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Effect of Tumor Derived TGF-β on the Efficacy of Dendritic Cell Vaccines

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As antigen presenting cells capable of inducing strong cytotoxic T lymphocyte (CTL) responses to specific antigens, dendritic cells (DCs) have become prime candidates for use in cancer immunotherapy. It has been shown that treatment of DCs with tumor cell supernatants results in reduced expression of MHC class II and reduced ability to induce a CTL response. These findings have led to the suggestion that tumors secrete soluble factors that inhibit the antigen presenting functions of DCs. In the clinical setting, immunization with DCs is well tolerated, but is unable to produce significant clinical responses. Taken together, these findings suggest that the tumors secrete immunosuppressive factors that interfere with the efficacy of DC immunotherapy. One such factor is transforming growth factor-β (TGF-β). TGF-β is a known suppressor of T cell function and recently has been implicated in decreasing the function of antigen presenting cells. Breast cancer cells secrete TGF-β and are less sensitive to TGF-β mediated growth arrest. Furthermore in breast cancer patients TGF-β immunostaining has been correlated with tumor progression. These findings suggest an important role for tumor-derived TGF-β in the progression of mammary tumors in animals and humans. The hypothesis to be tested is that tumor-derived TGF-β mitigates the efficacy of DC vaccines. The objective is to improve the efficacy of DC based vaccines by decreasing the suppressive effects TGF-β has on DCs. The specific aims are to assess 1) the effect of TGF-β on the antigen processing and presenting functions of DCs, and 2) the effect of tumor-derived TGF-β on the efficacy of DC vaccines.

14. SUBJECT TERMS
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Table of Contents

Cover ........................................................................................................................................ 1
SF 298...................................................................................................................................... 2
Table of Contents .................................................................................................................. 3
Key Accomplishments ........................................................................................................... 4
Reportable Outcomes ............................................................................................................ 5
Appendix 1 ............................................................................................................................ 6-38
Key accomplishments

1. Determination of the effect of TGF-β on the ability of dendritic cells to uptake and present antigen.

2. Evaluation of the effect of TGF-β on the ability of dendritic cells to induce tumor-sensitized T lymphocytes.


For detailed description of key accomplishments refer to Appendix 1.
Reportable Outcomes

Funding received based on research supported by this grant
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Presentations


Manuscript
Appendix 1

"TGF-β inhibits the antigen presenting functions and anti-tumor activity of dendritic cell vaccines."

Revised and Resubmitted Manuscript. Cancer Research
TGF-β inhibits the antigen presenting functions and anti-tumor activity of dendritic cell vaccines


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4 The abbreviations used are: APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; TIDC, tumor infiltrating dendritic cell; DLN, draining lymph node; IFN-γ, interferon-gamma; s.c., subcutaneous; FBS, fetal bovine serum; GM-CSF, granulocyte / macrophage colony-stimulating factor; IL-4, interleukin-4; TNF-α, tumor necrosis factor – alpha; FACS, fluorescence -activated cell sorter; PE, r-phycoerythrin; FITC, fluorescein isothiocyanate; OVA, ovalbumin; i.t., intratumorally; SLC, secondary lymphoid chemokine; RANTES, regulated on activation normally T cell expressed and secreted; MCP-1, macrophage chemoattractant protein-1.

Keywords: dendritic cell, tumor, TGF-β, 2G7, mammary, T cell
Running title: TGF-β neutralizing antibody and DCs
Abstract

Dendritic cell (DC)-based vaccines have exhibited minimal effectiveness in treating established tumors, likely due to factors present in the tumor microenvironment. One such factor is Transforming Growth Factor - β (TGF-β), a cytokine which 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 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 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 DCs 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 APCs 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 (reviewed in 6). The insensitivity of established tumors to DC therapy is likely due to factors present within the tumor microenvironment which are inimical to the optimal induction of an antitumor immune response (reviewed in 7). Examination of circulating and TIDCs 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 downregulation of MHC and co-stimulatory molecules (10, 11) and tumor-induced apoptosis of DCs (12).

