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Award Number: DAMD17-01-1-0126

TITLE: Tumor-Mediated Suppression of Dendritic Cell Vaccines

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REPORT DATE: March 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE March 2005	3. REPORT TYPE AND DATES COVERED Final (1 Apr 2001 - 28 Feb 2005)	
4. TITLE AND SUBTITLE Tumor-Mediated Suppression of Dendritic Cell Vaccines			5. FUNDING NUMBERS DAMD17-01-1-0126	
6. AUTHOR(S) Emmanuel Akporiaye, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Arizona Tucson, Arizona 85721-0158 E-Mail: akporiay@email.arizona.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The unique ability of dendritic cells to potently stimulate lymphocytes in an antigen-specific fashion has made them prime candidates for cancer immunotherapy. A number of tumor-derived products have been suggested to promote tumor establishment and progression by interfering with DC functions. One of the best characterized of these factors is Transforming growth factor-beta (TGF- β), a multifunctional cytokine that exerts potent suppressive effects on cells of the immune system. TGF- β specifically interferes with DC maturation, chemotaxis, antigen recognition and T cell activation. These findings strongly suggest that a strategy that protects DCs from the harmful effects of TGF- β should enhance the effectiveness of DC-based vaccines. In this study we show that TGF- β suppression of DC vaccines can be mitigated by inhibiting TGF- β gene expression in tumor cells and by neutralization of secreted TGF- β with specific antibody. We demonstrate that a novel small molecule TGF- β type 1 receptor kinase inhibitor abrogates tumor-derived TGF- β -mediated signaling and epithelial-mesenchymal transition characteristic of a more invasive phenotype. The inhibitor enhanced the effectiveness of DC vaccines in controlling the growth of established 4T1 tumors. Taken together, our studies demonstrate the usefulness of eliminating immunosuppressive tumor-derived products in order to improve the effectiveness of dendritic cell-based anti-cancer vaccines.				
14. SUBJECT TERMS Dendritic cell, TGF- β , tumor, vaccine			15. NUMBER OF PAGES 37	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

TABLE OF CONTENTS

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	18
Reportable Outcomes.....	19
Conclusions.....	20
References.....	21
Appendices.....	21

Introduction

The unique ability of dendritic cells to potently stimulate naïve lymphocytes in an antigen-specific fashion has made them prime candidates for cancer immunotherapy. A number of tumor-derived products have been suggested to promote tumor establishment and progression by interfering with numerous DC functions required for the induction of a potent antitumor response. One of the best characterized of these tumor-derived factors is transforming growth factor-beta (TGF- β), a multifunctional cytokine that exerts potent suppressive effects on cells of the immune system. TGF- β specifically interferes with DC maturation, chemotaxis, antigen recognition and T cell activation. By so doing, TGF- β is capable of inhibiting the generation of effective DC-mediated anti-tumor immunity. As a corollary, strategies that protect or render DC resistant to the deleterious effects of TGF- β would be expected to improve the effectiveness of DC-based immunotherapy of cancer. The goal of this study is to protect DCs from the direct actions of tumor-derived TGF- β in order to improve their effectiveness as cancer vaccines. The hypothesis to be tested is that interference with TGF- β signal transduction in DCs will abrogate tumor-derived TGF- β -mediated immunosuppression leading to more effective DC vaccines. The specific aims of this study are to: 1) determine the effect of tumor-derived TGF- β on antigen presentation and *in vivo* migration of DCs. 2) evaluate the impact of tumor-derived TGF- β on DC vaccines. 3) protect DCs from TGF- β -mediated immunosuppression by blocking TGF- β signal transduction.

SPECIFIC AIM #1 Determine the effect of tumor-derived TGF- β on DC antigen presentation and the migration

a) Effect of tumor-derived TGF- β on DC antigen presentation

Antigen presentation by DC is suppressed by TGF- β

The presentation of antigen by DCs to T cells is dependent on the expression of MHC I, MHC II, and co-stimulatory molecules. Previous studies have shown that TGF- β reduces expression of co-stimulatory molecules on DCs, therefore we evaluated its effects on the ability of DCs to stimulate T cells. Treatment of immature DCs with TGF- β significantly reduced their ability to stimulate the proliferation of allogeneic T cells (Figure 1A). Immature DCs were also evaluated for their ability to present OVA peptide to, and stimulate the subsequent proliferation of naive OVA-specific CD4⁺ T cells, which express a T cell receptor specific for the OVA MHC class II peptide. Treatment of immature DCs with TGF- β for 6 days significantly impaired their ability to stimulate OVA-specific T cells (Figure 1B).

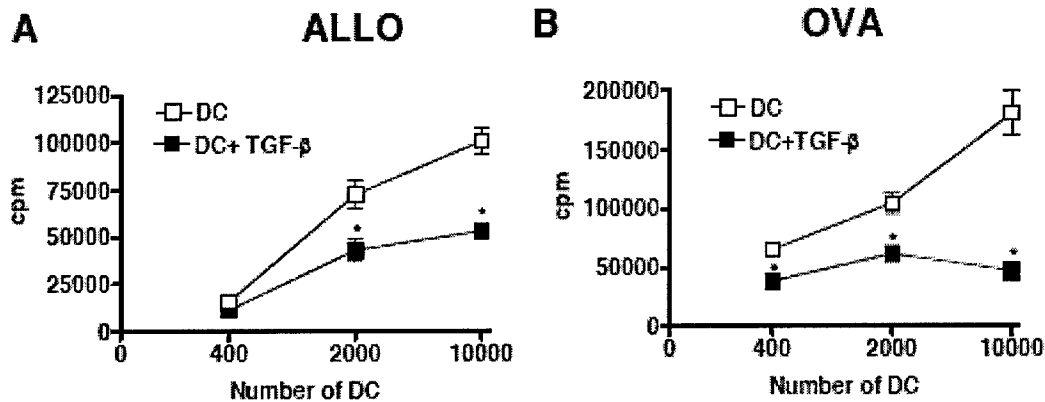


Figure 1. TGF- β suppresses the ability of DCs to present antigen. Dendritic cells were incubated in the absence or presence of 10 ng/ml of TGF- β for 6 days. **(A)** DCs were incubated with 2×10^5 splenic T cells isolated from C57/BL6 mice for 5 days with the addition of [3 H]-thymidine for the last 18 hours of culture. Values represent mean $\pm$ SEM of six replicates. **(B)** DCs were collected and incubated with 2×10^5 splenic T cells isolated from BALB/c-TgN (DO11.10) 10 Loh mice in the presence of OVA peptide for 5 days with the addition of [3 H]-thymidine for the last 18 hours of culture. Values represent mean $\pm$ SEM of four replicates. Results are from one experiment that is representative of three independent experiments. Asterisk (*) refers to statistical significance between groups ($p < 0.05$).

TGF- β reduces the ability of DCs to stimulate tumor-sensitized T lymphocytes

Observing that TGF- β inhibits the antigen uptake and presentation capacities of DCs, we evaluated its effect on the stimulation of tumor-sensitized T lymphocytes. Mature DCs were more effective at stimulating the proliferation of tumor-sensitized T lymphocytes than immature DCs. TGF- β -treatment significantly reduced the immunostimulatory ability of mature DCs (Figure 2A). In contrast, TGF- β did not alter the immunostimulatory ability of immature DCs, which was already lower than that of mature DCs (Figure 2A). The ability of immature and mature DCs to stimulate IFN- γ production by tumor-sensitized lymphocytes was also significantly inhibited following TGF- β exposure by 60% and 22% respectively (Figure 2B). Together these results demonstrate that TGF- β exposure reduces the ability of DCs to stimulate anti-tumor immune responses.

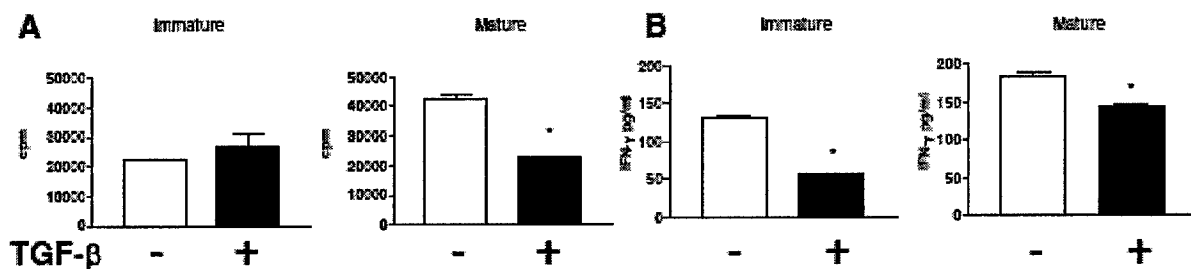


Figure 2. Stimulation of tumor-sensitized T lymphocytes by TGF- β treated DCs. Dendritic cells were pulsed with tumor cell lysate for 48 hours in the presence or absence of 10ng/ml of TGF- γ (immature DC), then cultured in TNF- γ in the presence or absence of TGF- β for 48 hours (mature DC). **(A)** Ten thousand DCs were incubated with 2×10^5 splenic T cells isolated from mice bearing 4T1 tumors for 5 days and [3 H]-thymidine was added for the last 18 hours of culture. **(B)** One million T cells isolated from inguinal lymph nodes draining 4T1 tumors were incubated with 2.5×10^5 DCs for 48 hours. Following incubation, supernatant was analyzed for IFN- γ production. Numbers are mean $\pm$ SEM of triplicate samples. Results are from one experiment representative of two independent experiments. Asterisk (*) refers to statistical significance between groups ($p < 0.05$).

b) Effect of tumor-derived TGF- β on DC migration

***In vitro* TGF- β treatment reduces DC migration to draining lymph nodes *in vivo*.**

Following antigen internalization at the tumor site, it is advantageous for the dendritic cell to migrate to draining lymph nodes (DLNs) where tumor-specific T lymphocytes can be stimulated. The ability of dendritic cells to migrate from the periphery to secondary lymphoid organs following TGF- β exposure was evaluated. Dendritic cells were labeled with PKH-67, a green fluorescent dye and injected subcutaneously (s.c.) into the hind flank of mice. Forty-eight hours following the injection, draining inguinal lymph nodes were removed. Migrated dendritic cells were enumerated by scanning laser cytometric analysis of cytopsin preparations of the lymph node cell suspensions. The ability of mature dendritic cells to migrate to draining lymph nodes (DLNs) was significantly ($p < 0.05$) inhibited following exposure to TGF- β (Table 1).

	DCs per 10^6 DLN cells	
	mDC	mDC+TGF- β *
experiment 1	1137	727
experiment 2	309	54
experiment 3	466	305
experiment 4	5500	5200

Table 1. Effect of *in vitro* TGF- β treatment on *in vivo* migration. DCs were cultured in the presence of 200 U/ml TNF- α with or without the addition of 10ng/ml of TGF- β for 48 hours. DCs were then labeled with PKH-67 membrane dye and injected s.c. into two naive BALB/c mice per group. Forty-eight hours following injection inguinal lymph nodes were harvested from mice and disaggregated. Lymph node cells were fixed and stained with Propidium Iodide/RNase I solution and analyzed by laser scanning cytometry. Data are from four independent experiments. Asterisk (*) refers to statistical significance between groups ($p < 0.05$).

***In vivo* exposure to tumor-derived TGF- β inhibits DC migration to tumor draining lymph nodes.**

Observing that *in vitro* exposure to TGF- β reduced the ability of DCs to migrate to DLNs we postulated that tumor-derived TGF- β within the tumor microenvironment may have a similar effect, thus preventing DC migration from the tumor site to DLNs. To reduce the amount of tumor-derived TGF- β within the tumor microenvironment, we utilized the 4T1-asT tumor cell line, which produces 90% less TGF- β as compared to the 4T1-N (vector control) cell line. PKH-67 labeled DCs were injected intratumorally (i.t.) into established 4T1-N or 4T1-asT primary tumors. Forty-eight hours later, the tumor DLNs were removed, and the number of PKH-67⁺, CD11c⁺ double-positive DCs were determined by flow cytometry. DCs injected into 4T1-asT tumors exhibited an enhanced ability to migrate to tumor DLNs as compared to DCs injected into 4T1-N tumors (Figure 3). These results suggest that tumor-derived TGF- β reduces the ability of DCs to migrate to tumor draining lymph nodes thereby interfering with their capacity to stimulate tumor-specific T lymphocytes.

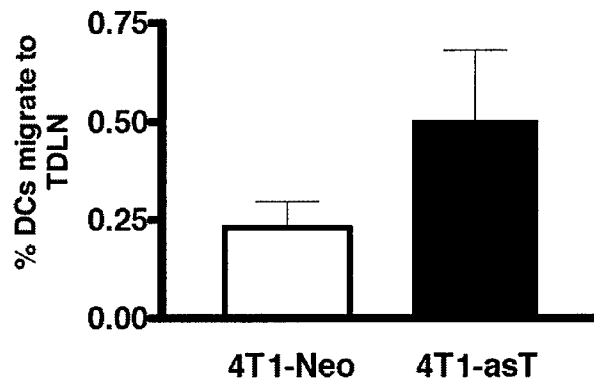


Figure 3. Migration of DCs following intratumoral injection. Ten million PKH-67⁺ DCs were injected into 4T1-N or 4T1-asT tumors. Tumors were removed 48 hours following injection and the percentage of PKH-67⁺ CD11c⁺ DC migrating from the tumor to DLNs was determined by flow cytometry. Values represent the mean $\pm$ SEM of three independent experiments.

