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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 chemotherapeutic agent, α-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 α-TOS and vesiculated α-TOS in inducing apoptosis in tumor cells *in vitro* and *in vivo*, **2)** determine the efficacy of the drug 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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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 both α-TOS, and a more soluble hydrophilic form of the drug, vesiculated α-TOS (Vα-TOS). The hypothesis to be tested is that α-TOS and 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 α-TOS and 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

1. α-TOS induces killing of 4T1 tumor cells.

In order to demonstrate the susceptibility of 4T1 tumor cells to α-TOS, cells were treated with 40μg/ml α-TOS for 24 hours and clonogenicity and apoptosis assays were performed.

a) For the clonogenicity assay, viable cells obtained after treatment with α-TOS were plated and evaluated for their ability to proliferate and form colonies. The data (Figure 1a) show that, although 50% of the cells were viable after α-TOS treatment only 15% of them had the ability to form colonies as compared with 90% of the ethanol treated (control) cells. This shows that in addition to directly killing tumor cells, α-TOS also suppresses the proliferative potential of surviving cells.

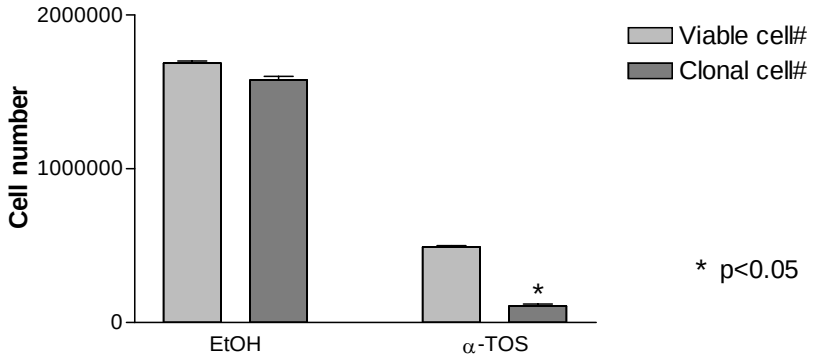


Figure 1a. Effect of α-TOS on clonogenic ability of tumor cells. 4T1 tumor cells were plated at 10^5 cells per well overnight. After 24h the non-adherent and adherent cells were collected and viability assessed using AO/PI. Viable cells were plated out at different dilutions and left undisturbed for 10 days. The clones obtained were fixed with methanol, stained with Giemsa and counted. The graph represents the total number of viable cells compared to the number of clones obtained from those cells.

b) For the apoptosis assay, 4T1 cells were treated with α-TOS, stained with Annexin V/ Propidium iodide (PI) using the Annexin-V-FLUOS staining kit (Roche Applied Sciences) and analyzed by flow cytometry. Annexin binds the phosphatidyl-serine moiety externalized in cells undergoing apoptosis and PI stains dead cells. The data (Figure 1b) show that α-TOS induces apoptosis as a function of time. At 4 hours, 69% of the cells were apoptotic (annexin V positive), which increased to 83% after treatment with α-TOS for 24 hours. In contrast control cells treated with sodium succinate (NaS) did not undergo significant apoptosis.

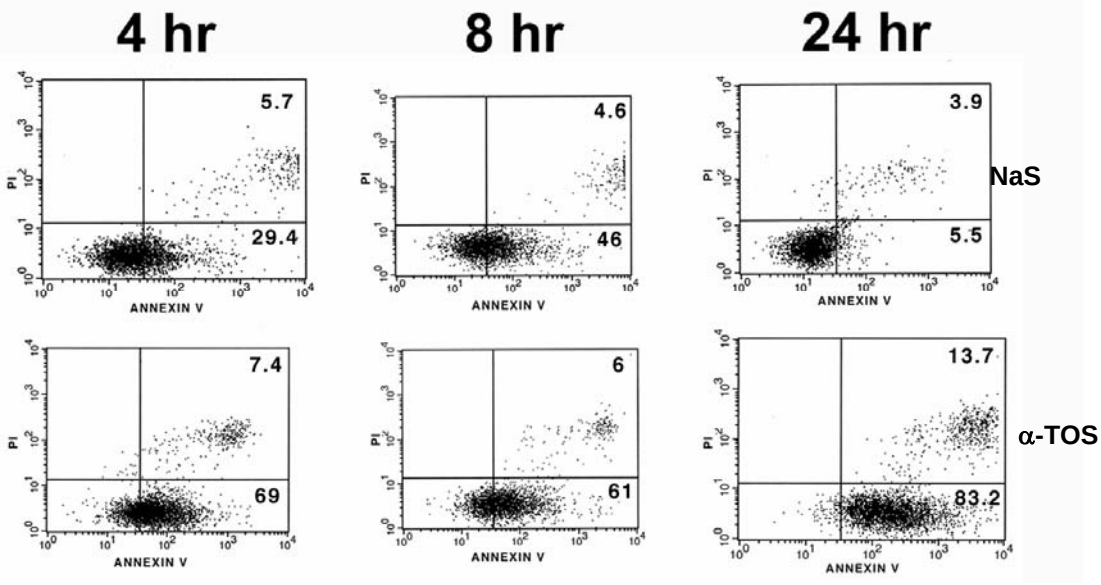


Figure 1b. Apoptosis assay. 4T1cells were plated at 10^5 cells per well in 6-well tissue culture plates overnight. The cells were treated with 40μg/ml α-TOS after 24h. At each time point (4h, 8h, 24h), adherent and non-adherent cells were collected and stained for detection of apoptosis using Annexin V-PI. The numbers in the dot plots represent the percentage of early apoptotic cells (lower right quadrant) and secondary necrotic cells (upper right quadrant) respectively

2. α-TOS is non-toxic to immune cells *in vivo.*

Since α-TOS kills tumor cells *in vitro,* it was important to determine whether it also impaired the functions of immune cells that are required for eliciting an immune response. For the purpose, we injected naïve mice three times with 4mg α-TOS every 4 days. T cells and dendritic cells were isolated from the spleens of the mice 48 hours after the last injection and evaluated for their functional activities. The data (Figure 2) show that α-TOS does not impair the ability of DCs to secrete IL-12 which is a cytokine secreted by mature activated DCs. α-TOS also does not inhibit the proliferation of T cells.

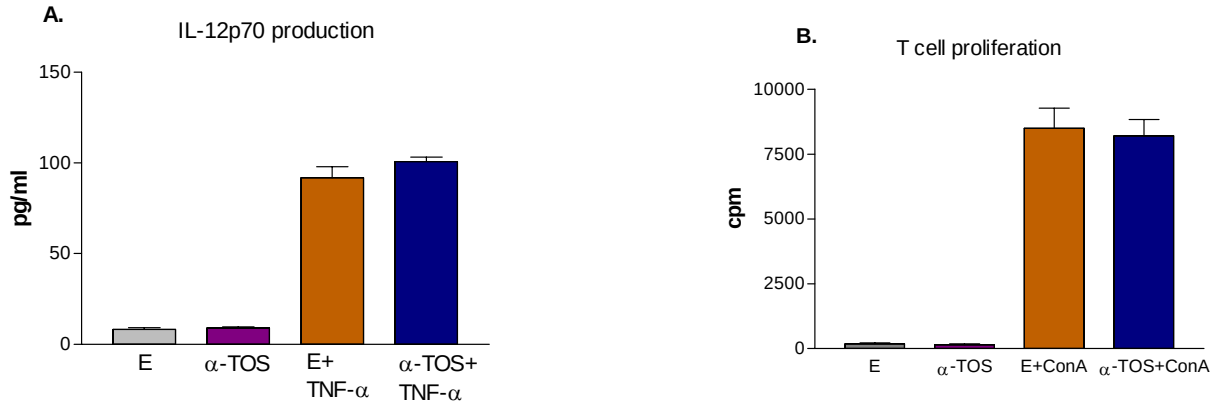


Figure 2. **Effect of α-TOS on immune cells *in vivo*.** Mice were injected three times with 4mg α-TOS every 4 days. Forty-eight hours after the last injection, splenocytes were isolated from the mice and DCs and T cells were purified using immunomagnetic beads. **A.** $5x10^5$ DCs were set up in triplicate 48-well plates with or without 20ng/ml TNF-a for 24 hours. Supernatants were then collected and assayed for the production of IL-12p70. **B.** $5x10^5$ purified T cells were set up in triplicate in 96-well plates with or without 2mg/ml ConA for 5 days. 1 μCi of [^{3}H]-thymidine was added to each well for the last 18 hours of incubation. Proliferation of T cells was determined by measuring thymidine uptake

3. α-TOS potentiates the anti-tumor activity of DC vaccines on pre-established mammary (4T1) tumors.

We next wanted to evaluate the ability of α-TOS to synergize with DC vaccines in controlling the growth of pre-established 4T1 tumors. For the purpose, mice with palpable 4T1 tumors were injected three times with 4mg of α-TOS every four days (day 14, 18 and 22). The mice were also injected with 10^6 immature DCs on days 16, 20 and 24.Tumor volume was monitored by measuring the tumor using calipers. The data (Figure 3) show that tumor growth was significantly inhibited by a combination of intraperitoneal injection of α-TOS plus subcutaneous injection of DC as compared to α-TOS alone or DC alone. Thus α-TOS alone does not affect tumor growth in an *in vivo* setting but has the ability to synergize with dendritic cells to create an effective anti-tumor response.

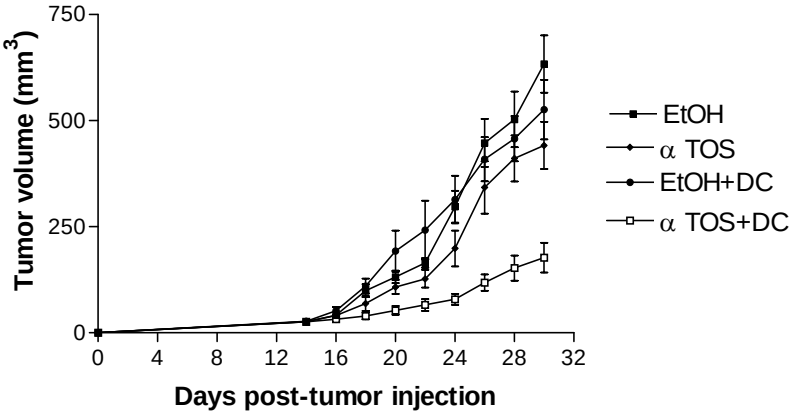


Figure 3. Effect of combined injection of α-TOS and DC on tumor growth *in vivo*. Balb/c mice were injected with $5x10^4$ tumor cells. On development of palpable tumors (day 14), they were injected i.p. with 4mg of α-TOS on days 14, 18 and 22. The mice were injected s.c with 10^6 immature DC on days 16, 20 and 24 and tumor growth was monitored. The figure represents the mean tumor volume± SEM of 7 mice per group

4. Combination therapy with α-TOS + DC induces increased production of IFN-γ by splenic lymphocytes.

Since α-TOS in combination with DCs inhibited the growth of pre-established tumors; we wanted to see if the observed clinical response correlated with elicitation of an enhanced immune response. For the purpose, splenocytes were isolated from mice of each of the treatment groups and re-stimulated with tumor lysate pulsed, TNF-α matured DCs for 48 hours. The supernatants were then collected and assayed for the production of IFN-γ by ELISA. The data (Figure 4) show that splenocytes isolated from mice treated with α-TOS + DC produced significantly higher levels of IFN-γ as compared to cells from mice treated with α-TOS alone or DC alone. This correlates with the tumor growth inhibition seen with the combination treatment.

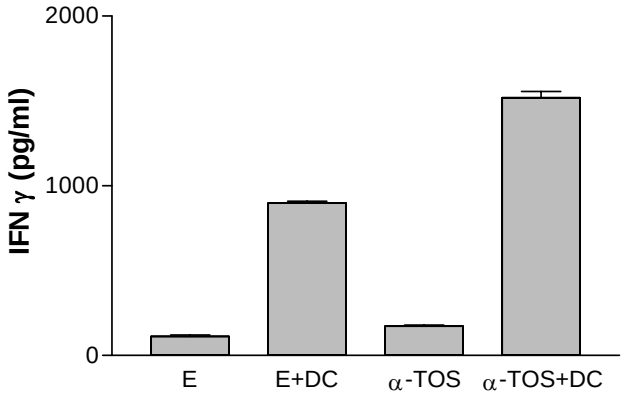


Figure 4. Effect of combined injection of α-TOS and DC on IFN-γ production *in vivo*. Spleens were isolated from 3 mice in each treatment group and pooled. The cells were then re-stimulated with tumor lysate-pulsed, TNF-α matured DC for 48 hours. The supernatants were assayed for the production of IFN-γ by ELISA. The data represent mean ± SEM of triplicate samples.

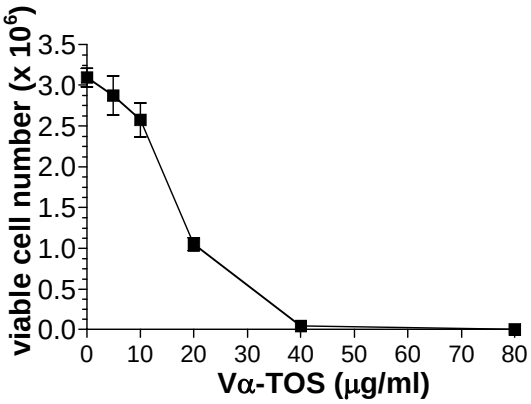
In the previous results we observed that α-TOS, when used in combination with non-matured dendritic cells (nmDC) to treat pre-established murine mammary tumors, acts as an effective adjuvant. One of the major limitations of using α-TOS is its insolubility in aqueous solvents. Unlike α-TOS which is soluble only in organic solvents like sesame oil, dimethylsulfoxide (DMSO) or ethanol (1-4), 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 (5). This circumvents the toxicities associated with the long-term use of DMSO or ethanol that are commonly used to solubilize α-TOS for parenteral administration making Vα-TOS better suited for long-term use in humans .In the following studies, we have used vesiculated α-TOS (Vα-TOS), in combination with dendritic cells to treat pre-established murine mammary tumors as well as residual metastasis following resection of the primary tumor.

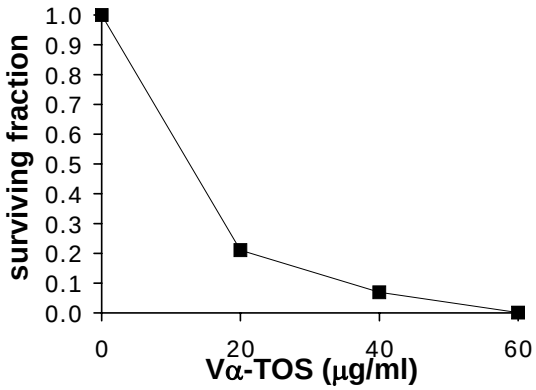
5. Vα-TOS is toxic to tumor cells and induces apoptosis *in vitro*

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 5A). 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 5B).

Vα-TOS-induced 4T1 tumor cell death was at least partially due to apoptosis as determined by Annexin V staining (Figure 5C). 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.

5A. **5B.**





5C.

