Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment


Department of Clinical Oncology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands

Running Title: Effect chemotherapy on tumor microenvironment

Keywords: chemotherapy, cervical cancer, ovarian cancer, COX-2, IL-6

Footnotes:

• 1 Correspondence to Prof. Dr. S.H. van der Burg, department of Clinical Oncology, K1-P, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Phone: +31 71 5261180, Fax: +31 71 5266760, Email: shvdburg@lumc.nl

• MW was financially supported by a grant from the Dutch Cancer Society 2009-4400; BT and RG are supported by NWO 40-00812-98-09012

• Word count: 5489

• Number of figures and tables: 7

Conflict of interest statement: authors report no conflicts of interests
Abstract

Current therapy of gynecological malignancies consists of platinum-containing chemotherapy. Resistance to therapy is associated with increased levels of interleukin-6 (IL-6) and prostaglandin E2 (PGE2), two inflammatory mediators known to skew differentiation of monocytes to tumor-promoting M2 macrophages. We investigated the impact of cisplatin and carboplatin on 10 different cervical and ovarian cancer cell lines as well as on the ability of the tumor cells to affect the differentiation and function of co-cultured monocytes in vitro. Treatment with cisplatin or carboplatin increased the potency of tumor cell lines to induce IL-10-producing M2 macrophages which displayed increased levels of activated signal transducer and activator of transcription-3 (STAT3) due to tumor-produced IL-6 as well as decreased levels of activated STAT1 and STAT6 related to the PGE2-production of tumor cells. Blockade of canonical NFκB signaling showed that the effect of the chemotherapy was abrogated, preventing the subsequent increased production of PGE2 and/or IL-6 by the tumor cell lines. Treatment with the cyclooxygenase (COX)-inhibitor indomethacin and/or the clinical monoclonal antibody against IL-6 receptor (IL-6R), tocilizumab, prevented M2-differentiation. Importantly, no correlation existed between the production of PGE2 or IL-6 by cancer cells and their resistance to chemotherapy-induced cell death, indicating that other mechanisms underlie the reported chemoresistance of tumors producing these factors. Our data suggests that a chemotherapy-mediated increase in tumor-promoting M2 macrophages may form an indirect mechanism for chemoresistance. Hence, concomitant therapy with COX-inhibitors and/or IL-6R antibodies might increase the clinical effect of platinum-based chemotherapy in otherwise resistant tumors.
Introduction

Current treatment of advanced cervical and epithelial ovarian cancer includes platinum-based multimodality therapy (1, 2). Many gynecological cancer patients develop resistance to platinum drugs, thereby limiting further treatment options and decreasing overall survival rates (1, 3). Gynecological cancers are generally known as immunogenic and prominent correlations exist between the infiltration of tumors by immune cells and clinical outcome (4, 5). Macrophages are the most abundant immune cells present in the tumor microenvironment. Macrophages originate from monocytic precursors in the blood and undergo specific differentiation depending on cues in the local tissue. Two extreme polarization states of macrophages are known, M1 and M2 of which the latter has poor antigen-presenting capacity, prevents T-cell activation, contributes to suppressing dendritic cell (DC) functions as well as enhances angiogenesis and metastasis (6). The presence of M2 macrophages in tumors is correlated to poor prognosis in several human cancers (7, 8). Previously, we and others demonstrated the influence of cervical (9) and ovarian cancer cells (10, 11) on differentiation of monocytes into DC or macrophages. The majority of cancer cells either hampered monocyte to DC differentiation or skewed their differentiation towards M2-like macrophages, depending on their ability to produce prostaglandin E2 (PGE$_2$) and/or interleukin-6 (IL-6). Blocking these cytokines completely restored their differentiation towards DC (9-11). Interestingly, in gynecological malignancies up-regulation of the cyclooxygenase (COX) enzymes has been associated with platinum drug resistance (12, 13). In addition, high levels of IL-6 in sera and ascites of patients with these gynecological malignancies have also been related to chemoresistance and poor clinical outcome (14, 15). IL-6 is one of the major immunoregulatory cytokines present in the tumor microenvironment and induces several pathways leading to tumor proliferation, angiogenesis and chemoresistance (16, 17). One important pathway stimulated by IL-6 is activation of signal transducer and activator of transcription-3 (STAT3) by phosphorylation. High levels of phosphorylated STAT3 are found in tumors (18) and tolerogenic antigen presenting cells (APC) (19) and consequently, STAT3 signaling in APC is linked to the induction of T-cell tolerance (20).
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In view of the observations that PGE$_2$ and IL-6 are associated with chemoresistance and with the tumor-induced differentiation of tumor-promoting M2 macrophages as well as recent literature indicating that chemotherapy may bear impact on the function and thereby the efficacy of tumor-infiltrating immune cells (21), we investigated the potential effects of platinum-based chemotherapeutics on the differentiation and function of APC under the influence of cervical and epithelial ovarian cancer cells in vitro.

