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Tumors Promote Altered Maturation and Early Apoptosis of Monocyte-Derived Dendritic Cells[1]

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Tumors produce a number of immunosuppressive factors that block the maturation of $CD34^+$ stem cells into dendritic cells (DC). We hypothesized that tumors might also interfere with the maturation and/or function of human monocyte-derived DC. In contrast to stem cells, we found that $CD14^+$ cells responded to tumor culture supernatant (TSN) by increasing expression of APC surface markers, up-regulating nuclear translocation of RelB, and developing allostimulatory activity. Although displaying these characteristics of mature DC, TSN-exposed DC lacked the capacity to produce IL-12, did not acquire full allostimulatory activity, and rapidly underwent apoptosis. The effects of TSN appeared to be specific for maturing DC, and were not reversed by Abs against known DC regulatory factors including IL-10, vascular endothelial growth factor, TGF-β, or PGE_2. Supernatants collected from nonmalignant cell sources had no effect on DC maturation. The altered maturation and early apoptosis of monocyte-derived DC may represent another mechanism by which tumors evade immune detection. ***The Journal of Immunology*****, 2000, 164: 1269–1276.**

Tumors employ a variety of mechanisms to evade detection and elimination by the immune system (1–4). Dendritic cells (DC)[3], which play a pivotal role in the development of antitumor immunity (5–7), appear susceptible to tumor-mediated immunosuppression. Both circulating and tumor-infiltrating DC are functionally impaired in tumor-bearing animals (8, 9) and in cancer patients (10–13). In vitro, $CD34^+$ cells cultured with tumor supernatant (TSN) fail to mature into fully functional DC (14, 15). In these cells, TSN inhibits NF-κB activation (16) as well as the normal acquisition of APC molecules such as CD80, CD86, and HLA-DR. Blocking and reconstitution studies suggest that tumor-secreted vascular endothelial growth factor (VEGF), M-CSF, and IL-6 mediate these effects (14, 15).

In addition to $CD34^+$ stem cells, $CD14^+$ monocytes provide another source for generating functional DC (17, 18). We hypothesized that for tumor-induced suppression to be effective, factors present in the tumor environment must also interfere with the maturation and/or function of monocyte-derived DC. Human adherent PBMC were cultured with GM-CSF and IL-4 in the presence or absence of culture supernatants collected from a variety of tumor cell lines. In contrast to the responses reported for $CD34^+$ precursors, DC generated from monocytes in the presence of TSN appeared mature, expressing essential cell surface molecules, enhanced translocation of RelB to the nucleus, and early development of T cell stimulatory activity. However, these cells did not develop full allostimulatory activity, had a diminished capacity to produce IL-12 and IL-10, and rapidly underwent apoptosis following this early, defective maturation process. While mediated by different mechanisms, TSN appears to effectively suppress the function of both monocyte- and stem cell-derived DC.

Materials and Methods

Cell lines and reagents

The A549 non-small cell lung carcinoma, the IMR90 lung fibroblast, the MCF-7 breast carcinoma (all from American Type Culture Collection, Manassas, VA), the A427 lung adenocarcinoma (obtained from Dr. J. A. Radosevich, Northwestern University, Chicago, IL), the R11 renal cell carcinoma (generous gift of Dr. A. Belldegrun, UCLA School of Medicine, Los Angeles, CA), and the UCLA-SOM-M14 (M14) melanoma cell lines were maintained in complete medium composed of RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA), penicillin-streptomycin-Fungizone (Life Technologies, Grand Island, NY), and 10 mM HEPES (Sigma, St. Louis, MO). Cytokines used for DC generation and stimulation included GM-CSF (sp. act., 1.13×10^7 U/mg; provided by Schering-Plough Research Institute, Kenilworth, NJ), IL-4 (sp. act., 8×10^6 U/mg; R&D Systems, Minneapolis, MN), CD40L (provided by Immunex, Seattle, WA), and IFN-γ and IL-12 (Peprotech, Rocky Hills, NJ). Abs used for phenotyping included fluorescently conjugated anti-HLA-DR and anti-CD80 (Becton Dickinson, San Jose), anti-CD14 and anti-CD40 (Caltag Laboratories, Burlingame, CA), anti-CD83 (Serotec, Raleigh, NC), and anti-CD11c and anti-CD86 (PharMingen, San Diego, CA). Abs used to purify DC (anti-CD3, anti-CD19, anti-CD56) and T cells (anti-CD14, anti-CD16, anti-CD19) were obtained from PharMingen (no azide, low endotoxin). Neutralizing Abs were used at 5–10 μg/ml and included anti-IL-10, anti-VEGF, anti-TGF-β (all from R&D Systems), and anti-PGE_2 (generously provided by J. Portanova, G. D. Searle, Skokie, IL).

