Differentiation Tumor-Induced Inhibition of Dendritic Cell Differentiation


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Prostanoids Play a Major Role in the Primary Tumor-Induced Inhibition of Dendritic Cell Differentiation

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Production of immunosuppressive factors is one of the mechanisms by which tumors evade immunosurveillance. Soluble factors hampering dendritic cell (DC) development have recently been identified in culture supernatants derived from tumor cell lines. In this study, we investigated the presence of such factors in 24-h culture supernatants from freshly excised solid human tumors (colon, breast, renal cell carcinoma, and melanoma). While primary tumor-derived supernatant (TDSN) profoundly hampered the in vitro DC differentiation from CD14+ plastic-adherent monocytes or CD34+ precursors (based on morphology and CD1a/CD14 phenotype), the effects of tested tumor cell line-derived supernatants were minor. Cyclooxygenase (COX)-1- and COX-2-regulated prostanoids present in the primary TDSN were found to be solely responsible for the observed hampered differentiation of monocyte-derived DC (MoDC). In contrast, both prostanoids and IL-6 were found to contribute to the TDSN-induced inhibition of DC differentiation from CD34+ precursor cells. While the addition of TDSN during differentiation interfered with the ability of CD34+ derived DC to stimulate a primary allogeneic T cell response, it actually increased this ability of MoDC. These opposite effects were correlated to different effects of the TDSN on the expression levels of CD86 and HLA-DR on the DC from the different precursor origins. Although TDSN increased the T cell-stimulatory capacity of MoDC, TDSN inhibited the IL-12 production and increased the IL-10 production of MoDC, thus skewing them to a type-2 T cell-inducing phenotype. In conclusion, this study demonstrates that primary tumors negatively impact DC development and function through COX-1 and -2 regulated factors, whereas tumor-derived cell lines may lose this ability upon in vitro propagation. The Journal of Immunology, 2002, 168: 4333–4343.

The importance of dendritic cells (DC)2 in the induction and possibly also in the effector phase of antitumor immune responses has been amply demonstrated, both by in vivo tumor rejection models and by correlations between the number of tumor-infiltrating DC and improved clinical outcome (1, 2). A number of recent publications has drawn attention to tumor-induced dysfunction of DC as a mechanism to escape immunosurveillance (3–9). Indeed, a reduced number of DC in primary tumors has been observed for breast cancer, basal cell skin cancer, cervical cancer, and colon cancer, among others (10). In addition, advanced cancer is associated with impaired differentiation and Ag-presenting functions of DC and their precursor cells (11). A direct involvement of tumor-derived factors in this phenomenon is strongly suggested by a recent study, showing breast tumor-infilt-rating DC to have an immature phenotype, while DC in surrounding stromal fields adopt a mature phenotype (12). A more systemic cancer-related defect in DC differentiation is evidenced by the presence of an increased number of relatively immature CD11c-negative myeloid DC precursor cells in the peripheral blood of cancer patients (13). This defect results in reduced absolute numbers of relatively immature blood DC, as observed in patients with breast cancer or other types of adenocarcinoma (14).

Detrimental effects of several soluble immunosuppressive factors on the differentiation and maturation of DC from monocytes or CD34+ myeloid stem cell progenitors have been demonstrated (15). Among these factors are a number of cytokines that are often produced by malignant cells, i.e., IL-10 (16), TGF-β1 (15), IL-6 (17), and vascular endothelial growth factor (VEGF) (18). Recent studies have revealed the involvement of tumor-derived IL-6 and VEGF in the inhibition of DC differentiation from both CD3+ and monocytyc precursors. However, these studies used superna-tants from tumor-derived cell lines rather than supernatants from primary tumor cell cultures (11, 17–19). To more accurately assess the putative involvement of tumor-derived immunosuppressive factors in the hampered DC differentiation in vivo we have generated supernatants from primary tumor cell cultures. To this end, single-cell suspensions were prepared from colon carcinoma samples that were collected for tumor cell-based vaccination purposes (20). Supernatants were collected after 24 h of culture. The effects of these tumor-derived supernatants (TDSN) were tested on the in vitro differentiation of DC from monocytes and from CD34+ stem cells. Although our studies mostly concentrated on colon tumors, their results were also extended to other solid tumors, i.e., breast and renal cell cancer and melanoma. In contrast to previously published data on tumor-derived cell lines (11, 17), our data on primary tumors reveal a predominant role of cyclooxygenase (COX)-1- and -2-regulated prostanoids (i.e., arachidonic acid metabolites) in the tumor-associated inhibition of DC differentiation.
As COX-2 dysregulation, resulting in, notably, an increased production of PGE$_2$, is a common feature of many tumors (21, 22), it may well be the primary cause of the widespread down-modulation of DC development and functions observed among cancer patients.