TGF- β impairs the chemotactic migration of DCs.

The migration of DCs from an inflammatory site such as a tumor to DLNs is regulated in part by the responsiveness of DCs to the chemokines (RANTES, MIP-1 α , MCP-1) produced within the tumor microenvironment and within DLNs (SLC, MIP-3 β). We evaluated whether the impairment of DC migration is a result of TGF- β altering their responsiveness to various chemokines. Treatment of DCs with TGF- β *in vitro* did not substantially alter their expression of chemokine receptor mRNA (Figure 4A). However, TGF- β treatment reduced DC chemotaxis toward SLC and MIP-3 β (Figure 4B), which may be the underlying mechanism for poor migration to DLNs.

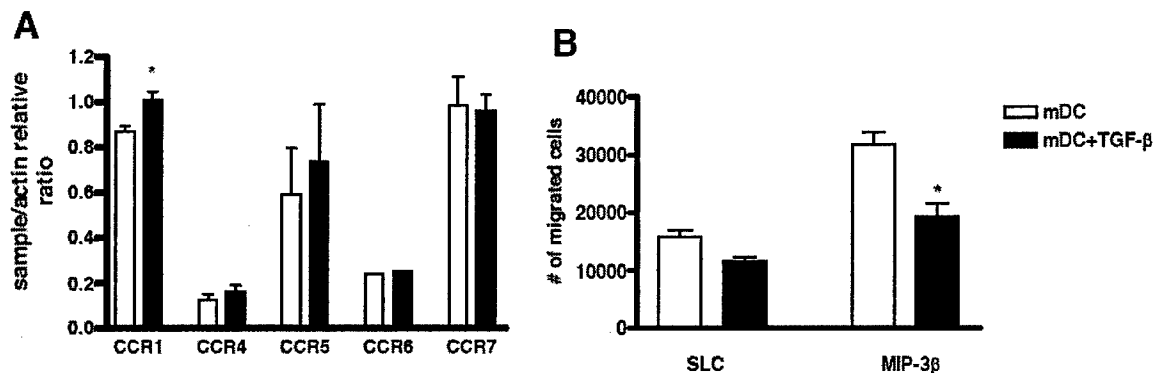


Figure 4. Chemokine receptor expression and *in vitro* chemotaxis. DCs were matured with TNF- α in the absence or presence of TGF- β for 48 hours. **(A)** mRNA was isolated from DCs and chemokine receptor expression was analyzed by RT-PCR. Data are expressed as ratios compared with actin as determined by densitometry. **(B)** DC were added to the upper chamber of a transwell migration chamber and evaluated for chemotactic migration. Cells migrating to the bottom chamber were recovered and analyzed by flow cytometry for CD11c expression. The results are mean $\pm$ SEM of three independent experiments. Asterisk (*) refers to statistical significance between groups ($p < 0.05$).

Phenotype of intra-tumorally injected DCs

Because it has been shown that *in vitro* exposure to TGF- β reduces the expression of co-stimulatory molecules on DCs, we postulated that TGF- β present in the tumor microenvironment might have the same effect on intra-tumorally injected DCs. In order to

address this possibility, PKH-67-labeled DCs were injected into established 4T1-Neo or 4T1-asT primary tumors. Forty-eight hours later, the tumors were removed and the numbers of cells positive for PKH-67 and either CD11c, I-A^d, CD40, CD80, or CD86 were determined. Only a small fraction ($\approx 10\%$) of the PKH-67⁺ cells injected into the tumor were recovered. This may be the result of DC migration to other tissues, an inefficient recovery process, or DC demise in the tumor microenvironment. We did not observe any substantial differences in the numbers of CD11c⁺, I-A^d⁺, CD40⁺, CD80⁺ or CD86⁺ cells recovered from 4T1-Neo or 4T1-asT tumors (Figure 5A). Analysis of tumor draining lymph nodes revealed that increased numbers of DCs migrated from 4T1-asT tumors as compared to 4T1-Neo tumors. As a result, an increased number of cells positive for both PKH-67 and either CD11c, I-A^d, CD40, CD80, or CD86 were present in DLNs from 4T1-asT tumor bearing mice as compared to those bearing 4T1-Neo tumors (Figure 5B).

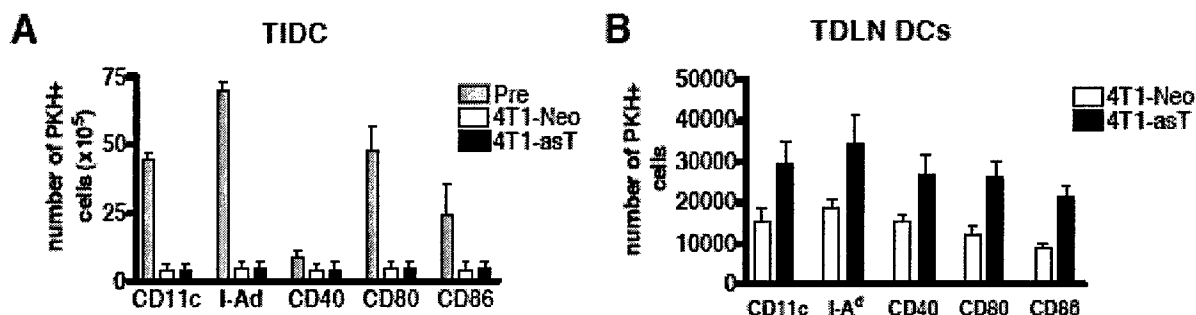


Figure 5. Phenotype of intratumorally injected DCs. Ten million PKH-67⁺ DCs were injected into 4T1-Neo or 4T1-asT tumors. Forty-eight hours following i.t. injection, tumors (A) and DLNs (B) were removed. The number of cells present was determined by FACS analysis. Values represent mean $\pm$ SEM of at least two independent experiments.

SPECIFIC AIM # 2 Determine the effect of tumor-derived TGF- β on the efficacy of dendritic cell-based vaccines

a) Treatment of Established Tumors with DC and Neutralizing TGF- β Antibody

Effectiveness of *in vivo* antibody neutralization of TGF- β in improving DC immunizations

The impairment of critical DC functions including antigen uptake, antigen presentation, tumor-specific T lymphocyte stimulation, and *in vivo* migration by TGF- β suggested that inhibition of TGF- β production in tumor-bearing animals might improve the efficacy of DC vaccines. To test this possibility, TGF- β production was suppressed by transfer of an antisense TGF- β transgene into 4T1 cells (4T1-asT). Expression of the transgene resulted in $>90\%$ inhibition of TGF- β production (0.083 ± 0.003 ng/ml in 4T1-asT compared with 1.244 ± 0.188 ng/ml in mock transduced cells (4T1-Neo). Treatment of 4T1-asT tumors with 2G7 mAb alone or DCs plus 2G7 mAb significantly inhibited tumor growth as compared with 4T1-asT tumors treated with DCs alone (Figure 6). Furthermore, complete tumor regression occurred in 40% (2/5) of 4T1-asT-tumor bearing mice that were treated with DCs plus 2G7 mAb. Similarly, mock transduced (4T1-Neo) tumors responded significantly better ($p < 0.05$) to treatment with DCs plus 2G7 mAb as compared to

treatment with DCs alone; however tumor growth inhibition in this group was inferior to that observed in animals bearing 4T1-asT tumors (Figure 6). Mice that exhibited complete tumor regression were re-challenged with parental 4T1 tumor cells to determine if they had developed long-term anti-tumor immunity. These mice failed to develop tumors (0/2); however all control naive mice challenged with tumor cells developed tumors (3/3). These data suggest that neutralization of TGF- β in mice bearing TGF- β secreting tumors enhances the effectiveness of DC vaccines in treating established tumors.

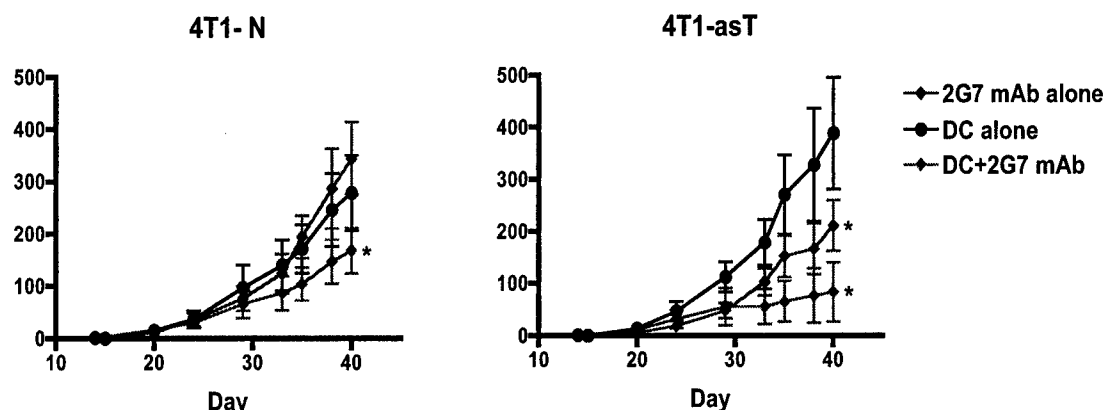


Figure 6. Treatment of established tumors. BALB/c mice (5 mice per group) with established 4T1-Neo (mock-transfected) and 4T1-asT (transfected with antisense TGF- β gene) tumors were vaccinated intratumorally on days 15, 20, and 25 with 1.5×10^6 tumor cell lysate-pulsed mature DCs alone or in combination with 100 μ g i.t and 300 μ g i.p of 2G7, TGF- β neutralizing antibody. Control mice were treated with 2G7 antibody alone. Mice were monitored for tumor growth. Graph represents mean tumor volume $\pm$ SEM. Asterisk (*) indicates significant ($p < 0.05$) difference as compared to mice treated with DC alone on day 40.

SPECIFIC AIM #3 Abrogation of TGF- β mediated immunosuppression of DC by transfer of Smad7 or mutant TGF- β Type II receptor genes

a) Abrogation of TGF- β mediated immunosuppression of DC by transfer of Smad7

AdSmad7 mediates expression of Smad7 protein in DCs

Our results show that tumor-derived TGF- β significantly impairs the ability of DCs to treat established 4T1 tumors. To protect DC from the immunosuppressive effects of TGF- β we constructed an adenoviral vector that contains the Smad7 gene linked to the FLAG fusion protein (AdSmad7). Smad7 inhibits the TGF- β signaling pathway by preventing the binding of the TGF- β receptor to Smad2. We successfully infected DC and demonstrated expression of the recombinant Smad7 protein by Western blot analysis. (Figure 7). DCs infected with the AdSmad7 express substantially higher levels of Smad7 protein as compared to those infected with AdLacZ (control vector).

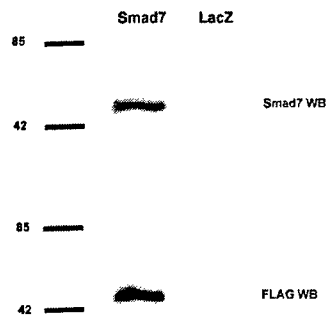


Figure 7. Expression of recombinant Smad7 protein. DCs were infected with AdSmad7 or AdLacZ. Forty-eight hours post-infection DCs were collected and cell lysate was prepared. Proteins (25 μ g) from the cell lysates were resolved by 8% SDS-PAGE and electrotransferred to PVDF membrane. Smad7 was detected by incubating the membrane with anti-Smad7 antibody. The membrane was then stripped and incubated with anti-FLAG antibody.

AdSmad7-infected DCs exhibit reduced TGF- β signaling

To determine if infection of DCs with AdSmad7 reduces TGF- β signaling, we evaluated the expression of the phosphorylated Smad2 (pSmad2) protein. Smad2 is an intermediate in the TGF- β signaling pathway, and its phosphorylation is prevented by Smad7. DCs infected with AdSmad7 expressed less pSmad2 as compared to AdLacZ infected DCs, following treatment with up to 5ng/ml of TGF- β (Figure 8). Expression of total Smad2 protein was not affected by AdSmad7 infection.

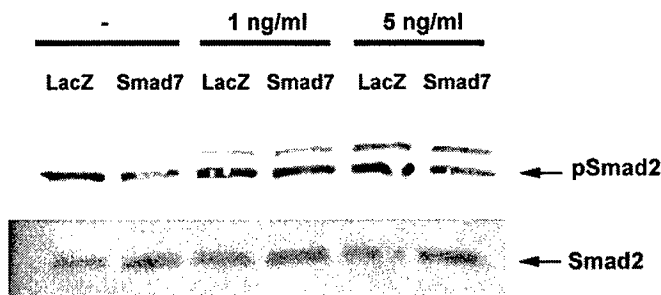


Figure 8. Reduced pSmad2 expression in AdSmad7-infected DCs. DCs were infected with AdSmad7 or AdLacZ. Forty-eight hours post-infection DCs were treated with 1 or 5 ng/ml of TGF- β for 45 minutes and cell lysate was prepared. Proteins were resolved as indicated above. pSmad2 was detected by incubating the membrane with anti-pSmad2 antibody. The membrane was then stripped and incubated with anti-Smad2 antibody.

