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Tumor-Derived TGFβ-1 Induces Dendritic Cell Apoptosis in the Sentinel Lymph Node

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Lymphatic flux from a primary tumor initially flows into a tumor-draining lymph node (LN), the so-called sentinel LN (SLN). Carried by the lymph fluid are a variety of mediators produced by the tumor that can influence immune responses within the SLN, making it a good model with which to investigate tumor-related immunology. For instance, dendritic cell (DC) numbers are reduced in SLNs from melanoma and breast cancer patients. In the present study, we investigated the mechanism by which DC numbers were reduced within SLNs from patients with non-small cell lung cancer. We found that the incidence of apoptosis among DCs was higher in SLNs than in non-SLNs, as were levels of TGFβ-1. In contrast, levels of TGFβ-1 mRNA did not differ between SLNs and non-SLNs, but were 30 times higher in tumors than in either LN type. In vitro, incubation for 2 days with TGFβ-1 induced apoptosis among both cultured DCs and DCs acutely isolated from normal thoracic LNs, effects that were blocked by the TGFβ-1 inhibitor DAN/Fc chimera. Taken together, these results suggest that tumor-derived TGFβ-1 induces immunosuppression within SLNs before the movement of tumor cells into the SLNs, thereby facilitating metastasis within those nodes. ***The Journal of Immunology,*** **2006, 176: 5637–5643.**

Evidence suggests that, for some types of cancer, lymph fluid and tumor cells from the primary tumor initially flow into a tumor-draining lymph node (LN),[2] the so-called sentinel LN (SLN), before flowing into more distal LNs (1–8). If correct, then when metastasis is not found in an SLN, it most likely will not be present in more distal nodes; and, in fact, the adverse effects of lymphadenectomy have been avoided in cases of breast cancer (1, 2) and melanoma (3, 4) based on that idea. There is also evidence of the existence of SLNs in non-small cell lung cancer (NSCLC) (5–7) as well as in colon and gastric cancers (8). Initially, the SLN concept was considered only in the context of surgical treatment. However, more recently, it also has been considered in the context of the pathophysiology of cancer, in particular, tumor immunity. For example, it has been reported that, in both breast cancer and melanoma, numbers of dendritic cells (DCs), an APC population thought to play key roles in the initiation of Ag-specific T cell proliferation—are lower in SLNs than in other LNs (9, 10). This finding is not entirely surprising, because it is well known that tumor cells release a variety of cytokines and other biologically active materials (11–15). These various mediators must flow into the SLN, making it easy to imagine that they could influence the response to tumor cells within the SLN, for instance, via a reduction in DC number.

The mechanisms by which tumor immunity is affected within the SLN are not fully understood. However, it is well known that tumor cells produce various immunosuppressive factors, including TGFβ (11–13), PGE_2 (14), vascular endothelial growth factor (14, 15), and IL-10 (14). Among these, overexpression of TGFβ is closely associated with a poor prognosis in patients with malignant tumors (16–18). TGFβ suppresses $CD4^+$ T cell production stimulated by IL-2, IFN-γ, or TNF-α (19) and induces apoptosis among peripheral blood T cells (20). In contrast, the effect of TGFβ on DCs is interestingly equivocal. On one hand, TGFβ-1 is required for the generation of Langerhans cell-type DCs from $CD34^+$ hemopoietic progenitor cells (21, 22), which express CD1a but not CD83 and are arrested at an immature differentiation stage (23), and TGFβ-1 mRNA is expressed in both Langerhans cell-type DCs and germinal center-type DCs (24). On the other hand, TGFβ suppresses the differentiation of mouse bone marrow-derived DCs as well as their capacity to secrete the Th1-polarizing cytokine IL-12 (25), to present Ag, to stimulate tumor-sensitized T lymphocytes, and to migrate into tumor-draining LNs (26), all of which suggests that TGFβ suppresses the immune response of non-Langerhans cell-type, monocyte-derived DCs. Therefore, our principle aim in the present study was to investigate the cause of the reduction in DC numbers within SLNs from NSCLC patients; in particular, we were interested in the role played by TGFβ.

Materials and Methods

SLN identification

This study was approved by the institutional review boards at Akita University School of Medicine and University Hospital. NSCLC patients were enrolled in the study after obtaining signed informed consent. LNs showing no evidence of metastasis were obtained from each patient, after which SLNs were identified using the method developed at our institute (5). Briefly, a magnetic tracer (ferumoxides) was injected around the lung tumor during surgery, after which the magnetic force within the LNs was measured using a highly sensitive handheld magnetometer, and LNs in which magnetic force was detected were defined as SLNs. Non-SLNs served as a control. The definition of non-SLN in this study was a magnetic force-negative LN from a matched station level based on the classification by Naruke et al. (27). We also obtained normal thoracic LNs from patients

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[2] Abbreviations used in this paper: LN, lymph node; SLN, sentinel LN; DC, dendritic cell; AEC, 3-amino-9-ethylcarbatole; NSCLC, non-small cell lung cancer; DAB, 3–3′-diaminobenzidine tetrahydrochloride.

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with pneumothorax after obtaining informed consent. The normal LNs were used to study the effect of TGFβ-1 on apoptosis among DCs and T lymphocytes.

Materials

Abs specific for S100β (rabbit polyclonal Ab; FabGennix), CD1a (mouse mAb clone O10; NeoMArkers), CD83 (mouse mAb clone HB15e; BD Biosciences), Lineage Cocktail 1 (CD3 clone SK7, CD14 clone MφP9, CD16 clone 3G8, CD19 clone SJ25C1, CD20 clone L27, CD56 clone NCAM16.2; FITC-conjugated mouse mAb clone SK7;, BD Immunocytometry Systems), CD11c (PE-conjugated mouse mAb clone As-HCL-3; BD Immunocytometry Systems), CD123 (PE-conjugated mouse mAb clone 9F5; BD Immunocytometry Systems), CD80 (PE-Cy5-conjugated mouse mAb clone B7.5; BD Biosciences), CD3 (FITC-conjugated mouse mAb clone SK7; BD Immunocytometry Systems), CD4 (PE-conjugated mouse mAb clone SK3; BD Biosciences), CD8 (PE-conjugated mouse mAb clone SK1; BD Immunocytometry Systems), HLA-DR (R-PE-conjugated and PE-Cy5-conjugated mouse mAb clone G46-6; BD Biosciences), CD40 (CyChrome-conjugated mouse mAb clone 5C3; BD Biosciences), TGFβ-1 (mouse mAb clone TB21; Chemicon International) were used. Recombinant human TGFβ1 and the TGFβ inhibitor DAN/Fc chimera (28), were purchased from Sigma-Aldrich.