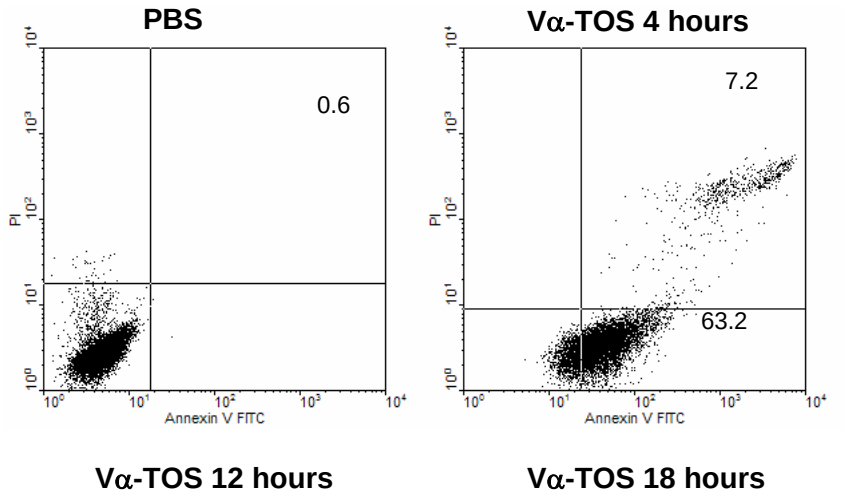
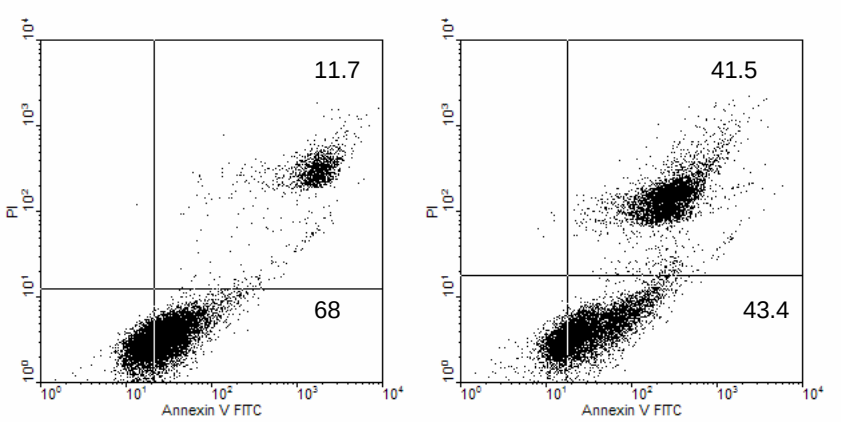


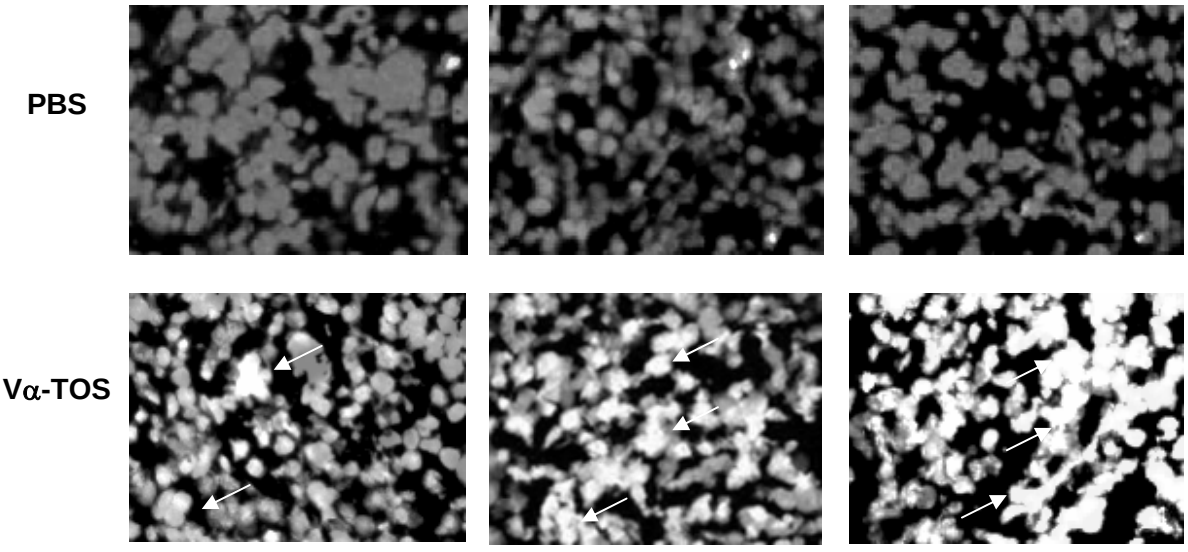
Figure 5. Effect of Vα-TOS treatment on 4T1 tumor cells *in vitro* and *in vivo*. 4T1 cells were allowed to adhere overnight in 6-well tissue culture plates. The cells were then treated with none (PBS), 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, or 80 μg/ml of Vα-TOS (in PBS). After a 24 h exposure, non-adherent 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. 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.



6. 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 indicated. 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 6). Maximum apoptosis was observed after 6 Vα-TOS injections.

6.



No. of Vα-TOS injections	2	4	6

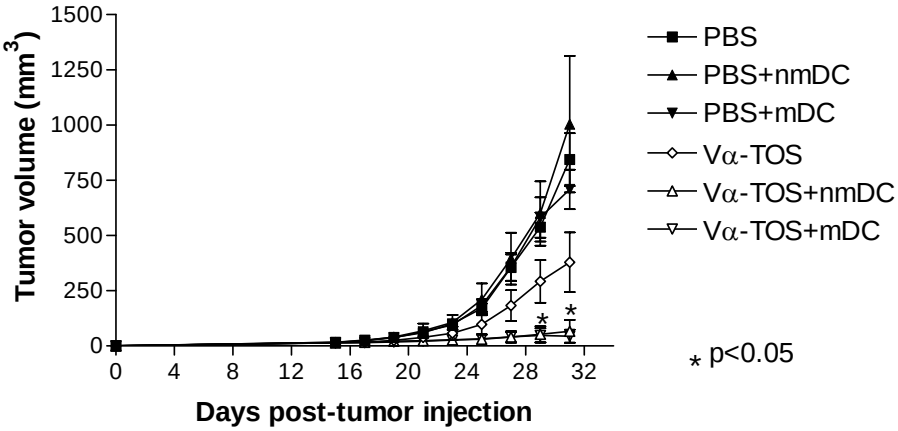
Figure 6. Effect of Vα-TOS on induction of apoptosis in tumors *in vivo.* Mice with pre-established tumors were injected with Vα-TOS on days 15, 17, 19, 21, 23 and 25. Twenty-four hours after every two Vα-TOS injections (days 18, 22 and 26), tumors were resected, frozen, sectioned and stained with the TUNEL /reaction mixture. The bright white regions (depicted by arrows) represent TUNEL positive regions and the gray regions represent TUNEL negative regions (Magnification = 400X).

7. 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 (4, 6). 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 7A) 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 7B). 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.

7A.



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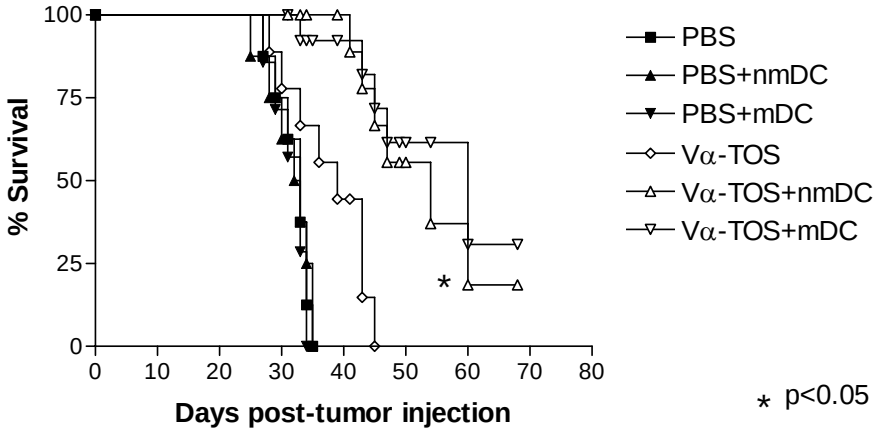


Figure 7. Effect of Vα-TOS plus DC immunotherapy on pre-established 4T1 tumors. Mice were injected orthotopically in the mammary pad with $5x10^4$ 4T1 tumor cells. On development of established tumors (day 15) mice were injected i.p. with 4 mg of Vα-TOS on alternate days for a total of 9 injections. 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).

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

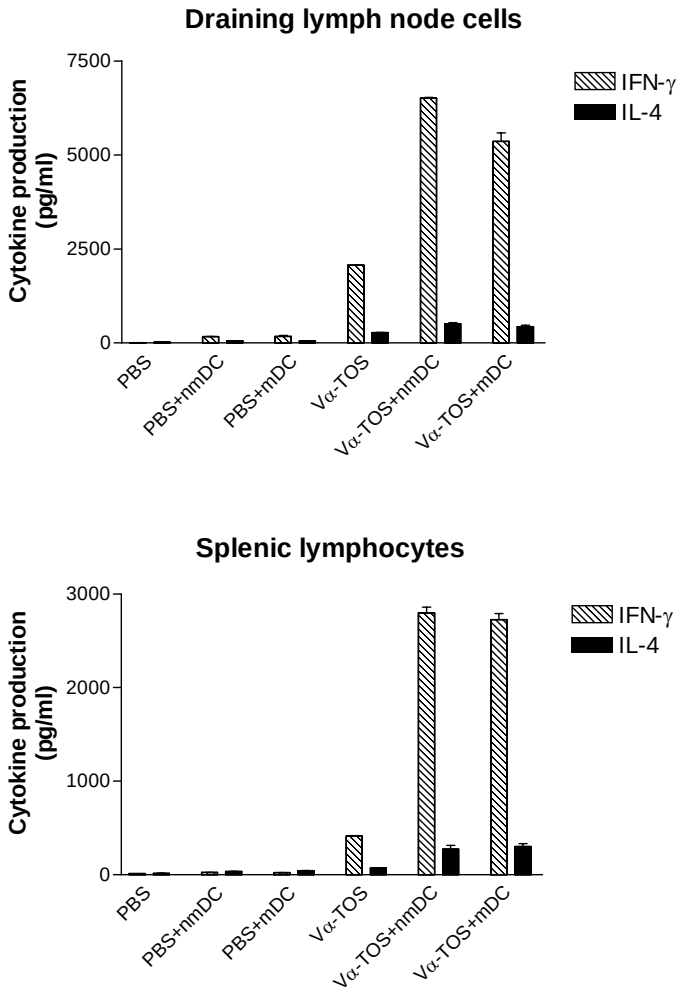
In order to determine whether the anti-tumor effect of Vα-TOS plus DC vaccination was associated with an enhanced immune response, cells were isolated from draining lymph nodes (DLN) as well as spleens of mice of the various treatment groups and evaluated for IFN-γ and IL-4 production by ELISA. Figure 8A 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 8B) 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 8A). 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 8B) which was significantly 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

8A.

the combination therapy suggests polarization towards a T_H1 mediated immune response



9. The 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 (7), 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 9) 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.

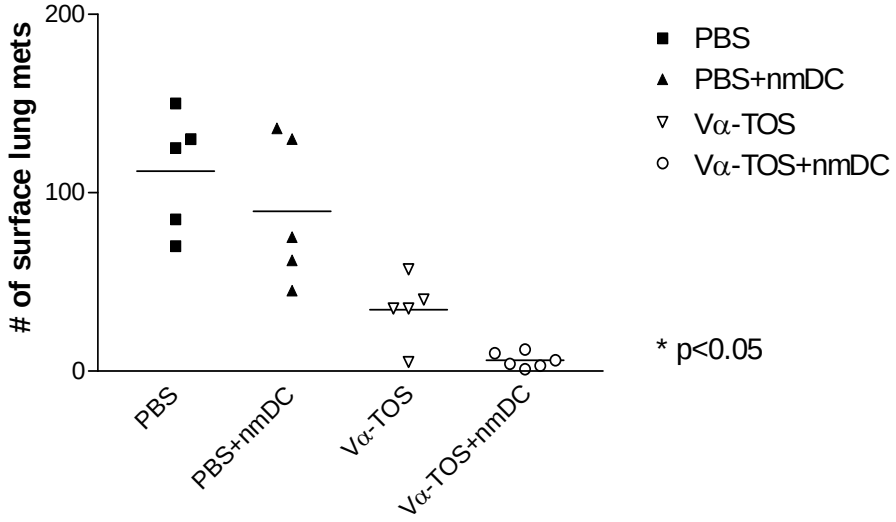


Figure 9. Effect Vα-TOS plus nmDC combination treatment on lung metastasis in the residual disease setting. Mice were injected orthotopically in the mammary fat pad with $5x10^4$ 4T1 tumor cells. Primary tumors were surgically resected on day 21. Starting on day 22, mice were injected i.p. with 4 mg 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.

10. 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 10). 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.

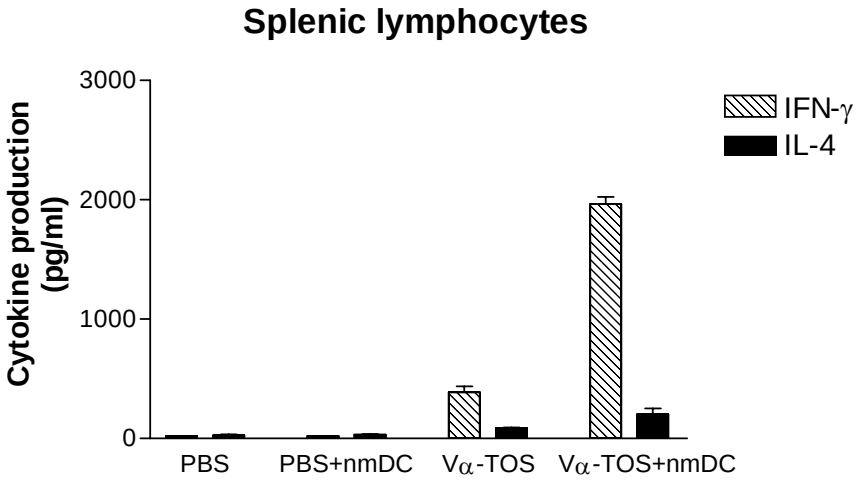


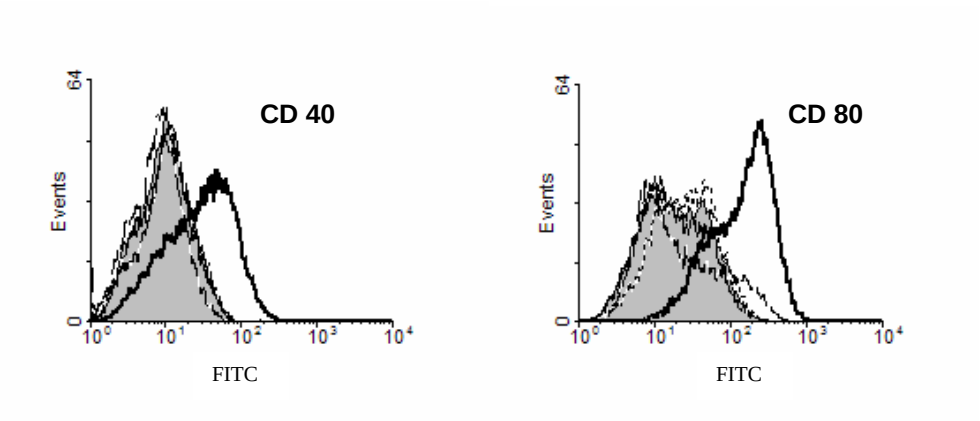
Figure 10. Effect of treatment with Vα-TOS plus DC on IFN-γ and IL-4 secretion by splenic lymphocytes in the residual disease model. Spleens were isolated on day 31 post-tumor injection from mice in each treatment group and pooled (5 mice per group). Splenic lymphocytes were separated by layering over 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.

11. Vα-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 Vα-TOS-derived tumor supernatant collected by high-speed centrifugation of supernatant fluid from tumor cells that were treated with Vα-TOS for 24 h. Following incubation with this fraction consisting of non-adherent cells, cellular debris and substances secreted by the tumor cells, DCs were assessed for the expression of the DC maturation markers CD40, CD80 and CD86. The data (Figure 11A) show that co-incubation of Vα-TOS-derived tumor 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 the factors produced by Vα-TOS-treatment of tumor cells caused DC maturation, we evaluated IL-12p70 secretion by DCs incubated with the high speed-spin fraction derived from supernatant of Vα-TOS-treated tumor cells. The data (Figure 11B) show that IL-12p70 secretion by DCs was significantly increased ($p < 0.001$) only when co-incubated with the Vα-TOS-derived tumor supernatant.

11A.



