CHAPTER 5.2

Modulation of tumor associated macrophages

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Ongoing work

ABSTRACT

CxCa patients with recurrent disease are treated with cisplatin or carboplatin. These chemotherapeutics may not only affect tumor cells but also the local immune cells. Previously we showed that CxCa can skew monocyte differentiation toward type 2 macrophages (M2) by secretion of PGE2 and IL-6. Therefore we studied the effects of these drugs on soluble factor mediated effects of tumor cells lines on APC differentiation.

Tumor induced M2 can be skewed towards pro-inflammatory M1 by interaction with IFNγ+ CD4+ T cells. In CxCa-patients however, a considerable proportion of tumor antigen specific T cells display an non-Th1 phenotype. To study the activation of tumor supernatant skewed M2 by Th2 or Th17 cells we activated APC with CD40-L and several T-cell derived cytokines.

We show that only CD40-L+IFNγ can skew M2 cells into activated M1 cells. Furthermore while CD40-L stimulated M2 macrophages induced strong T cell proliferation, only when stimulation was in the presence of IFNγ, the responding T cells were polarized into Th1. Treatment tumorcells with cisplatin, and to a lesser extent carboplatin, resulted in increased production of PGE2 and IL-6, resulting in induction of more M2 cells. This is an unwanted immunological effect and could be prevented by treating the tumor cells with COX-inhibitors.

INTRODUCTION

Currently the therapy for advanced, persistent or recurrent cervical cancer (CxCa) consists of cisplatin or carboplatin treatment although many tumors are resistant and the response rate does not exceed 30% (1,2). One described predictor of poor response to treatment is high expression of COX-2 protein in the tumor (3). COX-2 , which is the inflammation induced rate limiting enzyme for the conversion of arachnoid acid into prostaglandin E2 (PGE2) is found in several human solid tumors and we recently showed that PGE2 skews local antigen presenting cells (APC) into type 2 macrophages (M2) (4). The presence of M2 macrophages in tumors is correlated to poor prognosis in several human cancers (5-7).

Platinum based therapeutics act by binding to DNA. This activates various signal transduction pathways including those for DNA repair, cell cycle arrest and apoptosis. As a result it may have immune-modulating side effects (8). In a mouse tumor model oxaliplatin caused immunogenic cell death, thereby activating local APC and enhancing anti-tumor T-cell responses. Cisplatin triggered the release of the TLR-4 stimulating protein HMGB-1 (9,10). In addition, several platinum based drugs down regulated surface molecules in tumor and human APC via regulation of STAT6 (11).

In contrast to TLR-agonists or CD40-L help, a signal of CD40-L+IFNγ supplied through interaction with Th1 CD4+ T cells can switch tumor cell-induced M2 macrophages (TSN-skewed APC) into activated M1 macrophages (4). Studies on murine macrophages confirmed macrophage plasticity by showing that exposure to a type 1 cytokine environment before activation directs the macrophages towards M1 macrophages, whereas exposure to IL-10 directed them to M2 (12,13). In line with this Dulluc *et al.* comprehensively showed that IFNγ can prevent and reverse M2 differentiation of human monocytes by ascites of ovarian cancer *in vitro* (14). Together this indicates that IFNγ is a strong polarizing cytokine for macrophages. However in several human tumors not only Th1 (IFNγ/TNFα) CD4+ T cells but also Th2 (IL-5/IL-4), Th17 (IL-17) and regulatory T cells (TGFβ, IL-10) play a role. Especially in cervical cancer draining lymph nodes considerable numbers of tumor-antigen specific T cells are found that do not express a clear type 1 cytokine profile.

Subsequently we questioned the impact of platinum based chemotherapy on the micro milieu of cervical tumors. We focused on APC differentiation influenced by chemotherapy treated tumor cell lines as well as the impact of cognate interaction with several types of T cells in our in vitro system geared to result in M2 macrophages. To study the potential role of such differently polarized CD4+ T-cell subsets in the activation of locally present APC we cultured mo-DC without or with supernatant of CxCa cell lines (TSN-skewed APC) with CD40-L expressing cells in the presence of several Th-cytokines to mimic interaction with the separate T cell subsets.

MATERIAL AND METHODS

Media and reagents

APCs and CxCa cell lines were cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% FCS (Greiner Bio-one, Germany), 2 mM L-glutamine (Cambrex, USA), 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM β-mercaptoethanol (all Invitrogen), also referred to as complete or control medium. Adherent cell lines were harvested using trypsin/EDTA (Invitrogen).

The following factors were used to culture APCs: 500 U/mL IL-4 (Invitrogen), 800 U/mL GM-CSF (Immunotools, Germany), 5 ng/mL PGE2 (Sigma-Aldrich, Germany), 5 ng/ml IL-6 (immunotools) or 25 ng/ml M-CSF. The following was used to activate APCs. CD40-L expressing J558 mouse plasmacytoma cells ratio J558:APC 1:5 (The J558 cells were a kind gift of dr. E. de Jong, Amsterdam and dr. P. Lane, Birmingham); 10 µg/mL R848(CL097) 500 U/mL IFNγ (Bender Medsystems, Austria, this is approximately 1000 pg/ml), 2 ng/ml TNFα, 5 ng/ml IL-5, 2 ng/ml IL-17 (all immunotools). These concentrations were based on levels found in DC:T cell clone co-cultures or TIL cultures like described in chapter 6.

