Cellular Vaccines Modified with Hyper IL6 or Hyper IL11 Combined with Docetaxel in an Orthotopic Prostate Cancer Model

JACEK MACKIEWICZ[1,2,3], URSZULA KAZIMIERCZAK[1], MAREK KOTLARSKI[1], EWELINA DONDAJEWSKA[1], ANNA KOZŁOWSKA[1], ELIZA KWIATKOWSKA[1,2], ANITA NOWICKA-KOTLARSKA[1], HANNA DAMS-KOZŁOWSKA[1,2], PIOTR JAN WYSOCKI[1] and ANDRZEJ MACKIEWICZ[1,2,4]

[1]*Medical Biotechnology, University of Medical Sciences, Poznan, Poland;*
[2]*Department of Diagnostics and Cancer Immunology, Greater Poland Cancer Centre, Poznan, Poland;*
[3]*Department of Medical and Experimental Oncology, Clinical Hospital of Poznan University of Medical Sciences, Poznan, Poland;*
[4]*BioContract Sp z o.o., Poznan, Poland*

Abstract. *Background: Whole-cell-based vaccines modified with Hyper-IL-6 (H6) and Hyper-IL-11 (H11) have demonstrated high activity in murine melanoma and renal cancer models. Materials and Methods: H6 and H11 cDNA was transduced into TRAMP cells (TRAMP-H6 and TRAMP-H11). An orthotopic TRAMP model was employed. The efficacy of TRAMP-H6 and TRAMP-H11 in combination with docetaxel was evaluated. Immune cells infiltrating tumors were assessed. Results: Immunization with TRAMP-H6 and TRAMP-H11 vaccines extended OS of mice. Addition of docetaxel to TRAMP-H6 and TRAMP-H11 vaccines further extended OS of the animals. Vaccination with TRAMP-H6 alone and TRAMP-H11 combined with docetaxel augmented tumor infiltration by activated $CD8^+$ and $CD4^+$ T-cells and attracted higher number of activated, mature DCs infiltrating tumors. Addition of docetaxel to TRAMP-H6, TRAMP-H11, TRAMP-Adv700 vaccines enhanced the infiltration of the tumor by NK cells. Conclusion: Addition of docetaxel to modified TRAMP vaccines improved clinical benefit of treated mice and enhanced anti-tumor immune response.*

Whole-tumor-cell cancer vaccines deliver a wide spectrum of tumor antigens and successfully activate anti-tumor responses (1,2). At the vaccination site, dendritic cells (DCs) phagocyte tumor antigens released from irradiated tumor cells, and migrate to the regional lymph nodes where they present them in the context of major histocompatibility complex (MHC) class I and II molecules into T-cells (3). Modification of vaccine cells with genes encoding immunostimulatory molecules, including cytokines, increases their immunogenicity.

Interleukin (IL) 6 and IL11 act on cells through IL6 receptor (IL6R) and IL11R complex, respectively. These receptors have similar structures. They consist of two membrane-bound subunits, α, specific for IL6 or IL11, and β (GP130) common for both. The β subunit is expressed on every human or animal cell (4), in contrast to α subunits, which are present on particular cell fractions. Soluble variants of IL6Rα (sIL6R) and sIL11R exist and are functional, thus complexed with corresponding ligands (IL6 or IL11) can bind to GP130 and activate the target cells (5, 6). Thus the spectrum of biological activities of IL6/sIL6R and IL11/sIL11R complexes is much broader than those of IL6 and IL11 alone. sIL6R is present in human serum, while sIL11R has been found only in mouse serum so far. In order to stabilize IL6/sIL6R and IL11/sIL11R soluble complexes, artificial fusion cytokines hyper-IL6 (H6) and hyper-IL11 (H11) were designed and generated by linking of IL6 or IL11 with their respective soluble receptors α (7, 8). H-6 and H-11 demonstrate 10- to 1,000-fold higher biological activities than their soluble counterparts (7). Recent discoveries of tumor-related local and systemic immunosuppression, leading to abrogation of specific anticancer responses, indicate that cancer vaccination needs combination with other therapeutic modalities, which may include inhibition of immune check-points using antibodies against cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) or programmed death 1 protein (PD1) (9). Another option is the combination of vaccines with cytotoxic drugs which may display certain immunomodulatory properties. Low doses of cyclophosphamide prior to cancer vaccination enhanced immune response through suppression of

Correspondence to: Jacek Mackiewicz, MD, Ph.D., Chair of Medical Biotechnology, University of Medical Sciences, 15, Garbary St., 61-866 Poznan, Poland. Tel: +48 618850665 (secretary), Fax: +48 618528502, Mobile: +48 606228277, e-mail: jmackiewicz@ump.edu.pl

Key Words: Cancer vaccine, docetaxel, immunotherapy, murine model, prostate cancer.

$CD4^+/CD25^+$ cells in a murine prostate cancer model (10). Moreover, docetaxel administered after cancer vaccine inoculation was linked to enhanced antitumor response of $CD8^+$, $CD4^+$ and natural killer (NK) cells (11).

Accordingly, we evaluated the therapeutic efficacy of irradiated whole-cell vaccines consisting of transgenic adenocarcinoma of mouse prostate cells (TRAMP), genetically modified to secrete H6 or H11 combined with docetaxel in a murine orthotopic TRAMP model.

Materials and Methods

Animals. In all experiments, male C57BL6/J mice, aged 8-12 weeks, were used. The animals were purchased from the Polish Academy of Sciences (Lublin, Poland). Animals were housed in rooms with a 12-h day/night cycle with unlimited access to food and water. All experiments were carried out according to the guidelines approved by the Local Ethical Committee for Animal Research (No 9/2009) at the University of Medical Sciences (Poznań, Poland).

Tumor cells. Murine prostate cancer (TRAMP-C2) cell line was used throughout *in vitro* and *in vivo* experiments. TRAMP-C2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium DMEM (Invitrogen Corp., Carlsbad, CA, USA) with 4 mM L-glutamine (Invitrogen Corp.) adjusted to contain 1.5 g/l sodium bicarbonate (Invitrogen Corp.) and 4.5 g/l glucose (Invitrogen Corp.) supplemented with 0.005 mg/ml bovine insulin (Gibco, Grand Island, NY, USA) and 10 nM dehydroisoandrosterone (Invitrogen Corp.), enriched with 5% fetal bovine serum, and 5% of Nu-Serum IV (Invitrogen Corp.). 293 cell line (Manassas, VA, USA) was used for adenovirus propagation.

Cells were cultured in 78 cm^2 culture plates at 37˚C in a fully humidified atmosphere of 5% CO_2/95% air and passaged every 2-3 days.

Recombinant adenoviral vectors carrying H6 and H11. An E1-deleted adenoviral recombinant of human strain 5 was modified to encode H6 (AdH6) or H11 (AdH11) was constructed at the Department of Cancer Immunology at the University of Medical Sciences (Poznan, Poland). Mock-transduced E1-deleted adenoviral vector (AdV700), used as control, was kindly provided by Dr H.Ertl (Wistar Institute, Philadelphia, PA, USA). The viruses were propagated and titrated on E1-transfected 293 cells as described previously (12).

Transduction of TRAMP cells with H6 or H11. Gene-modified prostate cancer vaccine consists of TRAMP-C2 (TRAMP) cells transduced with cDNA-encoding Hyper-IL6 (TRAMP-H6) or Hyper-IL11 (TRAMP-H11). TRAMP cells at 85% confluence were incubated with AdH6 or AdH11 or AdV700 vector for 48 h. Following transduction cells were harvested and irradiated with a dose of 80 Gy (Gamma-Cell 1000, RTA, Ottawa, ON, Kanada). After irradiation, cells were aliquoted and stored in liquid nitrogen until use. A working cell bank was created (all experiments were carried-out using single transduced cells).

