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ORIGINAL RESEARCH

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Cisplatin inhibits frequency and suppressive activity of monocytic myeloid-derived suppressor cells in cancer patients

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ABSTRACT

Cancer immunotherapies have induced long-lasting responses in cancer patients including those with melanoma and head and neck squamous cell carcinoma (HNSCC). However, the majority of treated patients does not achieve clinical benefit from immunotherapy because of systemic tumor-induced immunosuppression. Monocytic myeloid-derived suppressor cells (M-MDSCs) are implicated as key players in inhibiting anti-tumor immune responses and their frequencies are closely associated with tumor progression. Tumor-derived signals, including signaling via STAT3-COX-2, induce the transformation of monocytic precursors into suppressive M-MDSCs. In a retrospective assessment, we observed that survival of melanoma patients undergoing dendritic cell vaccination was negatively associated with blood M-MDSC levels. Previously, it was shown that platinum-based chemotherapeutics inhibit STAT signaling. Here, we show that cisplatin and oxaliplatin treatment interfere with the development of M-MDSCs, potentially synergizing with cancer immunotherapy. *In vitro*, subclinical doses of platinum-based drugs prevented the generation of COX-2$^+$ M-MDSCs induced by tumor cells from melanoma patients. This was confirmed in HNSCC patients where intravenous cisplatin treatment drastically lowered M-MDSC frequency while monocyte levels remained stable. In treated patients, expression of COX-2 and arginase-1 in M-MDSCs was significantly decreased after two rounds of cisplatin, indicating inhibition of STAT3 signaling. In line, the capacity of M-MDSCs to inhibit activated T cell responses *ex vivo* was significantly decreased after patients received cisplatin. These results show that platinum-based chemotherapeutics inhibit the expansion and suppressive activity of M-MDSCs *in vitro* and in cancer patients. Therefore, platinum-based drugs have the potential to enhance response rates of immunotherapy by overcoming M-MDSC-mediated immunosuppression.

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Cancer; MDSC; platinum-based chemotherapy; cisplatin; immunotherapy

Introduction

Immunotherapy is a promising and proven therapeutic strategy to treat a number of cancer types including melanoma, non-small cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC).[1] In particular, the administration of immune checkpoint inhibitors against programmed cell death protein 1 (PD-1), PD-1 ligand (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) has greatly increased the survival of patients with melanoma and lung cancer.[2] Unfortunately, the reality is that the majority of cancer patients do not respond to currently available immunotherapies.[3] Several mechanisms exploited by advanced tumors to resist against therapeutic immune responses have been identified. These include downregulation of MHC class I molecules, expression of co-inhibitory molecules and recruitment of immunosuppressive cells.[4] Monocytic myeloid-derived suppressor cells (M-MDSCs) are immature myeloid cells capable of inhibiting anti-tumor immunity. The frequency of these cells is expanded during cancer progression driven by tumor-derived signals such as GM-CSF, IL-6, and PGE-2.[5,6] The ability to suppress T cells is the main defining feature of M-MDSCs and phenotypically they are defined by high expression of CD11b, CD33, and CD14 plus low to no expression of the MHC class II molecule HLA-DR.[7,8] M-MDSC levels are elevated in cancer patients and correlate to advanced disease and impaired efficacy of immunotherapies such as immune checkpoint inhibitors, DC vaccination, and adoptive cell transfer therapy.[9–12] Therefore, M-MDSCs are a prime target for drugs to overcome immunosuppression in the context of cancer immunotherapy. In this regard, signal transducer and activator of transcription 3 (STAT3) is an attractive target protein for inhibition because its signaling function plays a central role in M-MDSC expansion and suppressive activity.[5,13] Direct transcriptional targets of STAT3 are the enzymes NADPH oxidase 2 (NOX2), arginase-1 (ARG-1) and cyclo-oxygenase 2 (COX-

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2).[14–16] These enzymes play key roles in the ability to inhibit T cell function. The first two enzymes function to actively inhibit T cell proliferation and activation, whereas COX-2 produces inflammatory mediator PGE-2 which is involved in inflammation and in development of MDSCs.[5,17,18] Previously, it was reported that platinum-based chemotherapeutics such as cisplatin, oxaliplatin, and carboplatin can inhibit STAT signaling by binding to the SH2 domain in STAT proteins.[19] This observation was validated in HNSCC patients, where cisplatin-based concomitant chemoradiotherapy had increased patient survival in STAT3 and STAT6 positive tumors compared to patients with STAT3/6 negative tumors.[19] In addition, platinum-based compounds were found to have immunostimulatory effects through the induction of immunogenic cancer cell death, sensitizing tumors to immune attack and promoting effector immune functions.[20,21] In particular, the blocking of STAT6-dependent PD-L2 expression in platinum-treated tumor cells and dendritic cells contributed to increased T cell responses.[22]

Considering the importance of STAT signaling during expansion and activation of M-MDSCs in cancer patients,[13] we hypothesized that platinum drugs would modulate these suppressive myeloid cells thereby potentially contributing to the effect of these drugs on immune responses. Here, we report for the first time that platinum drugs specifically inhibit the generation and suppressive activity of human M-MDSCs *in vitro* and in HNSCC patients. These results support the use of platinum drugs to overcome immunosuppressive mechanisms in cancer patients to increase clinical benefit of cancer immunotherapy.

Material and methods

Healthy donor blood and patient blood

Healthy donor buffy coats were obtained from the Karolinska University Hospital blood center (ethical permit: #20010305,01–50) and Sanquin, Nijmegen, the Netherlands. Blood collection of patients was approved by the local Institutional Review Board (Regional Ethical Review Board in Stockholm #20010305,01–50 and Committee on Research involving Human Subjects Arnhem-Nijmegen #2014-083) and in accordance with the declaration of Helsinki. Written informed consent was obtained from all patients. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences) or lymphoprep (Axis-Shield).