We found that the treatment of tumor cells with cisplatin or carboplatin increased the potency of some tumor cell lines to skew monocytes to M2-like macrophages. These M2-like macrophages displayed IL-6-mediated increased levels of activated STAT3 and PGE$_2$-mediated decreased levels of activated STAT1 and STAT6, which are associated with immune potentiating pathways. The underlying mechanism was a cisplatin or carboplatin-induced enhanced activation of the NF$_{κB}$ pathway likely through the chemotherapy-induced DNA damage response (DDR). This resulted in an increased production of PGE$_2$ and IL-6 by cancer cells, but only if they already produced these factors, and an enhanced skewing of monocytes towards M2-like macrophages. Increased numbers of tumor-promoting M2-like macrophages may form an indirect mechanism for chemoresistance suggesting that concomitant therapy with COX inhibitors and/or blocking of IL-6R might increase the antitumor effect of chemotherapy.
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Materials and methods

Media and reagents

APC and cancer cell lines were cultured as described earlier (9) in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS Greiner Bio-one, Germany), 2 mM L-glutamine (Cambrex, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM β-mercaptoethanol (Invitrogen), also referred to as complete or control medium. Adherent cells were harvested using trypsin/EDTA (Invitrogen). The following factors were used to culture APC: 500 U/mL IL-4 (Invitrogen), 800 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunotools, Germany), 25 ng/mL macrophage colony-stimulating factor (M-CSF; R&D, Minneapolis, USA), 1-10 ng/mL PGE$_2$ (Sigma-Aldrich, Germany) and 1-50 ng/mL IL-6 (Immunotools, Germany). The toll-like receptor (TLR) ligand 0.25 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich, Netherlands) was used to activate APC; to mimic T cell interaction, APC were stimulated with irradiated CD40 ligand (CD40L)-expressing mouse fibroblasts. The following cytokines were used to induce STAT phosphorylation: 500 U/mL interferon gamma (IFN-γ; Bender Medsystems, Austria), 10 ng/mL IL-10 (Peprotech, USA) or 500 U/mL IL-4.

Chemotherapeutics, COX inhibitor, monoclonal antibody against IL-6(R) and Bay 11-7082

Cells were treated with 0.2-50 μg/mL cisplatin or 2-500 μg/mL carboplatin (Pharmachemie, Netherlands) and/or 25 μM indomethacin (Cayman Chemical, USA) dissolved in DMSO or as a control only with the corresponding concentration of DMSO. The monoclonal antibody used to block the (s)IL-6R was tocilizumab, 5-50 μg/mL (RoActemra, Roche BV, Netherlands). We used 0.02-200 μg/mL of the selective inhibitor of kappa B alpha (IkBα) inhibitor Bay 11-7082 (Sigma-Aldrich, Netherlands).

Tumor cell line culture

The human cervical cancer cell lines HELA, CASKI, CSCC1, CSCC7 and CC8 were typed and cultured as described earlier (9). Ovarian cancer cell lines SKOV3 and A2780 were purchased from the European Collection of Cell Cultures (ECACC). CAOV3 was obtained from the American
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Type Culture Collection (ATCC) and OVCAR3 and COV413B were kindly provided by the department of Clinical Pathology of the Leiden University Medical Center. All human ovarian cancer cell lines were of epithelial origin. Cell lines were authenticated every half year by short tandem repeat (STR) DNA markers as described previously (22). In brief, polymerase chain reaction (PCR) amplification of eight highly polymorphic microsatellite STR loci and gender determination were measured and the uniqueness of DNA profiles was compared for identity control within the STR database of ATCC (23). Stock vials were thawed and cultured for 10 passages and routinely tested for the presence of mycoplasma.

Cell lines were grown in culture flasks at 80–90% confluence and harvested with trypsin/EDTA and cultured in 6- or 12-wells plates (Corning, USA) for 24h, treated with chemotherapeutics and/or COX inhibitors and/or tocilizumab as indicated. After 24h of treatment, cells were washed carefully and medium was refreshed. Tumor supernatants (TSN) were harvested after an additional 24h of culture and stored at -20ºC. Cancer cell lines were cultured in the presence of 2 µg/ml cisplatin or 20 µg/ml carboplatin for 24h. These doses are estimated levels of chemotherapy in the tumor tissue since in patients the maximum doses for cisplatin or carboplatin as measured in the blood are 5-6 µg/ml and 40-80 µg/ml, respectively. Assuming that the levels in (poorly vascularized) tumor tissue are somewhat lower, the doses used in vitro are representative for the in vivo situation (24-29).

