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ORIGINAL ARTICLE

A novel murine model of allogeneic vaccination against prostate cancer

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Abstract Prostate cancer continues to be a major cause of death in men. Surgical and medical treatments of the disease have improved, but metastasic disease remains a significant clinical problem. Novel therapies such as whole cell vaccination offer the potential of treating disease by stimulating the immune system. To study the efficacy of a whole cell vaccine in prostate cancer two strains of mice were used: C57BL/6 (H-2Kb) and C3H/HeJ (H-2K^k) in combination with four different cell lines. Thus, a model was constructed of allogeneic and syngeneic vaccine, as well as a challenge tumour for each strain. Two novel cell lines were developed during this study. Firstly, the non tumourigeneic PMC-1 was derived from a normal mouse prostate and immortalized with HPV16. Secondly, the tumourigeneic PMC-1 C6ras1p1 was transformed with human *ras* gene which formed tumours in both SCID and C3H/HeJ mice. Protection, and the nature of the immune response to syngeneic and allogeneic vaccine, in males and females was examined in both strains. Vaccination with both syngeneic and allogeneic irradiated whole cell vaccines induced protection from syngeneic challenge in females. However, no protection was observed when allogeneic vaccine was given to male mice. This correlated with the immune response. Two types of cellular immune responses were generated in females. A NK-mediated response was observed in C57BL/6 mice, whilst C3H/HeJ mice developed a CTL response. Little or no cellular immune response was observed in males. The cytokine profile in C3H/HeJ females was a mixture of Th1 and Th2 whilst a mainly Th1 profile was observed in C57BL/6 mice. Male mice showed a diminished cytokine secretion compared to females which was further depressed after challenge. The difference in immunity was largely as expected, since tolerance to prostate antigens should not normally develop in female mice. However, this makes this model particularly relevant clinically since it directly mimics the human situation and thus may accelerate the development of whole cell vaccines for clinical use.

Keywords Vaccine · Prostate · Mouse · CTL · NK

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Introduction

Prostate cancer is the second most commonly diagnosed malignancy in men in the UK and remains the second leading cause of cancer deaths among this population after lung cancer [25]. Early and localized disease is usually treated by radical prostatectomy and radiation therapy. However, until recently, no treatment has been shown to prolong survival once the hormone-refractory phase has been reached with a typical survival of 2–3 years from the time of diagnosis of metastatic disease [16]. Docetaxel

based treatments have recently been reported to increased survival by 2 months but only 35% had reduction of pain and 22% improvement in quality of life [39]. However, there is a clear need for non toxic treatments.

Whole cell vaccines have been tested in many types of cancer, including melanoma [21] and renal carcinoma [18]. Commonly, an autologous approach is used [3, 17], in which cells derived from the patient's own tumour are reinjected after in vitro expansion and irradiation to prevent replication.

However, an alternative approach is to employ allogeneic whole cell vaccination, which is an attractive methodology due to the difficulty in obtaining sufficient amounts of autologous tumour cells. This is especially true for prostate cancer, because tumours are slow growing both in vivo and ex vivo [27]. Several studies show that tumours from the same origin share common antigens [9, 12, 19, 30]. Hence, after antigen processing, antigens shared between vaccine and tumour may induce protection in vivo [40], regardless of HLA tissue-type.

Studies show that allogeneic vaccines can elicit protection and induce cellular immune responses involving CD8+ T cells in animal models [23, 24]. Cross-priming [6] has been suggested as a possible mechanism in which exogenous shared antigens are processed and then presented to the host immune system via the endogenous MHC I pathway. Hence, allogeneic tumour cells can be processed at the injection site by antigen presenting cells (APCs) and then presented via MHC class I to CD8+ T cells thus inducing a CTL response [5].

Whole cell vaccines have been evaluated in clinical trials for prostate cancer [20], however only a few studies have been done in rodents, due to paucity of animal models. The difficulty of studying prostate cancer in animals is that prostate tumours are rare in most species except in dogs, rats and in man [10]. The only species that develops spontaneous prostate cancer, similar to humans, is the dog. In the last 10 years, several models of murine prostate cancer have been developed such as the mouse prostate reconstitution (MPR) and transgenic adenocarcinoma mouse prostate (TRAMP) models, however these rely on highly immunogenic viral oncogenes to induce tumourigenicity.

The objective of this study was to develop a model of allogeneic whole cell vaccination for prostate cancer in mice which would allow high throughput and rapid optimization of vaccination protocols. The difficulty in obtaining prostate cancer specimens in humans, as well as in animals, led us to use both normal tissue and cancerous tissue to produce whole cell vaccines in an analogous fashion to human clinical samples. A new whole cell vaccine was developed and characterized, from a normal mouse prostate tissue with an HPV-based immortalization (PMC-1). This cell line was used as an allogeneic cell vaccine to a syngeneic challenge using the well-defined tumourigenic prostate line RM-9 [4].

This model has allowed us to gain unique insight into the mechanism by which allogeneic whole cell vaccines mediate protection in the prostate, with particular reference to the cellular immune response. Furthermore, it appears to manifest the features of immunological tolerance that are required for a valid model of human disease. In order to examine the generality of these models, a second model of mouse prostate cancer was developed in a second strain of mice. A new tumourigenic cell line was made from PMC-1 using mutated human *ras* transformation. Studies have suggested roles of both the activated *ras* oncogene and the human papillomavirus in prostate carcinogenesis [2, 33]. Previous reports have shown that increased expression of the *ras* p21 protein is associated with increasing histological grade in human prostatic cancer [37]. *Ras* mutations have been reported in human prostate adenocarcinoma but not in normal or benign prostate samples [37]. Furthermore, Rhim et al. [29] have shown that human prostate epithelium can be transformed from an immortalised cell into a tumourigenic cell by introduction of an activated Ki-*ras* oncogene. It has also been shown, that a rodent cell line can be transformed using human mutated *ras* oncogene [36]. Therefore, we used a plasmid containing the human *ras* oncogene mutated on codon 12 to transform the HPV 16 immortalised cell line PMC-1 into a tumourigenic cell line since we demonstrated that PMC-1 was non-tumourigenic.

These two models enabled us to compare the immune response and protection in different MHC backgrounds and tumours at different stages. We show the expected differences in male and female immunity, presumably attributable to tolerance, and also demonstrate differences in immune function dependant on genetic background. Furthermore, we have shown that whole cell vaccines are capable of protecting a subset of male mice from prostate cancer. All of these are desirable features of a useful model which will enhance the development of whole cell vaccines.

Materials and methods

Established tumour cell lines and animals

The RM-9 cell line is a mouse prostate cell line derived from a tumour initiated using the MPR model [4]. Cells were cloned by limiting dilution in our laboratory and then grown in DMEM + 10% FCS + 2 mM L-glutamine. A single clone was used in all experiments (RM-9 clone1). This was maintained in a sub-confluent monolayer at 37°C in humidified atmosphere containing 5% CO_2 and subcultured

every 3–5 days using 1× trypsin/EDTA 0.05% (PAA Lab, Wembley, UK).

The P815 cell line is a mouse mastocytoma (NK cell specific target) [26, 28] and was purchased from the American Type Culture Collection (ATCC, VA, USA). Cells were grown in suspension in RPMI-1640 (Sigma, Poole, UK) + 10% FCS + 2 mM L-glutamine.

C57BL/6, C3H/HeJ, SCID and nu/nu female and male mice were purchased from Harlan (UK) or bred in the Barrier Maintained Unit (BMU) St George's Hospital Medical School, London, UK and used at approximately 12–15 weeks old. Animal husbandry was carried out in accordance with the UK Home Office Animal Act of 1986.