Metastatic melanoma patients

Frozen PBMC fractions of metastatic melanoma patients were collected during clinical trials of DC cancer vaccination (Figure 4a). Patients were categorized as short or long survivors based on overall survival of <12 months or >24 months after start of DC vaccination, respectively. PBMCs were thawed and stained with viability dye eFluor® 780 followed by an antibody panel (Sup. Table 1). Samples were acquired on CyAn ADP analyzer using Summit Software (both Beckman Coulter) and analyzed with FlowJo software (TreeStar V10).

Table 1. Metastatic melanoma patients characteristics

	Short survivors (n=14)	Long survivors (n=8)
Age (years, median + range)	59 (31-72)	59 (30-69)
Gender		
Male	12 (86%)	4 (50%)
Female	2 (14%)	4 (50%)
Stage of disease (AJCCC 7th edition)		
Stage III	0 (0%)	1 (12,5%)
Stage IV	14 (100%)	7 (88%)
Localisation primary		
Skin	13 (93%)	7 (88%)
Eye	0 (0%)	1 (12,5%)
MUP	1 (7%)	0 (0%)
OS (months, median + range)	7 (3-11)	47 (24-124)
LDH (U/L, median + range)	387 (195-498)	361 (252-459)
DC vaccination type		
TAA-loaded MoDC	14 (100%)	8 (100%)
TAA loading method*		
Peptide class I mod	1 (7%)	1 (12,5)
Peptide class I wt	9 (64%)	2 (25%)
Peptide class I + II wt	2 (14%)	2 (25%)
mRNA	2 (14%)	3 (37,5%)
Route of administration		
IN (1x) or IV/ID	4 (29%)	1 (12,5)
IV/ID	5 (36%)	3 (37,5%)
IN	5 (36%)	4 (50%)

**Class I mod: HLA class I-restricted modified gp100-derived peptides 154–162 Q-A and 280–288A-V and HLA class I-restricted tyrosinase-derived peptide 369–377. Class I wt: HLA class I-restricted wild-type gp100-derived peptides 154–162 and 280–288 and HLA class I-restricted tyrosinase-derived peptide 369–377. Class II wt: HLA class II-restricted gp100-derived peptide 44–59 and tyrosinase-derived peptide 448–462 analog. mRNA: messenger RNA encoding full length gp100 and tyrosinase. MUP, melanoma of unkown primary; OS, overall survival; LDH, lactate dehydrogenase; DC, dendritic cell; TAA, tumor-associated antigen; moDC, monocyte-derived autologous DC; mod, modified; wt, wild type IN, intranodal; IV, intravenous; ID, intradermal*

Table 2. HNSCC patient characteristics (flow cytometry).

	HNSCC patients (n = 19)
Age in years (median + range)	61 (52–69)
Gender	
Male	12 (63%)
Female	7 (37%)
Primary tumor	
Oropharynx	13 (68%)
Supraglottic larynx	2 (11%)
Hypopharynx	2 (11%)
Nasal sinus	1 (5%)
Unknown	1 (5%)
T stage	
T0	1 (5%)
T1	2 (11%)
T2	1 (5%)
T3	5 (26%)
T4	10 (53%)
N stage	
N0	6 (32%)
N1	4 (21%)
N2a	2 (11%)
N2b	2 (11%)
N2c	3 (16%)
N3b	1 (5%)
Nx	1 (5%)

Table 3. HNSCC patient characteristics (functional assays).

Patient no.	Age	Gender	Primary tumor	T stage	N stage
1	59	m	oropharynx	T4a	N2a
2	59	m	oropharynx	T2	N1
3	54	m	oropharynx	T3	N0
4	67	m	oropharynx	T3	N2c
5	54	m	supraglottic larynx	T3	N2c
6	70	v	oropharynx	T4a	N2c
7	54	v	oropharynx	T1	N2b
8	66	m	nasal sinus	T4a	N1
9	62	m	oropharynx	T1	N1

Melanoma-induced *in vitro* M-MDSCs

The melanoma cell line THFR was established from a metastatic lesion of a patient at the oncology clinic of the Karolinska University Hospital, as approved by the local Ethics Committee and after signing a written informed consent in accordance with the Declaration of Helsinki. To exclude the influence of long-term *in vitro* culture only fourth passage melanoma cells were used. Cells were thawed to be grown confluent in T75 flasks (Corning Life Sciences) containing IMDM medium (Gibco) + 10% heat-inactivated fetal bovine serum (FBS). Upon harvest cells were trypsinized and used for tumor-monocyte co-cultures. Monocytes were purified from healthy donor PBMCs using CD14 microbeads and LS columns (Miltenyi Biotec) and subjected to the melanoma-based M-MDSC induction protocol as previously published.[23] In brief, 4×10^5 THFR melanoma cells were seeded in a 6-well plate and 10^6 CD14+ monocytes were added in a total volume of 3 ml IMDM medium + 10% human serum (HS). Monocytes cultured without tumor cells were used as control cells. CD14-PBMCs were cultured using the same medium in a separate 6-well plate in a density of 5×10^6 cells per ml. After 3 days, co-cultured monocytes (*in vitro* M-MDSCs) and control monocytes were both harvested and purified from the tumor cells using HLA-DR microbeads (Miltenyi Biotec) according to manufacturer's instructions. Autologous T cells were purified from the CD14-PBMCs using the Pan T cell Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. To evaluate the effects of platinum-based drugs on M-MDSC induction by melanoma cells, subclinical dosages (concentrations indicated in figures) of oxaliplatin (Fresenius Kabi) or cisplatin (Teva Pharmaceuticals) were added to the tumor-monocyte co-cultures. Suppression assays with purified monocytes were performed as described below.