**APC culture**

APC were differentiated as described earlier (9). A brief explanation is given in supplementary material and methods. To address the direct cytotoxic effect of chemotherapy on APC the cultures were supplemented with 20% TSN or control medium (monocyte-derived DC; mo-DC) and titrated doses of chemotherapeutics, as indicated. To investigate the capacity of chemotherapy to alter the differentiation of APC by acting through tumor cell-mediated mechanisms, the cultures were supplemented with 20% TSN of untreated and treated cancer cell lines or control medium (mo-DC).
**Transwell assay**

Monocytes were cultured in a 24-wells plate in complete medium with or without 2 μg/ml cisplatin or 20 μg/ml carboplatin. Cancer cells were cultured in the upper compartment of a transwell 0.4 μm pore insert (Corning). After 3 days, complete medium with cytokines was added. At day 6, cells were analyzed by flow cytometry.

**Cell survival**

To determine the survival of the cancer cells upon chemotherapeutic treatment, a MTT assay (Trevigen, USA) was performed, according to the manufacturer’s instructions. Cell survival was calculated as follows: (OD$_{570-655nm}$ for treated / OD$_{570-655nm}$ for untreated) x 100%. To determine APC survival upon chemotherapeutic treatment, cells were analyzed by flow cytometry. Cell survival was calculated as follows: (% of cells in live gate for treated / % of cells in live gate for untreated) x 100%.

**Phosphorylated STAT (pSTAT) analysis**

pSTAT analysis was performed according to Krutzik et al. (30). Cells were fixed in 1.5% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature, harvested and washed twice in PBS containing 2% FCS (PAA, Austria) and 0.02% sodium azide (AZL Pharmacy, Netherlands). Then, cells were permeabilized in 90% methanol (Sigma-Aldrich, Netherlands) for 10 min on ice, washed and stained for pSTAT1 (pY701), pSTAT3 (pY705) or pSTAT6 (pY641; all PE; all BD Biosciences). Expression was calculated as follows: Geometric mean of fluorescence intensity of condition of interest - geometric mean of fluorescence intensity of the corresponding unstained control. Relative expression (ratio) was calculated as follows: Expression of condition of interest / expression of control condition (mo-DC).

**Cytokine analysis**

IL-12p70 and IL-10 were analyzed using ELISA kits from BD Biosciences or by inflammatory cytometric bead array (CBA; BD Biosciences) according to the manufacturer’s instructions. To
evaluate the cytokines present in supernatant of cancer cells, VEGF, IL-1β, IL-6 and IL-8 were determined by ELISA or CBA and M-CSF by Bioplex (Bio-Rad). PGE₂ levels were measured using the competitive PGE₂ immunoassay kit (Enzo Life Sciences, Belgium).

**PRDX1 knockdown using shRNA**

Lentivirus expressing shRNA against peroxiredoxin 1 (PRDX1) or TurboGFP (sh control) were produced and used to infect the CC8 cells at multiplicity of infection (MOI) 5. Further details can be read in the supplementary material and methods.
Results

**DC differentiation is altered by cervical and ovarian carcinoma cell lines**

Previously, we showed that when the TSN of cervical cancer cells was added to monocyte cultures, this could skew the differentiation of monocytes to cells resembling M2 macrophages (M2-like macrophages) (9). We investigated if also ovarian cancer cells could have this effect. Therefore, not only 5 cervical cancer cell lines but also 5 ovarian cancer cell lines were tested. First, the phenotype of in vitro-differentiated monocytes cultured with TSN of cervical cancer and ovarian cancer cell lines was assessed by the use of a panel of APC markers at day 6 of differentiation. We found that TSN from the two ovarian cancer cell lines COV413B and CAOV3 skewed differentiation of monocytes towards a M2-like macrophage phenotype and we confirmed the M2-like macrophage skewing capacity of HELA, CC8 and CSCC7. We will refer to these as M2-like macrophages and the tumor supernatant inducing these cells as M2-TSN (HELA, CC8, CSCC7, COV413B and CAOV3). Monocytes cultured in the presence of cancer cell lines that hampered monocyte differentiation but did not induce M2-like macrophages are referred to as tumor APC and the supernatant inducing this phenotype as APC-TSN (CSCC1, CASKI, SKOV3, OVCAR3 and A2780) [Supplementary Table 1]. Then, the functionality of these cultured APC was tested by a subsequent stimulation with LPS (a TLR4 agonist) – most often used to stimulate tumor resident DC in vitro – or with CD40L expressing fibroblasts (CD40L) to mimic APC-T-cell interaction. The production of IL-12 and IL-10 was measured after 48h. In accordance with our previous results, monocytes skewed by the TSN of ovarian cell lines COV413B and CAOV3 towards an M2-like phenotype produced almost no IL-12, but instead high levels of IL-10 when compared to mo-DC. APC-TSN display a similar balance in IL-12/IL-10 production as mo-DC.

To understand which soluble products in the TSN of ovarian cancer cells drove the differentiation of M2 macrophages, we measured the production of TGFβ, IL-1β, IL-6, IL-8, VEGF, PGE2 and M-CSF [Supplementary Table 2] and found that COV413B produced large amounts of IL-6 (25ng/ml) but no PGE2, while CAOV3 produced both IL-6 (14 ng/ml) and PGE2 (2ng/ml). Blocking of both these cytokines revealed that they are responsible for induction of phenotypically and
functionally M2-like macrophages not only by cervical cancer cell lines (9), but also by ovarian cancer cell lines (not shown).