Identification of H6 and H11 secreted by vaccine cells. TRAMP-H6 and TRAMP-H11 cells were thawed and cultured for 24 h. Then the culture medium was collected and the concentration of transgenic protein was assayed using enzyme-linked immunosorbent assay (ELISA) for human sIL6R (R&D, Minneapolis, MN, USA) or human IL11 (Anogen, Mississanga, ON, Canada) and validated against sIL6R or IL11 protein standards, respectively. sIL6R is a part of H6 fusion protein and reacts with antibody against human sIL6R, while IL11 is a part of H11 fusion protein thus it reacts with antibody to human IL11. TRAMP-H6 cells secreted 1.5 μg/ml IL6R, while TRAMP-H11 cells secreted 0.08 μg/ml IL11 per 10^6 cells/24 h, respectively.

Chemotherapy. Docetaxel was purchased from Sanofi-Aventis (Paris, France) and diluted in phosphate buffered saline (PBS) for intraperitoneal injection.

In vivo studies. Each study group included 10 animals. All experiments were repeated twice. In order to establish an orthotopic tumor model, mice were inoculated with 2×10^5 TRAMP cells suspended in 20 μl PBS into the dorsolateral lobe of the prostate. Prior to tumor cell administration, mice were anesthetized with an intraperitoneal injection of 0.7 mg/g Avertin (Sigma-Aldrich, St. Louis, MO, USA) anesthesia. Experiments in the orthotopic murine prostate cancer rejection model were carried-out in prophylactic and therapeutic settings. Orthotopic and ectopic (subcutaneous tumors) models were used to determine the tumorigenicity of vaccine cells (tumorigenicity model). All animals that were alive at the end of the experiment were euthanized and autopsy was performed.

Tumorigenicity model: In the first set of experiments, the effect of H6 and H11 on tumorigenicity of vaccine cells was evaluated. Non-irradiated wild type (WT) TRAMP-WT, TRAMP-H6, TRAMP-H11 or TRAMP-AdV700 (TRAMP-AdV) cells (10^6) suspended in 100 μl PBS were administered *s.c.* into the left hip or directly into prostate. Tumor volume was calculated according to the formula $[(W^2 \times L)/2]$, where W is the shorter diameter and L is longer diameter of the tumor. The tumor growth was monitored twice a week.

In the second set of experiments, 6 weeks after the implantation of non-irradiated TRAMP-WT, TRAMP-H6, TRAMP-H11 and TRAMP-AdV, the mice were sacrificed and the tumor volume was assessed as above and the formation of metastases in the lungs was evaluated (macroscopic evaluation of lung tissue stained with dye ink).

Prophylactic setting: In this set of experiments, mice were immunized *s.c.* five times at 4-day intervals with 10^6 TRAMP-irr (irr; irradiated), TRAMP-H6, TRAMP-H11, TRAMP-AdV cells or PBS starting at day 0. At day 14 from the start of immunization TRAMP-WT cells were administered into the prostate of all animals. The primary end-point was overall survival (OS).

In the second set of experiments, 12 weeks from the start of immunization the mice were sacrificed and the prostate size was evaluated and the occurrence of metastases to the lungs was assessed as above. Tumor volume was calculated according to the formula presented above.

Therapeutic setting: In this set of experiments, mice were inoculated with TRAMP-WT cells into the prostate on day 0. After 24 h, *s.c.* immunization (eight times at 4-day intervals) with TRAMP-irr, TRAMP-H6, TRAMP-H11, TRAMP-AdV, or PBS was initiated. In the subsequent groups, mice vaccinated with TRAMP-H6 and TRAMP-H11 received docetaxel. Docetaxel was administered intraperitoneally at a dose of 0.5 mg on days 15, 17, 19, 21 and 23. In the remaining groups, mice received docetaxel alone. The animal's survival was monitored twice a week.

In the second set of experiments, 12 weeks following the beginning of immunization, the mice were sacrificed, the prostate size was evaluated and the formation of metastases in lungs was assessed as mentioned above. Tumor volume was calculated according to the formula presented above.

Immunological analyses. Mice were immunized *s.c.* three times at 7-day intervals with TRAMP-H6, TRAMP-H11, TRAMP-AdV with or without DXL (administered *i.p.* on the day of vaccination). Ten days after the last inoculation, mice received an intracutaneous injection of 100 μl Matrigel containing 1×10^6 of TRAMP-WT cells. Ten days later, mice were sacrificed and Matrigel nodules, and spleens were excised, minced, pooled (five mice per group) and infiltrating mononuclear cells were isolated by gradient centrifugation.

Analysis of anti-tumor cellular responses. The single-cell suspension was then stained with anti-CD3 (fluorescein isothiocyanate, FITC), anti-CD80 (phycoerythrin; PE), anti-CD4 (PE-Cy7), anti-CD8 (PE-Cy7), anti-CD86 (FITC), anti-CD40 (antigen-presenting cells; APCs), anti-IA/IE (PE), anti-CD4 (FITC), anti-CD154 (PE), anti-CD49b (PE), anti-CD45/B220 (APC-Cy7), anti-CD19 (FITC), anti-CD11b (PE), anti-GR1 (myeloid differentiation antigen; Ly-6G) biotinylated, anti-CD69 (PE) (BD Biosciences, San Jose, CA, USA); anti-CD62L (APC), anti-CD44 (PE), anti-CD25 (APC), anti-forkhead box P3 (FOXP3) (PE) (Biolegend, San Diego, CA, USA). The cells were subsequently analyzed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA).

Proliferation test. The isolated splenocytes were seeded into 96-well plate at concentration of 1×10^6 cells per well and were incubated in the presence or absence of either TRAMP cells (for CD8 assay) or cell lysate (CD4 assay) for 72 h. Upon [^{3}H] thymidine incorporation into DNA, cells were harvested and splenocyte proliferation was measured using a beta scintillation counter. Data were computed as the difference in counts per minute (cpm) of the arithmetic mean of stimulated and control splenocytes. The results are referred to as Δ cpm. In the above tests, TRAMP-AdV was used as vehicle (untreated control group).

Statistical analyses. Survival curves were analyzed by log-rank (Mantel-Cox) test. For the comparison of tumor take rate between groups, a χ^2 test was used.

Results

H6 and H11 reduce tumorigenicity of TRAMP cells in ectopic and orthotopic murine prostate cancer models. H6 and H11 molecular adjuvants delayed formation and reduced TRAMP tumor growth kinetics (Figure 1).

In the ectopic model, mice injected *s.c.* with non-irradiated TRAMP-H6 and TRAMP-H11 developed tumors after 35 (mean volume throughout the whole experiment was 0.05 cm^3) and 29 days (mean volume=0.1 cm^3), respectively. The time of tumor formation in mice inoculated with TRAMP-WT (mean volume=0.55 cm^3) and TRAMP-AdV (mean volume=0.1 cm^3) was the same and occurred after 20 days (Figure 1A).

