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CHAPTER 5.1

M2 macrophages induced by PgE2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells

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ABSTRACT

Monocytes attracted by tumor-induced chronic inflammation differentiate to antigen-presenting cells (APC), the type of which depends on cues in the local tumor milieu. Here, we studied the influence of human cervical cancer cells on monocyte differentiation and showed that the majority of cancer cells either hampered monocyte to DC differentiation or skewed their differentiation towards M2-like macrophages. Blocking studies revealed that M2-differentiation was caused by tumor-produced PgE2 and IL-6. TGF β , IL-10, VEGF or M-CSF 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 when compared to DC these M2-macrophages displayed a tolerogenic phenotype reflected by a lower expression of co-stimulatory molecules, an altered balance in IL-12p70 and IL-10 production and a poor capacity to stimulate T-cell proliferation and IFN γ production. Interestingly, upon cognate interaction with Th1 cells these tumor-induced M2-macrophages could be switched to activated M1-like macrophages that expressed high levels of co-stimulatory molecules, produced high amounts of IL-12 and low amounts of IL-10, as well as 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 APC via CD40 in the presence of IFN γ . Our data on the presence, induction and plasticity of tumor-induced tolerogenic APC in cervical cancer suggest that tumor-infiltrated Th1 cells can stimulate a tumor-rejecting environment by switching M2-macrophages to classical pro-inflammatory M1 macrophages.

INTRODUCTION

Cervical cancer (CxCa) is induced by human papilloma virus (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 T-helper 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 antigen presenting cells (APC) 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) 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 vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (M-CSF), transforming growth factor (TGF β 1), IL-10, IL-6 and prostanooids (e.g. PgE2) (12). Cervical cancers are known also to secrete immunomodulatory compounds but their effect on APC is yet unknown (15-19).

Therefore, we studied the effect of cervical cancer cells on monocyte differentiation and function. We found that DC differentiation was hampered or even skewed towards the tolerogenic M2 macrophages by tumor-derived PgE2 and IL-6. Subsequently, we assessed the effects of APC activation by several different Toll like receptor (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 co-stimulatory molecules and producing high amounts of IL-12p70. Our data suggests that a highly immune-stimulatory local microenvironment might be achieved by utilizing COX-inhibitors and IL-6 blocking antibodies to prevent M2-differentiation and vaccine-mediated stimulation of Th1 cells to switch M2 macrophages to tumor-rejecting M1 macrophages.

MATERIAL AND METHODS

Immunofluorescent staining of tumor tissue

Ten patients with cervical cancer with FIGO stage I and II underwent radical hysterectomy (type III) in 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 μ m tissue-sections of formalin-treated and paraffin embedded tumor material. Immunostaining was performed with a monoclonal antibody mix of anti-CD14 (clone 7, Abcam USA) and anti-CD163 (clone 10D6, Novocastra United Kingdom) and after incubation overnight stained with fluorescent antibody conjugates (IgG2a-Alexa Fluor 488 and IgG1- alexa Fluor 647, Invitrogen USA) (22). Control staining with only secondary antibodies were included to ensure specificity. Images were captured at 25 X magnification with a confocal laser scanning microscope (Zeiss LSM 510, Germany) in a multitrack setting.

Media and reagents

APC and tumor cell-lines were grown in RPMI (Invitrogen) supplemented with 10% Fetal Calf Serum (Greiner bio-one Germany), penicillin/streptomycin (Invitrogen) and L-glutamine (Cambrex USA). Adherent cell-lines were treated with trypsin/EDTA 1x (PAA, Austria). T cell clones were grown in IMDM (Lonza Switzerland) supplemented with 10% FCS (PAA), P/S and L-glutamine. The following factors and final concentrations were used to generate APC: 500 U/ml IL-4, 800 u/ml GM-CSF (Gibco, USA), ,10-100 ng/ml M-CSF (R&D systems USA), 50-1000 pg/ml TGF β 1 (BD biosciences USA), 1-50 ng/ml prostaglandin E2 (Sigma-Aldrich Germany). TLR ligands used for activation: 25 μ g/ml Poly(I:C), 10 μ g/ml R848/CL097 (all from Invivogen, USA) and 0.25 μ g/ml LPS (Sigma-Aldrich). Optimal concentrations were used based on maximal cytokine release in mo-DC. To mimic T-cell interaction APCs were stimulated with irradiated CD40-L expressing mouse fibroblasts (23).

Blockade of TGF β signaling was achieved with 1 μ M SB431542 hydrate (Sigma-Aldrich) after optimization of the dose. IL-6 was blocked by adding 2.5 ug/ml antibody to IL-6 receptor (clone B-R6) and 2.5 ug/ml antibody to IL-6 (B-E8) (Abcam, USA) 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 HPV 16 or 18 DNA using the INNO-LiPa HPV Genotyping procedure (Innogenetics). C5CC-1, C5CC-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, harvested with trypsin/EDTA. 100.000 cells were plated in 2 ml/well of 6-well culture plate and cultured for 5 days. Supernatant was stored at -20 degrees. 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 μ M Indomethacin or 5 μ M NS-398 (Cayman Chemical, USA) dissolved in DMSO or as a control only with the corresponding concentration of DMSO. Medium was replaced after 24 hr and then harvested after 24 hours of culture.

DC culture

PBMC were obtained from buffy coats of healthy donors. CD14+ monocytes (>95% purity) were isolated using the MACS cell separation (Miltenyi Biotec Germany) 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 or 0.5 million cells/well respectively in the presence of IL-4 and GM-CSF (mo-DC). After 2 days 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)-APC are 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. DC were activated at day 6 and after 48 hr the supernatant was harvested and stored at -20 $^{\circ}$ C for cytokine analysis and cells were stained for flow cytometric analysis.

Mixed Lymphocyte Reaction

Naïve CD4 cells were isolated from PBMC 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 days by [3H]thymidine incorporation (0.5 uCi/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 LN cells of a patient with a HPV16+ cervical tumor. Clones were stimulated every 2 weeks with B-LCL loaded with the cognate HPV peptide, feeder-cells, TCGF and IL-15. Clones were used for DC activation after 2.5 weeks resting period.

