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14. ABSTRACT

Dendritic cells (DC) are considered attractive candidates for cancer immunotherapy due to their ability to process and present antigens and stimulate the immune system. However DC have not been as effective in treating established disease in animal models. This provides the rationale for combining DC vaccines with a chemotherapeutic drug, which may act as an adjuvant for DC vaccines. Vitamin E succinate or alpha tocopheryl succinate (α-TOS) is a non-toxic, esterified analogue of Vitamin E that has been shown to be selectively toxic to tumor cell lines *in vitro* as well as inhibit the growth of tumors in animal models *in vivo*. The objective of this study is to enhance the effectiveness of DC vaccines by using it in combination with the non-toxic, more soluble vesiculated version of the chemotherapeutic agent, Vα-TOS to treat pre-established tumors of the highly metastatic murine mammary cancer cell line 4T1. The specific aims are to **1)** study the effect of Vα-TOS in inducing apoptosis in tumor cells *in vitro* and *in vivo*, **2)** determine the efficacy of Vα-TOS and DC combination therapy in treating **a)** pre-established murine mammary tumors and **b)** lung metastasis after resection of primary tumor in a residual disease setting, **3)** identify the mechanism involved in mediating the anti-tumor response.

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Chemo-immunotherapy, alpha-tocopheryl succinate, dendritic cells, vaccines

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INTRODUCTION

Dendritic cells (DC) are considered attractive candidates for cancer immunotherapy due to their ability to process and present antigens and stimulate the immune system. However DC have not been as effective in treating established disease in animal models. This provides the rationale for combining DC vaccines with a chemotherapeutic drug, which may act as an adjuvant for DC vaccines. Most of the commonly used chemotherapeutic drugs cause tumor cell death but at the same time are toxic to normal cells, which might compromise the ability of the DC to stimulate an effective immune response. Vitamin E succinate or α-TOS is a non-toxic, esterified analogue of Vitamin E that has been shown to be selectively toxic to tumor cell lines *in vitro* as well as inhibit the growth of tumors in animal models *in vivo*. The goal of this study is to enhance the effectiveness of DC vaccines by using it in combination with a non-toxic chemotherapeutic agent, α-TOS. In our studies we have used a more soluble hydrophilic form of the drug, vesiculated α-TOS (Vα-TOS). The hypothesis to be tested is that Vα-TOS will act as an adjuvant for DC vaccines and effectively inhibit the growth of pre-established 4T1 tumors. The specific aims are to 1) study the effect of Vα-TOS in inducing apoptosis in tumor cells *in vitro* and *in vivo*, 2) determine the efficacy of Vα-TOS and DC combination therapy in treating a) pre-established murine mammary tumors and b) lung metastasis after resection of primary tumor in a residual disease setting, 3) identify the mechanism involved in mediating the anti-tumor response by the combination therapy.

BODY

For a detailed description of the results please see the manuscript attached as Appendix 1.

CONCLUSIONS

In this study, we evaluated the efficacy of Vα-TOS plus DC chemo-immunotherapy to treat pre-established tumors of the highly metastatic murine mammary cancer cell line 4T1. We demonstrate that Vα-TOS in combination with non-antigen pulsed, non-matured dendritic cells significantly inhibits the growth of established tumors *in vivo* and prolongs the survival of treated mice. In addition, the combination treatment of Vα-TOS plus DC, when initiated after removal of the established primary tumor, dramatically inhibits the residual metastatic potential of 4T1. The superior effect of the combination therapy was correlated with increased IFN-γ and IL-4 production by splenic lymphocytes and draining lymph node cells. Interestingly, when used in combination with Vα-TOS, non-matured DC are as effective as matured DC at inhibiting the growth of pre-established tumors. Supernatant derived from Vα-TOS-treated tumor cells causes maturation of DC as evidenced by the up-regulation of co-stimulatory molecules and secretion of IL-12p70 which may be mediated by Vα-TOS-induced up-regulation of heat shock proteins 60, 70 and 90 in tumor cells.

For a more detailed discussion of conclusions please see attached manuscript in Appendix 1

Key research accomplishments

1. Demonstrate the ability of the vesiculated form of Vitamin E succinate or Alpha-tocopheryl succinate (Vα-TOS) to kill 4T1 tumor cells and induce apotosis *in vitro* and *in vivo*

2. Demonstrate the ability of Vα-TOS to act as an adjuvant for dendritic cell (DC) vaccines and inhibit the growth of pre-established 4T1 tumors

3. Demonstrate the effect of the combination therapy in inducing the production of IFN-γ and IL-4 by draining lymph node cells and splenic lymphocytes

4. Demonstrate the ability of Vα-TOS+DC combination treatment to inhibit metastasis to the lungs following primary tumor resection in a residual disease setting

5. Evaluate the effect of supernatant derived from Vα-TOS treated tumor cells on expression of co-stimulatory molecules on non-matured DCs

6. Determine the effect of Vα-TOS on heat shock protein expression in tumor cells

7. Demonstrate partial inhibition of co-stimulatory molecule expression on DCs on pre-incubation with alpha-2 macroglobulin

For detailed description of key accomplishments refer to manuscript attached as Appendix 1

Reportable outcomes

Presentations

Lalitha V. Ramanathapuram and Emmanuel T. Akporiaye. VITAMIN E SUCCINATE AS AN ADJUVANT FOR DENDRITIC CELL VACCINES. Poster, Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Philadelphia, Pennsylvania, July 8-11, 2005.

Manuscript

Lalitha V. Ramanathapuram, Tobias Hahn, Sharon M. Dial and Emmanuel T. Akporiaye. Chemo-Immunotherapy of Breast Cancer Using Vesiculated Alpha-Tocopheryl Succinate in Combination with Dendritic Cell Vaccination. Submitted to Nutrition and Cancer, July 2005.

APPENDIX 1

Chemo-Immunotherapy of Breast Cancer Using Vesiculated Alpha-Tocopheryl Succinate in Combination with Dendritic Cell Vaccination

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ABSTRACT

In this study, we evaluated the efficacy of vesiculated alpha-tocopheryl succinate (Vα-TOS) in combination with non-antigen pulsed, non-matured DC (nmDC) to treat pre-established tumors of the highly metastatic murine mammary cancer cell line, 4T1. We demonstrated that Vα-TOS in combination with non-antigen pulsed, non-matured dendritic cells significantly inhibits the growth of established tumors *in vivo* and prolongs survival of treated mice. In addition, when initiated after resection of the established primary tumor, the combination treatment dramatically inhibits residual metastatic disease. The clinical response achieved with the combination therapy was correlated with increased IFN-γ and IL-4 production by splenic lymphocytes and draining lymph node cells. Interestingly, when used in combination with Vα-TOS, non-matured DC were as effective as TNF-α matured DC at inhibiting the growth of pre-established tumors. Supernatant derived from Vα-TOS-treated tumor cells caused maturation of DC as evidenced by the up-regulation of co-stimulatory molecules and secretion of IL-12p70. These results demonstrate the potential usefulness of Vα-TOS plus DC chemo-immunotherapy in treating established primary mammary tumors as well as residual metastatic disease.

INTRODUCTION

Alpha tocopheryl succinate (α-TOS) is a semi-synthetic ester analog of vitamin E derived by substituting the hydroxyl group of vitamin E (α-tocopherol) with a succinyl group (1, 2). Unlike vitamin E, α-TOS has been shown to be a potent inducer of apoptosis of a wide range of human and murine cancer cells including human breast, cervical, endometrial, prostate, colon, lung and lymphoid cancer cells (3-7), while showing limited or no toxicity towards normal cells or transformed non-tumorigenic cells (1, 5, 6). In an *in vivo* setting using experimental tumor models, α-TOS has been demonstrated to inhibit the growth of melanoma (8), breast (9), lung (10) and colon (3, 11) cancers. One of the major limitations of using α-TOS is its insolubility in aqueous solvents, which makes it unsuitable for use in humans. Unlike α-TOS which is soluble only in organic solvents like sesame oil, dimethylsulfoxide or ethanol (3, 8-10), vesiculated α-TOS (Vα-TOS) is hydrophilic and is generated by the addition of sodium hydroxide and sonication in PBS to form a colloidal suspension (12). This formulation of α-TOS has been shown to inhibit the progression of tumors as well as prolong the survival of tumor-bearing mice (12, 13).

Dendritic cells (DC) are potent antigen presenting cells with the ability to efficiently prime T cells making them prime candidates for cancer immunotherapy (14, 15). Once loaded with antigen, DCs need to be matured, express critical co-stimulatory molecules and migrate to the draining lymph node in order to induce an effective immune response (14). Pre-clinical and clinical studies have employed DCs pulsed with defined peptides or proteins to elicit potent anti-tumor T cell responses (16-22). Although antigen-pulsed DCs have been shown to be capable of

suppressing tumor growth (18, 23), they have been less effective in abrogating established disease in various animal models (18, 23-25). The limited success of DC vaccines in treating established disease provides a rationale for combining it with other treatment modalities such as chemotherapy in order to improve its anti-cancer activity.

In a previous report using a murine lung carcinoma model we demonstrated that non-antigen pulsed, non-matured DCs (nmDC) in combination with Vα-TOS was effective at inhibiting the growth of pre-established tumors (13). Also, supernatant derived from Vα-TOS treated tumor cells enhanced the expression of maturation markers on nmDC. This finding suggested that when used in conjunction with Vα-TOS, adoptively transferred nmDC undergo maturation *in vivo* in order to mediate an anti-tumor immune response.

In this study we investigated the effect of Vα-TOS plus DC combination therapy on a poorly immunogenic, highly metastatic murine mammary cancer cell line (4T1). We demonstrate that Vα-TOS by itself induces apoptosis of 4T1 tumor cells and that the combination of Vα-TOS and nmDC, inhibits the growth of pre-established 4T1 tumors and dramatically reduces the number of lung metastases after primary tumor resection. Supernatant derived from Vα-TOS-treated tumor cells caused DC maturation evidenced by up-regulation of co-stimulatory molecule (CD40, CD80, CD86) expression and IL-12p70 production. These findings suggest that tumor growth suppression by Vα-TOS is likely due to its combined effects of tumor cell killing and activation of dendritic cells.

