ORIGINAL ARTICLE

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

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

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Introduction

Several groups have recently reported on the safety and biologic effectiveness of DNA vaccines for immunization against pathogen or tumor antigens.^{1–4} The use of PSA-recombinant vaccinia virus has also been found to be safe and lead to an immune response.^{5,6} The main effectors in antitumor immunity after DNA immunization are CD8⁺ cytotoxic T cells that recognize tumor or tumor-associated antigen-derived peptides expressed in association with MHC class I molecules.⁷ A rate limiting step in loading the latter with peptides seems to be the antigen

supply.⁸ We have found that newly synthesized proteins that are purposefully retained in the cytosol are targeted for proteasomal degradation.⁹ Transfection with truncated DNA constructs (tVacs) that lack sequences encoding the leader peptide of a secreted protein, or the membrane domain of type II protein, leads to expression of such products. In our immunotherapeutic studies, we have targeted the prostate-specific membrane antigen (PSMA; gamma carboxyl peptidase II, GCP_{II}). PSMA expression is normally restricted to the prostate gland, brain tissue, jejunum and proximal kidney tubules.^{10–12} Its expression is increased nearly 10-fold in prostate cancer cells and is also found in tumor but not normal neovasculature.^{13,14} Elevated PSMA serum levels have been observed in healthy males and females, as well as in patients with benign prostate hypertrophy, prostate and breast cancer.^{15–17} It is a type II integral membrane protein^{18,19} whose main portion is extracellular. We have cloned the cDNA encoding the extracellular portion (AA 44–750) of the human PSMA (XC-PSMA) and included it in a mammalian expression vector (hPSMA_t). Dendritic cells, transfected with hPSMA_t, stimulate *in vitro* autologous T-cell proliferation and cytotoxicity against the target antigen.⁹ We speculate that, similarly to results from *in vitro* experiments, *in vivo* immunization with this vector will result in enhanced cytotoxicity and protection from tumor development.^{9,20}

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

Materials and methods

Rats and immunizations and tumor cell inoculation

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

For immunizations, 100 µg plasmid DNA with or without rat recombinant GM-CSF (R&D Systems, Minneapolis, MN), were brought to 100 µl with sterile saline for injection. The immunizations were performed at 10-day intervals at the right rear flank using insulin syringes. All injections were intradermal, taking caution that a blister was formed and the infusate was retained at the epidermal–dermal junction.

For tumor cell inoculation, 10⁵ of AT3B-1^{PSMA} cells (the YM2 clone, see ‘AT3B-1 transfection with hPSMAs’) were resuspended in 100 µl sterile saline and injected subcutaneously in the opposite rear flank.

Plasmid DNA vaccines

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

For the R (rat) ‘PSMA’-K plasmid (rPSMA_t), cDNA encoding for a rat analogue of the human extracellular

PSMA was cloned from rat brain tissue and included in a pVAX1 backbone. The cDNA had 85.66% homology with the human PSMA at the nucleotide level. The plasmid size was 5.1 kb. Products expressed from hPSMA_t or rPSMA_t plasmids were retained in the cytosol and degraded in the proteasome.⁹

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

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

AT3B-1 transfection with hPSMAs

Monolayers of AT3B-1 cells (CRL-2375; ATCC, Manassas, VA) were transfected with the hPSMAs using the FuGENE6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) and assayed for PSMA production by Western blot. The cells were seeded in 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 fetal bovine serum (FBS). COS-1 or AT3B-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 min at room temperature. Cells were then grown for 72 h in complete DMEM and then harvested.

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

Electrophoresis and immunoblotting

For electrophoresis and Western blotting, cells were harvested by gentle scraping, washed twice with 2 ml of cold PBS and lysed with 0.25 ml cold lysing buffer (0.5 M NaCl, 1% triton X-100, 0.2% Tween 20, 50 mM HEPES, pH 7.0). Lysates were transferred to Eppendorf tubes and homogenized by repeated pipetting on ice. Immunoblotting and electrophoretic assays of cell lysates and culture supernatants were performed using NuPage BisTris electrophoretic system (Invitrogen, Carlsbad, CA). Protein samples in loading buffer were heated at 70°C

for 10 min and loaded on 10% BisTris gels. After electrophoresis and electrotransfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 min. The following primary antibodies were used: Y-PSMA1 and Y-PSMA-2 (Yes Biotech Laboratories Limited, Mississauga, Ontario, Canada), rabbit polyclonal anti-GCPII antibodies, anti-GCPII monoclonal antibodies A5, E1, E7, E12 and H2 (all developed in the Institute of Organic Chemistry and Biochemistry, Prague, the Czech Republic). The membranes were probed with poly- or monoclonal anti-PSMA Abs for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma, St Louis, MO) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce, Rockford, IL) in accordance with the manufacturer's recommendations.