Clone 214 recognized HPV16E6 aa61-82, clone 238 recognized HPV16 aa61-82 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 antigen-specific activation. HLA-class II matched APC were loaded with an irrelevant or the cognate peptide for CD4+ Th1 clones and co-cultured at different DC:T-cell ratio's in medium containing 20% TSN. After 48 hours supernatant was analyzed and APC were phenotyped.

Flow cytometry.

Mouse monoclonal antibodies to human CD80, CD86, HLA-DR, CD206, CD11c(FITC) and CD83, CD86, CD14, CD16, CD163 (PE) and CD14, HLA-DR (PERCP) or CD11c, CD1a, CD4 (APC) (all from BD-biosciences) and CD163 (R&D systems) and PD-L1 (Ebioscience) were used. Cells were recorded (20.000/live gate) using a BD-FACS calibur with Cellquest software (BD-biosciences) and analyzed by Flowjo software (Tree star, inc. USA).

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 was measured with ELISA (Sanquin, the Netherland). To evaluate the cytokines present in supernatant of tumor cells IL-6, IL-8 and IL-10 were determined by CBA, M-CSF by Bioplex (BioRad). PgE2 was measured with the prostaglandin E2 parameter assay kit (R&D systems), TGF β -1 with the human TGF β 1 ELISA from Ebioscience. Samples were tested with and without acidic treatment to determine active and latent TGF β 1 in the cultures.

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 tumor supernatant (TSN) derived from five early passage CxCa cell lines (Table 1) (17,24) and the two well known cell lines CaSKI and HeLa. GM-CSF and IL-4 differentiated monocyte-derived DC (mo-DC) are defined as antigen presenting cells 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 7 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, DC, MDSC and macrophages. Mo-DC cultures typically contain >80% CD1a+ cells but when monocytes were differentiated in the presence of TSN from CC11-, CSCC1 or CaSKI

Table I. Immuno-suppressive factors produced by CxCa cell-lines.

Cell-line	Passage [†]	HPV [§]	cytokine production (pg/ml) [†]			
			IL-8	TGFβ-1**	PgE2	IL-6
CC-10B	P80	45	14000	0	0	10
CSCC-1	P22	16	1500	221	0	20
CC11 -	P32	67	425	398	403	3438
CaSKi	x	16	324	410	298	2335
CSCC-7	P9	16	9306	313	2781	263
CC-8	P41	45	1222	250	3662	2228
HeLa	x	18	4500	548	5389	12000

(†)number of passages since origin - HeLa and CaSKi not known

(§)integrated DNA found to be present in the tumor (24)

(*)100.000 cells/2ml cultured for 5 days in RPMI/10%FCS

(**)latent form of TGF-β1 measured by ELISA after acidic treatment

x, HeLa and CaSKi not known

this percentage dropped, reflecting poor DC-differentiation (Fig. 1a). TSN of the cell lines CCSC-7, CC-8 and HeLa did not only hamper CD1a expression but skewed the differentiation of monocytes towards 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, while CD1b and CD1c were absent (Fig.1b and not shown). This profile is highly similar to that of *in vitro* M-CSF-induced M2-macrophages and distinct from monocyte derived DC (Supplemental Fig. 1) (13,25). TSN of cell line CC-10B did not overtly alter the differentiation of monocytes to DC, 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 MDSC (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 M2-macrophages (CD14⁺CD163⁺) (26,27). Figure 1c shows the presence of CD14 single positive cells (macrophages), CD163 single positive cells but also CD14+CD163+ M2-polarized macrophages.

Functional impairment of APC by TSN.

Next we assessed the capacity of these tumor-modulated APC to respond to 5 different TLR agonists or CD40-L expressing fibroblast cells (CD40-L) to mimic T-cell interaction. Since the supernatant of CSCC-7, CC-8 and HeLa induced strikingly induced these M2-macrophages (TSN-M2), which are known to foster immune tolerance, we focused on these cell lines for further evaluation and compared the results to those obtained with normal differentiated mo-DC.

Stimulation of mo-DC with LPS or R848 for 48 hours resulted in a strong increase in the expression of CD86, CD80, CD83, HLA-DR and PD-L1 (Fig. 2a and not shown). PolyI:C was the least potent TLR-agonist. TSN-M2 expressed higher basal levels of CD86, HLA-DR and PD-L1 but stimulation with R848 or PolyI:C did not raise their expression level to that of mo-DC (Fig. 2a and not shown).

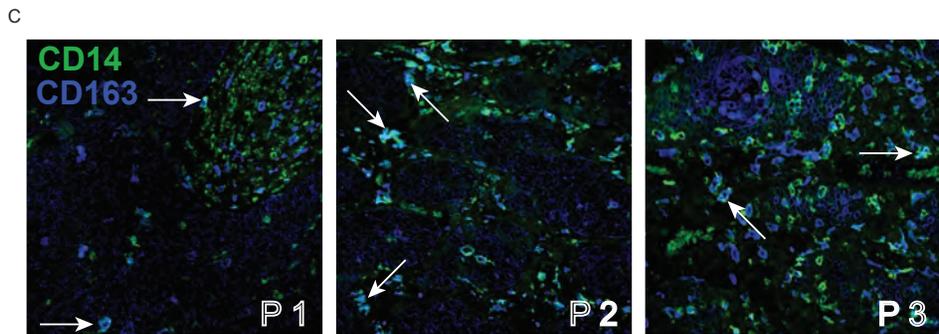
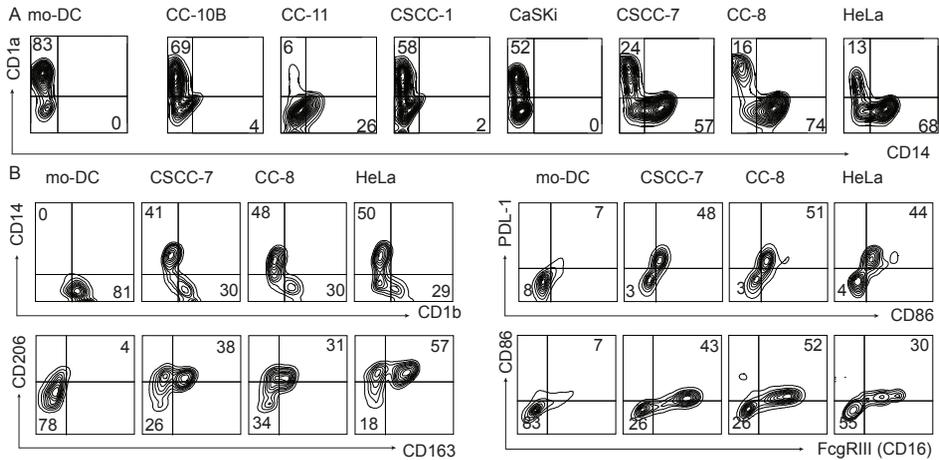