Preparation of TSN and TSN-exposed DC

TSN were prepared by seeding 25-cm^2 flasks with 1×10^6 tumor cells in 10 ml of complete medium. The culture supernatants were collected after 24 h, centrifuged to remove cells, and stored at −80°C. DC were generated from the adherent fraction of peripheral blood, as previously described (17, 19). Briefly, PBMC were allowed to adhere for 2 h in culture flasks, nonadherent cells were gently removed, and the remaining cells were cultured

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[3] Abbreviations used in this paper: DC, dendritic cells; LFI, linear fluorescence intensity; TSN, tumor supernatant; VEGF, vascular endothelial growth factor; CD40L, CD40 ligand.

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in complete medium supplemented with 800 U/ml (71 ng/ml) GM-CSF and 500 U/ml (63 ng/ml) IL-4. The effects of TSN on DC maturation were examined by replacing 0–50% of the culture medium with TSN at the initiation of culture. In some experiments, the TSN was washed out after 24 h and replaced with fresh medium and cytokines. In other experiments, IL-12 was added in addition to GM-CSF and IL-4. After either 4 or 7 days in culture, all free or loosely adherent cells were collected by vigorous rinsing. DC were further purified by negative depletion using an Ab mixture in conjunction with anti-mouse Ig-conjugated immunomagnetic beads (17) (Dynal, Lake Success, NY). To examine the effects of TSN on mature DC, control DC were collected on day 7 and cultured for 40 h in fresh medium and cytokines, in the presence or absence of 50% TSN.

DC phenotype and function

DC were analyzed by three-color FACS analysis using a FACScan II flow cytometer and CellQuest software (Becton Dickinson) with 5,000–10,000 events acquired for each sample. Control and TSN-DC collected on days 4 and 7 were assayed for their ability to stimulate allogeneic T cells in an MLR. Purified DC were cocultured with 1×10^5 $CD3^+$ T cells from an unrelated donor at ratios of 1:20, 1:50, and 1:100 DC:T cells. Allogeneic $CD3^+$ T cells were prepared by negative depletion of nonadherent PBMC with an Ab mixture and immunomagnetic beads. The assays were performed in triplicate in round-bottom 96-well plates. No TSN was present during the MLR assay. After 5 days of coculture, the wells were pulsed for 18 h with 1 μCi/well tritiated thymidine (Amersham, Arlington Heights, IL). Endocytosis was assessed by uptake of FITC-dextran (20). DC were incubated for 1 h at 37°C in the presence of 1 mg/ml FITC-dextran (40,000 m.w.; Molecular Probes, Eugene, OR). Control cells were incubated on ice for 1 h. After extensive washing, the cells were analyzed by single-color FACS analysis. Cytokine production was assessed by stimulating purified DC (3×10^5 cells/ml) for 24 h with CD40L (1 μg/ml) in the presence or absence of IFN-γ (1000 U/ml) (21). DC supernatants were then analyzed by ELISA for IL-12 p70 (Genzyme, Cambridge, MA), PGE_2 (Ab pairs; Cayman Chemical, Ann Arbor, MI), and IL-10 (Ab pairs; PharMingen).

Western blot analysis of RelB

DC collected on days 4 and 7 were pelleted, washed, and lysed (4°C, 15 min; 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% Nonidet P-40). After centrifugation (6000 rpm, 10 min), the supernatant (cytoplasmic protein) was collected. The pelleted nuclei were mixed with buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol) and kept on ice with occasional vortex mixing. After centrifugation (13,000 rpm, 10 min), the supernatant (nuclear protein) was collected. For Western blotting, 20 μg protein samples were resolved on a 12% SDS-PAGE gel and transferred to Hybond-ECL membranes (Amersham Life Sciences, Arlington Heights, IL). The Western analysis was performed using anti-RelB and anti-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA) and the Amersham Life Science enhanced chemiluminescence protocol.

Apoptosis

DC were assessed for apoptosis by two methods. The exposure of phosphatidylserine residues on the cell surface was measured using annexin-FITC, with dead cells identified with propidium iodide (Bender MedSytems kit, distributed by Biosource International, Camarillo, CA). These results were confirmed using a FACS-based TUNEL assay (APO-BrdU kit; PharMingen). For both of these assays, 10,000 events were collected on the FACScan II cytometer and analyzed with CellQuest software.

Results

To evaluate effects of the tumor environment on the maturation of monocyte-derived DC, adherent PBMC were cultured with GM-CSF and IL-4 in the presence or absence of TSN from the A549 lung cancer cell line. Maturing DC were assessed at days 4 and 7 of culture for expression of APC surface molecules by FACS, T cell stimulatory activity in an allogeneic MLR, endocytotic capacity, translocation of RelB to the nucleus, and the ability to produce cytokines in response to stimulation by CD40L.