Chemotherapeutics and COX inhibitors

Cells were treated with 0.2-50 µg/mL cisplatin or 2-100 µg/mL carboplatin (Pharmachemie, NL) or the corresponding volume of PBS as well as with 25 µM indomethacin or the corresponding volume of DMSO.

Tumor cell line culture

The CxCa cell lines HeLa, CaSki, CSCC-1, CSCC-7, and CC-8 were typed and cultured as described earlier (4,15). Cell lines were cultured in 6- or 12- well plates (Corning, USA) for 24h, and treated with chemotherapeutics and/or COX inhibitors, as indicated. After 16h of treatment, cells were washed carefully and medium was refreshed. Supernatants were harvested after 24h and stored at -20ºC.

Only for RNA isolation cells were treated with chemotherapeutics for 18 hr, carefully washed and RNA was extracted. cDNA was prepared and gene expression was analyzed using pre-designed taqman probes of Applied Biosystems for GAPDH, IL-6, COX-1 (PTGS-1) and COX-2 (PTGS-2).

Transwell assay

Monocytes were cultured in a 24 well plate in complete medium containing IL-4 and GM-CSF and 0.5 or 2 µg/mL cisplatin, or PBS control. CC-8 cells (50,000 cells in 100 µL complete medium) were cultured in the upper compartment of a transwell 0.4 µm pore insert (Corning). To control wells, without transwell inserts, 100 µL control medium or tumor supernatant from untreated CC-8 or HeLa cells was added. After 3 days, complete medium with cytokines but without cisplatin was added. At day 6, cells were analyzed by flow cytometry.

Cell survival

To determine tumor cell line survival upon chemotherapeutics treatment, a MTT assay (Trevigen, USA) was performed according to the manufacturer's instructions. Cells were cultured as described above, in a 96 well flat bottom culture plate (Corning), but instead of harvesting the supernatant, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added. Absorbance was determined at 570nm and corrected for absorbance at 655 nm. Cell survival was calculated as follows: ($OD_{570\text{-}655nm}$ for treated / $OD_{570\text{-}655nm}$ for untreated) x 100%. To determine APC survival upon chemotherapeutics treatment, cells were analyzed by flow cytometry (see below). Cell survival was calculated as follows: (% of cells in live gate for treated / % of cells in live gate for untreated) x 100%.

Flow cytometry

APC were phenotypically analized as described earlier (4).

Cytokine analysis

ELISA was used to quantify cytokine levels of IFNγ, IL-10 (Sanquin, NL) IL-12p70 (BD Biosciences) and chemokines CXCL-10 and CCL-22 (RndD systems), according to the manufacturer's instructions. Further analysis of inflammatory and T cell cytokines was performed by cytometric bead array (BD Biosicences). PGE2 levels were measured using the competitive PGE_2 immunoassay kit (R&D systems).

RESULTS

IFNγ is the only cytokine that skews M2 to activated M1

We found that in LN of CxCa patients tumor-antigen specific T cells produce IFNγ, TNFα, IL-5 or IL-17. Therefore we assessed whether these type of T cells also could activate TSN-skewed APC. Monocytes were cultured with IL-4 and GM-CSF in the presence of supernatant of HELA or CSCC-7 cells (TSN-skewed APC). Routinely this resulted in 30-40% cells expressing typical M2 markers (4). Addition of the cytokines IFNγ, TNFγα, IL-5 or IL-17 at quantities produced by patient derived T cell clones in antigen specific tests did not result in the production of IL-12, IL-10 or IL-23 by mo-DC or TSN-skewed APC. Only IFNγ induced the upregulation of costimulatory molecules CD86, CD80, CD83 and PD-L1 on APC(not shown).

To mimic T-cell help but to ensure no other receptors were involved we made use of the J558 cell line transfected with CD40-L (CD40-L). Upon stimulation with CD40-L the mo-DC and TSN-skewed APC expressed high co-stimulatory molecules and lost CD163 expression (not shown). Addition of cytokines did not induce any change in co-stimulatory molecule expression compared to CD40-L stimulation only. Upon CD40 stimulation, TSN-skewed APC produced lower levels of IL-12 but high IL-10 compared to mo-DC upon CD40-L stimulation. Only addition of the cytokine IFNγ to CD40-L stimulation resulted in increased IL-12 production indicating a switch from M2 into M1-like cultures (Figure 1a). No phenotypic or functional effects were observed upon TNFα, IL-5 or IL-17 addition to CD40-L. Also several combinations of cytokines were tested but no additive effect to IFNγ was seen (not shown).

APC secrete chemokines to attract other inflammatory cells. CXCL-10 is the ligand for $CXCR\text{-}3^+$ type 1 effector T cells whereas CCL-22 attracts CCR-4 expressing Th2 and regulatory T cells. CCL-22 was shown to be induced in human APC when activated in the presence of PGE2 (16). Unstimulated mo-DC or TSN-skewed APC did not produce any detectable CXCL-10 but did produce high levels of CCL-22 (mo-DC cultures 40 ng/ml or more). Interestingly, HELA-supernatant APC produced only half the quantities CCL-22 of mo-DC ($p < 0.043$ paired t-test). When APC were stimulated with IFNγ CXCL-10 production was increased almost 10-fold and this was the same for all APC cultures (fig 2b). No chemokine induction upon stimulation with TNFα, IL5 or IL-17 was seen.

