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ORIGINAL ARTICLE

The chemotherapeutic drug oxaliplatin differentially affects blood DC function dependent on environmental cues

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Abstract It has become evident that the tumor microenvironment plays a pivotal role in the maintenance of cancerous growth. One of the acquired functions of the tumor microenvironment is the suppression of immune responses. Indeed, blocking the inhibitory pathways operational in the microenvironment results in enhanced T-cell-dependent, anti-tumor immunity. Chemotherapeutic drugs not only directly kill tumor cells but also shape the tumor microenvironment and potentiate anti-tumor immunity. Here, we demonstrate that the chemotherapeutic compound oxaliplatin acts as a double-edged sword. Besides killing tumor cells, oxaliplatin bolsters immunosuppressive pathways, resulting in decreased activation of T cells by human plasmacytoid dendritic cells (pDCs). Exposure to oxaliplatin markedly increased expression of the T-cell inhibitory molecule programmed death receptor-ligand 1 (PD-L1) on human pDCs and also TLR9-induced IFNα secretion. Furthermore, oxaliplatin decreased TLR-induced STAT1 and STAT3 expression, and NF-κB-mediated responses. The oxaliplatin induced upregulation of PD-L1 and downregulation of costimulatory molecules CD80 and CD86 resulted in decreased T-cell proliferation. Our results demonstrate that platinum-based anticancer drugs adapt TLR-induced signaling in human pDCs and myeloid DCs (mDCs), thereby downgrading their immunostimulatory potential.

Keywords Blood DC subsets · PD-L1 · TLR · Oxaliplatin

Abbreviations

DC	Dendritic cell
moDC	Monocyte-derived DC
mDC	Myeloid DC
pDC	Plasmacytoid DC
PD-1	Death receptor 1
PD-L1	Program death ligand 1

Introduction

Dendritic cells (DCs) are the most potent professional antigen-presenting cells (APC) of the immune system. Upon infection or inflammation, immature DCs are activated and differentiate into mature DCs that instruct and activate B and T lymphocytes, the mediators of adaptive immunity [1]. Two major types of DCs circulate in peripheral blood, myeloid DCs (mDCs) and plasmacytoid (pDCs). These DC subsets express a divergent set of surface molecules and have distinct functions [2]. In addition to scavenging pathogens and presenting antigens, DCs

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secrete a large array of cytokines, which are all vital for proper functioning of both the acquired and the innate immune system.

Although these DC subsets reside in the peripheral blood, they also infiltrate solid tumors, such as breast cancer [3], head and neck cancer [4] and ovarian cancer [5]. Soluble factors secreted by the tumor and necrotic tumor material prevent differentiation and activation of infiltrating DCs, including pDCs [6–8]. These findings have been correlated with poor prognosis [3, 9]. Furthermore, Curiel et al. demonstrated that in human ovarian cancer, infiltrating DCs express the immunosuppressive receptor program death ligand 1 (PD-L1 also known as B7-H1 or CD274) [10]. PD-L1 negatively regulates the delicate balance between T-cell activation and inhibition through interaction with its receptor program death receptor 1 (PD-1) resulting in suppressed T-cell activation [11, 12] and maintenance of the immunosuppressive environment. The negative regulation of T-cell activation via PD-1/PD-L1 is also supported by studies using blocking antibodies to PD-L1 performed on DCs [10, 13–15]. The importance of this pathway is further highlighted in studies showing that blockade of the PD-1/PD-L pathway results in enhanced tumor-specific T-cell expansion and activation [16, 17]. PDCs and mDCs circulating in the blood do not or hardly express PD-L1. However, upon TLR-induced activation or stimulation with type I or type II IFNs, PD-L1 is upregulated on pDCs and mDCs [18]. This upregulation has been linked to decreased T-cell proliferation, IFN-γ and IL-10 secretion [11] and the induction of tolerance and anergic T cells [12, 19]. Blocking these inhibitory mechanisms resulted in enhanced anti-tumor immunity [16, 17].

Recent data show that chemotherapeutic drugs affect not only the tumor cells but also the immunological tumor microenvironment resulting in a more robust anti-tumor response [20, 21]. Widely used cytotoxic drugs such as doxorubicin, paclitaxel and gemcitabine have been shown to cause enhanced antigen cross-presentation, T-cell expansion and T-cell infiltration of tumors by to date unknown molecular mechanisms [22, 23]. Furthermore, our own studies show that oxaliplatin increased the T-cell stimulatory potential of monocyte-derived DCs (moDCs) by impairing the IL-4 induced upregulation of PD-L2 through dephosphorylation of STAT6 [24].

In the present study, we disclose that oxaliplatin has a different effect on blood DC subsets. We observed that exposure to platinum-based chemotherapeutics increased the T-cell inhibitory molecule PD-L1 on TLR9-activated human pDCs resulting in decreased T-cell proliferation. Furthermore, platinum-based chemotherapeutics markedly increased TLR9-induced type I IFN secretion and decreased the expression of STAT1 and STAT3 and the secretion of IL-6 and TNFα by human pDCs.

Material and methods

Cells

Buffy coats were obtained from healthy volunteers with informed consent according to institutional and international guidelines. PDCs were purified by positive isolation using anti-BDCA-4-conjugated magnetic microbeads, and BDCA-1 mDCs were purified using anti-CD1c-conjugated microbeads (both Miltenyi Biotec, Bergisch-Gladbach, Germany) after B cell depletion. Thereafter, both cell types were adjusted to 10^6 cells/ml in X-VIVO-15 (Lonza, Verviers, Belgium) supplemented with 2% human serum (Sanquin, Nijmegen, the Netherlands). PDC and mDC purity was routinely up to 95%, as assessed by double staining with BDCA-2/CD123 or CD11c/CD1c (all Miltenyi Biotec). PDCs were activated through the addition of 5 μg/ml ODN-CpG-C (M362, Axxora, San Diego, CA) or 4 μg/ml R848 (Axxora, San Diego, CA). Monocyte-derived DCs (moDCs) were generated from adherent peripheral blood mononuclear cells (PBMCs), by culturing in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml) (both Cellgenix, Freiburg, Germany). Cells were cultured in X-VIVO 15 medium supplemented with 2% of human serum and harvested on day 6 as immature DC. Immature DC or mDCs were activated through the addition of 4 μg/ml R848 and/or 20 μg/ml Poly I:C (Sigma-Aldrich). Oxaliplatin was added during DC activation, as indicated.

