Their cytotoxicity was then tested against LNCaP cells or control Malme M3 melanoma cells. Both tPSMA DCs and sPSMA DCs primed and supported development of T cells that are cytotoxic against LNCaP cells (Fig 4).

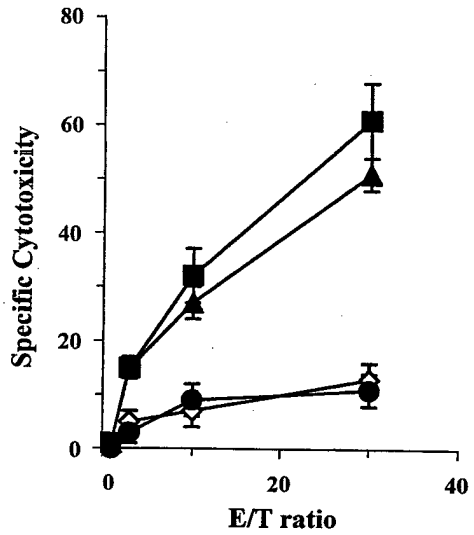

Figure 4 Generation of CTL by tPSMA DCs (squares) or sPSMA DCs (triangles). Nonadherent (T-cell-enriched) HLA A2(+) PBMCs were primed and then boosted twice at 8-day intervals with autologous monocyte-derived DCs that were transiently transfected with either tPSMA or sPSMA plasmid. The medium was changed, 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).

tPSMA DCs, but not sPSMA DCs, prime T cells that are reactive to subdominant PSMA epitopes

Since proteasomal degradation is the main source of antigenic fragments destined for MHC class I presentation,[14] and since purposeful cytosolic retention of non-glycosylated and misfolded newly synthesized tumor-associated antigens enhances such degradation (Fig 2), we reasoned that tPSMA-transfected DCs will have a greater potential to prime T cells specific for subdominant PSMA epitopes. To check this hypothesis, we primed the T-cell-enriched fraction from each leukapheresis with mature tPSMA DCs or sPSMA DCs and boosted them twice, at 8-day intervals, with autologous monocytes pulsed with one of several PSMA-derived peptides (Table 1). At 20 days after priming, cytotoxicity was tested against T2 cells pulsed with the same peptide used for boosting. Unlike the DCs transfected with the sPSMA plasmid, the tPSMA DCs prime T cells to all four PSMA-derived peptides in four of the five donors (Fig 5).

CTLA-4 inhibition during sPSMA DC priming leads to stimulation of T cells reactive to subdominant epitopes

Lack of responses to the subdominant PSMA epitopes when DCs are transfected with sPSMA may result from complete lack of such epitopes on the membrane of the