CD40 mediated activation did not induce significant CXCL-10 release but induced even more production of CCL-22 in mo-DC as well as TSN-skewed APC. These results show that IFNγ is the only T-cell produced cytokine that can activate TSN-skewed APC and reverse the cells to pro-inflammatory cytokine production and attraction of type 1 T cells.

Activation of APC by Th1 signals determines type of T-cell induction

Previously, we showed that CD40ligation of mo-DC resulted in the production of high levels of IL-12, a known strong type 1 directing stimulus (4). To assess whether the type of T cell

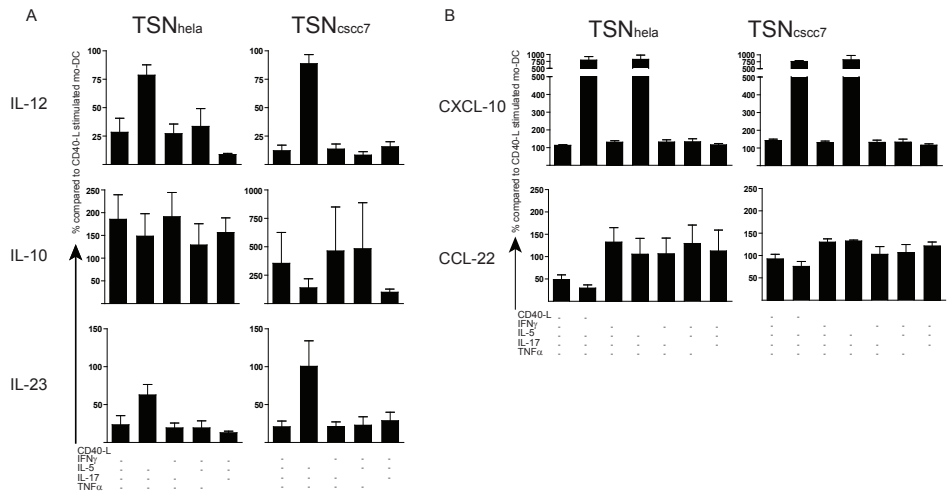

Figure 1. IFNγ activates tumor supernatant induced APC. Compared to mo-DC, supernatant induced cultures produce less IL-12 and more IL-10 upon CD40-L stimulation and only IFNγ can revert this balance. A) mo-DC or TSN-M2 (supernatant of HELA left, supernatant of CSCC-7 right) were activated with J558-CD40-L cells (ratio 5 DC:1 CD40-L cell) and recombinant cytokines (depicted at the x-axis). Supernatant was analyzed by ELISA after 48 hr. Mean cytokine production of CD40-L stimulated mo-DC: 24 ng/ml IL-12, 14 ng/ml IL-23 and 275 pg IL-10. Calculation: (activate TSN-M2 / Value mo-DC stimulated with CD40-L only)*100. Mean of 3 experiments + SEM. B) Chemokine production by cells described in A. Here also unstimulated and IFNγ only is shown. (In unactivated cultures no CXCL-10 was detected but CCL-22 was produced in all cultures.CCL-22 in mo-DC cultures >40 ng/ml) Mean of 3 experiments + SEM.

providing the activation signal may program the APC to subsequently instruct naïve T cells to adapt to a particular phenotype, we used a mixed lymphocyte reaction assay (MLR). Mo-DC or TSN-induced APC were activated for 48 hr with irradiated J558-CD40-L in the presence of recombinant cytokines. APC were extensively washed and cultured with allogeneic CD4+CD25- cells for 5 and 10 days. All APC types and activation signals induced comparable proliferation of the responder cells (fig 2. top left). However type 1 T-cell induction as reflected by the amounts of IFNγ and TNFα produced, was determined by the type of T cell providing the activation to the APC. Mo-DC activated with Th1 signals (CD40-L+IFNγ) clearly induced 4 fold higher type 1 cytokine production in the responder T cells. High IFNγ levels were also induced by TSN-induced M2 cultures stimulated with Th1 signals (figure 2, middle panels). Interestingly some type 2 cytokines were also detected in the cultures of T cells stimulated with CD40-L+IFNγ activated APC, but the production of these cytokines was more prominent in the T-cell cultures stimulated with TSN-skewed APC. Nine days after start of culture, the T cells were analyzed for expression of transcription factors. Figure 2 clearly shows that T cells display the highest T-bet expression following stimulation with mo-DC. Activation of APC in the presence of IFNγ resulted in higher T-bet expression in the responder T cells (figure 2 bottom left).