MATERIALS AND METHODS

Chemicals and reagents

Alpha-tocopheryl succinate (α-TOS), and alpha-2 macroglobulin (α_2M) were purchased from Sigma Chemical Co. (St. Louis, MO). Murine interleukin 4 (IL-4), granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumor necrosis-factor-alpha (TNF-α) were purchased from Peprotech (Rocky Hill, NJ). The antibodies for phenotyping DC (anti-CD11c, anti-I-A^d, anti-CD40, anti-CD80, anti-CD86) were purchased from BD Pharmingen (San Diego, CA) and Caltag Laboratories (Burlingame, CA). The heat shock protein (hsp)-specific antibodies (hsp60, 70 and 90) were purchased from Stressgen Biotechnologies (Victoria, BC, Canada). The ALEXA Flour 488 antibody was purchased from Molecular Probes (Eugene, OR). The goat anti-mouse HRP-conjugated antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The Annexin-V FLOUS staining kit and the APO-DIRECT TUNEL assay kit were purchased from Roche Applied Sciences (Indianapolis, IN) and BD Pharmingen (San Diego, CA) respectively. The mouse interferon-gamma (IFN-γ), IL-4 and IL-12p70 ELISA kits were purchased from Pierce Biotechnologies (Rockford, IL).

Preparation of vesiculated α-TOS

Vesiculated α-TOS (Vα-TOS) was generated as previously described (12). Briefly 40mg of α-TOS was dissolved in chloroform and a thin film was formed on the inside of a silanized 50 ml round bottom flask by rotary evaporation under a nitrogen atmosphere and dried overnight in a desiccator. Approximately 1.9 ml of

phosphate buffered saline (PBS), (10 mM, pH 8.0) was added to the dry thin film and sonicated for 25 min in a water-bath sonicator (Branson 3510, Branson Ultrasonic Corp. Danbury, CT). Subsequently, 80 μl of 1 M NaOH was added to a final concentration of 40 mM and the suspension was sonicated for 20 min and 20 μl of 1 M HCl was added to a final concentration of 8 mM before a final sonication for 30 min. The resultant solution (20 mg/ml Vα-TOS) was used for *in vitro* and *in vivo* experiments. The vesicles of α-TOS generated ranged in size from 25 - 300 nm with 75% of the vesicles being smaller than 60 nm as determined by transmission electron microscopic analysis of negatively stained samples (data not shown).

Cell culture

The metastatic murine mammary carcinoma cell line 4T1 was kindly provided by Dr. Fred Miller of the Michigan Cancer Foundation (Detroit, MI). The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Bovine Serum (FBS). For DC culture, bone marrow cells were harvested from flushed marrow cavities of femurs and tibiae of BALB/c mice under aseptic conditions and cultured with 100 U/ml GM-CSF and 100 U/ml IL-4 at 10^6 cells/ml in complete media (RPMI + 10% heat inactivated FBS) as previously described (26). On day 6, the non-adherent and loosely adherent cells were collected, washed three times with PBS before being used in various experiments. To obtain mature DCs, day 6 DC were incubated with 200 U/ml TNF-α for 48 hours (26).

Flow cytometric analysis of dendritic cells

Dendritic cells were identified by flow cytometry using the FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) on the basis of their expression of CD11c (26). These cells were 50-60% positive for CD11c expression and 70-80% positive for MHC class II (I-A^d) expression. Of the $CD11c^+$ cells, 2% were CD40 positive, 55% were CD80 positive and 38% were CD86 positive. Of the $CD11c^+$ cells after TNF-α induced maturation, 15%, 85% and 50% of the cells were positive for the expression of CD40, CD80 and CD86 respectively. Figure 1 depicts histograms of the expression of co-stimulatory molecules on day 6 non-matured DC (nmDC) and TNF-α-matured DCs (mDC).

Vα-TOS treatment and assessment of tumor cell viability, clonogenic potential and apoptotic cell death

For the *in vitro* cell viability assay, 4T1 tumor cells were plated at $2x10^5$ cells/well in 6-well tissue culture dishes (Sarstedt, Newton, NC) overnight. The cells were then treated with 0 μg/ml (PBS), 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml or 80 μg/ml of Vα-TOS (in PBS). Twenty-four hours later, non-adherent and adherent cells were collected and centrifuged at 200 x g for 5 min. Cell number and viability were determined by trypan blue dye exclusion. For the clonogenicity assay, 10^2, 10^3, 10^4, and 10^5 viable cells from each treatment group were plated in triplicate in 100 mm tissue culture dishes and incubated (7% CO_2, 37°C) for 10 days in IMDM containing 10% FBS. The resulting colonies were fixed in methanol and stained with Giemsa. Colonies containing >50 cells were counted and the surviving cell fraction was

determined using the following formula: surviving fraction = (# of colonies counted at a given concentration of Vα-TOS / # of cells plated at that concentration) / (# of control colonies counted (PBS) / # of control cells plated) (27).

For the apoptosis assay, tumor cells were treated with either 40 μg/ml Vα-TOS or PBS. After 4, 12, or 18 h, non-adherent and adherent cells were collected and stained with Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) following the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN). Briefly, following centrifugation and washing, tumor cells were re-suspended in Annexin V binding buffer and stained with Annexin V-FITC and PI for 20 min in the dark. Binding buffer was added to the samples prior to flow cytometric analysis using the FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The cells were gated on forward versus side scatter and bivariate scattergrams of Annexin V-FITC (excitation wavelength, 495 nm) versus PI fluorescence (excitation wavelength, 493 nm) were generated for analysis.

Co-culture of dendritic cells and tumor cells pre-treated with Vα-TOS

4T1 tumor cells were plated at $2x10^5$ cells/well in 6-well tissue culture plates in IMDM with 10% FBS. Twenty-four hours later, culture medium was removed and replaced with fresh medium containing 40 μg/ml Vα-TOS or PBS alone. After 24 h, the supernatant fluid was collected and centrifuged at 22,600 x g for 45 min. The pellet obtained was re-suspended in complete media and incubated with non-matured DC for 24 h. 4T1 tumor cell lysate generated by freeze-thaw (4 cycles) was added to a set of non-matured DC as a control. DCs were collected 24 h later,

phenotyped and evaluated for IL-12p70 production. For the phenotypic analysis, DCs were collected and washed with PBS and stained for the expression of CD11c, I-A^d and the co-stimulatory molecules CD40, CD80 and CD86. To evaluate IL-12p70 production, $5x10^5$ DCs were stimulated with 20 ng/ml TNF-α for 24 h in 48-well plates. The supernatant was collected and analyzed for IL-12p70 production by ELISA according to the manufacturer's protocol (Pierce Biotechnologies, Rockford, IL).

Expression of heat shock proteins by Vα-TOS-treated tumor cells

4T1 cells were plated at $2x10^5$ cells/well in 6-well tissue culture dishes. Twenty-four hours later, the medium was replaced with fresh culture medium containing 40 μg/ml of Vα-TOS and incubated for an additional 12 h (7% CO_2, 37°C). Non-adherent and adherent cells were collected, centrifuged at 200 x g for 5 min and washed twice with PBSB (phosphate buffered saline, 0.5% bovine serum albumin). The cells were then re-suspended in PBSB and labeled with mouse-derived monoclonal antibodies specific for hsp60, 70 and 90 for 45 min on ice. Controls included unlabeled cells and cells labeled with isotype IgG antibody. Cells were washed twice and stained with ALEXA FLOUR 488-conjugated goat anti-mouse secondary antibody for 45 min on ice. The cells were washed twice with PBSB before being finally re-suspended in PBSB for flow analysis using the FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The cells were gated on forward versus side scatter and histograms of ALEXA FLOUR 488 (excitation wavelength, 488 nm) were generated for analysis.

Western blot for hsp expression

4T1 tumor cells were plated at $2x10^5$ cells/well in 6-well tissue culture plates for 24 h in IMDM with 10% FBS. Twenty-four hours later, the culture medium was replaced with fresh medium containing 40 μg/ml Vα-TOS. After an additional 24 h, the non-adherent cells were collected and centrifuged (22,600 x g, 45 min). The cell pellets were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 μg/ml aproptinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The lysate was placed on a rocker at 4°C for 15 min and then forced five times through 25 gauge needles. The lysate was next centrifuged at 14,000 x g for 15 min at 4°C; the resultant supernatant recovered and protein content was determined using the BCA Protein Assay (Pierce Biotechnlogies, Rockford, IL). Proteins (30 μg) from the lysates were resolved by 10% SDS-PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubating the membrane in TBST/MLK (Tris buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk). The membrane was immunoblotted using mouse antibodies against either hsp60 (1:1000), hsp70 (1:500) or hsp90 (1:1000) (Stressgen Biotechnologies, Canada) and visualized with a goat anti-mouse HRP-conjugated secondary antibody (Upstate Biotechnologies, NY) using the Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnlogies, Rockford, IL).

Blockage of hsp binding to DC

In order to block the hsp receptor CD91, non-matured DC were incubated with or without 100 μg/ml of alpha-2 macroglobulin (α_2M), a natural ligand of CD91 (28), for 1 h in serum-free medium before addition of supernatant fluid from tumor cells treated with Vα-TOS. DCs were collected after 24 h and stained for the expression of CD11c, I-A^d, CD40, CD80 and CD86 and analyzed by flow cytometry as described before. To evaluate IL-12p70 production, $5x10^5$ DCs were stimulated with 20 ng/ml TNF-α for 24 h in 48-well plates. The supernatant was collected after 24 h and analyzed for IL-12p70 production by ELISA according to the manufacturer's protocol (Pierce Biotechnologies, Rockford, IL).

Animal studies

Six-week-old female BALB/c mice were purchased from The Harlan Laboratory (Indianapolis, IN). Mice were housed at the University of Arizona Animal Facilities in accordance with the Principles of Animal Care (NIH publication No. 85-23, revised 1985). For establishment of primary tumors, each mouse was injected orthotopically in the mammary fat pad with $5x10^4$ 4T1 tumor cells in 50 μl PBS. After tumors were established (20-25 mm^3) on day 15, the mice were randomized to ensure comparable tumor sizes in all groups and subjected to different treatment regimens. Mice were given 9 i.p. injections of Vα-TOS (4 mg/injection in 200 μl of PBS at 200 mg/kg body weight) on alternate days starting on day 15 after tumor cell injection. The control group consisted of mice injected with 200 μl of PBS. For the combination treatment, $1x10^6$ DC were injected s.c. on days 18, 22, and 26 in 50 μl

PBS. Tumor growth was monitored by measuring the tumor length and width with calipers and calculating the tumor volume according to the formula $V= (L \times W^2)/2$ (29). For the residual disease study, mice were injected with $5x10^4$ 4T1 tumor cells in the mammary fat pad. Primary tumors were excised 21 days after the initial tumor challenge. Mice were given 5 i.p. injections of Vα-TOS (4mg/injection in 200 μl PBS at 200 mg/kg body weight) or PBS (vehicle control) on alternate days starting on day 22. For the combination treatment, mice were injected with $1x10^6$ DC s.c. in 50 μl PBS on days 25 and 29. Mice were sacrificed on day 31 and visible metastatic lung nodules enumerated by staining with India ink and Fekete's solution as previously described (30).