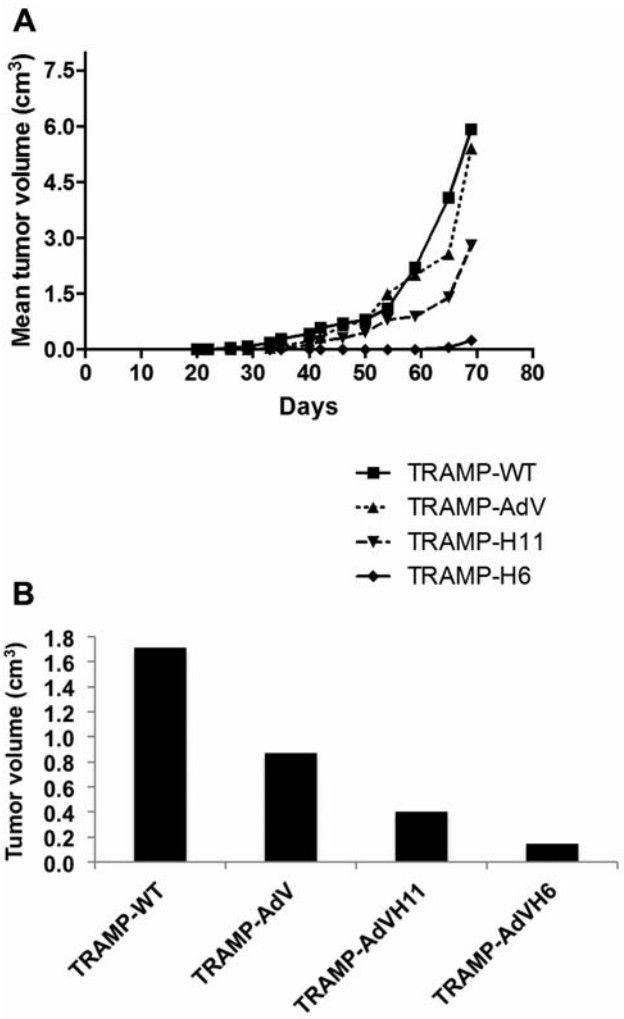
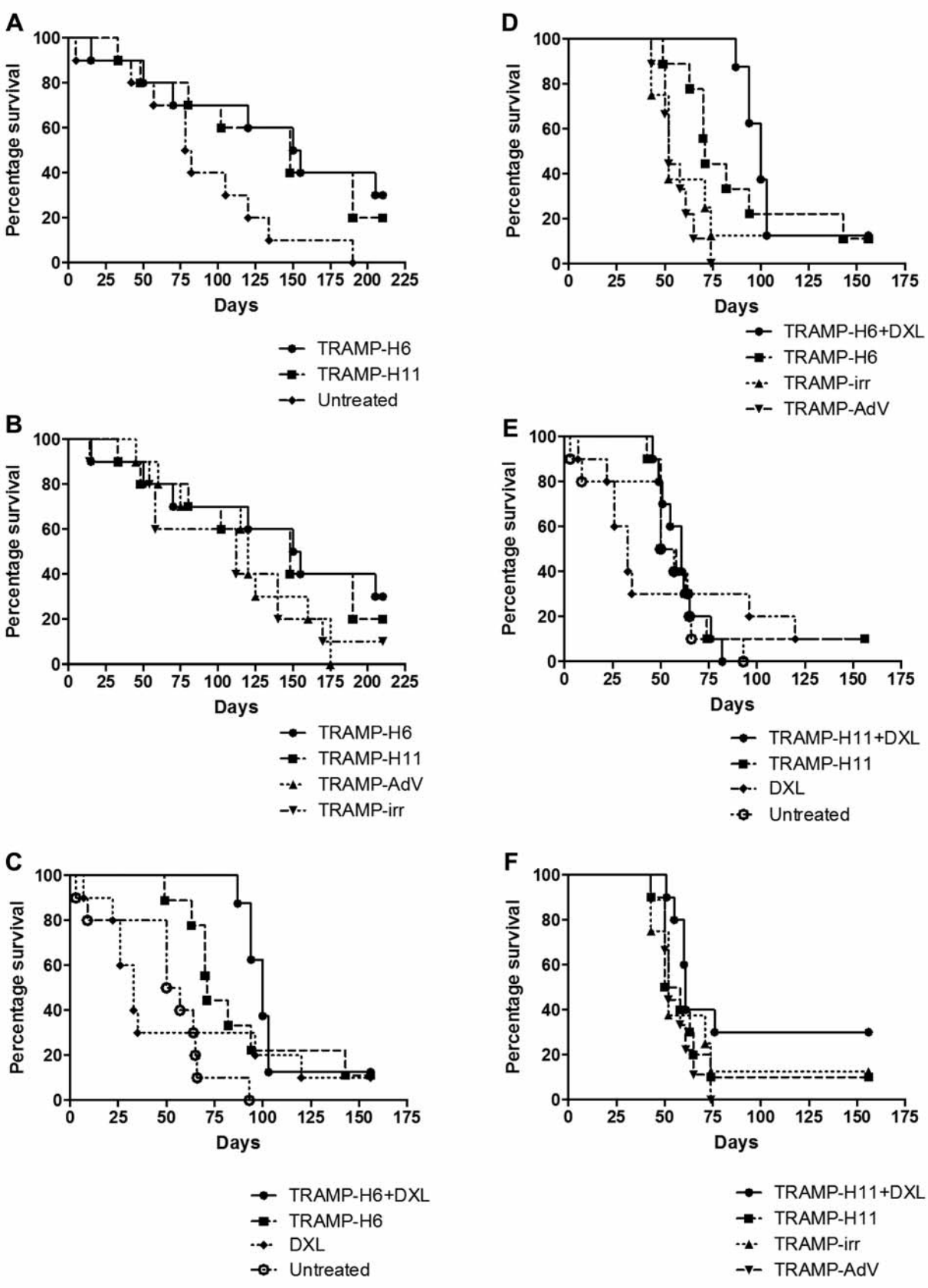


Figure 1. *Ectopic and orthotopic murine prostate cancer model. A: In the tumorigenicity model, ectopic tumor growth kinetics were assessed after s.c. implantation of non-irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock transduced E1-deleted adenoviral vector (TRAMP-AdV) or non-modified TRAMP cells (TRAMP-WT). B: In the orthotopic model, tumor volume was evaluated after six weeks from inoculation of TRAMP-H6, TRAMP-H11, TRAMP-AdV or TRAMP-WT into the prostate.*

In the orthotopic model, 6 weeks from TRAMP-WT, TRAMP-H6, TRAMP-H11 or TRAMP-AdV inoculation, the mice were sacrificed and the tumor volume was assessed. The smallest tumor volume was observed in mice receiving TRAMP-H6 and TRAMP-H11, with mean values of 0.15 cm^3 and 0.4 cm^3, respectively. The highest mean volume was noted in mice bearing TRAMP-WT cells (1.71 cm^3). The mean volume in mice inoculated with TRAMP-AdV was 0.87 cm^3 (Figure 1B). None of the mice developed lung metastases.


A
Percentage survival
Days
TRAMP-H6
TRAMP-H11
Untreated
B
Percentage survival
Days
TRAMP-H6
TRAMP-H11
TRAMP-AdV
TRAMP-irr
C
Percentage survival
Days
TRAMP-H6+DXL
TRAMP-H6
DXL
Untreated
D
Percentage survival
Days
TRAMP-H6+DXL
TRAMP-H6
TRAMP-irr
TRAMP-AdV
E
Percentage survival
Days
TRAMP-H11+DXL
TRAMP-H11
DXL
Untreated
F
Percentage survival
Days
TRAMP-H11+DXL
TRAMP-H11
TRAMP-irr
TRAMP-AdV

Vaccination with TRAMP cells modified with H6 and H11 prevents tumor growth. Mice were immunized with TRAMP-irr, TRAMP-H6, TRAMP-H11, TRAMP-AdV at day 0. The control group did not receive any treatment (untreated). At day 14, TRAMP-WT cells were administered into the prostate. The longest median OS was observed in mice treated with TRAMP-H6 (152 days), while in mice vaccinated with TRAMP-H11, TRAMP-AdV, TRAMP-irr and in untreated controls, the median OS was 148, 120, 112 and 80 days respectively (Figure 2).

In the subsequent experiment, mice were vaccinated according to the same scheme. TRAMP-WT cells were administered as above. After 12 weeks, mice were sacrificed and tumor volume was assessed. All untreated mice developed tumors in the prostate. Six, 7, 8 and 8 mice immunized with TRAMP-H6, TRAMP-H11, TRAMP-AdV and TRAMP-irr, respectively developed tumors in the prostate. The smallest tumor volume was observed in mice receiving TRAMP-H6 and TRAMP-H11, with mean values of 0.15 cm^3 and 0.45 cm^3, respectively. The highest mean volume was noted in mice vaccinated with TRAMP-irr (2.5 cm^3) and untreated mice (2.8 cm^3). The mean volume in mice inoculated with TRAMP-AdV was 2.0 cm^3 (Figure 3A). None of the mice developed lung metastases.