Different cytokines result in different macrophages in vitro

In vitro, macrophages can be obtained by culturing monocytes with only GM-CSF, M-CSF or as we reported previously with PgE2 and/or IL-6. Of note, the latter protocol induced

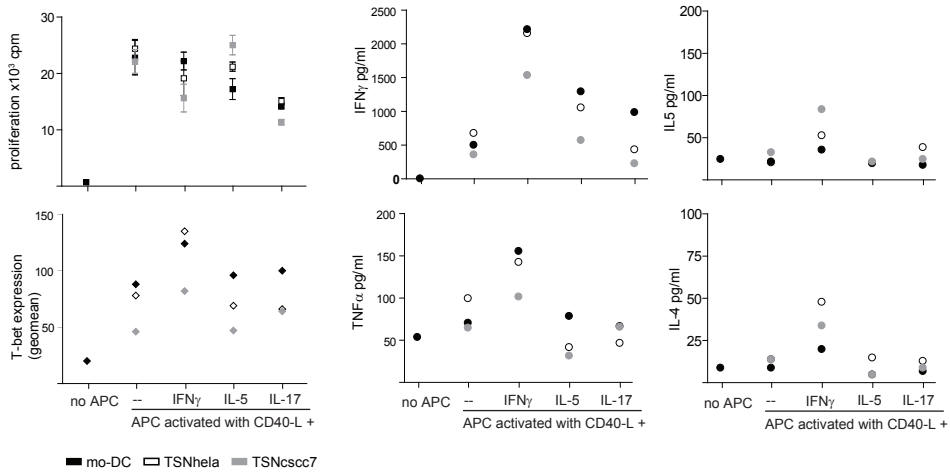

Figure 2. Th1 derived activation of APC instructs for type 1 T cell induction. Activation of APC in the presence of IFNγ results in higher T-bet expression and more type 1 cytokines. Allogeneic responder cells were cultured with mo-DC or TSN-skewed APC that were prior to co-culture activated with irradiated CD40-L only or in combination with cytokines (IFNγ, IL-5 or IL-17) as depicted at the x-axis. Top panel left; proliferation of T cell cultures at day 5 (initial ratio APC:T cell 1:5, triplo mean+SEM). Lower left panel: expression of the intra-cellular transcription factor T-bet at day 9 of T cell culture determined by intra-cellular staining and flowcytometry. Middle and right panels; cytokine levels in T-cell cultures at day 5 of co-culture. One experiment of 2 performed with IFNγ and IL-4 derived from MLR 17, IL-4 and TNFα from MLR 16.

macrophages even in the presence of the DC-skewing cytokines GM-CSF and IL-4. All cultures expressed macrophage marker CD14 and CD163 at day 6 with the latter being most prominently expressed by M-CSF induced M2 (data not shown). Since all these *in vitro* cultured macrophages display a similar phenotype, we tested whether they responded equally to T-cell interaction. Only the cytokine IFNγ upregulated co-stimulatory molecules on macrophages. CD40-L is a strong activator for mo-DC, less strong for GM-CSF or PgE2+IL-6-differentiated APC but hardly activated M-CSF-induced macrophages (Fig.3a). Compared to mo-DC, all macrophage subsets produced no to low levels of IL-12 upon activation with the indicated stimuli. In contrast IL-10 was produced by all cultures but PgE2+IL-6 derived macrophages produced extreme levels of IL-10 following activation via CD40 (fig 3b). Also here IFNγ was the only cytokine that clearly worked in synergy with CD40-L for activation of macrophages and the production of IL-12.

Platinum based chemotherapy of tumor lines increase M2 induction

Patients with recurrent or advanced disease are treated with cisplatin or carboplatin. It is reported that expression of the PgE2 converting enzyme COX-2 in tumors favors a poor response to this treatment (3,17). In view of recent literature indicating that chemotherapy may also influence the response of immune cells we assessed the impact of chemotherapeutics on tumor cells and the effect on APC differentiation. First monocytes that differentiate to DC as well as several tumor cell lines were treated with creasing doses of cisplatin or carboplatin. Figure 4a shows that all cell lines survive up to concentrations of 2 μg/ml for cisplatin and 20 μg/ml for carboplatin. In vitro treatment of APC revealed that these cells could tolerate the same