**Cisplatin and carboplatin alter APC differentiation and function by affecting cancer cells**

First, in order to address the cytotoxic effect of cisplatin and carboplatin on our panel of cancer cell lines, we applied an MTT assay [Supplementary Figure 1]. We observed that the PGE$_2$ and/or IL-6 producing cell lines HEla and CC8 were most sensitive for chemotherapy, while COV413B, CAOV3 and CSCC7, producing PGE$_2$ and/or IL-6, were most resistant to chemotherapy. In addition, SKOV3 was chemoresistant while the cells did not produce PGE$_2$ and/or IL-6 [Supplementary Figure S1]. This showed that there was no direct relation between the production of IL-6 and/or PGE$_2$ and chemoresistance of cancer cells.

Furthermore, the direct cytotoxic effect of cisplatin and carboplatin on the different types of APC (mo-DC, APC-TSN and M2-TSN) was addressed. Monocytes were cultured in the presence of TSN and increasing doses of chemotherapy. Following treatment, cells were analyzed by flow cytometry. Interestingly, M2-like macrophages were more vulnerable to chemotherapy than tumor APC or mo-DC [Figure 1A]. Subsequently, the sensitivity of classical M1 macrophages (monocytes cultured in the presence of GM-CSF) and classical M2 macrophages (monocytes cultured in the presence of M-CSF) to cisplatin and carboplatin was tested (31). The classical M2 macrophages were also more sensitive to chemotherapy than M1 macrophages and DC [Figure 1B].

We previously showed that IL-6 and PGE$_2$ promoted the differentiation of M2 macrophages (9), cells which are associated with a worse response to therapy (7,8). Therefore, we studied the effect of chemotherapy treatment on tumor cells focusing on the tumor-induced differentiation of APC. Tumor cells were incubated with a dose of chemotherapy representative for the level within the tumor microenvironment. Subsequently, monocytes were cultured in the presence of TSN isolated from untreated, cisplatin or carboplatin-treated cancer cells. A clear increase in the percentage of CD1a-CD14+CD206+CD163+ M2 macrophages was observed when M2-TSN from treated cancer cells was used compared to M2-TSN from untreated cells. This effect was not observed for tumor cells producing APC-TSN [Figure 2], excluding the possibility that cell
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debris would have been the cause for the effects observed with the M2-TSN of chemotherapy
treated tumor cells. In order to mimic the natural situation, we used a transwell system to culture monocytes and cancer cells together in the presence of cisplatin or carboplatin. This set-up confirmed that platinum-containing chemotherapeutics did not influence the differentiation of the APC directly as the phenotype of mo-DC remained the same upon treatment. However, addition of platinum chemotherapy to the culture system resulted in an increased percentage of M2-like macrophages [Figure 2A].

As expected (9), stimulation of differentiated monocytes with LPS or CD40L resulted in a strong cell surface expression of the markers CD80, CD83, CD86, HLA-DR and PD-L1 (not shown). Furthermore, chemotherapeutic treatment resulted in a significant (p<0.01) functional enhancement of M2-like macrophage function (measured by indirect as well as transwell assay). These M2-like macrophages produced significant less IL-12 and more IL-10 upon activation with LPS (not shown) and CD40L after chemotherapy compared to their untreated controls [Figure 2C].

**Platinum-containing chemotherapy enhance PGE2 and IL-6 production by cancer cells**
The capacity of cervical and ovarian cancer cells to skew monocytes to M2-like macrophages depends mainly on their ability to produce PGE2 and/or IL-6 (9-11). Treatment of tumor cells with cisplatin resulted in an increased production of PGE2 and IL-6 in cell lines that already produced these cytokines: CASKI and COV413B (IL-6), CSCC7 (PGE2), CC8, HELA, CAOV3 (both PGE2 and IL-6). Carboplatin displayed a significant effect on PGE2 levels in HELA and CAOV3 as well as enhanced the production of IL-6 in all IL-6 producing cell lines [Figure 3]. As expected by the increased cytokine levels, the expression of both COX-2 and/or IL-6, but not COX-1 mRNA levels were increased upon platinum-based treatment in accordance with the cytokine profile of the cell lines [Supplementary Figure 2]. Notably, cisplatin or carboplatin treatment of the cancer cells did not induce or enhance the production of TGFβ, IL-1β, IL-8, VEGF and M-CSF [Supplementary Table 2].