Figure 1 CxCa-secreted factors skew monocyte differentiation towards M2 macrophages. Monocytes were cultured for 6 days with GM-CSF and IL4 in culture medium with 20% tumor supernatant (TSN) of indicated CxCa cell lines or 20% control medium (mo-DC) and (A) analyzed for expression of CD14 and CD1a. TSN reduced CD1a expression and in 3 cases induced CD14⁺ expression. (B) 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 5. (C) *In situ* immunofluorescence stainings of cervical tumor tissue of 3 out of 10 different patients for CD14 (green) and CD163 (blue) and measured by confocal microscopy (25 x magnification). CD14⁺CD163⁺ display as turquoise, white arrows indicate M2 macrophages.

For the induction and polarization of cytotoxic T cells and Th1 cells the secretion of IL-12p70 by APC is essential (28,29). Mo-DC produced the biologically active IL-12p70 upon stimulation with LPS, R848, or CD40-L cells. The amounts varied per donor of whom the mo-DC were prepared (Fig. 2b, Supplemental Fig. 2). Relatively to stimulated mo-DC, the TSN-M2 almost completely lacked the ability to produce IL-12p70 when stimulated with TLR agonists. The strong activation signal induced by CD40-L cells allowed TSN-M2 to produce IL-12p70, albeit at significantly lower concentrations than CD40-stimulated mo-DC. In contrast, the production of the cytokine IL-10 – which varied greatly between donors – was at least 2-fold increased when TSN-M2 were stimulated with TLR agonist or with CD40-L cells in 2 out of 3 experiments (Fig. 2b, Supplemental Fig. 2). The alterations in APC function were imprinted during the differentiation

of the monocytes since activation of TSN-M2 cells in fresh medium without additives gave similar results (data not shown).

Subsequently, the capacity of TSN-M2 to induce proliferation and cytokine production of T cells was compared to mo-DC. Graded doses of APC were co-cultured with a fixed number of allogeneic CD4+CD25- T cells. Clearly, the activated TSN-M2 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 APC (30), was not clearly altered after 10 days of culture with TSN-M2 (data not shown). These results indicated that TSN-skewed APC were both phenotypically and functionally shifted towards that of M2 macrophages and that TLR mediated activation of TSN-M2 reinforced their tolerogenic profile.

Mediators of altered APC differentiation

Numerous mediators may cause the altered differentiation of monocytes to DC, including TGF β , PgE2, IL-6, IL-8, IL-10 and M-CSF (12). TSN 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 5 cell lines. IL-8 was present in all TSN, of which CC-10B produced the highest levels (Table 1). Since the tumor supernatant of CC10B did not affect monocyte to DC differentiation, IL-8 was not further evaluated. IL-10 and M-CSF were not detected in the tumor supernatants.

The three likely candidates, TGF β , PgE2 and IL-6 were further evaluated. The addition of TGF β during differentiation of monocytes to DC did not induce the expression of CD14 but resulted in higher expression of CD1a. 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-DC differentiated in the presence of 1-50 ng/ml PgE2 resulted in CD14+ 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-enzyme inhibitors. After treatment, the tumor cells were washed and incubated with fresh medium to obtain COX-blocked tumor supernatant. This procedure was chosen to avoid interaction of the inhibitor with COX in APC. Indeed, PgE2 production was totally abrogated by inhibition of COX 1 and 2 using Indomethacin or COX-2 by 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 towards DC was only partly restored as indicated by the percentage of CD14⁺CD1a⁺ APCs that was still lower than observed in mo-DC cultures (Fig. 3d). Furthermore, the capacity to produce IL-12p70 upon activation was restored while that of IL-10 was lowered (Fig. 4ab). The most pronounced effect of COX-inhibition was shown for C5CC-7 as the resulting APC from this COX-blocked tumor supernatant were completely comparable to mo-DC. The effects of COX-inhibition in lines CC-8 and HeLa on the function of TSN-altered APC was predominantly shown in CD40-activated APC. The functional restoration was reflected also by partial up regulation 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. 4ab).