Treatment of established 4T1 tumors with AdSmad7 DCs

Having demonstrated the reduced responsiveness of DC infected with AdSmad7 to TGF- β , we hypothesized that these DCs will be more effective stimulators of the anti-tumor immune response when injected into a TGF- β -containing tumor microenvironment. Mice bearing established mock transfected (4T1-N) or anti-sense TGF- β -expressing (4T1-asT) tumors received i.t. injections of AdSmad7 DCs or AdLacZ DCs alone, or in combination with the TGF- β neutralizing antibody, 2G7. Unfortunately we did not observe a significant difference in tumor growth rates between the control and treatment groups (Figure 9). However, treatment of the 4T1-asT tumor-bearing mice with Smad7-overexpressing DC enhanced production of IFN- γ by their tumor draining lymph node cells, indicating an enhanced immunostimulatory capacity (Table 2).

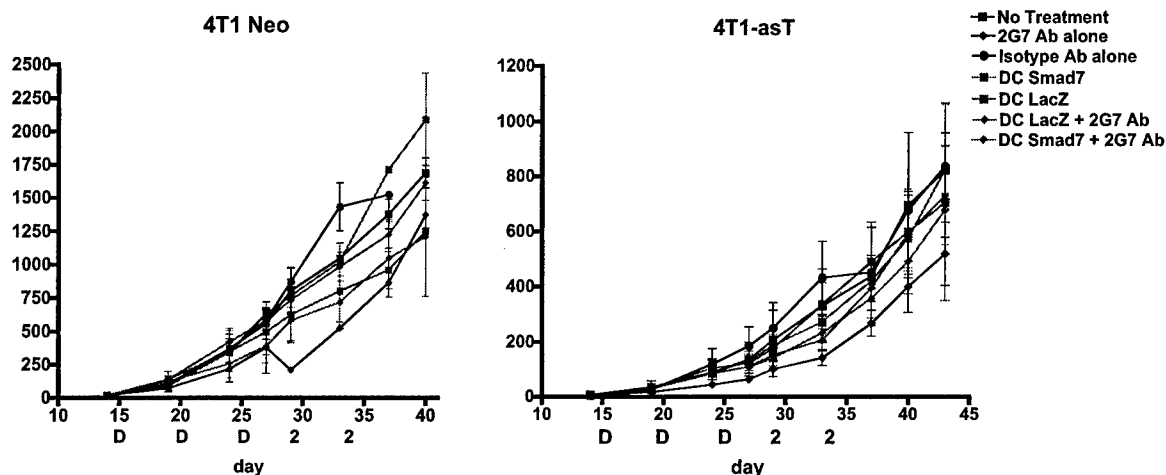


Figure 9. Treatment of established 4T1 tumors with Smad7-overexpressing DC. Mice bearing established 4T1-N or 4T1-asT primary tumors received intratumoral (i.t.) vaccinations with tumor lysate-pulsed, matured AdSmad7 or AdLacZ DCs alone or in combination with 300 μ g of 2G7 Ab intraperitoneally (i.p.) and 100 μ g i.t. on days 15, 20, and 25. Mice also received 300 μ g of 2G7 Ab i.p. the day before DC vaccination and on days 29 and 33. Data represents mean $\pm$ SEM of five mice per group.

	IFN- γ pg / ml / 10 ⁶ cells
No Treatment	2,053 $\pm$ 76
Isotype Ab	2,762 $\pm$ 76
2G7 Ab	12,239 $\pm$ 329
DC LacZ	7,526 $\pm$ 355
DC Smad7	62,207 $\pm$ 228
DC LacZ + 2G7 Ab	49,487 $\pm$ 507
DC Smad7 + 2G7 Ab	57,722 $\pm$ 583

Table 2. IFN- γ production by tumor draining lymph nodes (TDLN) cells following vaccination with Smad7-overexpressing DC. TDLNs were removed from 4T1-asT tumor bearing mice (5 mice per group) following treatment as indicated above. One million TDLN cells were cultured for 48 hours in each well of a 24 well plate pre-coated with anti-CD3 antibody. Supernatants were removed and the amount of IFN- γ present was determined by ELISA. Data represent mean $\pm$ SEM of triplicate samples.

b) Abrogation of TGF- β mediated immunosuppression of DC using a small molecule TGF- β inhibitor

Since DC transduced with Smad7 were ineffective at treating 4T1 tumors, we pursued an alternative strategy that could potentially be more effective in rendering DC resistant to TGF- β signaling. This approach involved the use of a recently described TGF- β receptor type I (TGF β -RI) small molecule kinase inhibitor, HTS466284 (1). Unlike Smad7, HTS466284, interferes with TGF- β signal transduction at the earliest step of the pathway by binding to the ATP pocket of TGF- β R1 and preventing its phosphorylation and activation.

HTS466284 is not toxic towards 4T1 cells *in vitro*

The potential toxicity of HTS466284 towards the highly metastatic 4T1 murine mammary carcinoma cell line was evaluated *in vitro* prior further studies to establish feasibility of use. 4T1 cells were treated with 1, 5, and 10 μ M of HTS466284. Forty-eight hours after treatment the cells were harvested and counted for viability. HTS466284 treatment had no significant effect on cell viability up to 10 μ M (Figure 10).

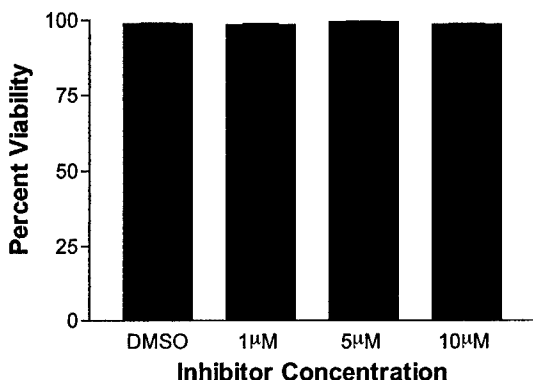


Figure 10. Effect of HTS466284 treatment of 4T1 cell viability *in vitro*. 4T1 cells were cultured for 48 hours in the presence of 1, 5, or 10 μ M of the TGF- β RI inhibitor HTS466284 dissolved in DMSO (final concentration 0.1% DMSO/well). Following treatment the cells were harvested and counted for viability using acridine orange/propidium iodide staining. Data represents the mean $\pm$ SEM of three independent experiments.

HTS466284 blocks recombinant human TGF- β 1 signaling *in vitro*

The ability of HTS466284 to block recombinant human TGF- β 1(rhTGF- β) signaling was evaluated using the TGF- β inducible reporter PAI-luciferase. Mv1Lu cells that were transfected with p3TPLux as described previously were treated with HTS466284 for 30 minutes followed by the addition of rhTGF- β 1 (2ng/ml) for 18 hours. Extracts were then prepared and assayed for luciferase activity using the LucLite Plus kit (Perkin-Elmer, Boston, MA). Light emission was detected with a Packard Lumicount. HTS466284 inhibited rhTGF- β 1-induced expression of a transfected PAI-luciferase reporter in a dose dependent manner (Figure 11).

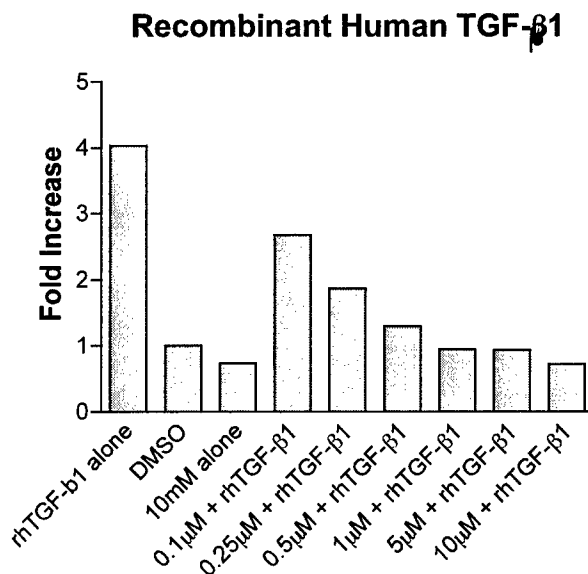


Figure 11. Effect of HTS466284 on the expression of the TGF- β inducible gene reporter PAI-luciferase in response to rhTGF- β . Mv1Lu transfected with the p3TPLux reporter were treated with 0.1, 0.25, 0.5, 1, 5, and 10 μ M of HTS466284 for 30 minutes followed by 18 hour treatment with 2ng/ml of rhTGF- β 1. Cell

extracts were prepared using the LucLite Luciferase Assay kit (Perkin-Elmer, Boston, MA) and light emission was detected with a Packard Lumicount top-count luminometer. Data represents mean $\pm$ SEM of triplicate samples.

HTS466284 blocks 4T1 tumor-derived TGF- β signaling *in vitro*

Observing that HTS466284 could block rhTGF- β 1 mediated signaling, we next wanted to determine if the drug could block the effects of 4T1 tumor-derived TGF- β . Supernatants were collected from 24 hour 4T1 cultures and concentrated 15X using Centricon-10 concentrators (Amicon, Inc, Beverly, Mass.). The supernatants were treated with 1.2M HCl for one hour to activate any TGF- β present and TGF- β 1 levels were quantified by ELISA (R&D Systems). Mv1Lu cells that were transfected with p3TPLux as described previously were treated with HTS466284 for 30 minutes followed by the addition of equivalent amounts of acid activated 4T1 derived TGF- β for eighteen hours. Extracts were then prepared and assayed for luciferase activity using the LucLite Luciferase Assay kit (Perkin-Elmer, Boston, MA). Light emission was detected with a Packard Lumicount. HTS466284 inhibited tumor-derived, TGF- β -induced expression of a transfected PAI-luciferase reporter in a dose dependent manner (Figure 12).

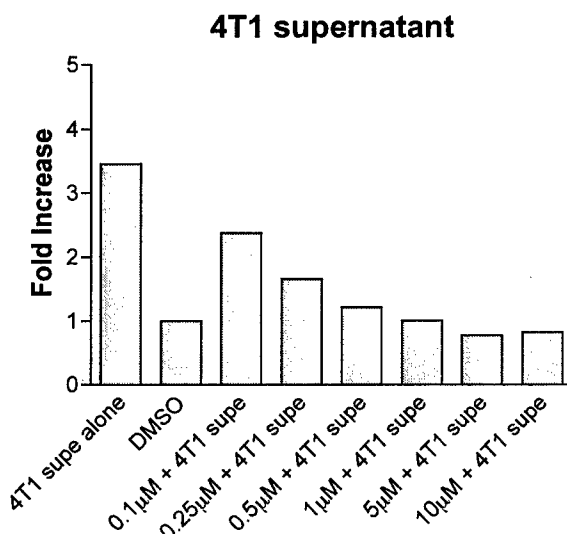


Figure 12. Effect of HTS466284 on the expression of the TGF- β inducible gene reporter PAI-luciferase in response to 4T1 derived TGF- β . Mv1Lu transfected with the p3TPLux reporter were treated with 1, 5, and 10 μ M of HTS466284 for 30 minutes followed by 18 hour treatment with 1ng/ml of 4T1 derived TGF- β 1. Cell extracts were prepared using the LucLite Luciferase Assay kit (Perkin-Elmer, Boston, MA) and light emission was detected with a Packard Lumicount top-count luminometer. Data represents mean $\pm$ SEM of triplicate samples.

HTS466284 blocks rhTGF- β 1-mediated morphological changes in 4T1 cells *in vitro*

We have previously observed that TGF- β treatment induces morphological changes in 4T1 cells indicative of epithelial to mesenchymal transition (EMT). These changes have been shown to be characteristic of highly malignant breast cancer cells. Therefore we evaluated the ability of HTS466284 to block TGF- β mediated EMT. 4T1 cells were treated with 1, 5, or 10 μ M of HTS466284 for thirty minutes prior to the addition of 2ng/ml of rhTGF- β for 48 hours. Following treatment, the cells were observed for morphological changes. The 4T1 mammary tumor cell line normally adheres to plastic and grows in

rounded clumps (Figure 13A), but upon incubation with 2ng/ml rhTGF- β the cells became elongated, less clumped and assumed a fibroblast-like appearance (Figure 13B). HTS466284 treatment was able to block TGF- β mediated EMT in 4T1 cells at all concentrations tested and these cells retained their normal clumped morphology (Figure 13C-D). TGF- β mediated EMT in the 4T1 cells could be restored if the HTS466284 treated 4T1 cells were washed with phosphate buffered saline (PBS) prior to the addition of rhTGF- β (Figure 14A-C).