DC phenotype and function

As shown in Fig. 1, exposure of maturing DC to TSN enhanced their early expression of cell surface molecules associated in Ag

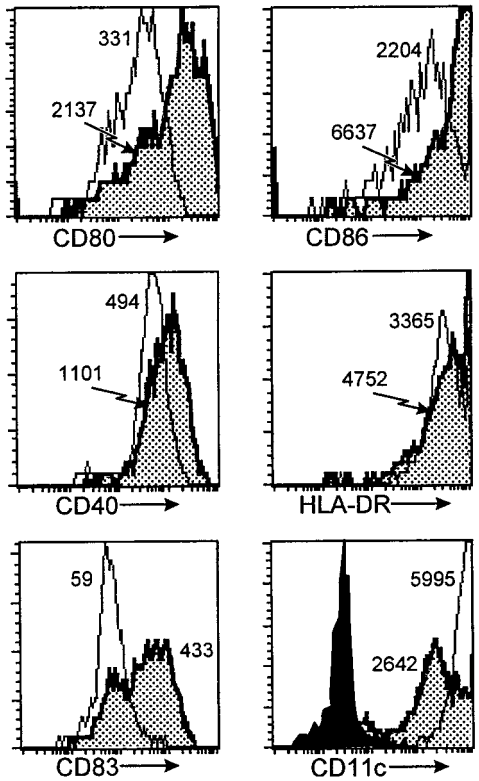

FIGURE 1. TSN promoted early phenotypic maturation of monocyte-derived DC. Cells were collected after 4 days of culture in GM-CSF and IL-4 in the presence or absence of 50% TSN, and stained with mAbs directed against APC surface molecules. The white histograms represent control DC, while the shaded histograms represent TSN-DC. Numerical values denote the mean fluorescence intensity, which was calculated by subtracting the autofluorescence of unstained cells (black histogram, *bottom right panel*) from the fluorescence intensity of the Ab-labeled cells. The data shown are representative of five similar experiments.

presentation, including CD80 (B7-1), CD86 (B7-2), CD40, and HLA-DR. A marker expressed on both immature and mature DC, CD11c, was reduced in cells cultured with TSN, while the same cells expressed higher than control levels of CD83, a marker of more mature DC. Between days 4 and 7, the expression of APC markers, as measured by mean linear fluorescence intensity (LFI), was maintained (CD80, 331 vs 313 LFI; CD83, 59 vs 63 LFI; CD11c, 5995 vs 5913 LFI) or continued to increase (CD86, 2204 vs 3293 LFI; CD40, 494 vs 576 LFI; HLA-DR, 3365 vs 4734 LFI) on control DC. In contrast, APC marker expression remained relatively constant (CD86, 6637 vs 6108 LFI; HLA-DR, 4752 vs 4990 LFI) or significantly decreased (CD80, 2137 vs 1290 LFI; CD40, 1101 vs 715 LFI; CD83, 433 vs 102 LFI; CD11c, 2642 vs 910 LFI) on DC grown in the presence of TSN. To evaluate the difference between tumor-related and non-tumor-related effects of cell culture supernatants, adherent PBMC also were cultured with GM-CSF and IL-4 in the presence of supernatants from the non-malignant lung fibroblast cell line IMR90, or from cultured allogeneic PBMC. These supernatants had no effect on maturing DC phenotype (results not shown).

A number of tumor-derived factors with the capacity to alter the maturation of $CD34^+$ DC precursors (VEGF, TGF-β) (14) or $CD14^+$ precursors (IL-10, PGE_2) (22–24) are produced by A549 (25–27). The role of these factors in TSN-induced phenotypic changes was evaluated by adding neutralizing mAbs to the culture medium during DC maturation. Neutralization of these cytokines

Table I. *Neutralizing Abs to VEGF, TGF-β, PGE_2, and IL-10 do not block the effects of TSN on DC phenotype and apoptosis*[a]

	Phenotype				Apoptosis (% annexin binding)
	B7-2	HLA-DR	CD40	B7-1	
Expt. 1					
No TSN	2502.6	1539.8	337.0	61.6	29.3
TSN	3567.9	1308.0	563.7	587.4	50.1
TSN + anti-VEGF	4120.2	1557.4	551.3	814.3	46.6
TSN + anti-TGF-β	4815.5	1855.6	554.9	898.1	40.6
TSN + anti-PGE_2 and α-IL-10	6637.5	2609.8	1025.8	2333.7	53.0
Expt. 2					
No TSN	2177.2	1423.1	306.4	39.9	
TSN	5923.8	2105.7	1154.4	923.1	
TSN + anti-PGE_2	5456.5	2052.3	838.2	753.7	
TSN + anti-IL-10	7548.7	3088.7	1072.8	1875.6	
TSN + anti-PGE_2 and α-IL-10	7519.0	2909.1	1225.8	2224.4	

[a] Cells were collected after 4 days of culture in GM-CSF and IL-4 in the presence or absence of 50% TSN, with neutralizing Abs to the noted factors. Neutralizing Abs were used at 5–10 μg/ml. APC cell surface molecule expression was assessed by staining with mAbs. Numerical values denote the mean fluorescence intensities. Apoptosis was measured by annexin binding and is expressed as percentage of positive cells.

did not prevent the premature phenotypic maturation of monocyte-derived DC, and in the case of anti-IL-10, appeared to enhance the TSN effect (Table I).