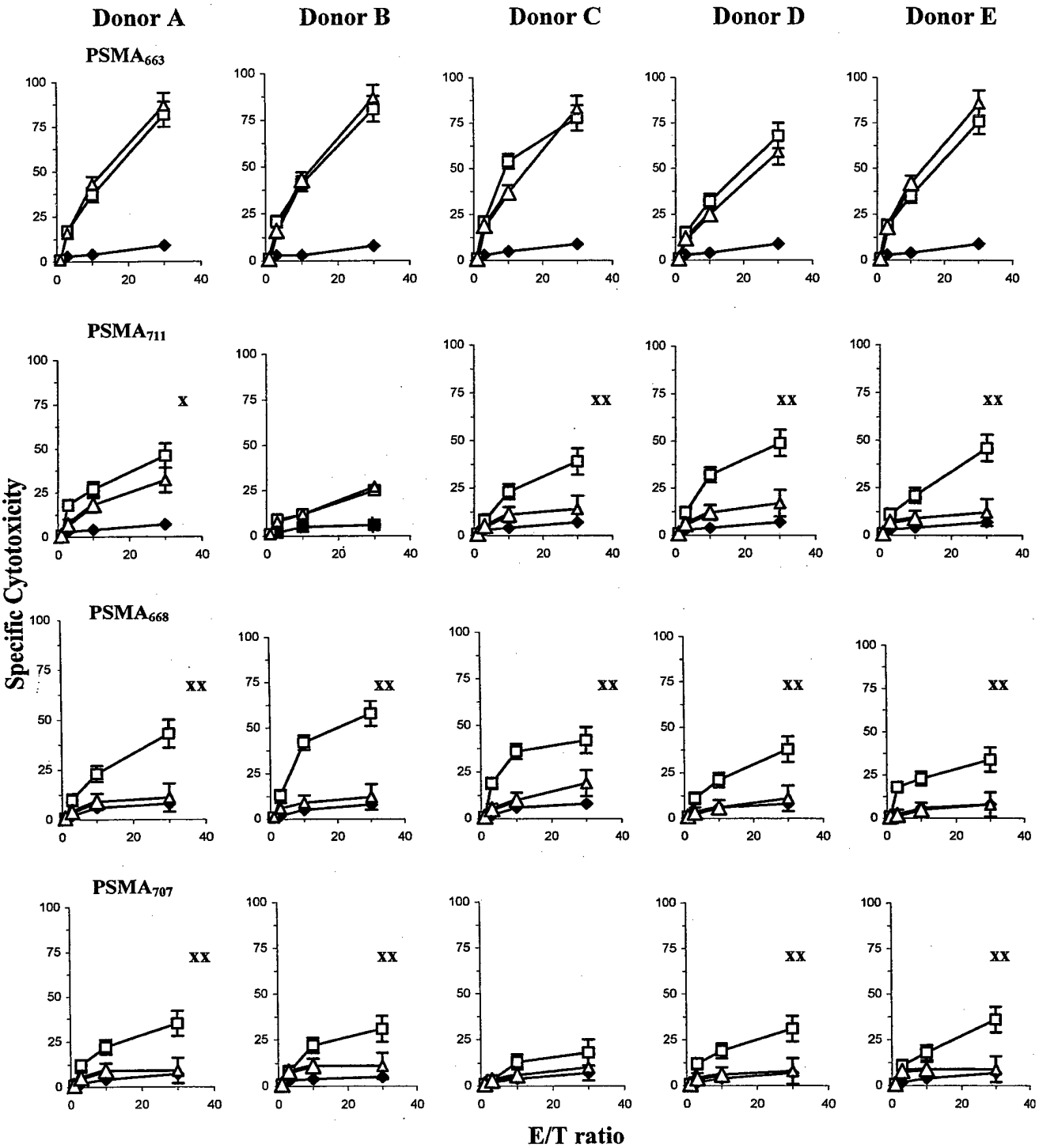

Figure 5 Induction of CTL responses with autologous DCs that have been transfected with the extracellular domain of PSMA. PBMCs that had been depleted of monocytes were primed with autologous DCs transfected with either sPSMA (triangles), tPSMA (squares) or control (mock, empty) plasmids (diamonds). Responding cultures were then boosted with monocytes pulsed with one of four HLA-A2-restricted PSMA-derived peptides (Table 1) and their cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during boosting, or with a control (influenza derived) peptide. Data points for the control peptide are not shown but are identical to those obtained with empty plasmid-transfected DCs. Each point represents the mean and SD from three different experiments. Both tPSMA DCs- and sPSMA DCs-primed T cells are cytotoxic against T2 cells pulsed with $PSMA_{663}$ peptide. Values for cytotoxicity of either tPSMA DCs- or sPSMA DC-primed T cells against T2 targets pulsed with $PSMA_{711}$, $PSMA_{668}$ or $PSMA_{707}$ peptides were compared. Significant differences at the 30:1 E/T ratio are indicated with xx ($P<0.01$) or with x ($P<0.05$). Levels of cytotoxicity for sPSMA DC-primed T cells against T2 cells pulsed with either $PSMA_{711}$ (donors C, D and E), $PSMA_{668}$ (donors A, B, C, D and E) or $PSMA_{707}$ (donors A, B, C, D and E) are identical to controls (T cells primed with empty plasmid-transfected DCs).

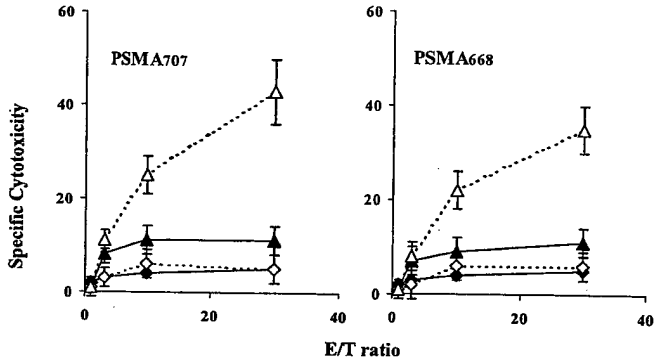

Figure 6 CTLA-4 inhibition during sPSMA DC priming enhances stimulation to subdominant epitopes. HLA A2(+) PBMCs that had been depleted of monocytes were primed with autologous DCs transfected with either sPSMA plasmid (triangles) or control empty plasmid (diamonds) in the presence (open symbols) or absence (closed symbols) of blocking anti-CD152 Fab fragments. The Fab fragments were added at the initiation of the culture (final concentration 0.02 mg/ml). After 3 days, and then two more times at weekly intervals, the responding cultures were boosted with monocytes pulsed with one of two HLA-A2-restricted, PSMA-derived peptides ($PSMA_{707}$ or $PSMA_{668}$). Cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during boosting, or with a control (influenza) peptide. Data points for the control peptide are not shown but are identical to those obtained in the control experiments with T cells primed with empty plasmid-transfected DCs. Each point represents the mean and SD of triplicate experiments. One representative from six independent experiments is shown. T cells primed in the presence of anti-CTLA-4 antibody are cytotoxic against specific targets at the 10:1 and 30:1 E/T ratio ($P<0.01$).

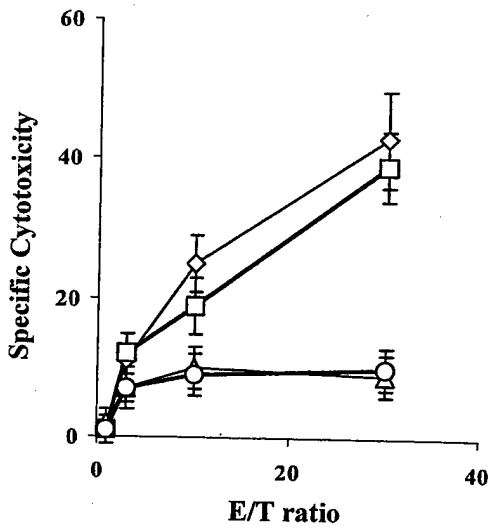

Figure 7 Early but not late CTLA-4 inhibition during sPSMADCs priming enhances T-cell stimulation to subdominant epitopes. HLA A2 (+) PBMCs depleted of monocytes were primed in the presence of blocking anti-CD152 Fab fragments with autologous DCs that had been transfected with sPSMA plasmid. The Fab fragments (0.02 mg/ml) were added either at the beginning (diamonds), or 6 (squares), 10 (triangles) or 18 (circles) hours after the initiation of culture. Lymphocytes were then cultured for 3 days, then washed, resuspended in IL-2 and IL-7 AIM-V medium and boosted twice at 1-week intervals with autologous $PSMA_{707}$-pulsed monocytes. Cytotoxicity was tested against T2 cells pulsed with the same peptide. Each point represents the mean and SD of triplicate experiments. Representative data from three independent experiments with cells from one donor are shown.

APCs. Alternatively, the epitopes may be generated, but factors other than T-cell receptor (TCR) signaling, such as the CTLA-4/B7 pathway, could be contributing to the ineffective proliferation of T cell to subdominant epitopes.

Currently, there is a general consensus that effective activation of naive T cells requires two signals: one dependent on the engagement of the TCR by peptide–MHC complexes, and the second costimulatory signal that is provided by interactions between cell surface molecules on the T cell and the APC.[27] Numerous studies have indicated that the CD28 molecule, expressed on T cells, provides a potent costimulatory signal following engagement with its ligands, B7-1 and B7-2.[28] The costimulatory function of CD28 is counterbalanced by the existence of a second higher-affinity receptor for B7, termed CTLA-4.[29] The latter inhibits T-cell activation at instances of weak T-cell receptor engagement,[30] and we speculated that we could enhance T-cell stimulation to existing subdominant epitopes if we inhibited CTLA-4 signaling with anti-CTLA-4 Fab fragments. Indeed, CTLA-4 inhibition during sPSMA DC priming led to stimulation of T cells reactive to subdominant epitopes. We interpret this as evidence that subdominant epitopes are generated in DC transfected with the sPSMA plasmid but the response can be detected only after CTLA-4 inhibition (Fig 6).

Since CTLA-4 was not expressed in resting T cells, we determined the time points during the 72-hour priming phase that the inhibitory control by CTLA-4 became apparent. The anti-CD152 antibody Fab fragments (20 μg/ml) were added either at the beginning, or at 6, 10 or 18 hours after the start of the culture. CTLA-4 inhibition was effective when performed early (0 or 6 hours) after initiation of culture (Fig 7).