**Altered levels of phosphorylated STAT1, 3 and 6 in TSN- differentiated macrophages**
To elucidate the mechanisms underlying the effect of chemotherapy on APC differentiation and function, we studied the involvement of STAT. Therefore, the intracellular pSTAT levels of APC were determined [Figure 4A]. Compared to mo-DC, APC cultured in the presence of all IL-6-producing cancer cell lines (CASKI, CC8, HELA, COV413B and CAOV3), had significantly increased levels of pSTAT3 [Figure 4B and not shown]. A number of APC cultures displayed a decrease in the levels of pSTAT1 and pSTAT6. This was associated with the presence of PGE2 in the TSN [Figure 4B and not shown]. Monocytes cultured in the presence of cancer cell lines that did not produce PGE2 nor IL-6 (CCSC1, SKOV3, OVCAR3 and A2780) did not show alteration in pSTAT levels. To test whether these factors were responsible for the altered levels of phosphorylated STATs, we differentiated monocytes in the presence of increasing doses of PGE2 and IL-6. Indeed, the levels of pSTAT1 and pSTAT6 were dose-dependently decreased in response to PGE2, whereas the level of pSTAT3 increased upon increasing amounts of IL-6 [Figure 4C]. We hypothesized that the observed increase in M2-skewing capacity of tumor cells treated with cisplatin or carboplatin would correlate with further alteration of pSTAT levels. Indeed, the use of M2-TSN isolated from cancer cell lines treated with cisplatin and carboplatin resulted in M2-like macrophages displaying significantly higher pSTAT3 and decreased pSTAT1 and pSTAT6 levels than M2-like macrophages cultured with M2-TSN from untreated cancer cells in almost all cases [Figure 4C]. Notably, M2-like macrophages cultured with TSN of CAOV3 already displayed low levels of pSTAT1 and pSTAT6 compared to mo-DC and when the TSN of CAOV3 treated with carboplatin was used no significant further decrease in pSTAT1/6 levels was found, despite the increase in PGE2 production.

**Targeting PGE2 and (s)IL-6R prevents chemotherapy-enhanced M2-like macrophage skewing**

We blocked the effect of IL-6 with tocilizumab, a human monoclonal antibody against (s)IL-6R that is clinically successful in the treatment of rheumatoid arthritis and Castleman disease (32, 33). The effect of PGE2 was blocked by treating the cancer cells with the COX (1 and 2) inhibitor indomethacin to inhibit PGE2 production via COX-2 [Figure 5A]. Restoration to the full phenotype
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of mo-DC [Figure 5B] as well as function – measured by the production of IL-12 and IL-10 – could be obtained when the cancer cells were treated with indomethacin and/or tocilizumab [Figure 5C]. Targeting COX-2 and (s)IL-6R also had a clear effect on the levels of pSTAT. As expected from our previous experiments, treatment with tocilizumab decreased the levels of pSTAT3, while indomethacin treatment restored pSTAT1 and pSTAT6 levels similar to that observed in mo-DC [Figure 5C].

*Activation of the NFκB pathway via the DNA damage response is required for the chemotherapy enhanced PGE₂ and IL-6 production*

In order to find the underlying mechanism of the enhanced PGE₂ and IL-6 production within the tumor cells upon treatment with cisplatin and carboplatin, we investigated two hypotheses. Previously, Wang et al. showed that cisplatin can selectively crosslink a complex of the chaperone protein PRDX1 and the nuclear factor kappa B complex (NFκB) at the NFκB promotor site of the COX-2 promotor region, thereby promoting COX-2 expression (34). Therefore, the expression of PRDX1 was blocked by shRNA, in order to prevent this protein to form a complex and hence, to prevent the increased production of PGE₂. We successfully downregulated the expression of PRDX1, but this did not result in decreased COX-2 mRNA-levels upon chemotherapy treatment [Supplementary Figure S3].

The second mechanism that could play a role in the increased production of PGE₂ and IL-6 by cancer cells is the activation of DNA damage response (DDR) pathway as this can lead to NFκB activation and subsequently to the production of PGE₂ and IL-6 (35, 36). To block the NFκB pathway, Bay 11-7082, a selective inhibitor of IκB phosphorylation and as such also of the canonical NFκB pathway, was used. Both untreated and cisplatin-treated cancer cells were incubated with increased doses of Bay 11-7082. Indeed, the cisplatin induced increase in PGE₂ and IL-6 production was blocked by Bay 11-7082 in a dose-dependent fashion [Figure 6].
Discussion

Here we showed two potential effects of platinum-based chemotherapy of gynecological cancers on the immune system. First, we showed that M-CSF-induced M2 macrophages and M2-like macrophages are most vulnerable for chemotherapy. Monocyte-derived DC and M1 macrophages were only affected at higher doses, suggesting a selective survival benefit of these cells. This can be considered beneficial for the patient. However, while it is clear that a sufficient dose of cisplatin or carboplatin to kill M2-like macrophages will be reached within the bloodstream, one may question whether this also occurs within the tumor microenvironment. The second effect concerns tumors in which the NFκB pathway, leading to the production of PGE2 and/or IL-6, is already activated. Treatment of these tumors with cisplatin and carboplatin resulted in an increased production of these two inflammatory mediators and subsequently in a more pronounced skewing of monocyte differentiation towards the tumor promoting M2-like macrophages, reflected by their production of large amounts of IL-10, decreased production of IL-12, activation of tolerogenic STAT3 pathway and a decrease in the immune potentiating STAT1 and STAT6 pathways. This effect should be considered detrimental to patients as it implies that, upon treatment with platinum-based regimens of PGE2 and/or IL-6-producing tumors, the number of local tumor-promoting M2 macrophages may increase, helping the tumor to defy the chemotherapeutic treatment. This would fit well with the existing literature showing that chemoresistance of cervical and ovarian cancer is associated with increased levels of PGE2 and IL-6 (12-15).