Primary cell culture

A male C3H/HeJ prostate was removed under sterile conditions. Tissues were put in RPMI-1640 + 10% FCS + 2 mM L-glutamine + penicillin/streptomycin (Life Technologies, Paisley, UK), minced to small pieces with a scalpel, centrifuged at 300×*g* for 3 min and resuspended in RPMI-1640 + 10% FCS + 2 mM L-glutamine + penicillin/streptomycin. The pieces were plated on collagen-coated six well plates (Becton Dickinson, Oxford, UK) in RPMI-1640 + 10% FCS + 2 mM L-glutamine + penicillin/streptomycin overnight. The medium was changed the next day to K-SFM (Life Technologies) + 2% FCS + 2.5 μg epidermal growth factor (EGF) (Life Technologies) + penicillin/streptomycin and then twice weekly thereafter.

Immortalization of primary prostate cells with HPV16 vector

After 13 days of culture, the primary cells isolated above were transfected with a retroviral construct containing the E6/E7 genes (a gift from Professor N. Maitland, York) from HPV16 and 8 μg/ml of polybrene (Sigma, Poole, UK) final concentration. This preparation was left overnight on the cells. The next day, 2 ml of K-SFM + 2% FCS + 2.5 μg EGF + penicillin/streptomycin were added to the preparation. The resulting cell line was designated PMC-1. PMC-1 was maintained in a sub-confluent monolayer at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured using 1× trypsin/EDTA 0.05%. PMC-1 was subsequently grown in DMEM + 10% FCS + 2 mM L-glutamine.

Transfection of PMC-1 with the purified pIRES puro2 *ras* val 12 plasmids

Approximately, 2×10^5 PMC-1 clones were seeded in 2 ml of DMEM + 10% FCS + 2 mM L-glutamine in a 35 mm tissue culture plate (Corning Incorporated). Cells were then incubated at 37°C in a 5% CO_2 incubator until they reached 40–60% confluency. They were then transfected with the purified pIRES puro2 *ras* (val 12) plasmids using lipofectin. Successful transfection was assessed by PCR. The result cell line was designated PMC-1C6 ras1.

Development of PMC-1 C6ras1p1

PMC-1 C6ras1 was injected into four C3H/HeJ female mice. Only one mouse out of four developed a tumour which was excised and grown in vitro for several months. The resulting clone will henceforth be referred to as, PMC-1 C6ras1p1. When C3H/HeJ female mice were challenged with 5×10^6 PMC-1 C6ras1p1/mouse the tumours developed tumours in 30–40 days.

Determination of androgen dependence of PMC-1 and PMC-1 C6 ras1 p1 in vitro

PMC-1 and PMC-1 C6 ras1 p1 were seeded at 5×10^4 cells/well of a six well plates in DMEM + 10% FCS + 2 mM L-glutamine overnight. The next day, medium was aspirated and the cells were washed once with HBSS. Then, DMEM + 2% charcoal depleted calf serum (Sigma, Poole, UK) + 10 pM of testosterone (Sigma, Poole, UK) or DMEM + 2% charcoal depleted calf serum were added to the cells. Cells were trypsinized and counted every 24 h with a hematocytometer and 0.1% trypan blue. The cell growth in each media was determined. Each condition was performed in triplicate.

Androgen treatment

PMC-1 and PMC-1 C6ras1p1 cells were plated in a T25 cm^2 flask (Greiner) with DMEM + 10% FCS + 2 mM L-glutamine. When they reached 80% confluency, PMC-1 and PMC-1 C6ras1p1 cells were washed once with HBSS. DMEM + 10% charcoal depleted calf serum and 10 pM of testosterone were added to the cells for 24 h. Charcoal depleted calf serum was used to achieve deprivation of hormones and growth factors. Cells were then trypsinized, washed once and cytospun at 200×*g* for 3 min onto positively charged microscope slides (Superfrost plus) (BDH laboratories supplies, Lutterworth, UK). The slides were kept at –80°C until required.

Immunocytochemistry

PMC-1 and PMC-1 C6ras1p1 were spun onto positively charged microscope slides. Specimens were fixed with ICC

staining kit fixative (Nexell Therapeutics Inc., Irvine, CA, USA) for 10 min at room temperature. They were blocked with 10% normal serum (Dako, Ely, UK) corresponding to the species of secondary antibody for 15 min; 100 µl of pre-titrated primary antibody [Cytokeratin 18 (RDI, Flanders, USA), Desmin (RDI), Vimentin (Santa Cruz, Wembley, UK), Androgen receptor (Santa Cruz), Anti-cytokeratin cocktail (ICC staining kit) (Nexell Therapeutics Inc.), MOPC21 isotype control (Dako), normal mouse IgG isotype control (ICC staining kit) (Nexell Therapeutics Inc.) was then added and incubated for 45 min at room temperature; 100 µl of pre-titrated secondary antibody [Goat anti-mouse (Jackson Immunology Research), Rabbit anti-goat (Jackson), Goat anti-rabbit (Jackson), Goat anti-mouse (ICC staining kit) (Nexell)] conjugated with alkaline phosphatase was added and incubated for 30 min. After two washes with TBS (Tris buffer solution), component A (Naphtol AS phosphate and Levamisole in Tris buffer, pH8.5) and B (Fast Red Violet salt) from the ICC staining kit were mixed, applied and incubated for 20 min at room temperature. Samples were washed twice and Haematoxylin [ICC staining kit (Nexell)] used to counterstain. Each immunostaining assay contained a negative control.

Flow cytometry and staining procedure

Cells were washed with 100 µl staining buffer [FACSflow (Becton Dickinson) containing 1% normal mouse serum (Sigma) and 0.01% azide (Sigma)], in a 96 well round bottom plate, and then incubated with 10 µl of the pre-titrated monoclonal antibodies [anti- H-2K^k PE (MHC class I), anti-I-A^k PE (MHC class II), anti-CD80 PE (B7.1), anti-CD86 PE (B7.2), Anti CD54 PE (ICAM-1), Anti CD106 FITC (VCAM-1)] or their respective isotype controls (Murine IgG2a PE, Murine IgG2a PE, Hamster IgG2 PE, Rat IgG2a PE, Hamster IgG1 PE, Rat IgG1 FITC) for 20 min at 4°C. They were washed once with staining buffer and resuspended in 200 µl of 1% paraformaldehyde (Sigma) and stored in the dark at 4°C until analysis. Analysis was carried out using a FACScan (Becton Dickinson). All antibodies were from Pharmingen (Oxford, UK), except CD106 FITC (Serotec, Oxford, UK).

Tumourigenicity studies

Trypsinized PMC-1, PMC-1 C6ras1, PMC-1 C6ras1p1 or RM-9 clone1 (positive control) were washed three times in PBS and resuspended at 5×10^6 cells/200 µl of saline. Five males and females nu/nu mice, SCID mice or female C3H/HeJ mice were injected once subcutaneously with 200 µl of the cell suspension (Table 1). Animals were observed for up to 2 months. Mice were sacrificed when tumour size exceeded 15 mm in either axis.

Immunization and survival

C57BL/6 model

RM-9 clone1 and PMC-1 were harvested with 1× trypsin/EDTA 0.05%, washed three times with sterile PBS and irradiated at 150 Gy. Five mice per group were injected subcutaneously with 1×10^6 irradiated RM-9 clone1, irradiated PMC-1 or saline respectively in a volume of 200 µl of saline, on the right flank, three times with 1-week interval. A week after the last vaccination, they were challenged with 5×10^4 RM-9 clone1 on the contra-lateral flank. The animals were monitored for palpable tumour and were sacrificed once a size of 15 mm in either axis had been reached.