Flow cytometry

Purity of pDCs and mDCs after isolation and the phenotype of the pDC populations were determined by flow cytometry. The following primary monoclonal antibodies (mAbs) and the appropriate isotype controls were used: anti-BDCA2-PE and CD123-APC (all Miltenyi Biotec); mIgG1-PE, mIgG1-APC, anti-HLA-ABC-PE (W6/32), anti-HLA-DR/DP-FITC (Q5/13), anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, anti-CD86-APC, anti-PD-L1-PE, anti-PD-L2-PE, (all BD Bioscience Pharmingen, San Diego, CA, USA); anti-CD83-PE (Beckman Coulter, Mijdrecht, the Netherlands). *Intracellular staining for STAT proteins:* PDCs were harvested using TEN harvest buffer and fixed with 4% formaldehyde by 10-min incubation at 37°. Cells were permeabilized by incubating with ice-cold 90% methanol for 30 min on ice. Permeabilized cells were washed and incubate with the following primary antibodies: rabbit-polyclonal-anti-STAT1, rabbit-polyclonal-anti-pSTAT1, rabbit-polyclonal-anti-STAT2, rabbit-polyclonal-anti-pSTAT2, rabbit-monoclonal-anti-STAT3, rabbit-monoclonal-anti-pSTAT3, rabbit-polyclonal-anti-STAT6 (all from Cell Signaling) and goat-polyclonal-anti-pSTAT6 (BD Bioscience).

Cells were washed twice and incubated with goat-anti-mouse-Alexa647 or goat-anti-rabbit-Alexa647 as secondary antibodies. Mean fluorescence intensity and percentage of positive cells were determined by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA, USA).

Mixed lymphocyte reaction

Allogeneic peripheral blood lymphocytes (PBLs) were co-cultured with differently matured pDCs, mDCs and moDCs in a 96-well round-bottom plate (pDC/PBL ratio 1:20 with 1 * 10^5 PBL). After 4 days of culture, 1 μCi/well ([0.037 MBq]/well; MP Biomedicals, Amsterdam, the Netherlands) of tritiated thymidine was added for 8 h and incorporation was measured in a beta-counter. In some experiments, blocking antibodies against PD-L1 (e-Bioscience, San Diego, USA) were added to the culture at a final concentration of 10 μg/ml. Normal mouse serum was used as isotype control. Antibodies against PD-L1 were preincubated with pDCs for 30 min before adding PBLs. T-cell proliferation data were normalized to account for intra-experimental differences.

Cytokine detection

PDCs and mDCs were cultured overnight at a concentration of 10^5 DCs/100 μl/well in a 96-well round-bottom plate. Supernatants were collected from DC cultures after 16 h of activation, and IFNα and IL-6 production was analyzed by murine monoclonal capture and HRP-conjugated anti-IFNα antibodies (Bender MedSystems, Vienna, Austria) or anti-IL-6 Abs (Sanquin, Amsterdam, the Netherlands) using standard ELISA procedures. TNFα, RANTES, IP-10 and MIP-1α production was measured using a human Multiplex kit (Bender MedSystems) according to manufacturer's instructions.

Preparation of protein lysates and Western blotting

2×10^5 cells were lysed in 20 μl lysis buffer containing 10 mM Tris/HCl pH 7.8, 5 mM EDTA, 50 mM NaCl, 1 mM Na_3VO_4 10 mM pyrophosphate, 50 mM NaF, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1X Roche protease inhibitor cocktail (Roche Diagnostics Nederland BV, Almere, the Netherlands). Samples were subjected to polyacrylamide gel electrophoresis and further processed for Western blot analysis. After blocking, membranes were incubated with one the following antibodies: mouse-monoclonal-anti-β-actin (1:20,000; Sigma-Aldrich, St. Louis, MO), purified mouse-anti-PTP1C/Shp-1 (1:250; BD), rabbit-polyclonal-anti-STAT1, rabbit-polyclonal-anti-pSTAT1, rabbit-monoclonal-anti-STAT3 and rabbit-monoclonal-anti-pSTAT3 (all from Cell Signaling). After washing, the membranes were incubated with one of the goat-anti-mouseIRDye800CW (LI-COR Biosciences, Lincoln, NE) or polyclonal goat-anti-rabbit-tAlexaFluor-680 (Molecular Probes, Eugene, OR) as secondary antibody and analyzed with the LICOR Odyssey Imaging system (LI-COR Biosciences). Integrated intensities were analyzed using Excel (Microsoft Corp., Redmond, WA).

Statistics

All experiments were performed at least three times and results are shown as the mean ± SEM. Data sets were either tested by a Student's *t* test or by one-way ANOVA followed by Newman–Keuls or Dunnett's multiple comparison test.

Results

Oxaliplatin differentially regulates the allostimulatory capacity of activated human DC subsets

When moDCs were exposed to the platinum-based chemotherapeutic compound oxaliplatin during activation, significantly higher T-cell proliferation was induced compared to moDCs activated without the addition of oxaliplatin (Fig. 1a and [24]). Since oxaliplatin is given intravenously to cancer patients, this finding prompted us to investigate the effect of this drug on blood DC function.

In contrast to what we observed for moDCs, both Poly I:C (TLR3)- and R848 (TLR7/8)-activated $CD1c^+$ mDCs induced lower allogeneic T-cell proliferation when oxaliplatin was present during activation (Fig. 1b). In line with TLR-activated mDCs, we observed that pDCs activated with the TLR9 agonist CpG-C in the presence of oxaliplatin displayed a significantly decreased capacity to induce allogeneic T-cell responses (Fig. 1c). Intriguingly, this effect was only observed when pDCs were activated through the TLR9 signaling pathway using CpG-C but not through TLR7/8 signaling using the single stranded RNA analog R848 (Fig. 1c). Oxaliplatin-treated pDCs displayed similar viability compared to oxaliplatin-treated moDCs (Suppl. Figure 1A, B), showing that the decreased T-cell proliferation is not caused by toxicity. Thus, oxaliplatin impairs the ability of TLR-activated blood DCs but not TLR7-activated pDCs to induce allogeneic T-cell responses.

