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M2 Macrophages Induced by Prostaglandin E2 and IL-6 from Cervical Carcinoma Are Switched to Activated M1 Macrophages by CD4⁺ Th1 Cells

Moniek Heusinkveld,* Peggy J. de Vos van Steenwijk,† Renske Goedemans,* Tamara H. Ramwadhdoebe,* Arko Gorter,‡ Marij J. P. Welters,* Thorbald van Hall,* and Sjoerd H. van der Burg*

Monocytes attracted by tumor-induced chronic inflammation differentiate to APCs, the type of which depends on cues in the local tumor milieu. In this work, we studied the influence of human cervical cancer cells on monocyte differentiation and showed that the majority of cancer cells either hampered monocyte to dendritic cell differentiation or skewed their differentiation toward M2-like macrophages. Blocking studies revealed that M2 differentiation was caused by tumor-produced PGE₂ and IL-6. TGF-β, IL-10, VEGF, and macrophage colony-stimulating factor did not play a role. Notably, these CD14⁺CD163⁺ M2 macrophages were also detected in situ. Activation of cancer cell-induced M2-like macrophages by several TLR-agonists revealed that compared with dendritic cells, these M2 macrophages displayed a tolerogenic phenotype reflected by a lower expression of costimulatory molecules, an altered balance in IL-12p70 and IL-10 production, and a poor capacity to stimulate T cell proliferation and IFN-γ production. Notably, upon cognate interaction with Th1 cells, these tumor-induced M2 macrophages could be switched to activated M1-like macrophages that expressed high levels of costimulatory molecules, produced high amounts of IL-12 and low amounts of IL-10, and acquired the lymphoid homing marker CCR7. The effects of the interaction between M2 macrophages and Th1 cells could partially be mimicked by activation of these APCs via CD40 in the presence of IFN-γ. Our data on the presence, induction, and plasticity of tumor-induced tolerogenic APCs in cervical cancer suggest that tumor-infiltrated Th1 cells can stimulate a tumor-rejecting environment by switching M2 macrophages to classical proinflammatory M1 macrophages. The Journal of Immunology, 2011, 187: 000–000.

C ervical cancer (CxCa) is induced by human papillomavirus (HPV) (1). In many cases, the development of CxCa is associated with a weak systemic and local immune response to HPV, reflected by low numbers of tumor-infiltrating T cells that comprise functionally impaired Th cells and regulatory T cells (2–7). When the tumor-specific immune response is stronger and more in favor of a Th1/CTL response, this is associated with an improved prognosis (4, 8–10).

Tumors foster a tolerant microenvironment by the activation of a plethora of immunosuppressive mechanisms, including the modulation of APCs that otherwise may stimulate adaptive immunity against cancer (11). Monocytes are attracted by the chronic inflammation caused by tumors and differentiate into a variety of tumor-associated macrophage (M2 macrophage) and dendritic cell (DC) subtypes depending on local mediators (12–14). Factors secreted by tumor cells that have been implicated in the prevention or modulation of DC differentiation and/or function are VEGF, macrophage colony-stimulating factor (M-CSF), TGF (TGF-β1), IL-10, IL-6, and prostanooids (e.g., PGE₂) (12). CxCAs are known also to secrete immunomodulatory compounds, but their effect on APCs is yet unknown (15–19).

Therefore, we studied the effect of CxCa cells on monocyte differentiation and function. We found that DC differentiation was hampered or even skewed toward the tolerogenic M2 macrophages by tumor-derived PGE₂ and IL-6. Subsequently, we assessed the effects of APC activation by several different TLR agonists, which are currently used or tested for the treatment of cancer in human beings (20), CD40 stimulation, or cognate interactions with Th1 cells. Notably, the interaction with Th1 cells resulted in a switch to activated M1-like macrophages expressing high levels of costimulatory molecules and producing high amounts of IL-12p70. Our data suggest that a highly immune-stimulatory local microenvironment might be achieved by using cyclooxygenase (COX) inhibitors and IL-6 blocking Abs to prevent M2 differentiation and vaccine-mediated stimulation of Th1 cells to switch M2 macrophages to tumor-rejecting M1 macrophages.

Materials and Methods

Immunofluorescent staining of tumor tissue

Ten patients with CxCa with FIGO stage I and II underwent radical hysterectomy (type III) at our hospital. Patients had not received radiotherapy or chemotherapy before surgery. Tumors were HPV typed by PCR and sequencing, as described previously (21). The use of clinical material was...
approved by the institutional review board according to the guidelines of the Dutch Federation of Medical Research Associations.

Staining was performed on 4-μm tissue sections of formalin-treated and paraffin-embedded tumor material. Immunostaining was performed with an mAb mix of anti-CD14 (clone 7; Abcam) and anti-CD163 (clone 10D6; Novocastra) and, after incubation overnight, staining with fluorescent Ab conjugates (IgG2a–Alexa Fluor 488 and IgG1–Alexa Fluor 647; Invitrogen) (22). Control staining with only secondary Abs was included to ensure specificity. Images were captured at ×25 magnification with a confocal laser scanning microscope (Zeiss LSM 510) in a multitrack setting.