We studied two possible mechanisms responsible for this effect and showed that activation of the NFκB pathway was required, which occurred most likely via the DNA damage response pathway. Indeed platinum-containing chemotherapeutics act by binding to and causing crosslinking of DNA and thus may trigger the DDR pathway. Recently, several studies reported that upon DNA damage response, NFκB activation may result in the production of PGE2 and IL-6 (35, 36). More recently, it was shown that excessive DNA damage induced NFκB activation promoted expression of IL-6 in HELA cells (37). Upon chemotherapy-induced DDR, the IκB kinase (IKK)
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complex phosphorylates IkBα for ubiquination and proteasomal degradation, thus allowing the NFκB complex to translocate to the nucleus (18, 38). As the DDR pathway-activated NFκB-mediated production of PGE₂ and IL-6 only occurred in tumor cell lines originally producing sufficient amounts of IL-6 and/or PGE₂, an autocrine role for these cytokines can be envisaged (39) [Figure 7].

STATs represent central regulators of cancer-associated inflammation and influence interactions between cancer cells and their immune microenvironment that determine whether the inflammation promotes or inhibits cancer. The majority of gynecological tumors have high STAT3 activity and this was associated with poor survival and chemoresistance (40). Interestingly, it has been reported that COX-2 is a transcriptional target of STAT3 signaling (41). Furthermore, high COX-2 expression results in enhanced STAT3 phosphorylation in an IL-6 dependent manner (42). Together, this may form an autocrine mechanism. Notably, STAT3 and NFκB interact at multiple levels, thereby promoting pro-tumorigenic inflammatory conditions in the tumor microenvironment (increased PGE₂ and IL-6 production), increasing tumor cell proliferation and survival as well as chemoresistance, tumor angiogenesis and metastasis (18). Our data suggest that the autocrine signaling loops of PGE₂ and IL-6 in the tumor cells are enhanced by the NFκB-activating signals of the DDR pathway induced by platinum treatment.

Tumor-produced IL-6 induced the activation of the STAT3 signaling pathway in M2-macrophages, a signaling pathway that is known to be activated in tolerogenic APC (19, 20) In contrast, STAT1 and STAT6 can support anti-tumor immunity (18). We found a reduction of pSTAT1 and pSTAT6 in M2-like macrophages and showed that this was PGE₂-dependent. These effects were enhanced when cancer cells were treated with cisplatin and carboplatin and sustain the notion that platinum-based chemotherapy may indirectly skew the local immune environment to a more tolerogenic and tumor-promoting milieu in cancers actively producing PGE₂ and/or IL-6. In a mouse tumor model, platinum-based therapy caused immunogenic cell death, thereby activating local APC and enhancing anti-tumor T-cell responses. Cisplatin triggered the release of the TLR4 stimulating protein HMGB-1 (43, 44). We previously showed (9), and confirm here that tumor-
induced macrophages respond to the TLR4 agonist LPS as reflected by the production of IL-10. Therefore, it is highly likely that chemotherapy mediated release of HMGB-1 may not only activate DC, but activate local tumor-promoting macrophages as well. Since tumor-associated macrophages can easily outnumber tumor-infiltrating DC, the overall effect may be less beneficial in case there is an excess of M2 macrophages.