C3H/HeJ model

RM-9 clone1 and PMC-1 were harvested with 1× trypsin/EDTA 0.05%, washed three times with sterile PBS and irradiated at 150 Gy. Five mice per group were injected subcutaneously with 1×10^6 irradiated RM-9 clone1, irradiated PMC-1 or saline respectively in a volume of 200 µl of saline, on the right flank, three times with 1-week interval. A week after the last vaccination, they were challenged with 5×10^5 PMC-1 C6ras1p1 on the contra-lateral flank. The animals were monitored for palpable tumour and were sacrificed once a size 15 mm in either axis had been reached.

Generation of CTL in vitro

C57BL/6

Four C57BL/6 female or male mice per group were injected subcutaneously with 1×10^6 irradiated (150 Gy)

Table 1 Summary of tumorignicity study

	nu/nu mice	SCID mice	C3H/HeJ
RM-9	+	n/a	n/a
PMC-1	–	n/a	n/a
PMC-1C6ras1	n/a	+	+
PMC-1C6ras1p1	n/a	n/a	+

+ Tumour is growing, – tumour is not growing, n/a not applicable

Table 2 Summary of immunization schedule

	C57BL/6	C3H/HeJ
Week 1	Vaccination	Vaccination
Week 2	Vaccination	Vaccination
Week 3	Vaccination	Vaccination
Week 4	Challenge/spleen for CTL	Challenge/spleen for CTL
Week 5	/	/
Week 6	Spleen for CTL	/
Week 7	/	Spleen for CTL

RM-9 clone1, PMC-1 or saline (Steri-Amp, Norton) in the right hip. This procedure was repeated three times with 7-day intervals between doses. A week after the third injection, the mice were challenged subcutaneously with 5×10^4 live RM-9 clone1 cells on the contra-lateral hip. Two mice were selected randomly from each group of animals, sacrificed and spleens removed a week after the third injection and again after at least 15 days post-challenge (Table 2). The spleens were mashed through a 0.70 μm cell strainer (BD Falcon, Oxford, UK). The cell suspension was carefully put on top of 4 ml of Histopaque 1083 (Sigma) to separate red cells from splenocytes. Tubes were then spun at 800×*g* for 25 min. The interface between the Histopaque and the medium was collected and washed once with RPMI-1640 + 10% FCS + 2 mM L-glutamine + 1% penicillin/streptomycin + 5 μM 2-mercaptoethanol (Sigma) for 10 min at 800×*g*. Splenocytes were then resuspended in RMPI + 10% FCS + 2 mM L-glutamine + 1% penicillin/streptomycin + 5 μM 2-mercaptoethanol and counted. Stimulator cells (the syngeneic tumour, RM-9 clone1) were treated for 24 h with 0.01 μg/ml mouse recombinant interferon-γ (mrIFN-γ) (R&D Systems, Abingdon, UK) to up-regulate MHC class I [1] and irradiated at 50 Gy. Splenocytes were cultured with irradiated (50 Gy) RM-9 clone1 mrIFN-γ treated at different ratios of effector:stimulator (100:1 or no stimulator cell) in RMPI + 10% FCS + 2 mM L-glutamine + 1% penicillin/streptomycin + 5 μM 2-mercaptoethanol + 10 U/ml of mrIL-2 for 5 days in 24 well plates (Greiner).

C3H/HeJ

Four C3H/HeJ female or male mice per group were injected subcutaneously with 1×10^6 irradiated (150 Gy) RM-9 clone1, PMC-1 or saline on the right hip. This procedure was repeated three times with 7-day intervals between doses. A week after the third injection, the mice were challenged subcutaneously with 5×10^5 live PMC-1 C6ras1p1 cells on the contral-lateral hip. Two mice were selected randomly from each group, sacrificed and spleens removed a week after the third injection and at least three weeks after the challenge (Table 2). Splenocytes were isolated as described above. Splenocytes (2×10^6cells/ml) were cultured with irradiated (50 Gy) PMC-1 C6ras1p1 (6×10^4cells/ml) in RPMI-1640 + 10% FCS + 5 μM 2 mM L-glutamine + 1% penicillin/streptomycin + 2-mercaptoethanol + 10 U/ml of rmIL-2 for 5 days in 24 well plates (ratio 100:1 or no stimulator cells).

Cytotoxic assay

After 5 days, the effector cells described above were used in a standard Chromium release assay. RM-9 clone1 target cells were treated for 24 h with 0.01 μg/ml rmIFN-γ to up-regulate MHC class I. All target cells RM-9, PMC-1 C6ras1p1 and P815 were labelled with 150 μCi of $Na_2Cr^{51}O_4$ (Amersham, Buckinghamshire, UK) at 37°C for 1 h. Cells were washed three times and resuspended in medium at 1×10^5 cells/ml. Cultured splenocytes were serially diluted (100:1, 50:1, 25:1, 12.5:1 and 6:1) in duplicate in 100 μl of medium (RPMI-1640 + 10% FCS + 2 mM L-glutamine) in 96 V-well plates (Greiner) and 100 μl of labelled target cells were added. Plates were incubated for 4 h at 37°C in a 5% CO_2 atm. Then, 25 μl of supernatant was removed from each well and added to 175 μl of scintillation fluid (Optiphase Supermix, Fisher chemicals, Loughborough, UK). Chromium release was measured with a Wallac MicroBeta counter (Perkin Elmer Life Sciences). Specific lysis was calculated from the following formula:

$$\%\,\text{Specific release} = \frac{(\text{Experimental release (cpm)} - \text{mean spontaneous release})\ (\text{cpm}) \times 100}{(\text{Mean total release (cpm)} - \text{mean spontaneous release})\ (\text{cpm})}$$

Spontaneous release and total release were measured by incubating labeled target cells with medium alone and 1% Tween 20 (Sigma), respectively.

Cytometric bead array (CBA)

The assay was carried out according to manufacturer's guidelines (BD Biosciences). After incubation, the plate was washed twice using 100 μl of the wash buffer from the

CBA kit. Finally the beads in each well were resuspended in 200 μl of wash buffer and analyzed on a FACSCalibur using the Multiwell Automated Sampler (MAS) (BD Biosciences). A total of 10,000 events were collected thus ensuring at least 2,000 replicates of each sample. Acquired data was analyzed using CBA analysis software (BD Biosciences). This is a fully quantitative method.

Statistics

Survival of mice was analyzed using the logrank test (Prism 3.0, GraphPad Software. Inc.). All animal vaccination experiments were repeated at least twice with a minimum of five animals per group. All immunological studies were repeated at least twice with two animals per time point, unless otherwise stated.

Results

Development and determination of PMC-1 and PMC-1 C6 ras1p1 tumourogenicity

The PMC-1 cell line was established from a 16-week-old C3H/HeJ male mouse. Pieces of normal prostate tissue attached to collagen type I coated 6-well plates, which then encouraged epithelial cell outgrowth. After 2 weeks of culture in vitro, epithelial cells were transfected with a retroviral construct containing E6/E7 genes from HPV16. This resulted in an immortalized epithelial cell line designated PMC-1. This cell line has been in continuous culture for more than 24 months, been passaged over one hundred times and the cells grow as an epithelial monolayer.