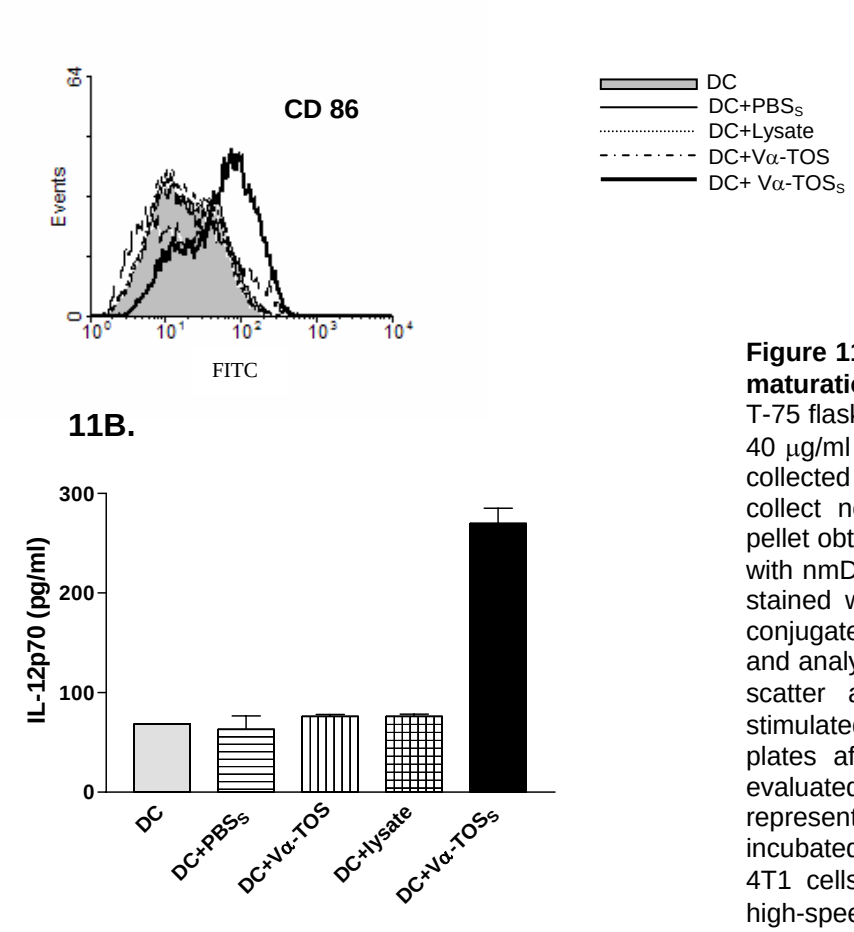


Figure 11. Effect of Vα-TOS treated tumor cells on DC maturation. 4T1 cells were allowed to adhere overnight T-75 flasks at 2.5x10^6 cells per flask and then treated with 40 μg/ml Vα-TOS or PBS for 24 h. The supernatant was collected and centrifuged at 22,600 x g for 45 min to collect non-adherent cells 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 high-speed spin fraction from PBS-treated 4T1 cells; DC+Vα-TOS$_S$ represents DC incubated with high-speed spin fraction 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.

12. 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 (8-11) and provide danger signals that may lead to activation and maturation of DCs (8-10, 12-16). Therefore, we postulated that the DC maturation by Vα-TOS-induced cellular factors that we have observed may be mediated at least in part, by hsps. First we needed to determine whether Vα-TOS treatment up regulated hsp expression on 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 12A) 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. The differential induction of hsps on tumor cells following Vα-TOS treatment was confirmed by Western blot analysis of the high speed-spin fraction derived from supernatant of Vα-TOS-treated cells (Figure 12B). This supernatant contained non-adherent cells, cellular debris and substances secreted by the tumor cells.

After we had shown that hsps are indeed up regulated on tumor cells in response to Vα-TOS treatment and are present in the high-speed spin fraction of Vα-TOS-treated tumor cells, we wanted to determine the involvement of hsps in the maturation of DC. For this purpose we blocked the cognate hsp receptor CD91 (17) on nmDC by pre-treatment with α_2-macroglobulin (α_2M). Subsequently, the DCs were co-incubated with the high speed-spin fraction of Vα-TOS-treated tumor cells as described above. The data (Figure 13A) 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 the cellular fraction 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-derived tumor cells (Figure 13B).

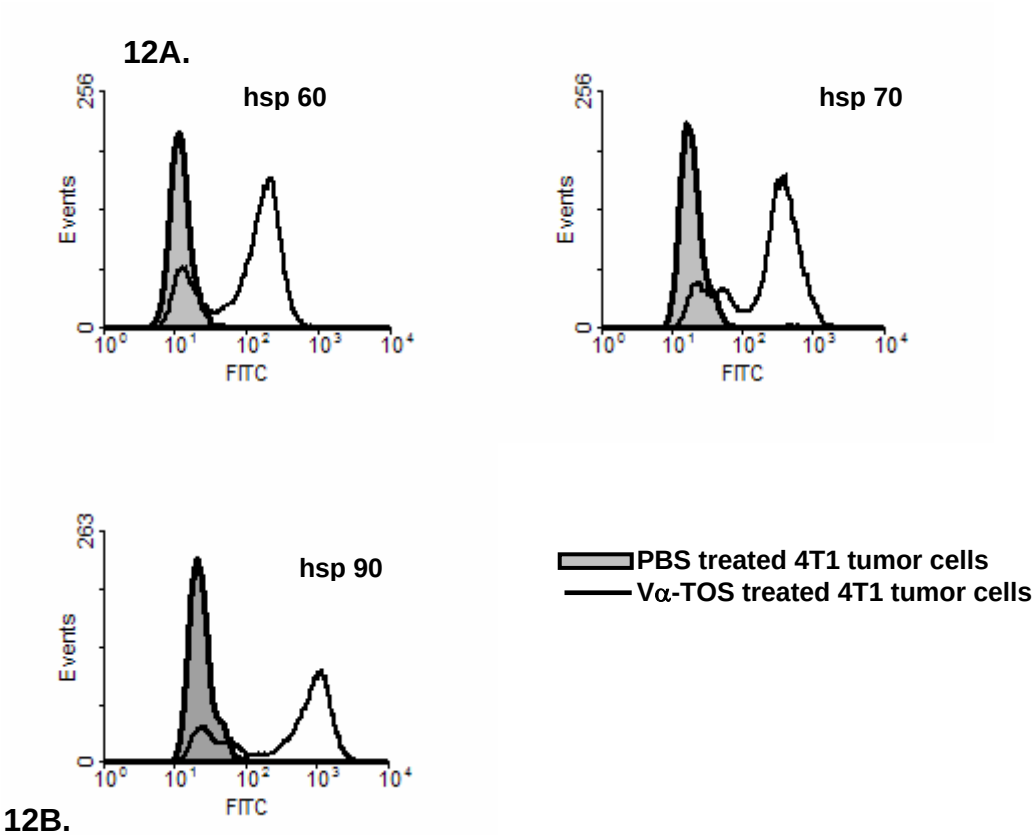
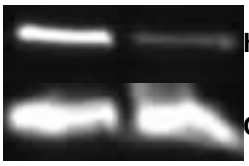
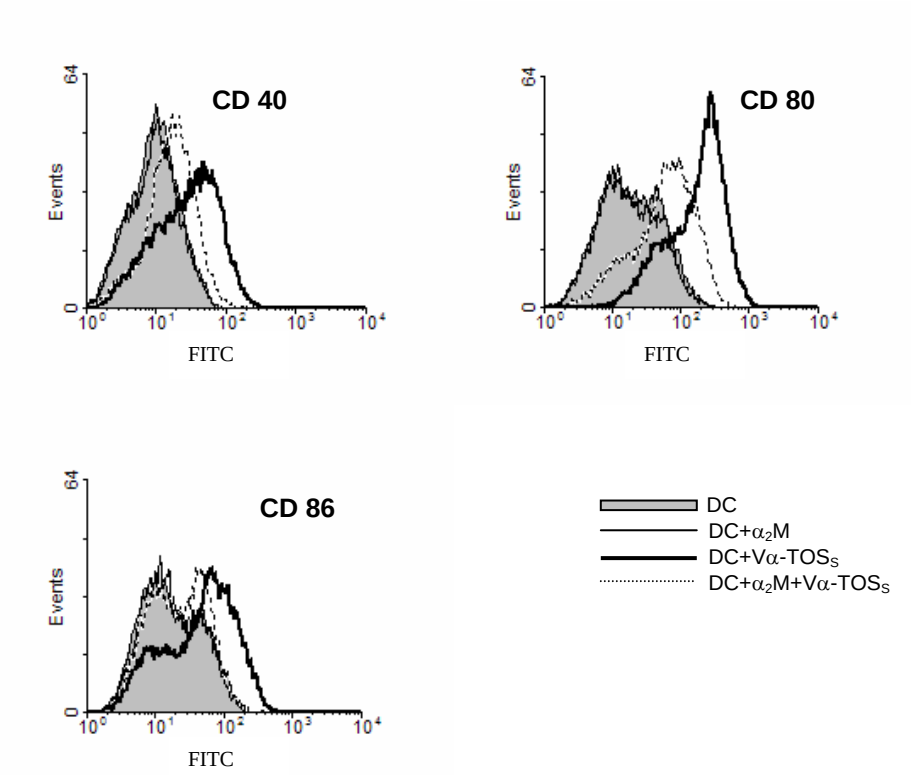


Figure 12. Heat shock protein expression in tumor cells after treatment with Vα-TOS. 4T1 cells were allowed to adhere overnight in 6-well tissue culture plates at $2x10^5$ cells per well and then treated with either 40 μg/ml Vα-TOS or PBS (vehicle). After 12 h, non-adherent and adherent cells were collected, washed twice with PBS and stained with antibodies against hsp60, 70 or 90. Goat anti-mouse IgG-ALEXA-FLUOR 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. (**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. Vα-TOS$_S$ represents lysate derived from high-speed spin fraction of Vα-TOS-treated 4T1 cells, PBS$_S$ represents lysate derived from high-speed spin fraction of PBS-treated 4T1 tumor cells.

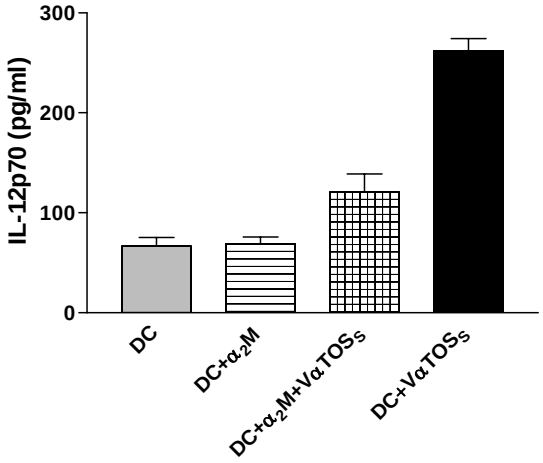


13A.



13B.

Figure 13. 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 high-speed spin fraction from Vα-TOS-treated 4T1 cells, DC+α_2M+Vα-TOS_S represents DC pre-treated



Non-matured DC have the ability to constantly sample their environment and pick up antigens by endocytosis and/or phagocytosis (18-20). In contrast, mature DC down regulate their antigen-uptake machinery, up regulate adhesion and co-stimulatory molecules and stabilize peptide-MHC complexes on their cell surface for antigen presentation (18).Having observed that Vα-TOS-derived tumor supernatant causes DC maturation, we wanted to study its effect on antigen uptake and presentation ability of DC, in order to further investigate and understand the immunomodulatory effects of alpha-tocopheryl succinate.

14. DCs Matured with Vα-TOS-derived Tumor Supernatant Retain their Endocytic and Phagocytic Abilities

In order to study antigen uptake, DC pre-treated with Vα-TOS-derived 4T1 tumor supernatant, PBS-tumor supernatant, TNF-α or left untreated, were used to study the uptake of FITC-labeled dextran beads by endocytosis or FITC-labeled *E.coli* particles by phagocytosis. The data (Figure 14A) show that DC matured with Vα-TOS-derived 4T1 tumor supernatant, retain the ability to endocytose FITC-labeled dextran beads similar to non-matured DC. These DC were also equally efficient in phagocytosing FITC-labeled *E.coli* particles as compared to nmDC (Figure 14B). In contrast DC matured with TNF-α had a significantly lesser ability to perform endocytic and phagocytic functions.

A.

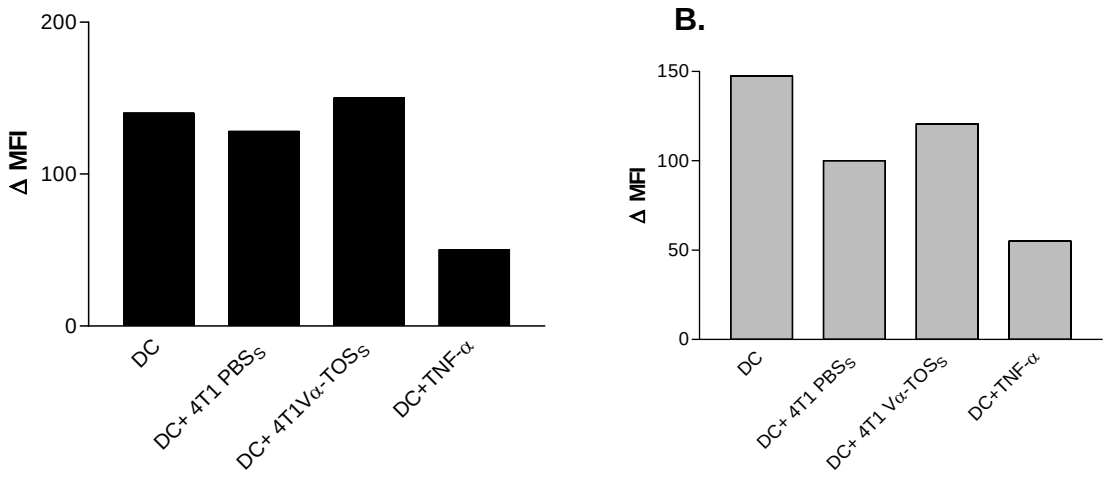


Figure 14. Effect of Vα-TOS-derived tumor supernatant on endocytosis and phagocytosis by DC. Supernatant from 4T1 or 3LL or B16 tumor cells treated with Vα-TOS (40 μg/ml) was collected and centrifuged at 22,600 x g for 45 min. The pellet obtained ($V\alpha\text{-}TOS_S$ or PBS_S) was re-suspended and incubated with non-matured BALB/c for 24 h. Subsequently DCs were collected and incubated with FITC-conjugated dextran beads for endocytosis **(A)** or FITC-labeled *E.coli* particles for phagocytosis **(B)** at 4°C and 37°C for 30 min or 60 min respectively. Cells were then washed and stained with CD11c-PE, fixed and analyzed by flow cytometry. Values represent difference in mean fluorescence intensity (MFI) at 37°C and 4°C of cells gated on CD11c. Data are representative of 2 independent experiments.

15. DCs Matured with Vα-TOS-derived Tumor Supernatant have Enhanced Antigen-Presenting Ability

Following antigen uptake, DC process the antigens and present them on MHC molecules to effector T-cells to initiate an immune response (20-22). Antigen presentation is a primary function of mature DC. Having observed that DC matured by Vα-TOS- derived tumor supernatant still retain the ability for antigen uptake by endocytosis and phagocytosis, we wanted to determine if these DC could function as effective antigen presenting cells. For the purpose, we performed an Allogenic-Mixed lymphocyte reaction (ALLO-MLR) by co-incubating BALB/c DC treated with or without Vα-TOS-derived tumor supernatant with allogeneic (C57BL/6) splenocytes for 4 days, with the addition of [^{3}H]thymidine for the last 18 h of culture. The cells were then harvested and radioactivity measured. DC matured with TNF-α was used as a positive control. The data (Figure 15) show that DC matured with Vα-TOS-derived tumor supernatant stimulates the proliferation of allogeneic splenocytes similar to that of TNF-α matured DC thus demonstrating that the DC are not only phenotypically but also functionally mature. In contrast DC incubated with PBS derived tumor supernatant or untreated DC had a significantly ($p<0.001$) lesser ability to induce proliferation.

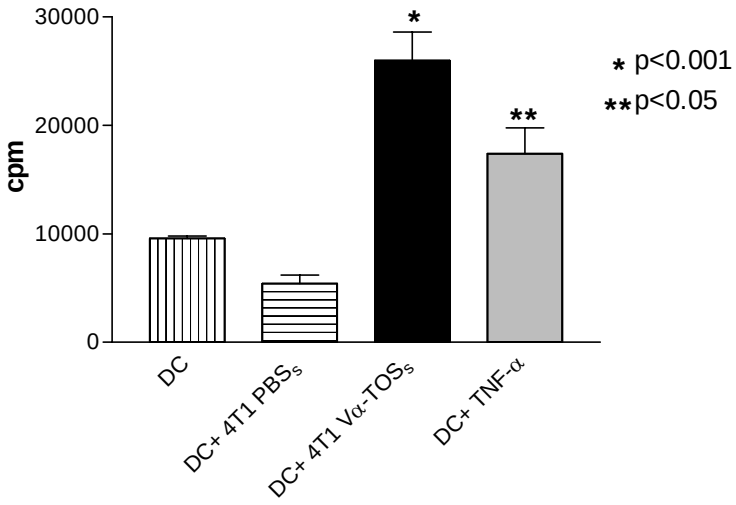


Figure 15. Effect of DC matured with Vα-TOS-derived tumor supernatant on stimulating Allogenic splenic lymphocytes. DC from BALB/c mice were incubated with Vα-TOS-derived tumor supernatant (Vα-TOS$_s$) or PBS-derived tumor supernatant (PBS$_S$), TNF-α or left untreated for 24 h. Subsequently 1x10^4 irradiated DCs from each group were incubated in a 96-well plate with 2x10^5 allogenic splenic lymphocytes for 4 days with the addition of [^{3}H]thymidine for the last 18 h of culture. The plate was harvested and amount of radioactivity measured. Data represents mean ± S.D. of triplicate samples per group.