An allogeneic MLR was used to compare the T cell stimulatory activity of control and TSN-exposed DC (Fig. 2). Corresponding to the early and heightened expression of APC markers, DC exposed to TSN also displayed higher allostimulatory activity at day 4 compared with control DC. However, despite this early increase, the degree of allostimulatory activity exhibited by TSN-DC did not reach the maximal level exhibited by control DC, and decreased significantly by day 7. In contrast, DC cultured under normal conditions showed a consistent increase in allostimulatory activity between days 4 and 7, corresponding to their continued maturation over the 7-day culture.

Heightened endocytotic activity is characteristic of cytokine-derived DC and their enhanced capacity to capture and process Ags. We measured mannose receptor-mediated endocytosis by the temperature-dependent uptake of FITC-labeled dextran (Fig. 3). Compared with control DC, TSN-exposed DC internalized only 63% as much FITC-dextran on day 4, and only 50% as much FITC-dextran by day 7.

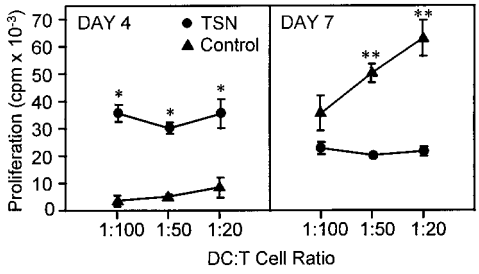

FIGURE 2. TSN promoted the development of early, but ultimately less potent, allostimulatory activity in monocyte-derived DC. Control and TSN-DC were collected and purified on days 4 and 7 of culture. Purified DC were added at various ratios to 1×10^5 allogeneic $CD3^+$ T cells. After 5 days, proliferation was assessed by the addition of tritiated thymidine for 18 h. The data shown are the average cpm of three replicates ± SD, and are representative of three similar experiments. *, $p < 0.002$ compared with day 4 control DC. **, $p < 0.006$ compared with day 4 TSN-DC, and $p < 0.0005$ compared with day 7 TSN-DC.

Nuclear translocation of RelB

Translocation of RelB to the nucleus, an event associated with DC maturation (28, 29), was assessed by resolving nuclear and cytoplasmic proteins on SDS-PAGE gels, followed by Western blotting. Consistent with the increased allostimulatory activity of the TSN-DC on day 4, there were high levels of RelB protein in both the cytoplasm and nucleus, while only minimal levels were observed in control DC on day 4 (Fig. 4). However, by day 7, the levels of RelB were greatly reduced in TSN-DC, while control DC showed enhanced nuclear translocation of RelB, consistent with their slower maturation.

Cytokine production by DC

The ability of DC to produce IL-12 when stimulated through CD40 is important for the generation of protective immunity. Stimulation with the combination of CD40L and IFN-γ resulted in production of IL-12 p70 (the bioactive form of IL-12) by the control cells, but not by DC exposed to TSN (Table II). The DC exposed to TSN

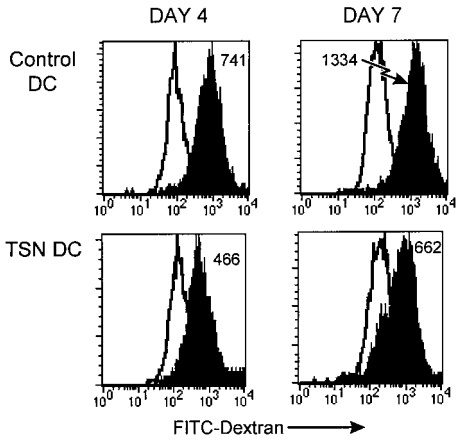

FIGURE 3. DC exposed to TSN had a decreased ability to take up FITC-dextran. Control and TSN-DC were collected and purified on days 4 and 7 of culture. Cells were incubated with FITC-dextran for 1 h, washed extensively, and analyzed by FACS. The open histograms represent uptake at 0°C, whereas the filled histograms represent uptake at 37°C.

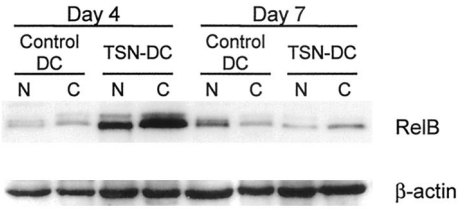

FIGURE 4. TSN increased levels of RelB in both the nucleus and cytoplasm of monocyte-derived DC. Control and TSN-DC were collected and purified on days 4 and 7 of culture. Nuclear and cytoplasmic proteins were resolved by SDS-PAGE, and Western blots were performed with anti-RelB and anti-actin Abs. The data shown are representative of three similar experiments.

also were impaired in their ability to produce IL-10, but did respond to stimulation by producing PGE_2 at levels equal to or greater than that produced by control DC.