Human DC cultures

Human DCs were cultured using the method of Shurin (29) with some modification. Briefly, peripheral blood was obtained from healthy volunteers, after which PBMCs were separated by Histopaque (1.007 g/ml; Sigma-Aldrich) density centrifugation (400 × *g* for 30 min at room temperature) and washed in PBS. Residual erythrocytes were removed with hypotonic treatment using 0.2% NaCl. The isolated PBMCs were washed twice in PBS and plated at 10^7 cells per well in six-well plates and incubated for 2 h in 2 ml of AIM V medium (Invitrogen Life Technologies) at 37°C under a humidified 5% CO_2/95% air atmosphere. Nonadherent cells were then removed, and adherent monocytes were gently washed with warm AIM V medium and then incubated overnight in RPMI 1640. $CD14^+$ monocytes were then selected using Dynabeads M450 CD14 (Dynal Biotech) according to the manufacturer's instructions and cultured for 7 days with recombinant human GM-CSF (1000 U/ml; R&D Systems) and IL-4 (1000 U/ml; R&D Systems) in RPMI 1640 medium supplemented with 10% autologous human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM HEPES (Invitrogen Life Technologies).

Immunohistochemical staining and TUNEL

SLNs and non-SLNs were fixed in periodate-lysine-paraformaldehyde solution (2% paraformaldehyde, 75 mM disodium hydrogen orthophosphate, 13.5 g/L lysine, and 2.14 g/L sodium periodate) for 2 h at 4°C and embedded in soft paraffin. After preparing 3-μM sections, the TUNEL method was used to identify apoptotic cells using an ApopTag Peroxidase In Situ Apoptosis Detection kit (S7100; Chemicon International) and developed using a 3-3′-diaminobenzidine tetrahydrochloride (DAB) or ApopTag Plus Fluorescein In Situ Apoptosis Detection kit (S7111; Chemicon International) according to the manufacturer's instructions.

In addition, DC markers (S100β, CD1a; mature DCs, CD83) were stained so that the number of DCs in SLNs and Non-SLNs could be counted. In those experiments, the TUNEL was omitted. Instead, sections in Target Retrieval Solution (pH 9.0; DakoCytomation) were either irradiated with microwaves for 5 min at 500 W (for CD1a and CD83 staining) and then cooled for 20 min, or they were left untreated (for S100β staining) in the same buffer. The sections were then double-labeled with anti-S100β (1/50 dilution), anti-CD1a (1/20 dilution), or anti-CD83 (1/20 dilution) primary Abs for 1 h at room temperature and rinsed five times with TBS. To stain for S100β, the cells were then incubated with alkaline phosphatase-labeled polymer anti-mouse/rabbit IgG (DAKO EnVision system; DakoCytomation) according to the manufacturer's instructions, after which the sections were developed with fuchsin. To stain for CD1a and CD83, the cells were incubated with a Catalyzed Signal Amplification System (DakoCytomation) according to the manufacturer's instructions, after which the sections were developed with 3-amino-9-ethylcarbatole (AEC). Because of the heterogeneous distribution of DCs within LNs, the numbers of $S100\beta^+$, $CD1a^+$, and $CD83^+$ cells were counted in five randomly selected high-power fields in the paracortical areas of the LNs under ×400 magnification. This approach has been used by other investigators when analyzing the expression of mature DCs (30). Each slide was examined on at least two separate occasions by at least two individuals.

Cells were also dissociated from normal lymph nodes and incubated for 2 days in the presence of TGFβ-1 at concentrations of 20 or 40 ng/ml. Cell monolayers were then prepared on slides using a Cytospin (Thermo Electron), after which the monolayers were fixed with acetone for 5 min at −20°C and incubated first with anti-S100β Ab (1/50 dilution) for 1 h at 37°C, then with alkaline phosphatase-labeled polymer anti-mouse/rabbit IgG (DAKO EnVision system; DakoCytomation) according to the manufacturer's instructions, and developed with fuchsin. To detect apoptosis, TUNEL was conducted using an ApopTag Peroxidase In Situ Apoptosis Detection kit (S7100; Chemicon International) and developed with DAB

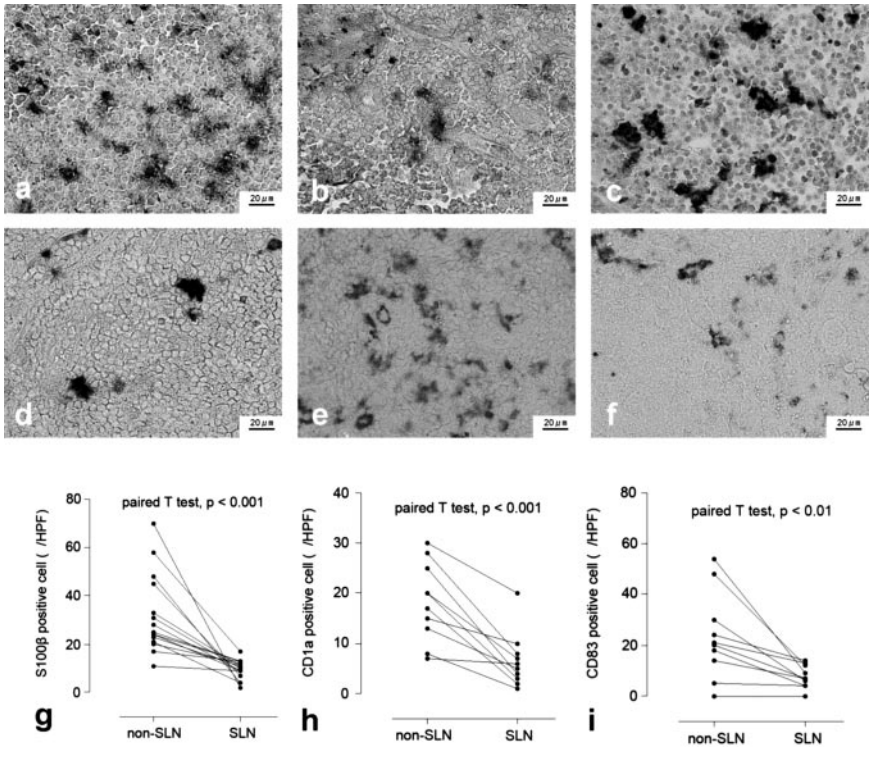

FIGURE 1. Histological analysis showing a reduction in DC levels within SLNs. SLNs and non-SLNs were immunohistochemically stained with anti-S100β Ab (*a* and *b*, respectively), anti-CD1a Ab (*c* and *d*, respectively), or anti-CD83 Ab (*e* and *f*, respectively), after which the numbers of stained cells were counted. *g–I*, Numbers of DCs labeled with the indicated Abs in SLNs and non-SLNs. Numbers of DCs were significantly higher in non-SLNs than SLNs.