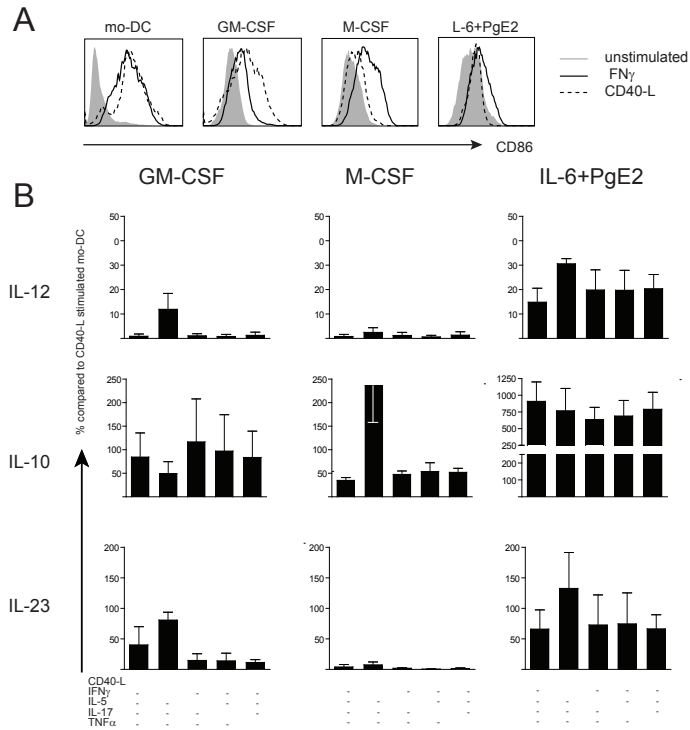

Figure 3. Different responses by cytokine induced macrophages. Macrophages were cultured with GM-CSF only, M-CSF only or to mimic tumorsupernatant IL-4/GM-CSF and 5 ng/ml IL-6+ 5 ng/ml PgE2 and compared to mo-DC. Cells were activated and analyzed after 48 hr for activation markers and cytokine production. A) CD86 expression unactivated or after activation, IFNγ only or CD40-L only. All other conditions as depicted in B did not induce CD86 expression. B) Depicted is the percentage of cytokine production compared to mo-DC in the same experiment stimulated with CD40-L only. Calculation: (Value TSN-M2 / Value mo-DC stimulated with CD40-L only)*100. Mean of 3 experiments + SEM.

doses (2 μg/ml cisplatin, 20 μg/ml carboplatin) with less than 10% dead cells but cell death was rapidly increasing at higher doses. No effect was seen on APC phenotype if chemotherapeutics were directly added during mo-DC or TSN-skewed APC differentiation.

Tumorcells were cultured and treated for 16 hr with cisplatin or carboplatin, carefully washed and cultured for another 24 hr. This supernatant was harvested and used in monocyte cultures. As shown earlier, TSN of line CC-8 and HELA skewed DC differentiation towards M2 macrophages. Strikingly, TSN from tumor cells treated with cisplatin induced a higher percentage of M2 macrophages. Carboplatin had the same effect on CC-8 cells but not on HELA cells (fig 4b). Remarkably, TSN of line CSCC-7 treated with cisplatin or carboplatin did not induce a higher percentage of M2 macrophages. The TSN of treated control lines CASKI and CSCC-1 did not show this effect (fig. 1c).

I*n vivo* tumor cells and immune cells are in the same microenvironment when treated with platinum. To better resemble the natural situation, monocytes and tumor cells were cultured together, using a transwell system in which chemotherapeutics were added at day