Materials and Methods

mAbs and recombinant cytokines

The following mAbs and recombinant cytokines were purchased from R&D Systems (Minneapolis, MN): anti-human IL-6 (MAB206), anti-human IL-10 Ab (MAB217), biotinylated anti-human TGF-β Ab (BAF240), anti-human TGF-β, capture Ab (MAB240), anti-human M-CSF Ab (MAB216), recombinant human (rh)VEGF165, and rhTGF-β1 (240-B). HiL-6 was purchased from IKT Diagnostics (Uithoorn, The Netherlands). Mouse mAbs with the following (anti-human) specificities were purchased from BD Biosciences (Mountain View, CA): CD14, CD80, CD86 (B7.1), CD86 (B7.2), CD54 (ICAM), and anti-human HLA-DR. Anti-human mAbs and recombinant human IL-10 and IL-6 were purchased from Becton Dickinson (San Jose, CA).

Inhibition of prostaglandin production

Production of prostaglandins by tumor or nontumor cells was blocked by the use of COX inhibitors. TDSN were prepared, as described above, in the presence or absence of 10 µM indomethacin (Merck, Darmstadt, Germany), a nonselective COX-1/COX-2 inhibitor. The selective COX-1 inhibitor SC560 (10 µM; Cayman Chemicals, Ann Arbor, MI) was similarly used.

PBMC and CD34+ stem cell isolation

PBMC were prepared from buffy coats obtained from healthy donors by density gradient centrifugation over Hypaque Lymphoprep (Nycomed, Oslo, Norway) and cryopreserved as previously described (24). Cells bearing CD34 were isolated from peripheral blood mononuclear fractions of G-CSF-mobilized patients through positive selection by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), using anti-CD34+ mAbs and goat anti-mouse IgG-coated microbeads (88–95% purity).

Generation of DC

DC were prepared from PBMC or CD34+ progenitor cells as described (17, 25).

MoDC. PBMC were thawed and resuspended in IMDM containing 10% FCS, 50 U/ml penicillin-streptomycin, 1.6 mM l-glutamine, and 0.01 mM 2-ME, and allowed to adhere to six-well tissue culture plates (3 × 10$^5$ cells/ml) for 2 h at 37°C. Nonadherent cells were removed and adherent cells were cultured in complete medium supplemented with 100 ng/ml GM-CSF (sp. act. 1.11 × 10$^8$ IU/mg; Schering-Plough, Madison, NJ) and 1000 U/ml IL-4 (sp. act. 1 × 10$^7$ U/mg; CLB). Monocytederived DC (MoDC) were collected at day 7.

CD34+ progenitor-derived DC. CD34+ cells were seeded at 5 × 10$^5$ cells/ml in 24-well plates and cultured in complete medium supplemented with 100 ng/ml GM-CSF, 2.5 ng/ml rhTNF-α (sp. act. 5 × 10$^8$ U/mg; CLB), and 20 ng/ml recombinant human stem cell factor (Peprotech, Rocky Hill, NJ). At day 9 of culture 1000 U/ml IL-4 was added. Cells were collected at days 12–14. Semiaherent DC were harvested with 0.05 mM EDTA. TDSN were added at the onset (day 0) of DC culture and were present during the entire differentiation period (7 days for MoDC and 11–14 days for CD34-derived DC).

Immunocytochemistry

Cytoplasts were prepared in a cytocentrifuge, loading 1 × 10$^5$ cells per cytoplast. Cells were centrifuged for 5 min at 500 rpm. Cytoplasts were dried overnight at room temperature and fixed with acetone for 10 min. Cells were blocked with normal rabbit serum (1:50; DAKO) for 10 min. Subsequently, slides were incubated with mAbs to CD1a (1:2000) or CD14 (1:100) for 1 h. Slides were washed with PBS and incubated with biotin-labeled rabbit anti-mouse (1:150; Zymed Laboratories, San Francisco, CA) for 1 h, washed again, and stained with streptavidin-IR (1:500; Zymed Laboratories). The peroxidase activity was visualized by aminethyl carbazol (0.4 g/L in sodium acetate buffer (0.1 M, pH 5) containing 0.05% H$_2$O$_2$). Staining was stopped with tap water and cells were counterstained with hematoxylin.

Cytokine and PGE$_2$ detection

Commercial kits were used to measure human IL-10, IL-6 (detection limits of 10 and 12 pg/ml, respectively; CLB) VEGF, M-CSF (detection limits of 16 and 31 pg/ml, respectively; Quantikine; R&D Systems), and PGE$_2$ (detection limit 1 pg/ml; Biotrak; Amersham Pharmacia Biotec, Piscataway, NJ). TGF-β$_1$ was quantitated using a combination of capture and secondary Abs that were paired for this application (clone 9016.2 (MAB240) as capture Ab and anti-TGF-β1 Ab; detection of 16 pg/ml; R&D Systems). TGF-β was quantitated after ELISA by acidification as previously described (26). Human IL-12 was detected in a capture ELISA as previously described (27).