In the process of performing experiments for the study, parts of it were developed expanding on results obtained. Thus, there might appear to be certain deviations from the original statement of work in terms of understanding the mechanism of action of the combination treatment of α-TOS and DC. Also, there were technical difficulties in obtaining the required statistically significant number of transgenic SCID mice and CD4/CD8 knockout mice to perform experiments mentioned in specific Aim 2 which could therefore not be performed.

Key research accomplishments

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

2. **Demonstrate the ability of α-TOS and 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**

8. **Evaluate the immunomodulatory effect of Vα-TOS-derived tumor supernatant by studying its effect on antigen uptake and presentation by DC**

Reportable outcomes

Degree obtained

I successfully defended my dissertation which was based on the work outlined in this report, supported by the Pre-doctoral training grant from the Department of Defense on May 11th2006. Degree will be awarded in August 2006.

Presentations

1) α-Tocopheryl Succinate Sensitizes Established Tumors to Vaccination with Non-matured Dendritic Cells. Lalitha V. Ramanathapuram, James J. Kobie, Claire M. Payne, Katrina Trevor and Emmanuel T. Akporiaye.
Poster, International Society for Biological Therapy of Cancer, 18th Annual Meeting. Bethesda, Maryland, October 30- November 2, 2003.

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

Manuscripts

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. Nutrition and Cancer, 2005; 53(2):177-93.

Lalitha V. Ramanathapuram, Tobias Hahn, Mikhal Gold and Emmanuel T. Akporiaye. Vesiculated Alpha-Tocopheryl Succinate Stimulates Maturation And Antigen Presenting Activity Of Dendritic Cells. Manuscript in preparation

CONCLUSIONS

Dendritic cells are nature's adjuvants that trigger and control immunity (21, 22). They are thus

attractive targets for therapeutic manipulation of the immune system to increase otherwise insufficient immune responses against tumor antigens (23). Although the use of DC vaccines in animal studies and human clinical trials has resulted in anti-tumor immune responses, eradication of establsihed tumor has been infrequently observed (24-27). DC-based immunotherapy approaches will likely benefit from coupling to other therapies, immune based and otherwise. Making tumor antigens available to DCs so that they can process and present them to elicit potent T cell responses is one such approach.

In these studies we have used a combination of relatively non-toxic chemotherapy and DC vaccines. The rationale for this approach is that drug-induced apoptotic death of tumor cells will make tumor-associated antigens available to exogenously injected DCs for processing and presentation to tumor-specific T cells. The chemotherapeutic drug that we have used, alpha-tocopheryl succinate (α-TOS), has certain advantages over other conventional chemotherapeutic agents in that it selectively targets cancer cells and is minimally toxic to normal cells (28-35). In addition, killing of tumor cells by α-TOS eliminates the secretion of tumor-derived effectors that contribute to immune evasion (36, 37). These attributes make α-TOS an attractive candidate for use in combination with DCs.

In these studies, we evaluated the efficacy of α-TOS and the more soluble analogue Vα-TOS plus DC chemo-immunotherapy to treat pre-established tumors of the highly metastatic murine mammary cancer cell line 4T1. Both α-TOS and Vα-TOS kill tumor cells in vitro partly mediated by apoptosis. We demonstrate that α-TOS and Vα-TOS in combination with non-antigen pulsed, non-matured dendritic cells significantly inhibits the growth of established 4T1tumors *in vivo* and prolongs the survival of treated mice. 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. On re-challenging the mice that showed complete tumor regression with a 10-fold higher dose of tumor cells, they did not develop any tumors indicative of the acquisition of memory immune response.

Apart from its inhibitory effect on the growth of established tumors, we also demonstrated that Vα-TOS plus DC treatment dramatically reduces lung metastasis, when treatment is initiated after primary tumor resection. This finding demonstrates the promise of Vα-TOS+DC therapy as an effective modality for treating residual metastatic disease, which is the primary cause of mortality in humans.

An interesting part of the study was the observation that non-antigen pulsed, non-matured DC were as effective as TNF-α matured, non-pulsed DC when used in combination with α-TOS or Vα-TOS. This finding suggested that Vα-TOS treatment may induce DC maturation. To pursue this possibility, we incubated immature DC with Vα-TOS-derived tumor supernatant for 24 h. Following incubation with this fraction that contained non-adherent cells, cellular debris and factors secreted by the tumor cells, DCs were assessed for the expression of co-stimulatory molecules and IL-12 secretion. The data showed that co-incubation of Vα-TOS-derived tumor supernatant with nmDC caused an up regulation of the maturation markers CD40, CD80 and CD86 on DC. Direct incubation of nmDC with Vα-TOS did not cause an increase in the expression of these markers. The up regulation of co-stimulatory molecules correlated with increased IL-12p70 production which is an important cytokine secreted by mature DCs that favors the development of a T_H1 immune response *in vivo.* In addition, we showed that Vα-TOS induces apoptosis of tumor cells leading to secondary necrosis. Taken together, these findings corroborate earlier studies that demonstrated that exposure of DC to stressed apoptotic tumor cells, tumor lysates or supernatants of necrotic transformed cell lines leads to maturation of human and murine dendritic cells (8-10, 16, 38).

The possibility that Vα-TOS treatment may induce the maturation of DC *in vivo* is significant, as it would facilitate the translation of this combination treatment approach to the clinic by obviating the need for additional *ex-vivo* manipulations of DC.

We also observed that Vα-TOS treatment of tumor cells induces expression of heat shock proteins 60, 70 and 90. Heat shock proteins are one of the most abundant soluble intracellular

molecules that function as molecular chaperones (39). They have essential roles in protecting cells from potentially lethal effects of stress and proteotoxicity (39). The presence of hsps in the extracellular environment acts as a “danger signal” that alerts antigen presenting cells including DC of potential damage or infection leading to their activation (8-10, 12, 13, 15, 38-40). Activated DCs are very effective antigen presenters, which migrate to secondary lymphoid organs where they initiate anti-tumor T cell responses (21).

In trying to understand the role of hsps, we blocked the cognate hsp receptor CD91 (17) on DC with α_2-macroglobulin and assessed the expression of the co-stimulatory molecules that are up regulated in activated mature DC. The data demonstrate that the blocking of hsp binding to the CD91 receptor resulted in partial inhibition of expression of the maturation markers CD40, CD80 and CD86 when DC were co-incubated with Vα-TOS-derived tumor supernatant. However, the absence of complete inhibition of co-stimulatory molecule expression may indicate the involvement of additional hsp receptors such as the scavenger receptor CD36, LOX-1 and toll-like receptor 4 (41) and/or other hsps including gp96 and calreticulin. Taken together, our results suggest a direct role for hsps in Vα-TOS-mediated DC activation.

Based on these findings we can speculate that intratumoral injection of Vα-TOS could directly kill tumor cells, leading to the release of hsps, which can cause maturation of tumor infiltrating immature DCs. These DCs could then migrate to the draining lymph node, present tumor antigens and stimulate an anti-tumor immune response.

In trying to further investigate the immunomodulatory role of Vα-TOS, we observed that although Vα-TOS-derived tumor supernatant causes DC maturation, these DC are still efficient at both antigen uptake and presentation.

In summary, the results of this study demonstrate the adjuvant effect on DC-based vaccines of a chemotherapeutic agent that is selectively toxic to tumor cells in controlling the growth of pre-established

tumors. Since α-TOS/ Vα-TOS preferentially kills tumor cells (29), it is potentially likely to induce less severe adverse side effects compared to conventional apoptosis-inducing chemotherapeutic drugs (29, 42, 43) and may therefore be clinically useful for enhancing anti-tumor immune responses.

The dramatic anti-metastatic effect of the Vα-TOS+DC combination treatment bodes well for the use of this approach in controlling micrometastatic disease. This is particularly relevant in breast cancer where mortality is due to disease recurrence at metastatic sites.

Taken together, our results suggest that alpha-tocopheryl succinate 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 treatment is effective in the treatment of established tumors 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.

REFERENCES

1. Malafa, M. P. and Neitzel, L. T. Vitamin E succinate promotes breast cancer tumor dormancy. J Surg Res, *93:* 163-170, 2000.
2. Malafa, M. P., Fokum, F. D., Mowlavi, A., Abusief, M., and King, M. Vitamin E inhibits melanoma growth in mice. Surgery, *131:* 85-91, 2002.
3. Weber, T., Lu, M., Andera, L., Lahm, H., Gellert, N., Fariss, M. W., Korinek, V., Sattler, W., Ucker, D. S., Terman, A., Schroder, A., Erl, W., Brunk, U. T., Coffey, R. J., Weber, C., and Neuzil, J. Vitamin E succinate is a potent novel antineoplastic agent with high selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) in vivo. Clin Cancer Res, *8:* 863-869, 2002.
4. Ramanathapuram, L. V., Kobie, J. J., Bearss, D., Payne, C. M., Trevor, K. T., and Akporiaye, E. T. Alpha-tocopheryl succinate sensitizes established tumors to vaccination with nonmatured dendritic cells. Cancer Immunol Immunother, *53:* 580-588, 2004.
5. Kogure, K., Manabe, S., Hama, S., Tokumura, A., and Fukuzawa, K. Potentiation of anti-cancer effect by intravenous administration of vesiculated alpha-tocopheryl hemisuccinate on mouse melanoma in vivo. Cancer Lett, *192:* 19-24, 2003.
6. Ramanathapuram, L. V., Hahn, T., Graner, M. W., Katsanis, E., Akporiaye, E. T. Vesiculated Alpha-tocopheryl Succinate Enhances the Anti-tumor Effect of Dendritic Cell Vaccines. Cancer Immunol Immunother In press, 2005.
7. Wu, R. S., Kobie, J. J., Besselsen, D. G., Fong, T. C., Mack, V. D., McEarchern, J. A., and Akporiaye, E. T. Comparative analysis of IFN-gamma B7.1 and antisense TGF-beta gene transfer on the tumorigenicity of a poorly immunogenic metastatic mammary carcinoma. Cancer Immunol Immunother, *50:* 229-240, 2001.
8. Basu, S., Binder, R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol, *12:* 1539-1546, 2000.
9. Feng, H., Zeng, Y., Graner, M. W., and Katsanis, E. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. Blood, *100:* 4108-4115, 2002.
10. Somersan, S., Larsson, M., Fonteneau, J. F., Basu, S., Srivastava, P., and Bhardwaj, N. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. J Immunol, *167:* 4844-4852, 2001.
11. Buttiglieri, S., Galetto, A., Forno, S., De Andrea, M., and Matera, L. Influence of drug-induced apoptotic death on processing and presentation of tumor antigens by dendritic cells. Int J Cancer, *106:* 516-520, 2003.
12. Basu, S. and Srivastava, P. K. Fever-like temperature induces maturation of dendritic cells through induction of hsp90. Int Immunol, *15:* 1053-1061, 2003.
13. Bethke, K., Staib, F., Distler, M., Schmitt, U., Jonuleit, H., Enk, A. H., Galle, P. R., and Heike, M. Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. J Immunol, *169:* 6141-6148, 2002.
14. Chen, W., Syldath, U., Bellmann, K., Burkart, V., and Kolb, H. Human 60-kDa heat-shock protein: a danger signal to the innate immune system. J Immunol, *162:* 3212-3219, 1999.
15. Flohe, S. B., Bruggemann, J., Lendemans, S., Nikulina, M., Meierhoff, G., Flohe, S., and Kolb, H. Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. J Immunol, *170:* 2340-2348, 2003.
16. Zheng, H., Dai, J., Stoilova, D., and Li, Z. Cell surface targeting of heat shock protein gp96 induces dendritic cell maturation and antitumor immunity. J Immunol, *167:* 6731-6735, 2001.
17. Basu, S., Binder, R. J., Ramalingam, T., and Srivastava, P. K. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity, *14:* 303-313, 2001.
18. Timmerman, J. M. and Levy, R. Dendritic cell vaccines for cancer immunotherapy. Annu Rev Med, *50:* 507-529, 1999.

19. Mellman, I. and Steinman, R. M. Dendritic cells: specialized and regulated antigen processing machines. Cell, *106:* 255-258, 2001.
20. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. Immunobiology of dendritic cells. Annu Rev Immunol, *18:* 767-811, 2000.
21. Banchereau, J. and Steinman, R. M. Dendritic cells and the control of immunity. Nature, *392:* 245-252, 1998.
22. Steinman, R. M. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol, *9:* 271-296, 1991.
23. Banchereau, J. and Palucka, A. K. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol, *5:* 296-306, 2005.
24. Fields, R. C., Shimizu, K., and Mule, J. J. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proc Natl Acad Sci U S A, *95:* 9482-9487, 1998.
25. Coveney, E., Wheatley, G. H., 3rd, and Lyerly, H. K. Active immunization using dendritic cells mixed with tumor cells inhibits the growth of primary breast cancer. Surgery, *122:* 228-234, 1997.
26. Gabrilovich, D. I., Ciernik, I. F., and Carbone, D. P. Dendritic cells in antitumor immune responses. I. Defective antigen presentation in tumor-bearing hosts. Cell Immunol, *170:* 101-110, 1996.
27. Gabrilovich, D. I., Nadaf, S., Corak, J., Berzofsky, J. A., and Carbone, D. P. Dendritic cells in antitumor immune responses. II. Dendritic cells grown from bone marrow precursors, but not mature DC from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. Cell Immunol, *170:* 111-119, 1996.
28. Neuzil, J., Svensson, I., Weber, T., Weber, C., and Brunk, U. T. alpha-tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation. FEBS Lett, *445:* 295-300, 1999.
29. Neuzil, J., Weber, T., Gellert, N., and Weber, C. Selective cancer cell killing by alpha-tocopheryl succinate. Br J Cancer, *84:* 87-89, 2001.
30. Neuzil, J., Kagedal, K., Andera, L., Weber, C., and Brunk, U. T. Vitamin E analogs: a new class of multiple action agents with anti-neoplastic and anti-atherogenic activity. Apoptosis, *7:* 179-187, 2002.
31. Prasad, K. N., Kumar, B., Yan, X. D., Hanson, A. J., and Cole, W. C. Alpha-tocopheryl succinate, the most effective form of vitamin E for adjuvant cancer treatment: a review. J Am Coll Nutr, *22:* 108-117, 2003.
32. Yu, W., Simmons-Menchaca, M., Gapor, A., Sanders, B. G., and Kline, K. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. Nutr Cancer, *33:* 26-32, 1999.
33. Yu, W., Sanders, B. G., and Kline, K. RRR-alpha-tocopheryl succinate-induced apoptosis of human breast cancer cells involves Bax translocation to mitochondria. Cancer Res, *63:* 2483-2491, 2003.
34. Yu, W., Israel, K., Liao, Q. Y., Aldaz, C. M., Sanders, B. G., and Kline, K. Vitamin E succinate (VES) induces Fas sensitivity in human breast cancer cells: role for Mr 43,000 Fas in VES-triggered apoptosis. Cancer Res, *59:* 953-961, 1999.
35. Israel, K., Yu, W., Sanders, B. G., and Kline, K. Vitamin E succinate induces apoptosis in human prostate cancer cells: role for Fas in vitamin E succinate-triggered apoptosis. Nutr Cancer, *36:* 90-100, 2000.
36. Seliger, B. Strategies of tumor immune evasion. BioDrugs, *19:* 347-354, 2005.
37. Kobie, J. J., Wu, R. S., Kurt, R. A., Lou, S., Adelman, M. K., Whitesell, L. J., Ramanathapuram, L. V., Arteaga, C. L., and Akporiaye, E. T. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Res, *63:* 1860-1864, 2003.