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

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

Cell proliferation

Standard [^3H]thymidine incorporation assays were performed to assess spleen cell proliferative responses. Splenocytes from immunized rats were resuspended at 5×10^6 cells/ml in RPMI 1640 containing 10% FBS. In all, 100 μl of cell suspension were added to each well in 96-well plates with 20, 10, 5, or 0 $\mu\text{g}/\text{ml}$ of recombinant PSMA. After 4 days of culture, 1 μCi [^3H]thymidine (Amersham Biosciences, Piscataway, NJ) was added to each well. Following a 16 h incubation, cells were harvested on glass filter paper and radioactivity was measured in a Beckman LS 2501 liquid scintillation counter. The stimulation index was calculated as: counts per minute (cpm) with Ag stimulation/background cpm without Ag.

Cell cytotoxicity

Cell cytotoxicity was determined as previously described.²⁶ Briefly, target cells (AT3B-1 or YM2 cells) were grown overnight in complete medium supplemented with 1 μCi [^3H]thymidine (Amersham Biosciences, Piscataway, NJ), then washed and resuspended in complete medium. Cells in different target to effector cell ratio were plated in 96 round bottom well plates for 6 h at 37°C in a humidified incubator. Cells were then harvested on glass

filter paper, and radioactivity was measured in a liquid scintillation counter. The killing was detected as a fall in counts per minute due to DNA fragmentation in cell samples undergoing apoptosis. All of the *E:T* ratios were tested in triplicate. Cell cytotoxicity was normalized to control samples that contained target cells only and expressed as specific lysis. Spontaneous cytotoxicity was determined in medium alone without effector cells.

Tumor development

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

Antibody determination

For Western blot, SDS gel electrophoresis of cell lysates, cell supernatants or GCP II were performed as described above. After electrotransfer and blocking, the nitrocellulose membranes were stained with sera from immunized rats or anti-PSMA antibodies for an hour. Secondary staining with antibodies conjugated to horseradish peroxidase (Sigma, St Louis, MO; dilution 1:10 000; for 1 h) was performed after three washes with TBS, 0.05% Tween 20. Finally, the blots were developed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to BioMax MR film (Kodak, New Haven, CT).

In some cases, serum anti-PSMA antibody titers from immunized rats were measured by a direct ELISA. A total of 96-well plates coated overnight with 100 $\mu\text{l}/\text{well}$ of 10 $\mu\text{g}/\text{ml}$ recombinant GCPII in PBS were blocked for 2 h with PBS containing 2% BSA and 0.05% Tween 20. Sera were then added in serial dilutions and incubated for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h with 1/5000 dilution of a peroxidase-conjugated anti-rat kappa-lambda chain secondary antibody (Jackson Laboratories, Bar Harbor, ME). The plates were then washed three times, developed with tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD), stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader (Dynatech Laboratories, Alexandria, VA).

Statistical analyses

Significant differences in cytotoxicity of spleen cells or antibody titers were assessed by Student's *t*-test. The difference between groups was considered statistically significant when the *P*-value was lower than 0.05. A nonparametric test of statistical significance for bivariate tabular analysis (χ^2) was performed for data accumulated when tumor development was assayed.

Results

Transfection of AT3B-1 cells with hPSMAs

We find that both rat prostate tissue and AT3B-1 cell lysates express low level of PSMA when tested by PCR (data not shown). Since PSMA expression is upregulated in human prostate cancer cells, we decided to transfect the

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

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

Male Copenhagen rats were immunized with the plasmid vaccines alone (Figure 2a), or with a cocktail that, in addition to the plasmid vaccine, contained $9 \mu\text{g}/\text{m}^2$ recombinant rat GM-CSF (R&D, Minneapolis, MN)

Figure 1 Detection of PSMA in AT3B-1 cells transfected with hPSMAs. $\pm$ L, presence or absence of lactacystin; 1 and 2, AT3B-1 cell clone YM1, stably transfected with hPSMAs; 3 and 4, AT3B-1 cell clone YM2, stably transfected with hPSMAs; 5, supernatant from YM2; 6, supernatant from YM1; 7, recombinant GCPII.