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**FACS analysis**

Immunophenotypic analysis was performed using FACS. In short, cells (0.25 x 10^6) were washed in PBS supplemented with 1% BSA and 0.02% NaN_3 (PBA) and incubated for 30 min at room temperature with PE- or FITC-conjugated specific mAbs in the presence or absence of 1000 U/ml rhIFN-γ (CLB) in 200 μl culture medium. After 24 h, the supernatants were collected and stored at −20°C. IL-12 and IL-10 concentrations were determined by capture ELISA (see cytokine and PGE_2 detection).

**IL-10 and IL-12 (p70) release**

Differentiated MoDC were analyzed for functional (p70) IL-12 and IL-10 release as described previously (27). Briefly, 4 x 10^5 DC were incubated with 4 x 10^5 J558-CD40L cells in the presence or absence of 1000 U/ml rhIFN-γ (CLB) in 200 μl complete medium. After 24 h, the supernatants were collected and stored at −20°C. IL-12 and IL-10 concentrations were determined by capture ELISA (see cytokine and PGE_2 detection).

**Results**

**Primary colon carcinoma-derived supernatants inhibit the differentiation of DC from monocytic origin**

To study the effect of tumor-derived soluble factors on DC development, TDSN were prepared from 24-h cultures of colon tumor cell lines and single cell suspensions of primary colon tumors. During the generation of DC from monocytes in the presence of GM-CSF and IL-4, TDSN were added to the culture medium in a concentration range of 0.3–30%. Using de novo CD1a expression as a measure for DC development, primary TDSN effected a total inhibition at concentrations of 3–30% (n = 3), whereas this was not yet achieved at a concentration of 30% using colon tumor cell line-derived supernatants (tested cell lines: WiDr, A2233, HT29, Colo320, and SW620). In all subsequent experiments, 10–30% of TDSN were added to culture medium to achieve an optimal suppression of DC differentiation. Besides CD1a neo-expression, the down-regulation of CD14 and the acquisition of a veiled morphology were also used throughout as hallmarks of DC differentiation.

The addition of primary tumor cell supernatants to the MoDC cultures led to an inhibition of CD1a expression, resulted in a maintained CD14 expression, and hampered the development of a typical DC morphology. In contrast, inclusion of cell line-derived supernatants could not completely prevent the monocytes from adopting DC properties. The effects of primary tumor- and cell line-derived supernatants on CD1a expression and DC morphology are shown in Fig. 1A. The superior suppressive effect on MoDC development of primary tumor supernatants, as compared with tumor cell line supernatants, is further demonstrated by FACS analysis of CD1a neo-expression, CD14 expression, and DC morphology (with the typical veiled appearance of DC translating into high side light scatter (SSC) values) (Fig. 1B).

Supernatants from a total of 30 primary colon tumors were tested at a concentration of 10% in the culture medium and the neo-expression of CD1a (in mean fluorescence intensity (MFI)) was reduced by 68% on average (range, 26–98%), while higher continued CD14 expression was observed in 26 of the 30 cases tested, both compared with medium controls (p < 0.00001 and p = 0.0006, respectively). In contrast, cell line-derived supernatants inhibited CD1a expression by only 14.7 ± 4.4% (n = 5, p < 0.002) as compared with the medium control and had no effect on CD14 expression.

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**FIGURE 1.** Primary colon tumor or cell line-derived soluble factors hamper MoDC development. A, Immunocytochemical analysis of CD1a expression and morphology of MoDC after culture with primary colon tumor supernatant (10% v/v) and colon tumor cell line supernatant (Colo320, 30% v/v), compared with medium control (magnification, ×400). B, FACS analysis of MoDC generated under the same conditions. CD1a and CD14 expression and SSC and forward light scatter (FSC) characteristics are shown. Primary TDSN results are representative of 30 primary colon tumors tested and the colon tumor cell line supernatant results are representative of five cell lines tested. C, Titration analysis of PGE_2 and IL-6 on MoDC differentiation, expressed as a percentage of CD1a-positive cells.
Identification of DC development-inhibitory factors in the colon TDSN: a dominant role for prostanoids in the suppression of MoDC differentiation

Dose response relationships for tumor-associated factors, which were previously reported to hamper the differentiation of DC, i.e., IL-10, TGF-β1, VEGF, IL-6, M-CSF (all in a range of 0–125 ng/ml), and PGE2 (in a range of 0–350 ng/ml), were determined in the MoDC system (results for PGE2 and IL-6 are shown in Fig. 1C). Indeed, the expected inhibitory effect on DC development (as judged on the basis of CD1a expression) was found for all these agents. However, only IL-10, IL-6, and PGE2 were found to be present in active concentrations in the tested TDSN (exceeding the IC20; Table I). However, the maintained expression of CD14 (as observed with the primary tumor supernatants) was found only for PGE2 and IL-6, while the inhibitory effect on the acquisition of DC morphology (based on SSC) was observed only for PGE2 (data not shown). Thus, PGE2 appeared to most accurately mimic the observed DC-inhibitory effects of the primary TDSN.