Media and reagents

APCs and tumor cell lines were grown in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Greiner bio-one), penicillin/streptomycin (Invitrogen), and L-glutamine. The following factors and final concentrations were used to generate APCs: 500 U/ml IL-4, 800 U/ml GM-CSF (Life Technologies), and 1–50 ng/ml PGE2 (Sigma-Aldrich). TLR ligands used for activation were 2.5 μg/ml polyinosinic:polycytidylic acid [poly(I:C)], 10 μg/ml R848/CL097 (all from Invivogen), and 0.25 μg/ml LPS (Sigma-Aldrich). Optimal concentrations were used based on maximal cytokine release in monocyte-derived DCs (mo-DCs). To mimic T cell interaction, APCs were stimulated with irradiated CD40L expressing mouse fibroblasts (23).

Blockade of TGF-β signaling was achieved with 1 μM SB431542 hydrate (Sigma-Aldrich) after optimization of the dose. IL-6 was blocked by adding 2.5 μg/ml Ab to IL-6 receptor (clone B-R6) and 2.5 μg/ml Ab to IL-6 (B-E8; Abcam) to the culture.

Supernatant of cervical cancer cell lines

To confirm the origin of the established lines HeLa and CaSkI, lines were tested for the presence of integrated HPV16 or HPV18 DNA using the INNO-LiPa HPV Genotyping procedure (Innogenetics). CSCC-1, CSCC-7, CC-8, CC-10B, and CC-11 were typed and cultured as described earlier (24). Stock ampoules were thawed and cultured for 10 passages and tested for the presence of mycoplasma monthly.

Cell lines were grown in flasks at 80–90% confluence and harvested with trypsin/EDTA. One hundred thousand cells were plated in 2 ml well of 6-well culture plate and cultured for 5 d. Supernatant was stored at −20°C. In case cultures were treated with COX inhibitors, 250,000 cells in 2 ml were plated in 6-well plates in the presence of 25 μM indomethacin or 5 μM NS-398 (Cayman Chemical) dissolved in DMSO or as a control only with the corresponding concentration of DMSO. Medium was replaced after 24 h and then harvested after 24 h of culture.

DC culture

PBMCs were obtained from buffy coats of healthy donors. CD14+ monocytes (>95% purity) were isolated using MACS cell separation (Miltenyi Biotec) and stored in liquid nitrogen until further use. Monocytes were thawed and cultured in 48- or 24-well plates in a density of 0.25 million or 0.5 million cells/well, respectively, in the presence of IL-4 and GM-CSF (mo-DC). After 2 d, fresh medium with cytokines was added. At day 5–6, the cells were analyzed for differentiation by flow cytometry and activated in the culture medium or harvested, washed, and activated in fresh medium. Tumor supernatant (TSN)-APCs were cultured as described above, but 20% supernatant of tumor cell lines or medium was added. Titrations showed that 20% supernatant gave the best reproducible results between donors. DCs were activated at day 6, and after 48 h the supernatant was harvested and stored at −20°C for cytokine analysis, and cells were stained for flow cytometric analysis.

Table I. Immunosuppressive factors produced by CxCa cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Passage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cytokine Production (pg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-10B</td>
<td>P80</td>
<td>45</td>
<td>IL-8: 14,000, TGF-β1: 0, PGE&lt;sub&gt;2&lt;/sub&gt;: 0, IL-6: 0</td>
</tr>
<tr>
<td>CCSC-1</td>
<td>P22</td>
<td>16</td>
<td>IL-8: 1,500, TGF-β1: 221, PGE&lt;sub&gt;2&lt;/sub&gt;: 0, IL-6: 2</td>
</tr>
<tr>
<td>CC-11</td>
<td>P32</td>
<td>67</td>
<td>IL-8: 425, TGF-β1: 398, PGE&lt;sub&gt;2&lt;/sub&gt;: 403, IL-6: 3,438</td>
</tr>
<tr>
<td>CaSkI</td>
<td>x</td>
<td>16</td>
<td>IL-8: 324, TGF-β1: 410, PGE&lt;sub&gt;2&lt;/sub&gt;: 298, IL-6: 2,335</td>
</tr>
<tr>
<td>CCSC-7</td>
<td>P9</td>
<td>16</td>
<td>IL-8: 9,306, TGF-β1: 313, PGE&lt;sub&gt;2&lt;/sub&gt;: 2,781, IL-6: 263</td>
</tr>
<tr>
<td>CC-8</td>
<td>P41</td>
<td>45</td>
<td>IL-8: 1,222, TGF-β1: 250, PGE&lt;sub&gt;2&lt;/sub&gt;: 3,662, IL-6: 2,228</td>
</tr>
<tr>
<td>HeLa</td>
<td>x</td>
<td>18</td>
<td>IL-8: 4,500, TGF-β1: 548, PGE&lt;sub&gt;2&lt;/sub&gt;: 5,389, IL-6: 12,000</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of passages since origin.
<sup>b</sup>Integrated DNA found to be present in the tumor (24).
<sup>c</sup>Latent form of TGF-β1 measured by ELISA after acidic treatment.