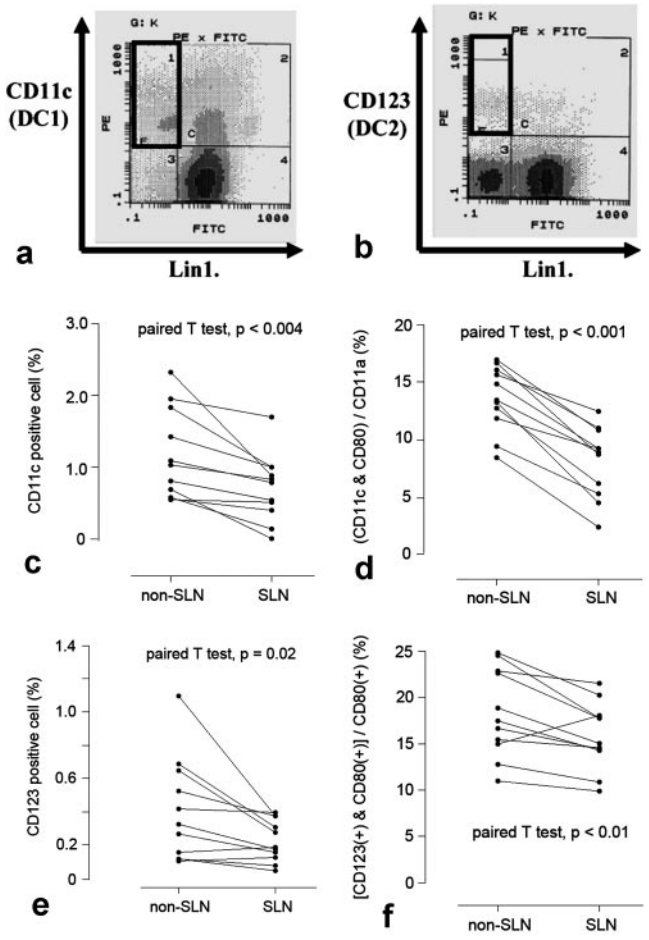

FIGURE 2. Flow cytometric analysis showing a reduction in DC levels within the SLNs. DCs were negatively selected using Lineage Cocktail 1, (FITC-conjugated Ab mixture against CD3, CD14, CD16, CD19, CD20, and CD56), after which DC1s and DC2s were resolved using PE-conjugated anti-CD11c (*a*) and anti-CD123 (*b*), respectively. The number (*c*) and activity (*d*) of DC1s were measured by staining CD11a and CD80, respectively. The number (*e*) and activity (*f*) of DC2 were measured by staining CD123 and CD80, respectively. The numbers and activities of DC1s and DC2s were higher in non-SLNs than in SLNs ($p < 0.005$).

according to the manufacturer's instructions. The slides were counterstained with hematoxylin. Numbers of apoptotic cells and DCs were then counted in five randomly selected high-power fields (×400).

Flow cytometry

After preparing single-cell suspensions from lymph nodes, flow cytometric analysis was conducted to analyze cell number, activity, and incidence of apoptosis among DCs, $CD4^+$ T lymphocytes, and $CD8^+$ lymphocytes. DCs were negatively selected using Lineage Cocktail 1 (FITC-conjugated Ab mixture against CD3, CD14, CD16, CD19, CD20, and CD56). Thereafter, myeloid and lymphoid DCs (DC1s and DC2s, respectively) were detected using PE-conjugated Ab against CD11c and CD123, respectively. After detecting the cells, their activities were evaluated based on expression of surface Ag CD80 using PE-Cy5-conjugated anti-CD80 Ab. T cells were selected using FITC-conjugated anti-CD3 Ab, after which $CD4^+$ and $CD8^+$ T cells were detected using PE-conjugated anti-CD4 and anti-CD8 Abs. The activities of $CD4^+$ and $CD8^+$ T cells were evaluated based on their expression HLA-DR detected using PE-Cy5-conjugated anti-HLA-DR Ab. Apoptosis among $CD4^+$ and $CD8^+$ T cells in single-cell suspensions made from normal LNs was analyzed using double-color flow cytometry. After treatment with 20 or 40 ng/ml TFGβ1 for 2 days, the cells were stained with PE-conjugated anti-CD4 or anti-CD8 Ab, after which apoptotic cells were stained using FITC-conjugated TUNEL reaction mixture provided with an In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Apoptosis among cultured DCs treated with 20 or 40 ng/ml TGFβ-1 for 2 days was analyzed using triple-color flow cytometry. Briefly, the $CD14^+$ cells cultured as described above were stained with CyChrome-conjugated anti-CD40 Ab and R-PE-conjugated anti-HLA-DR Ab. Apoptotic cells were then stained using FITC-conjugated TUNEL reaction mixture provided in an In Situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer's instructions. Cells that were $CD40^+$, $HLA\text{-}DR^+$, and $TUNEL^+$ were deemed to be apoptotic DCs.

Quantification of TGFβ by ELISA

Levels of TGFβ-1, -2, and -3 in tumor specimens and SLNs were measured using sandwich ELISAs according to the manufacturer's protocol (Quantikine human TGFβ-1, Quantikine human TGFβ-2, DuoSet human TGFβ-3; R&D Systems). The values were expressed as nanograms per milligram of protein.

Real-time semiquantitative RT-PCR analysis of TGFβ-1

Fresh tumor and LN samples were immediately stored in liquid nitrogen until use. For real-time semiquantitative RT-PCR, total RNA was isolated from the samples using a Fast RNA kit Green (Qbiogene) according to the manufacturer's instructions. After quantifying the isolated RNA using a spectrophotometer, 1-μg aliquots were reverse transcribed by incubation with Superscript II reverse transcriptase (Invitrogen Life Technologies) and 0.5 μg of oligo$(dT)_{12-18}$ for 50 min at 42°C, and then for 15 min at 70°C. The primer sequences used to amplify TGFβ-1 mRNA (GenBank accession no. BC000125) were 5′-GCGTCTGCTGAGGCTCAAGT-3′ (forward) and 5′-CTCAACCACTGCCGCACAA-3′ (reverse). PCR was conducted in a LightCycler using a Light Cycler-FastStart DNA Master SYBR Green 1 Kit (Roche Diagnostics). Thermocycling was done in a final volume of 20 μl containing 1 μl of cDNA sample (or standard), 3 mM $MgCl_2$, 0.5 μM each primer, and 2 μl of LightCycler-FastStart DNA Master SYBR Green 1. After 10 min of initial denaturation at 95°C, the cycling protocol entailed 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 7 s. As an internal control, we also amplified the mRNA for GAPDH using primers 5′-AACGTGT CAGTGGTGGACCTG-3′ (forward) and 5′-AGTGGGTGTCGCTGTT GAAGT-3′ (reverse). The reaction mixture was the same as used to amplify TGFβ-1 mRNA, except that 4 mM $MgCl_2$ was used; the thermocycling protocol was the same, except annealing was conducted at 62°C. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. After proportional background adjustment, the fit point method was used to determine the cycle in which the log-linear signal was distinguishable from the background, and that cycle number was used as the crossing-point value. The software then produced a standard curve by measuring the crossing point for each standard sample and plotting them against the logarithmic values of the concentrations. Levels of TGFβ-1 mRNA were then normalized to those of GAPDH.