TUNEL assay

Mice with established tumors were injected with Vα-TOS or PBS on days 15, 17, 19, 21, 23, and 25. Twenty-four hours after every two injections (days 18, 22 and 26 respectively) tumors were resected, embedded in optimal cutting temperature (OCT) compound and frozen using dry ice and 2-methylbutane. Sections of frozen tumor (4μm thick) were prepared and stained using the APO-DIRECT kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, sections were fixed in 1% paraformaldehyde, washed in PBS and immersed in 70% ethanol for 30 min. The sections were then reacted with staining solution containing terminal deoxinucleotidyl transferase (TdT) enzyme and FITC-labeled deoxyuridine triphosphate (dUTP). After incubating the slides for an hour at 37°C, the stain was washed off and slides incubated for 10 min with RNase/PI solution. The slides were

rinsed with PBS and sections mounted using DAKO fluorescent mounting medium (Dako Corporation, Carpenteria, CA). The presence of apoptosis in the tumor sections was evaluated by fluorescence microscopy (Nikon Eclipse TE2000-S, Nikon, Japan).

Cytokine production

Mice from the various treatment groups were sacrificed and the spleens and inguinal draining lymph nodes (DLN) were pooled from 3 animals from each treatment group. For the spleen cells, following red blood cell lysis, the cells were layered over a Ficoll Hypaque gradient to isolate the lymphocytes. The cells were then incubated in 24-well tissue culture plates for 48 hours and supernatants were collected and evaluated by ELISA for the production of IFN-γ or IL-4 following the manufacturer's protocol (Pierce Biotechnlogies, Rockford, IL).

Statistical analysis

Statistical significance of differences among data sets of treatment groups were assessed by one-way ANOVA including Tukey-Kramer post tests for multiple comparisons using the Prism software (GraphPad, San Diego, CA). Probability values (p) of ≤ 0.05 were considered indicative of significant differences between data sets. Log-rank tests were performed on the Kaplan-Meier survival curves of Vα-TOS+/- DC-treated and control (sham-treated) animals.

RESULTS

Vα-TOS is toxic to tumor cells and induces apoptosis *in vitro*

We first evaluated the cytotoxic activities of Vα-TOS on 4T1 tumor cells. For this purpose, we determined the viable cell number as well as the clonogenic potential of 4T1 cells after a 24 h exposure to different concentrations of Vα-TOS. The data show that Vα-TOS caused death of 4T1 tumor cells in a dose dependent manner (Figure 2A). Treatment of cells with 20 μg/ml Vα-TOS caused 67% cell death, which increased to 96% and 100% when treated with 40 μg/ml and 80 μg/ml of the drug respectively. The IC_{50} value of Vα-TOS was 18 μg/ml. In addition, tumor cells that survived the 24 h Vα-TOS treatment were significantly impaired in their ability to proliferate and form colonies in a dose-dependent manner (Figure 2B).

Vα-TOS-induced 4T1 tumor cell death was at least partially due to apoptosis as determined by Annexin V staining (Figure 2C). Phosphatidyl serine translocation to the cell surface (Annexin V positive) signifying early apoptosis was observed after a 4 h exposure to Vα-TOS and progressively increased with time leading to loss of membrane integrity signifying secondary necrosis (Annexin V and PI positive) by 18 h.

Vα-TOS induces apoptosis in tumors *in vivo*

In order to determine whether Vα-TOS induces apoptosis in tumors *in vivo*, we analyzed tumor sections by TUNEL assay. Mice with established tumors (~25mm^3) were injected with Vα-TOS or PBS (control) as described in "Materials and Methods." Twenty-four hours after every two Vα-TOS injections, tumors were

resected, frozen, sectioned and evaluated for apoptosis by TUNEL assay. Vα-TOS caused significant apoptosis in tumors as compared to tumors treated with PBS (Figure 2D). Maximum apoptosis was observed after 6 Vα-TOS injections.

Vα-TOS potentiates the anti-tumor activity of DC vaccines on the growth of established 4T1 tumors

In an earlier study we showed that α-TOS as well as Vα-TOS enhances the anti-tumor effect of adoptively transferred non-matured DC in treating pre-established 3LL tumors (10, 13). In this study, we compared the effectiveness of non-matured, unpulsed DC and TNF-α matured DC in combination with Vα-TOS in controlling pre-established 4T1 tumors. The data (Figure 3A) demonstrate that when used in combination with Vα-TOS, nmDCs are as effective as mDC in inhibiting 4T1 tumor growth compared to the controls (PBS, $p<0.001$; PBS+nmDC, $p<0.001$; PBS+mDC, $p<0.001$; Vα-TOS, $p<0.05$). The mean tumor volumes on day 31 post-tumor cell injection in mice receiving Vα-TOS plus either nmDC or mDC were 66.7 ± 51.2 mm^3 and 44.1 ± 30.2 mm^3 respectively. In contrast, the mean tumor volume in mice receiving Vα-TOS alone was 379.4 ± 135.3 mm^3 and the mean tumor volumes of the control groups (PBS, PBS+nmDC, PBS+mDC) ranged from 709 ± 251 to 1004 ± 348 mm^3. This is also reflected in the observation that Vα-TOS plus DC therapy significantly prolonged survival compared to PBS alone ($p<0.05$), DC (PBS+nmDC, $p<0.05$; PBS+mDC, $p<0.05$) or Vα-TOS alone ($p<0.05$) treated mice (Figure 3B). All control animals died because of large tumor burden (~1200 mm^3) by day 35. Mice injected with Vα-TOS alone died because of large tumor burden or

were sacrificed when tumor volumes reached ~1200 mm^3 by day 47. Five of the seven mice in the Vα-TOS + mDC group and six of the seven mice in the Vα-TOS + nmDC group were alive until day 60 when they were sacrificed as tumor volumes had reached 1200 mm^3.

However, two mice in the Vα-TOS + mDC group and one mouse in the Vα-TOS + nmDC group showed complete tumor regression by day 36. To ascertain the existence of long-term tumor immunity, these mice were challenged with ten times the original dose ($5x10^5$) of 4T1 cells. These mice did not develop 4T1 tumors (data not shown). In contrast, when challenged with the unrelated murine leukemia cell line 12B1 on the contralateral side, the tumors grew unhindered demonstrating specificity of the tumor immunity developed in these mice.

Combination treatment with Vα-TOS plus DC elicits increased IFN-γ and IL-4 production by draining lymph node cells and splenic lymphocytes

In order to determine whether the anti-tumor effect of Vα-TOS plus DC vaccination was associated with an enhanced immune response, cells were isolated from draining lymph nodes (DLN) as well as spleens of mice of the various treatment groups and evaluated for IFN-γ and IL-4 production by ELISA.

Figure 4A shows that DLN cells isolated from mice treated with Vα-TOS + nmDC or mDC produced significantly higher amounts of IFN-γ (6510.5±35.7 pg/ml and 5360.4±384.5 pg/ml respectively) compared to cells isolated from mice treated with PBS (10±0.96 pg/ml, $p<0.001$), PBS+nmDC (172.8±9.9 pg/ml, $p<0.001$), PBS+mDC (180.9±30.2 pg/ml, $p<0.001$) or Vα-TOS (2067±11.7 pg/ml, $p<0.001$).

Similarly, IFN-γ production by splenocytes isolated from mice treated with Vα-TOS plus nmDC or mDC was significantly higher (2801.7±151.6 pg/ml and 2749.8±146.7 pg/ml respectively) than that of splenocytes from control mice (13.5±0.8 - 25.2±2.8 pg/ml, $p<0.001$) (Figure 4B) or mice injected with Vα-TOS alone (437.1±55 pg/ml, $p<0.05$). The same pattern was observed for IL-4 production by DLN and spleen cells. DLN cells from mice injected with the combination treatment (Vα-TOS+nmDC or mDC) produced significantly higher amounts of IL-4 (508.6±50.2 and 437.1±55 pg/ml respectively) than DLN cells from mice treated with Vα-TOS alone ($p<0.001$) or from control mice (PBS, $p<0.001$; PBS+nmDC, $p<0.001$; PBS+mDC, $p<0.001$) (Figure 4A). Similarly splenocytes isolated from mice injected with the combination treatment produced 274±67.8 pg/ml (Vα-TOS + nmDC) and 303.7±51.2 pg/ml (Vα-TOS+mDC) of IL-4 (Figure 4B) which was significanlty higher ($p<0.001$) than IL-4 production by splenocytes from Vα-TOS treated or control mice.

The data show that Vα-TOS treatment alone resulted in an improved immune response. More importantly, the combination of Vα-TOS plus DC vaccination even further increased both the IFN-γ and IL-4 production by DLN cells and splenocytes, although the maturation status of the DCs used had no influence on cytokine secretion. Additionally, the high ratio of IFN-γ to IL-4 production in mice receiving the combination therapy suggests polarization towards a T_H1 mediated immune response (Table 1).

UThe combination of Vα-TOS plus non-matured DC effectively suppresses residual metastatic disease

In the clinical setting, it is often possible to surgically remove the primary tumor, which may prolong the survival of the patient but often fails to completely eradicate the disease. Since the primary cause of cancer recurrence and mortality is residual metastatic disease, we wanted to study the efficacy of the combination of Vα-TOS plus DC in treating residual metastatic disease after primary tumor resection. For this purpose, mice were injected orthotopically with $5x10^4$ 4T1 cells into the mammary fat pad. Twenty-one days post-tumor implantation, when the tumor had metastasized to the lungs (29), the primary tumors (~150 mm^3) were surgically removed. The mice were then treated with Vα-TOS+nmDC and evaluated for metastatic disease by enumerating the number of visible pulmonary nodules. The data (Figure 5) show that Vα-TOS treatment alone was able to significantly reduce the number of lung metastases compared to the controls (PBS, $p<0.01$; PBS+nmDC, $p<0.05$). However, more importantly, the combination therapy of Vα-TOS+nmDC was able to inhibit the development of lung metastasis even further, reducing the number of pulmonary surface nodules by 94% compared to PBS treatment alone.