Cell isolation from HNSCC patients

Nineteen HNSCC patients with lymph node metastases, but without distant metastases, were included. Treatment consisted of concomitant chemo-radiotherapy with weekly cisplatin (40 mg/m^2) for 6 weeks in the Radboudumc, Nijmegen (Table 2). Per patient, blood was collected at three time points being before the first round of treatment (before cisplatin), before the third round of treatment (during cisplatin), and two weeks after the 6th round of cisplatin (after cisplatin). Isolated PBMCs were stored overnight at 4° C. M-MDSCs from nine HNSCC patients were assessed in *ex vivo* suppression assays (Table 3). Therefore, T cells were isolated using the Pan T cell Isolation Kit and an LS column (Miltenyi Biotec) according to manufacturer's instructions. The T cell negative fraction was stained with antibodies against CD14 and HLA-DR (Sup. Table 1) in PBS with 0,1% bovine serum albumin (BSA) and 0,4% ethylenediaminetetraacetic acid (EDTA) for 30 minutes. CD14+ HLA-DRneg/low (M-MDSCs) were sorted with a FACSAria (BD Biosciences) using BD FACSDiva software.

T cell suppression assays

To assess the suppressive activity of melanoma-induced M-MDSCs, 10^5 autologous T cells, labeled with 1.4 μM carboxyfluorescein succinimidyl ester (CFSE; Biolegend), were co-cultured with M-MDSCs in ratios 1:2 and 1:4 (M-MDSCs: T cells) in a U-bottom 96-wells plate in IMDM medium supplemented with 10% HS. T cells were activated with 1 μl (4×10^4) beads CD3/CD28 Dynabeads® (Gibco) per 10^5 T cells in a total volume of 200 μl. After 4 days of incubation, cells were harvested and analyzed by flow cytometry as described below.

To assess the suppressive activity of M-MDSCs isolated from HNSCC patients undergoing cisplatin-based concomitant chemo-radiotherapy, two assay setups were performed defined by the origin of T cells (Figure 3a). In both setups 2.5×10^4 T cells were co-cultured with sorted M-MDSCs in ratios of 1:2 or 1:4 (M-MDSC: T cells) in X–VIVO 15 (Lonza) + 2% human serum (Sigma) in 96-well u-bottom plates in 3–6 technical replicates. T cells were activated with 5×10^3 CD3/CD28 Dynabeads® per 2.5×10^4 T cells (1 bead: 5 T cells). After 3 days, proliferation of T cells was assessed by incubating cells with 3 H-thymidine for 16 hours and

incorporation was measured with a β-counter. 75 µl of supernatant was stored at −20°C for determining IFNγ content by ELISA.

Flow cytometry

To determine the phenotype of melanoma-induced M-MDSCs compared to control cells, monocytes were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific) followed by a panel of antibodies (Sup. Table 1). Intracellular staining was performed to quantify the expression of COX-2 in melanoma-induced M-MDSCs compared to control monocytes. Cells were first stained for viability with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific), then fixed with BD Cytofix fixation buffer and subsequently permeabilized using BD Phosflow Perm Buffer III (BD Biosciences) according to manufacturer's instructions, before staining with COX2-PE antibody (Santa Cruz Biotechnology). Additionally, T cell proliferation after co-culture with melanoma-induced M-MDSCs was measured by flow cytometry based on CFSE intensities 7-AAD Viability Staining Solution (BioLegend) was used to exclude dead cells and CD3 was used to gate on T cells (Sup. Table 1).

PBMC fractions of healthy donors and HNSCC patients were blocked with PBA + 2% HS, stained with viability dye eFluor®506 (eBioscience) followed by separate antibody panels (Sup. Table 1). For intracellular staining cells were permeabilized using Fixation/Permeabilization Solution Kit (BD Biosciences) before staining for intracellular markers. Samples were acquired on a LSR II flow cytometer or FACSVerse (BD Biosciences) and analyzed with FlowJo software (Treestar V10).

Enzyme-linked immunosorbent assay for IFNγ secretion

Supernatant of suppression assays were taken after 3 days of co-culture and IFNγ content was quantified by a sandwich ELISA using the IFNγ human uncoated ELISA kit (Invitrogen) according to manufacturer's instructions. Absorption was measured using an iMark Microplate Reader (Bio-Rad).

Statistical analyses

Data were analyzed using Prism software version 5.0 (GraphPad Software). Significant differences between two means were analyzed by two-tailed student's t-tests or Mann–Whitney test in case of unequally distributed populations. Differences between three or more means were tested by one way-ANOVA. Error bars and sample sizes are stated in the figure legends. Overall survival (OS) was calculated from the time of diagnosis to death from any cause or last follow-up. Progression-free survival (PFS) was calculated from the time of diagnosis to disease progression, relapse, or death from any cause. Patients who remained alive or progression free were censored at last follow-up. Survival analysis was performed using the Kaplan–Meier method, and differences were compared using the log-rank (Mantel-Cox) test.