TSN induced early DC apoptosis

Viable cell yield was reduced in DC cultures containing TSN (70 ± 1.4% of control, $n = 4$), leading us to investigate the effect of TSN on DC apoptosis. As shown in Fig. 5, the percentages of both apoptotic (51.4 vs 6.7%) and dead (24 vs 12.5%) cells were higher in the day 4 TSN-DC. It is unlikely that the apoptosis-inducing effect of TSN was due to changes in cell culture pH or nutrient deprivation, since as little as 1% TSN produced substantial increases in annexin binding (Fig. 6, *left panel*). In addition, supernatants from neither allogeneic PBMC nor the nonmalignant lung fibroblast cell line IMR90 increased DC apoptosis. The effects of TSN on DC apoptosis were not neutralized by Abs against IL-10, VEGF, TGF-β, or PGE_2 (Table I).

In contrast to the effects on DC precursors, TSN did not induce apoptosis in resting or activated T cells. TSN was added at a 50% concentration to $CD3^+$ cells alone, or to $CD3^+$ cells undergoing activation with IL-2 or immobilized anti-CD3/CD28. Although the presence of IL-2 or anti-CD3/CD28 both induced a certain level of apoptosis (11.6% and 49.4%, respectively), no enhancement of this level was seen with the addition of TSN (IL-2 + TSN, 12.3%; anti-CD3/CD28 + TSN, 50.6%). Taken together, these data suggest that the apoptosis-inducing effects of TSN are specific for maturing DC.

TSN from a variety of tumor types induced DC apoptosis

TSN obtained from an additional lung cancer (A427), a breast cancer (MCF-7), a renal cell carcinoma (R11), and a melanoma (M14) cell line also were evaluated for their effect on DC maturation. At day 4 of culture, TSN from all of the tumor cell lines

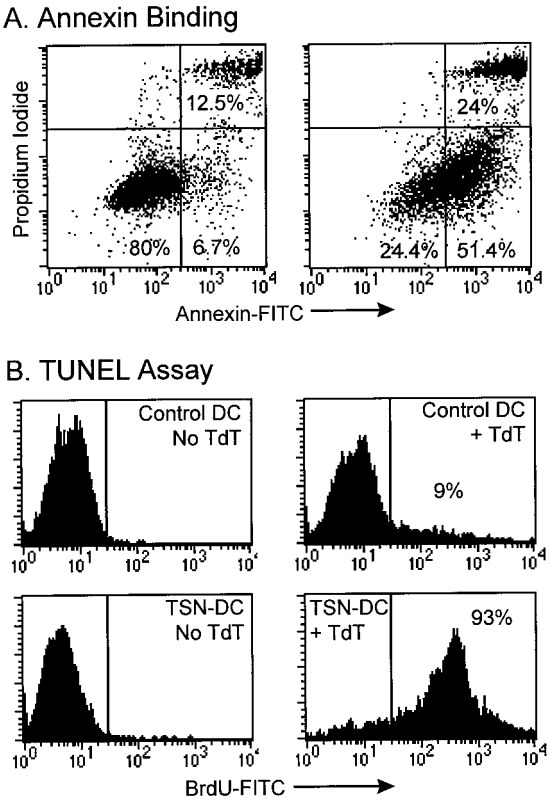

FIGURE 5. TSN induced apoptosis in developing monocyte-derived DC. Control and TSN-DC were collected and purified on day 4 of culture. Apoptosis was assessed by binding of annexin V-FITC (*A*), and by a FACS-based TUNEL assay utilizing Br-dUTP and anti-bromodeoxyuridine-FITC (*B*). TdT, the enzyme necessary for the incorporation of Br-dUTP into exposed 3′-OH DNA ends. The data shown are representative of three similar experiments.

induced similar levels of apoptosis in DC (Fig. 6, *right panel*). None of the day 6 TSN-treated DC were capable of producing IL-12 at the same levels as the control cells following stimulation with CD40L and IFN-γ (A427 and R11, <2 pg/ml; M14, 9 pg/ml), although MCF-7 TSN-treated DC did produce moderate amounts (630 pg/ml). In addition, TSN from all of the tumors enhanced expression of characteristic cell surface molecules (Fig. 7), and resulted in cells with enhanced allostimulatory activity at day 4 (data not shown), suggesting that the immunomodulating effects mediated by A549 are shared by a variety of tumor types.

Table II. *DC exposed to TSN have a decreased ability to produce IL-12 and IL-10*[a]

	IL-12 p70 (pg/ml)		IL-10 (pg/ml)		PGE_2 (pg/ml)	
	Control	+TSN	Control	+TSN	Control	+TSN
Day 4						
Unstimulated	<2	<2	10	12	480	700
CD40L	<2	<2	32	45	750	650
CD40L + IFN-γ	114	<2	107	10	1220	770
Day 6						
Unstimulated	<2	<2	13	9	600	590
CD40L	23	<2	442	12	920	1140
CD40L + IFN-γ	975	12	369	11	650	1336

[a] Control and TSN-DC were collected and purified on days 4 and 6 of culture. Purified DC (3×10^5 cells/ml) were stimulated for 24 h with CD40L (1 μg/ml) with or without IFN-γ (1000 U/ml). ELISA assays for IL-12 p70, IL-10, and PGE_2 were performed on the supernatants. The data shown are representative of two similar experiments.