Oxaliplatin differentially affects the TLR-induced cytokine secretion profile of blood DCs

Next, we determined whether oxaliplatin modulates TLR-induced proinflammatory cytokine and chemokine

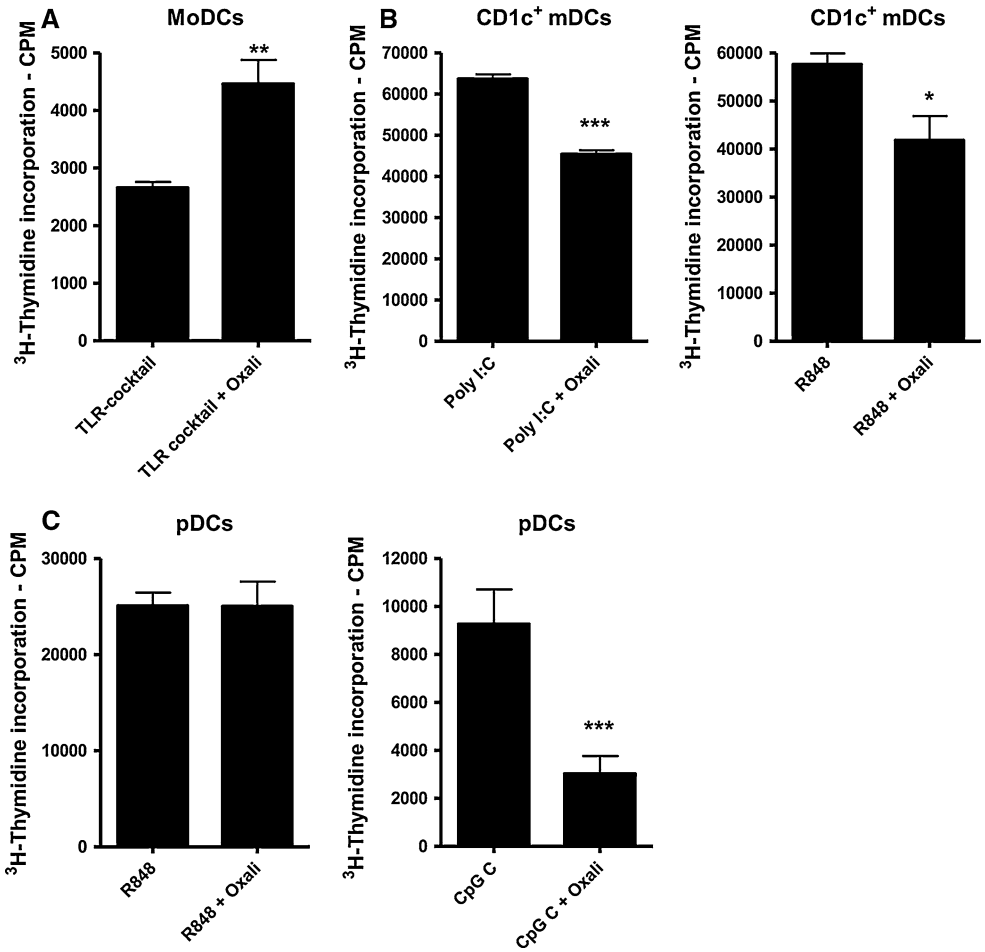

Fig. 1 Effect of oxaliplatin on DC allostimulatory capacity. Proliferation of T cells was measured by ^{3}H-thymidine incorporation and depicted as counts per minute. 1×10^5 peripheral blood leukocytes were stimulated for 4 days with 5×10^3 allogeneic **a** moDCs activated through the addition of R848 + Poly I:C or **b** CD1c$^+$ mDCs activated through the addition of either R848 or Poly I:C **c** pDCs activated through R848 or CpG-C. Where indicated, DCs were treated with oxaliplatin during activation. Data are the mean values ± SEM of six measurements of at least three independent experiments with different donors (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

secretion by pDCs and mDCs. Our results show that oxaliplatin negatively affected the secretion of IL-6, TNF-α, IP-10 and RANTES by TLR8-activated mDCs (Suppl. Figure 2A), TLR7-activated pDCs (Fig. 2a) and TLR9-activated pDCs (Fig. 2b). While oxaliplatin also negatively affected TLR3-induced IP-10 secretion by mDCs, it had no effect on other cytokine and chemokine secretion by TLR3-activated mDCs (Suppl. Figure 2B). Furthermore, TLR-induced MIP-1α by mDCs remained unaffected upon oxaliplatin treatment (Suppl. Figure 2A, 2B). By contrast, oxaliplatin treatment affected TLR-induced MIP-1α by pDCs (Fig. 2a, b). A putative mechanism for the observed effects could lie in the modification of upstream players controlling NF-κB activity, since it controls IL-6, TNFα, RANTES, MIP-1α and IP-10 secretion. Interestingly, we found that oxaliplatin had a divergent effect on the TLR-induced IFNα secretion by pDCs. On the one hand, oxaliplatin treatment had no effect on TLR7-induced IFNα secretion, but it markedly increased TLR9-induced IFNα secretion by human pDCs (Fig. 2a, b).

Oxaliplatin modulates the TLR-induced human blood DC phenotype

The observations that oxaliplatin affects TLR-induced cytokine secretion and decreased blood DC allostimulatory capacity prompted us to investigate whether oxaliplatin had an effect on the phenotype of blood DCs. As expected, CpG-C, R848 and Poly I:C induced a complete activated phenotype characterized by upregulation of CD40, CD80, CD86, CD83, and MHC class I and II (Fig. 3 and Suppl. Figure 3). Oxaliplatin modestly enhanced TLR-induced upregulation of CD40 on mDCs, but had no effect on TLR-induced upregulation of MHC class I and II (Suppl. Figure 3A, B). Oxaliplatin did not affect expression levels of CD40 and the molecules MHC class I and II on TLR-activated pDCs (Suppl. Figure 3C, 3D). However, oxaliplatin together with CpG-C significantly increased the expression levels of the maturation marker CD83 (Fig. 3d). By contrast, R848- and Poly I:C-induced CD83 expression was decreased after oxaliplatin treatment on pDCs and mDCs (Fig. 3a–c). While oxaliplatin enhanced R848- and