Therapeutic immunization with TRAMP-H6 and TRAMP-H11 combined with DXL is associated with prolonged OS. Mice were inoculated with TRAMP-WT cells into the prostate at day 0. After 24 h, mice were immunized with TRAMP-irr, TRAMP-H6, TRAMP-H11, or TRAMP-AdV. In the subsequent groups, mice received combination of TRAMP-H6 or TRAMP-H11 with docetaxel. Control mice did not receive any treatment. Mice vaccinated with TRAMP-H6, TRAMP-H11, TRAMP-AdV, TRAMP-irr or untreated controls demonstrated the following median OS of 71, 54, 52, 52 and 53 days, respectively.

The addition of docetaxel to TRAMP-H6 extended median OS to 100 days. Mice treated with TRAMP-H11 and

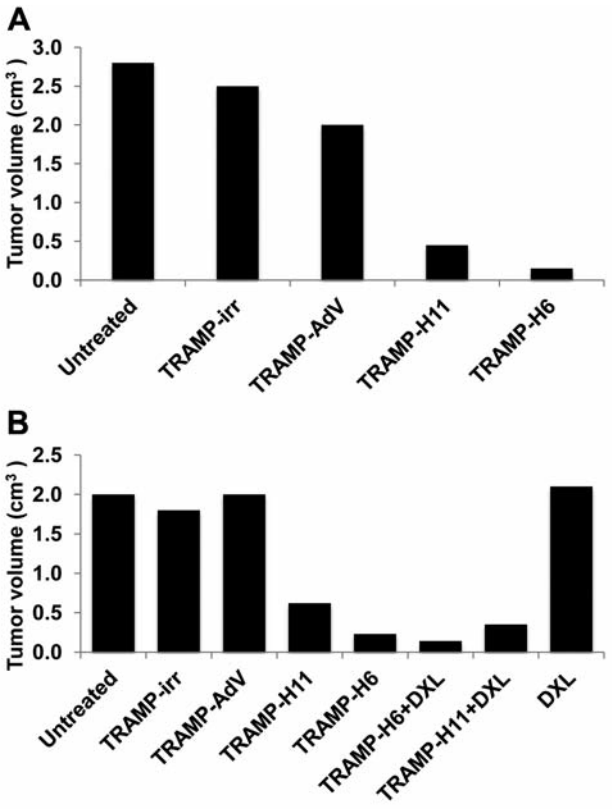


Figure 3. *Orthotopic murine prostate cancer model in the prophylactic (A) and therapeutic (B) setting: response assessment in treated mice. A: Fourteen days after the start of immunization with irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock transduced E1-deleted adenoviral vector (TRAMP-AdV) or non-modified cells (TRAMP-irr), mice were inoculated with non-irradiated, non-modified TRAMP cells (TRAMP-WT) into the prostate. Tumor volume consisting of TRAMP-WT was evaluated after 12 weeks from the start of immunization. The lowest mean tumor volume was observed in mice treated with TRAMP-H6 and TRAMP-H11. B: Mice were inoculated with TRAMP-WT cells into the prostate and after 24 h, immunization with TRAMP-irr, TRAMP-H6, TRAMP-H11, TRAMP-AdV, with or without docetaxel (DXL), was started. Twelve weeks after the beginning of vaccination, the tumor volume was assessed. The lowest mean tumor volume was observed in mice receiving TRAMP-H6, TRAMP-H11, TRAMP-H6 with DXL and TRAMP-H11 with DXL. In both experiments, control mice did not receive treatment.*

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Figure 2. *Orthotopic murine prostate cancer model – survival analysis in treated mice. A and B: In the prophylactic model, mice were immunized with irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock transduced E1-deleted adenoviral vector (TRAMP-AdV) or non-modified (TRAMP-irr) cells followed by orthotopic implantation of non-irradiated, non-modified TRAMP cells (TRAMP-WT) into the prostate. Control mice were untreated. The significant difference in median overall survival (OS) was only observed between group immunized with TRAMP-H6 and the untreated control (p=0.03). In the therapeutic model, mice were inoculated with TRAMP-WT cells into the prostate. Subsequently, mice were immunized with TRAMP-H6 with or without docetaxel (DXL), TRAMP-H11 with or without docetaxel, TRAMP-AdV or TRAMP-irr. An additional group received DXL alone. Control mice were untreated. A significant difference in median OS was observed in mice treated with TRAMP-H6 combined with DXL (p<0.0001) and with TRAMP-H6 alone (p=0.01) comparing to the untreated control (C). Differences in median OS were also noted in mice treated with TRAMP-H6 and DXL, and with TRAMP-H6 compared to those treated with TRAMP-AdV (p<0.0001; p=0.0055) or TRAMP-irr (p=0.04; p=0.35) (D). There was no statistically significant difference in median OS between any of the studied groups except those treated with TRAMP-H11 and DXL vs. TRAMP-AdV (p=0.03). However a trend towards extended survival was observed when comparing mice treated with TRAMP-H11 and DXL or TRAMP-H11 with the untreated control (E, F).*

Table I. *Flow cytometric analysis of activated T-lymphocytes infiltrating non-irradiated TRAMP cells (Matrigel nodules) in mice receiving irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock transduced E1-deleted adenoviral vector (TRAMP-AdV) with or with docetaxel (DXL).*

Population (% of cells)	Immunization					
	TRAMP-H6	TRAMP-H6 DXL	TRAMP-H11	TRAMP-H11 DXL	TRAMP-ADv700	TRAMP-ADv700 DXL
$CD8^+CD25^+$	41.9	29.0	37.0	41.8	19.5	10.5
$CD8^+CD69^+$	40.3	23.9	23.9	58.0	16.8	19.3
$CD4^+CD25^+$	7.8	6.6	7.6	14.9	4.0	5.2
$CD4^+CD69^+$	14.4	10.0	12.5	30.7	6.2	7.6

Table II. *Flow cytometric analysis of dendritic cells infiltrating Matrigel nodules containing non-irradiated TRAMP cells in mice receiving irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock transduced E1-deleted adenoviral vector (TRAMP-AdV) with or without docetaxel (DXL).*

Population (% cells)	Immunization					
	TRAMP-H6	TRAMP-H6 DXL	TRAMP-H11	TRAMP-H11 DXL	TRAMP-ADv700	TRAMP-ADv700 DXL
CD11c/CD80	22.7	5.0	4.5	23.0	3.8	2.6
CD11c/CD86	10.5	7.2	6.5	12.1	5.3	2.6
CD11c/CD40	10.7	4.9	5.8	9.7	8.8	4.5

docetaxel demonstrated a 61-day median OS. In mice receiving docetaxel alone, a median OS of 33 days was recorded (Figure 2C-F).

In the subsequent experiment, mice were vaccinated according to the scheme, as above. TRAMP-WT cells were administered into the prostate as described and mice were sacrificed 12 weeks after the beginning of vaccination. All untreated mice developed tumors in the prostate. Eight to ten mice vaccinated with TRAMP-H6+DXL, TRAMP-H6, TRAMP-H11+DXL, TRAMP-H11, TRAMP-AdV, TRAMP-irr, and docetaxe developed macroscopic tumors in the prostate (Figure 3B). None of the mice developed lung metastases.

Immunization with TRAMP-H6 and TRAMP-H11 combined with DXL leads to infiltration of TRAMP tumors by activated $CD8^+$ and $CD4^+$ T-lymphocytes. To elucidate the tumor-rejection mechanisms induced by vaccination with and without chemotherapy, we compared the number of activated $CD8^+$ and $CD4^+$ T-lymphocytes infiltrating tumors (Matrigel nodules containing TRAMP cells).