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

Maturation of potent CTL effectors requires repetitive boosting with target antigen. To determine the effect of a prime/boost vaccination strategy on the clonality of the T-cell response, tPSMA DC-primed cultures, known to contain CTLs to subdominant epitopes (Fig 5), were boosted with transfected or peptide-pulsed DCs or monocytes, and their cytotoxicity was tested against PSMA-peptide-pulsed T2 target cells. Boosting with either APCs that express multiple PSMA-derived epitopes (transfected DCs or DCs or monocytes pulsed with multiple peptides) restricts the immune response toward one immunodominant epitope (Table 2). A subdominant T-cell response could only be preserved if boosting is performed with an APC pulsed with the particular subdominant epitope.

Table 2 Boosting with DCs and monocytes that express multiple antigenic epitopes restricts the response to an immunodominant epitope

Prime/boost strategy[a]	*Number of patients developing CTL activity against PSMA peptide-pulsed T2 cells*			
	$PSMA_{663}$	$PSMA_{711}$	$PSMA_{668}$	$PSMA_{707}$
Prime: tPSMA DCs 2 boosts tPSMA DCs	5 of 5	1 of 5	0 of 5	0 of 5
Prime: tPSMA DCs 2 boosts sPSMA DCs	5 of 5	1 of 5	0 of 5	0 of 5
Prime: tPSMA DCs 2 boosts 1P DCs	5 of 5	4 of 5	5 of 5	4 of 5
Prime: tPSMA DCs 2 boosts 4P MCs	5 of 5	0 of 5	0 of 5	0 of 5
Prime: tPSMA DCs 2 boosts 1P MCs	5 of 5	4 of 5	5 of 5	4 of 5

[a]1P—pulsed with single peptide, 4P—pulsed with all four peptides, MCs—monocytes.

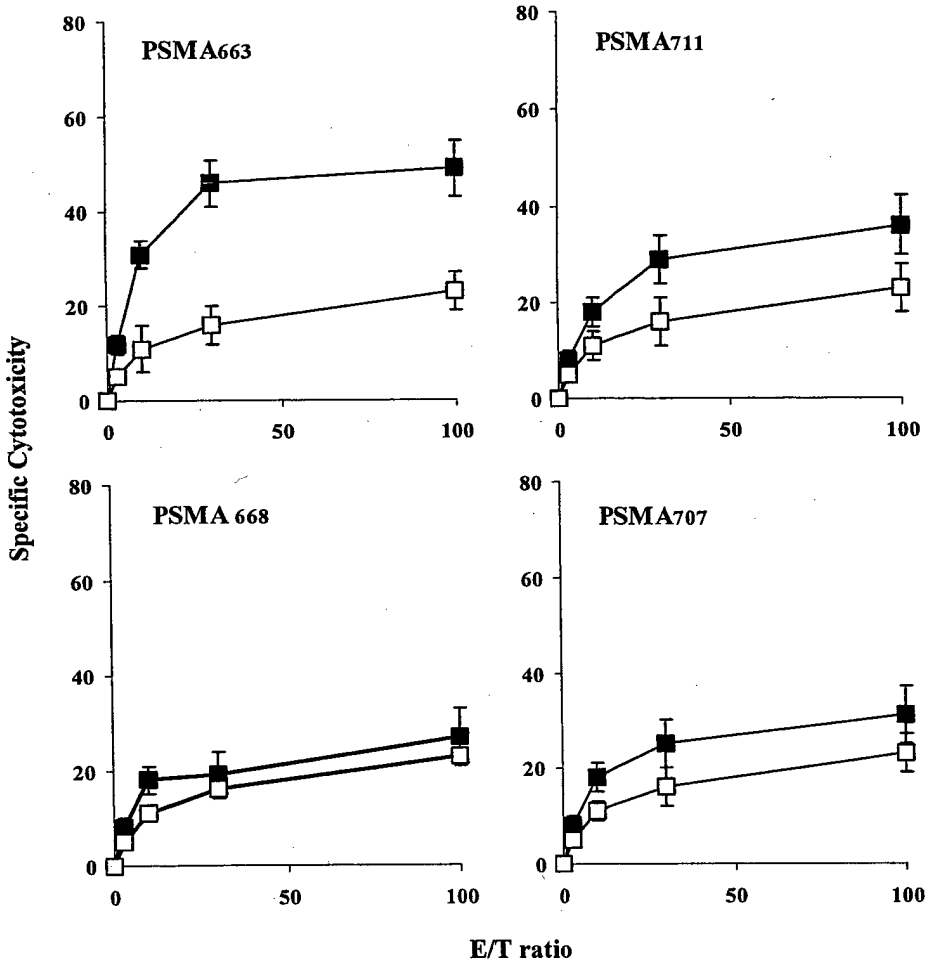

Figure 8 Antitumor reactivity of PSMA peptide-specific CTLs. Peptide-reactive CTL lines shown in Figure 5 were tested for their ability to recognize and kill prostate tumor cells. LNCaP cells (filled symbols) or Malme-3M melanoma cells (empty symbols) were used as targets. Each point represents the mean and SD of triplicate experiments. Significant differences ($P<0.01$) in cytotoxicity against specific and control targets exhibit T-cell lines reactive to $PSMA_{663}$ and $PSMA_{711}$. One representative result from five tested donors is shown.

Antitumor reactivity of PSMA-specific CTLs

The ability of the PSMA-reactive T-cell clones to recognize tumor cells that express PSMA was tested in an *in vitro* cytotoxicity assay. In all five donors, only two of the peptide-specific CTL lines ($PSMA_{663}$ and $PSMA_{711}$) were cytotoxic against nonmodified LNCaP cells (Fig 8).

Discussion

Despite being effective in breaking tolerance to a self-antigen or tumor-associated antigen, antitumor vaccination in its current mode of application leads to a narrow response, directed to few of the potential epitopes. Immunodominance is a natural control mechanism that

ensures the tight specificity of the immune reaction and prevents untoward autoimmunity.[19] Immunodominance, however, 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.[16,17] We have explored possibilities to extend the target epitope specificity of the immune response and this study was designed to test the ability of DCs transfected with different plasmid constructs to prime and boost cytotoxic T-cell responses.