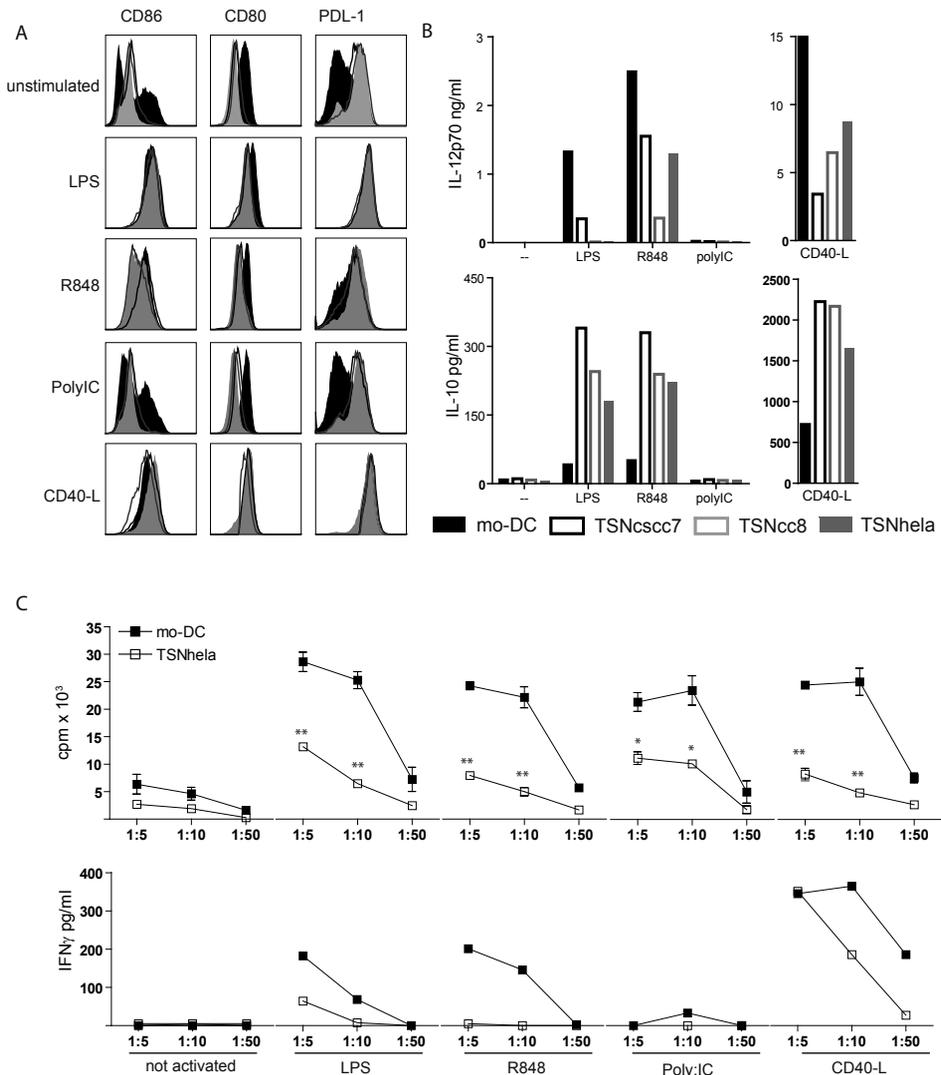


Figure 2 TSN-differentiated monocytes are phenotypically and functionally different from mo-DC.

(A) Monocytes were differentiated in the presence of the indicated TSN and activated with single TLR-ligands or CD40-L cells for 48 hr. TSN-M2 cells express higher basal levels of CD86 and PDL-1. R848 or Poly-IC can not 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 produce more IL-10 and less IL12 than mo-DC. (C) CD4+CD25- allogenic responder T cells were cultured with activated mo-DC or TSN-M2 (obtained with TSN from HeLa cells) at indicated ratio's (DC:T cell). Top row shows the higher proliferation of T cells when stimulated with upon activated mo-DC 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 3 experiments (A-C).

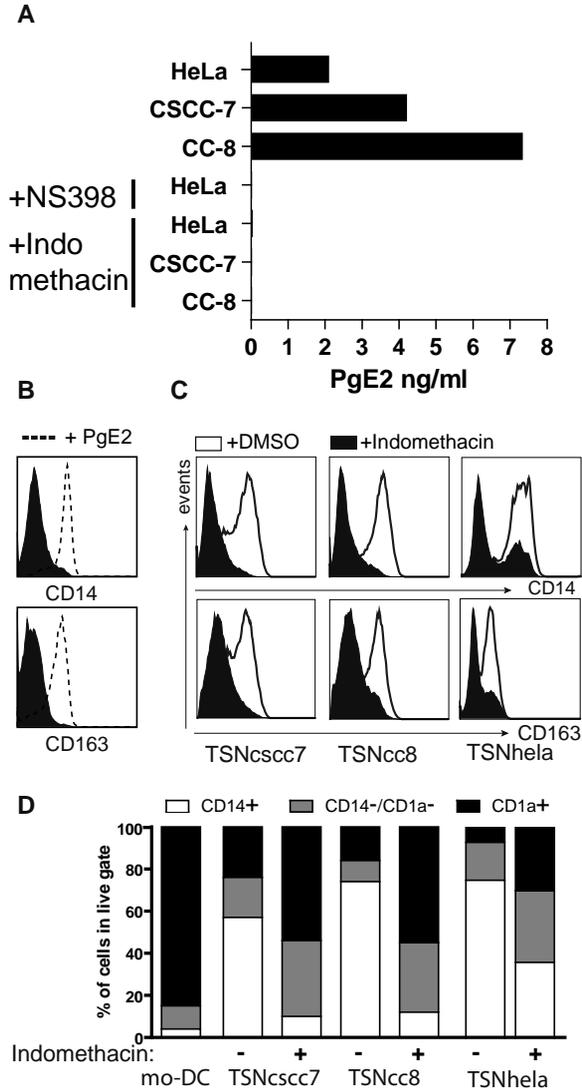


Figure 3 Cox-inhibition blocks PgE2 production and prevents M2-macrophage differentiation. (A)

Tumor cell lines were treated for 24 hr with DMSO (control) or Cox-inhibitor (NS398 or Indomethacin) followed by culturing in fresh medium without additives for 24 hrs. PgE2 levels were measured by ELISA. Inhibition of COX totally abrogates PgE2 production. $P < 0.005$ for all comparisons of PgE2 production by cells treated or not with indicated COX-inhibitor in 2 separate experiments (B) Culturing mo-DC (black fill) in the presence of 10 ng/ml PgE2 (dotted line) induce CD14+CD163+ expressing APC. (C) Flowcytometric analysis of CD14 and CD163-associated M2-macrophage marker expression on monocytes differentiated in the presence of TSN of Indomethacin-treated (black) tumor cells or TSN from non-treated tumor cells (white). (D) Comparison of CD1a and CD14 expressing populations following the differentiation of monocytes in the presence of TSN of Indomethacin-treated or non-treated tumor cells shows that TSN of COX-inhibitor treated cell lines induce less CD14+ cells and more CD1a+ DCs. Representative of 3 experiments (B-D).