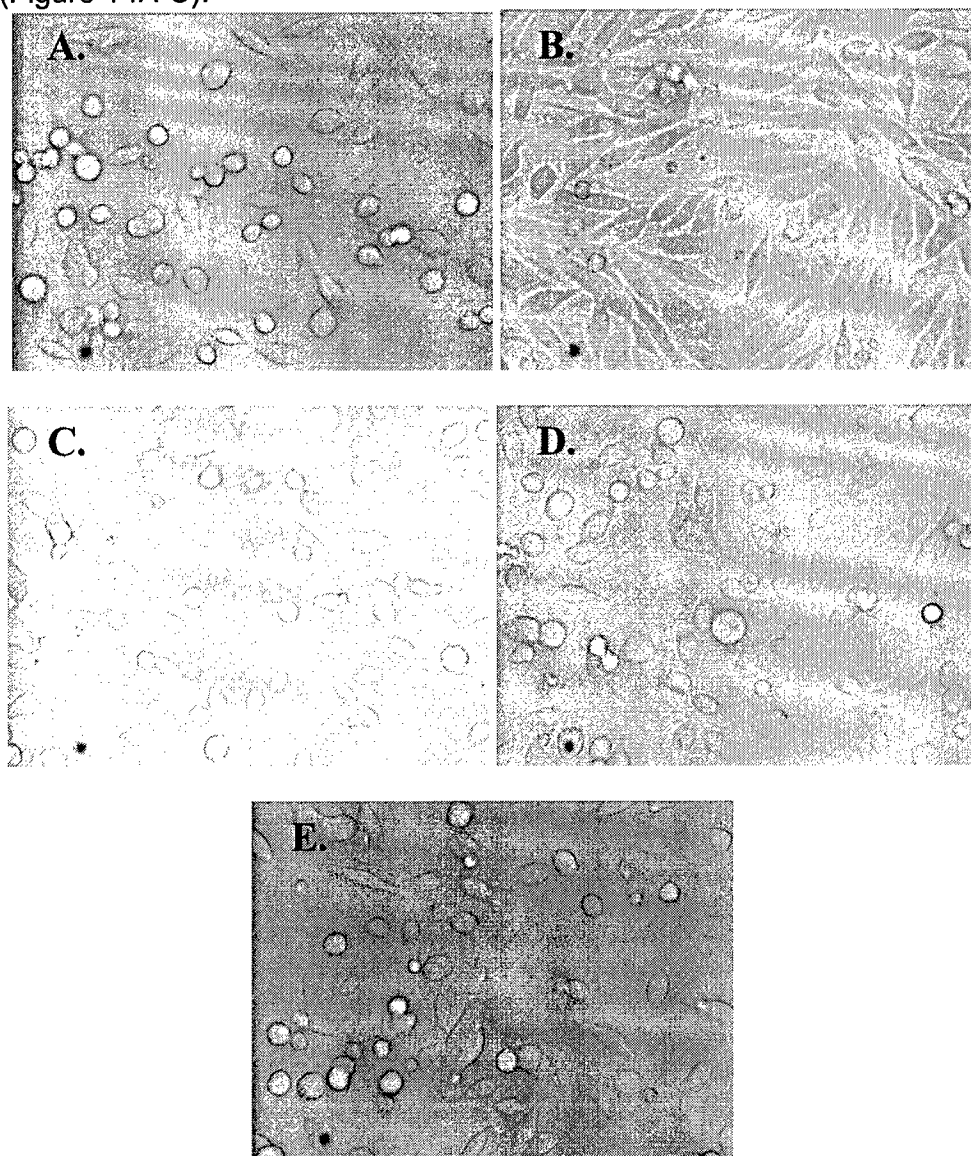


Figure 13. Inhibition of TGF- β mediated EMT in 4T1 cells by treatment with HTS466284. 4T1 cells were treated with 1, 5, or 10 μ M of HTS466284 for 30 minutes prior to the addition of 2ng/ml of rhTGF- β 1. After 48 hours the cells were observed for morphological changes. **A.** Cells Alone **B.** TGF- β Alone **C.** TGF- β + 1 μ M HTS466284 **D.** TGF- β + 5 μ M HTS466284 **E.** TGF- β + 10 μ M HTS466284

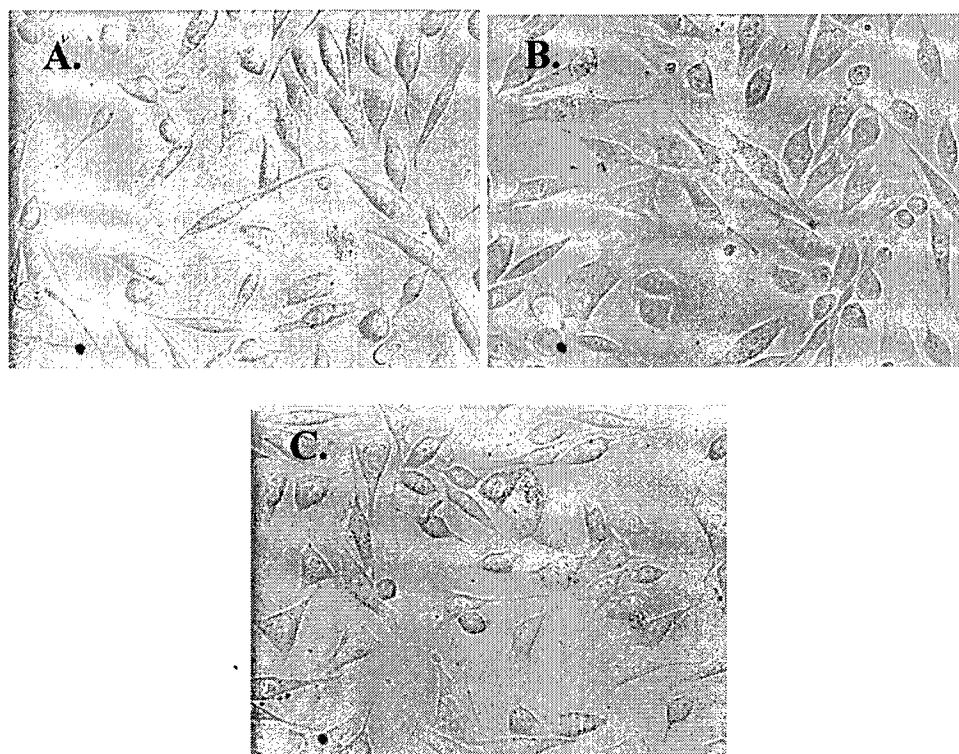


Figure 14. Restoration of TGF- β mediated EMT in 4T1 cells by removal of HTS466284. 4T1 cells were treated with 1, 5, or 10 μ M of HTS466284 for 30 minutes and then washed 3 times with PBS. Following the wash 2ng/ml of rhTGF- β 1 was added to the cells. After 48 hours the cells were observed for morphological changes. **A.** 1 μ M HTS466284 (washed) + TGF- β **B.** 5 μ M HTS466284 (washed) + TGF- β **C.** 10 μ M HTS466284 (washed) + TGF- β

HTS466284 inhibits Smad2 phosphorylation in 4T1 cells and DCs

Phosphorylation of the transcription factor Smad2 has been identified as an early step in the TGF- β signaling pathway. Therefore, blockade of TGF- β signal transduction by HTS466284 was assessed by Western blot analysis for phosphorylated Smad2 in 4T1 cells and DCs exposed to TGF- β in the presence or absence of the inhibitor. The data in Figure 15 show that HTS466284 inhibits Smad2 phosphorylation in a dose dependent manner.

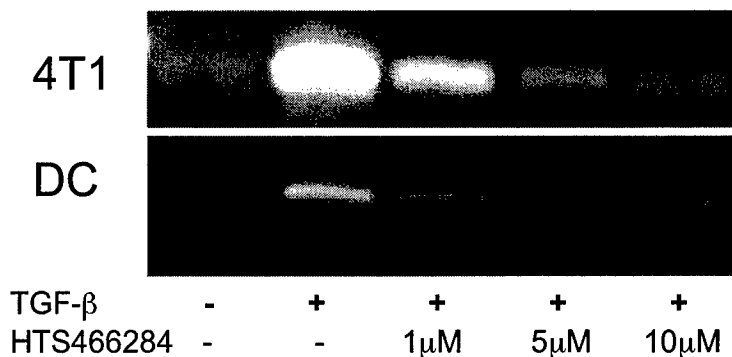


Figure 15. HTS466284 inhibits Smad2 phosphorylation in 4T1 cells and DCs. 4T1 cells or DCs were treated with 1, 5, and 10 μ M of HTS466284 for 30 min followed by exposure to rhTGF- β 1 for 45 min. Cell lysate was prepared, resolved by 10% SDS-PAGE, and electrotransferred to PVDF membrane. pSmad2 was detected by incubating the membrane in rabbit anti-human pSmad2 antibody (US Biological, Swampscott, MA).

Effect of combination DC + HTS466284 therapy on established 4T1 tumors *in vivo*.

After we established that HTS466284 inhibits TGF- β signaling *in vitro* we evaluated the ability of HTS466284 to inhibit tumor progression in the TGF- β -producing 4T1 murine mammary tumor model. For this purpose, HTS466284 was injected i.p. into BALB/c mice bearing pre-established 4T1 tumors. However, HTS466284 monotherapy did not significantly affect the growth of established 4T1 tumors *in vivo* (data not shown). Therefore, we evaluated if HTS466284 would be able to improve the effectiveness of DC vaccines in a TGF- β -producing tumor microenvironment by treating mice bearing established 4T1 tumors with TNF- α matured, 4T1 lysate-pulsed DCs alone or in combination with HTS466284. The data (Figure 16) show that HTS466284 treatment in combination with DC vaccination significantly inhibited the growth of established 4T1 tumor to the same degree as mice treated with interleukin-12 (IL-12) plus DC. Furthermore, the increased anti-tumor activity in the DC + HTS466284 and DC + IL-12 groups was associated with increased IFN- γ secretion by splenocytes compared to the control groups (Fig. 17). Taken together, these data indicate that neutralization of tumor derived TGF- β by HTS466284 enhanced the effectiveness of DC-based immunotherapy in treating established 4T1 tumors.

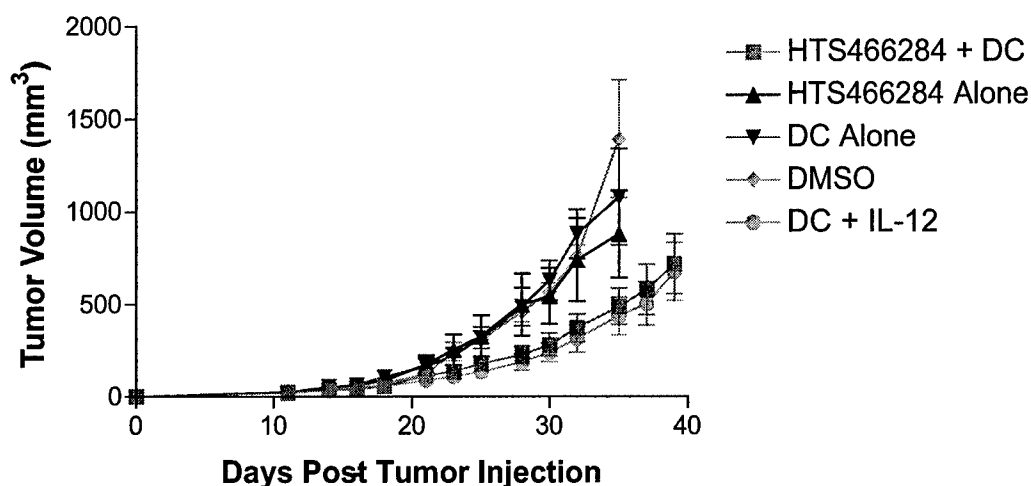


Figure 16. Treatment of established 4T1 tumors. Six-week-old female Balb/c mice were injected s.c. on Day 0 with 5×10^4 4T1 cells into the mammary fat pad. On Day 13 the mice were injected i.p. with 200 μ g of HTS466284 in 100 μ l of 50% DMSO. HTS466284 injections were continued daily until day 25 for a total of 13 injections. Control animals received 13 daily injections of 50% DMSO vehicle alone. Mice in the DC groups were injected s.c. near the tumor site with 2×10^6 4T1 tumor lysate pulsed, TNF- α matured DCs (70% CD11c positive) on days 15, 20, and 35. Some mice received 0.2 μ g (2000U) of IL-12 i.p. on 4 consecutive days following each DC injection. Mice were monitored for tumor growth and were sacrificed when tumors became ulcerated or reached an average size greater than 1000 mm³. Graphs represents mean $\pm$ SEM of 5 individual mice.

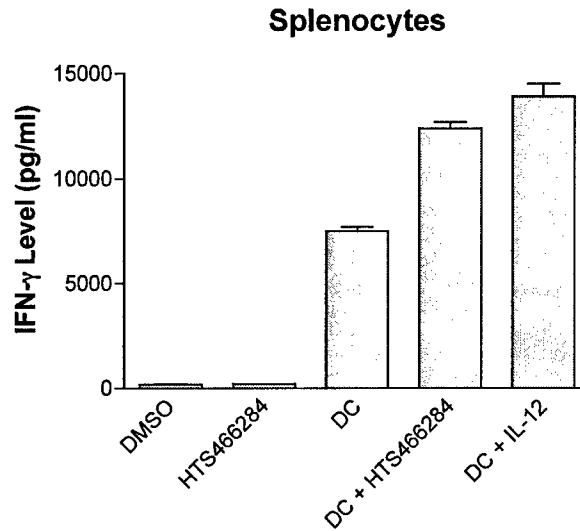


Figure 17. DC + HTS466284 and DC + IL-12 increased IFN- γ secretion by splenocytes. 2-3 mice from each treatment group were sacrificed and their spleens and tumor draining lymph nodes were harvested, pooled and disaggregated. Splenocytes and lymph node cells were purified on a Ficol density gradient (Atlanta Biological, Lawrenceville, GA) and 1×10^6 splenocytes were plated in 24-well plates for 48 hr. The supernatant was then collected and assayed for IFN- γ by ELISA (Pierce, Rockford, IL) according to the manufacturer's protocol. Graph represents mean $\pm$ SD of three separate samples.