A critical requirement for T cell activation is the engagement of TCRs with MHC molecules presenting antigenic peptides on the surface of an APC.[31] Efficient priming of multispecific CTL responses is limited by the poor immunogenicity of subdominant MHC class I-binding epitopes.[19] T cells binding with high affinity to the antigen compete with low-affinity T cells and inhibit their interaction with the same APC by downmodulation of peptide–MHC complexes on the APC.[32] Inhibition of responses to subdominant epitopes, therefore, could be partially overcome by simply increasing the amount of antigen present on the APCs.[33–35] We reasoned that, since proteasomal degradation is the main source of antigenic fragments destined for MHC class I presentation,[14] purposeful cytosolic retention of newly synthesized tumor-associated antigens in genetically manipulated APCs may enhance their proteasomal degradation and thus increase the quantity of such fragments. The transport from the cytosol into the endoplasmic reticulum (ER) is an important step in the biogenesis of many proteins, including secretory proteins and proteins of the plasma membrane. It is triggered by a signal sequence, which is normally located at the amino terminus of those polypeptides. For many membrane proteins, the signal sequence is identical with the first membrane anchor (signal-anchor sequence).[36] Since PSMA is a type II, bitopic integral membrane protein, we speculated that elimination of the sequence that encodes for its transmembrane domain will lead to the expression of a product that will not be translocated to the ER, but will be degraded by the proteasomes, presumably into peptides that will access the class I presentation pathway. Indeed, the product expressed following transfection of COS-1 cells with such "truncated" construct is not glycosylated, but is retained in the cytosol and rapidly degraded by the proteasomes. This leads to generation of PSMA peptides that are expressed via the class I MHC presentation pathway at density sufficient to prime CTLs to all four PSMA epitopes tested.

On the other hand, DCs transfected with DNA encoding for a product that is translocated to the ER, then glycosylated and secreted, do not prime against subdominant epitopes (Fig 5). Although all four PSMA epitopes are generated, T cells reactive to the subdominant ones could only be primed if CTLA-4 signaling is inhibited (Fig 6). Since high-affinity receptor T cells downmodulate peptide–MHC complexes from the APC membrane,[37] increased production of peptides after tPSMA transfection favors subdominant CTL priming. Stimulation of subdominant MHC–peptide complexes that are expressed at a lower density on the APC membrane following sPSMA transfection, however, requires CTLA-4 inhibition. A homologue of CD28, CTLA-4, also binds to the B-7 family members[38,39] but inhibits T-cell activation.[40] Mice lacking CTLA-4 reveal a striking phenotype of polyclonal T-cell activation and tissue infiltration, which results in death by 3–4 weeks of age, indicating a powerful regulatory role for CTLA-4.[41,42]

Our finding that priming to subdominant responses is enhanced by CTLA-4 inhibition may seem contradictory to the model proposed by others.[43] In that model, CTLA-4 signaling and not inhibition allows for a greater diversity in the T-cell response. Our results, however, support the idea originally proposed by Manzotti et al.[30] It proposes several operational mechanisms, but their clarification requires additional experimentation:

First, the intensity of TCR stimulation seems to be important with weak signals being easily inhibited.[30] In other words, weak signals being overwhelmed by inhibition through CTLA-4 will have no chance to prime CTL responses.

Second, CTLA-4 may act as a nonsignaling "decoy" receptor reducing the available ligand for CD28 costimulation.[44,45]

There is also a third possibility.[46,47] A small number of CTLA4+ T cells may exert a suppressive or regulatory effect on other T cells after stimulation through the CTLA-4 receptor. Such cells appear to be similar if not identical to the T regulatory cells.[48–50]

Experiments are currently in progress to validate any of these hypotheses.

Priming with tPSMA DCs leads to polyclonal CTL stimulation, but boosting with APCs that express both dominant and subdominant epitopes narrows the immune response to the dominant ones. Research from other groups has gained similar results.[51–55] In all these instances, boosting with polyepitope constructs has resulted in failure to expand polyepitope CTLs. A likely explanation is that competition between T cells for antigen on individual APC leads to obscuring of responses to subdominant epitopes when both the dominant and subdominant epitopes are present on the same APC.[55,56] New vaccines or new strategies employing modulation of costimulatory networks during boosting may need to be developed if the polyepitope response against a target is to be maintained.

Finally, not all PSMA-peptide-specific CTL clones exhibited tumor cell killing (Fig 8). A likely explanation for the lack of cytotoxicity is the downregulation of MHC. Unlike other tumor cells, LNCaP cells express low levels of MHC class I molecules.[57] However, neither the level of class I MHC expression, nor the percentage of killing, was influenced by pretreatment of the target cells with γ-IFN (data not shown). Alternatively, these subdominant epitopes may not be generated at sufficient quantity by LNCaP cells and further experimentation is necessary to test this possibility.

In conclusion, we have shown that:

(1) DCs transfected with plasmid DNA can successfully prime CTL responses *in vitro*.
(2) DCs transfected with a construct whose product is retained in the cytosol and degraded in the proteasome (tPSMA) prime to both dominant and subdominant epitopes. In contrast, sPSMA DCs prime to dominant epitopes only.
(3) CTLA-4 inhibition during priming *in vitro* enhances priming to subdominant epitopes generated following transfection of DCs with sPSMA.
(4) *In vitro* boosting with APCs that express both dominant and subdominant epitopes narrows the immune response to the dominant ones.

If confirmed in animal studies, these results will pose important questions on the design of vaccines and methods for reimmunization if a polyepitope response is to be maintained.

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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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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 s VacDCs, 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 (hPAP-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 *hPAP-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 (*hPSA-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 hPSA-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, hPSA-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.

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 ^3H 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).

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

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:

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).

Generation of mature DCs from peripheral blood monocytes

Immature DCs were generated from PBMC after 6 days of culture in GM-CSF- and IL-4-conditioned medium. DC appeared as non-adherent cells with the typical DC morphology and high expression of CD1a (80-90%). 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].

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 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 [28], 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 [29]. Since signaling through GITR has been found to downregulate the function of T regulatory cells and enhance the development of autoimmunity, [22, 23, 30, 31] 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, 32]

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,[33] 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 [34, 35]or creating opportunities for weak signals coming from sub-dominant epitopes otherwise prompt to inhibition by CTLA-4. [36]

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* [28], a possibility existed [37, 38] 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.[39-41] In murine models, suppression of auto-reactive T cells has been attributed to a population of spontaneously occurring CD4+CD25+ T cells.[42] Cells with similar phenotype and function have been found in healthy humans.[43-46] 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. [47, 48] One hypothesis is that depleting these CD4+CD25+ regulatory T cells in humans enhances a polyclonal T cell response.[21, 49]

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. Our current experimentation system, however, 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.[31] 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, 30, 31] On the other hand, GITR-GITR-L interaction could also provide a costimulatory signal for the antigen-driven proliferation of naive T cells.[50, 51] 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 and experimentation *in vivo* on animal models is worth pursuing.