38. Todryk, S., Melcher, A. A., Hardwick, N., Linardakis, E., Bateman, A., Colombo, M. P., Stoppacciaro, A., and Vile, R. G. Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. J Immunol, *163:* 1398-1408, 1999.
39. Mosser, D. D. and Morimoto, R. I. Molecular chaperones and the stress of oncogenesis. Oncogene, *23:* 2907-2918, 2004.
40. Srivastava, P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol, *20:* 395-425, 2002.
41. Binder, R. J., Vatner, R., and Srivastava, P. The heat-shock protein receptors: some answers and more questions. Tissue Antigens, *64:* 442-451, 2004.
42. Henderson, I. C. and Frei, E., 3rd Adriamycin cardiotoxicity. Am Heart J, *99:* 671-674, 1980.
43. Jo, M., Kim, T. H., Seol, D. W., Esplen, J. E., Dorko, K., Billiar, T. R., and Strom, S. C. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med, *6:* 564-567, 2000.

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Chemo-Immunotherapy of Breast Cancer Using Vesiculated α-Tocopheryl Succinate in Combination With Dendritic Cell Vaccination

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Abstract: *In this study, we evaluated the efficacy of vesiculated α-tocopheryl succinate (Vα-TOS) in combination with non-antigen pulsed, nonmatured dendritic cells (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 nmDC 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 interferon-γ and interleukin-4 (IL-4) production by splenic lymphocytes and draining lymph node cells. Interestingly, when used in combination with Vα-TOS, nmDC were as effective as tumor necrosis factor-α matured DC at inhibiting the growth of pre-established tumors. Vα-TOS–induced cellular factors collected by high-speed centrifugation of supernatant 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 + DC chemo-immunotherapy in treating established primary mammary tumors as well as residual metastatic disease.*

Introduction

α-Tocopheryl succinate (α-TOS) is a semisynthetic 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 toward 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. Unlike α-TOS, which is soluble only in organic solvents such as sesame oil, dimethylsulfoxide (DMSO), or ethanol (3,8–10), vesiculated α-TOS (Vα-TOS) is hydrophilic and is generated by the addition of sodium hydroxide and sonication in phosphate-buffered saline (PBS) to form a colloidal suspension (12). This circumvents the toxicities associated with long-term use of DMSO or ethanol, commonly used to solubilize α-TOS for parenteral administration, making Vα-TOS better suited for long-term use in humans. This novel aqueous 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, DC need to be matured, express critical co-stimulatory molecules, and migrate to the draining lymph node (DLN) to induce an effective immune response (14). Preclinical and clinical studies have employed DC pulsed with defined peptides or proteins to elicit potent antitumor T-cell responses (16–22). Although antigen-pulsed DC 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 to improve its anticancer activity.

In a previous report using a murine lung carcinoma model we demonstrated that treatment with non-antigen pulsed, nonmatured DC (nmDC) in combination with Vα-TOS was effective at inhibiting the growth of pre-established tumors (13). Also, cellular factors from 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 to mediate an antitumor immune response.

In this study we investigated the effect of Vα-TOS + DC combination therapy on a poorly immunogenic, highly meta-

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static 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. Vα-TOS–induced cellular factors collected by high-speed centrifugation of supernatant from Vα-TOS–treated tumor cells caused DC maturation evidenced by up-regulation of co-stimulatory molecule (CD40, CD80, CD86) expression and interleukin (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 DC.

Materials and Methods

Chemicals and Reagents

α-TOS and α-2 macroglobulin (α_2M) were purchased from Sigma Chemical Co. (St. Louis, MO). Murine IL-4, granulocyte/macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (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). ALEXA FLUOR 488 antibody was purchased from Molecular Probes (Eugene, OR). Mouse anti-glyceraldehyde phosphate dehydrogenase (GAPDH)-specific antibody was purchased from Chemicon International (Temecula, CA). Goat anti-mouse horseradish peroxide (HRP)-conjugated antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The Annexin-V FLOUS staining kit and the APO-DIRECT Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit were purchased from Roche Applied Sciences (Indianapolis, IN) and BD Pharmingen, respectively. Mouse interferon-γ (IFN-γ), IL-4, and IL-12p70 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Pierce Biotechnologies (Rockford, IL).

Preparation of Vesiculated α-TOS

Vα-TOS was generated as previously described (12). Briefly, 40 mg 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 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 to 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 nonadherent and loosely adherent cells were collected and washed three times with PBS before being used in various experiments. To obtain mature DC (mDC), Day 6 DC were incubated with 200 U/ml TNF-α for 48 h (26).

Flow Cytometric Analysis of Dendritic Cells

DC 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 nmDC and TNF-α-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 2×10^5 cells per well in six-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, nonadherent and adherent cells were collected and centrifuged at 200 *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

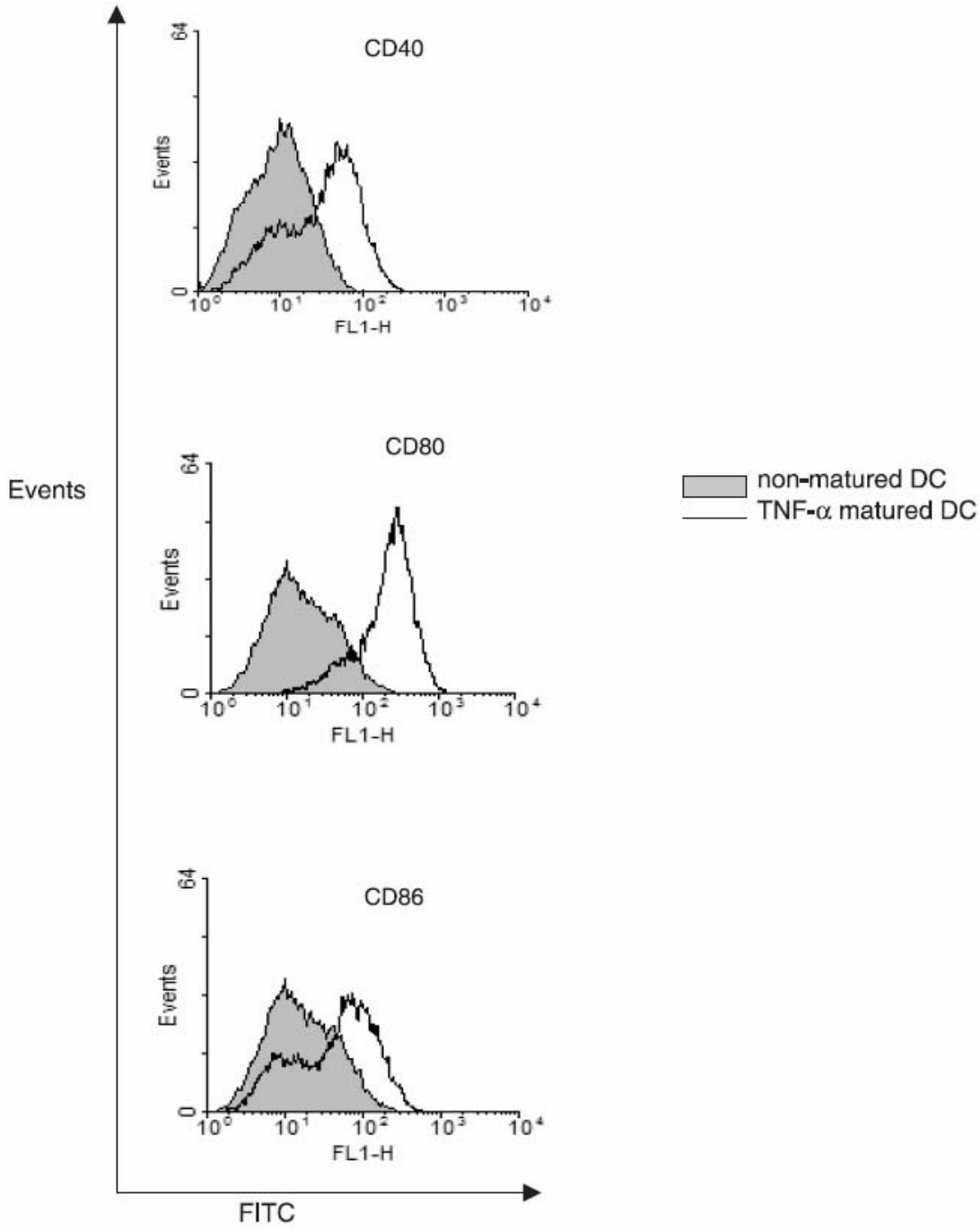


Figure 1. Expression of co-stimulatory molecules on Day 6 nonmatured dendritic cells (nmDC) and tumor necrosis factor-α (TNF-α) matured DC (mDC). DC were generated from bone marrow cells of BALB/c mice after culturing in granulocyte/macrophage colony-stimulating factor and interleukin-4 as described in **Materials and Methods**. On Day 6 or following TNF-α maturation, cells were collected and double stained with phycoerythrin (PE)-conjugated CD11c antibody and fluorescein isothiocyanate–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*, nmDC; *black line*, TNF-α mDC.

stained with Giemsa. Colonies containing >50 cells were counted, and the surviving cell fraction was determined using the following formula: surviving fraction = (no. of colonies counted at a given concentration of Vα-TOS/no. of cells plated at that concentration)/(no. of PBS control colonies counted/no. of PBS 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, nonadherent and adherent cells were collected and stained with Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) following the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN). Briefly, following centri-

fugation and washing, tumor cells were resuspended in Annexin V binding buffer and stained with Annexin V-FITC and propidium iodide (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). 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 with Vα-TOS Treatment–Induced Cellular Factors

4T1 tumor cells were plated at 2×10^5 cells per well in six-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 *g* for 45 min to collect the Vα-TOS treatment–induced cellular factors consisting of nonadherent cells, cell debris, and higher molecular weight factors secreted by the tumor cells. The resulting pellet was resuspended in complete media and incubated with nmDC for 24 h. 4T1 tumor cell lysate generated by freeze-thaw (four cycles) was added to a set of nmDC as a control. DC were collected 24 h later, phenotyped, and evaluated for IL-12p70 production. For the phenotypic analysis, DC 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, 5×10^5 DC 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).

Expression of Heat Shock Proteins by Vα-TOS–Treated Tumor Cells

4T1 cells were plated at 2×10^5 cells per well in six-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). Nonadherent and adherent cells were collected, centrifuged at 200 *g* for 5 min, and washed twice with PBS, 0.5% bovine serum albumin (PBSB). The cells were then resuspended 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 immunoglobulin G (IgG) antibody. Cells were washed twice and stained with ALEXA FLUOR 488–conjugated goat anti-mouse secondary antibody for 45 min on ice. The cells were washed twice with PBSB before being finally resuspended in PBSB for flow analysis using the FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems). The cells were gated on forward versus side scatter, and histograms of ALEXA FLUOR 488 (excitation wavelength, 488 nm) were generated for analysis.

Western Blot for hsp Expression

4T1 tumor cells were plated at 2×10^5 cells per well in six-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 supernatant containing nonadherent cells, cell debris, and factors secreted by the tumor cells was collected and centrifuged (22,600 *g*, 45 min). The 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; and 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 *g* for 15 min at 4°C; the resultant supernatant was recovered and protein content was determined using the BCA Protein Assay (Pierce Biotechnologies). Proteins (30 μg) from the lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubating the membrane in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk. The membrane was immunoblotted using mouse antibodies against either hsp60 (1:1,000), hsp70 (1:500), or hsp90 (1:1,000) (Stressgen Biotechnologies, Canada) and visualized with a goat anti-mouse HRP-conjugated secondary antibody (Upstate Biotechnologies, Lake Placid, NY) using the Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnologies). Subsequently, the membranes were stripped and reprobed with mouse anti-GAPDH–specific antibody (1:1,000) to confirm equal loading.

Blockage of hsp Binding to DC

To block the hsp receptor CD91, nmDC were incubated with or without 100 μg/ml of α_2M, a natural ligand of CD91 (28), for 1 h in serum-free medium before addition of the Vα-TOS–induced cellular factors generated as described previously. DC 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, 5×10^5 DC 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).

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 ac-

cordance 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 5×10^4 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 nine i.p. injections of Vα-TOS (4 mg per 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, 1×10^6 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 5×10^4 4T1 tumor cells in the mammary fat pad. Primary tumors were excised 21 days after the initial tumor challenge. Mice were given five i.p. injections of Vα-TOS (4 mg per 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 1×10^6 DC s.c. in 50 µl PBS on Days 25 and 29. Mice were sacrificed on Day 31, and visible metastatic lung nodules were 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 compound, and frozen using dry ice and 2-methylbutane. Sections of frozen tumor (4 µm thick) were prepared and stained using the APO-DIRECT kit (BD Pharmingen) 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 deoxynucleotidyl transferase enzyme and FITC-labeled deoxyuridine triphosphate. After incubating the slides for 1 h at 37°C, the stain was washed off and slides were incubated for 10 min with RNase/PI solution. The slides were rinsed with PBS and sections were 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 DLNs were pooled from three 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 h, and supernatants were collected and evaluated by ELISA for the production of IFN-γ or IL-4 following the manufacturer's protocol (Pierce Biotechnologies).

Statistical Analysis

Statistical significance of differences among data sets of treatment groups was assessed by one-way analysis of variance including Tukey-Kramer post-tests for multiple comparisons using 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 (Fig. 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 inhibitory concentration 50% (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 (Fig. 2B).

Vα-TOS–induced 4T1 tumor cell death was at least partially due to apoptosis as determined by Annexin V staining (Fig. 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 and signifying secondary necrosis (Annexin V and PI positive) by 18 h.

Vα-TOS Induces Apoptosis in Tumors In Vivo

To determine whether Vα-TOS induces apoptosis in tumors in vivo, we analyzed tumor sections by TUNEL assay. Mice with established tumors (~25 mm^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 compared with tumors treated with PBS (Fig. 2D). Maximum apoptosis was observed after six Vα-TOS injections.

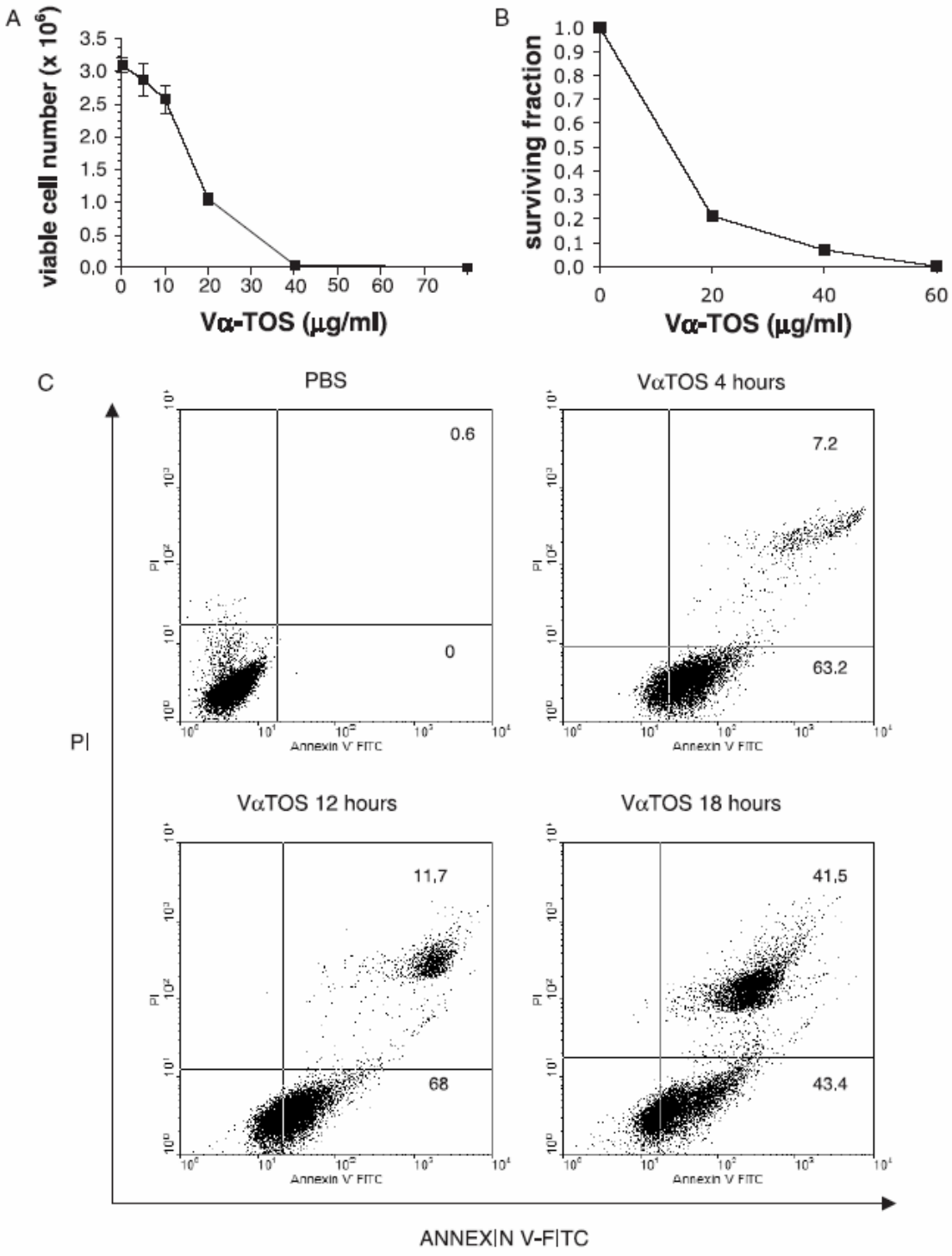


Figure 2. Effect of vesiculated α-tocopheryl succinate (Vα-TOS) treatment on 4T1 tumor cells in vitro and in vivo. 4T1 cells were allowed to adhere overnight in six-well tissue culture plates. The cells were then treated with none (phosphate-buffered saline, PBS), 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, or 80 μg/ml of Vα-TOS (in PBS). After a 24 h exposure, nonadherent and adherent cells were collected, and cell number and viability were determined by trypan blue dye exclusion. The data (A) are representative of two independent experiments, and the values denote mean ± SD of triplicate samples. 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 two independent experiments, and the values represent mean ± 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, nonadherent and adherent cells were collected and stained using Annexin V and propidium iodide. 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 three independent experiments. For the terminal deoxynucleotidyl transferase dUTP nick end labeling (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 bright white (arrow) regions in the tumor sections represent TUNEL-positive (apoptotic cells) and gray areas depict TUNEL-negative cells (magnification, 400×).