FIGURE 1. CxCa-secreted factors skew monocyte differentiation toward M2 macrophages. A. Monocytes were cultured for 6 d with GM-CSF and IL-4 in culture medium with 20% TSN of indicated CxCa cell lines or 20% control medium (mo-DC) and analyzed for expression of CD14 and CD1a. TSN reduced CD1a expression and in three cases induced CD14 expression. The cultures of monocytes differentiated with CD14+ cell-inducing TSN were analyzed for the expression of typical M2 macrophage markers. Numbers in quadrants represent percentage of cells within live gate. One representative of five. C. In situ immunofluorescence staining of cervical tumor tissue of 3 of 10 different patients for CD14 (green) and CD163 (blue) and measured by confocal microscopy (original magnification ×25). CD14+CD163+ display as turquoise; white arrows indicate M2 macrophages.
MLR
Naive CD4 cells were isolated from PBMCs by CD25+ cell depletion using MACS and subsequently isolation of CD4+ cells with the DynalBead system (Invitrogen) to a purity of >99%. These CD4+CD25+ cells were plated in a 96-well plate at 50,000 cells per well. Matured DCs were added at different doses up to 10,000 cells/well in triplicate. T cell proliferation was measured after 5 d by [3H]thymidine incorporation (0.5 µCi/well). Supernatant was taken at several time points and stored at −20°C for cytokine analysis.

CD4+ T cell helper clones
HPV-specific CD4+ T cell clones were obtained by limited dilution of lymph node cells of a patient with an HPV16+ cervical tumor. Clones were stimulated every 2 wk with B-LCL loaded with the cognate HPV peptide, feeder cells, TCGF, and IL-15. Clones were used for DC activation after a 2.5-wk resting period.

Clone 214 recognized HPV16E6 aa 61–82, clone 238 recognized HPV16 aa 61–82, and clone 16 recognized HPV16E6 aa 11–32, and all clones produced IFN-γ and IL-2 but only clone 238 produced IL-10 upon Ag-specific activation. HLA class II-matched APCs were loaded with an irrelevant or the cognate peptide for CD4+ Th1 clones and cocultured at different DC/T cell ratios in medium containing 20% TSN. After 48 h, supernatant was analyzed, and APCs were phenotyped.

Flow cytometry
Mouse mAbs to human CD80, CD86, HLA-DR, CD206, CD1c (FITC) and CD83, CD86, CD14, CD16, CD163 (PE) and CD14, HLA-DR (PerCP) or CD11c, CD1a, CD4 (allophycocyanin) (all from BD Biosciences) and CD83, CD86, CD14, CD163 (R&D Systems) and programmed cell death ligand 1 (PD-L1) (eBioscience) were used. Cells were recorded (20,000/live gate) using a BD FACSCalibur with CellQuest software (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Cytokine analysis
IL-12p40 and IL-12p70 were analyzed using ELISA kits from BD Biosciences or by inflammatory CBA (BD Biosciences). IL-10 and IFN-γ were measured with ELISA (Sanquin). To evaluate the cytokines present in

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** TSN-differentiated monocytes are phenotypically and functionally different from mo-DCs. A, Monocytes were differentiated in the presence of the indicated TSN and activated with single TLR ligands or CD40L cells for 48 h. TSN-M2 cells express higher basal levels of CD86 and PD-L1. R848 or poly(I:C) cannot induce equal expression of CD80 and CD86 in all APC types. B, Supernatants of the cultures described in A were tested for the presence of IL-12p70 and IL-10 revealing that TSN-M2 cells produce more IL-10 and less IL-12 compared with mo-DCs. C, CD4+CD25+ allogenic responder T cells were cultured with activated mo-DCs or TSN-M2 cells (obtained with TSN from HeLa cells) at indicated ratios (DC/T cell). Top row shows the higher proliferation of T cells when stimulated with activated mo-DCs at day 5 as measured by [3H]thymidine uptake. Test performed in triplicate; shown is mean with SEM. Means were compared by unpaired t test. *p < 0.05, **p < 0.005. Bottom row shows the IFN-γ production within these cultures measured in the supernatant isolated at day 4 by ELISA. One representative of three experiments (A–C).
Results

DC differentiation is altered by cervical carcinoma cells

To explore the effects of soluble factors secreted by CxCa on the differentiation of monocytes, in vitro cultures were set up to analyze the direct effect of TSN derived from five early-passage CxCa cell lines (Table I) (17, 24) and the two well-known cell lines CaSkii and HeLa. GM-CSF– and IL-4–differentiated mo-DCs are defined as APCs that lack the expression of CD14 but display the lineage marker CD11c, HLA class I and II, and CD1a. Healthy donor-derived monocytes were differentiated in the presence of 20% TSN of the seven tumor cell cultures. The presence of TSN had a striking effect on their differentiation as shown by evaluation of surface marker expression typical for monocytes, DCs, MDSCs, and macrophages. mo-DC cultures typically contain >80% CD14+ cells, but when monocytes were differentiated in the presence of TSN from CC-11, CSCC-1, or CaSkii, this percentage dropped, reflecting poor DC differentiation (Fig. 1A). TSN of the cell lines CSCC-7, CC-8, and HeLa not only hampered CD1a expression but also skewed the differentiation of monocytes toward the macrophage lineage as reflected by the high expression of CD14. Further evaluation of these CD14+ cells revealed that they expressed CD163 and CD206 as well. Notably, these TSN-induced cells expressed all human Fcγ receptors (CD16, CD32, and CD64) as well as PD-L1 and HLA class II, whereas CD11b and CD1c were absent (Fig. 1B and data not shown). This profile is highly similar to that of in vitro M-CSF–induced M2 macrophages and distinct from mo-DCs (Supplemental Fig. 1) (13, 25). TSN of cell line CC-10B did not overtly alter the differentiation of monocytes to DCs, indicating that CxCa supernatant does not per se result in phenotypical changes. Analysis of CD33, CD11b, and CD124 expression revealed no evidence for skewing of monocytes to MDSCs (data not shown).