Chemoresistance was thought for a long time to arise as a consequence of cell intrinsic genetic changes, including upregulation of drug efflux pumps, activation of detoxifying enzymes or apoptotic defects. Recent evidence suggests that resistance to chemotherapy can also result from cell extrinsic factors such as cytokines and growth factors (45), implying an important role for the tumor microenvironment. Our data support this hypothesis and suggest an immunological explanation for the correlation of high \textit{COX-2} expression and IL-6 levels with poor response to treatment. We found no correlation of PGE$_2$ and/or IL-6 production of the cancer cell lines with chemoresistance of the cancer cell lines, while several other studies show that high \textit{COX-2} expression and high levels of IL-6 in serum and ascites identified patients with a poor response to cisplatin and unfavorable prognosis (13, 17, 46). This effect was explained by enhanced apoptosis resistance of the cancer cells but this was not reflected by our \textit{in vitro} tests where cancer cell lines were incubated with different doses of cisplatin and carboplatin. Our observation that platinum-containing chemotherapy of cell lines with high \textit{COX-2} and/or \textit{IL-6} expression promoted the differentiation of monocytes to tumor-promoting M2-like macrophages substantiates the notion that also cell extrinsic factors such as cytokines play a role in chemoresistance (48). In our opinion, chemoresistance of a tumor may not only be mediated by the resistance of the cancer cells themselves but can occur through a skewed tumor microenvironment that is geared to promote tumor cell growth for instance through the action of M2 macrophages. This fits well with the finding that in glioma, the \textit{COX-2} pathway promotes gliomagenesis by supporting the development of myeloid suppressor cells in the tumor microenvironment (47). If so, blocking the tumor-promoting effects of PGE$_2$ and IL-6 might even enhance the sensitivity of otherwise more resistant tumors to chemotherapy, but this will require new studies.
Previously, we showed that monocytes differentiated in the presence of HELA, CC8 and CSCC7 or its supernatant induced a M2-like phenotype and that treatment with indomethacin (COX-inhibitor) and monoclonal antibodies (mAbs) against both IL-6 and IL-6R fully restored their phenotype and functionality towards that of DC. In recent clinical studies where the effects of IL-6 were targeted with the monoclonal antibody siltuximab against IL-6, the results were discordant (48-50). One explanation given was the fact that if tumors produce high levels of IL-6 it will be difficult to neutralize all the soluble IL-6 present. In our experiments, we used a monoclonal antibody against both the cell surface bound form and the soluble form of the IL-6 receptor called tocilizumab. This antibody, which successfully blocks the detrimental effects of IL-6 in the clinic when treating rheumatoid arthritis and Castleman disease (31, 32), was capable to fully block the effects of IL-6 produced by untreated or chemotherapy treated tumor cells. Pilot experiments revealed that this antibody to some extend also blocked IL-6 signaling in the tumor cells themselves [Supplemental Figure S4].

In summary, chemoresistance of tumors have long been associated with the activation of COX-2 and the production of IL-6. Our data showed that tumor cells do not produce the same levels of IL-6 and/or PGE2 and that there was no direct correlation between the efficacy of cisplatin and carboplatin to kill tumor cells and the levels of these cytokines. In contrast, we found that these chemotherapeutic compounds elicited the NFκB pathway leading to an increased production of PGE2 and IL-6 in tumors that actively produce these inflammatory mediators. As IL-6 or PGE2 by themselves or in combination can skew M2 macrophage differentiation, chemotherapeutic treatment of tumors will favor the differentiation of M2-like tolerogenic macrophages, despite the differences in levels produced by tumor cells. In the end this will result in a stronger immune suppressive tumor-promoting tumor microenvironment known to be associated with therapy resistance (8). Therefore, our data suggest that a chemotherapy induced increase in the number of intratumoral tumor-promoting M2 macrophages forms an indirect mechanism underlying chemoresistance. Successful blockers of PGE2 and the IL-6/IL-6R pathway are already in the Research.
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clinic. It will be of great interest to study the effects of a combined therapy of cytotoxic agents and these clinically available compounds in patients with apparently chemoresistant tumors.

Acknowledgements

We thank Dr. Thorbald van Hall and Bianca Querido for authenticating all cell lines.
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Tables and figures, legends

Figure 1. Survival of monocytes upon treatment with cisplatin and carboplatin
CxCa: cervical cancer cell line; OvCa: ovarian cancer cell line. A) Example of survival of mo-DC, APC-TSN (TSN CASKI) and M2-TSN (TSN CAOV3) upon treatment with increasing doses of cisplatin. B) Survival of different APC upon chemotherapy treatment. Tumor APC/M2-like macrophages reflect a mean of 3 experiments with 5 different APC-TSN and M2-TSN.

Figure 2. Platinum-based chemotherapy treatment of cervical and ovarian cell lines alters APC differentiation
Mean (with SEM) of 3 experiments. Treated tumor APC/M2-like macrophages were compared to untreated tumor APC/M2-like macrophages with a paired t-test; *p < 0.05; ** p < 0.01; *** p < 0.001. A) Typical example of a flow cytometric analysis. Numbers in quadrants represent the percentage of cells within the live gate. Phenotype of mo-DC is not affected by chemotherapy following direct incubation with chemotherapy. B) Bar plots showing the percentage of CD1a+, CD1a-CD14- and CD14+ cells induced by TSN of untreated and treated tumor cells. C) The production of IL-12 and IL-10 of the different types of APC depicted in figure B is shown. M2-like macrophages predominantly produce IL-10 whereas tumor APC produce IL-12 and lower amounts of IL-10 compared to mo-DC. This effect is significantly enhanced upon chemotherapy treatment in M2-like macrophages.

Figure 3. Alterations in phenotype and function of APC is associated with changes in PGE₂ and/or IL-6 production by tumor cells
Mean (with SEM) of 3 experiments. Treated tumor cells were compared to untreated tumor cells with a paired t-test; *p < 0.05; ** p < 0.01; *** p < 0.001.