To determine which tumor-derived factors were actually responsible for the observed suppression, TDSN were preincubated with saturating amounts of neutralizing Abs against IL-10, TGF-β1, VEGF, M-CSF, and IL-6 before addition to the MoDC cultures. While it proved possible to neutralize recombinant cytokines (at equivalent concentrations as present in the used supernatants) and thus abrogate their suppressive effects, the addition of the blocking Abs did not prevent the inhibition caused by the addition of TDSN (based on CD1a and CD14 expression patterns and SSC characteristics; data not shown). Not even a partial abrogation of inhibition of DC development was observed. In contrast, the addition of indomethacin (a combined COX-1 and -2 inhibitor) during the

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**FIGURE 2.** The impaired development of MoDC by primary TDSN can be abrogated by the COX-1/-2 inhibitor indomethacin. Primary colon TDSN, prepared in the presence or absence of 10 μM indomethacin (IM), were added to MoDC cultures (10% v/v). A, Representative FACS results for SSC/FSC values and CD1a/CD14 expression. B, MFI ± SD for CD1a and CD14 expression levels in the different culture conditions (n = 7).
culture of primary colon tumor cells to obtain conditioned supernatants led to the elimination of the primary tumor-induced inhibitory effects on the differentiation of DC from monocytes, signified by the presence of CD1a, the absence of CD14, and high SSC values (Fig. 2). Treatment with indomethacin did not affect the IL-6 and IL-10 concentrations in the TDSN, nor did the addition of IL-6 and IL-10 neutralizing mAbs to the TDSN, generated in the presence of indomethacin, further abrogate any inhibitory effects on MoDC development (data not shown). Thus, prostanoids (the production of which is dependent on COX-1/2 activity and of which PGE2 is the main constituent) were identified as the factors directly responsible for the observed inhibitory effect of primary TDSN in the MoDC differentiation model.

In contrast to the primary tumors, PGE2, M-CSF, IL-10, and IL-6 could not be detected in the five tested colon tumor cell line-derived supernatants. Only VEGF and TGF-β1 were detected (Table I), thus confirming the involvement of different mechanisms in the inhibition of DC development by primary tumors and the tested tumor-derived cell lines.

Both prostanoids and IL-6 contribute to tumor-induced inhibition of DC differentiation from CD34+ progenitor cells

To extend our findings to other pathways of DC differentiation, we next tested the effect of primary colon TDSN on the development of DC from peripheral blood CD34+ stem cells (G-CSF mobilized and isolated to 88–95% purity).

Similar to the MoDC model, differentiation of CD1a+ DC from the CD34+ cells could be prevented by the addition of primary colon tumor-conditioned supernatants (in equivalent concentrations). The percentage of CD1a-positive cells was reduced from 28.7% in the medium control to 6.4% (range, 5–8%) in the supernatant-containing conditions, while a simultaneous increase in the percentage of CD14+ cells was observed from 8.8 to 47.0% (range, 46–49%) (n = 4, p < 0.00001 for both markers). Again, we did not observe this effect on DC development by the cell line-derived supernatants (see Fig. 3A). In contrast to our findings in the MoDC model, addition of indomethacin during the generation of primary TDSN did not result in a total abrogation of the inhibitory effect on DC differentiation from CD34+ precursors (Fig. 3B). Further neutralization experiments revealed an additional role in the inhibition of DC differentiation for IL-6. While the use of indomethacin and neutralizing IL-6 Abs separately hardly had any effect, their combined use led to a near-complete abrogation of the primary tumor-induced suppression of DC differentiation from CD34+ progenitor cells (Fig. 3B).

**FIGURE 3.** Colon tumor-derived prostanoids and IL-6 both contribute to hampered DC differentiation from CD34+ progenitor cells. A. The DC differentiation from CD34+ progenitors is hampered by the presence of 10% (v/v) primary TDSN during the 2-wk culture period, but only a minor effect was observed after the addition of 30% (v/v) colon tumor-derived cell line (A2233) supernatant as shown by FSC/SSC and CD1a and CD14 expression. B, TDSN generated in the presence or absence of 10 μM indomethacin (IM) were added during CD34-DC development (10% v/v) with or without neutralizing Abs against IL-6 (anti-IL-6). CD1a and CD14 expression of the resulting DC are presented in MFI (n = 4).

**FIGURE 4.** Suppression of DC differentiation is mediated by CD45-negative tumor cell fractions and not by CD45+ tumor-infiltrating leukocytes. Tumor cells and infiltrating leukocytes from colon tumor single-cell suspensions were separated by indirect panning with anti-CD45 mAbs. Supernatants, derived from 24-h cultures of the CD45+ and CD45- cell fractions, were tested separately in MoDC differentiation cultures. The influence of supernatants from unseparated and separated cell fractions on DC differentiation was represented by the percentage of CD1a expression (fluorescence intensity determined by FACS analysis) relative to the medium control (mean ± SD of two separate experiments).