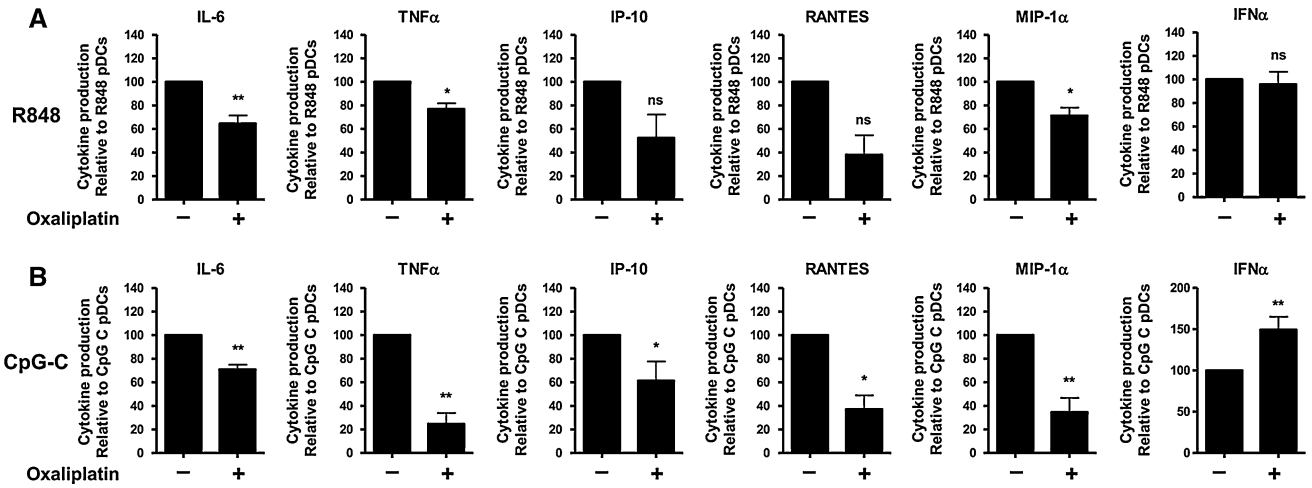

Fig. 2 Oxaliplatin alters the TLR-induced secretory profile in human pDCs. Supernatants of pDC cultures following incubation with R848 (**a**) or CpG-C (**b**) with or without 7 μg/ml oxaliplatin were analyzed for the presence IL-6, TNFα, IP-10, RANTES, MIP-1α and IFNα. The graphs show cytokine and chemokine production relative to pDCs activated in the absence oxaliplatin. Data shown are mean values ± SEM of at least three independent experiments with different donors (*$P < 0.05$; **$P < 0.01$)

Poly I:C-induced CD86 expression on mDCs (Fig. 3a, b), it impaired CpG-C-induced CD86 upregulation on pDCs (Fig. 3c, d). Furthermore, oxaliplatin also impaired R848- and CpG-C-induced CD80 expression on pDCs (Fig. 3d), although CD80 expression on mDCs and R848-activated pDCs remained unaffected (Fig. 3a–c). These findings demonstrated that oxaliplatin differentially modulated the TLR3-, TLR7/8- and TLR9-induced blood DC phenotype.

Enhanced PD-L1 expression on pDCs after oxaliplatin treatment results in impaired T-cell activation

Besides T-cell stimulatory mechanisms, DCs are also known to express and utilize a variety of T-cell inhibitory mechanisms. Previously, we demonstrated that the enhanced T-cell proliferation was due to the downregulation of PD-L2 receptor expression levels on moDCs [24]. Interestingly, as we evaluated the effect of oxaliplatin on TLR9-activated pDCs, we found that PD-L1 expression was increased upon exposure with oxaliplatin (Fig. 4a, b), while PD-L2 expression and other inhibitory molecules were not detected under any of the conditions tested (Fig. 4b and data not shown). By contrast, PD-L1 expression levels remained unaltered upon TLR3 and TLR7/8 stimulation and oxaliplatin treatment on pDCs and mDCs (Fig. 4a). Inhibition of PD-L1 signaling pathway by addition of anti-PD-L1 blocking antibodies rescued the lost capacity of platinum-treated CpG-C-activated pDCs to stimulate allogeneic T cells (Fig. 4c). These data support the observation that upregulation of PD-L1 has a significant role on the decreased T-cell stimulatory capacity of platinum-treated pDCs. Thus, enhanced PD-L1 and decreased CD80/CD86 expression on oxaliplatin-treated CpG-C-activated pDCs impairs T-cell proliferation.

IL-4 counterbalances oxaliplatin-induced TLR modulation in pDCs

Recently, we showed that pDCs stimulated with IL-4 together with a TLR9 agonist induced stronger allogeneic T-cell responses than pDCs stimulated solely through TLR9 [25]. Therefore, we sought to determine whether IL-4 could restore the decreased allostimulatory capacity of oxaliplatin-treated pDCs. In accordance with previous findings, Fig. 5a illustrates that IL-4 enhanced the allostimulatory capacity of TLR9-activated pDCs. Interestingly, the addition of IL-4 during oxaliplatin treatment could partially restore the low allogeneic T-cell proliferation by TLR9-activated pDCs (Fig. 5a). Additionally, we also observed that IL-4 was able to restore the oxaliplatin-impaired CD80 and CD86 expression to basal levels (Fig. 5b). This is in contrast to PD-L1 expression which remained high upon IL-4 stimulation (Fig. 5b). Besides inverting the allostimulatory capacity and expression of surface molecules, IL-4 also reduced the oxaliplatin-enhanced IFNα secretion (Fig. 5c). These data not only demonstrate that different environmental factors can modulate the functionality of TLR9-activated pDCs but also show that different environmental cues can counteract each other during TLR9 activation. Furthermore, these data also show that the balance between stimulatory and inhibitory signals provided by pDCs has a significant effect on T-cell proliferation (Fig. 5d).