To verify that these different cell types reside in the tumors of patients, paraffin-embedded tissue sections of 10 patients with FIGO stage I or II CxCa were stained for macrophages (CD14+) and data not shown). This profile is highly similar to that of in vitro M-CSF–induced M2 macrophages and distinct from mo-DCs (Supplemental Fig. 1) (13, 25). TSN of cell line CC-10B did not overtly alter the differentiation of monocytes to DCs, indicating that CxCa supernatant does not per se result in phenotypical changes. Analysis of CD33, CD11b, and CD124 expression revealed no evidence for skewing of monocytes to MDSCs (data not shown).

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Functional impairment of APCs by TSN

Next, we assessed the capacity of these tumor-modulated APCs to respond to five different TLR agonists or CD40L-expressing fibroblast cells (CD40L) to mimic T cell interaction. Because the supernatant of tumor cells, IL-6, IL-8, and IL-10 were determined by CBA and M-CSF by Bioplex (Bio-Rad), PGE2 was measured with the PGE2 parameter assay kit (R&D Systems) and TGF-β1 with the human TGF-β1 ELISA (eBioscience). Samples were tested with and without acidic treatment to determine active and latent TGF-β1 in the cultures.

DCs produced the biologically active IL-12p70 upon stimulation with LPS, R848, or CD40L cells. The amounts varied per donor from whom the mo-DCs were prepared (Fig. 2B, Supplemental Fig. 2). Relatively to stimulated mo-DCs, the TSN-M2 cells almost completely lacked the ability to produce IL-12p70 when stimulated with TLR agonists. The strong activation signal induced by CD40L cells allowed TSN-M2 cells to produce IL-12p70, albeit at significantly lower concentrations than those of CD40-stimulated mo-DCs. In contrast, the production of the cytokine IL-10, which varied greatly between donors, was increased at least 2-fold when TSN-M2 cells were stimulated with TLR agonist or with CD40L cells in two of three experiments (Fig. 2B, Supplemental Fig. 2). The alterations in APC function were imprinted during the differentiation of the monocytes, as activation of TSN-M2 cells in fresh medium without additives gave similar results (data not shown).

FIGURE 3. COX inhibition blocks PGE2 production and prevents M2 macrophage differentiation. A. Tumor cell lines were treated for 24 h with DMSO (control) or COX inhibitor (NS-398 or indomethacin) followed by culturing in fresh medium without additives for 24 h. PGE2 levels were measured by ELISA. Inhibition of COX totally abrogates PGE2 production. The p values were <0.005 for all comparisons of PGE2 production by cells treated or not with indicated COX inhibitor. B. Culturing mo-DCs (black fill) in the presence of 10 ng/ml PGE2 (dotted line) induces CD14+CD163+–expressing APCs. C. Flow cytometric analysis of CD14+ and CD163+–associated M2 macrophage marker expression on monocytes differentiated in the presence of TSN of indomethacin-treated tumor cells (black) or TSN from nontreated tumor cells (white). D. Comparison of CD1a+ and CD14+–expressing populations after the differentiation of monocytes in the presence of TSN of indomethacin-treated or nontreated tumor cells shows that TSN of COX inhibitor-treated cell lines induces fewer CD14+ cells and more CD1a+ DCs. Representative of three experiments (B–D).
FIGURE 4. Restored cytokine production when PGE2 production by tumor cells is blocked. A, The production of IL-12 and IL-10 by APCs differentiated in the presence of TSN of indomethacin-treated tumor cells (black) or TSN from nontreated tumor cells (white) upon 48 h activation with LPS, R848, or CD40L as measured by ELISA. Top row shows the cytokine production of untreated mo-DCs. One representative experiment of three. The blocking of PGE2 production by tumor cells alters the balance in IL-12 and IL-10 toward that observed in the corresponding mo-DCs. B, Cytokine production by APCs differentiated in the presence of TSN from nontreated tumor cells (white) compared with cytokine production by APCs differentiated in the presence of TSN from indomethacin-treated tumor cells (black) that were activated for 48 h (combined data of LPS, R848, and CD40L stimulated cells; n = 3 experiments). *p < 0.05, **p < 0.01. IL-12, all p < 0.003. IL-10, TSNcc7, p = 0.015; TSNcc8, p = n.s.; TSNhela, p = 0.026. C, mo-DCs or TSN-M2 cells obtained with TSN of untreated (+DMSO) or indomethacin-treated (+Indomethacin) HeLa cells were activated with LPS or R848 for 48 h and used to stimulate allogeneic responder cells. APCs differentiated in TSN of indomethacin-treated cells induce better T cell proliferation at day 5 of culture as compared with nontreated cells, indicating that TGF-β was not responsible for the observed effects of TSN (Supplemental Fig. 3).