Combination treatment with Vα-TOS plus DC elicits increased IFN-γ and IL-4 production by splenic lymphocytes in the residual disease setting

Since the suppression of pre-established 4T1 tumors with Vα-TOS plus DC treatment was correlated with an enhanced immune response, we wanted to

determine if this is also true in the residual disease setting. Therefore, splenocytes were isolated from mice of the various treatment groups and evaluated for IFN-γ and IL-4 production by ELISA (Figure 6). Similar to the IFN-γ production by splenocytes in the pre-established tumor setting, the combination treatment with Vα-TOS + nmDC caused significantly higher production of both IFN-γ (1963.5±106 pg/ml) and IL-4 (202.7±85 pg/ml) as compared to the controls (IFN-γ $p<0.01$; IL-4 $p<0.001$). Also the T_H1 to T_H2 ratio was higher in splenocytes from mice treated with Vα-TOS + nmDC as compared to the controls (Table 2).

UVα-TOS-treated tumor cells induce maturation of DCs *in vitro*

Our finding that the maturation status of the DCs had no influence on the tumor growth inhibition or cytokine production when combined with Vα-TOS, led us to hypothesize that Vα-TOS-treated tumor cells caused DC maturation. To examine this possibility, we incubated nmDC with supernatant derived from tumor cells exposed to Vα-TOS for 24 h and assessed the expression of the DC maturation markers CD40, CD80 and CD86. The data (Figure 7A) show that co-incubation of Vα-TOS-treated tumor cell supernatant with nmDC caused an increase in co-stimulatory molecule expression on DC. This increase in expression was comparable to that observed in DC matured with TNF-α (data not shown). In contrast, direct incubation of nmDC with Vα-TOS or nmDC with freeze-thawed tumor lysate for the same length of time did not cause an increase in the expression of these markers above background (nmDC alone or nmDC incubated with supernatant from PBS-treated tumor cells).

To provide additional evidence that Vα-TOS-treated tumor cells caused DC maturation, we evaluated IL-12p70 secretion by DCs incubated with supernatant derived from Vα-TOS-treated tumor cells. The data (Figure 7B) show that IL-12p70 secretion by DCs was significantly increased ($p < 0.001$) only when co-incubated with supernatant from Vα-TOS-treated tumor cells.

Vα-TOS induces the expression of heat shock proteins in tumor cells

Since heat shock proteins (hsp) are induced in response to apoptotic and/or necrotic cell death (31-34) and Vα-TOS induces apoptosis of 4T1 tumor cells, we wanted to determine if Vα-TOS treatment up-regulated hsp expression in tumor cells. For this purpose, 4T1 cells were exposed to 40 μg/ml Vα-TOS for 12 h and then stained with monoclonal antibodies specific for hsp60, 70 and 90 and analyzed by flow cytometry. The data (Figure 8A) show that the membrane expression of these heat shock proteins on 4T1 tumor cells was up-regulated following Vα-TOS treatment but not after vehicle (PBS) treatment. Western blot analysis of supernatant derived from Vα-TOS-treated cells (Figure 8B) confirmed the differential induction of hsps on tumor cells following Vα-TOS treatment.

DC maturation induced by Vα-TOS-treated tumor cells is mediated by heat shock proteins

It is well documented that heat shock proteins (hsps) are up-regulated during apoptotic or necrotic cell death (31-34) and provide danger signals that may lead to activation and maturation of DCs (31-33, 35-39). Since Vα-TOS caused apoptotic

cell death of 4T1 tumor cells and increased the expression of hsps, we postulated that DC maturation by supernatant derived from Vα-TOS treated tumor cells is mediated at least in part, by hsps. In order to address this possibility, we blocked the cognate hsp receptor CD91 (28) on nmDC by pre-treatment with α_2-macroglobulin (α_2M). Subsequently, the DCs were co-incubated with supernatant derived from tumor cells exposed to Vα-TOS for 24 h. The data (Figure 9A) show that pre-treatment with α_2M partially inhibited the expression of the maturation markers CD40, CD80 and CD86 on DCs. In contrast, the incubation of nmDC with α_2M alone did not cause any change in the expression of maturation markers. This also correlated with IL-12p70 production by the DCs. Pre-treatment of DCs with α_2M followed by incubation with supernatant derived from Vα-TOS treated tumor cells significantly inhibited ($p<0.001$) IL-12p70 secretion by DCs as compared to DC treated directly with Vα-TOS treated tumor supernatant.

DISCUSSION

In this study, we evaluated the efficacy of Vα-TOS plus DC chemo-immunotherapy in treating pre-established murine 4T1 breast cancer. The 4T1 tumor model closely resembles human breast cancer, because of its poor immunogenicity and ability to spontaneously metastasize to the lungs, liver, bone marrow and brain (29, 40). We demonstrated that Vα-TOS in combination with non-antigen pulsed, non-matured dendritic cells significantly inhibited the growth of established tumors and improved overall survival. We also observed that the combination treatment dramatically inhibited the formation of lung metastases when therapy was initiated

after primary tumor resection. The superior effect of the combination therapy was correlated with increased IFN-γ and IL-4 production by splenic lymphocytes and draining lymph node cells. The ratio of IFN-γ to IL-4 production suggests polarization towards a T_H1 mediated immune response indicating that the combination treatment enhanced a cell-mediated anti-tumor immune response. In this study we used a novel formulation of α-TOS that is formed by spontaneous self-assembly of α-TOS (12, 13, 41). This vesiculated form of α-TOS is more soluble in aqueous solvents (12, 41) and, unlike the liposomal preparations of α-TOS (27, 42), does not require the addition of lipids, making it a practical alternative for use in humans. Our finding that Vα-TOS significantly inhibits the growth of established tumors corroborates the findings of Lawson et al. (27) in which they used a liposomal formulation of α-TOS to treat a related murine mammary cancer (66cl-4). However in our studies the tumor size at the start of treatment ranged from 20-25 mm^3 compared to ~0.5mm^3 reported by Lawson et al (27). This observation suggests that the vesiculated formulation of α-TOS in combination with DC vaccines is more potent in treating established mammary tumors. In addition, we show here that Vα-TOS plus DC treatment, leads to a 94% reduction in the number of lung metastasis, demonstrating the promise of Vα-TOS+DC therapy as an effective treatment of residual disease after primary tumor resection.

The observation that nmDC mediated-tumor suppression was as effective as mDC *in vivo* when combined with Vα-TOS, suggests that Vα-TOS treatment of tumor cells induces DC maturation *in vivo*. This is supported by our finding that co-incubation of DC with supernatant fluid derived from Vα-TOS-treated 4T1 cells leads

to DC maturation *in vitro*. In addition, we show that Vα-TOS induces apoptosis of mammary tumor cells leading to secondary necrosis. Taken together, these findings corroborate earlier studies that report that exposure of DC to stressed apoptotic tumor cells, lysates or supernatants of necrotic transformed cell lines leads to maturation of human and murine dendritic cells (31-33, 39, 43). The possibility that Vα-TOS treatment may induce the maturation of DC *in vivo* is significant as it would facilitate the translation of our treatment approach to the clinic by removing the necessity for additional *ex-vivo* manipulations such as maturation and/or loading of DC with tumor antigens in order to generate DC capable of mediating anti-tumor activity *in vivo*.

In an effort to understand the mechanism by which Vα-TOS may cause DC maturation we looked for hsp expression by tumor cells after treatment with Vα-TOS. We demonstrate that Vα-TOS induces the expression of the heat shock proteins 60, 70 and 90 in 4T1 tumor cells and that co-incubation of DC with α_2-macroglobulin which competes with hsp60, 70 and 90 for binding to the cognate receptor CD91 (28) caused a partial reduction in the expression of co-stimulatory molecules when DC are incubated with supernatant fluid derived from Vα-TOS-treated 4T1 cells. However, the absence of complete inhibition of co-stimulatory molecule expression may indicate the involvement of additional hsp receptors and/or hsps including gp96 and calreticulin, which are currently under investigation in our laboratory. These results are consistent with our earlier study (13) that showed a similar maturation effect on DC using supernatant fluid derived from Vα-TOS-treated lewis lung (3LL) carcinoma cells. Previously it has been shown that heat shock protein 60, 70 and 90

induce the maturation of DC (31, 35, 36, 38, 43) and up-regulate the expression of pro-inflammatory cytokines (37, 44, 45). Heat shock proteins function as molecular chaperones and fulfill essential roles in protecting cells from potentially lethal effects of stress and proteotoxicity (46). However, hsps in the extracellular environment act as a "danger signal" alerting antigen presenting cells, including DC, leading to their activation (32, 33, 46, 47). Activated DCs are very effective antigen presenters, which migrate to secondary lymphoid organs where they initiate anti-tumor T cell responses (48). Therefore, our results extend these findings and may suggest a direct role for hsps in Vα-TOS-mediated DC activation.

Taken together, our results suggest that Vα-TOS may employ a two-pronged approach to potentiate DC-mediated immunotherapy of cancer; firstly, by direct killing of tumor cells whose antigens can be cross-presented by DC and secondly by maturation of DC via hsp-mediated "danger signals". Our finding that the combination of Vα-TOS with DC is effective in the treatment of established mammary cancer as well as metastasis after primary tumor resection, demonstrates the potential usefulness of this chemo-immunotherapeutic strategy that can be rapidly translated to the clinic.

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Figure 1. Expression of co-stimulatory molecules on day-6 non-matured DC (nmDC) and TNF-α matured DC (mDC). DC were generated from bone marrow cells of BALB/c mice after culturing in GM-CSF and IL-4 as described in Materials and Methods. On day 6 or following TNF-α maturation, cells were collected and double stained with PE-conjugated CD11c antibody and FITC-conjugated antibodies against CD40, CD80 and CD86 and analyzed by flow cytometry. Cells were gated on light scatter and CD11c-PE positive cells. *Shaded region* represents non-matured DC (nmDC) and *black line* represents TNF-α matured DCs.

Figure 2. Effect of Vα-TOS treatment on 4T1 tumor cells *in vitro* and *in vivo*. 4T1 cells were allowed to adhere overnight in 6-well tissue culture plates. The cells were then treated with none (PBS), 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, or 80 μg/ml of Vα-TOS (in PBS). After a 24 h exposure, non-adherent and adherent cells were collected and cell number and viability were determined by trypan blue dye exclusion. The data **(A)** are representative of 2 independent experiments and the values denote means ± SD of triplicate samples. In order to determine the clonogenic potential **(B)** 10^2, 10^3, 10^4, and 10^5 viable cells recovered after treatment with none (PBS), 20 μg/ml, 40 μg/ml, or 60 μg/ml of Vα-TOS for 24 h were plated in 100 mm tissue culture dishes and incubated for 10 days in culture medium. The resulting colonies were fixed and Giemsa stained. Colonies containing >50 cells were counted and the surviving cell fraction was determined as described in Materials and Methods. The data shown are representative of 2 independent experiments and the values represent means ± SD of triplicate samples. For the apoptosis assay **(C)** cells were treated with either 40 μg/ml Vα-TOS or PBS. At each time point, non-adherent and adherent cells were collected and stained using Annexin V and PI. Numbers represent the percentages of early apoptotic cells (lower right quadrant) and secondary necrotic cells (upper right quadrant) respectively. The data shown are representative of 3 independent experiments. For the TUNEL assay **(D)** mice with pre-established tumors were injected with Vα-TOS on days 15, 17, 19, 21, 23 and 25. Twenty-four hours after every two Vα-TOS injections (days 18, 22 and 26), tumors were resected, frozen, sectioned and stained with the TUNEL reaction mixture. The yellow and green regions in the tumor sections represent

TUNEL positive (apoptotic cells) and red areas depict TUNEL negative cells. (Magnification = 400X).