Compliance with ethical standards

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committees (Regional Ethical Review Board in Stockholm and Committee on Research involving Human Subjects Arnhem-Nijmegen) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Results

M-MDSC frequency is associated with short survival in metastatic melanoma patients after DC vaccination

To evaluate the relevance of M-MDSCs in the context of cancer immunotherapy, we assessed melanoma patients enrolled in clinical trials for DC vaccination to treat advanced stage melanoma (Table 1). M-MDSC frequencies were quantified by flow cytometry in blood samples from patients and healthy donors (Sup. Figure 1a). Before DC vaccination metastatic melanoma patients had increased levels of M-MDSCs among freshly isolated PBMCs (mean 7%) as compared to healthy donors (mean 3%), in line with previous studies[9,24,25] (Figure 1a). Next, we retrospectively divided patients after DC vaccination into short survivors (n = 14) and long survivors (n = 8) based on overall survival (OS) (less than 12 months versus more than 24 months) and thawed patient PBMCs for analysis. Although freezing and thawing of PBMCs led to an increase in absolute M-MDSC levels (Sup. Figure 4b), we observed after DC vaccination, that M-MDSCs were more frequent in short survivors (16%) relative to long survivors (10%) (Figure 1b). In line, when the OS and progression-free survival (PFS) of these 22 enrolled patients were stratified by M-MDSC frequency there was a significant correlation between increased M-MDSC frequencies and poor survival after DC vaccination (Figure 1c–f). These observations suggest that M-MDSCs counteract DC-induced anti-tumor immune responses thereby impairing clinical benefit of immunotherapy for patients. Overall, these data show that M-MDSCs are expanded in metastatic melanoma patients and associated with short survival after immunotherapy.

Platinum-based drugs inhibit the development and suppressive activity of melanoma-induced M-MDSCs *in vitro*

Previously, the role of melanoma tumor cells in generating M-MDSCs was demonstrated by co-culturing healthy donor monocytes with a melanoma patient-derived tumor cell line, THFR.[23] Here, we investigated the effect of platinum-based drugs on the development of melanoma-induced M-MDSCs

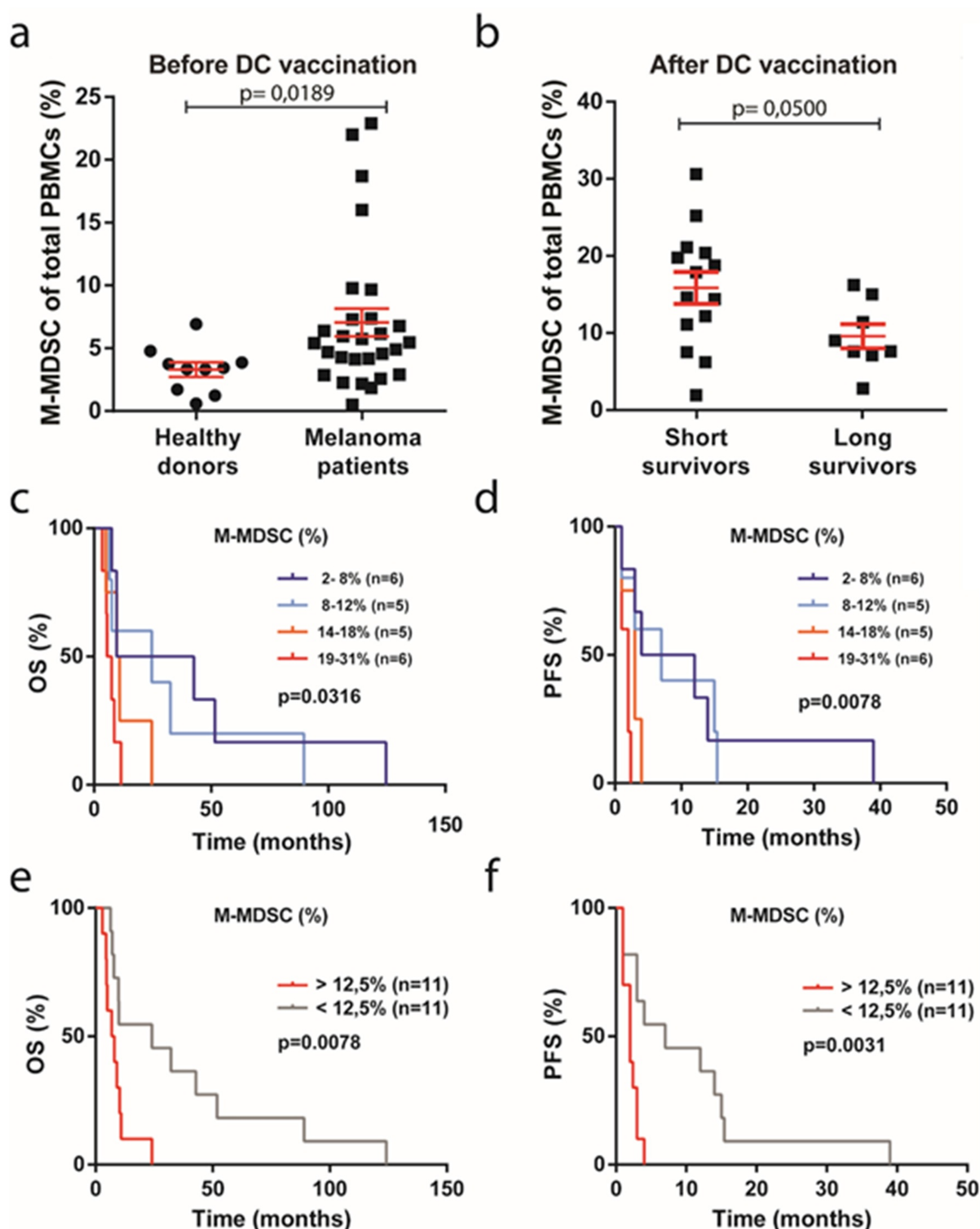

Figure 1. M-MDSC frequencies are increased in metastatic melanoma patients and associated to short survival after DC vaccination. Metastatic melanoma patient enrolled in clinical trials of DC vaccination were divided into short (<12 months) and long survivors (>24 months) based on overall survival. (a) Quantified frequency of M-MDSCs among freshly isolated total PBMCs in healthy donors (n = 10) and metastatic melanoma patients (n = 28) before DC vaccination. (b) Quantified frequency of M-MDSCs among frozen PBMCs in short (n = 14) and long (n = 8) survivors after DC vaccination. (c-d) Patients were divided into quartiles based on M-MDSCs frequency (low to high) and (e-f) divided into 2 groups based on above and below average M-MDSC frequency (12,5%). Increased M-MDSC frequencies correlated with significantly poor OS and PFS in 22 patients. Mann–Whitney test in panel b, unpaired t-test in panel c and log-rank (Mantel-Cox) test in panel c-f. Mean + SEM in panel a-b.