Mice immunized with TRAMP-H6, TRAMP-H11, TRAMP-AdV700 with or without docetaxel were inoculated intracutaneously with TRAMP cells in Matrigel. Ten days later, Matrigel and spleens were excised, and cells were isolated, stained and analyzed. The highest number of activated CD8+CD25+ tumor-infiltrating cells (Matrigel nodules) was observed in mice receiving TRAMP-H6 and TRAMP-H11 with docetaxel (Figure 4A). Much higher proportions of activated $CD8^+CD69^+$ (Figure 4B), $CD4^+CD69^+$ (Figure 4C, Table I) and $CD4^+CD25^+$ (Figure 4D, Table I) cells were noted in the mice treated with TRAMP-H6 compared to those receiving TRAMP-H6 with docetaxel, TRAMP-H11, TRAMP-Adv700, TRAMP-Adv700 with docetaxel. The highest proportions of $CD8^+CD69^+$, $CD4^+CD69^+$, and $CD4^+CD25^+$ cells were observed in mice vaccinated with TRAMP with docetaxel (Figure 4B-D, Table I).

Vaccination combined with docetaxel did not influence the proportion of the above listed cells in the spleens compared to those in mice administered vaccine alone.

Immunization with TRAMP-H6 and TRAMP-H11 with docetaxel provides a strong stimulatory signal for DCs. As the generation of specific antitumor response depends on priming of naïve $CD4^+$ and $CD8^+$ lymphocytes by professional APCs, we decided to evaluate the phenotype of DCs infiltrating the tumor site (Matrigel nodules).

The highest proportion of activated and mature DCs expressing co-stimulatory molecules such as CD40 (Figure 5A, Table II), CD80 (B7.1) and CD86 (B7.2) (Figure 5B, Table II) infiltrating the tumor site was observed in mice receiving TRAMP-H6 and TRAMP-H11 combined with docetaxel. The number of DCs in other groups was similar.

Addition of docetaxel to the vaccine enhances infiltration of NK cells at the tumor site. To evaluate the involvement of NK cells in tumor rejection in mice immunized with TRAMP-H6, TRAMP-H11, TRAMP-Adv700, TRAMP-Adv700 with and without docetaxel, we analyzed the expression of CD49b in cells infiltrating the TRAMP tumor (Matrigel nodule).

The treatment of mice with TRAMP gene-modified vaccine combined with docetaxel resulted in higher infiltration of the tumor by NK cells than did the vaccine alone.

Addition of docetaxel treatment to the TRAMP-H11 vaccination increased the proportion of WT tumor-infiltrating NK cells by two-fold compared to TRAMP-H11 treatment alone (16% *vs*. 8%, respectively). The proportion of NK cells infiltrating the tumor was also higher in mice receiving TRAMP-H6 with docetaxel (9.1%) than TRAMP-H6 alone (5.3%) (Figure 6).

Vaccination with TRAMP-H11 combined with docetaxel enhances infiltration of the tumor by B-lymphocytes. To assess the involvement of B-lymphocytes in the immune response in vaccinated mice with and without chemotherapy, we analyzed the expression of CD45/B220 and CD19 in cells infiltrating the WT tumor (Matrigel nodule).

The most significant fraction of B-lymphocytes infiltrating Matrigel nodules was observed in mice treated with combination of TRAMP-H11 with docetaxel (12.3%). The fractions of B-lymphocytes in other groups treated were similar and ranged between 4.4 and 6.7% (Figure 6).

Induction of immune response by cellular vaccine combined with docetaxel. To evaluate the induction of T-cell immunity, splenocytes were obtained following TRAMP-H6, TRAMP-H11, am TRAMP-AdV administration with and without docetaxel and analyzed for $CD4^+$ T-cell proliferation in response to vaccine antigens. Proliferation was observed in all groups treated with designer cytokines alone and in those combined with docetaxel. There was marginal proliferative response in the group which received TRAMP-AdV alone. The strongest splenocyte proliferation was observed in mice receiving TRAMP-H6 alone (~10 times stronger compared to control group) or TRAMP-H11 combined with docetaxel.

Discussion

There are seven major findings of this study: (i) modification of TRAMP cells with artificial molecular adjuvants, especially H6, increased the efficacy of TRAMP-based whole-cell vaccination; (ii) TRAMP-H6 and TRAMP-H11 vaccines extended the OS of mice in the orthotopic TRAMP model in both prophylactic and therapeutic settings; (iii) combination of TRAMP-H6 and TRAMP-H11 vaccination with docetaxel treatment further extended the OS of mice in the therapeutic orthotopic prostate cancer model; (iv) immunization with TRAMP-H6 alone or TRAMP-H11 combined with docetaxel augmented tumor infiltration by activated $CD8^+$ and $CD4^+$ T-lymphocytes leading to a higher proportion of activated, mature DCs infiltrating tumors; (v) addition of DXL to TRAMP-H6, TRAMP-H11, TRAMP-Adv700 vaccines enhanced the infiltration of the tumor by NK cells; (vi) vaccination with TRAMP-H11 combined with docetaxel enhanced tumor infiltration by B-lymphocytes; (vii) TRAMP-H6 immunization and TRAMP-H11 combined with docetaxel were linked to the strongest antigen-specific $CD4^+$ T-lymphocyte proliferation.

TRAMP mice develop prostate tumors with 100% frequency, and these metastasize to lymph nodes and lungs, and occasionally to kidney, adrenal gland and bones (13). The properties of the model has led to its wide employment in the discovery and development of human prostate cancer (adenocarcinoma) therapy. However, TRAMP mice consistently develop phyllodes-like epithelial-stromal tumors in seminal vesicles, which may become fully malignant (sarcoma, carcinosarcoma) and metastasize (14). Furthermore, neuroendocrine carcinoma arising in the prostate has also been reported in the TRAMP models (15, 16).

In order to use the most optimal strategy, we created an orthotopic murine prostate cancer model based on TRAMP-C2 cell line derived from prostate tumor of TRAMP mice obtained by modification with SV40 large T-antigen with the rat probasin promoter (17). After injection of TRAMP-C2 cells into the dorsolateral lobe of the prostate, all of the animals developed primary tumors with metastases (data not shown). However, we did not observe any metastases to bones or lungs, but rather locoregional growth of primary tumor with metastases to the regional lymph nodes.

For construction of model prostate cancer vaccines, we genetically modified the TRAMP cells to secrete H6 or H11. The efficacy of H6- and H11-modified whole tumor cell vaccines was shown in murine orthotopic renal cell carcinoma and melanoma models, including H6-vaccine in human melanoma trials (18-20). H6 possesses the ability to enhance $CD8^+$ T-cell trafficking to lymph nodes. In pre-clinical murine models, administration of H6 prior to adoptive transfer of tumor-specific effector $CD8^+$ T-cells delayed tumor growth (21). Amongst others, IL11 was found to induce antitumor response and stimulate B-lymphocytes to produce antibodies (22, 23). IL11 is active in mice and displays anti-melanoma activity when used as a molecular adjuvant in whole-cell melanoma vaccine formulation (24). In the induction phase of the immune response, H6 and H11 inhibited T-regulatory cell ($CD4^+CD25^+FOXP3$) formation and activated recruitment and maturation of DCs, and thus increased tumor antigen presentation. Downstream of immunization, tumors were densely infiltrated by $CD8^+$, $CD4^+$ and NK cells (8, 19).