Depletion of PGE2 in TSN by preventing its production revealed a striking effect on the DC differentiation of monocytes. The expression of CD14 and CD163 was completely reversed (Fig. 3C), but the phenotypic differentiation toward DC was only partly restored as indicated by the percentage of CD14+CD1a+ APCs that was still lower than that observed in mo-DC cultures (Fig. 2). Subsequently, the capacity of TSN-M2 cells to induce proliferation and cytokine production of T cells was compared with that of mo-DCs. Graded doses of APCs were cocultured with a fixed number of allogeneic CD4+CD25- T cells. Clearly, the activated TSN-M2 cells displayed a lower capacity to induce T cell proliferation and/or concomitant IFN-γ release (Fig. 2C). The percentage of CD25-Foxp3+ T cells, which can be increased upon stimulation with immature APCs (30), was not clearly altered after 10 d of culture with TSN-M2 cells (data not shown). These results indicated that TSN-skewed APCs were both phenotypically and functionally shifted toward that of M2 macrophages and that TLR-mediated activation of TSN-M2 cells reinforced their tolerogenic profile.

Mediators of altered APC differentiation

Numerous mediators may cause the altered differentiation of monocytes to DCs, including TGF-β, PGE2, IL-6, IL-8, IL-10, and M-CSF (12). TSNs of CxCa cell cultures were analyzed for these compounds. Latent TGF-β was produced by almost all cell lines, except for CC-10B. Three cell lines produced high amounts of PGE2, and significant amounts of IL-6 were produced by five cell lines. IL-8 was present in all TSNs, of which CC-10B produced the highest levels (Table I). Because the TSN of CC-10B did not affect monocyte to DC differentiation, IL-8 was not further evaluated. IL-10 and M-CSF were not detected in the TSNs.

The three likely candidates, TGF-β, PGE2, and IL-6, were further evaluated. The addition of TGF-β during differentiation of monocytes to DCs did not induce the expression of CD14 but resulted in higher expression of CD11b. Likewise, blocking of the TGF-β pathway in TSN–APC cultures did not restore the phenotype to that of mo-DC, indicating that TGF-β was not responsible for the observed effects of TSN (Supplemental Fig. 3).

Skewing of APC to a macrophage phenotype can occur at concentration of >2 pg/ml PGE2 (31). In fact, mo-DCs differentiated in the presence of 1–50 ng/ml PGE2 resulted in CD14+ CD163+ macrophages that are polarized to CD163+ M2-like macrophages (Fig. 3B). To test if PGE2 was the M2-inducing factor in the TSN, the tumor cells were treated with specific COX inhibitors. After treatment, the tumor cells were washed and incubated with fresh medium to obtain COX-blocked TSN. This procedure was chosen to avoid interaction of the inhibitor with COX in APCs. Indeed, PGE2 production was totally abrogated by inhibition of COX-1 and COX-2 using indomethacin or of COX-2 using NS-398 (Fig. 3A).

Depletion of PGE2 in TSN by preventing its production revealed a striking effect on the DC differentiation of monocytes. The expression of CD14 and CD163 was completely reversed (Fig. 3C), but the phenotypic differentiation toward DC was only partly restored as indicated by the percentage of CD14+CD1a+ APCs that was still lower than that observed in mo-DC cultures (Fig. 2).
Furthermore, the capacity to produce IL-12p70 upon activation was restored whereas that of IL-10 was lowered (Fig. 4A, 4B). The most pronounced effect of COX inhibition was shown for CSCC-7, as the resulting APCs from this COX-blocked TSN were completely comparable with mo-DCs. The effects of COX inhibition in lines CC-8 and HeLa on the function of TSN-altered APCs was predominantly shown in CD40-activated APCs. The functional restoration was reflected also by partial upregulation of their T cell stimulatory capacity (Fig. 4C). As a control, TSN of indomethacin-treated CaSKi cells—which hardly produce PGE2—was tested, and neither clear differences in the hampered differentiation of the APC nor in LPS-induced IL-12 production were observed (Supplemental Fig. 4A, 4B).