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

TITLES 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 - circles)
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 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 activated (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).

A

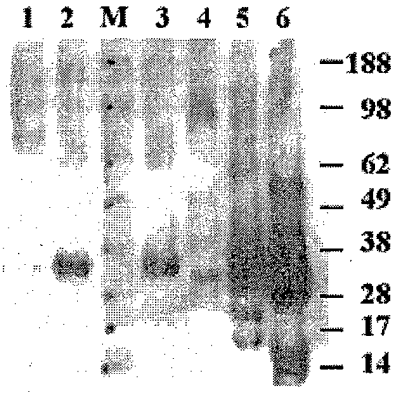

Fig. 1A

B

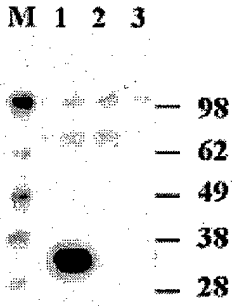

Fig. 1B

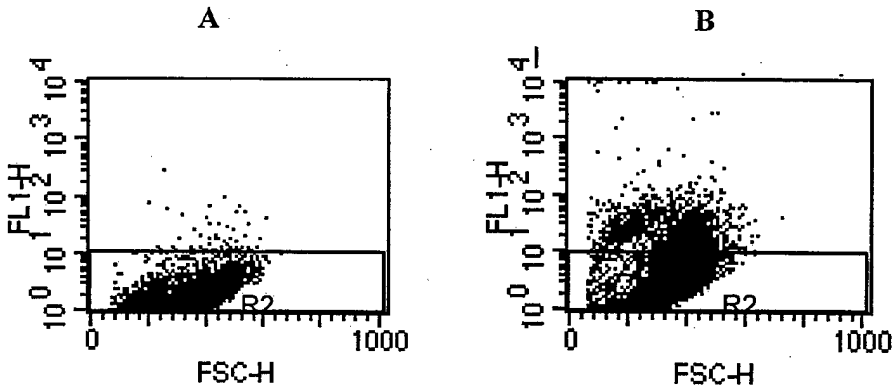

Fig. 2

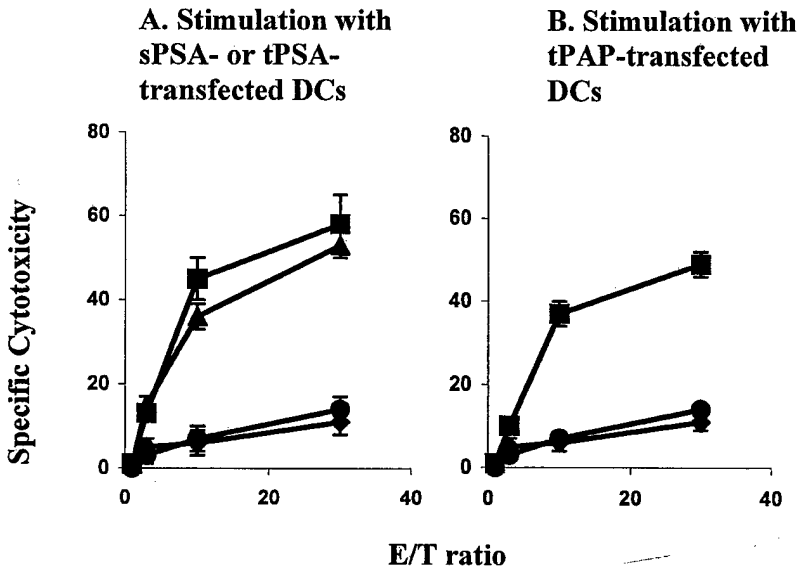
A. Stimulation with sPSA- or tPSA-transfected DCs
B. Stimulation with tPAP-transfected DCs
Specific Cytotoxicity
80
60
40
20
0
0
20
40
E/T ratio

Fig. 3A

Fig. 3B

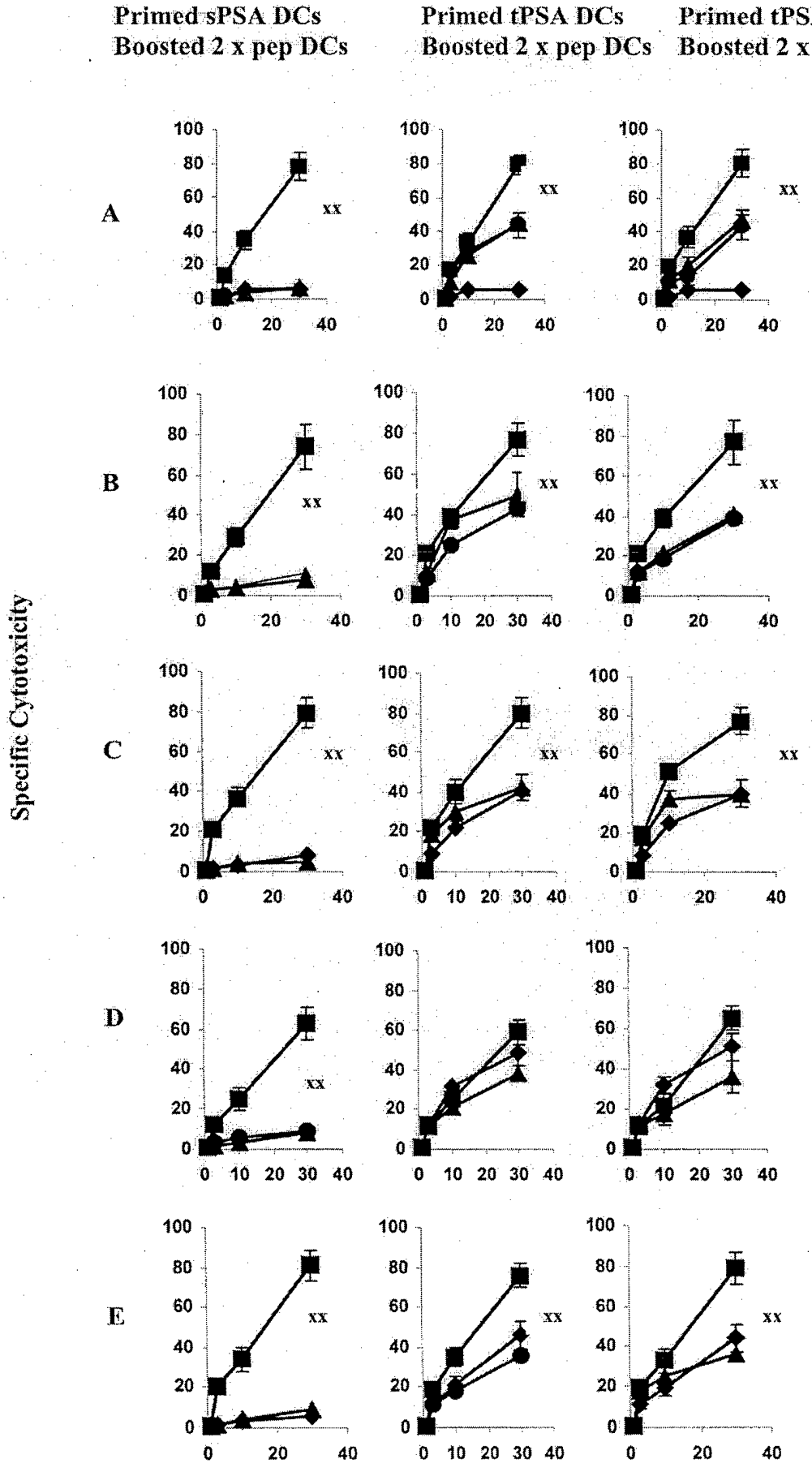
Primed sPSA DCs
Boosted 2 x pep DCs
Primed tPSA DCs
Boosted 2 x pep DCs
Primed tPSA DCs
Boosted 2 x pep MCs
A
B
C
D
E
xx
Specific Cytotoxicity
E/T ratio