Since restoration of the phenotype and function of APC 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 mediated also an effect. Mo-DC were cultured with or without 20% TSN of the non-treated or the COX-inhibitor treated HeLa cell line in the presence of monoclonal antibodies to IL-6 and IL-6-receptor (32,33). The differentiation of mo-DC, nor the production of cytokines was altered by the presence of these antibodies (Fig. 5). However, blocking of IL-6 showed a profound effect on the phenotype of TSN-M2 in that the cultures contained a higher percentage of CD14-CD1a+ APC. IL-6 blocking acted synergistically with the inhibition of COX since the combined treatment resulted in a complete phenotypical restoration of TSN-M2 to mo-DC (Fig. 5a). Blocking of IL-6 during the culture resulted in TSN-altered APC that after CD40-activation produced more IL-12p70 although this was not significant ($p=0.079$, $n=3$ experiments). There were no significant alteration in cytokine production ($p>0.05$, $n=3$ experiments) when the APC were activated by the TLR agonists (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. Since 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 DC 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-DC (Supplemental Fig. 4c). Together, these data showed that PgE2 predominantly influenced the expression of the macrophage markers, whereas IL-6 altered CD1a expression. While 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 CD40-L cells to mimic the interaction between T cells and TSN-M2. Since CD40-activation was the best stimulus to induce IL-12p70 production by APC we investigated the phenotypical and functional changes in TSN-M2 following cognate interactions with CD4+ T cells. Co-cultures of TSN-M2 and Th1 cells were performed in the same TSN-containing culture medium since tumor secreted factors may directly suppress T-cell function (34). Three different CD4+ Th1 clones were clearly able to fully activate mo-DC and TSN-M2 in an antigen dependent manner (Fig. 6). Notably, Th1-mediated activation of TSN-M2 resulted in a number of changes that suggested a shift from M2-like to M1-like macrophages. The levels of the co-stimulatory molecules readily increased to the same level as mo-DC (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-DC, as there is no significant difference ($p>0.05$, $n=3$ T-cell clones) between mo-DC or TSN-M2 (Fig. 6b and not shown) and much higher than previously observed after TLR- or CD40-activation (compare Fig. 2b and 6b). Strikingly, the production of IL-10 remained low and around the same level as the corresponding mo-DC cultures ($p>0.05$ for TSNcsc7 and TSNcc8, $p=0.03$ 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 were stimulated with LPS, R848 and CD40-L cells indicating that this is a reflection of APC activation (Supplemental Fig. 1bc). Furthermore, high amounts of T-cell produced IFN γ , TNF α and IL-2 were detected in the

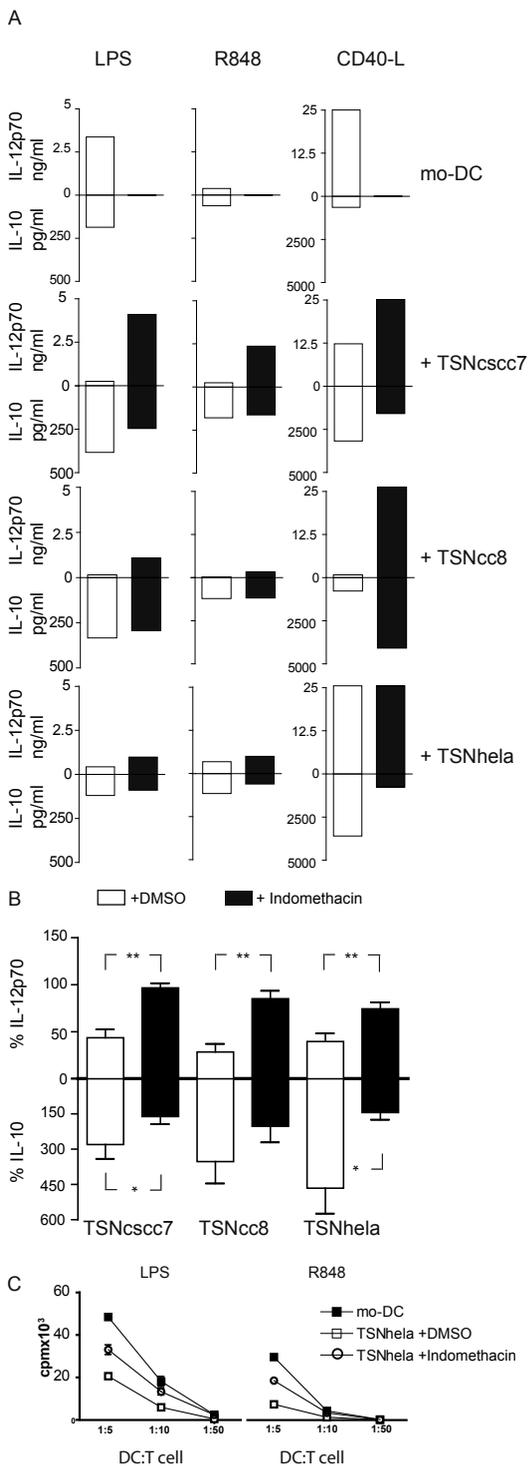


Figure 4 Restored cytokine production when PgE2 production by tumor cells is blocked.

(A) The production of IL-12 and IL-10 by APC differentiated in the presence of TSN of Indomethacin-treated (black) tumor cells or TSN from non-treated tumor cells (white) upon 48 hr activation with LPS, R848 or CD40-L as measured by ELISA. Top row shows the cytokine production of untreated mo-DC. One representative experiment of 3. The block of PgE2 production by tumor cells alters the balance in IL-12 and IL-10 towards that observed in the corresponding mo-DC. (B) Comparison of the cytokine production by APC differentiated in the presence of TSN from non-treated tumor cells (white) to Indomethacin-treated (black) tumor cells that were activated for 48 hours (combined data of LPS, R848 and CD40-L stimulated cells; n=3 experiments). IL-12, all $p < 0.003$. IL-10, TSNcsc7 $p = 0.015$; TSNcc8 $p = ns$; TSNhela $p = 0.026$. (C) Mo-DC or TSN-M2 obtained with TSN of untreated (+DMSO) or Indomethacin-treated HeLa cells (+Indomethacin) were activated with LPS or R848 for 48 hours and used to stimulate allogeneic responder cells. APC differentiated in TSN of Indomethacin-treated cells induce better T-cell proliferation at day 5 of culture as measured by $[^3H]$ -thymidine incorporation. Test performed in triplicate, shown is mean with SEM. Means were compared by unpaired t-test. Comparison of non versus indomethacin treated HeLa cells (1:5 ; 1:10) for LPS $p = 0.009$; $p = 0.024$, and for R848 $p = 0.0003$; $p = ns$. Comparison of mo-DC versus indomethacin treated HeLa cells (1:5 ; 1:10) for LPS $p = 0.004$; $p = ns$, and for R848 $p = 0.009$; $p = ns$. One representative out of 2 experiments.