Key research accomplishments

1. Determination of the effects of TGF- β on the antigen presentation capacity, ability to stimulate T cell responses and the migratory capacity of DCs *in vitro*.
2. Determination of the effects of tumor-derived TGF- β on the ability of DC to migrate and DC phenotype *in vivo*.
3. Evaluation of the effect of neutralization of tumor-derived TGF- β *in vivo* on the effectiveness of DC vaccines.
4. Mitigation of TGF- β signaling in DCs by infection with AdSmad7.
5. Evaluation of the ability of AdSmad7-DCs to treat established 4T1 tumors and stimulate an immune response.
6. Demonstration of ability of the novel TGF- β R1 small-molecule kinase inhibitor to abrogate TGF- β signaling and prevent EMT in tumor cells.
7. Evaluation of the ability of DC + HTS466284 combination therapy to treat established 4T1 tumors *in vivo*.

Reportable Outcomes

Publications

1. 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. TGF- β inhibits the antigen presenting functions and anti-tumor activity of dendritic cell vaccines. *Cancer Research.*, 63, (8), 1860-4, 2003.
2. Kobie, J. J., and Akporiaye, E. T.. Immunosuppressive Role of Transforming Growth Factor -Beta in Breast Cancer. *Clinical and Applied Immunology Reviews.*, 3, 277-287, 2003.

Presentations

1. Rausch, M., Mahadevan, D., and Akporiaye, E.T. Enhancement of DC-based immunotherapy using a small molecule TGF- β inhibitor. American Association for Cancer Research 96th Annual Meeting, April 16-20 2005. Oral presentation at mini symposium
2. Kobie, J.J., Ramanathapuram, L.V., and Akporiaye, E.T. Tumor-derived TGF- β inhibits the migratory and ant-tumor activity of dendritic cell-based vaccines. American Association for Cancer Research 94th Annual Meeting, April 2003. Poster presentation
3. Kobie, J. J., Wu, R. S., and Akporiaye, E. T. TGF- β inhibits the antigen presenting and anti-tumor activity of dendritic cell vaccines. Society of Biological Therapy 17th Annual Meeting, November 2002. Abstract selected for oral presentation.
4. Kobie, J.J., Mack, V., Lou, S., and Akporiaye, E.T. Effect of tumor-derived TGF- β on the efficacy of dendritic cell vaccines. Era of Hope, Department of Defense Breast Cancer Research Program Meeting. September, 2002. Poster presentation.

Conclusions

It is apparent from our research that tumor-derived TGF- β impairs the ability of DCs to stimulate anti-tumor immune responses. Previous research has demonstrated that TGF- β inhibits the ability of dendritic cells to internalize antigen, present antigen, stimulate tumor-sensitized T lymphocyte responses, and treat established 4T1 tumors. Our current research extends these findings and demonstrates that TGF- β impairs the ability of DCs to escape the immunosuppressive tumor microenvironment and migrate to DLNs. We demonstrate that TGF- β suppression of dendritic cell vaccines can be mitigated by inhibiting TGF- β gene expression in tumor cells and by neutralization of secreted TGF- β with specific antibody. Although, adenovirus-mediated transfer of the TGF- β pathway inhibiting protein Smad7 into dendritic cells failed to significantly enhance the antitumor activity of DC, we have been able to enhance their ability to stimulate an immune response in tumor bearing hosts. More importantly, we demonstrate that a novel small molecule TGF- β type 1 receptor kinase inhibitor (HTS466284) abrogates tumor-derived TGF- β -mediated signaling and epithelial-mesenchymal transition (EMT) characteristic of a more invasive phenotype without concomitant drug toxicity. *In vivo* experiments demonstrated that HTS466284 enhanced the effectiveness of DC vaccines in controlling the growth of established 4T1 tumors. Taken together, our studies so far demonstrate the usefulness of eliminating immunosuppressive tumor-derived products in order to improve the effectiveness of dendritic cell-based anti-cancer vaccines.

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Appendices

1. 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.. TGF- β inhibits the antigen presenting functions and anti-tumor activity of dendritic cell vaccines. *Cancer Research*. 63: 1860-1864, 2003.
2. Kobie, J. J., and Akporiaye, E. T.. Immunosuppressive Role of Transforming Growth Factor -Beta in Breast Cancer. *Clinical and Applied Immunology Reviews* 3: 277-287, 2003.

Transforming Growth Factor β Inhibits the Antigen-Presenting Functions and Antitumor Activity of Dendritic Cell Vaccines¹

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ABSTRACT

Dendritic cell (DC)-based vaccines have exhibited minimal effectiveness in treating established tumors, likely because of factors present in the tumor microenvironment. One such factor is transforming growth factor β (TGF- β), a cytokine that is produced by numerous tumor types and has been demonstrated to impair DC functions *in vitro*. We have evaluated the effect of TGF- β on the immunostimulatory activities of DCs. We demonstrate that TGF- β exposure inhibits the ability of DCs to present antigen, stimulate tumor-sensitized T lymphocytes, and migrate to draining lymph nodes. Neutralization of TGF- β using the TGF- β -neutralizing monoclonal antibody 2G7 enhanced the ability of DC vaccines to inhibit the growth of established 4T1 murine mammary tumors. Treatment of 4T1 tumors transduced with the antisense TGF- β transgene (4T1-asT) with the combination of DC and 2G7 monoclonal antibody inhibited tumor growth and resulted in complete regression of tumors in 40% of the mice. These results demonstrate that neutralization of TGF- β in tumor-bearing mice enhances the efficacy of DC-based vaccines.

INTRODUCTION

In recent years, DCs⁴ have become popular candidates in cancer vaccine development because of their crucial role in inducing T-cell responses. Upon antigen uptake, DCs residing in peripheral tissues internalize and process antigen and migrate to secondary lymphoid organs where they stimulate naïve T lymphocytes in the context of class I and class II MHC antigens (1). The effectiveness of DCs as antigen-presenting cells provides the rationale for their use as cancer vaccines with the objective of inducing durable antitumor immune responses. In numerous rodent models, vaccines consisting of tumor antigen-pulsed DCs are effective in inducing CTL responses and providing protection against subsequent tumor challenge (2-5). In contrast, DC vaccines have been less effective in abrogating established tumors in mice (2-5) and human cancer patients (6). The insensitivity of established tumors to DC therapy is likely because of factors present within the tumor microenvironment that are inimical to the optimal induction of an antitumor immune response (7). Examination of circulating and tumor-infiltrating DCs in tumor-bearing animals and in cancer patients has revealed that DCs are functionally impaired in their ability to induce T-cell responses (8-11). These deficits have been associated with down-regulation of MHC and

costimulatory molecules (10, 11) and tumor-induced apoptosis of DCs (12).

One of the factors produced within the tumor microenvironment that might interfere with DC functions is TGF- β . TGF- β is a pleiotropic cytokine produced by cancer cells of different histological types (13-16). Among the plethora of immunosuppressive effects of TGF- β (17) is the capacity to interfere with several DC functions. These include down-regulation of cell surface MHC antigens, costimulatory molecules, chemokine receptors, as well as impairment of *in vitro* chemotaxis (18-20).

Although the *in vitro* effects of TGF- β on DCs are relatively well known, the impact of TGF- β on the ability of DCs to migrate to secondary lymphoid organs and induce specific antitumor T-cell responses *in vivo* remains to be determined. In this study, we demonstrate that TGF- β inhibits DC migration to DLN and diminishes their capacity to stimulate IFN- γ secretion by tumor-sensitized T lymphocytes. Most importantly, we show that the combined use of antisense TGF- β gene transfer plus TGF- β -neutralizing antibody increases the efficacy of DC vaccines in treating established TGF- β -secreting 4T1 mammary tumors.

MATERIALS AND METHODS

Animals. Six-week-old female BALB/c and C57BL/6 (B6) mice were purchased from The Harlan Laboratory (Indianapolis, IN). Six-week-old female BALB/c-TgN (DO11.10) 10 Loh mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed at the University of Arizona Animal Facilities in accordance with the Principles of Animal Care (NIH publication no. 85-23, revised 1985).

Tumors. 4T1 murine mammary tumor cells were kindly provided by Dr. Fred Miller (Michigan Cancer Foundation, Detroit, MI) and maintained as described previously (21).

TGF- β -neutralizing Antibody (2G7). The 2G7 mouse IgG1 mAb was generated after immunization of BALB/c mice with recombinant human TGF- β_1 . 2G7 neutralized the growth inhibitory activity of TGF- β_1 , TGF- β_2 , and TGF- β_3 on Mv1Lu epithelial cells (22).

Generation of DCs and TGF- β Treatment. Bone marrow cells were harvested from flushed marrow cavities of femurs and tibiae under aseptic conditions and cultured with 100 units/ml granulocyte macrophage colony-stimulating factor and 100 units/ml interleukin 4 (Peprotech, Rocky Hill, NJ) at 10^6 cells/ml in complete media (RPMI 1640 containing 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 μ M sodium pyruvate, 2 mM L-glutamine, 100 μ g/ml streptomycin, 100 units/ml penicillin, 0.5 μ g/ml fungizone, and 5×10^{-5} M 2-mercaptoethanol). Cytokines were replenished on day 4. On day 6 of culture, DCs were collected and cultured at 10^6 cells/ml with granulocyte macrophage colony-stimulating factor and interleukin 4 with or without the addition of 10 ng/ml of recombinant human TGF- β_1 (R&D Systems, Minneapolis, MN) for 6 days. DCs were matured with 200 units/ml of TNF- α (Peprotech) for 48 h.

FACS Analysis. All antibodies used were purchased from Caltag Laboratories (Burlingame, CA) unless otherwise noted. For analysis of DCs, samples were stained with PE-conjugated anti-CD11c (BD PharMingen, San Diego, CA), FITC-conjugated anti-I-A^d (BD PharMingen), PE-conjugated anti-B7.1 (CD80), FITC-conjugated anti-B7.2 (CD86), or PE-conjugated anti-CD40. T cells were stained with PE-conjugated anti-CD3, FITC-conjugated anti-B220, PE-conjugated anti-CD8, or FITC-conjugated anti-CD4. Cells were analyzed

Received 5/28/02; accepted 2/18/03.

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¹ Supported by NIH Grants 1 RO1 CA9411-01 and DAMD 170010128 and DAMD 17010126 from the Department of Defense/United States Army.

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⁴ The abbreviations used are: DC, dendritic cell; DLN, draining lymph node; mAb, monoclonal antibody; FBS, fetal bovine serum; TGF- β , transforming growth factor β ; TNF- α , tumor necrosis factor α ; FACS, fluorescence-activated cell sorter; PE, r-phycoerythrin; OVA, ovalbumin; SLC, secondary lymphoid chemokine; MIP-3 β , macrophage inflammatory protein 3 β .

using a FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Induction of Allogeneic Mixed Lymphocyte Reactions. Spleen cells from B6 mice were harvested and enriched for CD3-positive cells using a T-cell enrichment column (R&D Systems). Cells were 80–95% CD3 positive as determined by FACS analysis. Varying numbers of DCs were incubated with 2×10^5 T lymphocytes for 5 days in 96-well tissue culture plates (Sarstedt, Newton, NC) with the addition of 1 μ Ci of [³H]thymidine (Perkin-Elmer Life Sciences, Boston, MA) for the final 18 h of culture.

OVA Peptide Presentation Assay. Spleen cells from DO11.10 OVA T cell receptor transgenic mice were enriched for CD3 positive cells as described above. Varying numbers of DCs were incubated with 2×10^5 T lymphocytes in the presence of 1 μ M of OVA peptide (ISQAVHAAHAEINEAGR; United Biochemical Research, Seattle, WA) in 96-well tissue culture plates for 5 days with the addition of 1 μ Ci of [³H]thymidine for the final 18 h of culture.

Endocytosis and Phagocytosis Assays. Endocytosis and phagocytosis assays were performed using modifications of previously described procedures (23). Endocytosis was measured by incubating 2×10^5 DC with 400 μ g of FITC-conjugated dextran beads, 40,000 MW (Molecular Probes, Eugene, OR) for 30 min at 4°C or 37°C. Phagocytosis was measured by incubating DCs with FITC-conjugated *Escherichia coli* (Molecular Probes) at a ratio of 100 *E. coli* particles to 1 DC for 60 min at 4°C or 37°C. After incubation, cells were washed extensively with PBS containing 0.5% bovine albumin and 0.1% sodium azide and analyzed by flow cytometry.

Stimulation of Tumor-sensitized T Lymphocytes. Bone marrow-derived DCs were pulsed with 4T1 tumor cell lysate at a ratio of 3 tumor cell

equivalents/DC for 24 h in the presence or absence of 10 ng/ml TGF- β_1 . DCs were then matured with 20 ng/ml TNF- α in the presence or absence of 10 ng/ml TGF- β_1 . Splenic T lymphocytes were purified from mice bearing 14 day 4T1 tumors as indicated above. Ten thousand DCs were incubated with 2×10^5 splenic T lymphocytes in 96-well tissue culture plates for 5 days with the addition of 1 μ Ci of [³H]thymidine for the final 18 h of culture. One million tumor DLN cells from mice bearing 14 day 4T1 tumors were incubated with 2.5×10^5 DCs for 48 h, and IFN- γ production was evaluated by ELISA (R&D Systems).