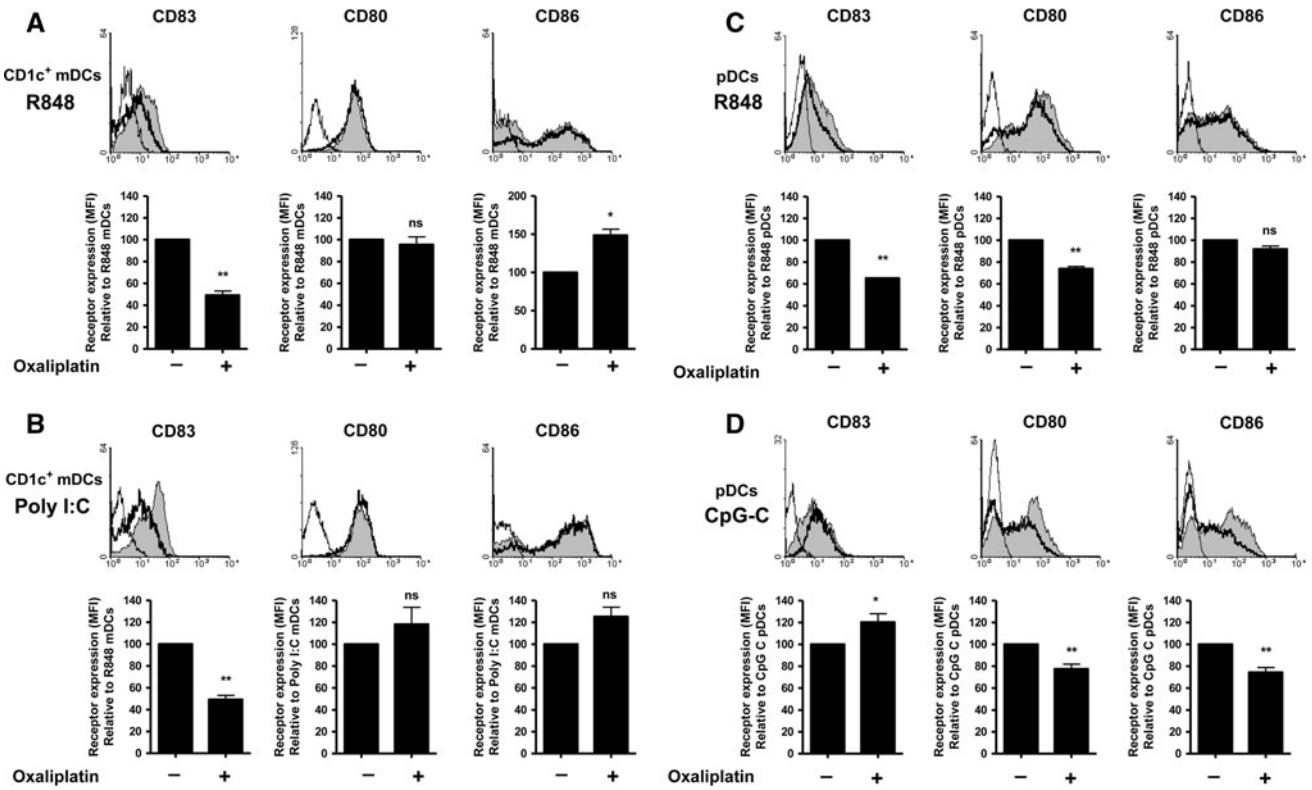

Fig. 3 Oxaliplatin modulates the TLR-induced human blood DC phenotype. Freshly isolated CD1c$^+$ mDCs and pDCs were activated overnight with either R848 or Poly I:C, for mDCs, and with R848 or CpG-C, for pDCs, with or without 7 μg/ml oxaliplatin. Histograms show the TLR-induced receptor expression levels of the surface molecules CD80, CD83 and CD86 on pDCs/mDCs (*gray filled*) compared to expression levels of oxaliplatin-treated TLR-activated pDCs/mDCs (*thick black lines*) and the appropriate isotype control (*thin black line*). Graphs show the receptor expression levels of the surface molecules CD80, CD83 and CD86 of activated pDCs/mDCs relative to R848 (**a**), Poly I:C (**b**) activated mDCs or R848 (**c**), CpG-C (**d**) activated pDCs. Data shown are mean values ± SEM of at least three independent experiments with different donors (*$P < 0.05$; **$P < 0.01$)

Oxaliplatin impairs TLR9-induced STAT1 and STAT3 expression

Recently, An et al. described that SHP-1 differentially regulates the production of proinflammatory cytokines and type I interferon [26]. Therefore, we studied the potential involvement of phosphatases in order to pinpoint the potential candidate proteins responsible for the observed dual function of oxaliplatin on pDCs: an enhanced IFNα secretion and a decreased NF-κB-induced response. We hypothesized that oxaliplatin treatment might increase SHP-1 expression in human TLR9-activated pDCs. In Fig. 6a, it is shown that SHP-1 expression is induced in human pDCs upon TLR9 activation with CpG-C. However, oxaliplatin in combination with CpG-C did not result in significant enhanced SHP-1 expression (Fig. 6a, b). Because the functional activity of SHP-1 does not have a linear relation with its expression [27], we also investigated the effect of SHP-1 inhibition using the inhibitor sodium stibogluconate. To exclude the involvement of other phosphatases, we also tested the broad range phosphatase inhibitor suramin [28, 29]. The presence of sodium stibogluconate reduced the oxaliplatin-induced IFNα secretion, but did not affect oxaliplatin-induced CD80, CD86 and PD-L1 expression (Fig. 6c). Interestingly, there was an increase in the effect of oxaliplatin in the presence of suramin hinting to the possibility that a phosphatase is involved (Fig. 6c). Another recent study reported that oxaliplatin activates the NOTCH-1 pathway by induction of the γ-secretase complex [30]. Therefore, we investigated the effect of pDC preincubation with a γ-secretase inhibitor on oxaliplatin-induced effects. However, this inhibitor did not result in abrogation of the oxaliplatin-induced effects (data not shown).

Previously, Lesterhuis et al. demonstrated that oxaliplatin was able to dephosphorylate STAT6 [24]. That prompted us to investigate whether oxaliplatin also affects STAT signaling in TLR9-activated pDCs. We showed by both Western blot and FACS analysis that oxaliplatin reduced expression levels of total STAT1 and STAT3 in TLR9-activated human pDCs (Fig. 6d, e). However, total STAT2,