Because restoration of the phenotype and function of APCs induced by PGE2-producing cell lines treated with indomethacin was not complete and the cell lines CC-8 and HeLa produced significant amounts of IL-6, we explored the possibility that IL-6 also mediated an effect. mo-DCs were cultured with or without 20% TSN of the nontreated or the COX inhibitor-treated HeLa cell line in the presence of mAbs to IL-6 and IL-6-receptor (32, 33). Neither the differentiation of mo-DCs nor the production of cytokines was altered by the presence of these Abs (Fig. 5). However, blocking of IL-6 showed a profound effect on the phenotype of TSN-M2 cells in that the cultures contained a higher percentage of CD14^+ CD1a^+ APCs. IL-6 blocking acted synergistically with the inhibition of COX, as the combined treatment resulted in a complete phenotypical restoration of TSN-M2 cells to mo-DCs (Fig. 5A). Blocking of IL-6 during the culture resulted in TNS-altered APCs that after CD40 activation produced more IL-12p70, although this was not significant (paired t-test, n = 3 experiments). There was no significant alteration in cytokine production (paired t-test, n = 3 experiments) when the APCs were activated by the TLR agonist LPS (Fig. 5B). No major synergistic effect of IL-6 blocking on IL-12p70 and IL-10 production was found when COX-inhibited TSN was used. Because the supernatant of CaSKi cells, which hampered DC differentiation and function (Supplemental Fig. 4), also contained high levels of IL-6, we blocked this cytokine during the differentiation of monocytes to DCs with TSNcaski. Blocking of IL-6 restored both CD1a expression and the balance between IL-12p70 and IL-10 to what is found for mo-DCs (Supplemental Fig. 4C). Together, these data showed that PGE2 predominantly influenced the expression of the macrophage markers, whereas IL-6 altered CD1a expression. Whereas both PGE2 and IL-6 affect the balance between IL-12p70 and IL-10, PGE2 had a more dominant negative effect. Blocking of these two mediators prevents M2 skewing and restores normal monocyte to DC differentiation. 

**CD4^+ Th1 T cells can switch tumor-induced M2 to activated M1 macrophages**

Initially, we had used CD40L cells to mimic the interaction between T cells and TSN-M2 cells. Because CD40 activation was the best stimulus to induce IL-12p70 production by APCs, we investigated the phenotypical and functional changes in TSN-M2 cells after cognate interactions with CD4^+ T cells. Cocultures of TSN-M2 and Th1 cells were performed in the same TSN-containing culture medium because tumor-secreted factors may directly suppress T cell function (34). Three different CD4^+ Th1 clones were clearly able to activate fully mo-DCs and TSN-M2 cells in an Ag-dependent manner (Fig. 6). Notably, Th1-mediated activation of TSN-M2 cells resulted in a number of changes that suggested a shift from M2-like to M1-like macrophages. The levels of the costimulatory molecules readily increased to the same level as that of mo-DCs (Fig. 6A). In addition, the expression levels of PD-L1 increased. The strong activation was also reflected by the high amounts of IL-12p70 produced, reaching IL-12 levels similar to that of mo-DCs as there is no significant difference (p > 0.05, n = 3 T cell clones) between mo-DCs or TSN-M2 cells (Fig. 6B and data not shown) and IL-12p70 production was much higher than previously observed after TLR or CD40 activation (compare Figs. 2B and 6B). Strikingly, the production of IL-10 remained low and around the same level as that of the corresponding mo-DC cultures (p > 0.05 for TSNcsc7 and TSNcsc8, p = 0.03 for TSNhela; n = 3 T cell clones). After the interaction with Th1 cells, the typical M2-like macrophage markers CD206 and CD163 were lost. This was also observed when TSN-M2 cells

**FIGURE 5.** Blocking of IL-6--induced and PGE2--induced altered differentiation restores monocyte to DC differentiation completely. A. Monocytes were differentiated in the presence of control (+DMSO) or COX-inhibited HeLa cells (+Indomethacin) without or with blocking Abs against IL-6 and IL-6-receptor. A combination of tumor cells treated with indomethacin and blocking of IL-6 during differentiation results in full DC differentiation reflected by high expression of CD1a and lack of CD14. B. Blocking of IL-6 during differentiation did not affect the production of IL-12 (ng/ml) and IL-10 (pg/ml) of mo-DCs upon activation by indicated agonists. CD40-activated TSN-M2 cells of which the IL-6 in TSN was blocked during differentiation produced more IL-12. One representative experiment of three.
were stimulated with LPS, R848 and CD40L cells indicating that this is a reflection of APC activation (Supplemental Fig. 1B, C).

Furthermore, high amounts of T cell-produced IFN-γ, TNF-α, and IL-2 were detected in the supernatant of the cocultures reflecting the activation of the T cells upon recognition of their cognate peptide (Fig. 6B and data not shown). Apart from IL-12p70 and IFN-γ for which it is clear that they are only produced by the APCs or T cells, respectively, IL-10 may be produced by both cell types, and this cannot be distinguished by ELISA. It is likely that the IL-10 detected in coculture with clone 238 is produced by the T cell clone, as mo-DCs stimulated with the other two clones do not produce IL-10. Our previous experiments indicated that ligation of CD40 could not switch M2 to M1 macrophages; therefore, we analyzed if one of the T cell-produced cytokines synergized with CD40L cell-mediated activation to switch M2 to M1-like macrophages. TSN-M2 cells were activated with CD40L and IFN-γ or TNF-α. This revealed that the combination of CD40L cells with IFN-γ but not CD40L cells or IFN-γ alone resulted in high levels of IL-12 not only in mo-DC but also in TSN-M2 cell cultures (Fig. 6B, right, and data not shown). This capacity of Th1 cells or CD40L cells plus IFN-γ to switch M2-like macrophages to M1-like macrophages could be reproduced in cocultures with M-CSF–induced M2 macrophages (data not shown).