To investigate PMC-1 tumourogenicity, tumour growth was examined in nu/nu mice. Approximately, 5×10^6 PMC-1 or RM-9 clone1 cells were injected once into five females and five males nu/nu mice. Mice injected with RM-9 clone1 developed tumour within 10 days whereas mice injected with PMC-1 did not form any tumours for up to two months. PMC-1 C6ras1 was injected once into five males or five female SCID mice to determine its tumouriginicity in vivo. PMC-1 C6ras1 clone grew in vivo in both all male and female SCID mice. PMC-1 C6ras1 was then injected once into four C3H/HeJ female mice. Only one mouse out of four developed a tumour and the resulting clone was designated as PMC-1 C6ras1p1. C3H/HeJ female mice challenged with 5×10^6 PMC-1 C6ras1p1/mouse developed tumours in 30–40 days (data not shown) (Table 1).

Both PMC-1 and its *ras* transfected contrepart, PMC-1 C6ras1p1 grew in vitro as an epithelial monolayer (data not shown).

Characterization of PMC-1 and PMC-1 C6ras1p1

To confirm the epithelial and prostatic origin of these two cell lines, immunocytochemistry was performed on cytospun samples from actively growing transformed cultures (Table 3). The epithelial origin of PMC-1 and PMC-1 C6ras1p1 was established by cytokeratin-18 and pan-cytokeratin expression. Desmin has not been found in normal epithelium or tumour epithelium [41] and hence negative desmin and fibroblast staining confirms epithelial origin of PMC-1 and PMC-1 C6ras1p1.

To characterize the prostatic origin of these cell lines, presence of androgen receptor (AR), and its up-regulation in response to androgen, was examined. Androgen receptor is present on PMC-1 cells and is up-regulated by culturing the cells with 10 pM of testosterone for a period of 24 h. PMC-1 C6ras1p1 was stained for AR and cultured with 10 pM of testosterone for a period of 24 h. PMC-1 C6ras1p1 stained positively for AR. The intensity of the staining with this specific antibody remained the same on the testosterone treated cells compared to the non-treated cells. These data suggest PMC-1 and PMC-1 C6ras1p1 are two epithelial prostatic cell lines.

To determine whether PMC-1 and PMC-1 C6ras1p1 was androgen dependent in vitro, PMC-1 and PMC-1 C6ras1p1 were cultured with 10 pM of testosterone for 4 days. The

Table 3 Characterisation of prostate cell lines PMC-1 and PMC-1 C6ras1p1

	PMC-1	PMC-1 C6ras1p1
Immunochemistry		
Cytokeratin-18	+	+
Pan-cytokeratin	+	++
Fibroblast	–	–
Desmin	–	–
Androgen receptor	+	+
Androgen receptor (testosterone treatment)	++	++
Cytofluorometry		
H-2K^k PE	++	++
I-A^k PE	–	–
CD80 PE	++	++
CD86 PE	–	–
CD54 PE	++	++
CD106 FITC	–	–

PMC-1 and PMC-1 C6ras1p1 were cytospun and stained for cytokeratin-18, pan-cytokeratin), fibroblast, desmin, androgen receptor and IgG control. Cells were treated with testosterone for 24 h, cytospun and then stained with androgen receptor monoclonal antibody

Surface markers on the immortalised prostate derived cell line PMC-1 and PMC-1 C6ras1p1 were analysed by cytofluorometry. The following monoclonal antibodies were used to stain the two cell lines: H-2K^k PE, I-A^k PE, CD80 PE, CD86 PE, CD54 PE, CD106 FITC

presence of testosterone did not increase the growth rate of the cells (Fig. 1a, b). Therefore, PMC-1 and PMC-1 C6 ras1p1 can be considered androgen independent in vitro.

PMC-1 and PMC-1 C6ras1p1 expressed significant surface levels of MHC class I, the costimulatory molecule B7.1 (CD80) and the adhesion molecule ICAM-I (CD54) as determined by flow cytometry (Table 1). They did not express detectable levels of MHC class II, B7.2 (CD86) or VCAM-1 (CD106) molecules. After treatment with IFN-γ, only a small up-regulation of MHC class I was observed (data not shown).

Determination of the survival in male and female in two different strains of mice after whole cell vaccination

C57BL/6 mice were vaccinated three times with cells and then challenged 7 days after the last injection with 1×10^5 live RM-9 clone1. Figure 1a shows that three vaccinations induced a protective response in 80% of females with the syngeneic vaccine RM-9 clone1 (H-2b), whilst the allogeneic vaccine PMC-1 (H-2k) gave 40% protection. However, the antitumour response induced by vaccination with either allogeneic or syngeneic vaccine was variable. Further experiments demonstrated that the protection induced by the vaccine ranged from 40 to 80% of animals in the syngeneic setting, whilst the protection induced by the allogeneic vaccine ranged from delay in onset of tumour to 40% survival.

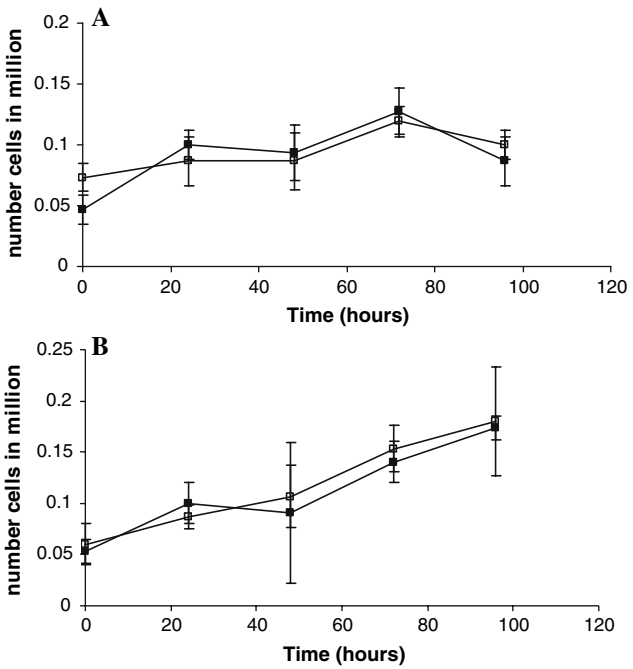

Fig. 1 Growth curve for PMC-1 **a** and PMC-1C6ras1p1 **b** cell lines treated with 10 pM of testosterone. Approximately, 5×10^4 cells per well plated in (*open square*) DMEM 10% FCS charcoal depleted or (*filled square*) DMEM 10% FCS charcoal depleted + 10 pM of testosterone in a six-well plate on day 0. Cell number was determined every 24 h. Each point is the average cell count for three wells

When the experiment was repeated in C57BL/6 males, after 25 days, all control male mice developed tumours. C57BL/6 males were not protected when they were vaccinated with the allogeneic cells ($P = 0.1476$), but as shown in females, 60% protection was obtained after syngeneic vaccination ($P = 0.005$) (Fig. 1b).

Consequently, we have shown syngeneic vaccination induces protection both in females and males, whereas allogeneic vaccination only protects females.

Similar experiments were carried out in C3H/HeJ mice. Female C3H/HeJ mice (H-2k) were vaccinated three times with irradiated either allogeneic RM-9 clone1 (H-2b), irradiated syngeneic PMC-1 (H-2k) or saline. A week after the third vaccination, mice were challenged with live syngeneic PMC-1 C6ras1p1 (H-2k). Protection was only 20% (compared to 40–80% in C57BL/6 mice) when females were vaccinated either with PMC-1 or RM-9 clone1. No protection was observed when C3H/HeJ males were vaccinated with either allogeneic or syngeneic vaccine (data not shown) (Fig. 2).