DNA fragmentation

DCs cultured with and without TGFβ-1 were lysed with 20 μl of lysis buffer provided in a Quick Apoptotic DNA Ladder Detection kit (Biovision). The DNA was then isolated according to the manufacturer's instructions, after which samples were electrophoretically separated on a 1% agarose gel containing 0.1 μg/ml ethidium bromide. DNA was visualized using a UV transilluminator, and the gels were photographed.

Statistics

Differences between the cell counts obtained with SLNs and non-SLNs were assessed using Student's paired *t* test. Group data were expressed as means ± SD and were analyzed using two-way ANOVA in combination with the least square method using the statistical software JMP IN 5.1.1 (SAS Institute). The significance of individual differences was evaluated using contrast tests. Values of $p < 0.05$ were considered significant.

Results

Apoptosis among DCs within SLNs from NSCLC patients

SLNs and non-SLNs obtained from patients with primary adenocarcinomas of the lung (NSCLC) showing no evidence of nodal metastasis were used in this study. After identifying SLNs and non-SLNs as described in *Materials and Methods*, we estimated the number of DCs ($S100\beta^+$, $CD1a^+$, and $CD83^+$ cells and mature DCs) within the nodes by evaluating immunohistochemically stained sections (Fig. 1). We found there to be significantly fewer

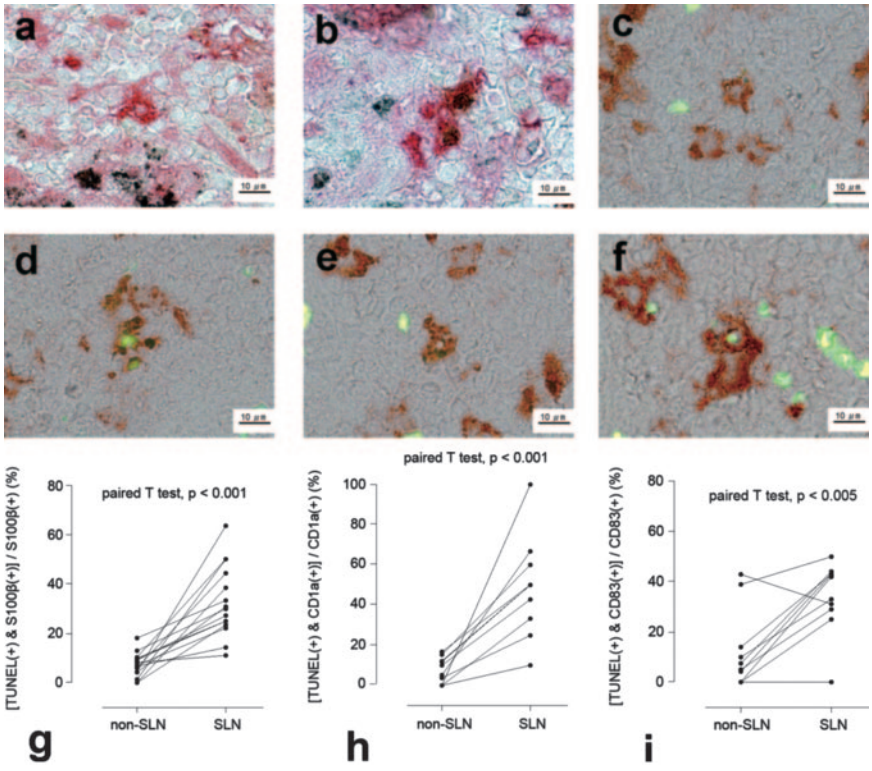

FIGURE 3. Incidence of apoptosis among DCs is increased within SLNs. TUNEL analysis of apoptosis among DCs (S100β^+, CD1a$^+$, and CD83$^+$ cells) within SLNs. *a* and *b*, S100β^+ cells are stained red (fuchsin); TUNEL-positive cells are stained dark brown (DAB). *c* and *d*, CD1a$^+$ cells were stained red (AEC); TUNEL-positive cells are stained green (FITC). *e* and *f*, CD83$^+$ cells are stained red (AEC); TUNEL-positive cells were stained green (FITC). *g*, Percentage of apoptotic (TUNEL$^+$) cells in SLN and non-SLNs; the percentage was significantly higher in SLNs than non-SLNs ($p < 0.05$).

DCs present within SLNs than non-SLNs; moreover, triple-color flow cytometry showed that both the number and activity (CD80$^+$) of both DC1s (CD11$^+$) and DC2s (CD123$^+$) were reduced in SLNs, as compared with non-SLNs (Fig. 2).

Hypothesizing that the reduced numbers of DCs within SLNs might reflect an increased incidence of apoptosis, we compared SLNs and non-SLNs by double-staining the cells using a specific DC marker (S100β, CD1a, or CD83) and TUNEL (Fig. 3, *a–f*). Consistent with our hypothesis, we found the incidence of apoptosis among DCs to be significantly higher in SLNs than in non-SLNs (Fig. 3, *g–i*), suggesting that apoptosis is a major factor underlying the reduction in DCs within SLNs.

Because TGFβ suppresses stimulated CD4$^+$ T cell production and induces apoptosis among PBMCs (19, 20), we next examined the extent to which TGFβ contributes to the induction of apoptosis among DCs within SLNs. Using ELISAs, we found that levels of TGFβ-1 were higher in SLNs than non-SLNs, whereas no significant difference was detected in the levels of TGFβ-2 or -3 in the two groups (Fig. 4, *a–c*). Moreover, immunohistochemical analysis of SLNs and primary NSCLS tumor specimens showed substantial amounts of TGFβ-1 to be present within the tumors, but little or no TGFβ-1 was detected in the SLNs, suggesting the tumor is the source of the cytokine affecting DCs within SLNs (Fig. 4, *d* and *e*). We subsequently confirmed that idea using semiquantitative RT-PCR, which showed expression of TGFβ-1 mRNA to be >30 times higher in the primary tumor than in SLNs or non-SLNs (Fig. 4*f*). Given that the SLN is the first LN into which the tumor drains it appears to us that tumor-derived TGFβ-1 flows into