TSN-M2 cells expressed higher levels of CCR7 than mo-DCs after their cognate interaction with CD4+ Th1 cells (Fig. 6C). As we had already found that IFN-γ synergized with CD40 activation to switch M2-like to M1-like macrophages, we tested the hypothesis that IFN-γ or TNF-α secreted by the T cell clone either alone or in combination with PGE2 present in TSN was responsible for the high levels of CCR7. Indeed, incubation with IFN-γ but not TNF-α induced the expression of CCR7 on mo-DCs and a very high expression on TSN–M2 cells. Furthermore, pretreatment of mo-DCs with PGE2 during the differentiation phase resulted in similar high expression of CCR7 as found on TSN-M2 cells (Fig. 6C).

Thus, cognate interaction with IFN-γ–producing T cells can switch the tumor-promoting M2-like polarized macrophages to activated classical M1-like macrophages that express high levels of costimulatory molecules, produce high amounts of IL-12, and gain the expression of the lymphoid homing receptor CCR-7.
Discussion

Our analysis on the effect of tumor-secreted factors from seven different CxCa cell lines on the differentiation of monocytes to DCs and their functional capacity revealed that these cancer cells can be sorted into two major categories: 1) cancer cells that hamper DC differentiation and function and 2) cancer cells that induce M2-like macrophages. These two categories comprised similar HPV types ruling out that the effects seen were HPV type specific. Tumor-secreted PGE2 and/or IL-6 were clearly responsible for these effects, whereas no role was found for TGF-β, IL-8, IL-10, or M-CSF. In vivo, such APCs are present at different levels of differentiation in stroma and epithelial compartments of HPV-induced CxCa, and these include next to immature DCs, mature DCs, macrophages, and M2 macrophages (Fig. 1) (10, 35).

Category 2 cancer cells stimulated the differentiation of CD14+, CD16+, CD206+, CD163+ M2-like macrophages. Consequently, these TSN-M2 cells displayed an altered cytokine profile and a poor capacity to stimulate T cells compared with mo-DCs. Careful evaluation of the expression of costimulatory molecules, cytokine production, and T cell stimulatory capacity of these TSN-M2 cells showed that stimulation with a number of clinically applicable TLR agonists or CD40L cells could not provoke the same phenotypical and functional activity as found for mo-DCs. Notably, unstimulated TSN-M2 cells expressed PD-L1 at higher levels than those of mo-DCs. While stimulation of mo-DCs and TSN-M2 cells resulted in an increased expression of PD-L1, the expression on TSN-M2 cells remained higher on TSN-M2 cells, suggesting that TSN-M2 cells display an altered costimulatory/inhibitory molecule ratio on the cell surface compared with that of mo-DCs. High levels of PD-L1 expression on monocytes have been shown to effectively suppress tumor-specific T cell immunity and to contribute to the growth of human hepatocellular carcinoma cells in vivo (36). Furthermore, knockdown of PD-L1 in activated DCs has been shown to increase the IFN-γ and IL-2 production of reacting T cells (37). We are currently investigating the role of PD-L1 expression level with respect to the lack of responsiveness of naïve T cells in our experiments. Notably, comparison of the two different agonists R848 and LPS to stimulate TSN-M2 cells revealed clear differences in their effects on costimulatory molecule expression and cytokine production, indicating that previous results reported with the TLR4 agonist LPS—most often used to stimulate tumor-induced DCs in vitro (16, 38)—cannot be translated to other TLR agonists. Earlier studies identified macrophages (CD68+) within the CxCa microenvironment and showed that an increase in macrophages is inversely correlated with survival (39). The presence of macrophages correlated with a high production of IL-6 by tumor cells, the latter of which was associated with poor survival (19). IL-6 was shown in vitro to hamper the DC differentiation and allogeneic T cell stimulatory capacity and could even switch monocyte differentiation from DCs to macrophages (32, 33, 40–42). Others showed that the overexpression of COX enzymes in HPV-induced lesions is associated with a loss in CD11c+ cells, and PGE2 was suggested to mediate this effect (16). Under our experimental conditions, IL-6 alone was able to hinder DC differentiation and function, but PGE2 was responsible for the conversion of monocytes to M2 macrophages. Differentiation of monocytes to M2 macrophages could be prevented by inhibition of the production of PGE2 in tumor cells and blocking IL-6 during the differentiation period of the monocyte.

Importantly, when fully polarized M2 macrophages present Ag to Th1 cells within the context of an M2-polarizing milieu—as represented by the M2-inducing TSN—this interaction not only results in repolarization of M2 to M1 macrophages, but it also activates these M1 macrophages to express high levels of costimulatory molecules, to produce IL-12, and to express the lymph node homing marker CCR7. The switch from M2 to M1 macrophages accords with the plasticity of macrophages to change their functional phenotype from classically activated macrophages to wound-healing or regulatory macrophages and vice versa (43, 44). This switch could be reproduced by stimulating TSN-M2 cells with CD40L cells and IFN-γ. Although activation via CD40 was enough to induce changes in the typical M2 markers, IFN-γ provided the necessary signals for the macrophages to produce IL-12 without additional IL-10 production. Mouse models have elegantly demonstrated the important role of the Th1–macrophage axis in antitumor immunity. Tumor-resident macrophages were shown to process and present tumor Ag to Th1 cells, which in turn activated these macrophages—through the local release of IFN-γ—to become tumoricidal and to induce a CD4+ T cell-dependent tumor protection (45, 46). Our data suggest that alteration of the suppressive tumor microenvironment by tumor-infiltrating Th1 cells—which change the tolerogenic M2 macrophage phenotype to that of activated M1 macrophages—could be one of the underlying mechanisms of this tumor protection system.