**Tumor-induced inhibition of DC development is infiltrate independent**

The colon carcinoma single-cell suspensions used to generate TDSN contained leukocyte infiltrate (10% on average). To address the possible role of this infiltrate in the observed DC suppression, the CD45+ tumor-infiltrating leukocytes were depleted from the single-cell suspensions by indirect panning with anti-CD45 mAbs. Unbound colon cells and bound leukocytes were separately obtained and CD45-negative supernatants at a concentration of 1 × 106 cells/ml over a period of 24 h. FACS analysis showed that the inhibition of MoDC development was associated with the addition of tumor cell-conditioned supernatants and not with the addition of leukocyte-conditioned supernatants (Fig. 4). Therefore, it can be concluded that the infiltrating immune effector cells were not responsible for the observed suppression. This corresponds with the presence of PGE2 in the CD45-negative supernatants (at similar levels to the supernatants of the unseparated cell suspensions) and its absence (i.e., below detection level) in the CD45+ leukocyte-conditioned supernatants (data not shown; n = 3). Similarly, IL-6 release, partly responsible for suppression in the CD34+ model system, was most likely tumor derived, as IL-6 could not be detected in the infiltrate-conditioned supernatant, while the CD45-negative cell-conditioned supernatants contained high concentrations of IL-6 (data not shown).

**Differential COX-1/COX-2 dependence of prostanoid-induced DC suppression between normal and malignant colon tissue**

To ascertain any restriction of prostanoid-induced DC suppression to tumor tissues, normal tissue samples were collected from colon...
tumor resection specimens. Both normal and tumor single-cell suspensions were thus generated from the same surgical samples. Supernatants derived from normal colon single-cell suspensions contained PGE$_2$ in active, but significantly lower, concentrations than their corresponding malignant counterparts (146 ng/ml (range, 57.6–319 ng/ml) vs 282 ng/ml (range, 142–424 ng/ml), respectively; $n = 9; p = 0.009$ in a paired t test). Accordingly, suppressive effects of normal colon cell-derived supernatants (NDSN) were clearly detectable, but were less profound than those of TDSN (Fig. 5). Testing the effect of coculture of the normal or tumor cells with the specific COX-1 inhibitor SC560 revealed the suppressive effect exerted by the NDSN to be fully COX-1 dependent (Fig. 5A). In contrast, the inhibitory effect of the TDSN could only partially be eliminated through coculture with SC560. Complete elimination of the suppressive effect required simultaneous COX-1 and COX-2 inhibition by the indiscriminate COX inhibitor indomethacin, indicating the additional involvement of COX-2 in the production of tumor-derived prostanoids (Fig. 5A). In Fig. 5B the mean values from four NDSN/TDSN couples are shown, revealing a significant difference in CD1a and CD14 expression between MoDC generated in the presence of NDSN or TDSN, which were derived from colon cultures with the COX-1 inhibitor SC560 ($p = 0.037$ and $p = 0.004$, respectively).

**Suppression of DC differentiation by tumor-derived prostanoids is not colon restricted**

Although the DC differentiation-inhibitory effect of IL-6 was previously reported for tumor cell lines derived from various tissue types, a similar effect for primary tumor-derived prostanoids is a novel finding. To exclude that the inhibitory effect of the tumor-derived prostanoids on the DC development was colon-restricted, supernatants conditioned by mamma ($n = 2$) and renal cell ($n = 2$) carcinomas, and by melanomas ($n = 2$), in the absence or presence of indomethacin, were similarly tested in the MoDC and CD34$^+$ model systems. Similar inhibitory effects were observed, regardless of the tissue origin of the tested tumors (Fig. 6). CD1a expression was inhibited, CD14 expression was maintained (in the MoDC model) or acquired (in the CD34$^+$ model), and SSC values remained low when TDSN conditioned in the absence of indomethacin were used (consistent with the conservation or acquisition of the monocyte phenotype and morphology). The opposite was observed after the addition of TDSN conditioned in the presence of indomethacin (evidence of the acquisition of DC phenotype and morphology). As before, prostanoids appeared to only partially contribute to the observed inhibition of differentiation in the CD34-derived DC model (Fig. 6B).

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**FIGURE 5.** DC-suppressive prostanoids derived from normal and malignant colon tissue are differentially regulated by COX-1 and COX-2. A, Effects of a NDSN and a corresponding TDSN (conditioned by tissues from one surgical resection specimen) on the development of MoDC in a 7-day differentiation culture. Also shown are the effects of the addition of the selective COX-1 inhibitor SC560 and the nonselective COX-1/2 inhibitor indomethacin during the generation of the NDSN and TDSN. B, The effect of addition of NDSN vs corresponding TDSN in MoDC cultures on CD14/CD1a expression (shown as a percentage positive cells; $n = 4$) and the effect of the addition of the selective COX-1 inhibitor SC560 during the generation of the NDSN and TDSN. Significant differences in the paired $t$ test was as follows: *, CD14 expression, TDSN vs NDSN, $p = 0.028$; **, CD14 expression, TDSN plus SC560 vs NDSN plus SC560, $p = 0.037$; ***, CD1a expression, TDSN plus SC560 vs NDSN plus SC560, $p = 0.004$.