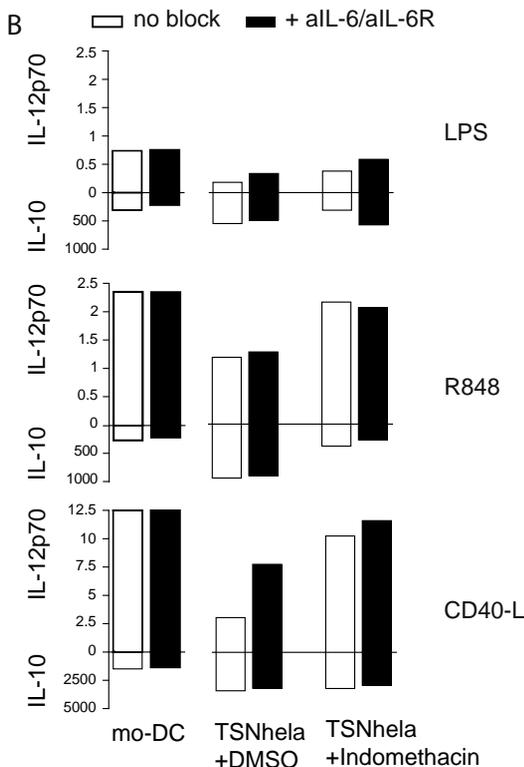
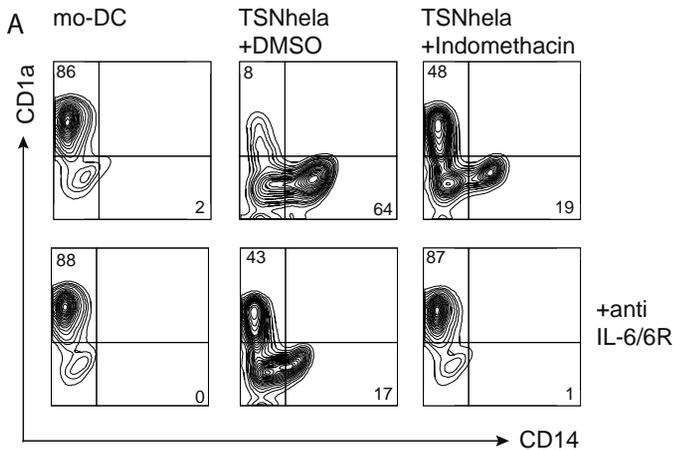


Figure 5 Blocking IL-6 and PgE2-induced altered differentiation restores monocyte to DC differentiation completely.

(A) Monocytes were differentiated in the presence of control(+DMSO) or COX-inhibited HeLa cells(+Indomethacin) without or with blocking antibodies against IL-6 and IL-6 receptor. A combination of tumor cells treated with Indomethacin and blocking IL-6 during differentiation results in full DC differentiation reflected by high expression of CD1a and lack of CD14. (B) Blocking IL-6 during differentiation did not affect the production of IL-12 (ng/ml) and IL-10 (pg/ml) of mo-DC 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 out of 3.

supernatant of the co-cultures reflecting the activation of the T cells upon recognition of their cognate peptide (Fig. 6b and not shown). Apart from IL-12p70 and IFN γ for which it is clear that they are only produced by the APC or T cells, respectively, IL-10 may be produced by both cell types and this can not be distinguished by ELISA. It is likely that the IL-10 detected in co-culture with clone 238 is produced by the T cell clone as mo-DC stimulated with the other 2 clones