Study of the immune response

Cellular immune response

C57BL/6 females were vaccinated three times with 1-week interval using RM-9 clone1, PMC-1 or saline. A week after the last vaccination, and 2 weeks after challenge, the ability of splenocytes to kill ^{51}Cr-labelled RM-9 clone1 target cells was determined. Mice injected with saline did not show any CTL or NK activity at any stage. Both RM-9 and PMC-1 induce NK cells to a similar level. Little or no CTL is detectable (Fig. 3a). This is unffacted by tumour challenge (Fig. 3b). Interestingly, when irradiated tumour cells were present as feeder cells in the culture in vitro, no lytic activity was observed (data not shown). The same experiment was repeated in C57BL/6 males. No cytolytic activity was observed after three vaccinations or after challenge with either target (data not shown), and also when tumour cells were present in culture in vitro (data not shown).

In C3H/HeJ female mice, immunization led to the generation of cytotoxic activity (Fig. 3) which was due to both NK and CTL lysis. We again see NK cells, but this time they are reduced post challenge (Fig. 3b). Also low, but detectable CTL are induced by PMC-1 (Fig. 3a, b), suggesting that syngeneic vaccination can induce adaptive immune responses and therefore, presumably, induce memory cells. No CTL or NK lysis was observed in the control group.

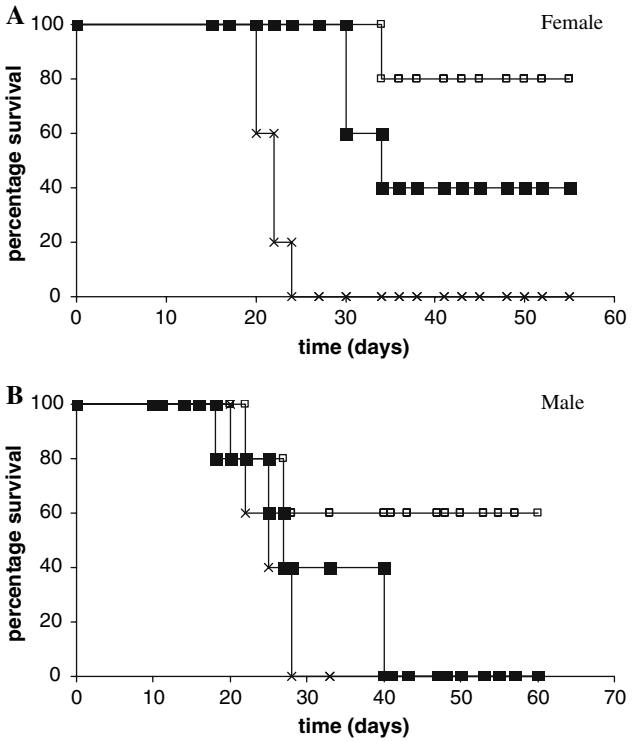

Fig. 2 Survival curve in C57BL/6 model. Groups of 5 C57BL/6 females **a** and males **b** were injected with 1×10^6 irradiated (*line width open square*) RM-9 clone1 (*line width filled square*) PMC-1 or (*line width cross symbol*) saline three times with a week interval. The mice were challenged 7 days after the last injection with 5×10^4 live RM-9 clone1. The animals were monitored for palpable tumour. Data are representative of two experiments

The same experiment was carried out in C3H/HeJ males. After three vaccinations, barely detectable amounts of CTL activity were observed which disappeared after challenge (data not shown). NK activity was not detected before or after challenge (data not shown).

The combined data suggest that the protective response induced by vaccination in females was likely to be mediated predominantly by CTL and, to a lesser extent, by NK cells. These data in males show that, even if the vaccination had induced a cellular response, the presence of the tumour eradicates completely the cellular immune response and thus could explain the lack of protection observed previously.

Cytokine secretion

In order to dissect the differences between male and female mice further, quantification of different cytokines by Cytometric Bead Array (CBA) was carried out. Splenocytes from vaccinated and control C57/BL6 mice were cultured in vitro for 5 days. Supernatants were then collected and analyzed (Fig. 4a). As expected, very low levels of cytokine secretion were detectable in the control group. Vaccination increased the production of several cytokines including IFN-γ, TNF-α, IL-5, IL-4 and IL-2, compared to the control. Secretion of IFN-γ was increased after challenge. In C57/BL6 females, all cytokine were increased after challenge after both allogeneic or syngeneic vaccination. The level of IL-4 was very low and often not detectable at all.

In the male C57/BL6 group, similar to females, cytokine secretion was at a basic level in the control group. All cytokine secretion was increased after challenge in syngeneic vaccination. However, contrary to the females, secretion of all cytokines was down-regulated after challenge when animals were vaccinated with the allogeneic vaccine. These results correlate with the survival results. Notably, male levels were considerly lower than female.

A similar experiment was performed in C3H/HeJ mice. Supernatants were then collected and analyzed with CBA as before (Fig. 4b). Once more low levels of cytokine secretion was detected in the control group after vaccination. The presence of IL-2 in the culture medium could not have interfered with the results since the secretion in the control group remained at a basic level. Cytokine secretion in the control group became higher after challenge in both males and females.

Vaccination induced the production of IFN-γ, TNF-α, IL-5, IL-2 and IL-4. As seen previously in the C57BL/6 model, cytokine secretion in females was higher than in males. Moreover, the secretion increased after challenge in females, whilst it decreased after challenge in males in both syngeneic and allogeneic vaccination. Unlike the C57BL/6 model, production of cytokines was not down-regulated when irradiated tumour cells were present in the culture (data not shown).

These data, taken together, shown that the cytokine profile in C3H/HeJ females is a mixture of Th1 and Th2 responses. In comparison, it was mainly Th1 in C57BL/6 mice. In males, cytokine secretion is much lower than in females, regardless of haplotype and is uniformly diminished after challenge.

Discussion

Three different cell lines PMC-1, PMC-1 C6ras1p1 and RM-9 clone1 were used as cell vaccines in this study. PMC-1 and PMC-1 C6ras1p1 were generated and characterized in our laboratory. They were isolated from a normal mouse prostate and immortalized with the E6/E7 gene from HPV 16. PMC-1 C6ras1p1 is a tumourigenic prostate cell line which has been derived from PMC-1 and transformed with a human mutated *ras* oncogene. Both lines show epithelial cell morphology and express specific epithelial