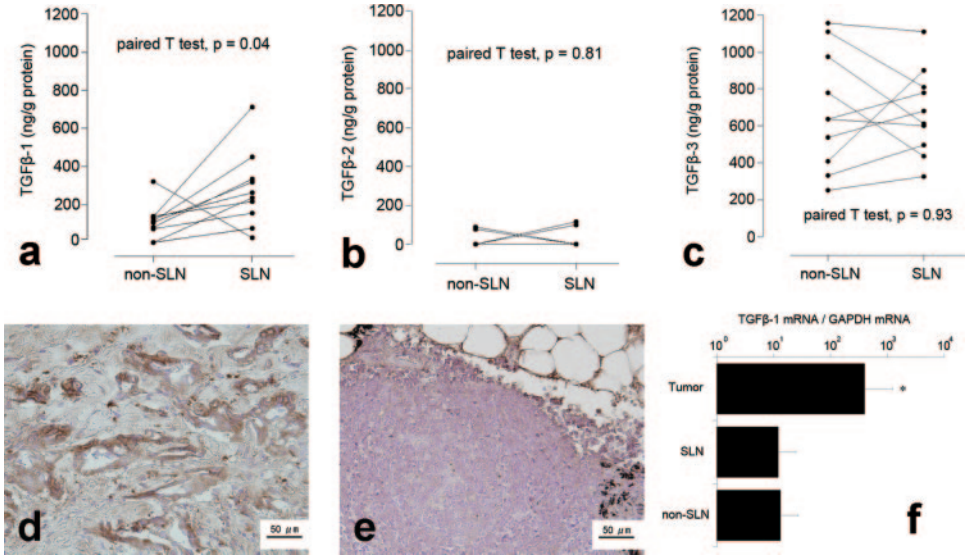

FIGURE 4. Levels of tumor-derived TGFβ-1 are increased within SLNs. TGFβ-1 (*a*), TGFβ-2 (*b*), and TGFβ-3 (*c*) content of SLNs and non-SLNs was measured using specific ELISAs. TGFβ-1 was higher in SLNs than non-SLNs ($p < 0.05$). Distribution of TGFβ-1 in a representative tumor specimen (*d*) and SLN (*e*) was analyzed with immunohistochemical staining. Positive staining for anti-TGFβ-1 Ab is shown in brown. *f*, Semiquantitative RT-PCR analyses of TGFβ-1 mRNA in tumor specimens, SLNs, and non-SLNs were performed. *, $p < 0.05$ vs SLNs and non-SLNs (ANOVA in combination with the Tukey-Kramer honest significant difference test; $n = 5$ in each group).

the SLN, where it induces apoptosis among the DCs, but it does not reach more distant LNs at levels sufficient to exert a significant effect.

In vitro effects of TGFβ-1 on cultured and acutely dissociated DCs

To further confirm that TGFβ-1 mediates the increase in apoptosis seen among DCs within SLNs, we next examined the in vitro effects of TGFβ-1 on the cultured DCs from normal LNs. The cells were incubated with TGFβ-1 (20 or 40 ng/ml) for 2 days, after which electrophoresis of the extracted DNA yielded the ladder pattern characteristic of apoptotic cells (Fig. 5*b*), and flow cytometry showed increased incidence of apoptosis (Fig. 5*c*). In addition, DAN/Fc chimera, a specific inhibitor of TGFβ-1, almost completely blocked the evoked apoptosis among cultured DCs. In similar fashion, when suspensions of cells acutely isolated from normal LNs were incubated with TGFβ-1 for 2 days and then double-stained with anti-S100β mAb as a marker of DCs and TUNEL to evaluate the apoptosis, TGFβ-1 was found to induce significant increases in the incidence of apoptosis and, again, the effect was largely blocked by DAN/Fc chimera (Fig. 6, *a* and *b*). In neither preparation did the tracer (magnetite) that we used for the detection of SLNs have any effect on DC apoptosis.

Effects of TGFβ-1 on the number and activity of T lymphocytes within SLNs

Because TGFβ is also known to suppress $CD4^+$ T cell production (19), we also evaluated the number and activity (reflected by HLA-DR expression) of $CD4^+$ and $CD8^+$ T cells in SLNs (Fig. 7). We found that the numbers of $CD4^+$ T cells were significantly lower in SLNs than in non-SLNs, although there was no significant difference in the numbers of $CD8^+$ T cells. In addition, HLA-DR expression by both $CD4^+$ and $CD8^+$ T cells was lower in SLNs than non-SLNs. These findings were confirmed using double-color flow cytometry to assess the effect of TGFβ-1 on apoptosis among $CD4^+$ and $CD8^+$ T cells in suspensions of cells acutely isolated

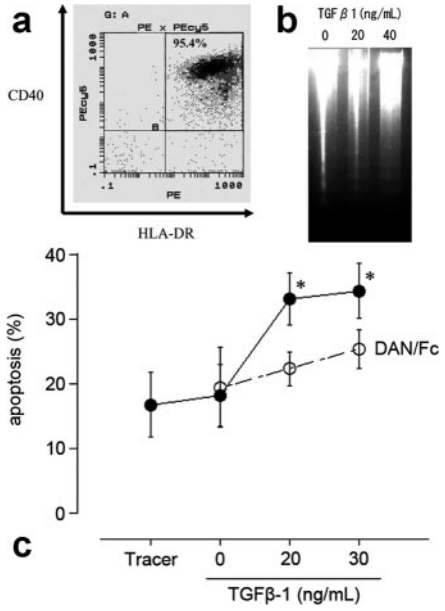

FIGURE 5. TGFβ-1 induces apoptosis among cultured DCs. Apoptosis among cultured DCs incubated for 2 days with TGFβ-1 (20 or 40 ng/ml) was analyzed using flow cytometry. *a*, Cells that were $CD40^+$, $HLA\text{-}DR^+$, and $TUNEL^+$ were deemed to be apoptotic DCs. *b*, The representative electrophoresis of DNA extracted from cultured DCs incubated for 2 days with the indicated concentration of TGFβ-1 is shown; note the ladder pattern of the fragmented DNA characteristic of apoptosis. *c*, Percentage of apoptotic cells was detected after 2 days in presence of the indicated concentration of TGFβ-1, without (●) or with (○) the inhibitor DAN/Fc chimera. *, $p < 0.05$ vs DAN/Fc chimera; DAN/Fc, DAN/Fc chimera (two-way ANOVA in combination with the least square method; the significance of individual differences was evaluated using contrast tests).

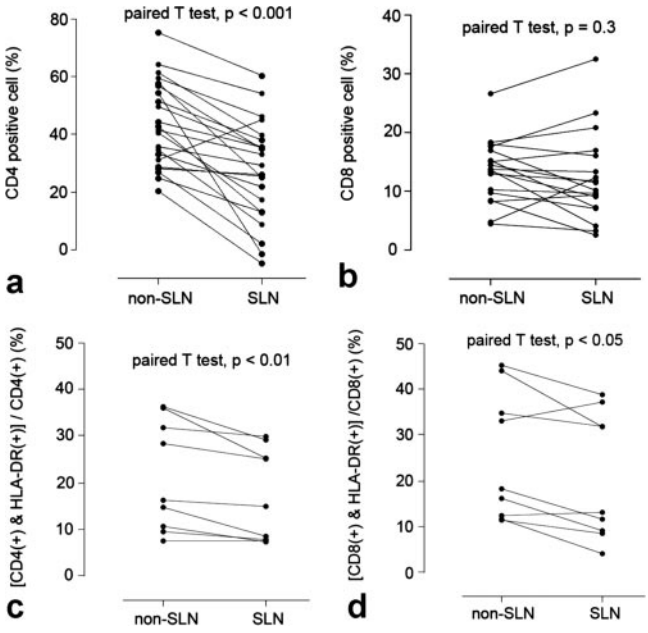

FIGURE 7. Numbers of $CD4^+$ T cells are reduced within SLNs. The numbers of $CD4^+$ (*a*) and $CD8^+$ (*b*) cells in SLNs and non-SLNs were analyzed using flow cytometry. To assess T cell activity, numbers of $CD4^+$ (*c*) and $CD8^+$ (*d*) cells that were also $HLA\text{-}DR^+$ were counted.