One of the factors produced within the tumor microenvironment which might interfere with DC functions is TGF-β. TGF-β is a pleiotropic cytokine produced by cancer cells of different histological types (13-16). TGF-β exerts
its effects on responsive cells via its interactions with serine/threonine kinase Type I and II TGF-β receptors on the cell surface (reviewed in 17). Engagement of TGF-β receptors by ligand leads to phosphorylation of receptor-regulated Smad2 and/or Smad3 proteins. This is followed by complex formation with the common mediator Smad4, and translocation to the nucleus where activation of target gene transcription occurs (18). Amongst the plethora of immunosuppressive effects of TGF-β (reviewed in 19) is the capacity to interfere with several DC functions. These include down-regulation of cell surface MHC antigens, co-stimulatory molecules, chemokine receptors as well as impairment of in vitro chemotaxis (20-22).

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 anti-tumor T cell responses in vivo remains to be determined. In this study we examined the effects of TGF-β on the immunostimulatory and in vivo migratory activities of DCs and evaluated the impact of TGF-β on the effectiveness of DC vaccines in treating established mammary tumors. 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. These results suggest that treatment strategies that inhibit or neutralize tumor-
derived TGF-β are likely to increase the efficacy of DC-based vaccines. These findings demonstrate the potential usefulness of a combined therapeutic approach to eliminate tumor-mediated immunosuppression in order to improve the effectiveness of DC-based vaccines.
Materials and Methods

Animals. Six-week-old female BALB/c and C57BL/6 (B6) mice were purchased from The Harlan Laboratory (Indianapolis, Indiana). Six-week-old female BALB/c-TgN (DO11.10) 10 Loh mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). 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 previously described (23).

TGF-β neutralizing antibody (2G7). The 2G7 mouse IgG1 monoclonal antibody was generated following immunization of Balb/c mice with recombinant human TGF-β1. 2G7 neutralized the growth inhibitory activity of TGF-β1, TGF-β2, and TGF-β3 on Mv1Lu epithelial cells (24).

Generation of DCs and TGF-β Treatment. Bone marrow (BM) cells were harvested from flushed marrow cavities of femurs and tibiae under aseptic conditions and cultured with 100 U/ml GM-CSF and 100 U/ml IL-4 (Peprotech, Rocky Hill, NJ) at 10⁶ cells/ml in complete media (RPMI 1640 containing 10% heat inactivated FBS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.5
µg/ml fungizone, and 5x10^{-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 GM-CSF and IL-4 with or without the addition of 10 ng/ml of recombinant human TGF-β₁ (R&D Systems, Minneapolis, MN) for 6 days. DCs were matured with 200 U/ml of TNF-α (Peprotech, Rocky Hill, NJ) for 48 hours.

**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, San Diego, CA), 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 using a FACStarPLUS flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

**Induction of Allogeneic Mixed Lymphocyte Reactions (MLR).** Spleen cells from B6 mice were harvested and enriched for CD3 positive cells using a T cell enrichment column (R&D Systems, Minneapolis, MN). Cells were 80-95% CD3 positive as determined by FACS analysis. Varying numbers of DCs were incubated with 2x10^6 T lymphocytes for 5 days in 96 well tissue culture plates (Sarstedt, Newton, NC) with the addition of 1 µCi of [³H]-thymidine (Perkin Elmer Life Sciences, Boston, MA) for the final 18 hours 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 2x10⁵ T lymphocytes in the presence of 1 μM of OVA peptide (ISQAVHAAHAEINEAGR, United Biochemical Research, Seattle, WA) in 96 well tissue culture plates for 5 days with the addition of 1 μCi of [³H]-thymidine for the final 18 hours of culture.