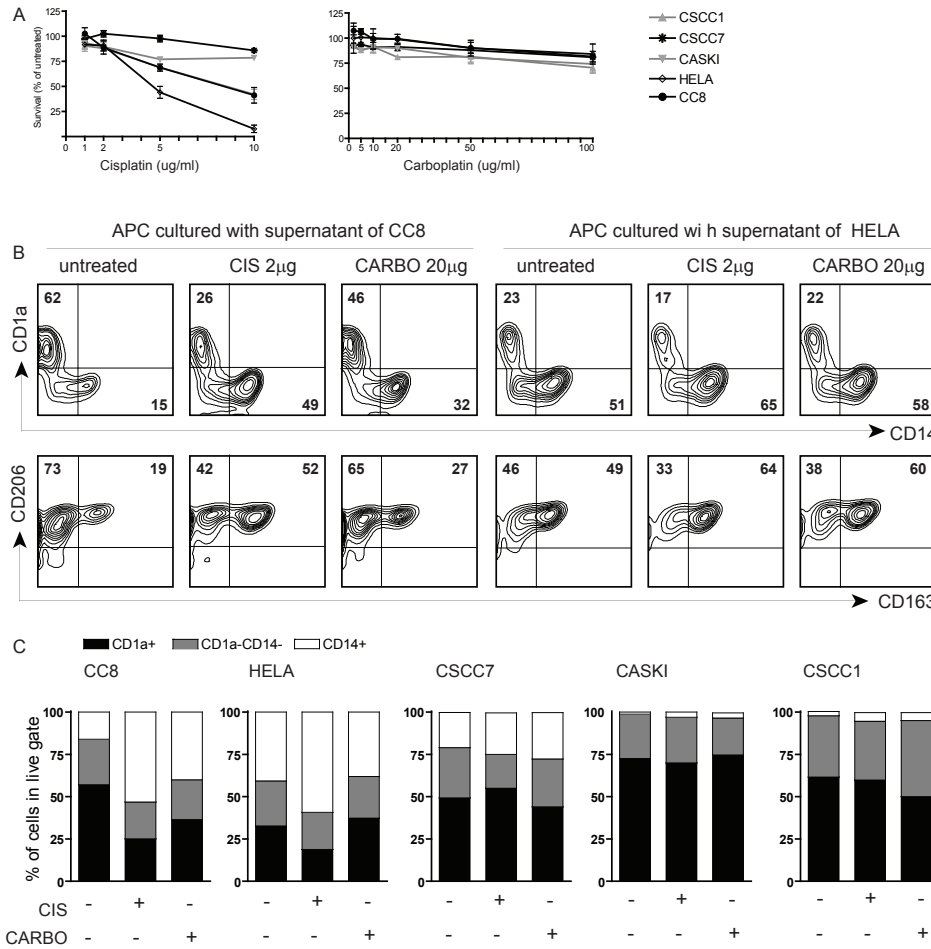

Figure 4. Platinum based chemotherapy treatment of CxCa Lines enhances M2 induction. Cxca cell lines that are treated with a dose of cisplatin or carboplatin that does not induce major cell death produce soluble factors that induce more type 2 macrophages. A) Survival of CxCa cell lines upon increasing doses of cisplatin (left) or carboplatin (right) measured in a MTT assay. Shown the mean of 3 experiments with SEM. Lines that induce M2 are shown in black. B) Monocytes were differentiated with IL-4 and GM-CSF in the presence of 20% tumor supernatant from CC-8 (left) or HELA(right) that were treated with 2 µg/mL cisplatin or 20 µg/mL carboplatin, or control medium (untreated). Differentiation was analyzed at day 5 by flow cytometry for expression of DC and M2 markers. Numbers in quadrants represent the percentage of cells within the live gate. Representatives of 5 independent experiments. C) $CD1a^+$, $CD1a^-CD14^-$ and $CD14^+$ subpopulations of cells within the live gate of APCs described in B. Mean of 3 (CC-8, HELA, CSCC7, CSCC1) or 2 (CASKI) experiments.

0 of culture. Again treatment with cisplatin resulted in an increased percentage of M2 cells when monocytes were cultured in the presence of cell line CC8 and cisplatin (fig.5a). However, chemotherapeutic treatment also resulted in a functional suppression of APC (fig. 5b). TSN induced APC produced less IL-12 and more IL-10 upon activation compared to mo-DC, but the production of both cytokines was lowered when tumor lines CC-8 and HELA were treated with

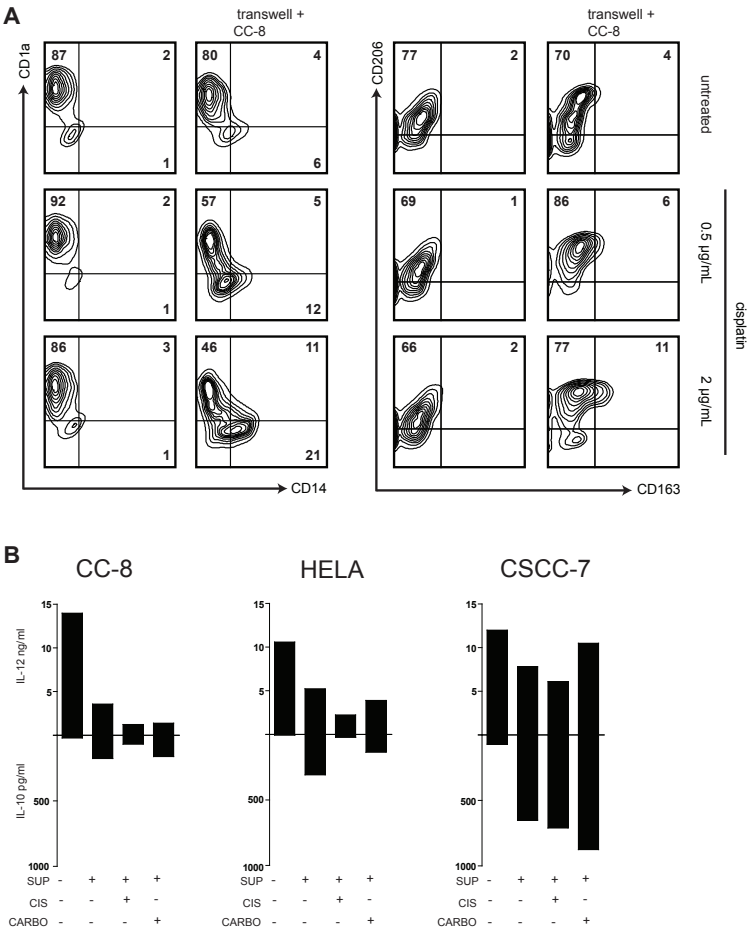

Figure 5. Platinum enhances macrophage induction and induce poor cytokine production. A) Monocytes were cultured with IL-4 and GM-CSF. In the upper chamber of a transwell tumor cells were added and in the lower chamber control (untreated) or cisplatin was added only at day 0 of culture. At day 3 fresh medium was added with IL-4 and GM-CSF. Cells were analyzed for phenotype at day 6. Numbers are percentages of cells in live-gate (livegate was similar for each condition). B) APC were cultured in the presence of supernatant of control or platinum treated tumor cells and stimulated with R848 for 48 hr. Cytokine production was measured by ELISA. All tumor lines were tested separately and this is one experiment of 2 of each.

chemotherapeutics. Line CSCC-7 did not induce more M2 macrophages after treatment and also the cytokine production upon activation by these APC was not affected (fig 5b, one of 2 experiments).

Chemotherapeutics enhance PgE2 and IL-6 production in tumor cells

M2 macrophage induction by CxCa cell lines depends on the levels of PGE2 and IL-6 (ref). Therefore we measured the amount of PGE2 and IL-6 produced by the cell lines upon platinum treatment. Cisplatin treatment increased the production of PGE2 2-7 fold by line CC-8 and HELA

but not by line CSCC-7. Carboplatin had no effect on PGE2 levels (fig 6a). Furthermore cisplatin and carboplatin increased the IL-6 production by CC-8 and HELA. Also IL-6 was induced in line CSCC-7, albeit at much lower levels compared to the other lines. The CASKI line, known to produce already high levels of IL-6 also responded to treatment with cisplatin by increasing the production of IL-6. Still this did not skew APC differentiation into M2 macrophages (fig 4c) (4). This indicates that both PGE2 and IL-6 in sufficient levels are needed to induce M2 differentiation.