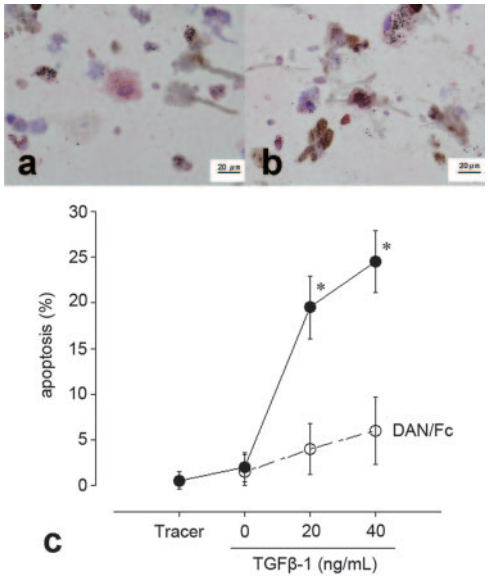

FIGURE 6. TGFβ-1 induces apoptosis in DCs within LNs. *a* and *b*, TUNEL analysis of TGFβ-1-induced apoptosis among cells acutely dissociated from normal LNs is shown. DCs ($S100\beta^+$ cells) are stained red (fuchsin), whereas $TUNEL^+$ cells are stained brown (DAB). *a* and *b*, Nonapoptotic DCs and apoptotic DCs are shown, respectively. Percentage of apoptotic cells was detected after 2 days in presence of the indicated concentration of TGFβ-1, without (●) or with (○) the inhibitor DAN/Fc chimera (*c*). *, $p < 0.05$ vs DAN/Fc chimera (two-way ANOVA in combination with the least square method; the significance of individual differences was evaluated using contrast tests).

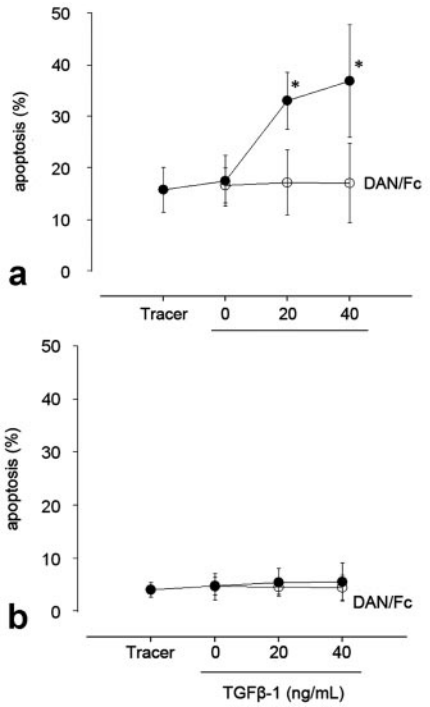

FIGURE 8. TGFβ-1 induces $CD4^+$ T cell apoptosis. TGFβ-1-induced apoptosis among $CD4^+$ (*a*) and $CD8^+$ (*b*) T cells acutely dissociated from normal LNs were analyzed using the TUNEL method. Percentages of apoptotic cells were determined after 2 days in presence of the indicated concentration of TGFβ-1, without (●) or with (○) the inhibitor DAN/Fc chimera. *, $p < 0.05$ vs DAN/Fc chimera; DAN/Fc, DAN/Fc chimera (two-way ANOVA in combination with the least square method; the significance of individual differences was evaluated using contrast tests).

from normal LNs. The results showed that exposure to TGFβ-1 (20 or 40 ng/ml) for 2 days induced a significant increase in apoptosis among $CD4^+$ but not $CD8^+$ T cells (Fig. 8) and, as with the DCs, the effect on $CD4^+$ T cells was blocked by DAN/Fc chimera; the inhibitor had no effect on $CD8^+$ T cells.

Discussion

Our findings that tumor-derived TGFβ-1 induces apoptosis among DCs and $CD4^+$ T lymphocytes within SLNs from NSCLC patients presenting with no evidence of nodal metastasis is consistent with an earlier report (31) and suggests that tumor-derived TGFβ-1 induces immunosuppression within SLNs before the movement of tumor cells into the node, thereby facilitating nodal metastasis. We were careful to select patients with no evidence of metastasis to avoid the effects of cytokines derived from metastatic tumor cells, because Poindexter et al. (32) reported that the number of mature $CD83^+$ DCs in tumor-free SLNs is higher than that in tumor-containing SLNs. In addition, our finding that numbers of $CD11^+$, $CD11^+CD80^+$, $CD123^+$, and $CD123^+CD80^+$ cells were reduced in SLNs indicates that not only the overall number but also the activity of both DC1s and DC2s was reduced in SLNs from NSCLC patients. It is also noteworthy that TGFβ reportedly inhibits DC migration into tumor-draining LNs (26), suggesting that, in addition to apoptosis, a TGFβ-induced reduction in DC migration into SLNs may also contribute to the decline in the numbers of DCs within SLNs. These effects on DCs within SLNs from NSCLC patients are consistent with observations reported for other malignant tumors, including breast cancer (9) and melanoma (10).

A comparison of our findings with those previously reported underscores concentration-dependence and tissue specificity of the effects exerted by TGFβ-1. At a concentration of 10 ng/ml, for instance, TGFβ-1 inhibits DC migration from tumors into draining LNs, as well as the ability of DCs to present Ag and stimulate T lymphocytes (26), though it reportedly promotes the growth and differentiation of monocyte-derived DCs into Langerhans cell-type DCs by protecting the viability of DC precursors (33, 34). In addition, Riedl et al. (35) reported that, at a lower concentration (0.5 ng/ml), TGFβ-1 inhibits DC apoptosis induced by TNF-α, although Kiertscher et al. (36) reported that a neutralizing anti-TGFβ mAb had no effect on DC apoptosis induced by the conditioned supernatant from tumor cell cultures. In contrast, Jacobsen et al. (37) showed that, at 2 ng/ml, TGFβ-1 induces apoptosis among immature murine progenitor cells, which is consistent with the finding of Radeke et al. (38) that, at 0.1–10 ng/ml, TGFβ induces expression of Smad3 mRNA, transient Smad3/4 oligomerization, and Smad3/DNA binding in Langerhans cell-type DCs. The activation of Smad3 by TGFβ-1 is an upstream event leading to activation of the Fas death pathway to apoptosis (39). The average serum TGFβ-1 levels in healthy volunteers and cancer patients were previously shown to be 50.8 ± 19.2 and 40.5 ± 12.1 ng/ml, respectively (40); we selected TGFβ-1 concentrations of 20 and 40 ng/ml for our in vitro studies based on that finding.