Endocytosis and Phagocytosis Assays. Endocytosis and phagocytosis assays were performed using modifications of previously described procedures (25). DCs were resuspended in 12x75 mm polystyrene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) in Phosphate Buffered Saline (PBS) (135 mM NaCl, 2 mM KCl, 2 mM KH₂PO₄, 8 mM Na₂HPO₄) containing 1% heat-inactivated FBS and 25 mM HEPES. Endocytosis was measured by incubating 2x10⁵ DC with 400 μg of FITC-conjugated dextran beads, 40,000 MW (Molecular Probes, Eugene, OR) for 30 minutes at 4°C or 37°C. Phagocytosis was measured by incubating DCs with FITC-conjugated E. coli (Molecular Probes, Eugene, OR) at a ratio of 100 E. coli particles to one DC for 60 minutes at 4°C or 37°C. Following 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 per DC for 24 hours in the presence or absence of 10 ng/ml TGF-β₁. DCs were then matured with 20 ng/ml TNF-α in the presence or absence of 10 ng/ml of TGF-β₁. Splenic T lymphocytes were purified from mice bearing 14 day 4T1 tumors as indicated above. Ten thousand DCs were incubated with 2x10^5 splenic T lymphocytes in 96-well tissue culture plates for 5 days with the addition of 1 µCi of [³H]-thymidine for the final 18 hours of culture. One million tumor draining lymph node cells from mice bearing 14 day 4T1 tumors were incubated with 2.5x10^5 DCs for 48 hours and IFN-γ production was evaluated by ELISA (R&D Systems, Minneapolis, MN).

In Vivo Migration Assay. Bone marrow-derived DCs were incubated in the presence or absence of 200 U/ml TNF-α with or without 10 ng/ml of TGF-β₁ for 48 hours. DCs were labeled with 10 µM PKH-67L, green fluorescent dye (Sigma, St. Louis, MO) as previously described (22). Naive mice were injected s.c. in the right flank with 5-8x10^6 DCs. Forty-eight hours 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 minutes. 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 while DC were identified by their green fluorescence
signal; 35,000 propidium iodide events were analyzed from each treatment
group per experiment. The identity of each DC detected during scanning was
confirmed visually by direct microscopic observation using the instrument's re-
location function.

Chemokine Receptor Expression. Bone marrow-derived DCs were
incubated in the presence or absence of 200 U/ml TNF-α with or without 10
ng/ml of TGF-β1 for 48 hours. mRNA was isolated from 10⁶ DCs using an
mRNA isolation kit (Qiagen, Chatsworth, CA). The absence of DNA
contamination was confirmed by PCR with mRNA prior to the reverse
transcription reaction. Complementary DNA was generated using random
hexamer primers (Promega, Madison, WI) and M-MLV reverse transcriptase
(Promega). An aliquot (3μl) of the resulting cDNA was subjected to semi-
quantitative PCR (94°C, 15 s, 59°C, 30 s, 72°C, 45 s) for 35 cycles using
chemokine receptor-specific primers on a MJ Research Thermocycler (MJ
Research, Waltham, MA). PCR products were analyzed on a 2% agarose gel
with ethidium bromide (Sigma). The intensity of the chemokine receptor
product was normalized to the respective intensity of the GAPDH product using
the Fluorchem 7700 advanced imaging system (Alpha Innotech Corp., San
Leandro, CA). The primers used included GADPH (212 bp) sense 5’
CAGGTTGTCTCCTGCGACTT 3’; antisense 5’ CTTGCTCAGTGTCTTGGCTG
3’; CCR1 (437 bp) sense 5’ AAGCCTACCCCAACTACAGA 3’; anti-sense
5' GGTGATGATGCCAAGAGTAAC 3'; CCR2 (380 bp) sense 5'
GTTCTCTTCTTGACCACCTTCC3'; anti-sense 5'
ACCACTGTCTTTTGACCACCTTCC 3'; CCR4 (527 bp) sense 5'
ATTCTGGTTGTTCTGGTCCT 3'; anti-sense 5'
GCAGTGTTGCAAGCTCTAATG 3'; CCR5 (582 bp) sense 5'
ACAGGGCTCTATCACATGGTT 3'; antisense 5'
GGCATAGATGACAGGGTTTACG 3'; CCR6 (307 bp) sense 5'
ACTTTAACTGTGGGAATAGCTGCT 3'; anti-sense 5'
ATAAACAGCAAAGGGGTGAAGA 3'; and CCR7 (453 bp) sense 5'
GACGGATACCTACCTGCTCAAC 3'; anti-sense 5'
TAGCAGAAAACATCATAGCCAGCA 3'.