in vitro (Figure 2a, Sup. Figure 2a). Cisplatin and oxaliplatin were added during the co-culture of monocytes and tumor cells in a dose that is well below the maximum concentration observed in patients after intravenous administration (C_{max}: 5 µg/ml, both).[26,27] These subclinical doses of platinum drugs did not affect the viability of monocytes and melanoma cells in the co-cultures (Sup. Figure 2b,c). Subsequently, monocytes were harvested and co-cultured with CD3/CD28 stimulated autologous T cells in a 2:1 or 4:1 ratio (T cell: Monocyte) for 4 days to assess their suppressive capacity. At a 2:1 ratio, monocytes co-cultured with melanoma cell-line THFR were able to significantly suppress T cell proliferation compared to mono-cultured control monocytes, indicating successful induction of M-MDSCs *in vitro* by the melanoma cell-line

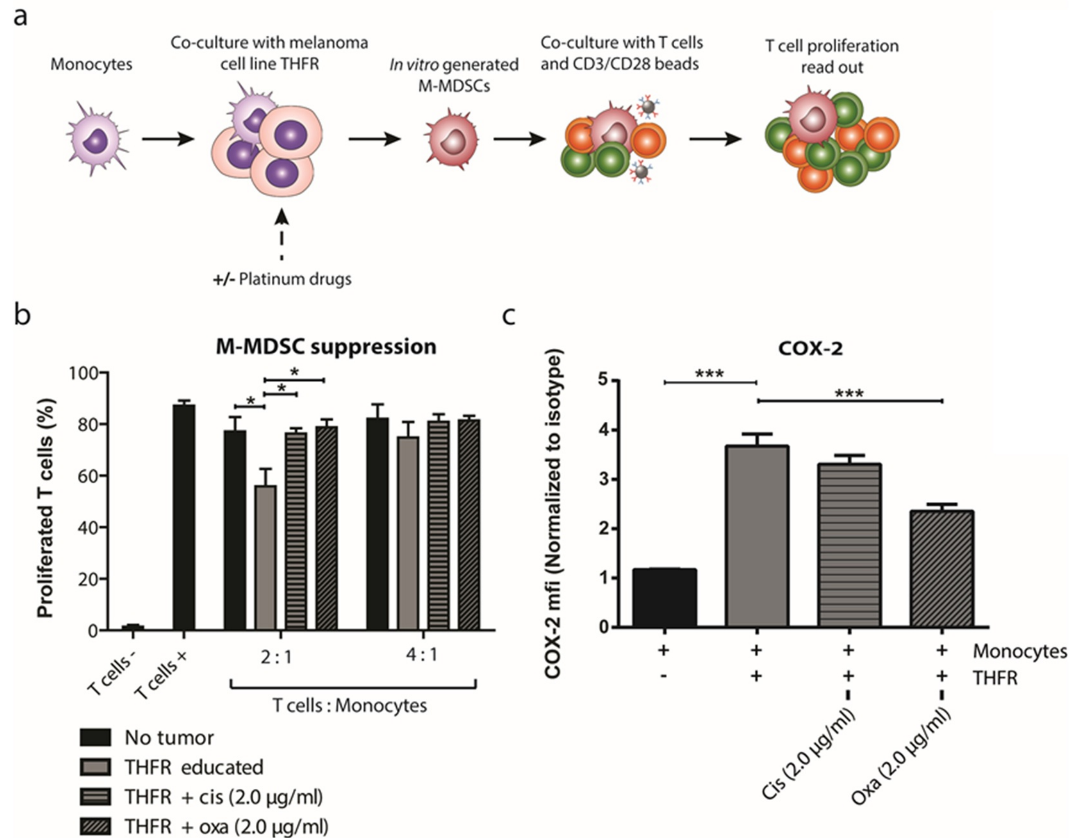

Figure 2. Platinum-based drugs inhibit the development and suppressive activity of melanoma-induced M-MDSCs *in vitro*.(a) Schematic overview of M-MDSC induction by THFR primary melanoma cells in the presence and absence of platinum drugs followed by autologous T cell co-culture. (b) Percentage of proliferated CD3 + T cell based on CFSE signal after 4 day co-culture with conditioned monocytes (n = 4). (c) Intracellular COX-2 expression by conditioned monocytes was quantified by flow cytometry. Data are depicted as fold increase of COX-2 MFI compared to isotype control (n = 4). One way ANOVA was used to test significance. Mean + SEM in both graphs *** $p = <0.001$, ** $p = <0.01$, * $p = <0.05$.

THFR (Figure 2b). Treatment of monocytes with low-dose cisplatin and oxaliplatin during the co-culture with melanoma cell-line THFR completely abrogated the suppressive activity of *in vitro* generated M-MDSCs to the level of control monocytes. Melanoma-induced M-MDSCs did not significantly inhibit the proliferation of autologous T cells in a 4:1 ratio, hence effects of platinum drugs could not be measured. It was previously shown that the development and suppressive activity of melanoma-induced M-MDSCs is dependent on a positive feedback loop of STAT3-COX-2 signaling.[23] In brief, melanoma-derived PGE-2 induces STAT3 signaling in monocytes that activates the expression of suppressive machinery and COX-2, which in turn leads to more PGE-2 production. To explore whether STAT3 inhibition by platinum-based drugs could explain their inhibitory effect on M-MDSCs *in vitro*, intracellular COX-2 expression was measured in melanoma-induced M-MDSCs. Indeed, the acquired suppressive capacity of these M-MDSCs coincided with an increased expression of intracellular COX-2 as compared to control monocytes, indicative of active STAT3 signaling (Figure 2c). In addition, treatment of monocytes with oxaliplatin during melanoma co-culture significantly reduced COX-2 expression suggesting inhibition of STAT3 signaling. Taken together, our observations show that subclinical doses of cisplatin and oxaliplatin prevent the transformation of monocytes into melanoma-induced M-MDSCs *in vitro*.