**FIGURE 6.** The dominant role of tumor-derived prostanoids in the inhibition of DC differentiation is not restricted to tumors of colon origin. Phenotypic FACS analysis is shown of the CD1a and CD14 expression on MoDC (A) and CD34-derived DC (B), generated in the presence of TDSN conditioned by mamma and renal adenocarcinoma, or melanoma cells, in the presence or absence of the COX-1/2 inhibitor indomethacin. The results shown are representative of two tested tumors for each tissue origin.
T cell-stimulatory capacity of DC differentiated in the presence of TDSN: the effect of tumor-derived prostanoids and IL-6. A, MoDC or CD34-DC were differentiated in the presence or absence of TDSN (n = 3) and were added at the indicated ratios to 50,000 allogeneic plastic-nonadherent PBL. B, TDSN were generated in the presence or absence of indomethacin (IM) and were added to DC cultures (10% v/v) with or without neutralizing Abs against IL-6 (anti-IL-6). Thus generated MoDC or CD34-DC were tested in the MLR. Results shown are from the highest PBL:DC ratio (50,000:3,000) and are representative of three experiments. After 5 days T cell proliferation was determined. Results are means ± SD of triplicate cultures.

**FIGURE 7.** Allogeneic T cell-stimulatory capacity of DC generated in the presence of TDSN: the effect of tumor-derived prostanoids and IL-6. A, MoDC or CD34-DC were differentiated in the presence or absence of TDSN (n = 3) and were added at the indicated ratios to 50,000 allogeneic plastic-nonadherent PBL. B, TDSN were generated in the presence or absence of indomethacin (IM) and were added to DC cultures (10% v/v) with or without neutralizing Abs against IL-6 (anti-IL-6). Thus generated MoDC or CD34-DC were tested in the MLR. Results shown are from the highest PBL:DC ratio (50,000:3,000) and are representative of three experiments. After 5 days T cell proliferation was determined. Results are means ± SD of triplicate cultures.

**FIGURE 8.** The expression levels of HLA-DR, CD86, and CD54 on MoDC and on CD34-DC are differentially affected by TDSN: the effect of tumor-derived prostanoids and IL-6. CD86, HLA-DR, and CD54 expression (in fluorescence intensity) are shown for MoDC (A) and CD34-DC (B) generated under the indicated conditions. The markers indicate the corresponding isotype controls. Results are representative of three experiments.
of IL-12 production by MoDC differentiated in the presence of TDSN, after stimulation by CD40L and IFN-γ. However, this effect could only be partially prevented by indomethacin (complete prevention was observed in one of the three TDSN tested). In contrast, IL-10 release by MoDC was increased as a result of the addition of TDSN during differentiation, and in all cases this could be prevented by indomethacin during the generation of TDSN (Fig. 9A). IL-12:IL-10 ratios clearly demonstrate the favored development of DC that are more skewed toward the induction of type-2 T cell responses under the influence of tumor-derived prostanoids (Fig. 9B). CD40L stimulation without IFN-γ resulted in the production of considerably lower amounts of IL-12, which dropped below detection levels in the TDSN conditions, and higher amounts of IL-10, but the same effects of the (indomethacin-conditioned) TDSN were observed in both cases (data not shown).

Discussion

Despite the demonstration that a variety of tumor cell lines have the potential to hamper the development of DC through the action of soluble factors (15, 17–19), until now no studies have assessed whether this is also the case for primary tumor cells. In this study, we have demonstrated that the suppression by primary TDSN is mediated mainly through prostanoids. In our model system, the tested colon tumor cell line-derived supernatants showed only a minor ability to prevent monocytic or CD34⁺ precursor cells from adopting DC properties, and they could not prevent the concomitant loss of CD14 expression. In contrast, at equivalent concentrations primary colon TDSN completely blocked GM-CSF- and IL-4-induced DC differentiation so that CD14⁺ precursors were arrested in an early stage of development. Prostanoids present in the colon TDSN were found to play a dominant role in this process. The identification of prostanoids as the major culprit in the primary tumor-induced inhibition of DC differentiation is a novel finding.