(Figure 2b and c). In separate experiments, rats were primed with the hPSMA GM-CSF, but boosted with the rPSMA GM-CSF cocktail (Figure 2d). Addition of GM-CSF to the immunization cocktail increased the efficacy of the immunization (Figure 2b and c). Rats immunized with hPSMA showed stronger spleen cell cytotoxicity to YM2 compared to those immunized with rPSMA, but the best results were obtained when priming was performed with hPSMA, while boosting with rPSMA (Figure 2d).

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

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

Copenhagen rats, inoculated subcutaneously with 10^5 syngeneic AT3B-1^{PSMA} YM2 tumor cells, develop

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

Figure 2 Cytotoxicity of rat spleen cell towards the YM2 clone. Copenhagen rats were immunized three times at 10-day intervals, euthanized 14 days later and their spleen cells tested for cytotoxicity against the YM2 clone. (a) Rats were injected with either rPSMA (circles), empty vector (squares) or saline (diamonds). (b) Rats were injected with rPSMA with (filled in circles) or without rat recombinant GM-CSF. (c) Rats were injected with hPSMA with (filled in triangles) or without rat recombinant GM-CSF. (d) Rats were primed and boosted with cocktails containing rat GM-CSF and either hPSMA (triangles) or rPSMA (circles), or primed with hPSMA and boosted twice with rPSMA (squares).

Table 1 Gene-based vaccination-induced tumor protection

Injection with	Number of animals with tumor (total number of animals per group)	Mean size of tumor
(A) 3 × hPSMA _t	2 (10)	0.5 ± 0.1 cm ³
(B) 3 × rPSMA _t	5 (10)	1.2 ± 0.23 cm ³
(C) 3 × hPSMA _s	7 (10)	1.6 ± 0.48 cm ³
(D) 3 × empty vector	8 (10)	> 2.3 ± 0.3 cm ³
(E) 3 × saline	9 (10)	> 2.44 ± 0.48 cm ³

Differences in the number of tumor developing rats. Difference between groups A and B is not statistically significant ($\chi^2 = 1.978$; $P < 0.2$). Differences between groups A and E is statistically significant ($\chi^2 = 5.05$; $P < 0.025$). Differences between groups D and E (combined) and A or B are statistically significant ($\chi^2 = 3.8$; $P < 0.01$ for A and $\chi^2 = 4.17$; $P < 0.05$ for B).

detectable tumors by days 10–12 following injection. To explore the role of preimmunization on tumor development, rats were immunized three times at 10-day intervals with either saline, empty plasmid vector, hPSMA_t or rPSMA_t (Table 1). No rat GM-CSF was included in the immunization cocktail. At 14 days after the last immunization, all animals received 10^5 of YM2 tumor cells in the right rear flank. Tumors, when developing, were palpable at day 12 following tumor cell inoculations. All animals were killed 15 days following tumor inoculations and tumors, when present, excised and measured. Only two of the 10 animals immunized against human PSMA and five of the 10 animals immunized against the rat analogue developed tumors. In contrast, 17 of the 20 animals in the control groups developed tumors. The tumors, developing in the group, immunized with the human or the rat construct, were smaller than the ones in the control group (Table 1).

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

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

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

The protection of the immunized animals from development of tumor depends on the amount of cells inoculated during tumor challenge. Partial tumor protection could be seen when 2×10^5 or 3×10^5 tumor cells are injected. All

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

Injection with	Number of animals with tumor (total number of animals per group)	Mean size of tumor
(F) 3 × hPSMA _t +rGM-CSF	0 (10)	—
(G) 3 × rPSMA _t +rGM-CSF	0 (10)	—
(H) 3 × hPSMA _s +rGM-CSF	2 (10)	1.1 ± 0.1 cm ³
(I) 3 × Empty vector+rGM-CSF	9 (10)	> 1.89 ± 0.34 cm ³

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

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

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

Rats immunized with 'truncated' constructs (hPSMA_t or rPSMA_t) did not develop antibodies to PSMA as detected by ELISA (Figure 5) or Western blot (data not shown), no matter whether GM-CSF was present or not. No antibodies against PSMA (detection by Western blot or ELISA) developed following immunization with plasmid, encoding the secreted PSMA (hPSMA_s), or with GCP II, when GM-CSF was absent from the immunization cocktail (Figure 5). Immunization with hPSMA_s in the presence of GM-CSF led to formation of antibodies against the native protein (detected by ELISA; Figure 5).