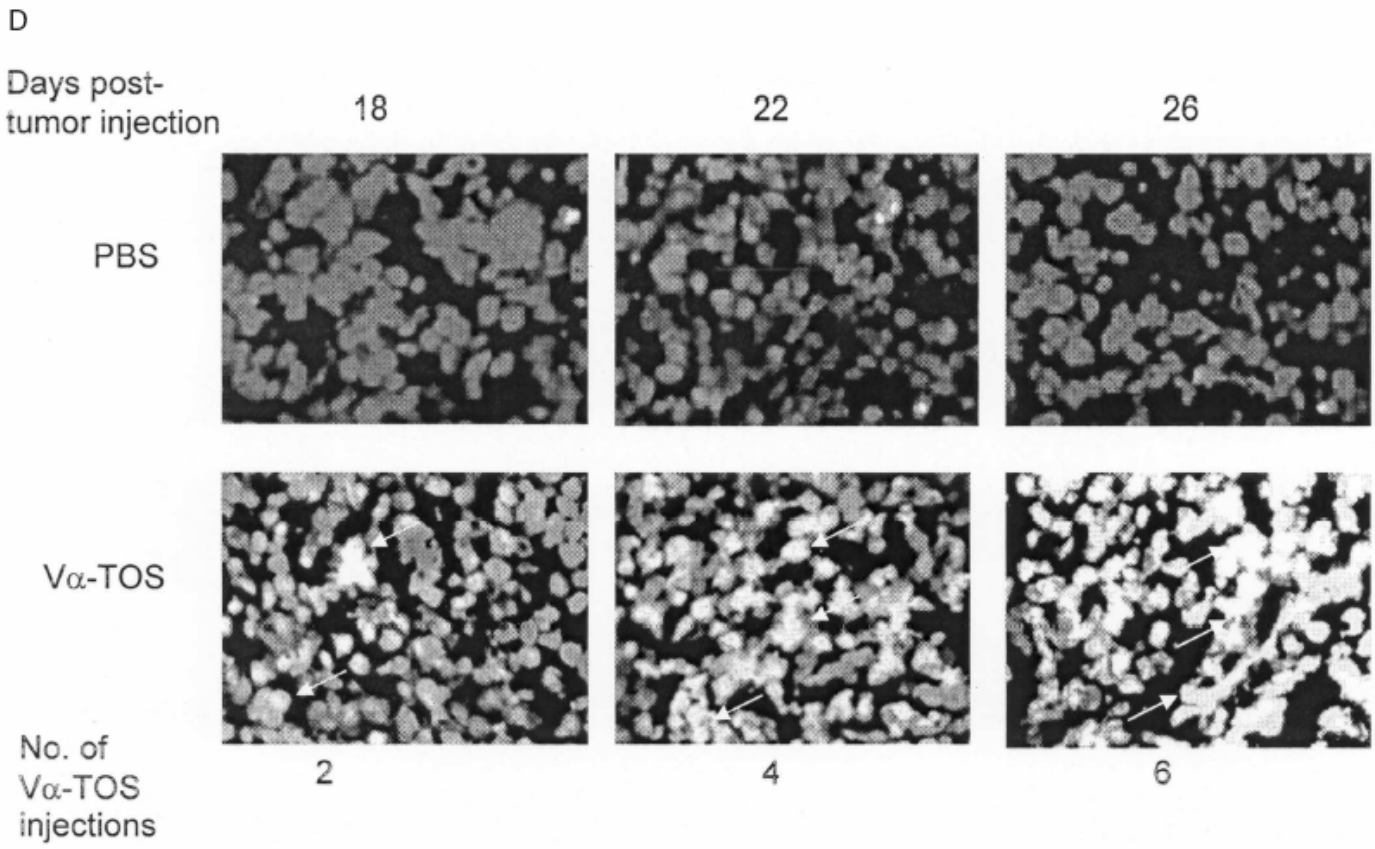


Figure 2. Continued.

Vα-TOS Potentiates the Antitumor 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 enhance the antitumor effect of adoptively transferred nmDC in treating pre-established 3LL tumors (10,13). In this study, we compared the effectiveness of unpulsed nmDC and TNF-α mDC in combination with Vα-TOS in controlling pre-established 4T1 tumors. The data (Fig. 3A) demonstrate that, when used in combination with Vα-TOS, nmDC are as effective as mDC in inhibiting 4T1 tumor growth compared with 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 + 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, and PBS + mDC) ranged from 709 ± 251 to 1,004 ± 348 mm^3. This is also reflected in the observation that Vα-TOS + DC therapy significantly prolonged survival compared with 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 (Fig. 3B). All control animals died because of large tumor burden (~1,200 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 1,200 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 10 times the original dose (5×10^5) 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 + Dendritic Cells Elicits Increased IFN-γ and IL-4 Production by Draining Lymph Node Cells and Splenic Lymphocytes

To determine whether the antitumor effect of Vα-TOS + DC vaccination was associated with an enhanced immune response, cells were isolated from DLNs 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 (6,510.5 ± 35.7 pg/ml and

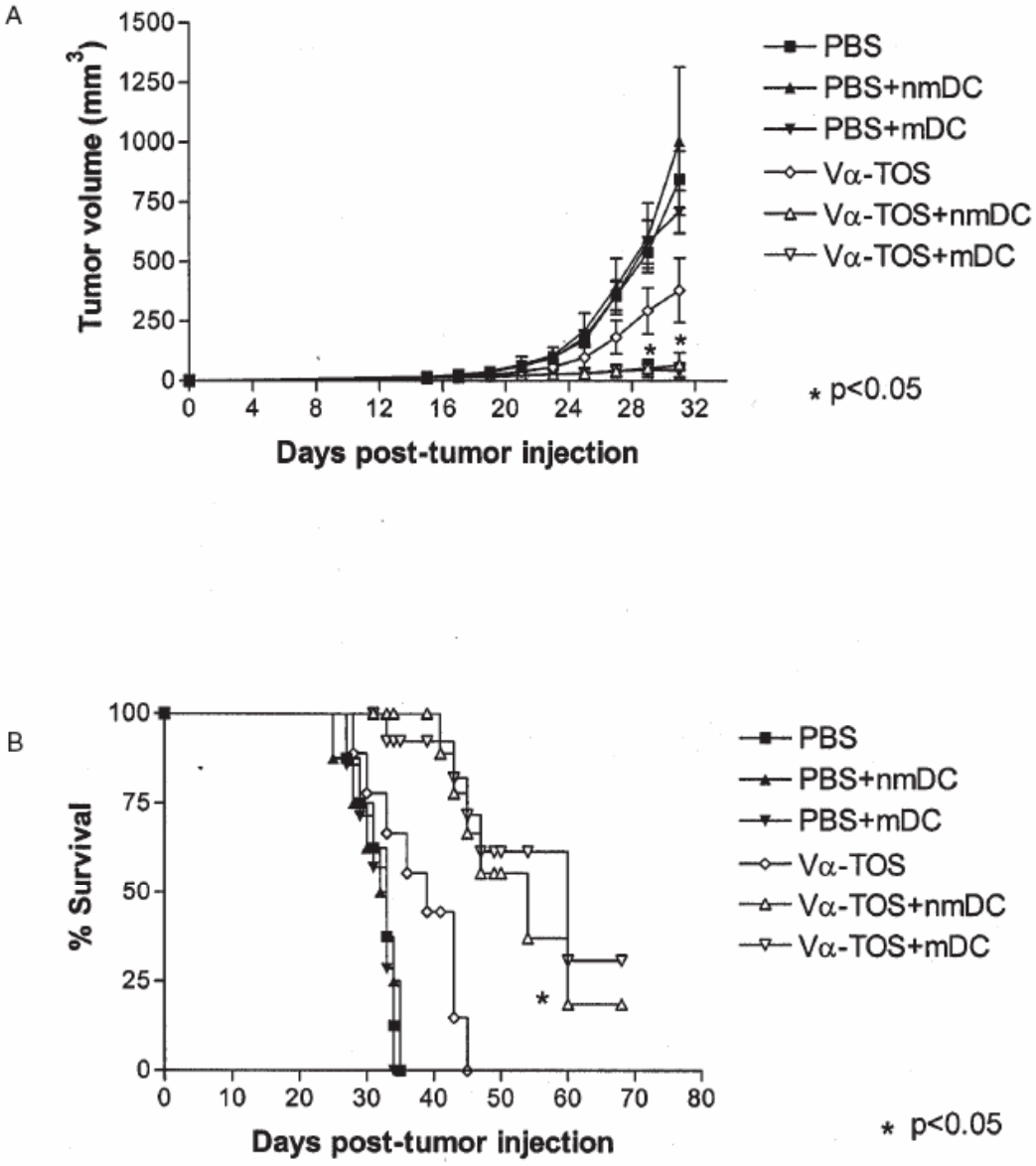
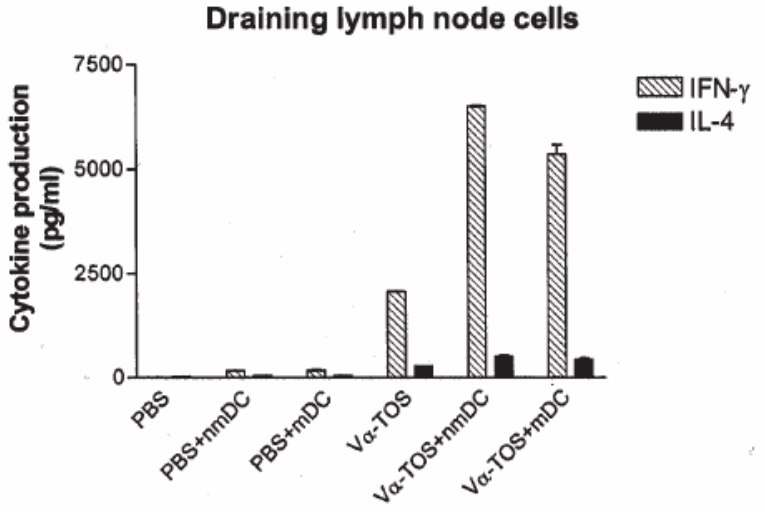


Figure 3. Effect of vesiculated α-tocopheryl succinate (Vα-TOS) + dendritic cell (DC) immunotherapy on pre-established 4T1 tumors. Mice were injected orthotopically in the mammary pad with 5×10^4 4T1 tumor cells. On development of established tumors (Day 15) mice were injected i.p. with 4 mg of Vα-TOS on alternate days for a total of nine injections. The mice were also injected in the contralateral mammary pad with either 10^6 nonmatured DC (nmDC) or tumor necrosis factor-α matured DC (mDC) on Days 18, 22, and 26. The data represent (A) mean tumor volumes ± SD and (B) percent survival of seven individual mice per group. All control animals died because of large tumor burden (~1,200 mm^3) by Day 35, and mice injected with Vα-TOS died naturally or were sacrificed when tumor volumes reached ~1,200 mm^3 by Day 47. In contrast, six mice in the Vα-TOS + nmDC group and five mice in the Vα-TOS + mDC group were alive until Day 60, when they were terminated as tumor volumes reached ~1,200 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 rechallenged with a 10-fold higher dose (5×10^5) of 4T1 tumor cells (data not shown).

5,360.4 ± 384.5 pg/ml, respectively) compared with 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 (2,067 ± 11.7 pg/ml; $P < 0.001$).

Similarly, IFN-γ production by splenocytes isolated from mice treated with Vα-TOS + nmDC or mDC was significantly higher (2,801.7 ± 151.6 pg/ml and 2,749.8 ± 146.7 pg/ml, respectively) than that of splenocytes from control mice (13.5 ± 0.8 to 25.2 ± 2.8 pg/ml; $P < 0.001$) (Fig. 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, $P <$

A



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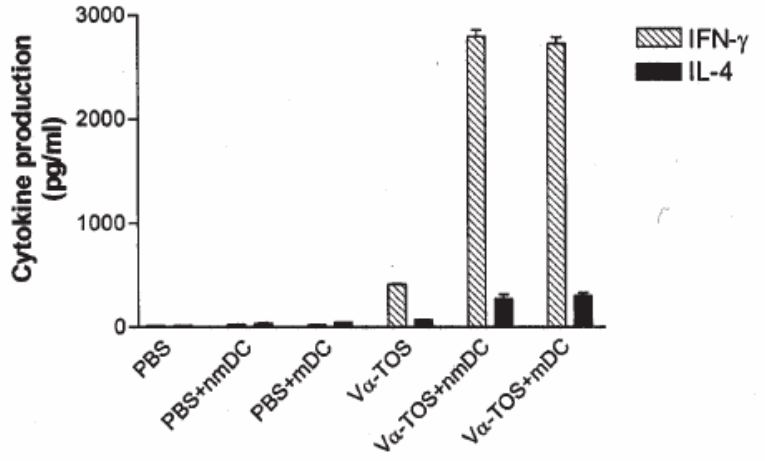

C

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 4. Effect of combination treatment with vesiculated α-tocopheryl succinate and dendritic cell on interferon-γ (IFN-γ) and interleukin-4 (IL-4) secretion by splenic lymphocytes and draining lymph node (DLN) cells. Spleens and DLNs 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 h. The supernatants were collected and evaluated by enzyme-linked immunosorbent assay for the production of IFN-γ and IL-4, respectively. Data are mean ± SD of triplicate samples. The table (C) depicts the T_H1/T_H2 (IFN-γ/IL-4) ratio for the various treatment groups.

0.001) (Fig. 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 (Fig. 4B), which was significantly 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 + DC vaccination even further increased both the IFN-γ and IL-4 production by DLN cells and splenocytes, although the maturation status of the DC 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 toward a T_H1-mediated immune response (Fig. 4C).

Combination of Vα-TOS + Nonmatured 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. Because the primary cause of cancer recurrence and mortality is residual metastatic disease, we wanted to study the efficacy of the combination of Vα-TOS + DC in treating residual metastatic disease after primary tumor resection. For this purpose, mice were injected orthotopically with 5×10^4 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 (Fig. 5) show that Vα-TOS treatment alone was able to significantly reduce the number of lung metastases compared with 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 with PBS treatment alone.

Combination Treatment with Vα-TOS + DC Elicits Increased IFN-γ and IL-4 Production by Splenic Lymphocytes in the Residual Disease Setting

Because the suppression of pre-established 4T1 tumors with Vα-TOS + 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 (Fig. 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 (1,963.5 ± 106 pg/ml) and IL-4 (202.7 ± 85 pg/ml) compared with the controls (IFN-γ, $P < 0.01$; IL-4, $P < 0.001$). Also the T_H1/T_H2 ratio was higher in splenocytes from mice treated with Vα-TOS + nmDC compared with the controls (Fig. 6B).