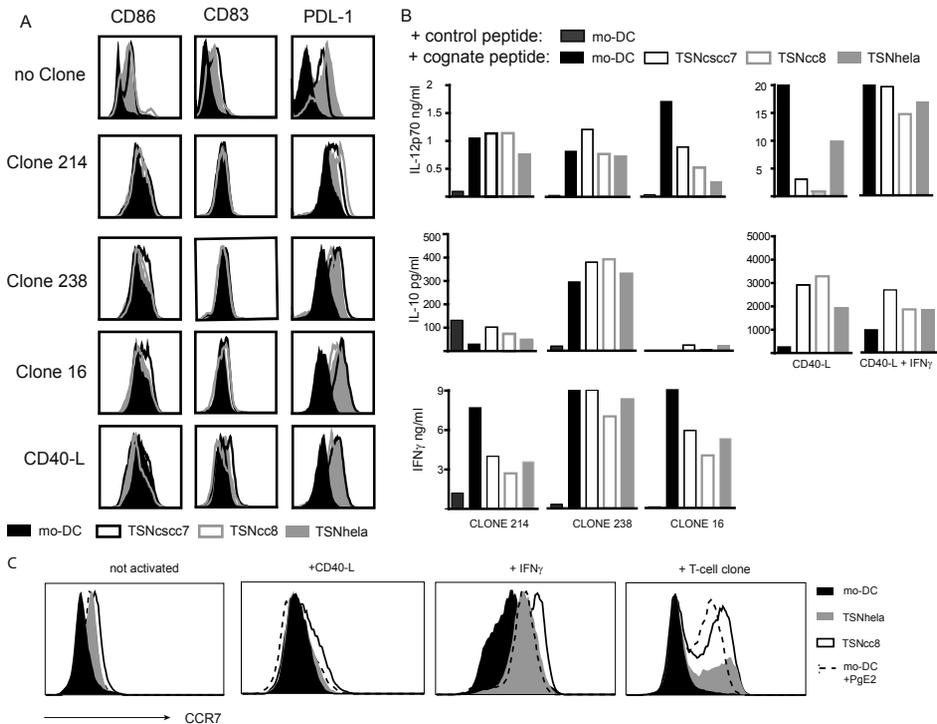


Figure 6 CD4⁺ T cells switch TSN-M2 to M1-like macrophages and activate them to produce IL-12p70. (A) Mo-DC or TSN-M2 were pulsed with the cognate Th1-peptide and co-cultured with HPV-specific CD4⁺ T-cell clones (2 DC:1 T cell) or CD40-L cells. T-cell interaction results in high expression of co-stimulatory molecules and PDL-1. One representative experiment out of 3. (B) Top row: Cognate interaction of Th1 cells with TSN-M2 results in high IL-12 production. Stimulation with CD40-L cells in combination with 500 pg/ml IFN γ induces IL-12p70 production in TSN-M2 cultures to the level of mo-DC. Middle row: Cognate interaction with Th1 cells induced the production of IL-10 in TSN-M2 cultures at similar low levels detected for mo-DC. The addition of IFN γ to CD40-stimulation results in similar production of IL-10 as after CD40-L cell stimulation. Bottom row: The Th1 clones produce IFN γ when stimulated with peptide-pulsed mo-DC and TSN-M2. Shown are the results of the experiment in which all conditions and 3 clones were combined. Similar results were obtained in experiments where individual clones and TSN-M2 combinations were tested (C) APC cultured in the presence of M2-inducing TSN or PGE2 were activated with CD4⁺ T-cell clone 16, CD40-L expressing fibroblasts or IFN γ and analyzed for the expression of CCR7 at 48 hr after activation. Cognate Th1 cell interaction induces high CCR-7 on TSN-M2 and this can be mimicked by activating DC with IFN γ . The level of CCR7 is enhanced when PGE2 is present during the differentiation of monocytes. One representative experiment out of 3.

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 CD40-L cell-mediated activation to switch M2 to M1-like macrophages. TSN-M2 were activated with CD40-L and IFN γ or TNF α . This revealed that the combination of CD40-L cells with IFN γ but not CD40-L cells or IFN γ alone resulted in high levels of IL-12 not only in mo-DC but also in TSN-M2 cultures (Fig. 6b right and not shown). This capacity of Th1

cells or CD40-L cells + IFN γ to switch M2-like macrophages to M1-like macrophages could be reproduced in co-cultures with M-CSF-induced M2 macrophages (not shown).

TSN-M2 expressed higher levels of CCR7 than mo-DC following their cognate interaction with CD4+ Th1 cells (Fig. 6c). As we had already found that IFN γ synergized with CD40-activation to switch M2- 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-DC and a very high expression on TSN-M2. Furthermore, pretreatment of mo-DC with PgE2 during the differentiation phase resulted in similar high expression of CCR7 as found on TSN-M2 (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 co-stimulatory 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 7 different cervical cancer cell lines on the differentiation of monocytes to dendritic cells 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 while no role was found for TGF β , IL-8, IL-10 or M-CSF. In vivo, such APC are present at different levels of differentiation in stroma and epithelial compartments of HPV-induced cervical cancer and these include next to immature DC, mature DC, macrophages and type II macrophages (Fig. 1) (10,35).

Category 2 cancer cells stimulated the differentiation of CD14⁺, CD16⁺, CD206⁺, CD163⁺ M2-like macrophages. Consequently, these TSN-M2 displayed an altered cytokine profile and a poor capacity to stimulate T cells when compared to mo-DC. Careful evaluation of the expression of co-stimulatory molecules, cytokine production and T-cell stimulatory capacity of these TSN-M2 showed that stimulation with a number of clinical applicable TLR-agonists or CD40-L cells could not provoke the same phenotypical and functional activity as found for mo-DC. Interestingly, unstimulated TSN-M2 cells expressed PD-L1 at higher levels than mo-DC. While stimulation of mo-DC and TSN-M2 resulted in an increased expression of PD-L1, the expression on TSN-M2 remained higher on TSN-M2, suggesting that TSN-M2 display an altered co-stimulatory/inhibitory molecule ratio on the cell surface as compared to mo-DC. High levels of PDL-1 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 DC 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 naive T cells in our experiments. Notably, comparison of the two different agonists R848 and LPS to stimulate TSN-M2 revealed clear differences in their effects on co-stimulatory molecule expression and

cytokine production, indicating that previous results reported with the TLR4 agonist LPS – most often used to stimulate tumor-induced DC in vitro (16,38)– can not 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 DC to macrophages (32,33,40-42). Others showed that the over-expression of COX-enzymes in HPV-induced lesions is associated with a loss in CD1a+ 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 antigen to Th1 cells within the context of a M2-polarizing milieu – as represented by the M2-inducing tumor supernatant – this interaction not only results in re-polarization of M2- to M1-macrophages but it also activated these M1 macrophages to express high levels of co-stimulatory molecules, to produce IL-12 and to express the lymph node homing marker CCR7. The switch from M2- to M1-macrophages is in concordance 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 with CD40-L cells and IFN γ . While 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 importance role of the Th1-macrophage axis in anti-tumor immunity. Tumor-resident macrophages were shown to process and present tumor antigen to Th1 cells which in return 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 antigen presented by these altered APC 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 auto-immune diseases and trials are now ongoing, with the aim to determine the effect of low dose NSAID on tumor prevention by disrupting the COX-2 mediated oncogenic pathways (49). Furthermore, monoclonal antibodies to IL-6 receptor are already in clinical use for the treatment of autoimmune diseases (50). It can be envisaged 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 cervical cancer(51,52).