**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 per dendritic cell for 24 hours. Following pulsing, DCs were matured with 200 U/ml of TNF-α for 48 hours. Mice were injected i.t. with $1.5 \times 10^6$ tumor cell lysate-pulsed, matured DCs in 50μl PBS on day 15 when tumors were palpable. Vaccination was repeated again on day 20 and day 25. Two hours prior to each vaccination mice received i.p. injections of 300μg of 2G7. In combination with DCs or alone, mice received 100μg of 2G7 antibody i.t.. Primary tumors were measured in 2 dimensions and tumor volume was calculated using the formula $v = (l \times w^2) / 2$, where $v =$ volume (mm$^3$), $l =$ long
diameter, and \( w \) = short diameter. Mice exhibiting complete tumor regression were challenged with ten-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). Probability values (\( P \)) of \(<0.05\) were considered to indicate significant differences between data sets.
Results

Antigen uptake and presentation by DC. 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 resulted in decreased expression of B7.1 (MFI of 136 for untreated DCs vs. 73 for TGF-β-treated DCs) and CD40 (MFI of 80 for untreated DCs vs. 50 TGF-β-treated DCs), surface molecules critical for antigen presentation and T lymphocyte stimulation (Figure 1A). This pattern was observed in each of 3 experiments conducted. Next, we evaluated the ability of DCs to endocytose dextran particles and phagocytose E. coli particles following TGF-β treatment. TGF-β exposure inhibited the endocytic and phagocytic capacities of DCs by 58% and 87% respectively (Figure 1B) a pattern that was observed in all 3 experiments conducted. To evaluate the effect of TGF-β exposure on antigen presentation, mixed lymphocyte reactions were performed. Stimulation of allogeneic T lymphocytes (Figure 1C) and presentation of OVA peptide (Figure 1D) by DCs was significantly suppressed following TGF-β exposure (p<0.05). 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 (Figure 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, following TGF-β exposure. Together these data demonstrate that TGF-β exposure reduces the ability of DCs to stimulate anti-tumor immune responses.

In Vivo Migration and Chemokine Receptor Expression. Since migration of DCs to secondary lymphoid organs is necessary for T cell priming (26), we assessed the effect of TGF-β treatment on the ability of mature DC to migrate to draining lymph nodes. For this purpose TGF-β-treated DCs were labeled with PKH-67 and injected s.c. into the hind flank of mice. Forty-eight hours 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 draining lymph nodes (p=0.06) (Figure 3A). To determine the mechanism responsible for decreased migration of mature DCs, chemokine receptor expression by DCs was analyzed by RT-PCR. TGF-β caused a 20% decrease in CCR7 expression (Figure 3B). In contrast, expression levels of CCR1, CCR4 and CCR5 were modestly increased by TGF-β treatment, while CCR6 expression was not affected (Figure 3B).
Treatment of Established Tumors with DC 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 previously described (23). 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 DCs plus 2G7 antibody inhibited tumor growth (Figure 4A) compared with 4T1-asT tumors treated with DCs or 2G7 antibody alone (Figure 4A). Furthermore, complete tumor regression occurred in 40% (2/5) of 4T1-asT-tumor bearing mice that were treated with DCs plus 2G7 antibody (Figure 4B). Similarly, mock transduced (4T1-N) tumors responded better to treatment with DCs plus 2G7 antibody; however tumor growth inhibition in this group was inferior to that observed in animals bearing 4T1-asT tumors. (Figure 4). Tumor growth was not affected by treatment with isotype control antibody (data not shown). To determine if 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 (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.
Discussion