Figure 4. Platinum-based chemotherapy treatment of cancer cell lines influences STAT phosphorylation in APC
A) Examples of pSTAT1, pSTAT3 and pSTAT6 expression of monocytes cultured in the presence of (un)treated supernatant of cancer cells. B) Overview of
pSTAT levels in M2-like macrophages. Mean (with SEM) of 3 experiments performed on 3 different donors. Treated tumor APC/M2-like macrophages were compared to untreated tumor APC/M2-like macrophages with a paired t-test; *p < 0.05; ** p < 0.01; *** p < 0.001. C) pSTAT1 and 6 are PGE2 dependent, whereas pSTAT3 is IL-6 dependent. Dotted lines indicate pSTAT-levels in mo-DC.

Figure 5 Targeting COX-2 and IL-6 restores phenotype/function/STAT phosphorylation of APC. Mean of 3 experiments. Blocked conditions were compared to unblocked conditions with a paired t-test; *p < 0.05; ** p < 0.01; *** p < 0.001 A) PGE2 and IL-6 production is increased upon cisplatin treatment. PGE2-production is decreased by inhibition by indomethacin. B) Phenotype of APC is restored by targeting COX-2 with indomethacin (HELA, CC8, CAOV3) and/or by blocking the (s)IL-6R with tocilizumab (COV413B, CAOV3). C) IL-10 production is decreased whereas IL-12p70 production is restored upon targeting both COX-2 and the IL-6R. D) Indomethacin treatment restores pSTAT1 and pSTAT6 levels to levels similar of mo-DC, whereas treatment with tocilizumab decreases the levels of pSTAT3.

Figure 6 Enhanced PGE2 and IL-6 production in cancer cells in response to NFκB activation. Increasing doses of the selective inhibitor of IκB phosphorylation – and such also of the canonical NFκB pathway – Bay 11-7082 were added one hour before platinum-treatment. Mean (with SEM) of 3 experiments.

Figure 7. Proposed mechanism of enhanced PGE2 and IL-6 production by cancer cells upon chemotherapy treatment. DNA damage caused by cisplatin and carboplatin phosphorylates IκBα for ubiquination and proteasomal degradation, allowing the NFκB complex to translocate to the nucleus. When STAT3 is active in tumors, NFκB prefers STAT3 – p300 interaction, thereby promoting the production of mediators, such as IL-6 and COX-2, which are associated with cancer-promoting inflammation. Figure adapted from Yu (18).
A. mo-DC

APC-TSN
CASKI
CxCa

M2-TSN
CAOV3
OvCa

FSC
SSC

0 µg/ml
0.5 µg/ml
2 µg/ml
5 µg/ml
10 µg/ml

Survival (% of cells in live gate)

B. Cisplatin (µg/ml) Carboplatin (µg/ml)

Survival (% of cells in live gate)

FIGURE 1.
<table>
<thead>
<tr>
<th>Cisplatin</th>
<th>Carboplatin</th>
</tr>
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<tbody>
<tr>
<td>HELA CxCa</td>
<td>- + - +</td>
</tr>
<tr>
<td>CC8 CxCa</td>
<td>- + - +</td>
</tr>
<tr>
<td>CSCC7 CxCa</td>
<td>- + - +</td>
</tr>
<tr>
<td>COV413B OvCa</td>
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<tr>
<td>OVCAR3 OvCa</td>
<td>- + - +</td>
</tr>
<tr>
<td>A2780 OvCa</td>
<td>- + - +</td>
</tr>
</tbody>
</table>

**FIGURE 3.**
**A.**

Unstained mo-DC and M2-TSN show pSTAT1, pSTAT3, and pSTAT6 expression.

**B.**

Bar graphs showing M2/mo-DC ratios for different cell lines (HELA, CC8, COV413B, CAOV3) under the influence of Cisplatin and Carboplatin.

**C.**

Bar graphs showing APC-PGE2 and APC-IL-6 levels across different IL-6 and PGE2 concentrations.
**FIGURE 5.**

A. Graph showing the concentration of PGE2, IL-6, IL-12, and IL-10 in different cell lines treated with Cisplatin and Indomethacin.

B. Bar chart showing the percentage of cells in the live gate in different cell lines treated with Cisplatin and Indomethacin.

C. Bar chart showing the effect of Cisplatin and Indomethacin on IL-12 and IL-10 production.

D. Bar chart showing the effect of Cisplatin and Indomethacin on M2-TSN and mo-DC in different cell lines.
Figure 6.
DNA damage (Cisplatin/Carboplatin)

IL-6

JAK

STAT3

IkBa

Ub

IkBa

NFkB

IKK

Nucleus

Pro-inflammatory

NFkB

Oncogenic and chronic inflammation

Anti-tumor inflammatory mediators

Pro-cancer inflammatory mediators eg PGE2 and IL-6

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Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment

Eveline M. Dijkgraaf, Moniek Heusinkveld, Bart Tummers, et al.

Cancer Res Published OnlineFirst February 22, 2013.

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