In Vivo Migration Assay. Bone marrow-derived DCs were matured with 200 units/ml TNF- α with or without 10 ng/ml TGF- β_1 for 48 h. DCs were labeled with 10 μ M PKH-67L, green fluorescent dye (Sigma, St. Louis, MO) as described previously (20). Naive mice received s.c. injections in the right flank with $5-8 \times 10^6$ DCs. Forty-eight h after injection, mice were sacrificed, and inguinal lymph nodes were harvested and disaggregated. Lymph node cells were centrifuged (Shandon, Pittsburgh, PA) onto glass slides at 700 rpm for 4 min. The slides were fixed with 4% paraformaldehyde and stained with a propidium iodide/RNase solution (Phoenix Flow Systems, San Diego, CA). Slides were analyzed using a laser scanning cytometer (CompuCyte, Cambridge, MA). Detection and contouring of cells was keyed by propidium iodide signal, whereas DCs were identified by their green fluorescence signal; 35,000 propidium iodide events were analyzed from each treatment group/experiment. The identity of each DC detected during scanning was confirmed visually by direct microscopic observation using the instrument's relocation function.

In Vitro Chemotaxis Assay. Bone marrow-derived DCs were matured with TNF- α in the presence or absence of TGF- β_1 for 48 h as indicated above.

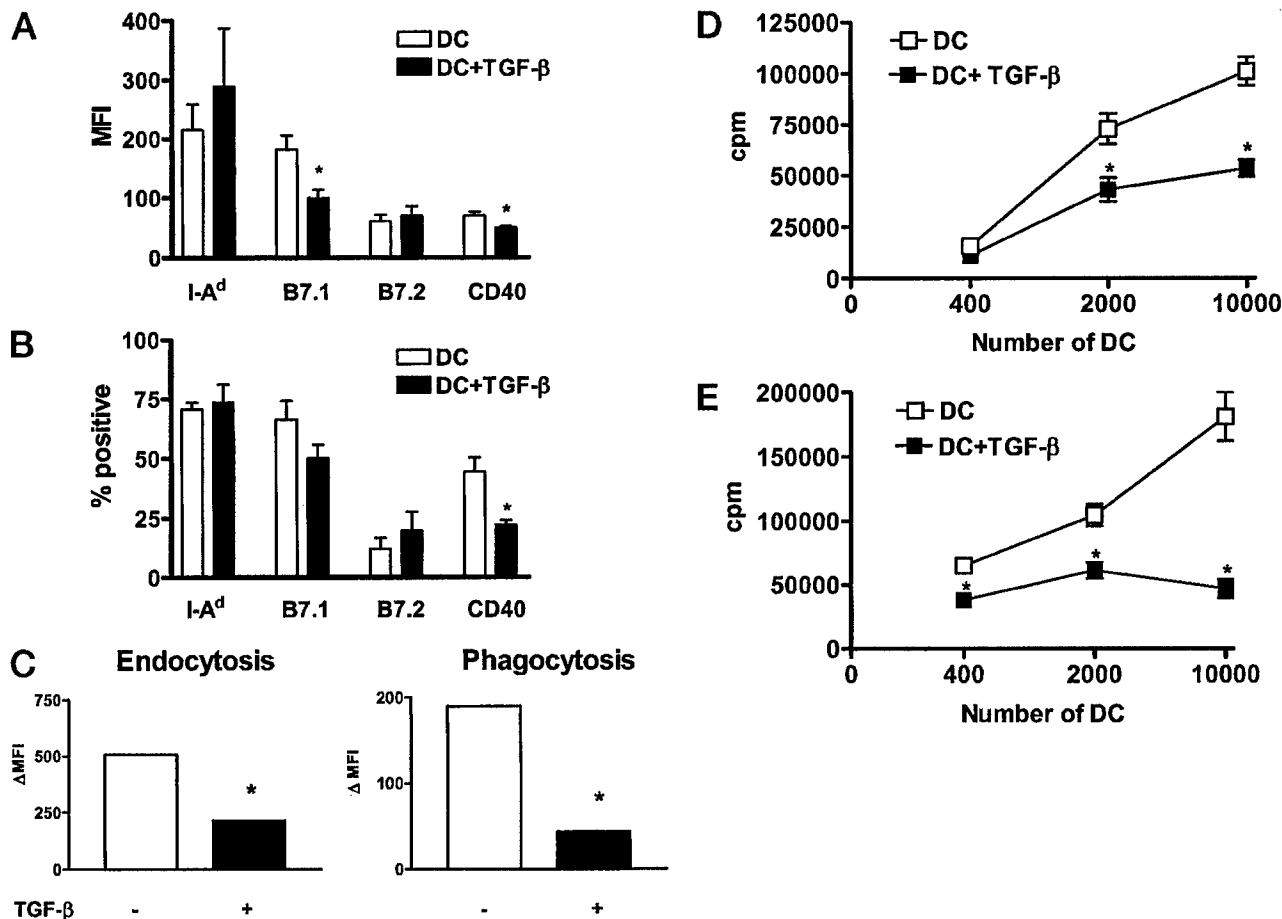


Fig. 1. Effect of TGF- β exposure on DC functions *in vitro*. DCs were incubated in the absence or presence of 10 ng/ml TGF- β for 6 days. Cytokines were replenished every 2 days. A and B, DCs were stained with anti-CD11c, anti-I-A^d, anti-B7.1, anti-B7.2, and anti-CD40 antibodies and analyzed by flow cytometry. The results are mean $\pm$ SE of three independent experiments. C, DCs were incubated with FITC-conjugated dextran particles or FITC-conjugated *E. coli* particles at 4°C or 37°C, fixed, and analyzed by flow cytometry. Values represent mean fluorescence intensity (MFI) at 37°C minus 4°C. D, DCs were incubated with 2×10^5 splenic T cells isolated from C57/BL6 mice for 5 days with the addition of [³H]thymidine for the last 18 h of culture. Values represent mean $\pm$ SE of six replicates. E, DCs were collected and incubated with 2×10^5 splenic T cells isolated from BALB/c-TgN (DO11.10) 10 Loh mice in the presence of OVA peptide for 5 days with the addition of [³H]thymidine for the last 18 h of culture. Values represent mean $\pm$ SE of four replicates. Results are from one representative experiment of three independent experiments. * refers to statistical significance between groups ($P < 0.05$).

An *in vitro* chemotaxis assay was performed as described previously (24). SLC (PeproTech) and MIP-3 β (R&D Systems) were diluted with serum-free media to a final volume of 600 μ l of 100 ng/ml chemokine and added to 24-well tissue culture plates (Corning Costar, Cambridge, MA). Transwell culture inserts (Corning Costar) with 6.5-mm diameter and 5.0- μ m pore-size were inserted into each well, and DCs (4×10^5 cells/each well) were added to the top chamber in serum-free media at a final volume of 100 μ l. After the plates were incubated at 37°C in 5% CO₂ for 4 h, the cells in the bottom chamber were recovered, the migrating cells were counted, and an aliquot was stained with anti-CD11c mAbs to be analyzed by FACS. Controls included wells with chemokine in both the top and bottom chambers. The number of migrated cells was determined by subtracting the number of migrated cells in control wells from the number of migrated cells in experimental wells.

Treatment of Established Tumors. Six-week-old BALB/c mice were orthotopically injected with 10^4 4T1-N or 4T1-asT tumor cells into the mammary gland. DCs were pulsed with 4T1 tumor cell lysate at a ratio of 3 tumor cell equivalents/dendritic cell for 24 h. After pulsing, DCs were matured with 200 units/ml TNF- α for 48 h. Mice were injected i.t. with 1.5×10^6 tumor cell lysate-pulsed, matured DCs in 50 μ l of PBS on day 15 when tumors were palpable. Vaccination was repeated on days 20 and 25. Two h before each vaccination, mice received i.p. injections of 300 μ g of 2G7 mAb. In combination with DCs or alone, mice received 100 μ g of 2G7 mAb i.t. Primary tumors were measured as reported previously (21). Mice exhibiting complete tumor regression were challenged with 10-fold more 4T1 tumor cells (10^5) and monitored for tumor growth.

Statistical Analysis. For all analyses, student *t* tests were performed using Prism software (GraphPad, San Diego, CA). *P*s of <0.05 were considered to indicate significant differences between data sets.

RESULTS

Antigen Uptake and Presentation by DCs. Before evaluating the effect of TGF- β *in vivo*, we evaluated its effects on several DC functions *in vitro*. Treatment of DCs with TGF- β caused a significant ($P < 0.05$) decrease in the levels of B7.1 and CD40 (Fig. 1A), as well as in the percentage of cells expressing CD40 (Fig. 1B). Next, we evaluated the ability of DCs to endocytose dextran particles and phagocytose *E. coli* particles after TGF- β treatment. TGF- β exposure

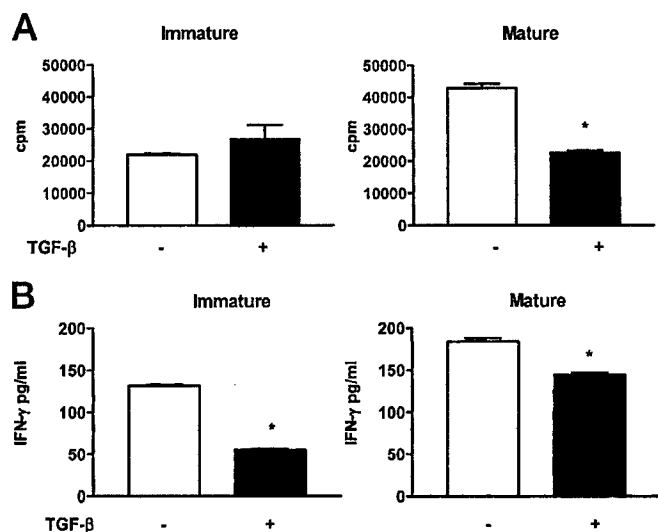


Fig. 2. Stimulation of tumor-sensitized T lymphocytes. DCs were pulsed with tumor cell lysate for 48 h in the presence or absence of 10 ng/ml TGF- β (immature), then cultured in TNF- α in the presence or absence of TGF- β for 48 h (mature). A, ten thousand DCs were incubated with 2×10^5 splenic T cells isolated from mice bearing 4T1 tumors for 5 days, and [³H]thymidine was added for the last 18 h of culture. B, one million T cells isolated from lymph nodes draining 4T1 tumors were incubated with 2.5×10^5 DCs for 48 h. After incubation, supernatant was analyzed for IFN- γ production. Numbers are mean $\pm$ SE of triplicate samples. Results are from one representative experiment of two independent experiments. * refers to statistical significance between groups ($P < 0.05$).

Table 1 Effect of TGF- β on *in vivo* migration

	DCs/ 10^6 LN cells	
	mDC	mDC + TGF- β *
Experiment 1	1137	727
Experiment 2	309	54
Experiment 3	466	305
Experiment 4	5500	5200

DCs were cultured in the presence of 200 U/ml TNF- α with or without the addition of 10 ng/ml of TGF- β for 48 hours. DCs were then labeled with PKH-67 membrane dye and injected s.c. into two naive BALB/c mice per group. Forty-eight hours following injection inguinal lymph nodes were harvested from mice and disaggregated. Lymph node cells were fixed and stained with Propidium Iodide/RNase I solution and analyzed by laser scanning cytometry. Data are from four independent experiments.

*refers to statistical significance between groups ($p < 0.05$).

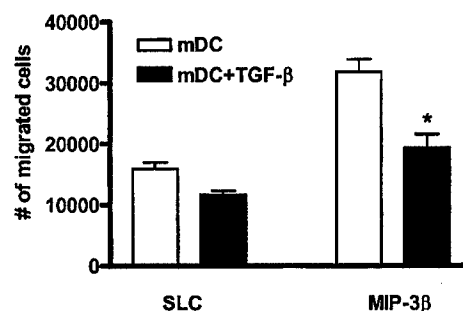


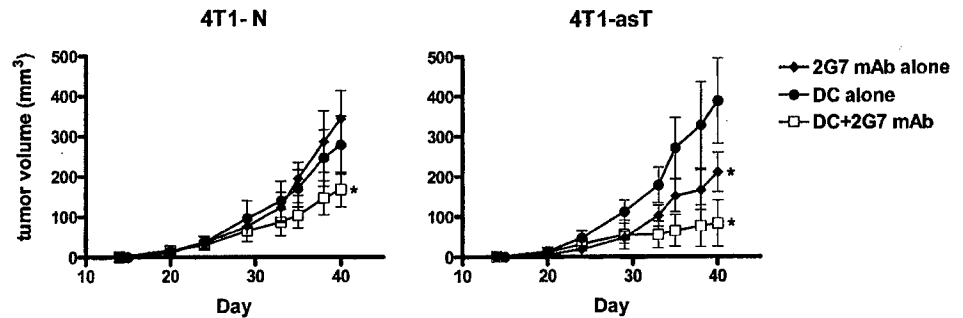
Fig. 3. Effect of TGF- β on *in vitro* chemotaxis. DCs were added to the upper chamber of a transwell migration chamber and evaluated for chemotactic migration. Cells migrating to the bottom chamber were recovered and analyzed by flow cytometry for CD11c expression. The results are mean $\pm$ SE of three independent experiments. * refers to statistical significance between groups ($P < 0.05$).

inhibited the endocytic and phagocytic capacities of DCs by 58% and 87%, respectively (Fig. 1C), a pattern that was observed in all three experiments conducted. To evaluate the effect of TGF- β exposure on antigen presentation, mixed lymphocyte reactions were performed. Stimulation of allogeneic T lymphocytes (Fig. 1D) and presentation of OVA peptide (Fig. 1E) by DCs was significantly ($P < 0.05$) suppressed after TGF- β exposure. These results demonstrate that TGF- β inhibits the uptake and presentation of antigens by DCs.