Our study also suggests that TGFβ-1 derived from primary tumors induces apoptosis among $CD4^+$ T cells within SLNs. By contrast, Chen et al. (41) reported that T cell apoptosis was enhanced in mice lacking TGFβ-1; moreover, a low concentration of TGFβ-1 (1 ng/ml) did not inhibit the synthesis and secretion of cytokines by $CD4^+$ T cells and acted synergistically with IL-2 to block apoptosis among $CD4^+$ T cells (42). We suggest that the discrepancy between our present findings and those earlier reports reflects the difference in the TGFβ-1 concentrations used. In fact, it has been shown that higher concentrations of TGFβ (5–25 ng/ml) induce $CD4^+$ T cell apoptosis (43, 44). Thus, TGFβ may suppress apoptosis among $CD4^+$ T cells at low concentrations, but induce it at higher concentrations.

In conclusion, we found that in NSCLC patients TGFβ-1 derived from the primary tumor induces apoptosis among both DC and $CD4^+$ T cells within SLNs which, at least in part, accounts for the decline in their numbers there. We suggest that tumors prepare SLNs for metastasis via this immunosuppressive mechanism, making the SLN a good model for investigating tumor immunity and its relationship to metastasis.

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Disclosures

The authors have no financial conflict of interest.

References

1. Giuliano, A. E., P. S. Dale, R. R. Turner, D. L. Morton, S. W. Evans, and D. L. Krasne. 1995. Improved axillary staging of breast cancer with sentinel lymphadenectomy. *Ann. Surg.* 222: 394–399.
2. Petrek, J. A., R. T. Senie, M. Peters, and P. P. Rosen. 2001. Lymphedema in a cohort of breast carcinoma survivors 20 years after diagnosis. *Cancer* 92: 1368–1377.
3. Morton, D. L. 2001. Lymphatic mapping and sentinel lymphadenectomy for melanoma: past, present, and future. *Ann. Surg. Oncol.* 8: 22S–28S.
4. Shen, J., A. M. Wallace, and M. Bouvet. 2002. The role of sentinel lymph node biopsy for melanoma. *Semin. Oncol.* 29: 341–352.
5. Nakagawa, T., Y. Minamiya, Y. Katayose, H. Saito, K. Taguchi, H. Imano, H. Watanabe, K. Enomoto, M. Sageshima, T. Ueda, and J. Ogawa. 2003. A novel method for sentinel lymph node mapping using magnetite in patients with non-small cell lung cancer. *J. Thorac. Cardiovasc. Surg.* 126: 563–567.
6. Liptay, M. J., S. C. Grondin, W. A. Fry, C. Pozdol, D. Carson, C. Knop, G. A. Masters, R. M. Perlman, and W. Watkin. 2002. Intraoperative sentinel