Vα-TOS–Treated Tumor Cells Induce Maturation of DC In Vitro

Our finding that the maturation status of the DC had no influence on 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 Vα-TOS–induced cellular factors collected by high-speed centrifugation of supernatant fluid from tumor cells that were

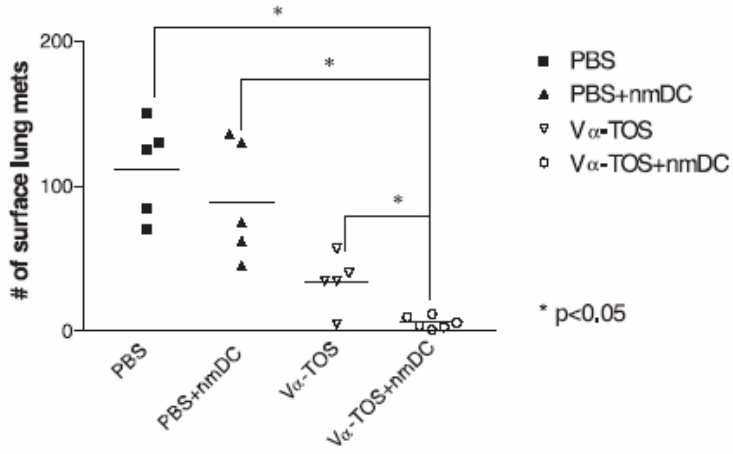

Figure 5. Effect of vesiculated α-tocopheryl succinate (Vα-TOS) + nonmatured dendritic cell (nmDC) combination treatment on lung metastasis in the residual disease setting. Mice were injected orthotopically in the mammary fat pad with 5×10^4 4T1 tumor cells. Primary tumors were surgically resected on Day 21. Starting on Day 22, mice were injected i.p. with 4 mg of Vα-TOS on alternate days for a total of five 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 were evaluated for visible metastatic nodules by staining with India ink and Fekete's solution.

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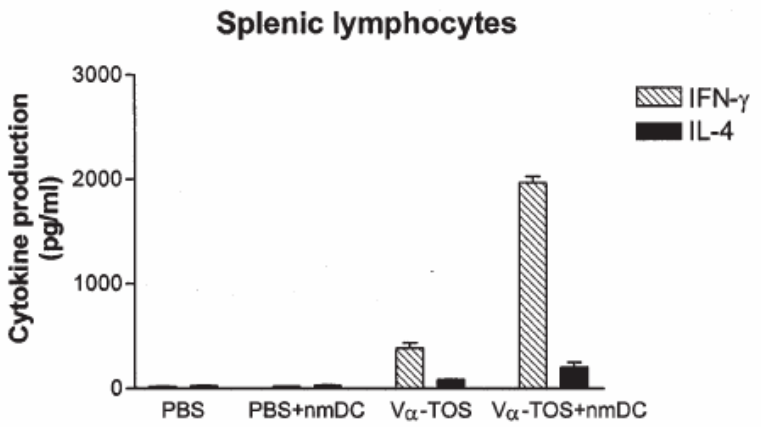

B

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 6. Effect of treatment with vesiculated α-tocopheryl succinate + dendritic cell on interferon-γ (IFN-γ) and interleukin-4 (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 (five 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 h. The supernatants were collected and evaluated by enzyme-linked immunosorbent assay for the production of IFN-γ and IL-4. Data are mean ± SD of triplicate samples. The table (B) depicts the T_H1/T_H2 (IFN-γ/IL-4) ratio for the various treatment groups.

treated with Vα-TOS for 24 h. Following incubation with this fraction consisting of nonadherent cells, cellular debris, and substances secreted by the tumor cells, DC were assessed for the expression of the DC maturation markers CD40, CD80, and CD86. The data (Fig. 7A) show that co-incubation of Vα-TOS–induced cellular factors with nmDC caused an increase in co-stimulatory molecule expression on DC. This increase in expression was comparable with 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 the factors produced by Vα-TOS treatment of tumor cells caused DC maturation, we evaluated IL-12p70 secretion by DC incubated with the high-speed spin fraction derived from supernatant of Vα-TOS–treated tumor cells. The data (Fig. 7B) show that IL-12p70 secretion by DC was significantly increased ($P < 0.001$) only when co-incubated with the Vα-TOS–induced cellular factors.

DC Maturation Induced by Vα-TOS–Treated Tumor Cells Is Mediated by Heat Shock Proteins

It is well documented that hsps are up-regulated during apoptotic or necrotic cell death (31–34) and provide danger signals that may lead to activation and maturation of DC (31–33,35–39). Therefore, we postulated that the DC maturation by Vα-TOS–induced cellular factors that we have observed may be mediated, at least in part, by hsps. First, we needed to determine whether Vα-TOS treatment up-regulated hsp expression on 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 (Fig. 8A) show that the membrane expression of these hsps on 4T1 tumor cells was up-regulated following Vα-TOS treatment but not after vehicle (PBS) treatment. The differential induction of hsps on tumor cells following Vα-TOS treatment was confirmed by Western blot analysis of the high-speed spin fraction derived from supernatant of Vα-TOS–treated cells (Fig. 8B). This supernatant contained nonadherent cells, cellular debris, and substances secreted by the tumor cells.

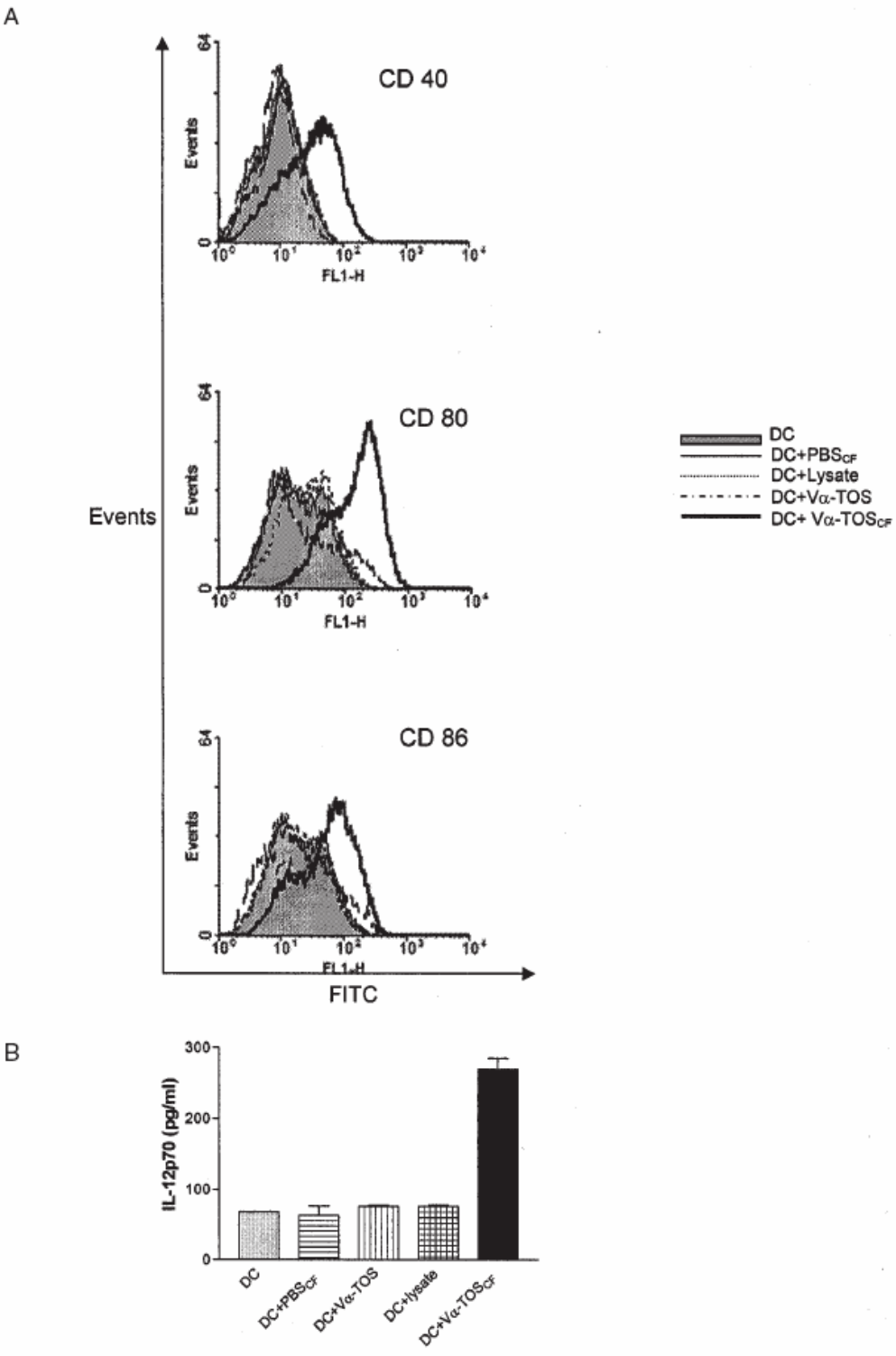

Figure 7. Effect of vesiculated α-tocopheryl succinate (Vα-TOS)–treated tumor cells on dendritic cell (DC) maturation. 4T1 cells were allowed to adhere overnight in six-well tissue culture plates at 2×10^5 cells per well and then treated with 40 μg/ml Vα-TOS or phosphate-buffered saline (PBS) for 24 h. The supernatant was collected and centrifuged at 22,600 *g* for 45 min to collect nonadherent cells and membrane debris. The pellet obtained was resuspended in media and incubated with nonmatured (nm) DC for 24 h. (A) DC were collected and double-stained with phycoerythrin (PE)-conjugated CD11c antibody and fluorescein isothiocyanate–conjugated antibodies against CD40, CD80, and CD86 and analyzed by flow cytometry. Cells were gated on light scatter and CD11c$^+$ cells. (B) DC were also restimulated with tumor necrosis factor-α for 24 h in 48-well tissue culture plates after which the supernatant was collected and evaluated for interleukin (IL)-12p70 production by enzyme-linked immunosorbent assay. DC, untreated DC; DC+PBS$_{CF}$, DC incubated with cellular factors from PBS-treated 4T1 cells; DC + lysate, DC incubated with freeze-thaw lysate of 4T1 tumor cells; DC+Vα-TOS, DC treated with 40 μg/ml Vα-TOS; DC+Vα-TOS$_{CF}$, DC incubated with cellular factors from Vα-TOS–treated 4T1 cells. The data are representative of three independent experiments.

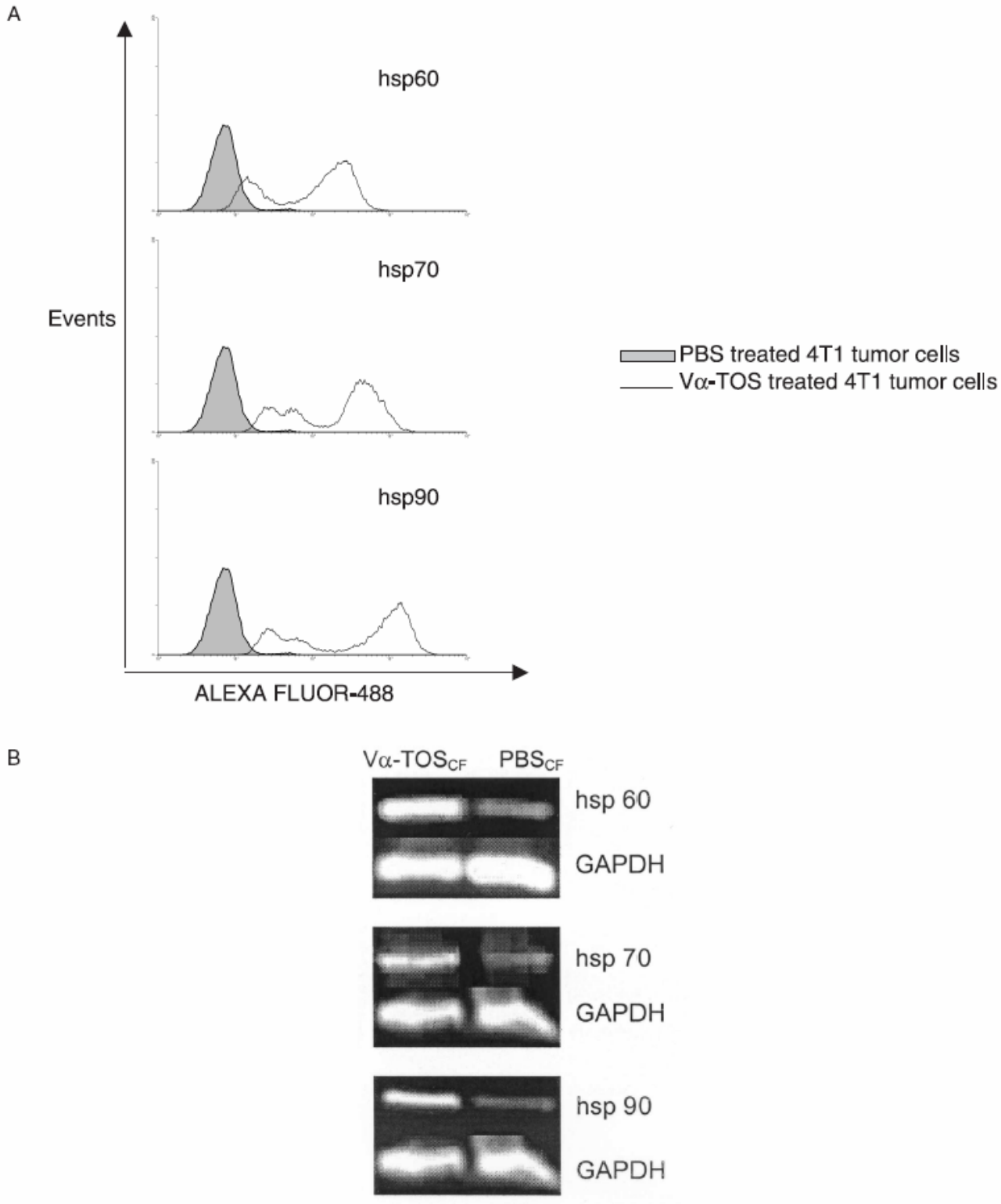

Figure 8. Heat shock protein (hsp) expression in tumor cells after treatment with vesiculated α-tocopheryl succinate (Vα-TOS). 4T1 cells were allowed to adhere overnight in six-well tissue culture plates at 2×10^5 cells per well and then treated with either 40 μg/ml Vα-TOS or phosphate-buffered saline (PBS, vehicle). After 12 h, nonadherent and adherent cells were collected, washed twice with PBS, and stained with antibodies against hsp60, 70, or 90. Goat anti-mouse IgG-ALEXA-FLUOR 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*, PBS-treated cells; *black line*, 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 *g* for 45 min. The pellet obtained was lysed, protein concentration measured and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes and stained with hsp60-, 70-, and 90-specific antibodies, respectively. Vα-TOS$_{CF}$, lysate derived from cellular factors from Vα-TOS–treated 4T1 cells; PBS$_{CF}$, lysate derived from cellular factors from PBS-treated 4T1 tumor cells.

After we had shown that hsps are indeed up-regulated on tumor cells in response to Vα-TOS treatment and are present in the high-speed spin fraction of Vα-TOS–treated tumor cells, we wanted to determine the involvement of hsps in the maturation of DC. For this purpose we blocked the cognate hsp receptor CD91 (28) on nmDC by pretreatment with α_2M. Subsequently, the DC were co-incubated with the high-speed spin fraction of Vα-TOS–treated tumor cells as described previously. The data (Fig. 9A) show that pretreatment with α_2M partially inhibited the expression of the maturation markers CD40, CD80, and CD86 on DC. 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 DC. Pretreatment of DC with α_2M followed by incubation with the cellular fraction derived from Vα-TOS–treated tumor cells significantly inhibited ($P < 0.001$) IL-12p70 secretion by DC compared with DC treated directly with Vα-TOS–induced cellular factors.