Induction of Tumor-sensitized T Lymphocytes. Observing that TGF- β inhibits the antigen uptake and presentation capacities of DCs, we evaluated its effect on the stimulation of tumor-sensitized T lymphocytes by tumor cell lysate-pulsed immature and mature DCs. TGF- β -treated mature DCs were significantly less effective ($P = 0.0002$) than untreated mature DCs at stimulating the proliferation of tumor-sensitized T lymphocytes (Fig. 2A). The ability of immature and mature DCs to stimulate IFN- γ production by tumor-sensitized lymphocytes was also significantly inhibited ($P < 0.0001$, $P = 0.0003$) by 60 and 22%, respectively, after TGF- β exposure. Taken together, these data demonstrate that TGF- β exposure reduces the ability of DCs to stimulate antitumor immune responses.

***In Vivo* Migration and *In Vitro* Chemotaxis.** Because migration of DCs to secondary lymphoid organs is necessary for T-cell priming (25), we assessed the effect of TGF- β treatment on the ability of mature DC to migrate to DLNs. For this purpose, TGF- β -treated DCs were labeled with PKH-67 and injected s.c. into the hind flank of mice. Forty-eight h later, draining inguinal lymph nodes were harvested, and infiltrating DCs were enumerated by scanning laser cytometry. TGF- β treatment resulted in a decrease in migration of mature DCs to the DLNs (Table 1). This was a significant ($P < 0.05$) trend observed over four independent experiments. To determine the mechanism responsible for decreased migration of mature DCs, *in vitro* chemotaxis toward SLC and MIP-3 β was evaluated. DCs migrating to the bottom chambers were recovered and analyzed by flow

Fig. 4. Treatment of established tumors. BALB/c mice with established 4T1-N (mock transfected) and 4T1-asT (transfected with antisense *TGF- β* gene) tumors were vaccinated intratumorally on days 15, 20, and 25 with 1.5×10^6 tumor cell lysate-pulsed mature DCs alone or in combination with 100 μ g i.t. and 300 μ g i.p. of 2G7, *TGF- β* -neutralizing antibody. Control mice were treated with 2G7 antibody alone. Mice were monitored for tumor growth. Graph represents mean tumor volume $\pm$ SE of 5 mice. * indicates significant ($P < 0.05$) difference as compared with mice treated with DC alone on day 40.



cytometry for CD11c expression. No significant difference in CD11c expression was observed between untreated and *TGF- β* -treated DCs (data not shown). *TGF- β* treatment caused reduced migration of DC toward both SLC and MIP-3 β , however, only migration toward MIP-3 β was significantly decreased ($P < 0.05$; Fig. 3).

Treatment of Established Tumors with DCs and Neutralizing *TGF- β* Antibody. The impairment of critical DC functions, including antigen uptake and antigen presentation, tumor-specific T lymphocyte stimulation, and *in vivo* migration by *TGF- β* , suggested that inhibition of *TGF- β* production in tumor-bearing animals may improve the efficacy of DC vaccines. To test this possibility, *TGF- β* production was suppressed by transfer of an antisense *TGF- β* transgene into 4T1 cells (4T1-asT) as described previously (21). Expression of the transgene resulted in $>90\%$ inhibition of *TGF- β* production (0.083 ± 0.003 ng/ml in 4T1-asT compared with 1.244 ± 0.188 ng/ml in mock-transduced cells (4T1-N)). Treatment of 4T1-asT tumors with 2G7 mAb alone or DCs plus 2G7 mAb significantly inhibited ($P < 0.05$) tumor growth compared with 4T1-asT tumors treated with DCs alone (Fig. 4). Furthermore, complete tumor regression occurred in 40% (two of five) of 4T1-asT-tumor-bearing mice that were treated with DCs plus 2G7 mAb (Fig. 4). Similarly, mock-transduced (4T1-N) tumors responded significantly better ($P < 0.05$) to treatment with DCs plus 2G7 mAb as compared with treatment with DCs alone; however, tumor growth inhibition in this group was inferior to that observed in animals bearing 4T1-asT tumors. (Fig. 4). Tumor growth was not affected by treatment with isotype control (IgG) antibody (data not shown). To determine whether the mice that exhibited complete tumor regression had developed long-term immunity, they were rechallenged with parental 4T1 tumor cells. These mice failed to develop tumors (zero of two); however, all control naive mice challenged with tumor cells developed tumors (three of three). These data suggest that neutralization of *TGF- β* in mice bearing *TGF- β* -secreting tumors enhances the effectiveness of DC vaccines in treating established tumors.

DISCUSSION

In this study, we evaluated the impact of *TGF- β* on *in vivo* migration and immunostimulatory activities of DCs. Our results demonstrate that *TGF- β* treatment diminishes the ability of DC to migrate to secondary lymphoid organs and to induce T-cell responses. Most importantly, suppression of tumor-derived *TGF- β* by antisense *TGF- β* gene transfer plus neutralization of secreted *TGF- β* with anti-*TGF- β* mAb significantly improved the antitumor activity of intratumorally injected tumor cell lysate-pulsed DCs. Previous studies have demonstrated that suppression of *TGF- β* by antisense gene transfer (13, 21, 26) or abrogation of *TGF- β* using neutralizing antibody (27, 28) increases tumor immunogenicity, leading to tumor growth inhibition or rejection. To our knowledge, this is the first study to evaluate the impact of both approaches simultaneously on DCs in

controlling established tumors. The finding that the antitumor effect was most evident when antisense *TGF- β* -expressing tumors were treated with DCs plus anti-*TGF- β* mAb directly implicates tumor-derived *TGF- β* in tumor progression. In our study, *TGF- β* within the tumor milieu could be promoting tumor growth by interfering with the antigen-presenting and effector functions of DCs at the tumor site, as well as preventing the emigration of injected DCs to DLNs to activate naive tumor-specific T lymphocytes. The former possibility is supported by the recent findings by Kirk *et al.* (29) who suggested that migration of i.t. injected DCs to DLNs is not required for the induction of an antitumor response. Using SLC gene-modified DCs, they demonstrated comparable tumor growth inhibition in normal mice and lymphotoxin $\alpha^{-/-}$ mice lacking peripheral lymph nodes (29). The latter possibility is supported by the decreased chemotactic response of *TGF- β* -treated DCs to MIP-3 β and SLC observed in our study. These chemokines produced in the lymph nodes recruit DCs via their interaction with the chemokine receptor, CCR7 (20, 30). A possible explanation is that CCR7 gene expression is inhibited in DC by *TGF- β* treatment as has been reported by others (19, 20).

As with previously published studies (29, 31, 32), only a minute fraction ($<1\%$) of s.c. injected DCs in our study migrated to DLNs. It is yet to be determined if these lymph node-infiltrating DCs represent a unique subpopulation capable of singularly stimulating the antitumor response or require the participation of endogenous DCs to achieve this goal. In the setting of DC vaccination to prevent or treat inaccessible micrometastases, it is desirable that adoptively transferred DCs are able to migrate to secondary lymphoid organs to stimulate naive T lymphocytes. Thus this LN-infiltrating, *ex vivo* manipulated DC population needs to be more actively studied.

In summary, this study demonstrates the potential usefulness of a combined therapeutic approach to eliminate immunosuppressive tumor-derived factors to improve the effectiveness of DC-based vaccines.

ACKNOWLEDGMENTS

We thank Vivian Mack for technical assistance and Barbara Carolus for flow cytometric analysis.

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Immunosuppressive role of transforming growth factor beta in breast cancer

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Received 2 December 2002; received in revised form 7 February 2003; accepted 17 February 2003

Abstract

Transforming growth factor beta (TGF- β) is a multifunctional cytokine, whose myriad of functions include its ability to potently suppress the immune system. Because of its ability to negatively modulate the inductive and effector phases of the immune response, TGF- β is thought to contribute to tumor progression and metastases formation. Immunosuppression by tumor-derived TGF- β is increasingly becoming recognized as an important factor in tumor progression and may, in part, explain the low response rates achieved in cancer patients undergoing immunotherapy for their disease. This review will focus on the immunosuppressive role of tumor-derived TGF- β in breast cancer. Due to the paucity of human studies, it will specifically address the actions of tumor-derived TGF- β on cells of the immune system in preclinical animal models, as well as discuss strategies to negate the deleterious effects of TGF- β in order to improve the anti-tumor immune response.

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Keywords: Transforming growth factor beta; Breast cancer; Tumor; Immunosuppression

1. Introduction

In the development of mammary cancer, transforming growth factor-beta (TGF- β) acts initially to inhibit the proliferation of normal epithelial cells [1]. However, following transformation, breast cancer cells, in addition to secreting TGF- β [2–4], become resistant to the

Abbreviations: BMP, bone morphogenic protein; CTL, cytotoxic T lymphocytes; DC, dendritic cell; DNA, deoxyribonucleic acid; FAST, forkhead activin signal transducer; IFN- γ , interferon- γ ; IL-2, interleukin-2; LAP, latency associated protein; LTBP, latent TGF- β binding protein; mRNA, messenger ribonucleic acid; NK, natural killer; OVA, ovalbumin; SARA, Smad anchor for receptor activation; T β RI, TGF- β receptor I; T β RII, TGF- β receptor II; TGF- β , transforming growth factor beta; TIDC, tumor-infiltrating dendritic cells.

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antiproliferative effects of this cytokine [1]. This phenotype results in a growth advantage of tumorigenic cells over normal nontransformed epithelial cells. Several mechanisms have been identified to explain the resistance of tumorigenic cells to TGF- β -mediated cell cycle arrest. These include downregulation of, and/or mutations in TGF- β receptors, signaling proteins and transcription factors [5]. Contrary to these findings of defective TGF- β signaling during tumor progression, several investigators have also reported increased production of TGF- β and retention of TGF- β signaling pathways in malignant cancer cells [6,7]. In the former case, tumor-derived TGF- β may promote tumor growth by suppressing several compartments of the immune system (summarized in Table 1, also reviewed in [8]), while in the latter case it can lead to increased invasiveness and metastasis of tumor cells [6].

Table 1
Effects of TGF- β on cells of the immune system

Cell type	Effects
T lymphocytes	inhibits IL-2 dependent proliferation [56,57] induces apoptosis [57,58] inhibits IFN- γ secretion [57]
CD4+	inhibits Th2 development [59] inhibits Th1 development [60] induces IL-10 production via GATA-3/Smad3 interaction [61] inhibits secondary immune responses [62] induces proliferation of antigen specific Th2 cells [62] inhibits activation and cytokine secretion by antigen specific memory Th1 cells [62]
CD8+	inhibits CD8 expression in cultured PBL [63] inhibits cytotoxic activity and IFN- γ production [64] induces suppressive CD8+ T cells [65]
B lymphocytes	inhibits antibody production [66] induces IgA synthesis [67] inhibits proliferation [68,69] induces apoptosis [70,71] inhibits expression of IgM, IgD, IgA, κ , and λ chains, and CD23 (Fc ϵ R2), transferrin receptor, and MHC class II expression [72]
Monocytes/Macrophages	induces expression of IL-1 receptor antagonist protein [73] inhibits expression of MHC class II [74] inhibits expression of Fc Receptor [75] inhibits nitric oxide production [76] induces IL-10 production [77,78] inhibits TNF- α production [78]
Dendritic Cells	inhibits chemokine receptor expression and chemotactic migration [79] inhibits maturation [80] inhibits expression of DC-SIGN (CD209) [81]
NK cells	inhibits proliferation [82] inhibits IFN- γ , TNF- α , and GM-CSF production [82]

This review will focus on the immunosuppressive role of tumor-derived TGF- β in mammary cancer progression in preclinical animal models and discuss strategies for counteracting the effects of TGF- β in order to enhance the antitumor immune response. To better understand the molecular basis of these therapeutic approaches, a brief review of the structure and signaling pathways of TGF- β is provided at the outset.

2. TGF- β structure and activation

TGF- β belongs to the TGF- β superfamily that is composed of numerous members including the TGF- β , activin/inhibins, and bone morphogenic protein (BMP) subfamilies. Several TGF- β isoforms have been identified including TGF- β_1 , which is the most extensively characterized. TGF- β_1 is secreted as a biologically inactive 290 kDa protein complex consisting of the TGF- β_1 homodimer (25 kDa), and the latency associated protein (LAP) (75 kDa), which is associated with the latent TGF- β_1 binding protein (LTBP) (190 kDa) glycoprotein via a disulfide bond. Activation of TGF- β_1 requires dissociation of the TGF- β_1 homodimer from the latency complex. In vitro, TGF- β can be activated by pH extremes [9,10], heat treatment [9], and alkylating agents [11]. Mechanisms of *in vivo* activation include proteolysis by several enzymes including plasmin [12–14], stromelysin-1 (MMP-3) [15], 72 kDa gelatinase (MMP-2), [15] matrix metalloproteinase-9 [16], binding of the TGF- β complex to the extracellular matrix protein thrombospondin [17,18], or the $\alpha_v\beta_6$ integrin [19].