Figure 3. Effect of Vα-TOS plus DC immunotherapy on pre-established 4T1 tumors. Mice were injected orthotopically in the mammary pad with $5x10^4$ 4T1 tumor cells. On development of established tumors (day 15) mice were injected i.p. with 4 mg of Vα-TOS on alternate days for a total of 9 injections. The mice were also injected in the contralateral mammary pad with either 10^6 non-matured DC (nmDC), or TNF-α matured DC (mDC) on days 18, 22 and 26. The data represent **(A)** mean tumor volumes ± SD and **(B)** % survival of seven individual mice per group. All control animals died because of large tumor burden (~1200 mm^3) by day 35 and mice injected with Vα-TOS died naturally or were sacrificed when tumor volumes reached ~1200 mm^3 by day 47. In contrast, six mice in the Vα-TOS + nmDC group and five mice in the Vα-TOS + mDC groups were alive until day 60 when they were terminated as tumor volumes reached ~1200 mm^3. Two mice in the Vα-TOS + mDC group and one mouse in the Vα-TOS + nmDC group showed complete tumor regression. These mice did not develop tumors when they were re-challenged with a ten fold higher dose ($5x10^5$) of 4T1 tumor cells (data not shown).

Figure 4. Effect of combination treatment with Vα-TOS and DC on IFN-γ and IL-4 secretion by splenic lymphocytes and draining lymph node cells. Spleens and draining lymph nodes were isolated on day 35 post-tumor injection from each of three mice in each treatment group and pooled. Splenic lymphocytes were separated by layering over a Ficol-Hypaque gradient. The cells were incubated in 24-well tissue culture plates for 48 hours. The supernatants were collected and evaluated by ELISA for the production of IFN-γ and IL-4 respectively. Data are mean ± SD of triplicate samples. Table 1 depicts theT_H1/T_H2 (IFN-γ/IL-4) ratio for the various treatment groups.

Figure 5. Effect Vα-TOS plus nmDC combination treatment on lung metastasis in the residual disease setting. Mice were injected orthotopically in the mammary fat pad with $5x10^4$ 4T1 tumor cells. Primary tumors were surgically resected on day 21. Starting on day 22, mice were injected i.p. with 4 mg of Vα-TOS on alternate days for a total of 5 injections. The mice were also injected s.c. with 10^6 nmDC on days 25 and 29. All the mice were sacrificed on day 31 and the lungs evaluated for visible metastatic nodules by staining with India Ink and Fekete's solution.

Figure 6. Effect of treatment with Vα-TOS plus DC on IFN-γ and IL-4 secretion by splenic lymphocytes in the residual disease model. Spleens were isolated on day 31 post-tumor injection from mice in each treatment group and pooled (5 mice per group). Splenic lymphocytes were separated by layering over a Ficol-Hypaque gradient. The cells were incubated in 24-well tissue culture plates for 48 hours. The supernatants were collected and evaluated by ELISA for the production of IFN-γ and IL-4. Data are mean ± SD of triplicate samples. Table 2 depicts theT_H1/T_H2 (IFN-γ/IL-4) ratio for the various treatment groups.

Figure 7. Effect of Vα-TOS treated tumor cells on DC maturation. 4T1 cells were allowed to adhere overnight in 6-well tissue culture plates at $2x10^5$ cells per well and then treated with 40 μg/ml Vα-TOS or PBS for 24 h. The supernatant was collected and centrifuged at 22,600 x g for 45 min to collect non-adherent cells and membrane debris. The pellet obtained was re-suspended in media and incubated with nmDC for 24 h. **(A)** DCs were collected and double-stained with PE-conjugated CD11c antibody and FITC-conjugated antibodies against CD40, CD80 and CD86 and analyzed by flow cytometry. Cells were gated on light scatter and $CD11c^+$ cells. **(B)** DCs were also re-stimulated with TNF-α for 24 h in 48-well tissue culture plates after which the supernatant was collected and evaluated for IL-12p70 production by ELISA. DC represents untreated DC, DC+PBS_s represents DC incubated with supernatant from PBS-treated 4T1 cells; DC+Vα-TOS_s represents DC incubated with supernatant from Vα-TOS-treated 4T1 cells; DC+Vα-TOS represents DC treated with 40 μg/ml Vα-TOS; DC+lysate represents DC incubated with freeze-thaw lysate of 4T1 tumor cells. The data are representative of 3 independent experiments.

Figure 8. Heat shock protein expression in tumor cells after treatment with Vα-TOS. 4T1 cells were allowed to adhere overnight in 6-well tissue culture plates at $2x10^5$ cells per well and then treated with either 40 μg/ml Vα-TOS or PBS (vehicle). After 12 h, non-adherent and adherent cells were collected, washed twice with PBS and stained with antibodies against hsp60, 70 or 90. Goat anti-mouse IgG-ALEXA-FLOUR 488 was used as the secondary antibody. Flow cytometric analysis **(A)** was performed on intact cells based on light scatter gates. The data are representative of two independent experiments. *Shaded region* represents PBS-treated cells and *black line* represents Vα-TOS-treated cells. **(B)** 4T1 cells were treated with either 40 μg/ml Vα-TOS or PBS for 24 h. Supernatant was then collected and centrifuged at 22,600 x g for 45 min. The pellet obtained was lysed, protein concentration measured and separated by 10% SDS-PAGE and transferred to PVDF membranes and stained with hsp60, 70 and 90-specific antibodies respectively. $V\alpha\text{-TOS}_s$ represents lysate derived from Vα-TOS-treated 4T1 tumor supernatant, PBS_s represents lysate derived from PBS-treated 4T1 tumor supernatant.

Figure 9. Effect of pre-treatment of non-matured DCs with α_2-macroglobulin on maturation induced by Vα-TOS treated tumor cells. Non-matured DCs were incubated in serum-free media with or without 100 μg/ml α_2M for one hour. 4T1 cells were treated with 40 μg/ml Vα-TOS or PBS for 24 h. The supernatant was collected and centrifuged at 22,600 x g for 45 min. The pellet obtained was re-suspended in media and added to the pre-treated DC for 24 h. **(A)** DCs were collected and stained with PE-conjugated CD11c antibody and FITC-conjugated antibodies against CD40, CD80 and CD86 and analyzed by flow cytometry. Cells were gated on light scatter and $CD11c^+$ cells. **(B)** DCs were also re-stimulated with TNF-α for 24 h in 48-well tissue culture plates after which the supernatant was collected and evaluated for IL-12p70 production by ELISA. DC represents untreated DC; DC+α_2M represents DC pre-treated with α_2M, DC+Vα-TOS_s represents DC incubated with supernatant from Vα-TOS-treated 4T1 cells, DC+α_2M+Vα-TOS_s represents DC pre-treated with α_2M and incubated with supernatant from Vα-TOS-treated 4T1 cells. The data are representative of 2 independent experiments.

Figure 1

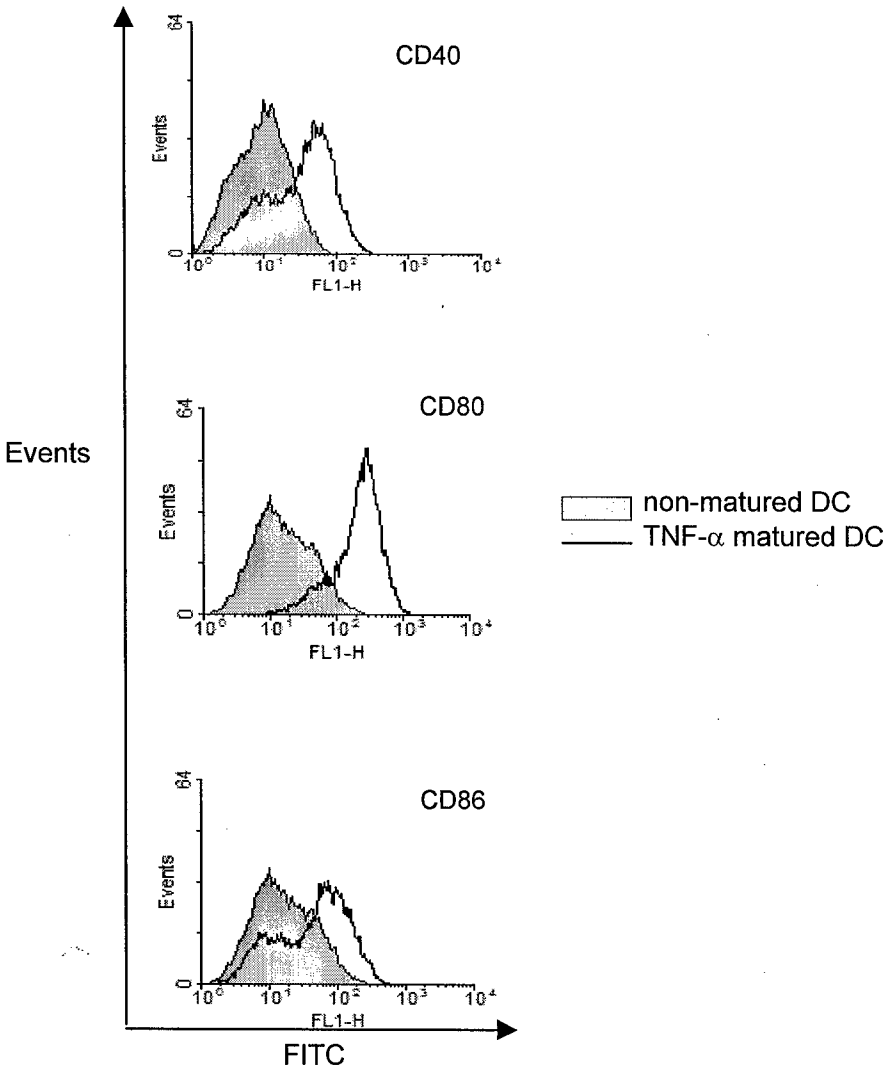
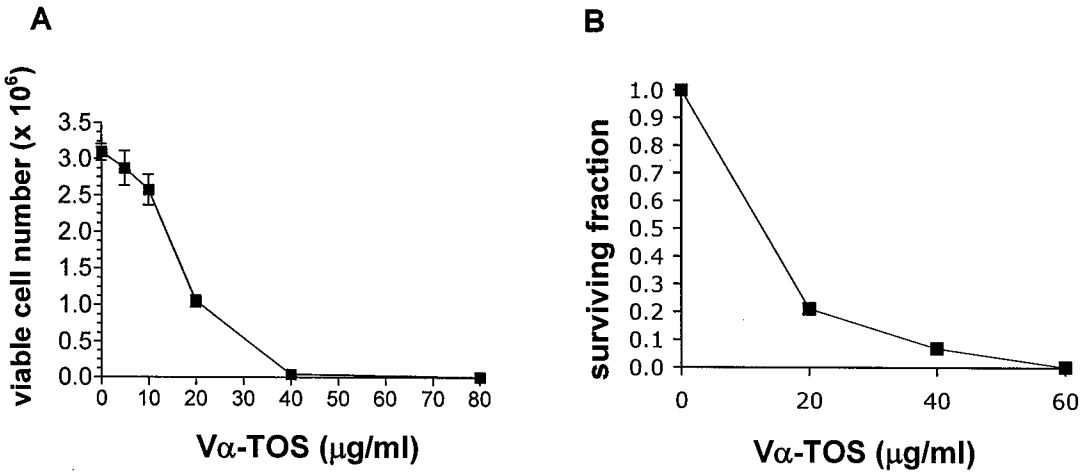