Fig. 4

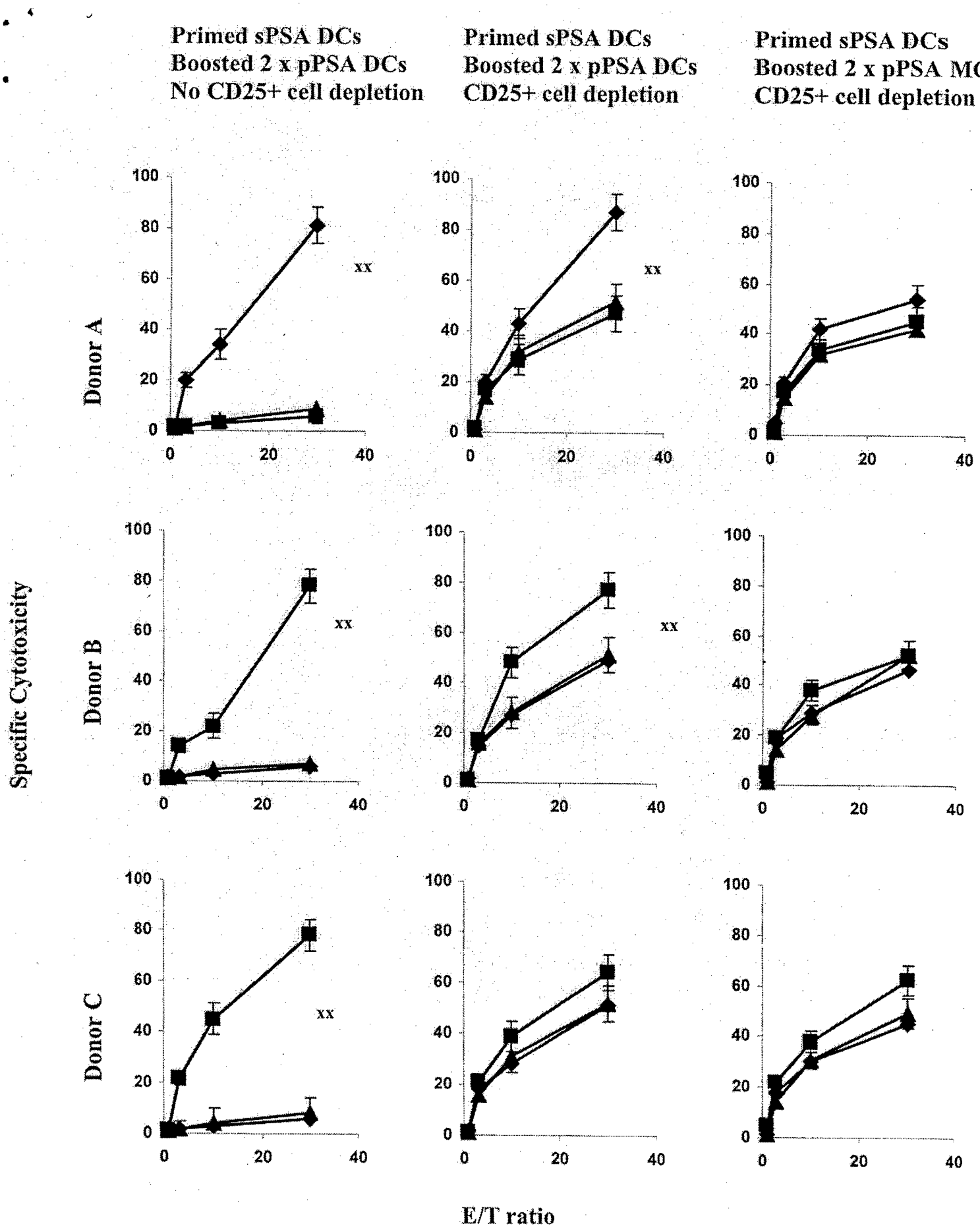
Primed sPSA DCs
Boosted 2 x pPSA DCs
No CD25+ cell depletion
Primed sPSA DCs
Boosted 2 x pPSA DCs
CD25+ cell depletion
Primed sPSA DCs
Boosted 2 x pPSA MCs
CD25+ cell depletion
Donor A
Donor B
Donor C
Specific Cytotoxicity
E/T ratio
xx
0
20
40
60
80
100