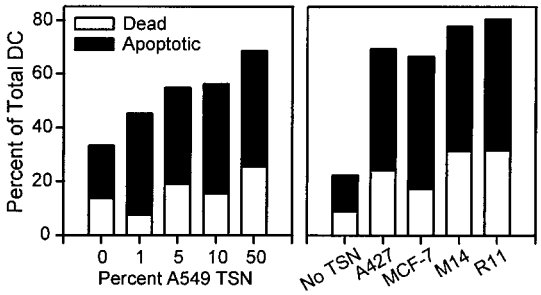

FIGURE 6. DC apoptosis was TSN concentration dependent and occurred with all tumors tested. DC were generated in the presence of 1–50% A549 TSN (*left panel*), or in the presence of 50% TSN from various tumor cell lines (*right panel*). Control and TSN-DC were collected and purified on day 4 of culture. The percentages of apoptotic and dead cells were determined by annexin binding and propidium iodide staining. The data shown are representative of three similar experiments.

TSN affected developing, but not mature DC

DC precursors exposed to TSN for only the first 24 h of culture demonstrated altered maturation and apoptosis similar to that observed with cells continuously exposed to TSN (Fig. 8). In contrast, DC precursors that were allowed to mature for 7 days in GM-CSF and IL-4 before exposure to TSN for 48 h resembled control DC (Fig. 9). These cells did not up-regulate HLA-DR, CD80, or CD86, indicating that TSN does not act as a final maturation factor like LPS, CD40L, and monocyte-conditioned medium (30–35). Therefore, the effects of TSN appear to be maturation dependent, acting only on DC precursors and not mature DC.

IL-12 did not protect against TSN-induced apoptosis of DC

It has been demonstrated that IL-12 protects DC from the induction of apoptosis by anti-Fas Ab in vitro (36), and is involved in the survival-enhancing effect of CD40 ligation in vivo (37). Coupled with the lack of production of IL-12 by the TSN-exposed DC, it is possible that exogenous IL-12 could have a similar effect on the induction of apoptosis by TSN. We added a range of IL-12 concentrations (0.2–100 ng/ml) to adherent PBMC at the initiation of DC culture. As shown in Fig. 10, addition of IL-12 did not decrease the level of apoptosis in the TSN-DC. No change was observed in the expression of APC cell surface molecules (data not shown).

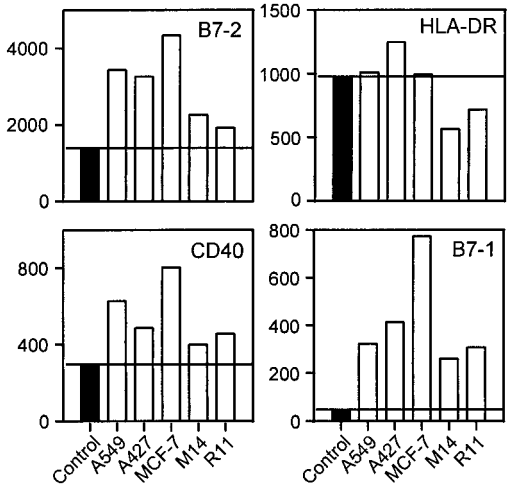

FIGURE 7. TSN from a variety of tumor types promoted early phenotypic maturation of monocyte-derived DC. DC were generated in the presence of 50% TSN from various tumor cell lines, as described in Fig. 6. Their phenotype was assessed by staining with mAbs directed against APC surface molecules, and is expressed as linear fluorescence intensities. The data shown are representative of two similar experiments.