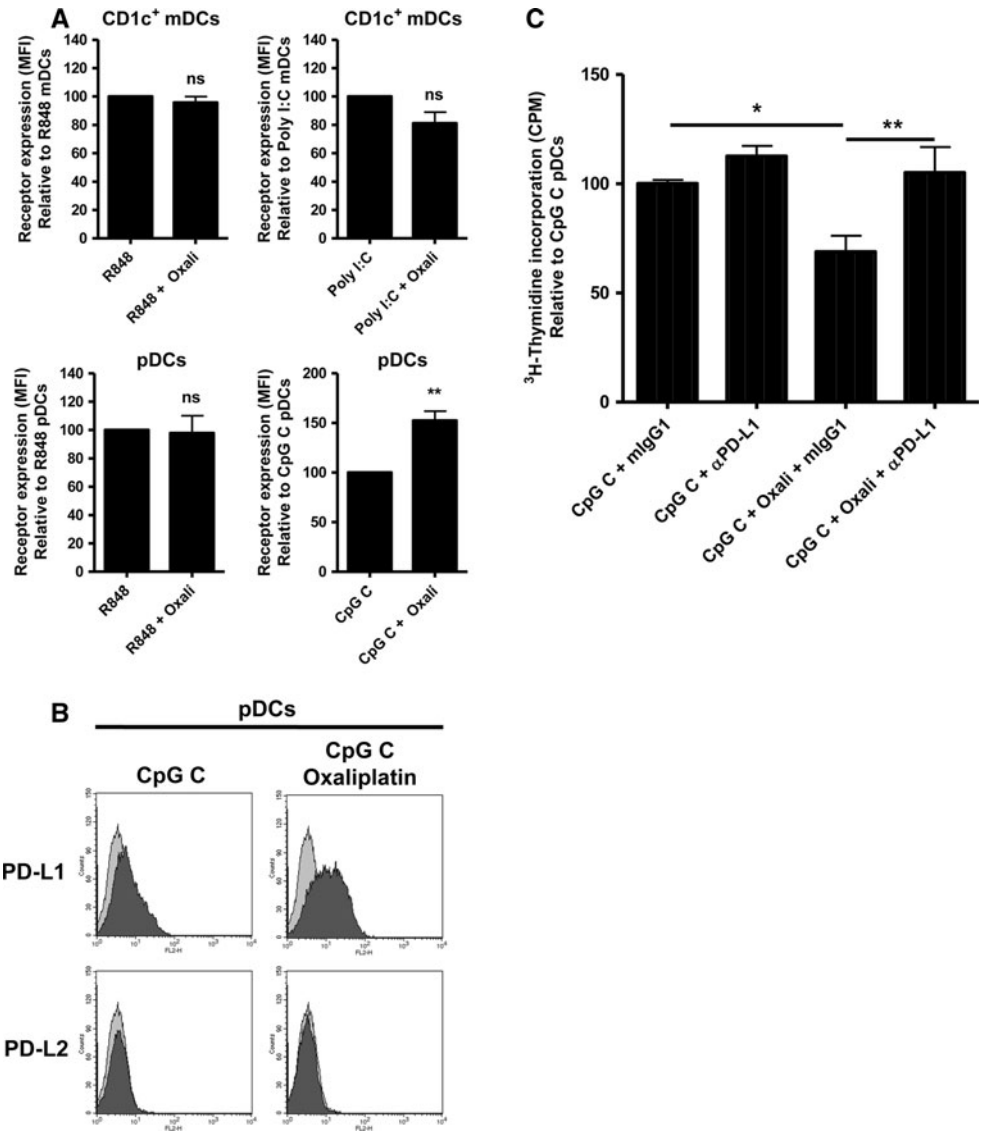

Fig. 4 TLR9-induced PD-L1 expression is enhanced by oxaliplatin on human pDCs. PD-L1 and PD-L2 expression by pDCs activated overnight with R848 or CpG-C with or without 7 μg/ml oxaliplatin. **a** Graphs show the PD-L1 receptor expression (MFI) by TLR-activated pDCs/mDC with or without oxaliplatin. Data shown are mean values of at least three independent experiments ± SEM. **b** Histograms show the expression levels of PD-L1 and PD-L2 (*dark gray filled histograms*) compared to isotype controls (*light gray filled histograms*) on TLR9-activated pDCs, of one representative experiment. **c** Mixed lymphocyte reaction with CpG-C-activated pDCs in the presence or absence of 7 μg/ml oxaliplatin in the presence of blocking antibodies against PD-L1 or control IgG. Data shown are mean values ± SEM of four independent experiments performed with different donors. The effect of PD-L1 blocking in the various conditions was compared to CpG-C-activated pDCs with IgG control antibodies that was set to 100% (*$P < 0.05$; **$P < 0.01$)

STAT6 and expression of all phosphorylated STATs remained unaffected upon oxaliplatin treatment in TLR9-activated pDCs (Fig. 6d, e). In addition to oxaliplatin, we also sought to determine the TLR9-induced STAT expression upon IL-4 exposure. As expected, IL-4 induced the phosphorylation STAT6 and STAT3, whereas expression and phosphorylation of other STATs remained unaltered (Fig. 6e). Besides reducing the expression of total STAT1 and STAT3, oxaliplatin also impaired the IL-4 induced phosphorylation of STAT6 and STAT3 (Fig. 6e). All together, these data show that oxaliplatin affects STAT1 and STAT3 expression and TLR9-induced signaling.

Discussion

Recent studies show that besides having a direct cytotoxic effect on tumor cells, chemotherapeutic drugs can also potentiate the immune system [20, 21]. Here, we show that the chemotherapeutic platinum compound oxaliplatin modulated TLR9-induced signaling in human pDCs. This modulation led to increased IFNα production, decreased inflammatory cytokine and chemokine secretion, and decreased STAT1 and STAT3 expression by human pDCs. Furthermore, we showed that oxaliplatin enhanced PD-L1 expression levels on pDCs, resulting in impaired T-cell proliferation depending on the maturation stimuli. Generally, oxaliplatin is used in combination with other cytotoxic drugs as a treatment regimen for gastrointestinal, colorectal, esophago-gastric and/or pancreatic cancer. However, it remains to be elucidated whether the other active compounds in these treatment regimens may also affect the immunogenicity of human DC subsets.

We studied STATs and protein tyrosine phosphatases, which are key regulators of intracellular signaling as an obvious mechanism exploited by oxaliplatin to modulate