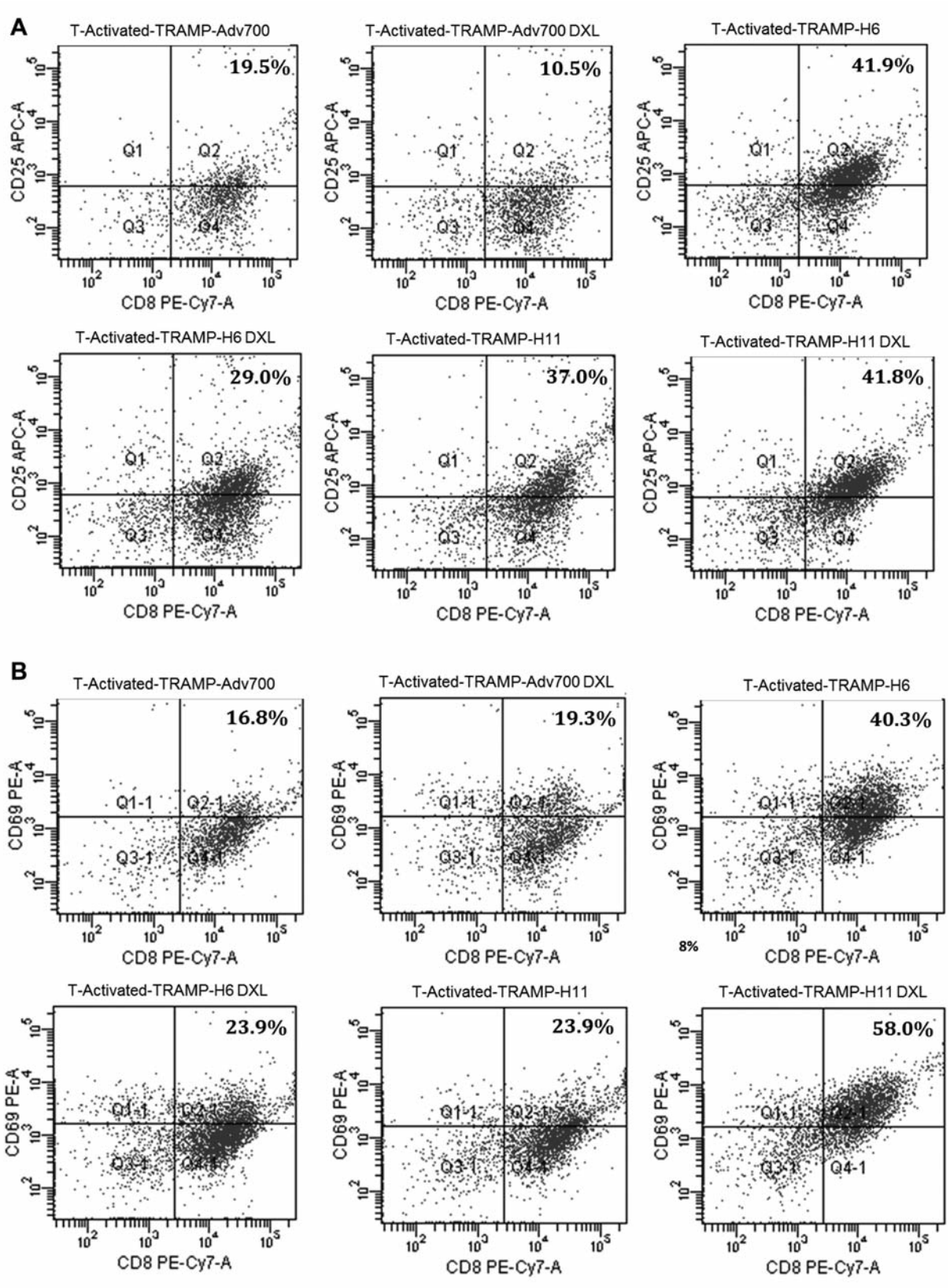

Figure 4. *Continued*

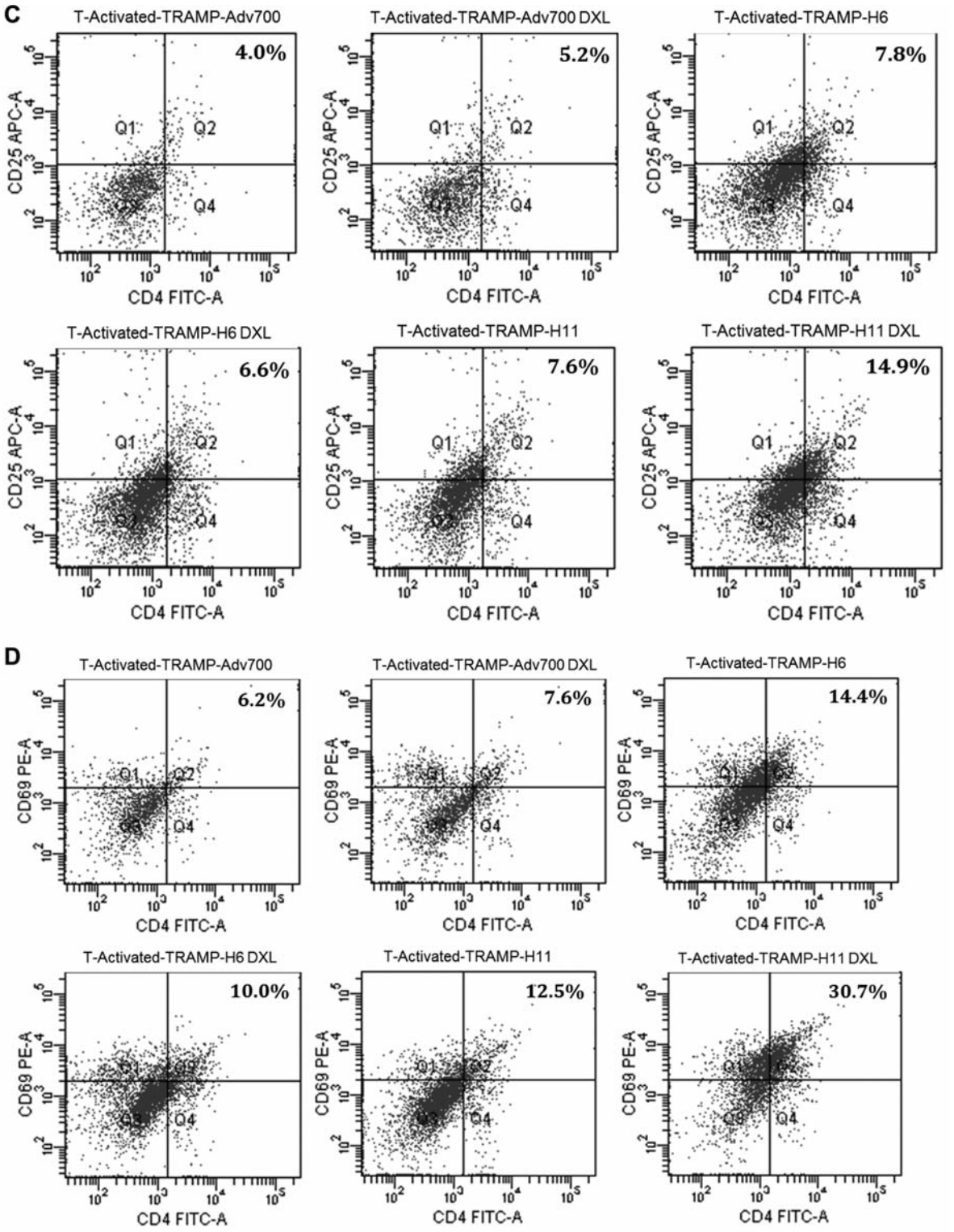

Figure 4. *$CD8^+$ and $CD4^+$ T-lymphocytes infiltrating tumors. Mice were immunized with irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock-transduced E1-deleted adenoviral vector (TRAMP-AdV) cells with or with docetaxel (DXL) followed by intracutaneous injection of non-irradiated, non-modified TRAMP cells (TRAMP-WT) suspended in Matrigel. After 10 days tumors were excised, pooled within each experimental group and tumor-infiltrating cells were isolated and analyzed with flow cytometry. At the site of tumor cell injection, an increased proportion of $CD8^+CD25^+$ cells in mice treated with TRAMP-H6 and TRAMP-H11 was observed (A). The highest number of $CD8^+CD69^+$ (B), $CD4^+CD69^+$ (C) and $CD4^+CD25^+$ (D) was noted in mice vaccinated with TRAMP-H11 with DXL (representative experiment).*