As expected by the increase in cytokine levels, gene expression analysis revealed that IL-6 as well as COX-2 were induced upon platin treatment in both CC8 and CSCC7 cell lines with highest induction in CC8 cells. Furthermore levels of COX-1 mRNA were not increased by platinum treatment in both lines (fig 6b middle panel). Thus, cisplatin in a non-lethal dose,

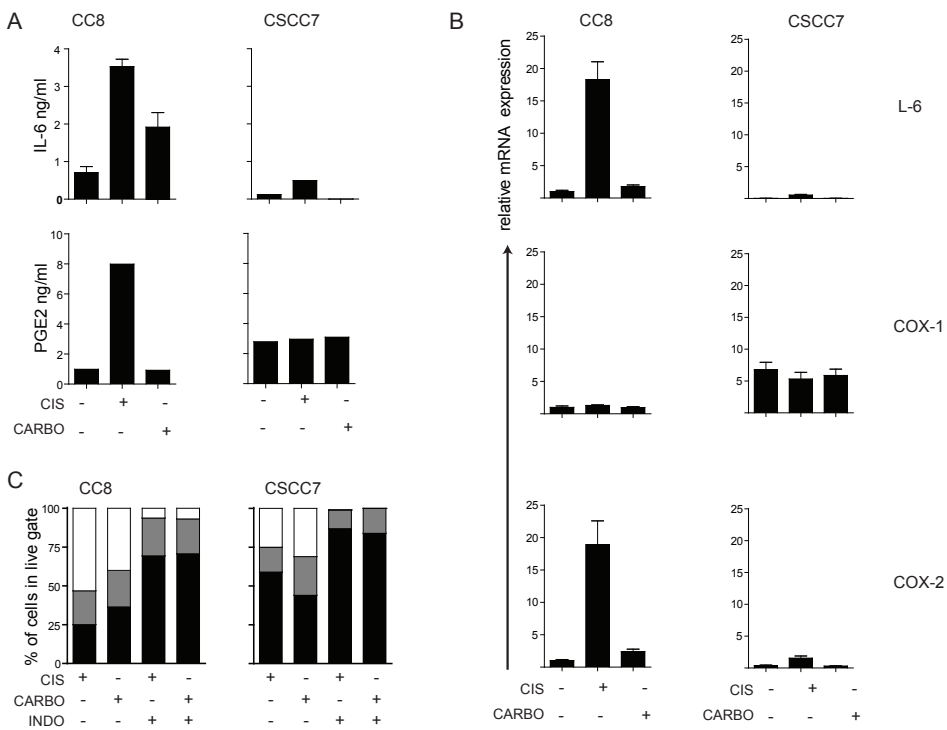

Figure 6. Induction of PgE2 and IL-6 by platinum based chemotherapy. Upon cisplatin production the levels of the M2-inducing factors PgE2 and IL-6 are increased in CxCa cell lines. A) Tumor cell lines were treated with cisplatin (2 ug/ml) or carboplatin (20 ug/ml) for 24 hours, carefully washed and cultured for another 24 hrs. This medium was analyzed for the production of IL-6 and PgE2 by ELISA. B) Tumor lines were treated as described in A, but after 18 hr total RNA was isolated and TaqMan gene expression assay was perfomed. Ct-values were corrected to GAPDH expression and depicted are relative mRNA levels compared to untreated CC-8 cells. Example of 1 cell culture, measured in triplo. C) Tumor Lines were treated with indomethacin (25 μM) and cis- or carboplatin for 24 hrs, washed and supernatant was used in APC cultures. APC cultured with supernatant were analyzed at day 5 and $CD1a^+$, $CD1a^-CD14^-$ and $CD14^+$ subpopulations of cells within the live gate are shown. M2 induction is abolished if cells are treated with COX enzyme inhibitor. Mean of 3 (CC-8) or one single experiment (CSCC7)

enhances the expression of COX-2 and IL-6 but not COX-1 and the production of PgE2 and IL-6 thereby inducing more M2 macrophages.

PgE2 is a critical factor in the *in vitro* skewing of monocytes to macrophages. If COX-enzymes are blocked with indomethacin in the tumorlines during chemo treatment, M2 induction is abolished (fig 6c). For line CC8 and HELA specific inhibition of COX-2 by NS398 resulted in abolished M2 induction, indicating that induced PGE2 levels upon platinum treatment are COX-2 mediated. For line CSCC-7 specific COX-2 inhibition was not yet tested.