Prostanoids are derived from membrane-associated arachidonic acids. COX proteins control the rate of prostanoid synthesis by catalyzing the conversion of arachidonic acid to PGH₂, the common PG precursor. COX-1 is constitutively expressed, while COX-2 is induced by a variety of proinflammatory signals (28). In various tumor types, including colon, breast, lung, and melanoma, overexpression of COX-2 has been observed (21, 22, 29–31). Moreover, high levels of COX-2 expression correlate to poor clinical outcome. In keeping with these findings, we found a significant contribution of COX-2-regulated prostanoids to primary colon tumor-induced inhibition of DC development. In contrast, the significantly less pronounced inhibitory effect of soluble factors derived from adjacent nonmalignant tissue could be completely blocked by the COX-1 inhibitor SC560. Numerous reports have drawn attention to a role for prostanoids (most notably for PGE₂) in the carcinogenesis of solid tumors. Nonsteroidal anti-inflammatory drugs, which inhibit their synthesis and of which indomethacin is an example, have been reported to reduce the risk of developing colorectal cancer (32). Our results indicate that prostanoids may also contribute to carcinogenesis through the inhibition of DC development, thus seriously crippling immune surveillance. This certainly ties in with numerous reports on hampered DC development in cancer patients and a negative prognostic
value of reduced numbers of tumor-infiltrating DC (12, 14, 33). Previous reports indicate that DC are most sensitive to the modulatory effects of PGE2 (34). Because this is the most prominently expressed prostanoid and its concentration in the tested supernatants correlated well with the observed effects (data not shown), it seems most likely that this is the agent responsible for the observed inhibition of DC differentiation in our model systems. Although PGE2 production by monocytes has also been observed, we clearly established that the intratumoral leukocyte infiltrate was not involved in the DC suppression. By the same token, PGE2-induced IL-10 release by tumor-infiltrating lymphocytes could not be responsible, in keeping with the finding that IL-10 neutralization could not abrogate the inhibition of DC development. A direct inhibition of DC differentiation by PGE2 was previously described (35). In contrast with this inhibitory ability, PGE2 can actually stimulate DC maturation. This may account for the observed increased allogeneic T cell stimulation by MoDC generated in the presence of TDSN, even though they were obviously stunted in their differentiation. This increased stimulatory ability was found to be related to a prostanoid-induced increase in expression levels of CD86 and HLA-DR. Recent studies also point to autocrine stimulation of DC by PGE2 and its importance in reaching full phenotypic and functional maturity (36). However, these autocrine production levels are ~1000-fold lower than the PGE2 concentration in TDSN. Moreover, we found no effect on DC differentiation of direct coculture with indomethacin (data not shown). A tumor-associated increase in phenotypic MoDC activation, characterized by an up-regulation of, among others, CD86 and HLA-DR, was also previously reported by Kiertscher et al. (19) and Menetrier-Caux et al. (37), both using tumor cell line-derived supernatants. Maturation induction of MoDC thus appears to be a common feature of soluble tumor-derived products from different sources, as reported by different research groups, and is in marked contrast to effects observed on CD34-derived DC (17, 37). Although tumor-derived soluble factors may increase maturation and T cell-stimulatory capacity of MoDC, the simultaneous reduction in IL-12 production and increase in IL-10 production will most likely lead to the generation of a deleterious type-2 antitumor T cell response. We found this tumor-induced shift in IL-12:IL10 ratio to be mostly prostanoid dependent. This is in keeping by findings of Kalinski et al. (38), who showed that PGE2-induced maturation skewed DC toward the preferential induction of a type-2 T cell response. In addition, Kiertscher et al. (19) reported the induction of apoptosis in MoDC through coculture with tumor cell line-derived supernatants. We were unable to reproduce this finding in our DC differentiation model systems (apoptosis determined by annexin V/pro-pidium iodide staining; data not shown) by coculture of cell line supernatants or of primary TDSN.

The maturational effect of TDSN appears to depend on the hematopoietic precursor origin of the DC, because TDSN inhibited rather than increased the allogeneic T cell-stimulatory ability of CD34-derived DC. Again, this effect correlated to CD86 and HLA-DR expression levels, which remained low in the presence of TDSN, in keeping with previous reports by Menetrier-Caux and colleagues (17, 37). The finding that CD54 showed opposite expression patterns from CD86 and did not correlate to T cell-stimulatory capacity of the DC is in keeping with previous reports that CD86 plays a more important role in the induction phase of the immune response (39).

The properties of prostanoids, and more specifically PGE2, with regard to the inhibition of DC differentiation and the skewing of type-1 to type-2 T cell responses, have been reported as essential to the maintenance of tolerance against food-derived Ags in the gut mucosa (40). Indeed, although significantly elevated in supernatants from malignant colon tumors, we also found high levels of PGE2 in supernatants from normal colon tissue cultures. To establish that the dominant effect of tumor-derived prostanoids on DC development was not merely a colon-associated trait, we also studied the effects of TDSN from breast, renal, and melanoma origin. A similarly overriding influence of prostanoids was found for these TDSN, in accordance with previous reports of elevated levels of PGE2 in these tumor types. Thus, prostanoid-induced inhibition of DC differentiation appears to be a generalized tumor-associated phenomenon.