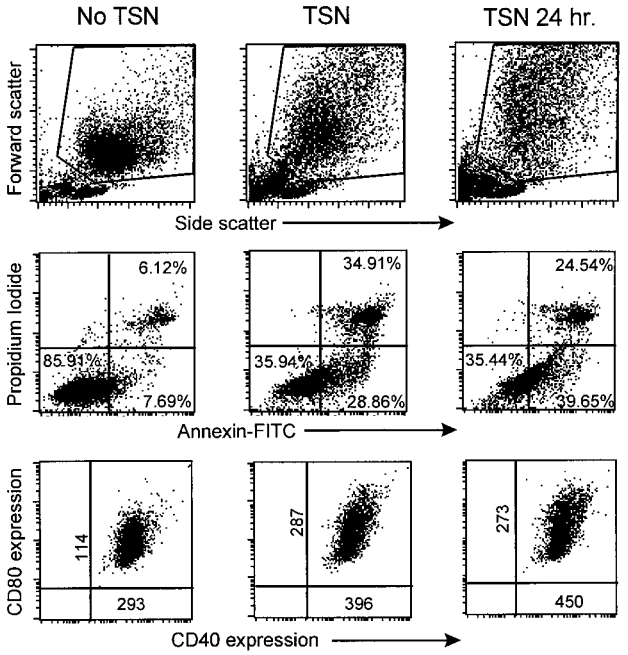

FIGURE 8. Exposure to TSN for the first 24 h of culture was sufficient to alter phenotypic maturation and enhance apoptosis in monocyte-derived DC. DC were generated with GM-CSF and IL-4 under three different conditions: no TSN added, TSN present for the entire culture period, or TSN removed after the first 24 h of culture. DC from the three groups were collected on day 4 and assessed for cell surface molecule expression and apoptosis. The percentages of apoptotic and dead cells were determined by annexin binding and propidium iodide staining. Phenotypic analysis was performed with fluorescently labeled anti-CD40 and anti-CD80, and the mean fluorescence intensities are noted. The data shown are representative of three similar experiments.

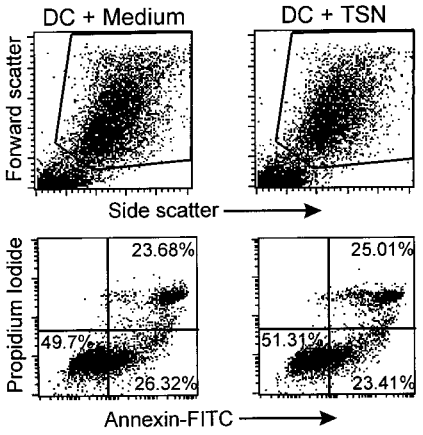

FIGURE 9. TSN did not induce apoptosis in mature monocyte-derived DC. DC were generated from adherent cells with GM-CSF and IL-4. After 7 days of culture, the DC were collected and purified, then cultured for an additional 40 h in fresh medium and cytokines, in the presence or absence of 50% TSN. The percentages of apoptotic and dead cells were determined by annexin binding and propidium iodide staining. The results shown are representative of four similar experiments.

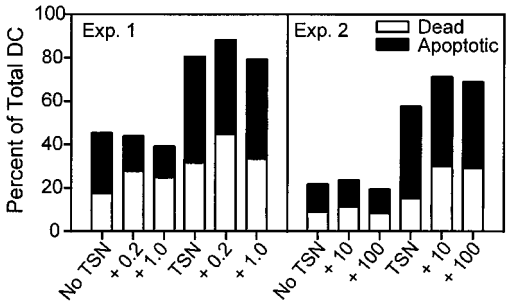

FIGURE 10. Exogenous IL-12 did not prevent TSN-induced DC apoptosis. Cells were cultured for 4 days in GM-CSF and IL-4 in the presence or absence of 50% TSN, with or without 0.2–100 ng/ml IL-12. The percentages of apoptotic and dead cells were determined by annexin binding and propidium iodide staining. The results shown are representative of three similar experiments.

Discussion

Circulating and tumor-infiltrating DC collected from cancer patients appear to be phenotypically and functionally defective (10–12). A variety of tumor-derived cytokines, including VEGF, IL-6, and M-CSF, have been shown to alter the maturation of DC from $CD34^+$ precursors in vitro (14, 15). Instead of producing functionally active DC, these factors promote the development of monocyte-like cells with little APC activity. These TSN effects led us to hypothesize that factors present in the tumor environment might similarly interfere with the maturation and/or function of monocyte-derived DC. Although we confirmed this hypothesis, the mechanism by which TSN acts on monocytic DC precursors appears to be quite unique. In contrast to its effects on $CD34^+$ precursors, we found that TSN induced an early maturation of monocyte-derived DC, as characterized by rapid up-regulation of CD80, CD86, and HLA-DR, translocation of RelB to the nucleus, and early development of allostimulatory activity. However, this maturation process was ultimately defective, resulting in DC that failed to produce IL-12 after stimulation by CD40L, exhibited reduced endocytotic activity, failed to develop full allostimulatory activity, and underwent rapid apoptosis.

DC maturation is a coordinated process involving up-regulation of cell surface molecules, cytokine production, and functional activity (28). DC that fail to develop properly may be unable to stimulate Ag-specific T cells, or may promote the development of T cell anergy (13). It appears that TSN disrupts the maturation process by inducing monocyte DC precursors to develop some, but not all, of the characteristic properties of DC. The resulting DC, while appearing phenotypically mature, have functional defects that impact on their ability to stimulate specific T cell responses. In addition, TSN-exposed DC undergo apoptosis at an early time point, reducing the window of time during which they might interact with T cells in vivo. These effects of TSN on monocyte DC precursors are clearly different from those reported for $CD34^+$ DC precursors. $CD34^+$ cells exposed to TSN during DC maturation do not up-regulate CD80 and CD86 expression, develop only minimal HLA-DR expression, and never acquire significant APC activity (14, 15). The differential effects of TSN on maturation of DC from $CD34^+$ and monocyte precursors suggest two distinct pathways by which tumors might suppress immune recognition.