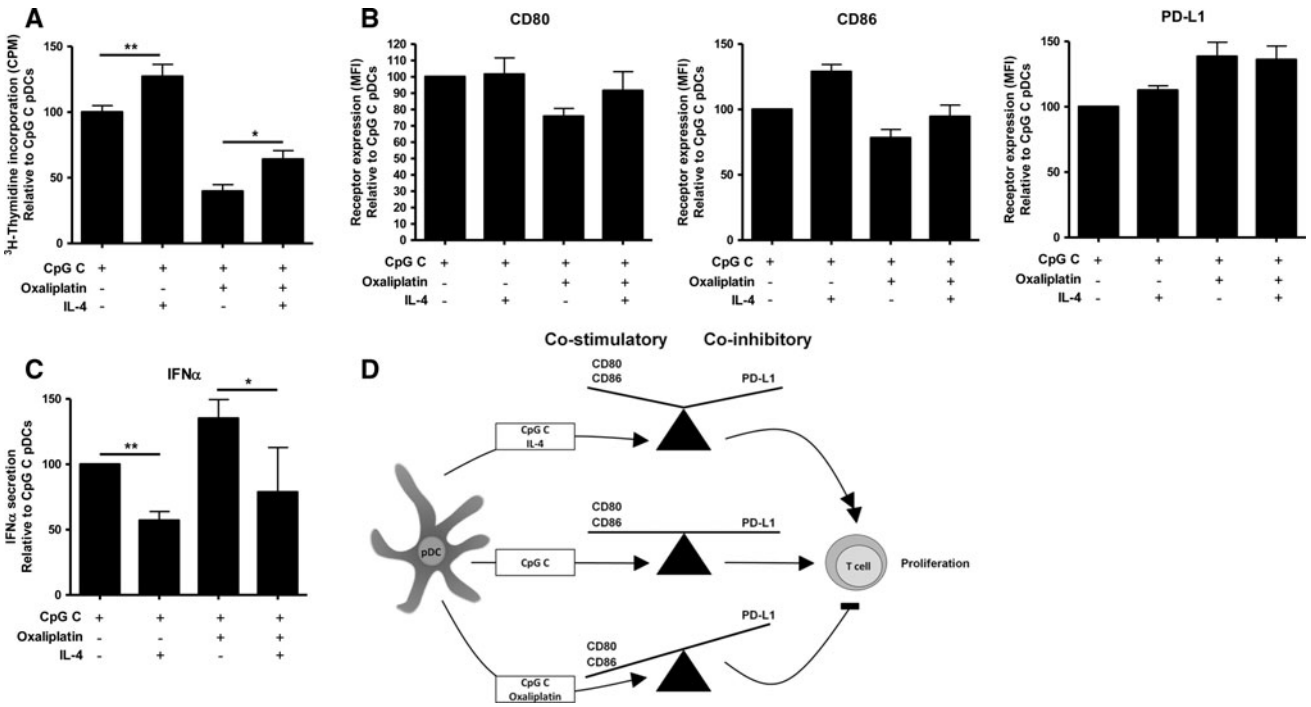

Fig. 5 IL-4 counterbalances oxaliplatin effects. Freshly isolated pDCs were activated overnight through CpG-C in the presence or absence of IL-4 and/or 7 μg/ml oxaliplatin. **a** Mixed lymphocyte reaction with CpG-C-activated pDCs in the presence and/or absence of oxaliplatin and IL-4. Data shown are mean values ± SEM of four independent experiments performed with different donors and normalized to CpG-C-activated pDCs. Graphs show the surface receptor expression of **b** CD80, CD86 and PD-L1 and **c** IFNα secretion by CpG-C-activated pDCs in the presence and/or absence of oxaliplatin and IL-4. **d** Model; TLR-9-induced activation of human pDCs can be altered by the presence of environmental cues, resulting in an enhanced or decreased expression profile of costimulatory and co-inhibitory molecules. This converted balance leads to more tolerogenic or immunogenic pDCs that largely influence the fate of the T-cell response (*$P < 0.05$; **$P < 0.01$)

TLR-induced signaling. The protein tyrosine phosphatases SHP-1 was reported to block TLR-induced production of proinflammatory cytokines by inhibiting the activation of NF-κB pathway and MAPKs by direct dephosphorylation [26, 31, 32]. Moreover, An et al. recently described that in mice SHP-1 increased TLR- and RIG-I-induced type I IFN production by directly binding to the kinase domain of IRAK1 and thus inhibiting IRAK1 activity in bone marrow DCs, peritoneal macrophages and splenic CD11c$^+$ DCs [26]. We obtained the same observations in the TLR9-induced cytokine secretion profile after oxaliplatin treatment; however, a role for SHP-1 was excluded based on experiments which showed that sodium stibogluconate had no effect. Interestingly, suramin, a broad phosphatase inhibitor, enhanced the oxaliplatin-induced effects. These data indicate that oxaliplatin impairs the activity of a certain phosphatase rather than increasing the expression of a phosphatase. Besides phosphatases, also kinases are involved in signaling upon ligand binding by TLRs [33]. Larangé et al. demonstrated that using specific inhibitors for JNK or JAK upon TLR7 or TLR8 stimulation, significant changes were observed in the expression of certain surface molecules and the production cytokines [33]. These findings underscore that inhibition of mediators involved in downstream signaling after TLR stimulation has subtle but significant influences on cell functionality. The observed increased expression of PD-L1 related to oxaliplatin treatment implicates undesired effects on the immunological elimination of tumor cells outcome in cancer patients. It was reported that tumor-infiltrating dendritic cells which express PD-L1 help to maintain the immunosuppressive environment [10]. PD-L1 expression has also been detected on the surface of a wide variety of human cancers, including lung, colon, breast, ovary, renal cell, glioma and melanoma [13, 34, 35], and has been correlated with poor prognosis [34, 36–39]. Tumors escape from the host immune system by attenuation of tumor-specific T-cell responses via PD-L1/PD-1 interactions. Normally, PD-L1 expression is controlled by STAT3 and becomes upregulated by tumor cells and other immune cells upon IFN or IL-6 stimulation [34, 40–43]. Recently, a study showed that chemotherapeutic agents also induced PD-L1 surface expression in breast cancer cells and promoted PD-L1-mediated T-cell apoptosis [44]. Additionally, we found that other platinum-based chemotherapeutic also induced PD-L1 and decreased CD80 and CD86 expression on pDCs, and that oxaliplatin also