CD40
CD80
CD86
Events
FL1-H
FITC
non-matured DC
TNF-α matured DC

Figure 2


A
viable cell number (x 10^6)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 10 20 30 40 50 60 70 80
Vα-TOS (μg/ml)
B
surviving fraction
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 20 40 60
Vα-TOS (μg/ml)

C

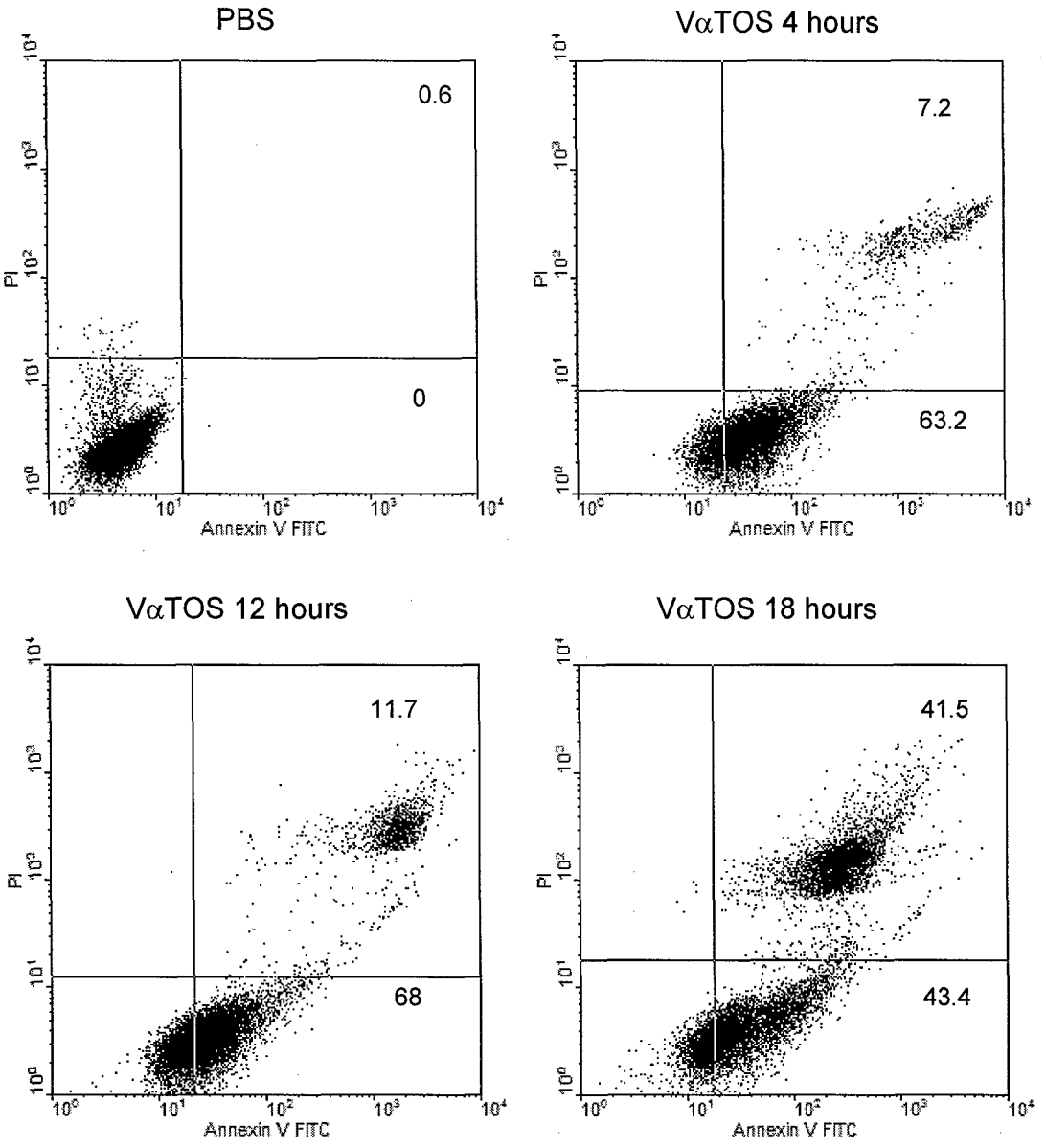

PBS
0.6
0
PI
Annexin V FITC
VαTOS 4 hours
7.2
63.2
PI
Annexin V FITC
VαTOS 12 hours
11.7
68
PI
Annexin V FITC
VαTOS 18 hours
41.5
43.4
PI
Annexin V FITC

Figure 2D

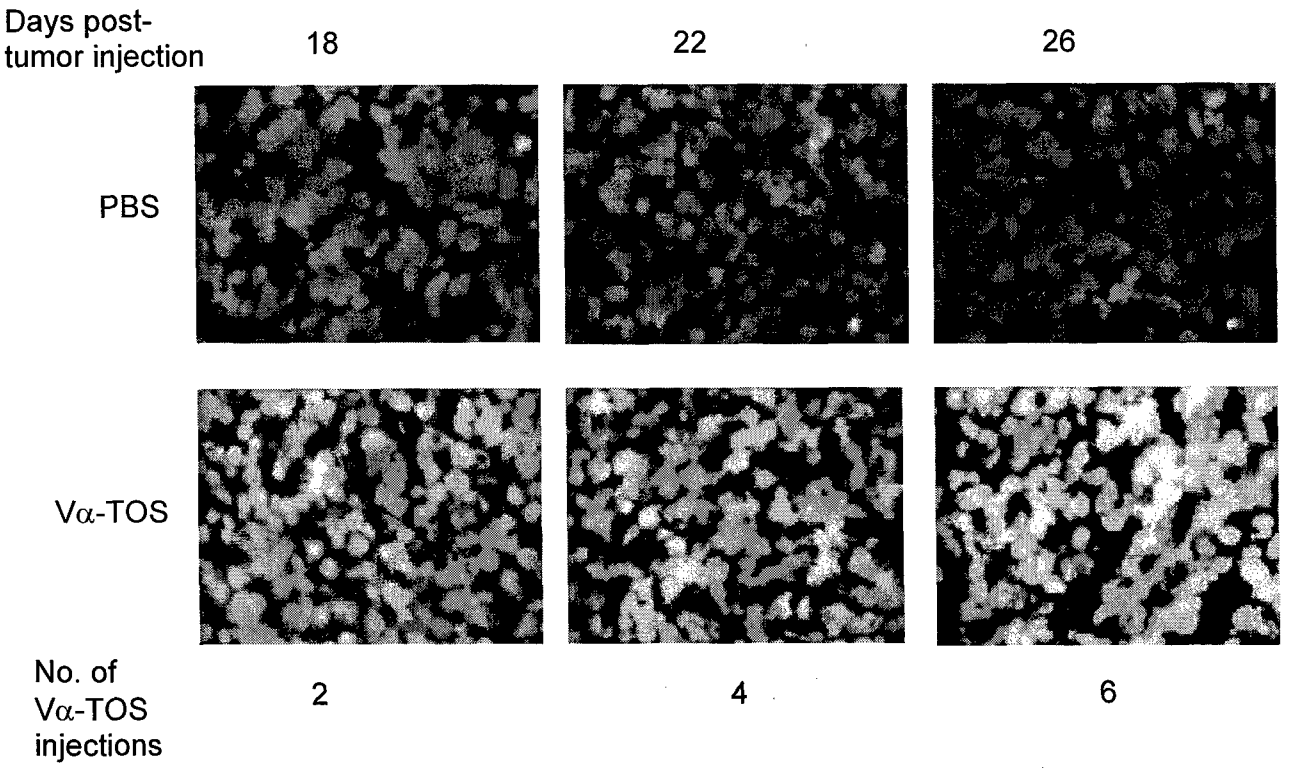
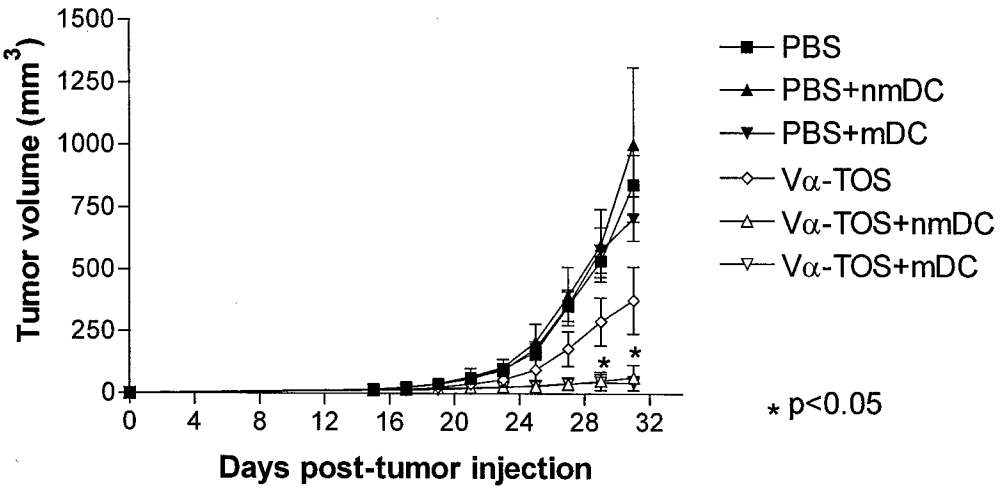

Days post-tumor injection
18
22
26
PBS
Vα-TOS
No. of Vα-TOS injections
2
4
6

Figure 3
A.