The highest titer of antibodies against PSMA was observed when priming was performed with GCPII or tVacs, and boosting was performed with the recombinant protein and GM-CSF was added as an adjuvant (Figures 5 and 6). Antibodies, developing following immunization

with hPSMAs or GCPII, were of mixed Th2 and Th1 type, since both IgG1 and IgG2a subtypes were detected. On the contrary, priming with tVacs (hPSMA_t or rPSMA_t) and boosting with hPSMAs or GCP II led to formation of Th1, cytotoxic antibodies (IgG2a and IgG2b) with relatively high titer.

Figure 4 Tumor development in rats following different prime-boosting regimens. All rats were immunized with a cocktail containing 100 μ g plasmid DNA and 9 μ g/m² body surface area rat GM-CSF. Circles: rats were primed with hPSMA_t and boosted with rPSMA_t; triangles, primed with hPSMAs and boosted with rPSMA_t; diamonds, primed and boosted with rPSMA_t; squares, primed and boosted with empty vector.

Discussion

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

Figure 5 Immunization with the native protein leads to development of antibodies against its native conformation (data from ELISA, 1:30 serum dilution) (a) priming with hPSMA_t, boosting with rPSMA_t (with GM-CSF); (b) three immunizations with hPSMAs (no GM-CSF); (c) three immunizations with hPSMAs (with GM-CSF); (d) three immunizations with GCPII (with GM-CSF); (e) prime with GCPII, boost with hPSMA_t and rPSMA_t (with GM-CSF); (f) prime with GCPII, boost with rPSMA_t (with GM-CSF); (g) prime with hPSMA_t, boost with GCPII (with GM-CSF); (h) prime with rPSMA_t, boost with GCPII (with GM-CSF).

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

- (a) Effective generation of antigen-derived peptide in the transfected cell cytoplasm for MHC class I presentation.
- (b) Presence of antigenic determinants that would more-likely break existing tolerance to autologous antigens targeted for cancer immunotherapy.

For this reason we compared vectors that encode for:

1. (a) Proteins that do not undergo post-translational modification in the ER but are degraded in the proteasome,^{9,28} that is tVacs.
2. (b) Xenogeneic rather than autologous (syngeneic) proteins.^{21,22,24}

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

An important issue for effective vaccines is the development of potent adjuvants that can facilitate induction or augmentation of immunity. Despite the presence of nonmethylated CpG motifs in plasmid DNA vaccines,^{29–35} naked DNA immunization of rats obviously requires an additional adjuvant, and GM-CSF could act as such (Figure 2, Table 2). GM-CSF enhances immune responses by inducing the proliferation, maturation, and migration of dendritic cells.³⁶ Animals, immunized with a GM-CSF-plasmid DNA cocktail, are fully protected against transplantable tumors. This result is in agreement with earlier observations both in animals and in humans.^{4,37–39} The expansion and differentiation of both T and B lymphocytes is affected³⁶ since the adjuvant effect of GM-CSF influences both cytotoxic T-cell generation (Figure 2) and antibody development (Figure 5).

It has been shown that immunization with xenogeneic rather than autologous proteins is more likely to break existing tolerance²¹ and we, as have others,²⁴ speculated that immunizations with plasmid DNA that encodes xenogeneic proteins will have the same advantage. This clearly is the case as far as priming is concerned (Figure 4). Addition of GM-CSF to the immunization cocktail, however, leads to easier break of immune tolerance to PSMA since good protection against tumor development

is observed even in those animals that were immunized with rPSMA^t (Table 2), a construct encoding the autologous protein.

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

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

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

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

protein antigen results in production of cytotoxic antibodies.

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