In this study we evaluated the impact of TGF-β on in vivo migration and immunostimulatory activities of dendritic cells. 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 antibody 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, 23, 27) or abrogation of TGF-β using neutralizing antibody (28, 29) 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 antibody 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 draining lymph nodes to activate naïve tumor-specific T lymphocytes. The former possibility is supported by the recent findings by Kirk et al. (30) who suggested that migration of intratumorally-injected DCs to draining lymph nodes is not required for the induction of an antitumor response. Using secondary lymphoid chemokine
(SLC)- gene-modified DCs they demonstrated comparable tumor growth inhibition in normal mice and lymphotoxin α/− mice lacking peripheral lymph nodes (30). The latter possibility is supported by the reduced gene expression of CCR7 in TGF-β-treated DCs, a finding which has also been reported by others (21, 22). Of interest in our study is the increased expression by DCs of CCR1, CCR4, and CCR5 chemokine receptors by TGF-β treatment. This finding correlates with the ability of 4T1 cells to produce RANTES and MCP-1 (31) which bind these receptors (reviewed in 32). Increased expression of these chemokine receptors on DCs would be expected to favor their retention within TGF-β-secreting 4T1 tumors.

As with previously published studies (30, 33, 34) only a minute fraction (<1%) of s.c. injected DCs in our study migrated to draining lymph nodes. 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 naïve 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 in order 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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dendritic cell migration in vivo upon subcutaneous and intravenous
Figure 1

A

CD11c  I-A^d

B7.1  B7.2  CD40

CELL NUMBER

FLUORESCENCE

B

Endocytosis  Phagocytosis

\[ \Delta \text{MFI} \]

TGF-\(\beta\)  -  +  -  +
Figure 1. Effect of TGF-β exposure on DC functions in vitro. Dendritic cells were incubated in the absence or presence of 10 ng/ml of TGF-β for 6 days. Cytokines were replenished every two days. (A) 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 gray line represents unstained cells, the thin black line represents untreated cells, and the thick black line represents TGF-β treated cells. (B) 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. (C) 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 hours of culture. Values represent mean ± SEM of six replicates. (D) 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 hours of culture. Values represent mean ± SEM of four replicates. Results are from one representative experiment of three independent experiments. * refers to statistical significance between groups (p<0.05).
Figure 2

A

Immature

Mature

TGF-β

-cpm-

-cpm-

B

Immature

Mature

TGF-β

-cpm-

-cpm-

IFN-γ pg/ml

IFN-γ pg/ml

-cpm-

-cpm-

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

A

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<td>MDC + TGF-(\beta)</td>
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B

![Graph showing sample/actin relative ratio for CCR1, CCR4, CCR5, CCR6, CCR7 for mDC and mDC+TGF-\(\beta\).]
Figure 3. Effect of TGF-β on in vivo migration and chemokine receptor expression. DCs were cultured in the absence or presence of 200 U/ml TNF-α with or without the addition of 10 ng/ml of TGF-β for 48 hours. (A) 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 results from three independent experiments. (B) mRNA was isolated from DCs and chemokine receptor expression was analyzed by RT-PCR. Data are expressed as ratios compared with GADPH as determined by densitometry.
**Figure 4**

A

4T1-N

4T1-asT

B

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<td>&gt;100 mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&lt;100 mm&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>2G7 Ab alone</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>DC alone</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>DC + 2G7 Ab</td>
<td>2</td>
<td>3</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tumor volume of individual mice

<sup>b</sup> 2 of 4 mice exhibited complete tumor regression
Figure 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 1.5x10⁶ tumor cell lysate-pulsed mature DCs alone or in combination with 100 μg i.t and 300 μg i.p of 2G7, TGF-β neutralizing antibody. Control mice were treated with 2G7 antibody alone. Mice were monitored for tumor growth. A. Graphs represent tumor growth of individual mice. B. Tumor volumes of individual mice were compared on day 35 following tumor challenge.