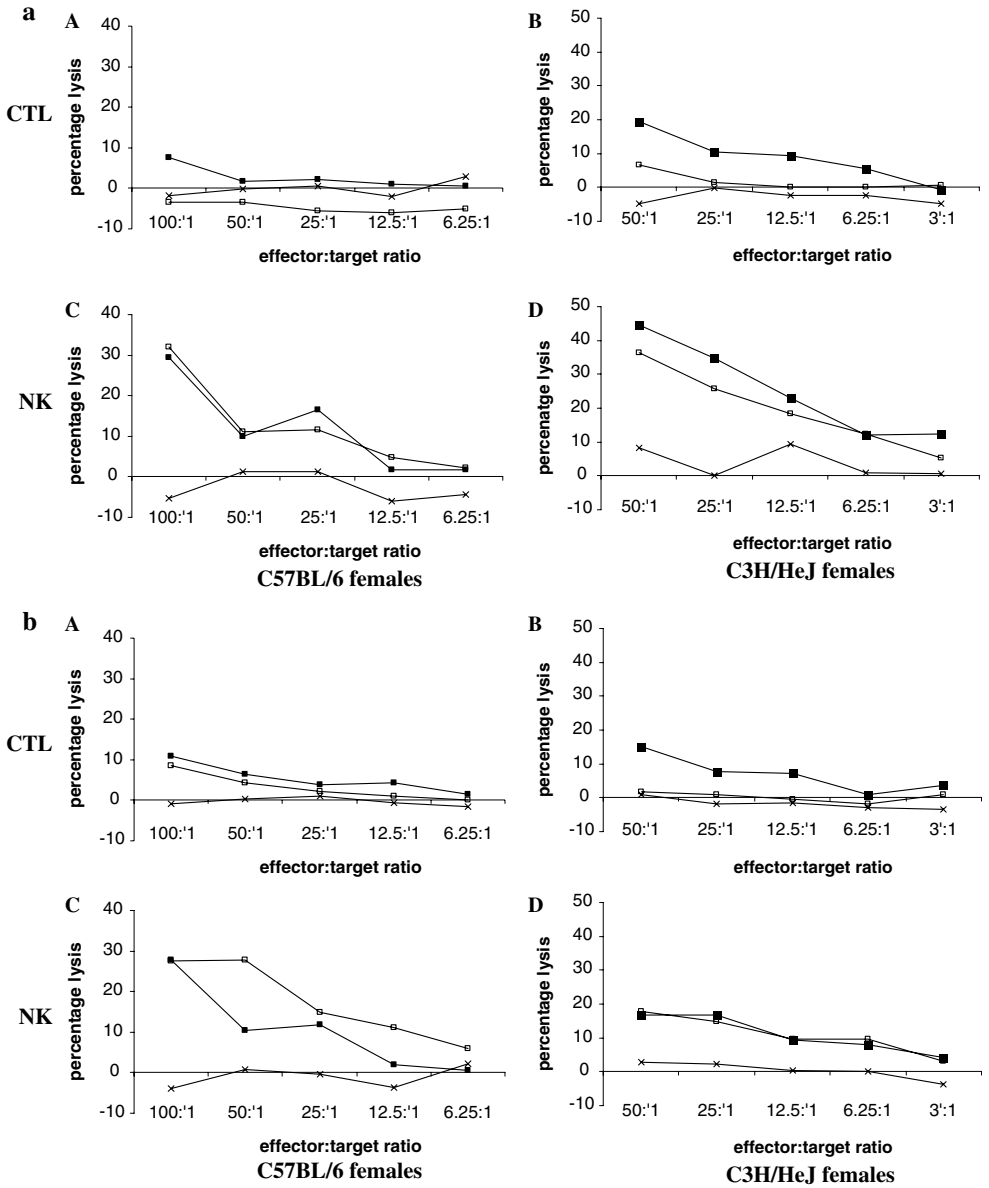

Fig. 3 Cytotoxic activity and NK activity in female C57BL/6 mice and in female C3H/HeJ mice after the third vaccination **a** and after challenge **b**. The graph shows a comparison of percentage lysis (y-axis) of ^{51}Cr labelled P815 target cells (**c**, **d**) or rmIFN-γ treated, ^{51}Cr labelled RM-9 clone1 target cells (**a**, **b**) by splenocytes from (*line width open square*) RM-9 clone1 (*line width filled square*) PMC-1 or (*line width cross symbol*) saline. Splenocytes were isolated from spleens of vaccinated females C57BL/6 mice (**a**, **c**) and female C3H/HeJ mice (**b**, **d**) after the third vaccination **a** and after challenge **b** and cultured in vitro for 5 days before use in a 4 h ^{51}Cr release assay. Data are representative of two different experiments

markers such as cytokeratin-18 and pan-cytokeratin. The androgen receptor present on PMC-1, and PMC-1 C6ras1p1, can be up-regulated in vitro when treated with testosterone. Both cell lines are androgen independent in vitro since they grow in the absence of androgen. Moreover when testosterone is added to the medium, doubling times remain similar. However, in vivo, PMC-1 C6ras1p1 appears to be androgen sensitive since fewer cells are required per mouse to grow a tumour in males than in females.

To study whole cell vaccination in prostate cancer, two models were developed. The first model used RM-9 clone1 as a tumour challenge in C57BL/6 mice. This cell line is an established mouse prostate cell line isolated from the MPR model [4]. It is very aggressive, causing tumours in mice in less than 15 days at a low seeding density (5×10^4 cells per mouse). It does not express MHC class I molecules and is androgen independent since tumour cells grow in both males and females. The second model uses PMC-1 C6ras1p1 as a tumour challenge in C3H/HeJ mice. This cell line is slow