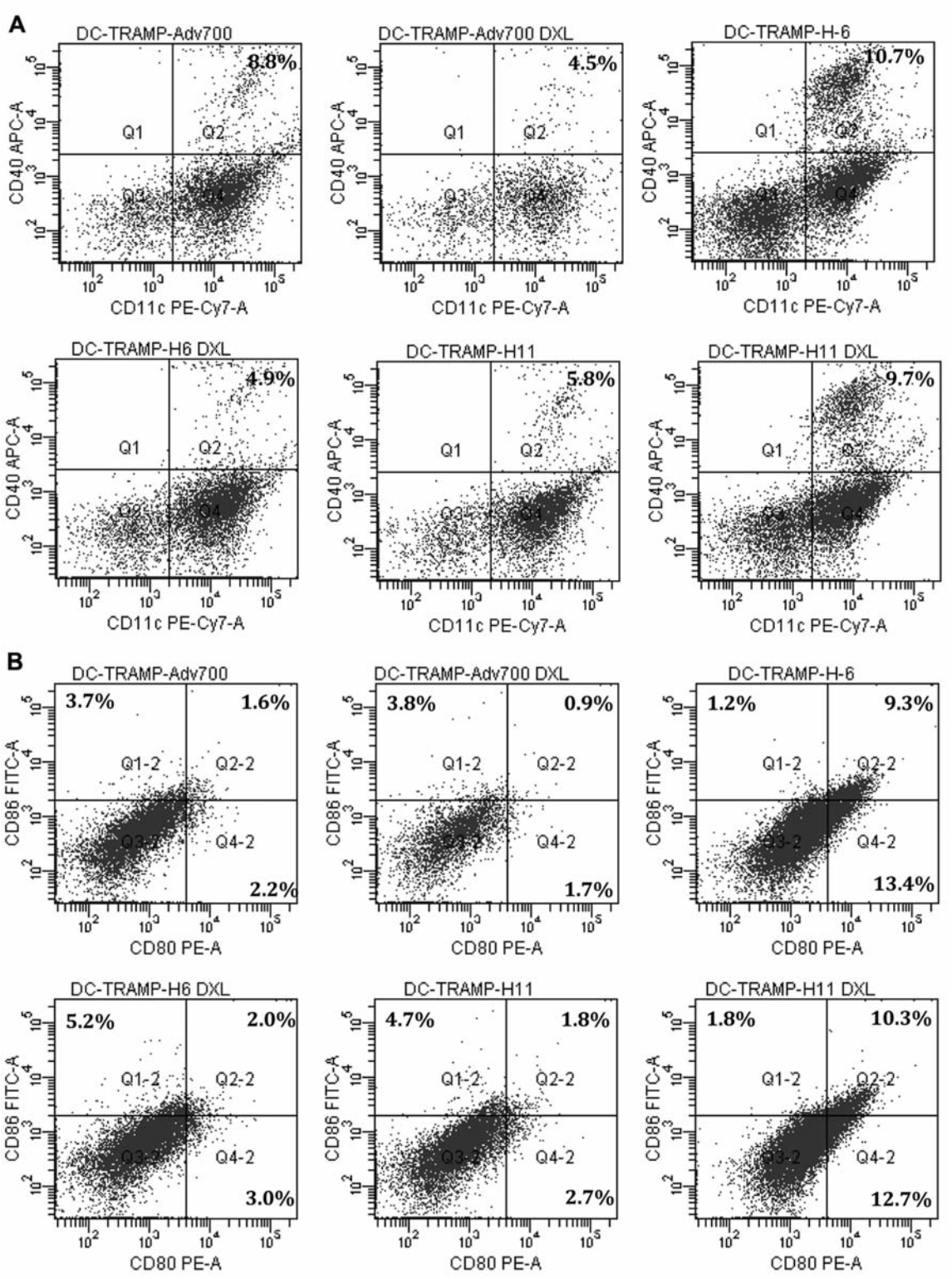

Figure 5. *Dendritic cells (DCs) infiltrating tumors. Mice were immunized with irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock-transduced E1-deleted adenoviral vector (TRAMP-AdV) cells with or with docetaxel (DXL) followed by intracutaneous injection of non-irradiated, non-modified TRAMP cells (TRAMP-WT) suspended in Matrigel. After 10 days, tumors were excised, pooled within each experimental group and tumor-infiltrating cells were isolated and analyzed with flow cytometry. At the site of tumor cell injection, an increased proportion of $CD40^+$ (A), $CD80^+$ [Q2+Q4] and $CD86^+$ [Q1+Q2] (B) DCs was observed in groups vaccinated with TRAMP-H6 and TRAMP-H11 with DXL (representative experiment).*