Fig. 5

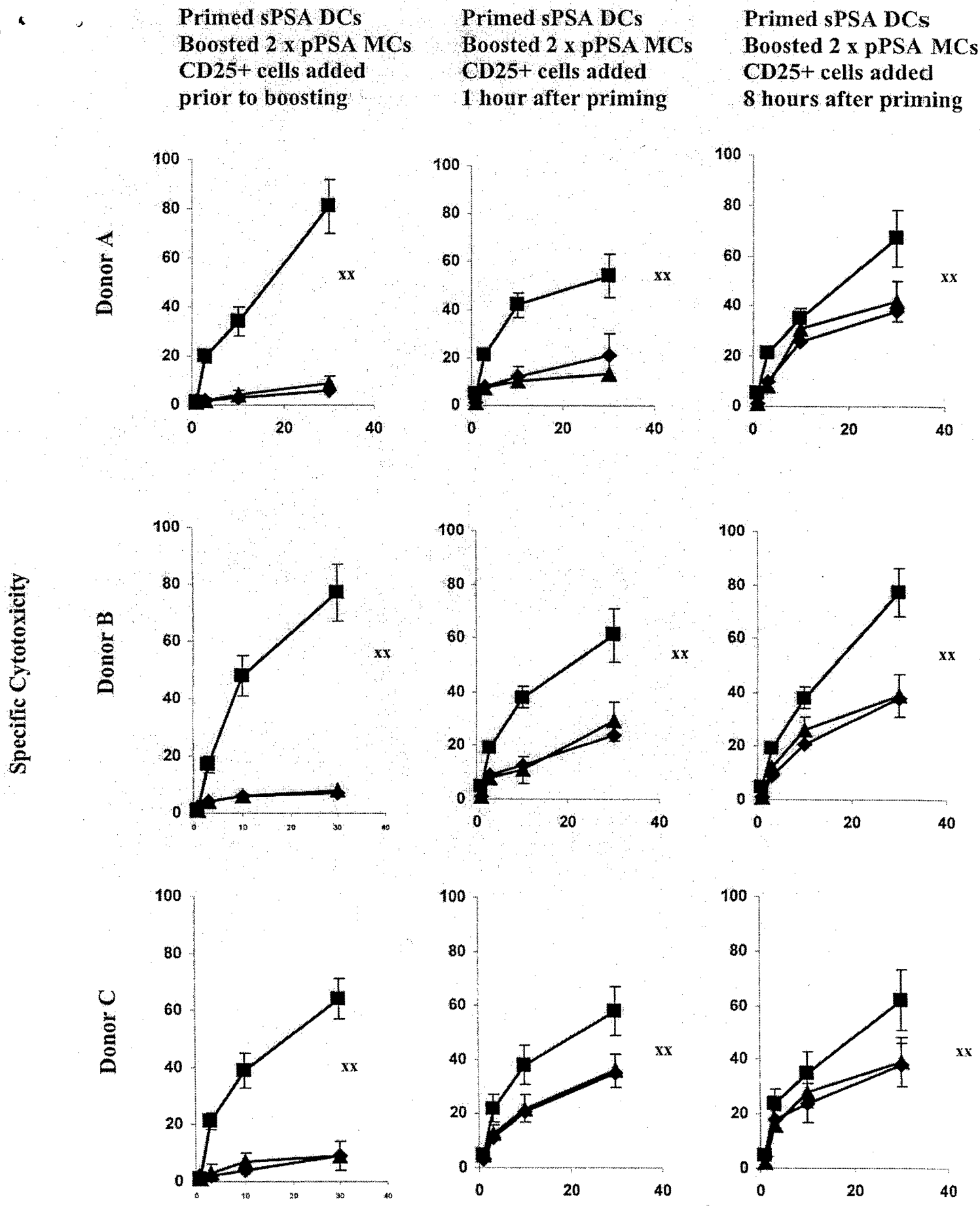
Primed sPSA DCs
Boosted 2 x pPSA MCs
CD25+ cells added
prior to boosting
Primed sPSA DCs
Boosted 2 x pPSA MCs
CD25+ cells added
1 hour after priming
Primed sPSA DCs
Boosted 2 x pPSA MCs
CD25+ cells added
8 hours after priming
Donor A
Donor B
Donor C
Specific Cytotoxicity
E/T ratio
xx

Fig. 6

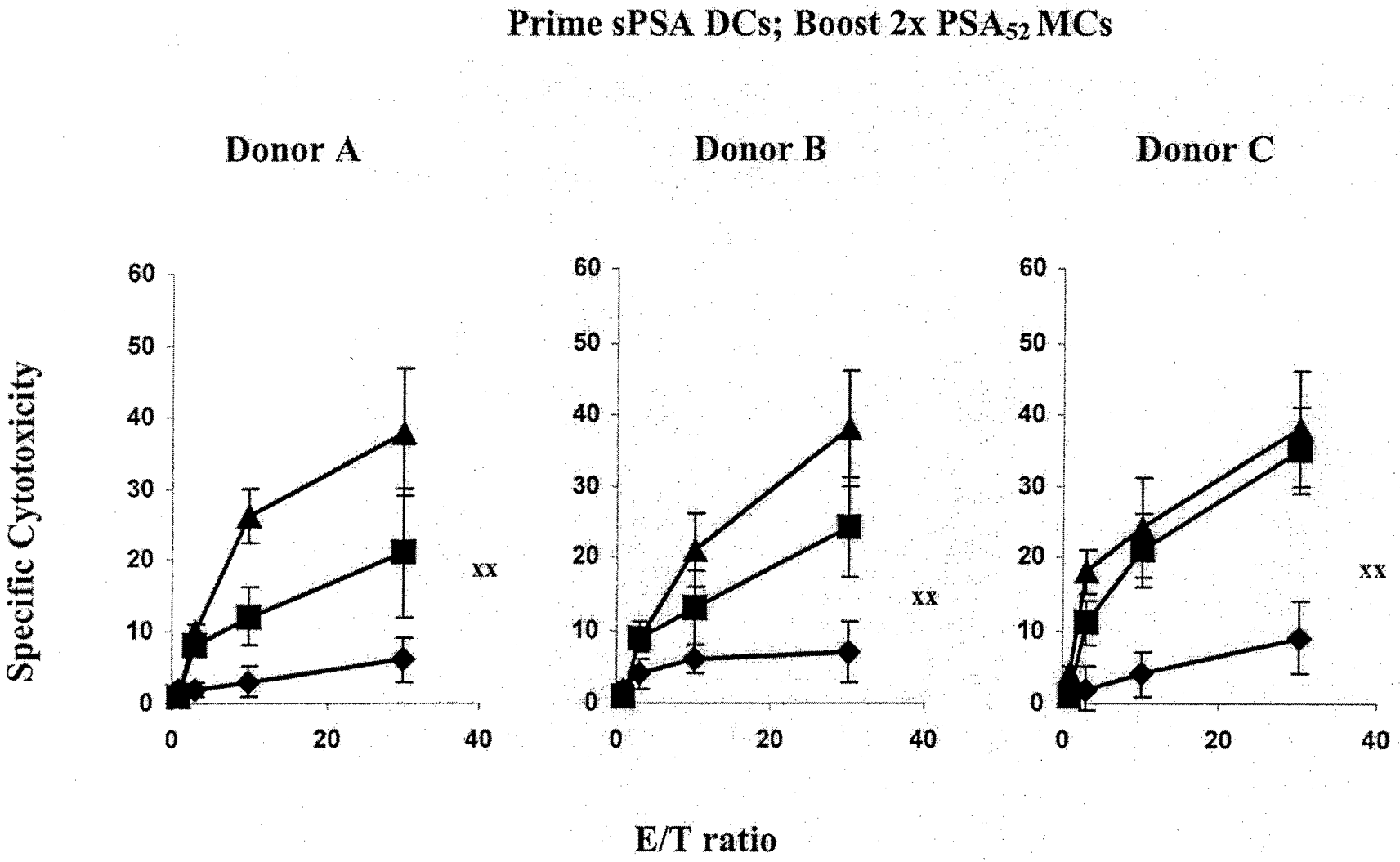
Prime sPSA DCs; Boost 2x PSA52 MCs
Donor A
Donor B
Donor C
Specific Cytotoxicity
60
50
40
30
20
10
0
0
20
40
xx
E/T ratio