Cisplatin diminishes M-MDSC frequency and lowers the expression of STAT3-controlled enzymes in M-MDSCs in HNSCC patients

Melanoma patients are not treated with platinum-based chemotherapy; therefore, we selected patients with HNSCC to investigate the ability of platinum-based drugs to deplete M-MDSC numbers *in vivo*. To this end, blood from 19 HNSCC patients undergoing chemo-radiotherapy with weekly cisplatin as the sole chemotherapeutic agent was collected (Table 2). Figure 3a illustrates the treatment schedule according to which blood samples from patients were collected. Blood was taken at three time points per patient: Before start, during week 3 (prior to third administration) and 2 weeks after six rounds of cisplatin administration. Of note, not all time points could be collected for each patient. The frequency of M-MDSCs and monocytes of PBMCs isolated from these HNSCC patients and 10 healthy donors was determined by flow cytometry (Sup. Figure 3a). There was an increased frequency of M-MDSCs in patients before treatment as compared to healthy donor PBMCs (mean 8% vs 2%) (Figure 2b).

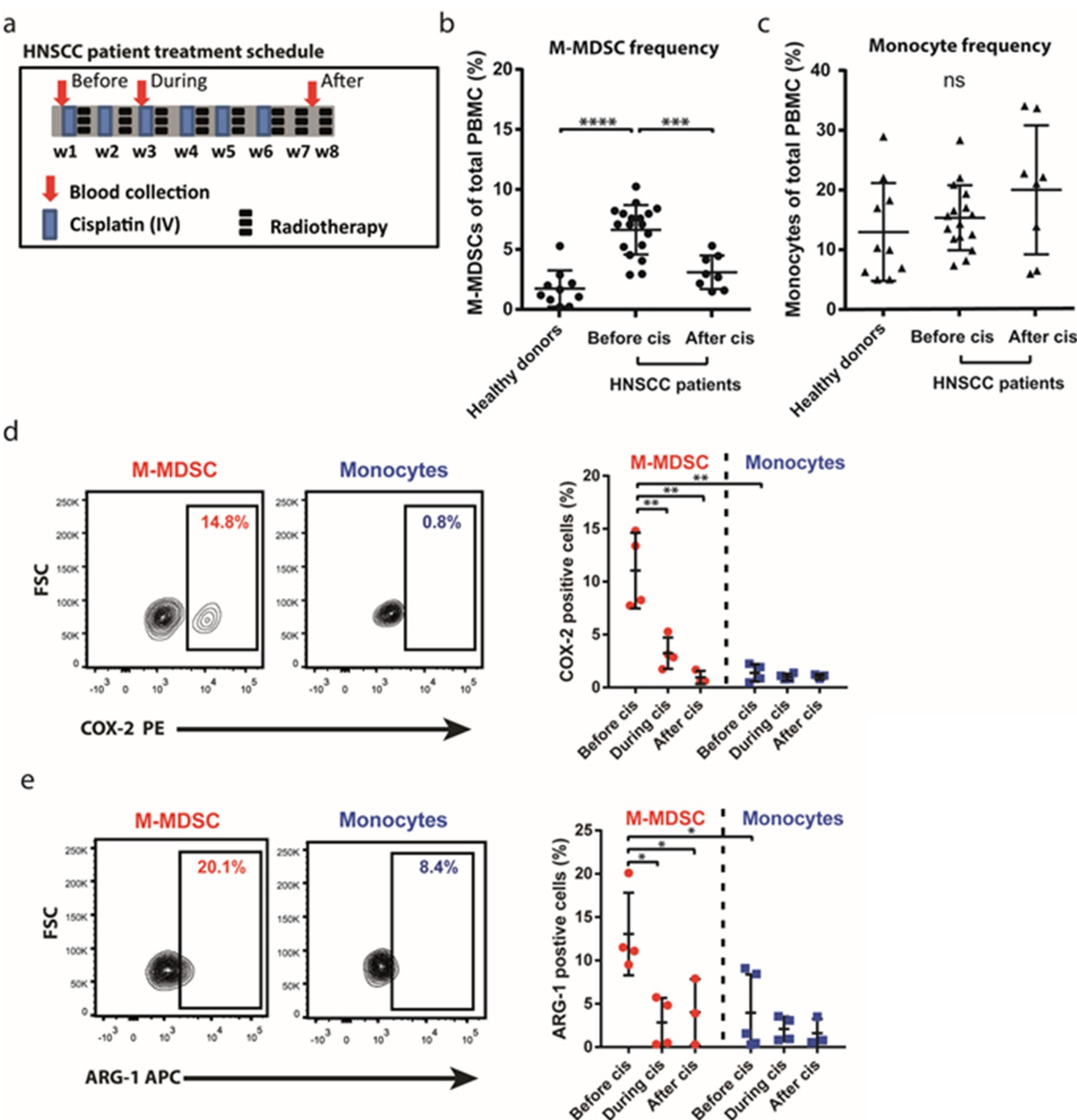

Figure 3. Cisplatin inhibits M-MDSC frequency and expression of STAT3-controlled enzymes in HNSCC patients. (a) Overview of treatment schedule for HNSCC patients undergoing cisplatin-based chemoradiotherapy and timepoints of blood collection for assessment of PBMCs by flow cytometry. Shown are (b) quantified frequency of M-MDSCs (n = 8–19) and (c) quantified frequency of monocytes (n = 8–19) of total viable PBMCs plus (d) a representative plot and quantified COX-2 positivity (n = 3–4) and (e) a representative plot and quantified ARG-1 positivity (n = 3–5) both within M-MDSCs and monocytes. One way ANOVA was used to test significance. Mean + SD in all graphs ****p = <0.0001 *** *p* = <0.001, ** *p* = <0.01, * *p* = <0.05, ns = non-significant.