DISCUSSION

Activation of mo-DC or TSN-skewed macrophages by CD40-L stimulation in the presence of helper T-cell produced cytokines all resulted in activation but no synergism of IL-5, IL-17 or TNFα with CD40-L was found for the induction of pro-inflammatory cytokines or chemokines. We showed that the presence of IFNγ clearly activates the TSN-skewed APC cultures to stimulate a type 1 inflammatory response reflected by the pre-dominant Th1 profile in subsequent stimulated T-cell cultures. In this model, we mimicked T-cell interaction with the use of CD40-L expressing cells that have a (artificially) high expression of this molecule. However when APC and T-cell crosstalk, more co-stimulatory molecules will be involved and therefore the activation is likely to be altered. T-cell receptor-peptide-MHC interaction results in the formation of immunological synapses –with locally high levels of cytokines- that is not mimicked in this system (18). The concentrations of cytokines used in our system were based on the amounts detected in patient derived HPV specific T-cell cultures. We did not find any response to IL-5, TNFα or IL-17 on mo-DC or TSN-skewed APC by upregulation of co-stimulatory molecules or IL12, IL23 or IL-10 production. Lack of IL-5 receptor may form one explanation as so far it was not reported to be present on unactivated myeloid cells (19,20). In contrast TNFα has been described in human monocytic cultures and mice DC to induce moderate activation (16,21) and high doses of IL-17 could activate human macrophages in vitro as well (22). Doses used in these studies outreached the amounts we detected in our in vitro T cell assays and used in our system.

PGE2 was described to induce CCL-22 production in human APC cultures if added during activation (16). Indeed in our cultures unactivated and activated APC produced high levels of CCL-22 but this was the same for mo-DC as well as TSN-skewed APC. Both HELA and CSCC7 supernatant contain PGE2 but addition of this supernatant induced CCL-22 production in the unactivated APC as high or lower then mo-DC cultured without PGE2.

Interestingly, IFNγ was the only stimulus that instructed mo-DC and TSN-skewed APC to produce the type 1 attracting chemokine CXCL-10. Thus, based on this limited study, the interaction of APC with antigen specific T cells may result in attraction of Th2/Treg cells which would contribute to shut down of the immune response. However when IFNγ is present, high levels of CXCL-10 are secreted which may attract more type 1 T cells.

In concordance with the IL-12/IL-10 cytokine balance produced by APC upon activation by CD40-L+IFNγ, induced naïve T cells become activated IFNγ-producing T cells even when APC were skewed into M2 by TSN.

Treatment of 2 out of 3 tumor cell lines that produce high PGE2 and IL-6 with a non-lethal dose of cisplatin resulted in an increased production of these inflammatory mediators and

subsequent M2 skewing in differentiating monocytes. Maximum doses in patients as measured in the blood for cisplatin or carboplatin are 5-6 ug/ml and 40-80 ug/ml respectively and assuming that levels in (poorly vascularized) tumor tissue is several times lower the used in vitro doses may be representative for the in vivo situation (23-25). Here we show that myeloid cells can survive the chemotherapeutic treatment but are affected by tumor released factors. The mRNA levels of COX-1 were higher expressed in line CSCC7 compared to line CC8 but were not influenced by treatment with cisplatin or carboplatin. In line CC8 the COX-2 and IL-6 mRNA levels clearly increased upon cisplatin treatment. This increase corresponds with the induction of M2 macrophages. The fact that supernatant of cisplatin treated CSCC7 did not result in more M2 induction correlates with the limited elevation of PGE2 and IL-6 upon treatment of this line. A possible explanation lies in the chemoresistence of this tumor cell line.

High COX-2 expression in CxCa identified the patients with a poor response to cisplatin and unfavorable prognosis (3). This effect was explained by enhanced apoptosis resistance of the tumor cells, but this was not reflected by our tested tumor cell lines. CCSC7 and CASKI cell lines were the most resistant to chemotherapy but did not express the highest levels of COX-2 and PGE2. Our data suggests an immunological explanation for the correlation of high COX-2 expression with poor response to treatment. Cisplatin increases the inflammatory mediators IL-6 and PGE2 in lines with high COX-2 expression, thereby promoting M2 macrophages and supporting tumor survival and growth. Interestingly these effects of cisplatin were only seen in the cell lines CC8 and HELA. Wang et al. showed that the chaperone protein PRDX-1 can join and stabilize the NF-kB complex at NF-kB promotor site of the COX-2 promotor region. This complex was shown to be selectively crosslinked to the DNA by cisplatin and therefore COX-2 gene expression is promoted (26).

Importantly, the effects of the supernatant on monocytes be reversed by inhibiting COX-enzymes in the tumor cells. The effects of IL-6 blocking is not yet addressed in this work.

Thus these results indicate that activation of tumor-skewed M2 by T cells without IFNγ results in IL-10 production and the induction of non-type 1 polarized T cells. Only CD40-L+IFNγ reverts TSN-skewed APC into Th1 inducing activated APC. Since spontaneous evoked T-cell responses often lack Th1 cytokines in patients with CxCa, therapeutic vaccination that can induce a strong type 1 T cell response is needed (4). This vaccine should not only aim for the induction of specific CTL but also strong IFNγ producing Th1 CD4+ T cells to activate the local macrophages and change the inflammatory balance in the tumor.

Concomitant treatment with cisplatin may have a devastating impact on local myeloid cells in tumors with high PGE2 and IL-6 production. Treatment of patients with COX-2 inhibiting drugs during chemotherapeutic treatment might prevent these unwanted immune related effects.

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