Fig. 7

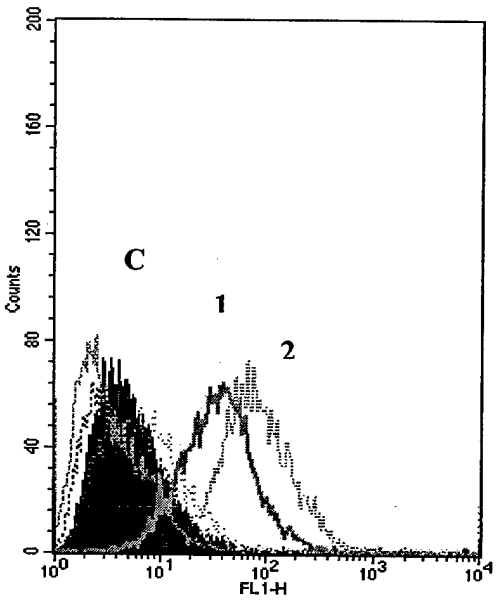

Fig. 8

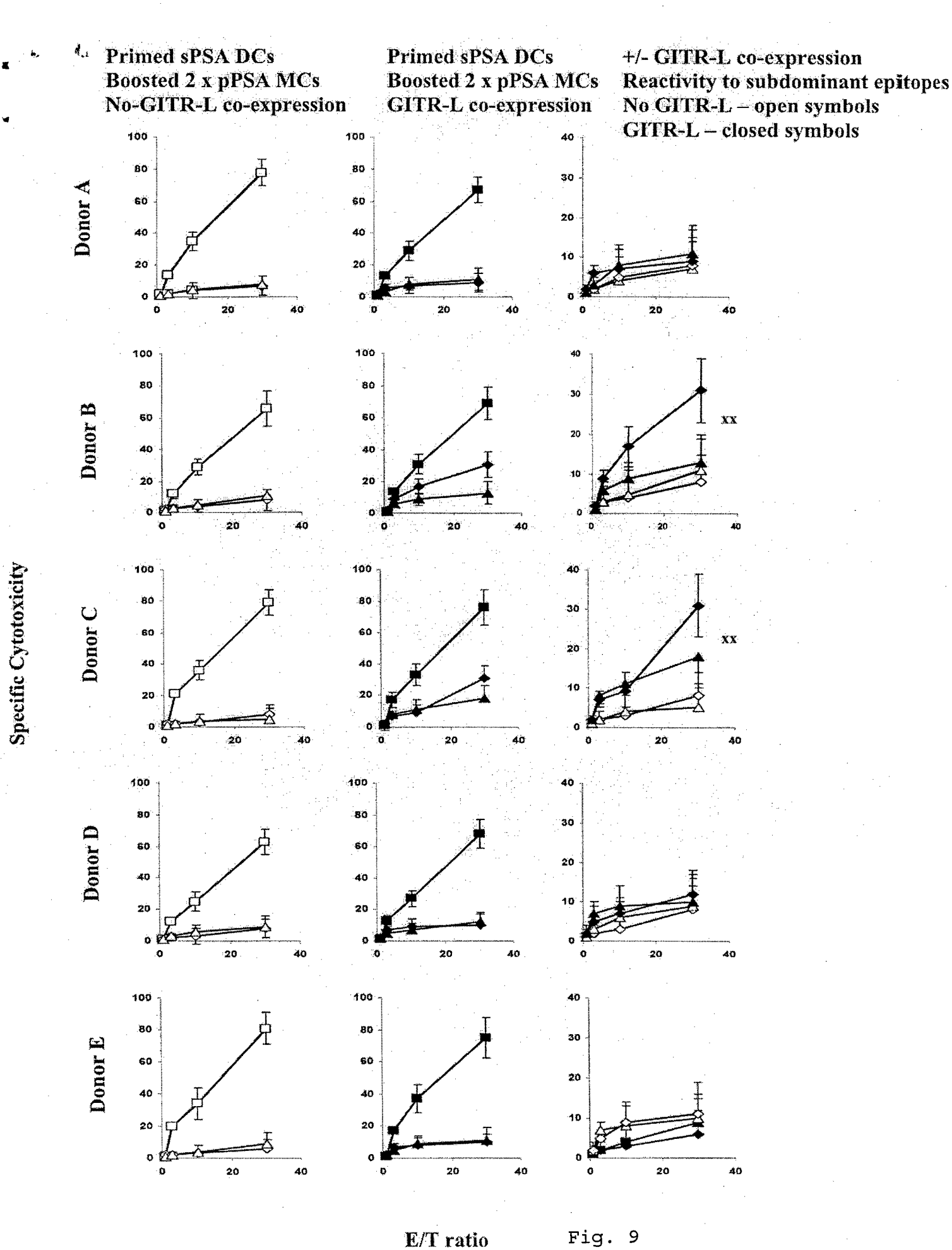
Primed sPSA DCs
Boosted 2 x pPSA MCs
No-GITR-L co-expression
Primed sPSA DCs
Boosted 2 x pPSA MCs
GITR-L co-expression
+/- GITR-L co-expression
Reactivity to subdominant epitopes
No GITR-L – open symbols
GITR-L – closed symbols
Donor A
Donor B
Donor C
Donor D
Donor E
XX
XX
Specific Cytotoxicity
E/T ratio

Fig. 9