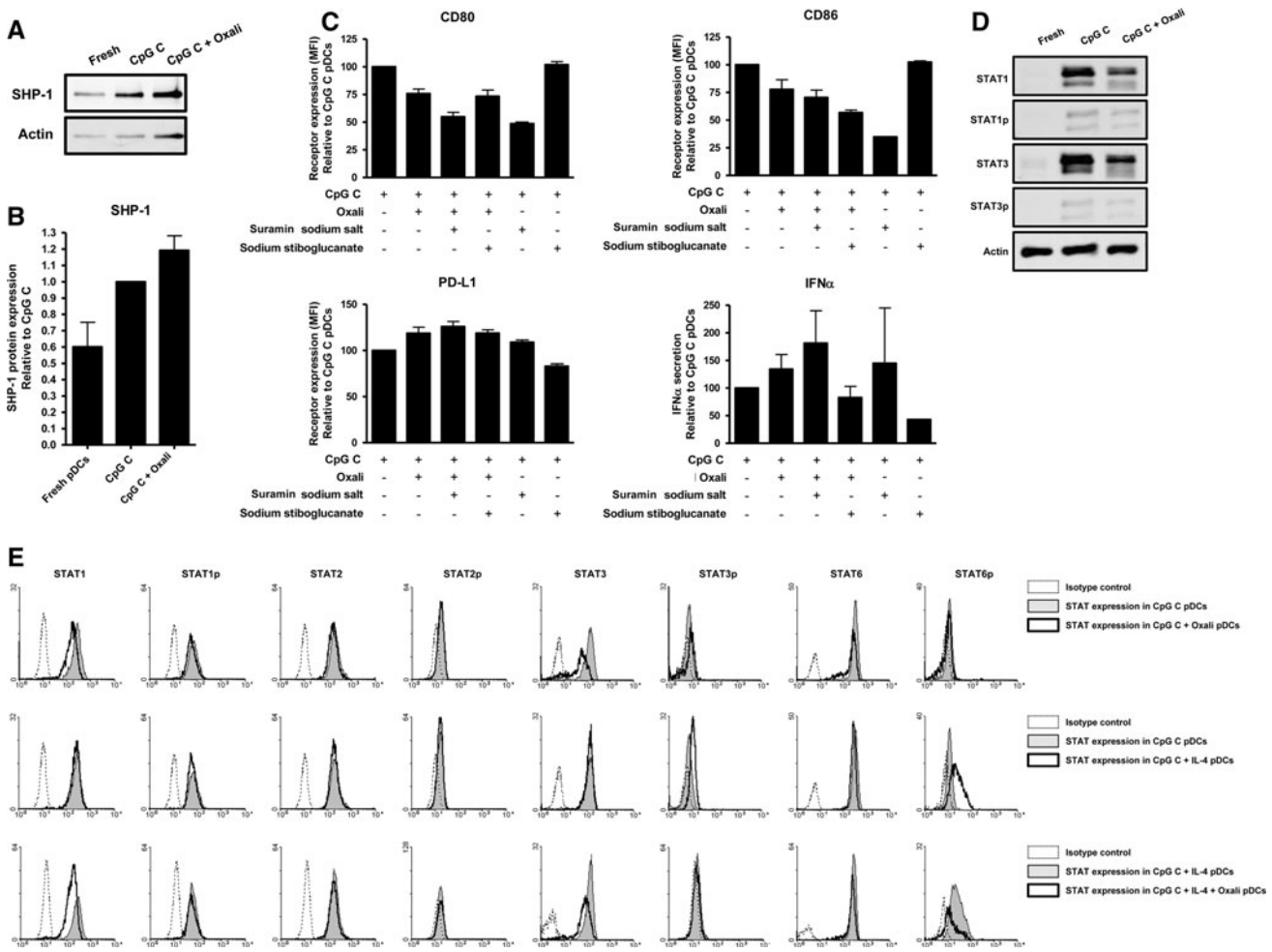

Fig. 6 Oxaliplatin decreases TLR9-induced STAT1 and STAT3 expression. **a** 2×10^5 cells were left untreated or activated with CpG-C or activated with CpG-C with or without oxaliplatin (7 μg/ml). After overnight culture, cells were lysed and SHP-1 expression was analyzed by Western blot. Data shown are of one representative experiment. **b** The graph shows SHP-1 expression by pDCs relative CpG-C-activated pDCs. SHP-1 expression relative to actin was quantified as described in methods. Data shown are mean values of three independent experiments ± SEM. **c** PDCs were activated overnight by CpG-C with or without oxaliplatin and phosphatase inhibitors where indicated. The graphs show CD80, CD86 and PD-L1 surface expression levels and IFNα secretion by pDCs relative to CpG-C-activated pDCs. Data shown are mean values of three independent experiments ± SEM. **d** Freshly isolated, CpG-C-activated ± oxaliplatin pDCs were lysed, and total and phosphorylated STAT1 and STAT3 expression was analyzed by Western blot. One representative experiment is shown. **e** Histograms show the expression of total or phosphorylated STAT1, STAT2, STAT3 and STAT6 after overnight activation with CpG-C (*gray filled*; *top* and *middle row*) in the presence of oxaliplatin (*thick black lines*; *top* and *bottom row*) or IL-4 (*thick black lines*; *middle row*: *gray filled; bottom row*). One representative experiment is shown

induced PD-L1 expression on tumor cells (Suppl. Figure 4). In summary, the expression of PD-L1 on tumors has a negative effect on the immunological outcome and is considered as a key feature of tolerogenic DCs [43].

In the present study, we demonstrated that human pDCs, treated with oxaliplatin in the presence of TLR9-induced type I IFN, markedly increased expression levels of PD-L1. Aspord et al. demonstrated that in breast cancer tumors $CD4^+$ T cells are present secreting IFNγ and IL-13 [45]. These T cells provide an additional source of IFNs which in combination with chemotherapeutic agents can lead to enhanced PD-L1 expression on either tumor cells or on tumor-infiltrating pDCs. Moreover, infiltration of pDCs and expression of PD-L1 in severable tumors correlated with poor prognosis. Recently, Lesterhuis et al. described that platinum compounds positively affect the immunostimulatory potential of myeloid DCs by impairing the upregulation of PD-L2 [24]. Together, this demonstrates that platinum compounds have dissimilar effects on different DC subsets dependent on the presence of environmental cues. In cancer patients, the therapeutic off-target effect of platinum compounds will depend on the presence and the amount of the different tumor-infiltrating DC subsets, the TLR expression on those DC subsets, and the micro-environment in which they reside. Based on the present study, when tumors are infiltrated with high

numbers of pDCs, it would be beneficial to provide additional TLR7-agonists rather than TLR9-agonists in combination with platinum-based chemotherapeutics. Combining chemo- with immunotherapy is already reported, showing that combining chemotherapy with immunotherapy improves patient survival compared to patients treated with only chemotherapy [46]. However, in order to become even more effective, therapeutic strategies should have direct cytotoxic effects but also be tailored based on the presence of tumor-infiltrating DCs and the TLR expression by those tumor-infiltrating DCs. This tailor-made combination of chemotherapy with additional immunotherapy has great potential in inducing superior anti-tumor responses and ultimately will lead to enhanced tumor clearance and patient survival.

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