The local presence of IFN-γ-producing T cells responding to Ag presented by these altered APCs may restore proper tumor-rejecting immune function, but such T cells are often absent in tumors (47, 48). COX-inhibiting drugs are widely used in the clinic for treatment of autoimmune diseases, and trials are now under way with the aim to determine the effect of low-dose nonsteroidal anti-inflammatory drug on tumor prevention by disrupting the COX-2–mediated oncogenic pathways (49). Furthermore, mAbs to IL-6 receptor are already in clinical use for the treatment of autoimmune diseases (50). It can be envisioned that a combination therapy consisting of COX inhibition, IL-6 blocking, and the induction of a strong Th1 T cell response by currently available vaccines may form the next generation of immunotherapy for the treatment of CxCa (51, 52).

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary fig 1. Phenotype of in vitro differentiated DC and M2 macrophages.
(A) Monocytes were cultured for 6 days with GM-CSF+IL-4 to obtain imm DC or M-CSF to obtain M2 macrophages and analyzed by flowcytometry for the expression of phenotypical markers. M2-differentiated macrophages typically display high expression of the scavenger receptor CD163 and the mannose receptor CD206. In contrast to monocyte derived DC M2-macrophages express all FcyReceptors (CD32, CD64 and CD16). (B+C) Stimulation of TSN-M2 for 48 hr with (B) indicated TLR-ligands and (C) Th1 cells or CD40-L cells results in a lowered expression of CD14 and CD163 as detected by flowcytometry.
Supplementary fig 2  Function of DC is hampered by TSN.
Compared to mo-DC, TSN-M2 cultures produce less IL-12 and more IL-10 upon depicted stimulus. We observed large variation in the amounts of cytokines produced by each donor to the different TLR-ligands, especially for IL-10. To compare 3 different donors with respect to the amounts of cytokine produced; the relative production of cytokines of each condition within one experiment was calculated. The amount of cytokines produced by mo-DC to the indicated stimulus was set to 100%. Three experiments combined, mean with SEM. For each stimulus means were compared to mo-DC by paired t test. * indicates p<0.05, ** indicates p<0.005.

Heusinkveld et al. supplement fig 2
Supplementary fig 3  Blocking of the TGFβ pathway in TSN differentiated APC.

(A) Mo-DC were cultured with increasing concentration of TGFβ and activated for 48 hrs with LPS. Flowcytometric analysis show that high levels of TGFβ make APC unresponsive to LPS as reflected by a failure to upregulate the expression of co-stimulatory molecules. One representative experiment of 3. (B) Mo-DC or APC differentiated in the presence of 500 pg/ml TGFβ-1 (TGFβ) or 20% TSN (obtained from HeLa or CaSKI) were treated without (white) or with the ALK-inhibiting compound SB431542 (black) during culture and activated with LPS for 48 hr. Although the effect of TGFβ on the unresponsiveness to LPS is completely abrogated by compound SB431541, no effect of this compound is found on the expression of CD1a, CD86 and CD83 by APC in TSN cultures. (C) The production of IL12p70 upon 48 h LPS stimulation of control (white) or compound SB431542 treated (black) cultures. The compound restores the IL12p70-production by TGFβ-treated cells to the same level as mo-DC, but does not restore IL-12 production by TSN-differentiated APC.
Supplementary fig 4 Blocking IL-6 in the TSN of CaSKi cells abrogates the suppressive effect on DC differentiation and function.

(A) A lower percentage of monocytes differentiated in the presence of 20% supernatant of caski-cells expresses CD1a after 6 days of culture, reflecting poor DC differentiation. Addition of IL-6 and IL-6Receptor blocking antibodies to the culture does not influence normal monocyte to DC differentiation but increases the percentage of CD1a expressing cells in cultures with TSNcaski. Treating the CaSKi-cells with COX-inhibitor indomethacin does not affect the effect on monocyte differentiation. Depicted is the percentage of all cells in live gate. One representative of 2 experiments.

(B) Monocytes were differentiated in the presence of control (+DMSO) or COX-inhibited CasKi supernatant and activated with LPS. Cytokine production was analyzed after 48 hr. (C) APC differentiated in the presence of TSNcaski produce more IL-10 and less IL-12 upon activation by TLR-ligands or CD40-L when compared to fully differentiated mo-DC. Blocking of IL-6 in the supernatant during differentiation of monocytes restores the capacity of the resulting cells to produce IL-12 while reducing the production of IL-10. To compare different donors, the amount of cytokine produces is depicted as a percentage of the cytokine when produced by mo-DC activated by the same stimulus without blocking of IL-6. Three experiments combined. Means were compared by the two-tailed unpaired t-test using Graphpad software. * indicates p<0.05, ** indicates p<0.005.

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