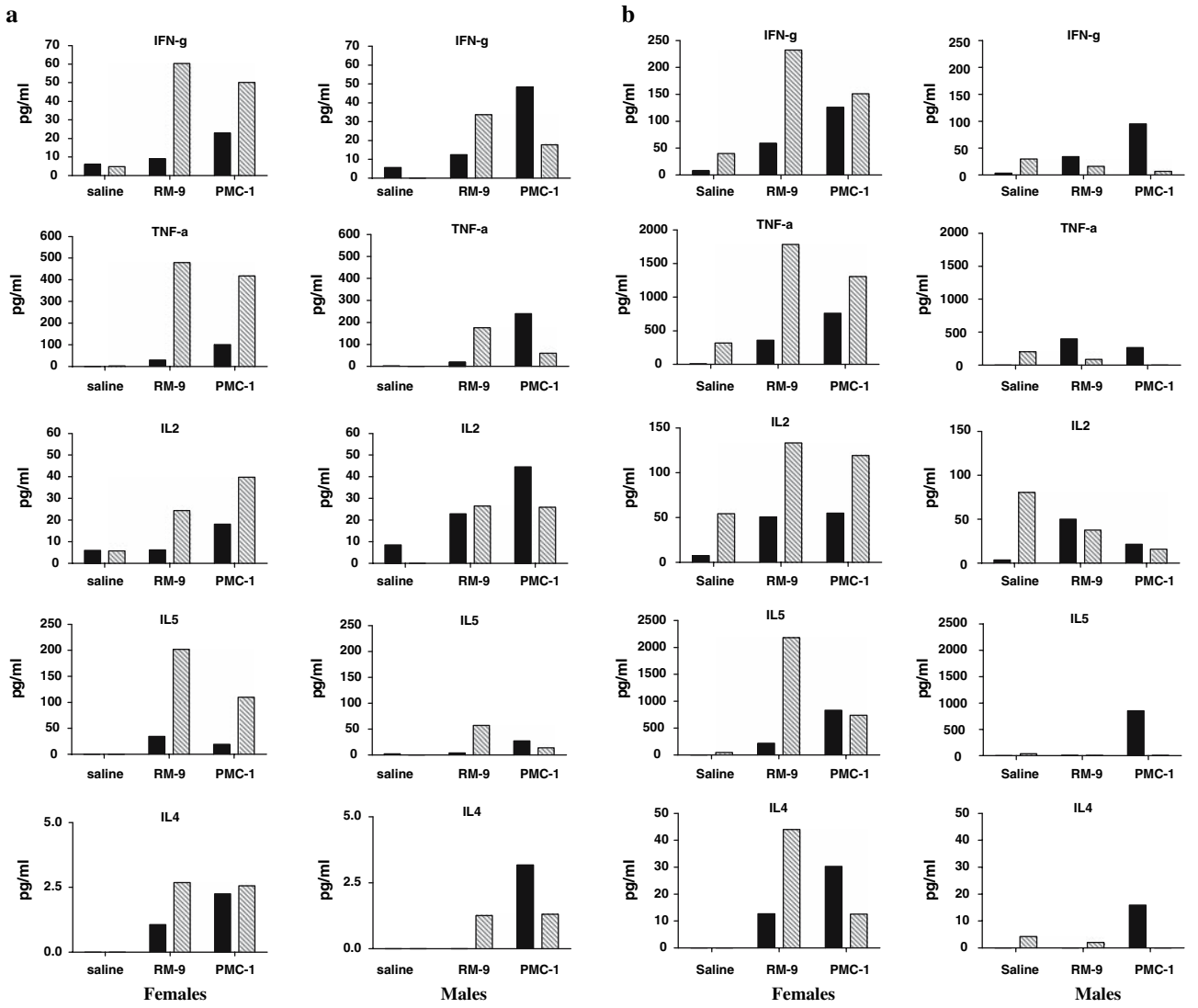

Fig. 4 **a** Cytokine quantification by CBA in C57BL/6 model. **b** Cytokine quantification by CBA in C3H/HeJ model Groups of C57BL/6 female and male mice were injected with 1×10^6 irradiated RM-9 clone1, PMC-1 or saline three times with a week interval and challenged with 5×10^4 live RM-9 clone1. Spleens were collected a week after the third vaccination (*filled square*) and 2 weeks after challenge (*open square*). Splenocytes were cultured for 5 days in vitro with 10 U/ml of IL-2. Supernatants were collected and detection of IFN-γ, IL-2, IL-5, IL-4 and TNF-α was performed by CBA assay was performed. Data are representative of two different experiments

growing and results in weak tumours in vivo. Mice develop tumours in about 30 days and require ten-fold higher cell seeding (5×10^5 cells per mouse). It expresses MHC class I molecules and is androgen independent since tumours grow equally well in both males and females. However, at a lower level of cells (1×10^5), tumours do not grow in females whilst males develop tumours (data not shown). It is likely that the presence of prostate antigens makes the cell more "foreign" for females. It is noteworthy that even if PMC-1 C6ras1p1 is androgen independent, it might be androgen sensitive and grow faster due to the presence of androgen in vivo. A possibly interesting further study would be to ovariectomize females to determine if removal of estrogenic influence also diminishes response to vaccination.

These two models represent two different stages of prostate cancer. The first model is characteristic of advanced hormone refractory disease, since the tumours do not express MHC class I molecules [8], are androgen independent [22] and grow rapidly in vivo. The second model appears to represent an earlier stage of the disease; tumours express MHC class I molecules [8], appear androgen sensitive at a low level in males [22] and grow very slowly in vivo [11].

The level of protection observed after allogeneic or syngeneic vaccination in females, differs in C57BL/6 and C3H/HeJ mice. When RM-9 clone1 is used as a tumour challenge in C57BL/6, syngeneic vaccination was more effective than allogeneic vaccine. When PMC-1 C6ras1p1

is used as a challenge in C3H/HeJ mice, allogeneic vaccination was more effective than syngeneic vaccine. In the C57BL/6 mouse, the RM-9 clone1 (challenge and syngeneic vaccine) used is derived from the MPR model which involves transfection with *myc* and *ras* oncogenes. The protection observed might be due to a stronger response to these two oncogenes, which are present on both challenge and on syngeneic vaccine, in addition to other tumour antigens present on the cells. It has been shown that when fibroblasts are transfected with *ras*, they can confer protection against B16/F10 in a melanoma model in C57BL/6 mice (J. Kayaga Ph.D. thesis, 1999, unpublished data).

In the C3H/HeJ mouse, PMC-1 (syngeneic vaccine) may be less efficient because it is derived from a normal prostate and thus has few tumour antigens. Moreover, being syngeneic, the vaccine is not seen as "foreign". Some of the protection observed here when PMC-1 is used as a vaccine may be due to the irradiation, which has been shown to make cells more immunogenic. It has been shown that radiation increases the expression of heat shock proteins [32, 35] and also induces production of apoptotic bodies by the vaccine cells [31]. Heat shock proteins have been shown to induce protection and immunogenicity [7, 15, 38]. Furthermore, production of apoptotic bodies is associated with the generation of a protective response [34]. However, protection may also be due to the presence of HPV 16.

Results detected in males after allogeneic vaccination were similar in both models. No protection was observed. When syngeneic vaccination was used, only a degree of protection was seen in C57/bl6 mice, whereas none was observed in C3H/HeJ mice. These results taken together suggest tolerance. The prostate is only present in males, therefore prostate antigens are seen as "self" by the male immune system while they are seen as "non-self" by the female immune system. The fact that RM-9 does confer protection in males is perhaps due to the expression of *myc* and *ras* oncogenes to which there is no tolerance. Tolerance depends on the level of expression of antigen [13] presented to the immune system and also on the presentation of the antigen by antigen presenting cells such as dendritic cells. It is possible that in the two other cell lines' expression of tumour antigens is too low or too high and thus they are tolerized by the immune system. The presence of allogeneic MHC class I molecules has been shown to have an adjuvant effect [14].

An obvious criticism of these experiments is that the protection seen in females may simply be due to HY antigens. To examine this, C57BL/6 females were vaccinated with RM-9 clone1 (mouse prostate cell isolated from male), B16/F10 (mouse melanoma cell isolated from female) or K1735 (mouse melanoma cell isolated from female). Then mice were challenged either with B16/F10 or RM-9 clone1. Protection was observed when mice were vaccinated with K1735 or with RM-9 clone1 and challenged with RM-9 clone1 or B16/F10. Therefore, if male antigens were involved, protection with K1735 would not have been observed when mice were challenged with RM-9 clone1. Therefore it is likely that tumour antigens must be shared between the two cell lines, regardless of the sexual origin of the cells (data not shown).

To dissect the mechanisms behind the protection observed, cytotoxic assays and cytokine secretion profiles were carried out. These two models gave different cellular responses in female mice. In C57BL/6 females, the cellular immune response was mainly NK mediated and the cytokine profile was Th1 dominated. In C3H/HeJ females, the immune response was mainly CTL and a mixture of Th1 and Th2. Cytokine secretion was much higher in this model than in the C57BL/6 model. In both models the response observed was up-regulated after challenge in females.

When similar experiments were repeated in males, no cellular immune response was detectable in C57BL/6. A low level of CTL activity was seen before challenge but disappeared after challenge in C3H/HeJ male mice. The levels of secreted cytokines were much lower in males than in females. Similar to the females, the level of cytokine detected in C57BL/6 was lower than in C3H/HeJ mice.

Here, several phenomena may be occuring. In C57BL/6 males, no cellular immune response was detected after vaccination. In C3H/HeJ males, an immune response was detected after vaccination. Cytokine levels and cellular immune responses remain the same in the presence of tumour cells in vitro. In the TRAMP model, it has been shown that when an ovalbumin sequence was inserted under the probasin promoter, peripheral tolerance was induced to this protein (Ratliff et al., personal communication). It is possible that peripheral tolerance may be involved in C57BL/6 mice, since no cellular immune response was detected after vaccination. Secretion of suppressive cytokines (such as TGF-β) by tumour cells may also be involved in the C57BL/6 mice since presence of tumour in vitro induced a decrease of cytokine secretion as well as immune response in females. In the C3H/HeJ mice, tolerance due to T regulatory cells could be present since the cellular immune response was only detectable before challenge.

The development of these two models is likely to be very useful for developing clinical strategies. The systems represent two different stages of disease (early and late stage). Furthermore, using females enables us to demonstrate and study two different immunological mechanisms involved. This may be related to the fact that at different stages of disease, different responses can occur. However, the different markers expressed on the surface of the cells, and the two different methods used to transform the cells, might

have also have an influence on the immunological response in vivo. These data may aid in the understanding of the immunological events that occur when normal cells transform into tumourigeneic cells during cancer development.