Tumor volume (mm3)
1500
1250
1000
750
500
250
0
0 4 8 12 16 20 24 28 32
Days post-tumor injection
PBS
PBS+nmDC
PBS+mDC
Vα-TOS
Vα-TOS+nmDC
Vα-TOS+mDC
* p<0.05

B.

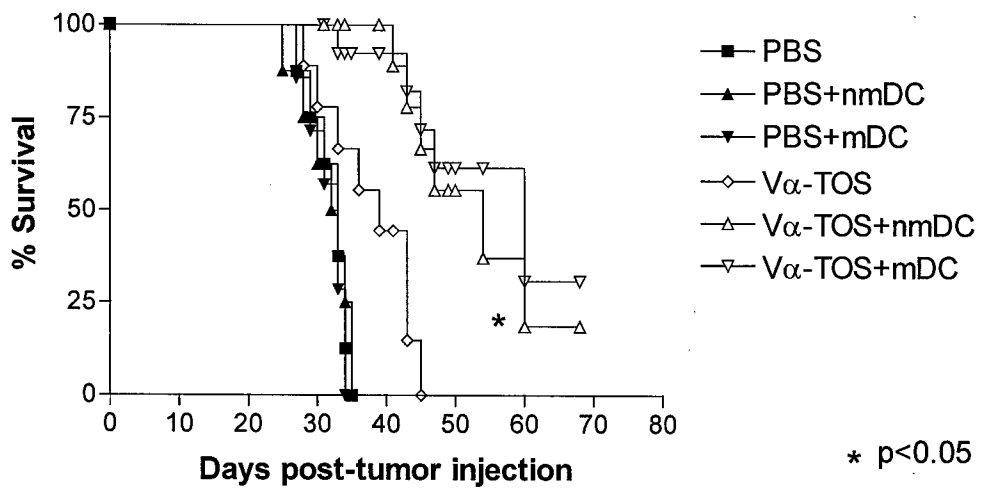

% Survival
100
75
50
25
0
0 10 20 30 40 50 60 70 80
Days post-tumor injection
PBS
PBS+nmDC
PBS+mDC
Vα-TOS
Vα-TOS+nmDC
Vα-TOS+mDC
* p<0.05

Figure 4

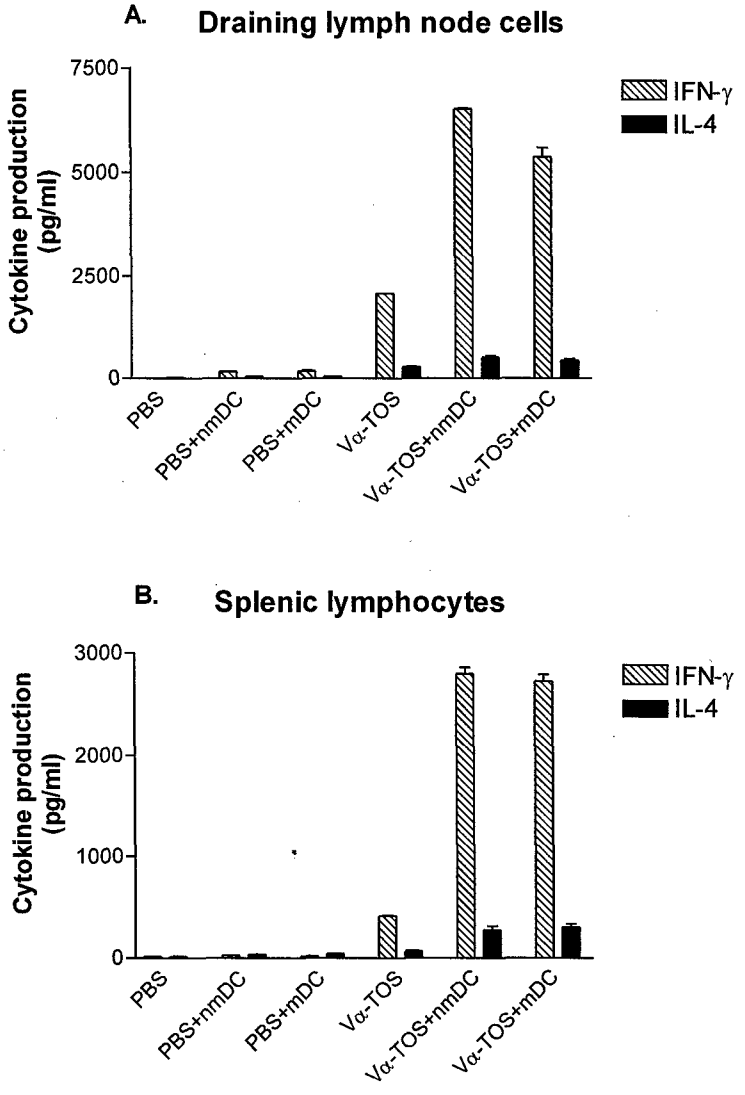


Table 1- TH1/TH2 (IFN-γ/IL-4) ratios

	Treatment Groups					
	PBS	PBS+nmDC	PBS+mDC	VαTOS	VαTOS+nmDC	VαTOS+mDC
Draining lymph node cells	0.5	3.2	3.4	7.5	12.8	12.3
Splenic lymphocytes	0.84	0.7	0.6	5.8	10.2	9.1

Figure 5

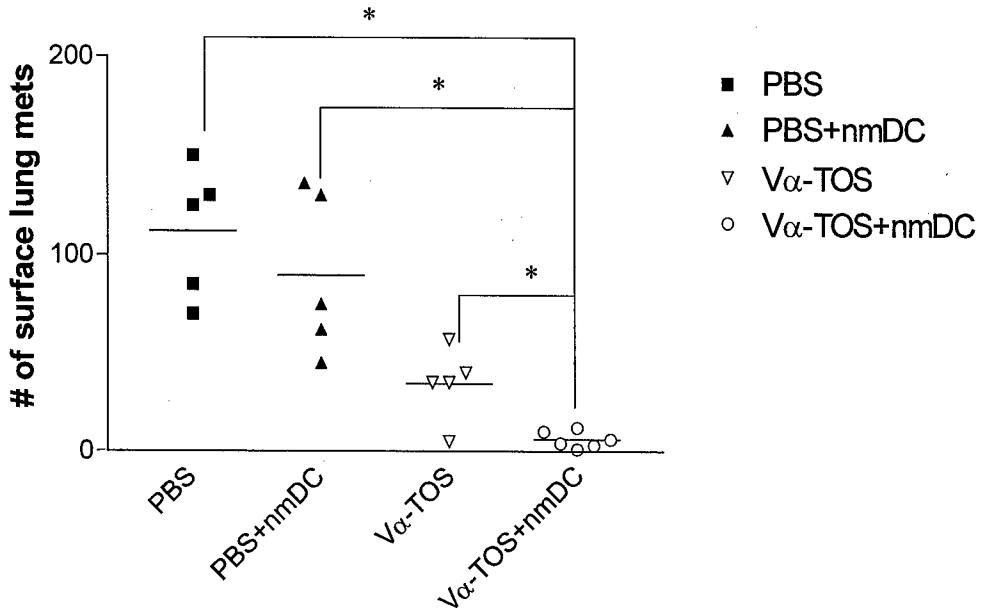

*
*
*
200
100
0
# of surface lung mets
PBS
PBS+nmDC
Vα-TOS
Vα-TOS+nmDC
PBS
PBS+nmDC
Vα-TOS
Vα-TOS+nmDC

Figure 6

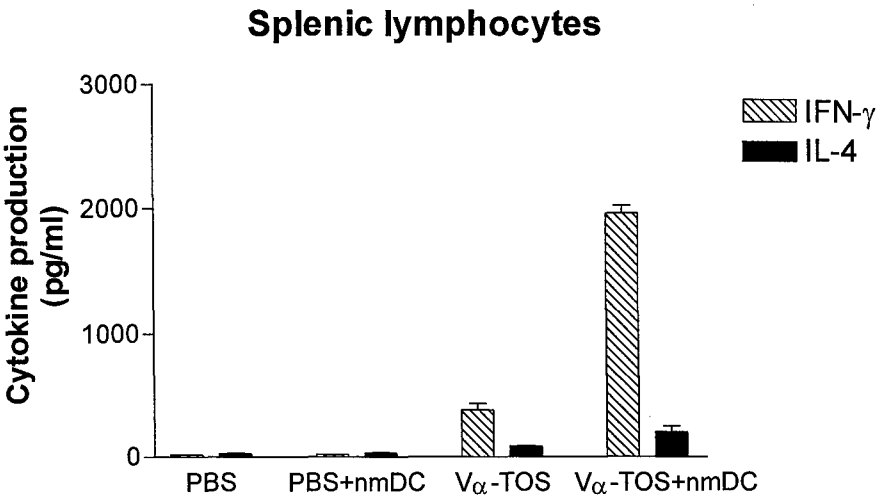
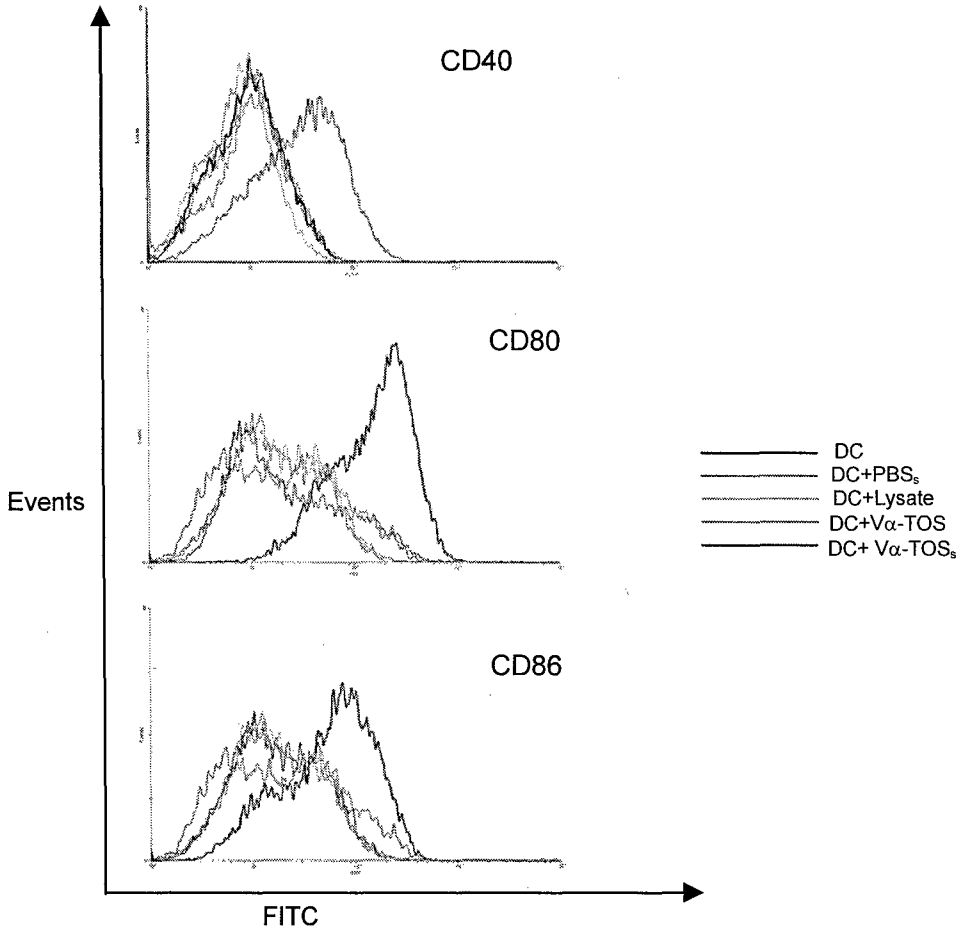