Interestingly, this M-MDSC frequency was drastically lowered in HNSCC patients after receiving six rounds of cisplatin treatment (mean 4%). In contrast, monocyte frequencies were similar between untreated HNSCC patients and healthy donors (mean 12% vs 14%) and did not significantly change after cisplatin treatment (mean 20%) (Figure 3c). These observations indicate that cisplatin treatment specifically impairs M-MDSC survival or the development of new M-MDSCs. To investigate whether STAT3-COX-2-dependent induction of M-MDSCs was inhibited by cisplatin, intracellular COX-2 expression was measured in M-MDSCs and monocytes from 4 HNSCC patients. COX-2 expression was highest in M-MDSC compared to monocytes before treatment (mean 11% vs 1% positive), suggestive of increased STAT3 signaling (Figure 3d). Strikingly, COX-2 expression in M-MDSCs was significantly decreased during treatment after two rounds of cisplatin (3% positive) and after six rounds of cisplatin (1% positive). In addition, intracellular expression of a second STAT3-controlled enzyme, ARG-1, followed the same pattern as COX-2 in M-MDSCs and monocytes during treatment. ARG-1 expression was highest in M-MDSCs before treatment as compared to monocytes (13% vs 4% positive) and significantly decreased in M-MDSCs during and after cisplatin treatment (3% and 4% positive) (Figure 3e). Collectively, our data show that cisplatin treatment of HNSCC patients specifically depletes M-MDSCs, but not monocytes, which could be