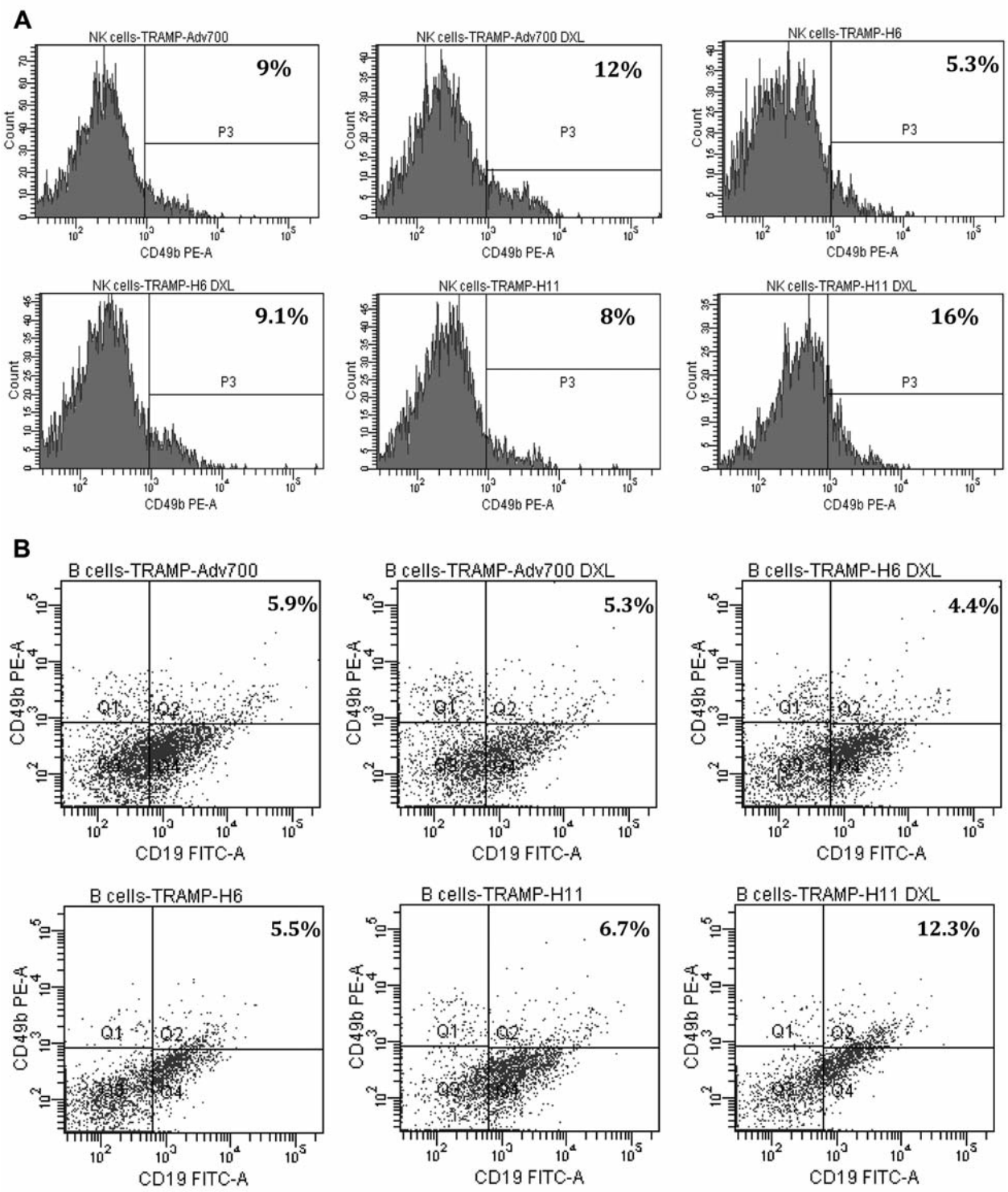

Figure 6. *Natural killer cells (NK) and B-cells infiltrating tumors. Mice were immunized with irradiated TRAMP cells modified with Hyper-IL-6 (TRAMP-H6) or Hyper-IL-11 (TRAMP-H11), or mock transduced E1-deleted adenoviral vector (TRAMP-AdV) cells with or with docetaxel (DXL) followed by intracutaneous injection of non-irradiated, non-modified TRAMP cells (TRAMP-WT) suspended in Matrigel. After 10 days, tumors were excised, pooled within each experimental group and tumor-infiltrating cells were isolated and analyzed with flow cytometry. A: At the site of tumor cell injection, the highest number of NK cells was observed in mice receiving TRAMP-H11 and DXL compared to all other experimental groups. B: The highest percentage of B-cells infiltrating Matrigel was observed in mice receiving TRAMP-H11 and DXL compared to all other experimental groups (representative experiment).*

We demonstrated an increased immunogeneicity of both TRAMP vaccines modified with designer cytokines in ectopic and orthotopic murine models, where delayed tumor formation and growth of non-irradiated TRAMP-H6 and TRAMP-H11 cells were observed. Furthermore, in the prophylactic setting, TRAMP-H6 and TRAMP-H11 vaccines were able to induce tumor rejection. Similar findings were observed in our earlier studies in the renal cell carcinoma (RENCA) model, where RENCA cells were transduced with H6 or H11 cDNAs. These studies demonstrated that RENCA-H6 and RENCA-H11 vaccines had very high therapeutic potential and significantly extended the OS of animals bearing orthotopic RENCA tumors (prophylactic and therapeutic settings) (18, 19). Moreover, TRAMP-irr cells mock-modified with adenovirus (AdV700) or non-modified TRAMP-irr cells can also induce an immune response, however, their immunogenicity is lower. It is generally accepted that the *in vivo* immunogenic potential of TRAMP cells is poor (17). However, due to irradiation, MHC I and II molecule expression is up-regulated in these cells (25). Earlier studies demonstrated that modification with adenoviral non-coding vector influenced the immunogenicity of TRAMP cells (26).

This study is one of the few reports of combining genetically-modified whole-cell cancer vaccines with chemotherapy. In the past, it was believed that the antitumor effect of cytotoxic agents was only due to their direct toxicity towards tumor cells. Today it is recognized that chemotherapy may also modulate the antitumor immune response and by combination with cancer vaccines in particular regimens and appropriate dosing, the efficacy of active immunotherapy can be enhanced. Examples include chemotherapy that specifically targets suppressor cells such as myeloid-derived suppressor cells (MDSC) or regulatory T-cells (27). Moreover, chemotherapy can lead to immunogenic death of tumor cells that may further activate T-cell killing of cancer cells (28-30).

Docetaxel is highly effective against various types of human cancer. Its mechanism of action involves blocking microtubule de-polarization, leading to cell apoptosis (31). However, docetaxel also displays immunomodulating features, including stimulation of the production of pro-inflammatory cytokines (tumor necrosis factor-α, IL1, and IL12) or induction of macrophage-mediated tumor killing, and increasing lymphokine_activated killer and NK cell antitumor activity (32-34). The above findings contributed to combining immunotherapy with docetaxel in pre-clinical studies. In a murine melanoma model, whole-cell vaccine modified with granulocyte-macrophage colony-stimulating factor combined with docetaxel extended mouse survival (35). In another study in a murine colon adenocarcinoma model, docetaxel combined with recombinant viral vaccine was superior to either agent used alone in reducing tumor burden. The study demonstrated that docetaxel modulated $CD4^+$, $CD8^+$, $CD19^+$, NK cell and regulatory T-cell populations in non tumor-bearing mice (36).

The therapeutic potential of TRAMP-H6 and TRAMP-H11 vaccines with or without docetaxel was evaluated using the scheme of immunization and dosing as in our earlier studies in melanoma and RENCA pre-clinical models (18, 19). Scheme and dosing of docetaxel (0.5 mg *i.p.*) was adopted from other studies conducted in various murine tumor models (36-38). In the study, we did not observe any serious toxicity or increased mortality of mice receiving docetaxel. Of great concern in chemotherapy are hematological adverse events, which occur very frequently in treated patients. Garnet *et al.* evaluated the effect of 0.5 mg docetaxel (given three times *i.p.* every 2 days) on bone marrow in mice. After 14 days post-treatment, neither myelosuppression nor anemia were seen (36).

Our immunization induced clinical responses and extended survival of mice studied. Addition of docetaxel to TRAMP-H6 and TRAMP-H11 further inhibited tumor growth and increased mouse survival. The longest OS was observed in mice receiving TRAMP-H6 in combination with docetaxel. However, survival of mice treated with TRAMP-H11 and docetaxel was not significantly extended.

Cancer vaccination with or without chemotherapy led to a broad spectrum of immunomodulatory activities of anticancer immune response *in vivo*. The highest proportions of activated $CD8^+$ and $CD4^+$ tumor-infiltrating T-cells were noted in mice receiving TRAMP-H6 alone and TRAMP-H11 with docetaxel. Interestingly, adding docetaxel to TRAMP-H11 highly increased the docetaxel had the opposite effect. The results obtained are consistent with our earlier studies in the RENCA model, where RENCA-H6 and RENCA-H11 vaccine administered alone induced higher infiltration of tumors by activated CD8 and CD4 T-lymphocytes compared to control groups (18, 19). We observed that addition of docetaxel to TRAMP-H11 increased by two-fold the infiltration of the tumor by NK cells than did the vaccine alone. Surprisingly, we did not observe enhanced tumor infiltration by NK cells in mice receiving TRAMP-H6 and TRAMP-H11 alone, which was very pronounced in the RENCA model. The above differences might be due to the different tumor cells used or different tumor microenvironments. It is also possible that humoral immune response might be involved in tumor rejection, while activated B-lymphocytes were found at the tumor site. However, only TRAMP-H11 combined with docetaxel seemed to stimulate B-cells. A very significant infiltration of tumors by mature DCs in mice vaccinated with TRAMP-H6 alone or TRAMP-H11 with docetaxel was also observed. In addition, vaccination with TRAMP-H6 induced the strongest proliferation of antigen-specific $CD4^+$ T-lymphocytes. The combination of TRAMP-H6 with docetaxel reduced the

proportion of proliferating $CD4^+$ cells, whereas combination of TRAMP-H11 with docetaxel was associated with an increased proportion of these cells. The enhancement of lymphocyte proliferation was also observed in mice receiving recombinant proxiviral vaccine combined with docetaxel (36).

The results of the present study show that TRAMP cells modified with H6 and H11 induce prostate tumor regression and increase mouse survival by stimulating the immune system. It remains unclear why docetaxel enhances the stimulation of the immune response when combined with TRAMP-H11 vaccine and supresses the response when combined with TRAMP-H6. Overall, the addition of docetaxel to TRAMP-H6 is associated with the highest clinical benefit in treated mice, while combination of TRAMP-H11 and docetaxel provides the most powerful stimulation of the immune system, with good clinical outcome in this murine prostate cancer model.

Acknowledgements

The study was supported by a grant from the Ministry of Science and Higher Education, Warsaw, Poland (N N401582038).

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Received March 9, 2015
Revised March 20, 2015
Accepted March 24, 2015