3. Mechanisms of TGF- β signaling

TGF- β mediates its biological activity by binding to TGF- β receptor II (T β RII) (Fig. 1). Upon binding to the ligand, T β RII recruits TGF- β receptor I (T β RI) to form a heteromeric complex [20]. Additionally, the TGF- β Type III receptor (betaglycan) and endoglin can bind TGF- β and facilitate its binding to T β RII [21–23]. Both T β RI and T β RII are serine-threonine kinases, composed of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic serine-kinase domain. Downstream signaling is mediated by a series of structurally related proteins, termed Smads, originally identified by their similarity to the *Drosophila melanogaster Mothers against decapentaplegic* (*Mad*) and the *Caenorhabditis elegans sma* gene products [24–26]. The binding of TGF- β to the TGF- β receptor complex results in the GS sequence of T β RI being phosphorylated by the constitutively activated kinase domain of T β RII [20,27,28]. Smad2 is recruited to the membrane and presented to the TGF- β receptor complex by the Smad anchor for receptor activation (SARA). Phosphorylation of T β RI activates its downstream kinase domain, which in turn phosphorylates Smad2. Smad2 is next released from the TGF- β receptor complex, binds to Smad4, and is translocated to the nucleus [29,30]. The Smad2–Smad4 complex is able to regulate gene expression by directly binding to promoter regions of genes containing the Smad-binding element consensus sequence GTCTAGAC or by interaction with various DNA-binding transcription factors including FAST-1 and FAST-2 [31].

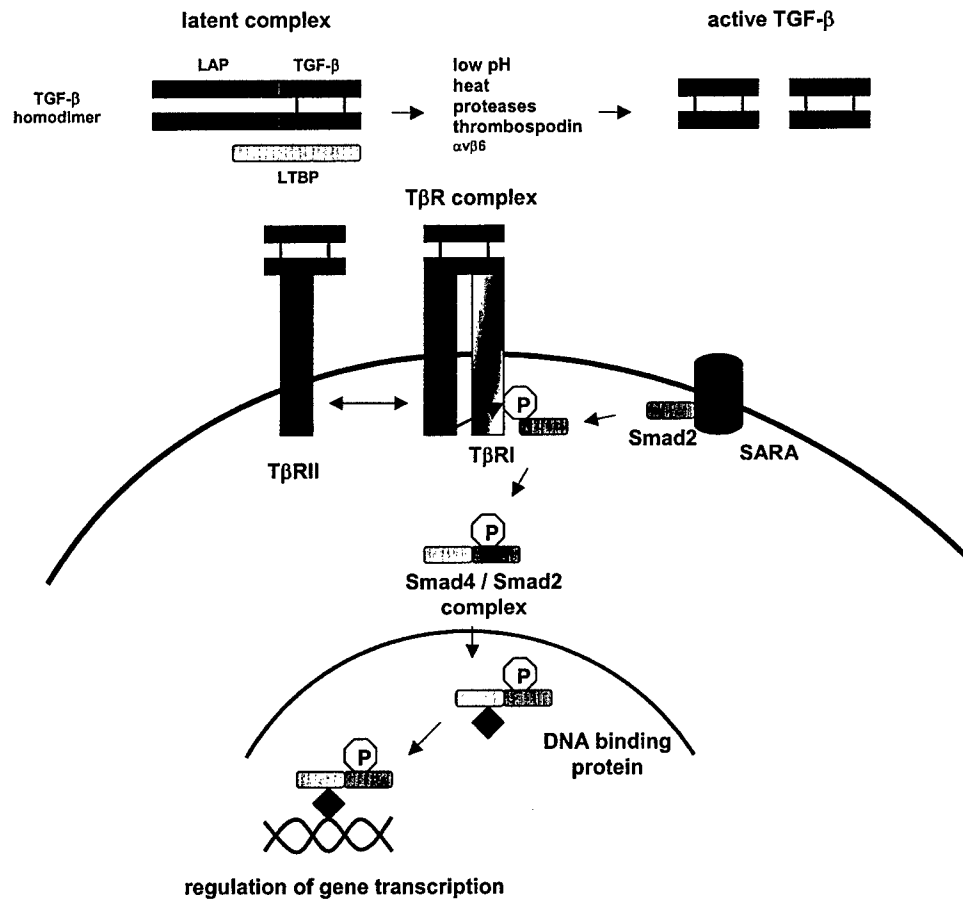


Fig. 1. TGF- β activation and signaling pathway. The latent TGF- β complex can be activated by a number of mechanisms. Active TGF- β binds to the T β R complex and the kinase domain of T β RI is activated by T β RII. Smad2 is recruited to the cell membrane and presented to T β RI by SARA. T β RI phosphorylates Smad2 and Smad2 complexes with Smad4. The Smad2–Smad4 complex translocates to the nucleus and regulates gene transcription.

4. Effects of TGF- β on the immune system in breast cancer

4.1. Preclinical animal model studies

Although numerous studies have documented secretion of TGF- β by breast cancer cells [3,32, reviewed in 33] only a handful of studies have sought to demonstrate a connection between the immunosuppressive properties of tumor-derived TGF- β and mammary tumor progression. In one of the earliest studies to address this issue, Arteaga et al. [34] demonstrated that inoculation of the human MDA-231 breast cancer cell line into nude athymic mice decreased natural killer (NK) cell activity in splenocytes isolated from these mice. They also showed that intraperitoneal injection of the TGF- β neutralizing antibody 2G7, restored NK function as well as suppressed intra-abdominal tumor and lung metastases. In contrast,

2G7 was unable to stimulate NK activity in NK-deficient beige nude mice. These observations led them to implicate TGF- β in the progression of mammary carcinomas via a mechanism that involved the suppression of NK cell activity. In a more recent study, Harthun et al. [35] demonstrated that tissue culture supernatant from a breast cancer cell line (BRC 173) suppressed interleukin-2-induced NK and lymphokine activated killer (LAK) cell activity of human peripheral blood lymphocytes, as well as the generation of tumor-reactive cytotoxic T lymphocytes (CTL). Addition of neutralizing TGF- β antibody or methylamine-activated α 2 macroglobulin (α 2M-MA), a plasma protein that binds with high affinity to TGF- β [36], almost completely restored the effector functions of these cells. Taken together, these results demonstrate that TGF- β modulates the development and functions of immune cells mediating both MHC-restricted and non-MHC restricted antitumor activity.

Using a TGF- β -secreting mouse mammary carcinoma cell line (EMT6), McAdam et al. [37] also showed that tumor cell-conditioned medium inhibited the development of alloreactive CTL *in vitro* and that this effect could be blocked by adding neutralizing TGF- β antibody or interleukin-2 (IL-2) to the cultures. Furthermore, they showed that transfer of the IL-2 gene into EMT6 cells caused tumor rejection. These results led them to propose that TGF- β contributed to the tumorigenic potential of EMT6 cells and that IL-2 can negate this activity. Since TGF- β downregulates IL-2 receptor (IL-2R) expression by T cells making them less responsive to IL-2-induced proliferation and activation [38], a likely mechanism for the overriding effect of IL-2 is the reinduction of IL-2R expression via an autocrine pathway [39]. Follow-up studies by us [40,41] using this cell line provided the first direct evidence for the immunosuppressive role of tumor-derived TGF- β in mammary cancer progression. We showed that transfer and expression of a full length antisense TGF- β ₁ cDNA in EMT6 cells significantly suppressed tumor growth [40] and that this effect could be enhanced by interferon- γ (IFN- γ) gene transfer [41]. *In vivo* depletion of T lymphocyte subsets revealed that CD8⁺ T-cells were required for manifestation of the antitumor response [41].

In another study by our group [42] using a poorly immunogenic, highly aggressive and metastatic mouse mammary tumor cell line (4T1), we demonstrated that ectopic expression of the antisense TGF- β transgene in these cells significantly inhibited the growth of primary tumors and the formation of lung, liver, and bone metastases. Histologic analyses revealed a predominantly lymphocytic infiltration of antisense TGF- β -expressing tumors compared to the primarily neutrophilic infiltrate observed in mock-transduced tumors. *In vivo* T cell depletion studies implicated CD4⁺ and CD8⁺ T cells as the primary immune effectors of the antitumor response. Furthermore, in a residual disease setting in which primary tumors were excised and animals were treated for residual metastatic disease, we showed that vaccination of mice with tumor cells modified to co-express antisense TGF- β and IFN- γ genes resulted in statistically significant prolongation of life [42]. IFN- γ may act in an autocrine manner to mitigate TGF- β signaling in tumor cells, by stimulating expression of the inhibitory Smad, Smad7, and promoting formation of Smad7–Smurf2 (Smad ubiquitination regulatory factor-2) complex formation that results in T β R degradation [43,44].

In more recent studies (unpublished data) we have shown that TGF- β also interferes with the inductive phase of the antitumor immune response by interfering with dendritic cell (DC) functions. Exposure to TGF- β inhibited the ability of DCs to present chicken egg ovalbumin (OVA) antigen, stimulate tumor-sensitized T lymphocytes, and migrate to draining lymph

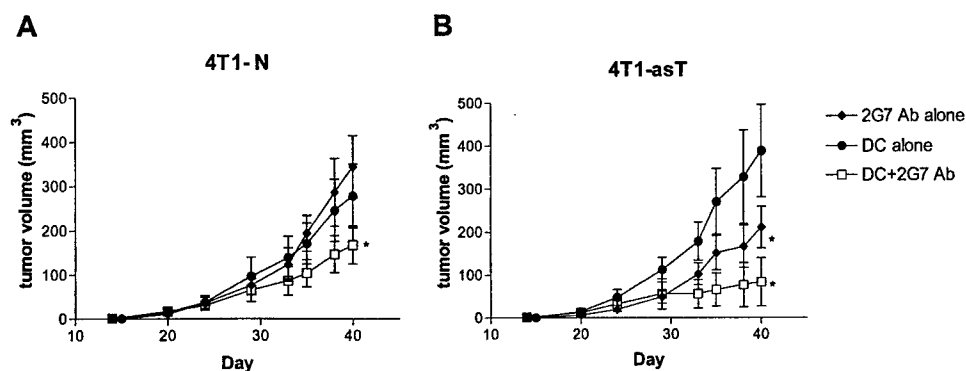


Fig. 2. Tumor-derived TGF- β inhibits the ability of DC vaccines to treat established 4T1 mammary tumors. Mice were challenged with 10^4 mock transduced (4T1-N) tumor cells (A) or antisense TGF- β transgene expressing (4T1-asT) tumor cells (B). On days 15, 20 and 25 mice received intraperitoneal injections of 2G7 antibody alone, intratumoral injections of 1.5×10^6 tumor cell-lysate pulsed matured DCs, or the combination of 2G7 antibody and DCs. Mice were monitored for primary tumor growth as a function of time. Data represents mean tumor volume $\pm$ SEM from 5 mice per group. *significant differences ($p < 0.05$) as compared to treatment with DCs alone on day 40 [81].

nodes [45]. *In vivo* studies also demonstrated that co-injection of 2G7 and tumor lysate-pulsed DCs into pre-established 4T1 tumors resulted in significant tumor growth inhibition with tumor regression occurring in a few animals compared to tumors injected with only 2G7 or DCs. (Fig. 2A). Furthermore, combination of 2G7 antibody treatment with inhibition of TGF- β production by tumor cells via antisense TGF- β transfer (4T1-asT) resulted in an improved antitumor effect (Fig. 2B). Taken together, these studies demonstrate that tumor-derived TGF- β contributes to mammary tumor progression by suppressing cellular immune responses.

4.2. Human studies

Unlike animal studies, little is known about the potential immunosuppressive role of TGF- β in human breast cancer. The available data are only correlative in nature. Elevated plasma levels of TGF- β in breast cancer patients have been reported by several investigators [46,47]. Other studies have also demonstrated that TGF- β immunoreactivity in tumor tissues positively correlates with lymph node involvement and disease progression [48–51]. Recently, Iwamoto et al. [52] demonstrated an inverse correlation between intratumoral TGF- β expression and content of mature (CD83+) tumor-infiltrating dendritic cells (TIDC). The number of CD83+ tumor-infiltrating DCs was associated with relapse-free and overall patient survival [48] suggesting that the state of maturation of TIDC may be of prognostic significance. However, no direct causal link between TGF- β and DC maturation was shown in the study. Contrary to the above findings, Marrogi et al. [53] reported a positive correlation of TGF- β mRNA expression in tumor tissue with survival of breast cancer patients but not with the clinical stage of their disease. Since a similar correlation was not observed with TGF- β protein expression, the prognostic value of this finding remains to be confirmed.

The association of TGF- β immunoreactivity of tumor tissue in invasive breast cancer with decreased survival and increased mortality suggest an immunomodulatory role for TGF- β in breast cancer progression. However, direct evidence of TGF- β -mediated immunosuppression in breast cancer patients is yet to be provided. Further clinical investigation of the role of TGF- β in the progression of human breast cancer is imperative.

5. Conclusions and future directions

It is clear from the knowledge gleaned from preclinical animal studies that TGF- β facilitates tumor progression, in part by interfering with the activities of several immune effectors. Consequently, treatment strategies that eliminate or neutralize tumor-derived TGF- β such as administration of TGF- β neutralizing antibodies [34], competing soluble TGF- β receptors [54], and antisense TGF- β gene transfer would be expected to improve patient responsiveness to immune-based therapies for established tumors or residual metastatic disease following tumor debulking. Given the other tumor promoting effects of TGF- β [reviewed in 55], these approaches will also be expected to have significant impact on tumor invasiveness and metastatic potential. One concern about such therapies that target tumor-secreted TGF- β is the potential to interfere with the normal physiologic role of TGF- β in maintaining homeostasis. Whether or not this will be a major concern awaits future clinical trials in humans.

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