Most importantly, although males are not protected very well, there is some protection. Thus, this model could be used to study the effects of different immunotherapies such as whole cell vaccines combined with a novel adjuvant for example. Clearly, any improvement in male protection could be easily translated into the clinical setting.

References

1. Abril E, Mendez RE, Garcia A et al (1996) Characterization of a gastric tumor cell line defective in MHC class I inducibility by both alpha- and gamma-interferon. Tissue Antigens 47:391–398
2. Anwar K, Nakakuki K, Shiraishi T, Naiki H, Yatani R, Inuzuka M (1992) Presence of ras oncogene mutations and human papillomavirus DNA in human prostate carcinomas. Cancer Res 52:5991–5996
3. Baars A, Claessen AM, van den Eertwegh AJ et al (2000) Skin tests predict survival after autologous tumor cell vaccination in metastatic melanoma: experience in 81 patients. Ann Oncol 11:965–970
4. Baley PA, Yoshida K, Qian W, Sehgal I, Thompson TC (1995) Progression to androgen insensitivity in a novel in vitro mouse model for prostate cancer. J Steroid Biochem Mol Biol 52:403–413
5. Berard F, Blanco P, Davoust J et al (2000) Cross-priming of naive CD8 T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. J Exp Med 192:1535–1544
6. Bevan MJ (1976) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med 143:1283–1288
7. Blachere NE, Udono H, Janetzki S, Li Z, Heike M, Srivastava PK (1993) Heat shock protein vaccines against cancer. J Immunother 14:352–356
8. Blades RA, Keating PJ, McWilliam LJ, George NJ, Stern PL (1995) Loss of HLA class I expression in prostate cancer: implications for immunotherapy. Urology 46:681–686
9. Boon T, van der BP (1996) Human tumor antigens recognized by T lymphocytes. J Exp Med 183:725–729
10. Bosland MC (1996) Hormonal factors in carcinogenesis of the prostate and testis in humans and in animal models. Prog Clin Biol Res 394:309–352
11. Bostwick DG, Qian J (2001) Effect of androgen deprivation therapy on prostatic intraepithelial neoplasia. Urology 58:91–93
12. Bremers AJ, Andreola S, Leo E et al (2000) T cell responses in colorectal cancer patients: evidence for class II HLA-restricted recognition of shared tumor-associated antigens. Int J Cancer 88:956–961
13. Carbone FR, Kurts C, Bennett SR, Miller JF, Heath WR (1998) Cross-presentation: a general mechanism for CTL immunity and tolerance. Immunol Today 19:368–373
14. Chen PW, Ananthaswamy HN (1993) Rejection of K1735 murine melanoma in syngeneic hosts requires expression of MHC class I antigens and either class II antigens or IL-2. J Immunol 151:244–255
15. Chen X, Tao Q, Yu H, Zhang L, Cao X (2002) Tumor cell membrane-bound heat shock protein 70 elicits antitumor immunity. Immunol Lett 84:81–87
16. Crawford ED, Blumenstein BA, Goodman PJ et al (1990) Leuprolide with and without flutamide in advanced prostate cancer. Cancer 66:1039–1044
17. Dillman RO, Barth NM, VanderMolen LA et al (2001) Treatment of kidney cancer with autologous tumor cell vaccines of short-term cell lines derived from renal cell carcinoma. Cancer Biother Radiopharm 16:47–54
18. Dillman RO, Beutel LD, Cornforth AN, Nayak SK (2000) Short-term tumor cell lines from renal cell carcinoma for use as autologous tumor cell vaccines in the treatment of kidney cancer. Cancer Biother Radiopharm 15:161–168
19. Duffour MT, Chaux P, Lurquin C, Cornelis G, Boon T, van der BP (1999) A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. Eur J Immunol 29:3329–3337
20. Eaton JD, Perry MJ, Nicholson S et al (2002) Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. BJU Int 89:19–26
21. Haigh PI, Difronzo LA, Gammon G, Morton DL (1999) Vaccine therapy for patients with melanoma. Oncology (Huntingt) 13:1561–1574
22. Hiipakka RA, Liao S (1998) Molecular mechanism of androgen action. Trends Endocrinol Metab 9:317–324
23. Kayaga J, Souberbielle BE, Sheikh N et al (1999) Anti-tumour activity against B16-F10 melanoma with a GM-CSF secreting allogeneic tumour cell vaccine. Gene Ther 6:1475–1481
24. Kircheis R, Kupcu Z, Wallner G, Rossler V, Schweighoffer T, Wagner E (2000) Interleukin-2 gene-modified allogeneic melanoma cell vaccines can induce cross-protection against syngeneic tumors in mice. Cancer Gene Ther 7:870–878
25. Landis SH, Murray T, Bolden S, Wingo PA (1998) Cancer statistics, 1998. CA Cancer J Clin 48:6–29
26. Lundak RL, Raidt DJ (1973) Cellular immune response against tumor cells. I. In vitro immunization of allogeneic and syngeneic mouse spleen cell suspensions against DBA mastocytoma cells. Cell Immunol 9:60–66
27. Peehl DM, Stamey TA (1986) Serum-free growth of adult human prostatic epithelial cells. In Vitro Cell Dev Biol 22:82–90
28. Plaut M, Lichtenstein LM, Gillespie E, Henney CS (1973) Studies on the mechanism of lymphocyte-mediated cytolysis. IV. Specificity of the histamine receptor on effector T cells. J Immunol 111:389–394
29. Rhim JS, Webber MM, Bello D et al (1994) Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV-18 and v-Ki-ras. Proc Natl Acad Sci USA 91:11874–11878
30. Rosenberg SA, Kawakami Y, Robbins PF, Wang R (1996) Identification of the genes encoding cancer antigens: implications for cancer immunotherapy. Adv Cancer Res 70:145–177
31. Russo V, Tanzarella S, Dalerba P et al (2000) Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. Proc Natl Acad Sci USA 97:2185–2190
32. Santin AD, Hermonat PL, Ravaggi A et al (1998) The effects of irradiation on the expression of a tumour rejection antigen (heat shock protein gp96) in human cervical cancer. Int J Radiat Biol 73:699–704
33. Sarkar FH, Sakr WA, Li YW, Sreepathi P, Crissman JD (1993) Detection of human papillomavirus (HPV) DNA in human prostatic tissues by polymerase chain reaction (PCR). Prostate 22:171–180
34. Scheffer SR, Nave H, Korangy F et al (2003) Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response in vivo. Int J Cancer 103:205–211
35. Sierra-Rivera E, Voorhees GJ, Freeman ML (1993) Gamma irradiation increases hsp-70 in Chinese hamster ovary cells. Radiat Res 135:40–45

36. Spandidos DA, Wilkie NM (1984) Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 310:469–475
37. Sumiya H, Masai M, Akimoto S, Yatani R, Shimazaki J (1990) Histochemical examination of expression of ras p21 protein and R 1881-binding protein in human prostatic cancers. Eur J Cancer 26:786–789
38. Tamura Y, Peng P, Liu K, Daou M, Srivastava PK (1997) Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. Science 278:117–120
39. Tannock IF, de WR, Berry WR et al (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351:1502–1512
40. Thomas MC, Greten TF, Pardoll DM, Jaffee EM (1998) Enhanced tumor protection by granulocyte–macrophage colony-stimulating factor expression at the site of an allogeneic vaccine. Hum Gene Ther 9:835–843
41. Truong LD, Rangdaeng S, Cagle P, Ro JY, Hawkins H, Font RL (1990) The diagnostic utility of desmin. A study of 584 cases and review of the literature. Am J Clin Pathol 93:305–314