The inability of the tested tumor cell lines to suppress DC development may be due to their inability to produce sufficient levels of prostanoids. Indeed, in one early passage tumor cell line generated from a primary colon tumor in our lab, we saw a decline in PGE2 levels with relatively low levels still detectable at passage number 4 (886 pg/ml). By passage number 6 PGE2 levels had dropped below the detection limit (data not shown). These observations indeed suggest that differences in PGE2 production between primary tumors and cell lines derive from in vitro propagation, which may result in methylation of the COX-2 gene, as previously described (21, 22). However, that this may not always be the case is indicated by previous reports of high-level prostanoid production by melanoma cell lines (21). Similarly, we observed only low levels of suppressive IL-6 in the five colon tumor cell lines we tested, while more heterogeneous levels were previously reported (17). Therefore, we conclude that expression of the various DC-inhibitory factors can be very heterogeneous among cell lines but appears to be more consistently high in primary tumors.

Previous studies using tumor cell lines implicated IL-6, M-CSF (17), VEGF (18), and recently gangliosides (41) as tumor-derived factors inhibiting the development of DC. This was observed with both MoDC and CD34-derived DC. However, our results clearly show that in primary TDSN prostanoids play a dominant, overriding role. Although present at potentially active concentrations, no role for either IL-10 or IL-6, determined by incubation with neutralizing mAbs, could be established in the inhibition of MoDC differentiation by primary TDSN, not even after the release of prostanoids was blocked by indomethacin. The presence of high concentrations of IL-4 (1000 U/ml) in our MoDC cultures may largely explain this. Indeed, Menetrier-Caux and colleagues (17, 37) demonstrated that the inhibition of differentiation of MoDC and CD34-derived DC by supernatants of renal cell carcinoma lines was directly mediated by IL-6, but that IL-4 protected against this inhibition. The inhibitory effect described by Menetrier-Caux and colleagues (17, 37) was related to up-regulation of the M-CSF receptor CD115 and down-regulation of the GM-CSF receptor CD116 on the developing DC. Again, our use of high concentrations of IL-4 during DC differentiation may explain why we did not observe these effects in our system, neither with the use of cell line supernatants nor using primary TDSN (data not shown). We did observe a clear IL-6-mediated effect in the CD34-derived DC model, even in the presence of IL-4. However, to completely abrogate inhibition of DC differentiation in this model, both prostanoids and IL-6 had to be eliminated from the primary TDSN. In contrast, we found an overriding role of primary tumor-derived IL-6 in the inhibition of DC development from CD34+ precursors in the absence of IL-4 in comparison to tumor-derived prostanoids, which is in keeping with findings by Menetrier-Caux and colleagues (17, 37) (C. Sombroek, F. van den Eertwegh, R. Scheper, and T. de Grujil, manuscript in preparation).

The relative contribution of the different suppressive factors to the cancer-associated inhibition of DC development in vivo remains to be assessed. Almand et al. (42) showed that decreased
numbers of functional DC in the blood and regional lymph nodes of cancer patients correlated well with tumor stage and duration of the disease, as well as with serum levels of VEGF, which is indicative of a suppressive systemic effect of VEGF. We were unable to establish an inhibitory effect of primary tumor-derived VEGF, which was present in the TDSN at subactive levels in vitro. However, the in vivo DC-inhibitory activity of VEGF has been reported at considerably lower concentrations than in vitro (11). Therefore, it is conceivable that the relatively low VEGF levels in the primary TDSN were not sufficient for an in vitro effect but might still be able to affect DC development in vivo. Similar studies correlating PGE2 serum levels to DC number and activation status in vivo need to be performed to establish a possible systemic effect of tumor-related prostanooid overproduction and hampered DC differentiation. Alternatively, prostanooids may exert a profound influence on local DC functions. In this regard, the contrasting phenotypic effects of prostanooids on the processes of DC differentiation and maturation of monocyctic or CD34+ progenitors deserve further study.

This study, as well as previous studies, determined the effects of soluble tumor factors on DC development. However, a recent report by Chomarat et al. (43) revealed that physical contact with tumor cells and/or fibroblasts skewed the differentiation of monocytes from T cell-stimulatory DC to scavenging macrophages without T cell-stimulatory capacities. This phenomenon was found to be IL-6 dependent. It is clear that interactions between soluble and contact-dependent factors will require further study to unravel the intricate processes leading up to the tumor-induced inhibition of DC development; however, it is equally clear that, besides tumor-derived cell lines, such studies should make use of primary tumor cells and TDSN to draw conclusions that more reliably reflect the in vivo situation. Based on our findings, an important role for primary tumor-derived prostanooids is certainly indicated. Clearly, the correct identification of the factors responsible for hampered DC development in cancer patients is of utmost importance for the selection of the appropriate immunotherapeutic approaches to overcome this defect (1, 44, 45).

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References