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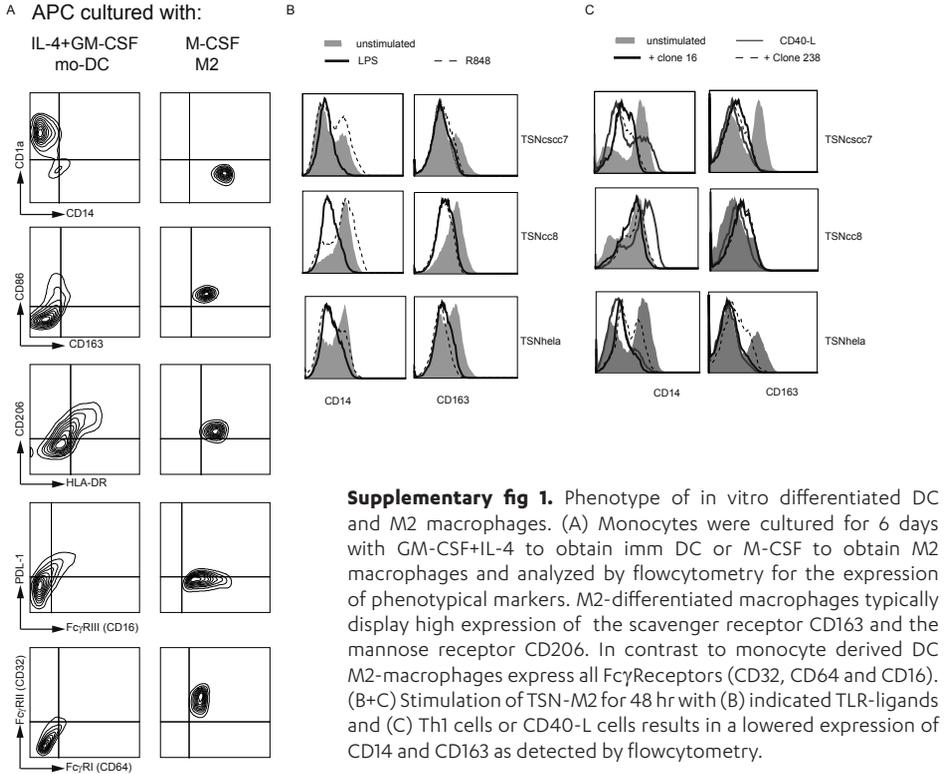
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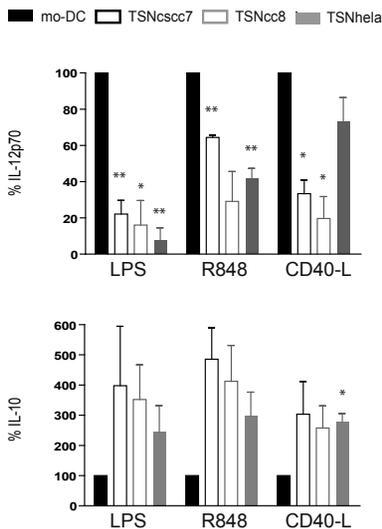
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SUPPLEMENTARY FIGURES

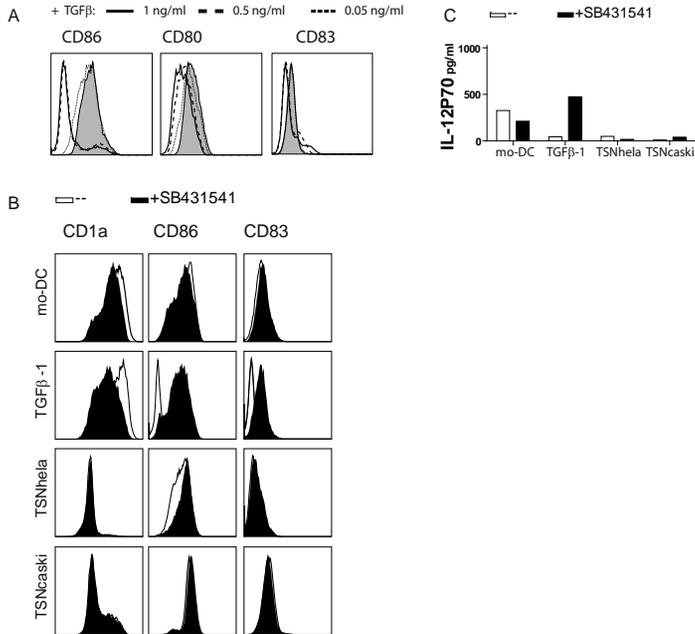


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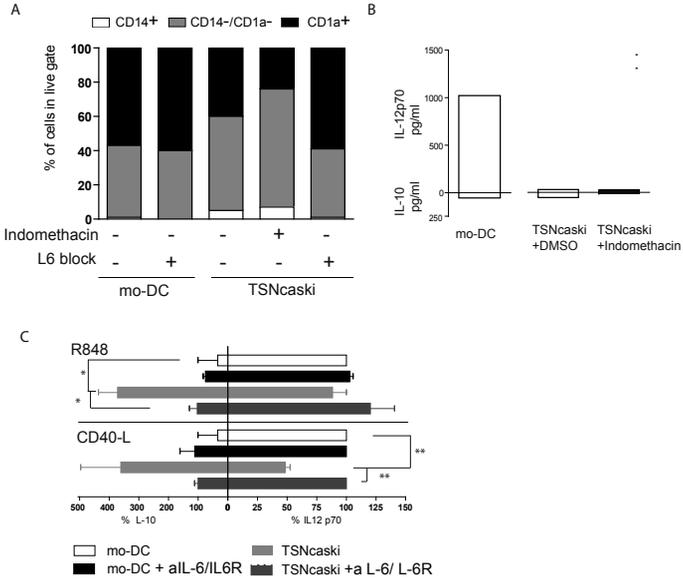
Supplementary fig 1. Phenotype of in vitro differentiated DC and M2 macrophages. (A) Monocytes were cultured for 6 days with GM-CSF+IL-4 to obtain imm DC or M-CSF to obtain M2 macrophages and analyzed by flowcytometry for the expression of phenotypical markers. M2-differentiated macrophages typically display high expression of the scavenger receptor CD163 and the mannose receptor CD206. In contrast to monocyte derived DC M2-macrophages express all Fc γ Receptors (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. 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 monocytes 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.



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 (TGFb) 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 TGFb-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. Depicted is the percentage of all cells in live gate. One representative of 3 experiments. (B) 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 $p < 0.005$.