lymph node mapping in non-small-cell lung cancer improves detection of micrometastases. *J. Clin. Oncol.* 20: 1984–1988.
7. Tiffet, O., A. G. Nicholson, A. Khaddage, N. Prevot, G. Ladas, F. Dubois, and P. Goldstraw. 2005. Feasibility of the detection of the sentinel lymph node in peripheral non-small cell lung cancer with radio isotopic and blue dye techniques. *Chest* 127: 443–448.
8. Lafreniere, R. 2004. What's new in general surgery: surgical oncology. *J. Am. Coll. Surg.* 198: 966–988.
9. Huang, R. R., D. R. Wen, J. Guo, A. E. Giuliano, M. Nguyen, R. Offodile, S. Stern, R. Turner, and A. J. Cochran. 2000. Selective modulation of paracortical dendritic cells and T-lymphocytes in breast cancer sentinel lymph nodes. *Breast J.* 6: 225–232.
10. Essner, R., and M. Kojima. 2002. Dendritic cell function in sentinel nodes. *Oncology* 16: 27–31.
11. Conrad, C. T., N. R. Ernst, W. Dummer, E. B. Brocker, and J. C. Becker. 1999. Differential expression of transforming growth factor β1 and interleukin 10 in progressing and regressing areas of primary melanoma. *J. Exp. Clin. Cancer Res.* 18: 225–232.
12. Amoils, K. D., and W. R. Bezwoda. 1997. TGF-β1 mRNA expression in clinical breast cancer and its relationship to ER mRNA expression. *Breast Cancer Res. Treat.* 42: 95–101.
13. Asselin-Paturel, C., H. Echchakir, G. Carayol, F. Gay, P. Opolon, D. Grunenwald, S. Chouaib, and F. Mami-Chouaib. 1998. Quantitative analysis of Th1, Th2 and TGF-β1 cytokine expression in tumor, TIL and PBL of non-small cell lung cancer patients. *Int. J. Cancer* 77: 7–12.
14. Yang, L., and D. P. Carbone. 2004. Tumor-host immune interactions and dendritic cell dysfunction. *Adv. Cancer Res.* 92: 13–27.
15. Gabrilovich, D. I., H. L. Chen, K. R. Girgis, H. T. Cunningham, G. M. Meny, S. Nadaf, D. Kavanaugh, and D. P. Carbone. 1996. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.* 2: 1096–1103.
16. Saito, H., S. Tsujitani, S. Oka, A. Kondo, M. Ikeguchi, M. Maeta, and N. Kaibara. 2000. An elevated serum level of transforming growth factor-β1 (TGF-β1) significantly correlated with lymph node metastasis and poor prognosis in patients with gastric carcinoma. *Anticancer Res.* 20: 4489–4493.
17. Ghellal, A., C. Li, M. Hayes, G. Byrne, N. Bundred, and S. Kumar. 2000. Prognostic significance of TGFβ1 and TGFβ3 in human breast carcinoma. *Anticancer Res.* 20: 4413–4418.
18. Hasegawa, Y., S. Takanashi, Y. Kanehira, T. Tsushima, T. Imai, and K. Okumura. 2001. Transforming growth factor-β1 level correlates with angiogenesis, tumor progression, and prognosis in patients with non-small cell lung carcinoma. *Cancer* 91: 964–971.
19. Yamamoto, N., J. P. Zou, X. F. Li, H. Takenaka, S. Noda, T. Fujii, S. Ono, Y. Kobayashi, N. Mukaida, K. Matsushima, et al. 1995. Regulatory mechanisms for production of IFN-γ and TNF by antitumor T cells or macrophages in the tumor-bearing state. *J. Immunol.* 154: 2281–2290.
20. Bright, J. J., L. D. Kerr, and S. Sriram. 1997. TGF-β inhibits IL-2-induced tyrosine phosphorylation and activation of Jak-1 and Stat 5 in T lymphocytes. *J. Immunol.* 159: 175–183.
21. Strobl, H., E. Riedl, C. Scheinecker, C. Bello-Fernandez, W. F. Pickl, K. Rappersberger, O. Majdic, and W. Knapp. 1996. TGF-β1 promotes in vitro development of dendritic cells from CD34$^+$ hemopoietic progenitors. *J. Immunol.* 157: 1499–1507.
22. Caux, C., C. Massacrier, B. Dubois, J. Valladeau, C. Dezutter-Dambuyant, I. Durand, D. Schmitt, and S. Saeland. 1999. Respective involvement of TGF-β and IL-4 in the development of Langerhans cells and non-Langerhans dendritic cells from CD34$^+$ progenitors. *J. Leukocyte Biol.* 66: 781–791.
23. Strobl, H., C. Bello-Fernandez, E. Riedl, W. F. Pickl, O. Majdic, S. D. Lyman, and W. Knapp. 1997. flt3 ligand in cooperation with transforming growth factor-β1 potentiates in vitro development of Langerhans-type dendritic cells and allows single-cell dendritic cell cluster formation under serum-free conditions. *Blood* 90: 1425–1434.
24. de Saint-Vis, B., I. Fugier-Vivier, C. Massacrier, C. Gaillard, B. Vanbervliet, S. Ait-Yahia, J. Banchereau, Y. J. Liu, S. Lebecque, and C. Caux. 1998. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J. Immunol.* 160: 1666–1676.
25. Lyakh, L. A., M. Sanford, S. Chekol, H. A. Young, and A. B. Roberts. 2005. TGF-β and vitamin D_3 utilize distinct pathways to suppress IL-12 production and modulate rapid differentiation of human monocytes into CD83$^+$ dendritic cells. *J. Immunol.* 174: 2061–2070.
26. Kobie, J. J., R. S. Wu, R. A. Kurt, S. Lou, M. K. Adelman, L. J. Whitesell, L. V. Ramanathapuram, C. L. Arteaga, and E. T. Akporiaye. 2003. Transforming growth factor β inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res.* 63: 1860–1864.
27. Naruke, T., K. Suemasu, and S. Ishikawa. 1978. Lymph node mapping and curability at various levels of metastasis in resected lung cancer. *J. Thorac. Cardiovasc. Surg.* 76: 832–839.
28. Kim, A. S., and S. J. Pleasure. 2003. Expression of the BMP antagonist Dan during murine forebrain development. *Brain Res. Dev. Brain Res.* 145: 159–162.
29. Shurin, M. R. 2003. Preparation of human dendritic cells for tumor vaccination. *Methods Mol. Biol.* 215: 437–462.
30. Laguens, G., S. Coronato, R. Laguens, E. Portiansky, and V. Di Girolamo. 2002. Human regional lymph nodes draining cancer exhibit a profound dendritic cell depletion as comparing to those from patients without malignancies. *Immunol. Lett.* 84: 159–162.
31. Cochran, A. J., D. L. Morton, S. Stern, A. M. Lana, R. Essner, and D. R. Wen. 2001. Sentinel lymph nodes show profound downregulation of antigen-presenting cells of the paracortex: implications for tumor biology and treatment. *Mod. Pathol.* 14: 604–608.
32. Poindexter, N. J., A. Sahin, K. K. Hunt, and E. A. Grimm. 2004. Analysis of dendritic cells in tumor-free and tumor-containing sentinel lymph nodes from patients with breast cancer. *Breast Cancer Res.* 6: R408–R415.
33. Borkowski, T. A., J. J. Letterio, A. G. Farr, and M. C. Udey. 1996. A role for endogenous transforming growth factor β1 in Langerhans cell biology: the skin of transforming growth factor β1 null mice is devoid of epidermal Langerhans cells. *J. Exp. Med.* 184: 2417–2422.
34. Nunez, R., N. Garay, A. Bruno, C. Villafane, E. Bruno, and L. Filgueira. 2004. Functional and structural characterization of two populations of human monocyte-derived dendritic cells. *Exp. Mol. Pathol.* 77: 104–115.
35. Riedl, E., H. Strobl, O. Majdic, and W. Knapp. 1997. TGF-β1 promotes in vitro generation of dendritic cells by protecting progenitor cells from apoptosis. *J. Immunol.* 158: 1591–1597.
36. Kiertscher, S. M., J. Luo, S. M. Dubinett, and M. D. Roth. 2000. Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. *J. Immunol.* 164: 1269–1276.
37. Jacobsen, F. W., T. Stokke, and S. E. Jacobsen. 1995. Transforming growth factor-β potently inhibits the viability-promoting activity of stem cell factor and other cytokines and induces apoptosis of primitive murine hematopoietic progenitor cells. *Blood* 86: 2957–2966.
38. Radeke, H. H., H. von Wenckstern, K. Stoidtner, B. Sauer, S. Hammer, and B. Kleuser. 2005. Overlapping signaling pathways of sphingosine 1-phosphate and TGF-β in the murine Langerhans cell line XS52. *J. Immunol.* 174: 2778–2786.
39. Kim, S. G., H. S. Jong, T. Y. Kim, J. W. Lee, N. K. Kim, S. H. Hong, and Y. J. Bang. 2004. Transforming growth factor-β1 induces apoptosis through Fas ligand-independent activation of the Fas death pathway in human gastric SNU-620 carcinoma cells. *Mol. Biol. Cell* 15: 420–434.
40. Yanagimoto, H., S. Takai, S. Satoi, H. Toyokawa, K. Takahashi, N. Terakawa, A. H. Kwon, and Y. Kamiyama. 2005. Impaired function of circulating dendritic cells in patients with pancreatic cancer. *Clin. Immunol.* 114: 52–60.
41. Chen, W., W. Jin, H. Tian, P. Sicurello, M. Frank, J. M. Orenstein, and S. M. Wahl. 2001. Requirement for transforming growth factor β1 in controlling T cell apoptosis. *J. Exp. Med.* 194: 439–453.
42. Zhang, X., L. Giangreco, H. E. Broome, C. M. Dargan, and S. L. Swain. 1995. Control of CD4 effector fate: transforming growth factor β1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. *J. Exp. Med.* 182: 699–709.
43. Lin, C. M., F. H. Wang, and P. K. Lee. 2002. Activated human CD4$^+$ T cells induced by dendritic cell stimulation are most sensitive to transforming growth factor-β: implications for dendritic cell immunization against cancer. *Clin. Immunol.* 102: 96–105.
44. Chang, C. J., C. H. Liao, F. H. Wang, and C. M. Lin. 2003. Transforming growth factor-β induces apoptosis in antigen-specific CD4$^+$ T cells prepared for adoptive immunotherapy. *Immunol. Lett.* 86: 37–43.