Activation of the NF-κB/RelB family and translocation of RelB from the cytoplasm to the nucleus are associated with the maturation of DC (28) and development of Ag-presenting activity (29). Factors that prevent DC maturation from $CD34^+$ precursors also inhibit NF-κB activity and decrease RelB and c-Rel activation, consistent with the failure of the cells to develop APC phenotype or functional activity (16). Monocyte DC precursors exposed to TSN demonstrated increased levels of both cytoplasmic and nuclear RelB. This increase coincided with the rapid up-regulation of APC cell surface molecules and activity, but levels diminished substantially by day 7, corresponding to a reduction in functional activity. In contrast, control DC reached their maximal levels of RelB expression at day 7, corresponding to their slow, progressive development of APC cell surface molecule expression and allostimulatory activity. Despite their differences in response to TSN, the expression and translocation of RelB appear to coincide with the acquisition of APC function in both monocyte and $CD34^+$-derived DC.

The number of DC infiltrating tumor sites has been correlated with prognosis and survival (38). Many tumors have reduced numbers of DC, and those DC that are present have low functional activity (8–13). Our data suggest that tumor-derived factors induce monocyte-derived DC to undergo apoptosis early during their maturation. Furthermore, the cells that remain viable at day 7 have reduced allostimulatory activity and do not produce IL-12, but rather produce high levels of PGE_2. The functional consequences of these cytokine changes may be quite important because a reduction in IL-12 levels may prevent stimulation of Th1 T cell responses, and skew responding T cells toward a Th2 response (39, 40). In addition, IL-12 has been reported to act as an antiapoptotic factor for DC (36, 37), and lack of production by DC may contribute to early cell death. However, in our hands, the addition of exogenous IL-12 was unable to prevent TSN-induced DC apoptosis. Although TSN-exposed DC do not produce IL-10, enhanced levels of PGE_2 in the lung tumor microenvironment have been shown to promote IL-10 production by T cells and tumor cells (41). In the absence of IL-12 production by the DC, increased levels of IL-10 may lead to the development of Ag-specific anergy (42, 43). Other investigators have compared the cytokine profiles of progressing and regressing metastases obtained from the same patient, and have reported that progressing metastases express high levels of IL-10, while regressing metastases express IL-1, IFN-γ, and IL-12. DC from progressing metastases induced anergy in $CD4^+$ T cells, which could be reversed by the addition of exogenous IL-12 or IL-2 (13).

Apoptosis is a tightly regulated process that occurs normally during cellular activation, maturation, and senescence. We observed DC apoptosis late in the maturation of control DC, and a similar outcome has been reported for activated T cells (44) and for DC induced to mature by bacterial engulfment (28). Apoptosis may serve an important role by removing cells after they have served their functional purpose and allowing the immune system to return to homeostasis. Our data suggest that tumor cells may take advantage of this process by promoting a premature apoptosis that destroys DC before they can effectively interact with T cells. Certain factors and events have been shown to promote the survival of mature activated DC, including exogenous IL-12, TRANCE, and CD40L (36, 45, 46), as well as survival signals provided by Ag-specific interactions with T cells (47). It is unclear whether these substances have the same antiapoptotic effect on developing DC, and an evaluation of their ability to counteract the effects of TSN will be the focus of further study.

The use of ex vivo generated DC to overcome the APC deficiencies in cancer patients is the rationale behind recent clinical studies. Methods have been developed to pulse DC with tumor Ags before readministration to the patient, with the hope that an increase in the number of functional tumor Ag-presenting DC will generate specific antitumor responses and immunity. Deleterious

effects of tumors on readministered DC could have serious consequences for the efficacy of these treatments. Encouragingly, our results suggest that DC reach a maturation point at which TSN has little or no effect. Although early exposure results in dysfunctional maturation and apoptosis of DC, addition of TSN to day 7 DC did not induce phenotypic changes or apoptosis. Reports that DC prepared ex vivo from monocyte precursors were able to stimulate antitumor responses in patients are consistent with this observation (48–50).

In summary, tumors have at least two mechanisms by which they can alter DC maturation and function. The first, mediated by tumor-derived VEGF, M-CSF, and IL-6, affects $CD34^+$ precursors by promoting the generation of immature DC that lack the cell surface phenotype and T cell stimulatory activity characteristic of mature, functional DC (14, 15). The second pathway, demonstrated in this study, is the ability of soluble tumor-derived factors to promote dysfunctional maturation and early apoptosis of DC developing from monocyte precursors. This ability was observed even at low TSN concentrations, and was common to a variety of tumor types. The cytokines IL-10, TGF-β, and VEGF, which have been shown to have immunomodulatory effects on $CD34^+$ and/or $CD14^+$ DC precursors, are produced in picogram quantities by A549. However, the addition of neutralizing Abs to these factors did not block TSN-induced changes in phenotype and apoptosis. The factor(s) and mechanism responsible for these effects of TSN remain to be determined and are the focus of ongoing study. By acting on both $CD34^+$ and monocyte DC precursors, tumors might effectively evade immune recognition and prevent the establishment of an antitumor T cell response.

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