Table 2- TH1/TH2 (IFN-γ/IL-4) ratios

	Treatment Groups			
	PBS	PBS+nmDC	Vα-TOS	Vα-TOS+ nmDC
Splenic lymphocytes	0.72	0.66	4.4	9.7

Figure 7

A.


CD40
CD80
CD86
Events
FITC
DC
DC+PBS$_s$
DC+Lysate
DC+Vα-TOS
DC+ Vα-TOS$_s$

B.

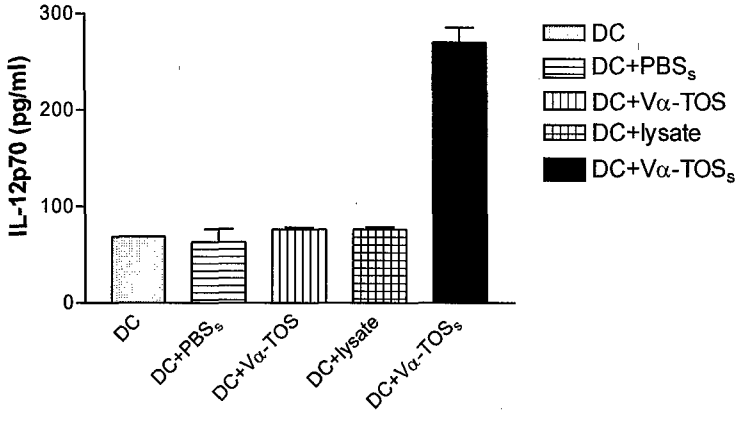
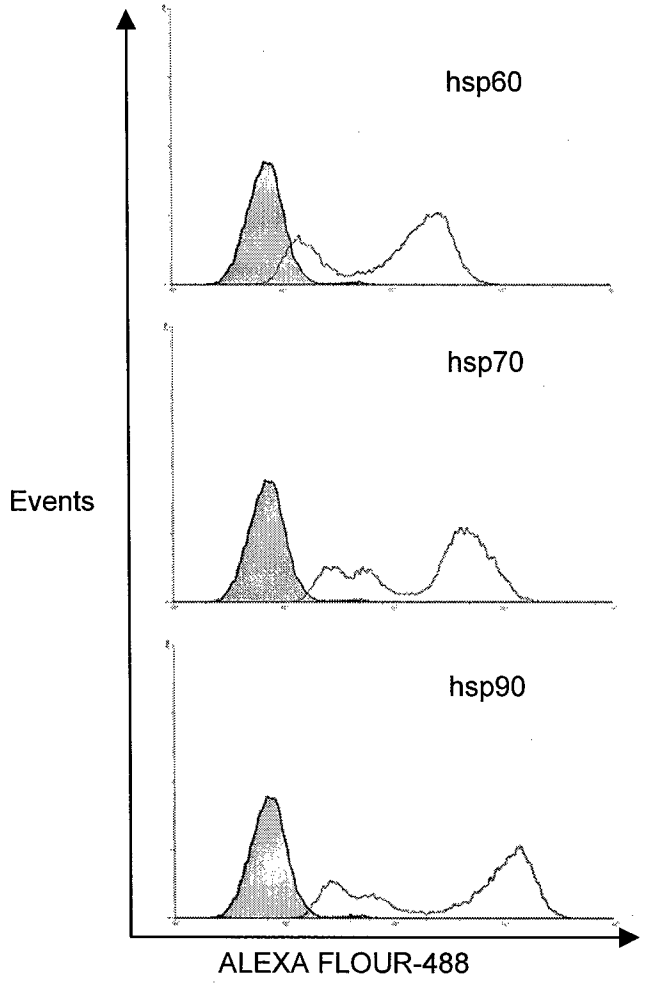

IL-12p70 (pg/ml)
300
200
100
0
DC
DC+PBS$_s$
DC+Vα-TOS
DC+lysate
DC+Vα-TOS$_s$

Figure 8
A.


hsp60
hsp70
hsp90
Events
ALEXA FLOUR-488

B.

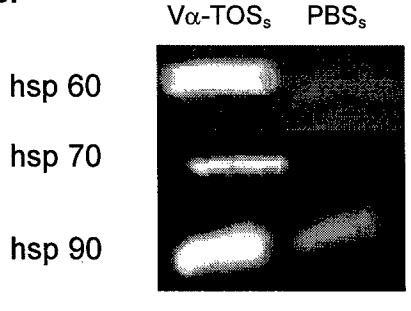
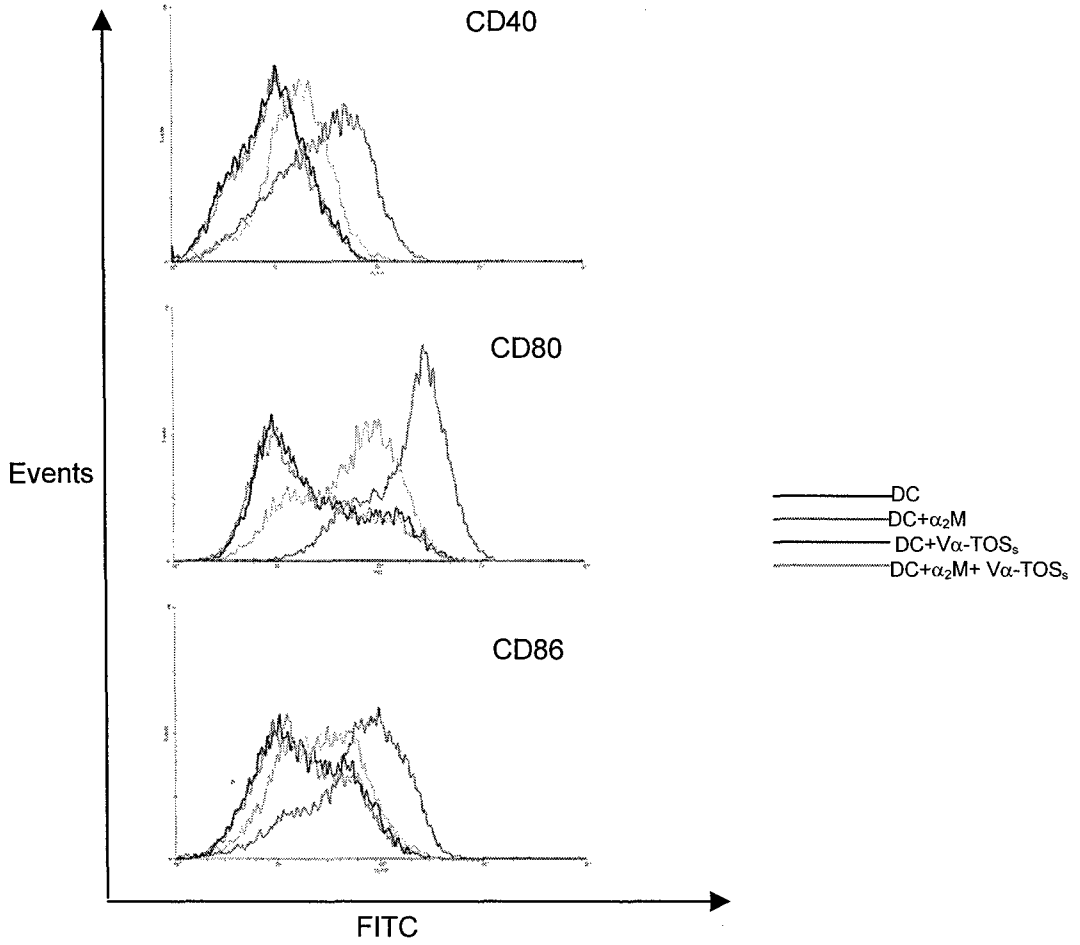
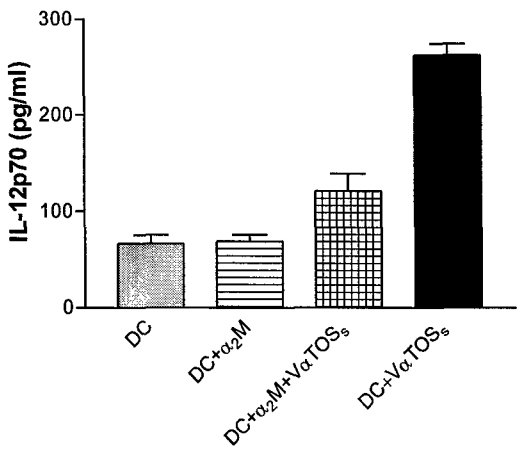

Vα-TOSs
PBSs
hsp 60
hsp 70
hsp 90

Figure 9

A.


CD40
CD80
CD86
Events
FITC
DC
DC+α2M
DC+Vα-TOSs
DC+α2M+ Vα-TOSs

B.


IL-12p70 (pg/ml)
300
200
100
0
DC
DC+α2M
DC+α2M+VαTOSs
DC+VαTOSs