Discussion

In this study, we evaluated the efficacy of Vα-TOS + 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 nmDC 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. This study corroborates and extends our earlier reports (10,13) using the Lewis lung carcinoma model in which we showed that α-TOS as well as Vα-TOS synergized with nmDC to suppress the growth of pre-established tumors. In the 4T1 tumor model, the superior effect of the combination therapy was correlated with increased IFN-γ and IL-4 production by splenic lymphocytes and DLN cells. The ratio of IFN-γ to IL-4 production suggests polarization toward a T_H1-mediated immune response, indicating that the combination treatment enhanced a cell-mediated antitumor immune response. Unlike α-TOS and liposomal preparations of α-TOS (10,27,41), Vα-TOS is more readily soluble in aqueous solvents (12,13,42). Vα-TOS is more practical for human use, particularly for long-term therapy, because the nontoxic nature of the vehicle (PBS) used to generate Vα-TOS circumvents the potential adverse effects of the commonly used solvents DMSO and ethanol. Although Vα-TOS is an improvement over α-TOS, its translational potential is still limited because of its susceptibility to cleavage by host-derived intestinal esterases (41,43). These esterases convert α-TOS to the parent compound vitamin E, which lacks anticancer activity. This renders Vα-TOS unsuitable for oral delivery, a highly desired property if vitamin E analogs were to be used as chemopreventative agents. To overcome this limitation, ongoing studies in our laboratory are evaluating the potencies of Vα-TEA, a nonhydrolyzable ether analog of vitamin E that can be delivered orally, as an anticancer agent.

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 to 25 mm^3 compared with ~0.5 mm^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 + 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 cellular factors 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 DC (31–33,39,44). 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 to generate DC capable of mediating antitumor activity in vivo.

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 hsps 60, 70, and 90 in 4T1 tumor cells and that co-incubation of DC with α_2M that competes with hsps 60, 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 Vα-TOS–induced cellular factors 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. We rule out the induction of reactive oxygen species (ROS) by Vα-TOS as a mechanism of DC maturation for two reasons. First, co-incubation of DC with Vα-TOS did not result in DC activation. Second, the high-speed spin fraction derived from supernatant of Vα-TOS–treated tumor cells is unlikely to contain ROS after the centrifugation, yet co-incubation of DC with these Vα-TOS induced cellular factors caused DC maturation (Figure7).

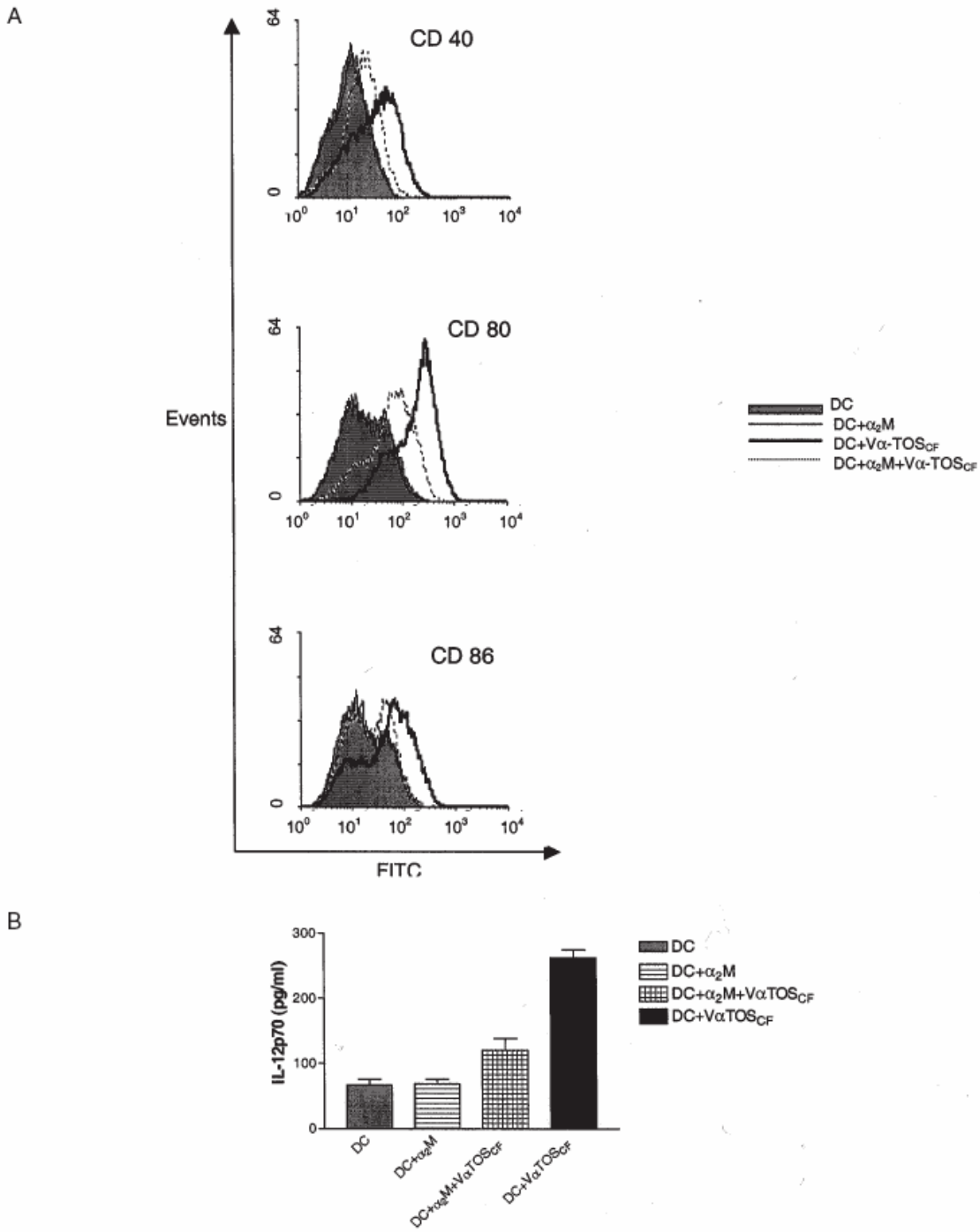

Figure 9. Effect of pretreatment of nonmatured dendritic cells (nmDC) with α-2 macroglobulin (α_2M) on maturation induced by vesiculated α-tocopheryl succinate (Vα-TOS)–treated tumor cells. nmDC were incubated in serum-free media with or without 100 µg/ml α_2M for 1 h. 4T1 cells were treated with 40 µg/ml Vα-TOS or phosphate-buffered saline for 24 h. The supernatant was collected and centrifuged at 22,600 *g* for 45 min. The pellet obtained was resuspended in media and added to the pretreated DC for 24 h. (A) DC were collected and stained with phycoerythrin (PE)-conjugated CD11c antibody and fluorescein isothiocyanate–conjugated antibodies against CD40, CD80, and CD86 and analyzed by flow cytometry. Cells were gated on light scatter and CD11c$^+$ cells. (B) DC were also restimulated with tumor necrosis factor-α for 24 h in 48-well tissue culture plates after which the supernatant was collected and evaluated for interleukin-12p70 production by enzyme-linked immunosorbent assay. DC, untreated DC; DC+α_2M, DC pretreated with α_2M; DC+Vα-TOS$_{CF}$, DC incubated with cellular factors from Vα-TOS–treated 4T1 cells; DC+α_2M+Vα-TOS$_{CF}$, DC pretreated with α_2M and incubated with cellular factors from Vα-TOS–treated 4T1 cells. The data are representative of two independent experiments.

Our results with the 4T1 mammary tumor model 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 hsps 60, 70, and 90 induce the maturation of DC (31,35,36,38,44) and up-regulate the expression of pro-inflammatory cytokines (37,45,46). Hsps function as molecular chaperones and fulfill essential roles in protecting cells from potentially lethal effects of stress and proteotoxicity (47). However, hsps in the extracellular environment act as a "danger signal," alerting antigen-presenting cells, including DC, leading to their activation (32,33,47,48). Activated DC are very effective antigen presenters, which migrate to secondary lymphoid organs where they initiate antitumor T-cell responses (49). 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: 1) by direct killing of tumor cells whose antigens can be cross-presented by DC and 2) 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.

Acknowledgments and Notes

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References

1. Neuzil J, Weber T, Gellert N, and Weber C: Selective cancer cell killing by alpha-tocopheryl succinate. *Br J Cancer* **84,** 87–89, 2001.
2. Prasad KN, Kumar B, Yan XD, Hanson AJ, and Cole WC: Alpha-tocopheryl succinate, the most effective form of vitamin E for adjuvant cancer treatment: a review. *J Am Coll Nutr* **22,** 108–117, 2003.
3. Webr T, Lu M, Andera L, Lahm H, Gellert N, et al.: Vitamin E succinate is a potent novel antineoplastic agent with high selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) in vivo. *Clin Cancer Res* **8,** 863–869, 2002.
4. Yu W, Sanders BG, and Kline K: RRR-alpha-tocopheryl succinate inhibits EL4 thymic lymphoma cell growth by inducing apoptosis and DNA synthesis arrest. *Nutr Cancer* **27,** 92–101, 1997.
5. Yu W, Simmons-Menchaca M, Gapor A, Sanders BG, and Kline K: Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. *Nutr Cancer* **33,** 26–32, 1999.
6. Israel K, Yu W, Sanders BG, and Kline K: Vitamin E succinate induces apoptosis in human prostate cancer cells: role for Fas in vitamin E succinate-triggered apoptosis. *Nutr Cancer* **36,** 90–100, 2000.
7. Dalen H and Neuzil J: Alpha-tocopheryl succinate sensitises a T lymphoma cell line to TRAIL-induced apoptosis by suppressing NF-kappaB activation. *Br J Cancer* **88,** 153–158, 2003.
8. Malafa MP, Fokum FD, Mowlavi A, Abusief M, and King M: Vitamin E inhibits melanoma growth in mice. *Surgery* **131,** 85–91, 2002.
9. Malafa MP and Neitzel LT: Vitamin E succinate promotes breast cancer tumor dormancy. *J Surg Res* **93,** 163–170, 2000.
10. Ramanathapuram LV, Kobie JJ, Bearss D, Payne CM, Trevor KT, et al.: Alpha-tocopheryl succinate sensitizes established tumors to vaccination with nonmatured dendritic cells. *Cancer Immunol Immunother* **53,** 580–588, 2004.
11. Barnett KT, Fokum FD, and Malafa MP: Vitamin E succinate inhibits colon cancer liver metastases. *J Surg Res* **106,** 292–298, 2002.
12. Kogure K, Manabe S, Hama S, Tokumura A, and Fukuzawa K: Potentiation of anti-cancer effect by intravenous administration of vesiculated alpha-tocopheryl hemisuccinate on mouse melanoma in vivo. *Cancer Lett* **192,** 19–24, 2003.
13. Ramanathapuram LV, Hahn T, Graner MW, Katsanis E, and Akporiaye ET: Vesiculated alpha-tocopheryl succinate enhances the anti-tumor effect of dendritic cell vaccines. *Cancer Immunol Immunother* **55,** 166–177, 2006.
14. Steinman RM: The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* **9,** 271–296, 1991.
15. Mule JJ: Dendritic cells: at the clinical crossroads. *J Clin Invest* **105,** 707–708, 2000.
16. Chang AE, Redman BG, Whitfield JR, Nickoloff BJ, Braun TM, et al.: A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. *Clin Cancer Res* **8,** 1021–1032, 2002.
17. Burdin N and Moingeon P: Cancer vaccines based on dendritic cells loaded with tumor-associated antigens. *Cell Biol Toxicol* **17,** 67–75, 2001.
18. Fields RC, Shimizu K, and Mule JJ: Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc Natl Acad Sci USA* **95,** 9482–9487, 1998.
19. Gatza E and Okada CY: Tumor cell lysate-pulsed dendritic cells are more effective than TCR Id protein vaccines for active immunotherapy of T cell lymphoma. *J Immunol* **169,** 5227–5235, 2002.
20. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, et al.: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* **4,** 328–332, 1998.
21. Porgador A and Gilboa E: Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J Exp Med* **182,** 255–260, 1995.
22. Zitvogel L, Mayordomo JI, Tjandrawan T, DeLeo AB, Clarke MR, et al.: Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* **183,** 87–97, 1996.
23. Coveney E, Wheatley GH III, and Lyerly HK: Active immunization using dendritic cells mixed with tumor cells inhibits the growth of primary breast cancer. *Surgery* **122,** 228–234, 1997.
24. Gabrilovich DI, Ciernik IF, and Carbone DP: Dendritic cells in antitumor immune responses. I. Defective antigen presentation in tumor-bearing hosts. *Cell Immunol* **170,** 101–110, 1996.
25. Gabrilovich DI, Nadaf S, Corak J, Berzofsky JA, and Carbone DP: Dendritic cells in antitumor immune responses. II. Dendritic cells grown from bone marrow precursors, but not mature DC from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. *Cell Immunol* **170,** 111–119, 1996.
26. Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK, et al.: Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res* **63,** 1860–1864, 2003.

27. Lawson KA, Anderson K, Simmons-Menchaca M, Atkinson J, Sun L, et al.: Comparison of vitamin E derivatives alpha-TEA and VES in reduction of mouse mammary tumor burden and metastasis. *Exp Biol Med* **229,** 954–963, 2004.
28. Basu S, Binder RJ, Ramalingam T, and Srivastava PK: CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* **14,** 303–313, 2001.
29. Wu RS, Kobie JJ, Besselsen DG, Fong TC, Mack VD, et al.: Comparative analysis of IFN-gamma B7.1 and antisense TGF-beta gene transfer on the tumorigenicity of a poorly immunogenic metastatic mammary carcinoma. *Cancer Immunol Immunother* **50,** 229–240, 2001.
30. McEarchern JA, Kobie JJ, Mack V, Wu RS, Meade-Tollin L, et al.: Invasion and metastasis of a mammary tumor involves TGF-beta signaling. *Int J Cancer* **91,** 76–82, 2001.
31. Basu S, Binder RJ, Suto R, Anderson KM, and Srivastava PK: Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* **12,** 1539–1546, 2000.
32. Feng H, Zeng Y, Graner MW, and Katsanis E: Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood* **100,** 4108–4115, 2002.
33. Somersan S, Larsson M, Fonteneau JF, Basu S, Srivastava P, et al.: Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* **167,** 4844–4852, 2001.
34. Buttiglieri S, Galetto A, Forno S, De Andrea M, and Matera L: Influence of drug-induced apoptotic death on processing and presentation of tumor antigens by dendritic cells. *Int J Cancer* **106,** 516–520, 2003.
35. Basu S and Srivastava PK: Fever-like temperature induces maturation of dendritic cells through induction of hsp90. *Int Immunol* **15,** 1053–1061, 2003.
36. Bethke K, Staib F, Distler M, Schmitt U, Jonuleit H, et al.: Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. *J Immunol* **169,** 6141–6148, 2002.
37. Chen W, Syldath U, Bellmann K, Burkart V, and Kolb H: Human 60-kDa heat-shock protein: a danger signal to the innate immune system. *J Immunol* **162,** 3212–3219, 1999.
38. Flohe SB, Bruggemann J, Lendemans S, Nikulina M, Meierhoff G, et al.: Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. *J Immunol* **170,** 2340–2348, 2003.
39. Zheng H, Dai J, Stoilova D, and Li Z: Cell surface targeting of heat shock protein gp96 induces dendritic cell maturation and antitumor immunity. *J Immunol* **167,** 6731–6735, 2001.
40. Aslakson CJ and Miller FR: Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* **52,** 1399–1405, 1992.
41. Anderson K, Simmons-Menchaca M, Lawson KA, Atkinson J, Sanders BG, et al.: Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E succinate and vitamin E analogue, alpha-TEA. *Cancer Res* **64,** 4263–4269, 2004.
42. Jizomoto H, Kanaoka E, and Hirano K: pH-sensitive liposomes composed of tocopherol hemisuccinate and of phosphatidylethanolamine including tocopherol hemisuccinate. *Biochim Biophys Acta* **1213,** 343–348, 1994.
43. Neuzil J, Weber T, Schroder A, Lu M, Ostermann G, et al.: Induction of cancer cell apoptosis by alpha-tocopheryl succinate: molecular pathways and structural requirements. *FASEB J* **15,** 403–415, 2001.
44. Todryk S, Melcher AA, Hardwick N, Linardakis E, Bateman A, et al.: Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. *J Immunol* **163,** 1398–1408, 1999.
45. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, et al.: HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* **6,** 435–442, 2000.
46. Jin Y, Fuller L, Ciancio G, Burke GW III, Tzakis AG, et al.: Antigen presentation and immune regulatory capacity of immature and mature-enriched antigen presenting (dendritic) cells derived from human bone marrow. *Hum Immunol* **65,** 93–103, 2004.
47. Mosser DD and Morimoto RI: Molecular chaperones and the stress of oncogenesis. *Oncogene* **23,** 2907–2918, 2004.
48. Srivastava P: Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu Rev Immunol* **20,** 395–425, 2002.
49. Banchereau J and Steinman RM: Dendritic cells and the control of immunity. *Nature* **392,** 245–252, 1998.