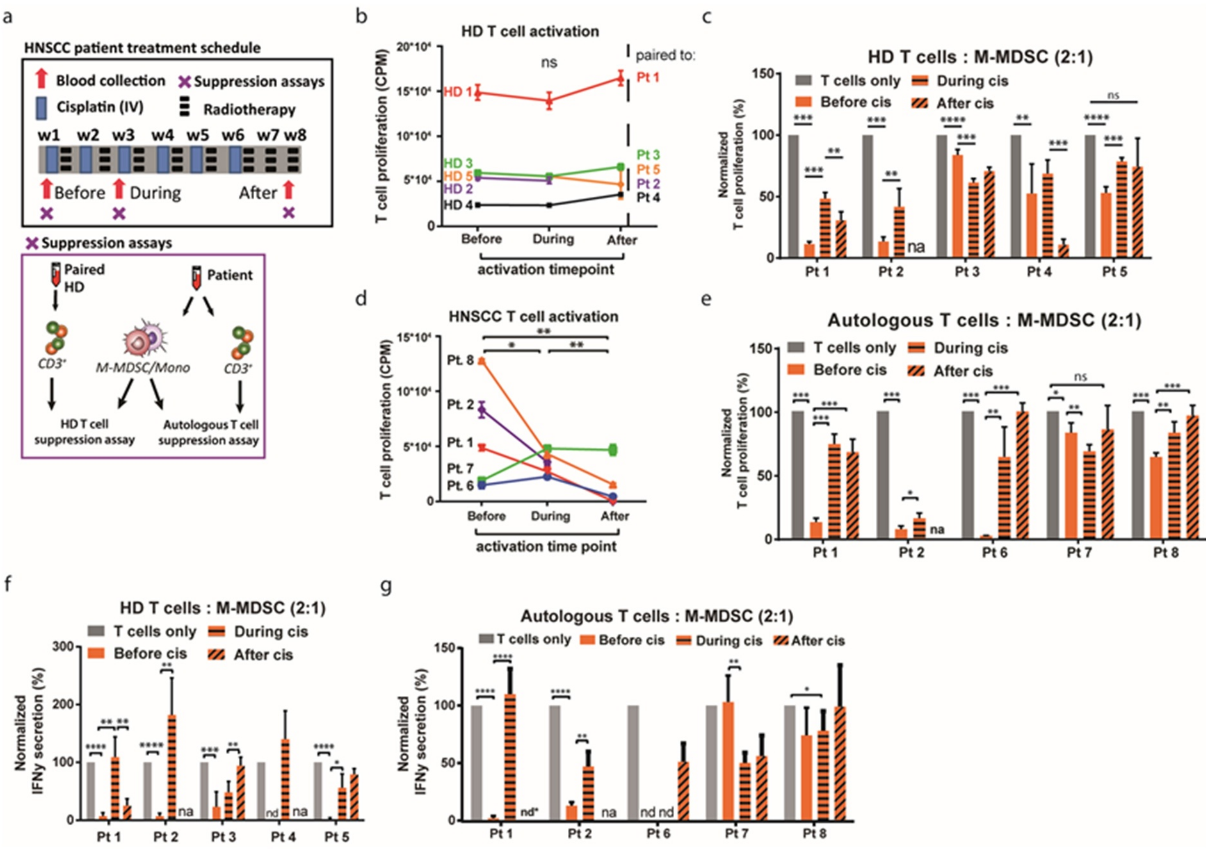

Figure 4. Cisplatin inhibits suppressive activity of M-MDSCs in HNSCC patients. (a) Overview of treatment schedule for HNSCC patients undergoing cisplatin-based chemoradiotherapy and timepoints of blood collection for assessment of suppressive capacity by M-MDSCs. Subpanel indicates two experimental setups of suppression assays performed at each time point. (b) Absolute proliferation of activated T cells in counts per minute after 3 days of culture followed by 16 h thymidine incorporation. T cells from a paired HD were thawed and stimulated for a suppression assay at three timepoints for each patient isolation (in panel b) ensuring similar T cell responses. (c) Proliferation of activated HD T cells was measured by thymidine incorporation after 3 day co-culture with M-MDSCs isolated from patients before, during and after intravenous cisplatin treatment. Proliferation was normalized to monocultured activated HD T cells at every timepoint. (d) Absolute proliferation of activated T cells in counts per minute after 3 days of culture followed by 16 h thymidine incorporation. Autologous T cells were isolated in parallel with M-MDSCs for a suppression assay at three timepoints for each patient (in panel e) simulating *in vivo* T cell responses. (e) Proliferation of activated autologous T cells was measured by thymidine incorporation after 3 day co-culture with M-MDSCs isolated from patients before, during and after intravenous cisplatin treatment. Proliferation was normalized to monocultured activated autologous T cells at every timepoint. (f) Secreted IFNγ after 3 day co-culture of activated HD T cells and M-MDSCs (from panel C). (g) Secreted IFNγ after 3 day co-culture of activated autologous T cells and M-MDSCs (from panel E). One way ANOVA was used to test significance. Mean + SD in all graphs. ****p = <0.0001 *** p = <0.001, ** p = <0.01, * p = <0.05, ns = non-significant, na = not available, nd = not detected, value below detection limit ELISA, nd* = IFNγ could not be detected in any sample including monocultured activated T cells.

explained by the inhibition of STAT3 signaling as indirectly evidenced by decreased expression of COX-2 and ARG-1 in platinum-treated M-MDSCs *in vivo*.

Cisplatin inhibits the suppressive activity of M-MDSCs in HNSCC patients

Next, we examined the suppressive capacity of M-MDSCs isolated from HNSCC patients undergoing cisplatin-based chemotherapy with concomitant radiotherapy (Figure 4a, Sup. Figure 3b). M-MDSCs were FACS-sorted from 5 HNSCC patients at three time points during cisplatin treatment and co-cultured with stimulated T cells from a healthy donor (HD) (Table 3). To minimize donor–donor variation, the same combination of patient-donor (M-MDSC-T cell) was used at each time point. This HD T cell suppression assay was set up to observe effects of cisplatin-treated M-MDSCs on T cells, not affected by tumor-induced or therapy-induced alterations in T cells. In line, the amount of proliferation by CD3/CD28 stimulated T cells derived from the paired HD was similar at every time point when M-MDSCs were isolated from the patient (Figure 4b). Before cisplatin treatment, M-MDSCs from all five patients had the capacity to inhibit stimulated HD T cells in a 2:1 ratio (T cell: M-MDSC) *ex vivo* (Figure 4c). Decreased to no suppressive activity by isolated M-MDSCs was observed in three out of five patients after two rounds of cisplatin and in two out of four patients after six rounds of cisplatin. Similar results were seen for 4:1 ratio (T cell: M-MDSC) co-cultures (Sup. Figure 4a). Next, M-MDSCs were FACS-sorted from 5 HNSCC patients and co-cultured with stimulated autologous T cells that were isolated in parallel with M-MDSCs from the patient at the 3 time points (Table 3). This autologous T cell suppression assay was setup to be more clinically relevant by assessing whether the effect of cisplatin-treated M-MDSCs toward T cells is conserved in the context of chemotherapy and active disease. Notably, the amount of T cell proliferation induced by CD3/CD28 stimulation was significantly decreased after two and six rounds of cisplatin illustrating

cisplatin-induced inhibition of T cell responses (Figure 4d, Sup. Figure 4b). M-MDSCs co-cultured with autologous stimulated T cells in a 2:1 ratio (T cell: M-MDSC) were suppressive in all five patients (Figure 4e). In addition, M-MDSCs were significantly less or non-suppressive when isolated after two rounds of cisplatin in four out of five patients and after six rounds of cisplatin in four out of four patients. A similar pattern was observed in 4:1 ratio co-cultures (Sup. Figure 4c) and when untreated autologous T cells (before cisplatin) were used at every time point (Sup. Figure 4d). Whether the rescue of T cell proliferation by cisplatin-treated M-MDSCs corresponds with improved T cell activation was assessed by measuring IFNγ secretion in the T cell – M-MDSC co-cultures. In line with the proliferation data, IFNγ secretion by both HD and autologous T cells was suppressed by M-MDSC *ex vivo* before cisplatin treatment and increased after treatment in 4 out of 5 patients (Figure 4e,g). Overall, cisplatin treatment significantly impaired the suppressive capacity of M-MDSCs as evidenced by the rescue of proliferation and activation of healthy donor and autologous T cells after *ex vivo* co-culture in 60–80% of treated HNSCC-patients.

Discussion

In this study, we investigated the effects of platinum-based chemotherapeutics on the development and suppressive capacity of M-MDSCs *in vitro* and in cancer patients. First, we observed that M-MDSCs frequency in metastatic melanoma patients was associated with short survival after DC vaccination, which was previously also shown for patients treated with adoptive cell therapy and immune checkpoint inhibitors.[9,11,12,28] Subsequently, we made use of a clinically relevant *in vitro* model that differentiates monocytes into suppressive cells that closely resemble human M-MDSCs through co-culture with a patient-derived melanoma cell line.[23,29] We showed that treatment of monocytes with non-cytotoxic doses of cisplatin and oxaliplatin during these co-cultures completely prevented the development of suppressive melanoma-induced M-MDSCs. This is in line with a recent study performed in a murine colon cancer model, where *in vitro* treatment with non-cytotoxic doses of oxaliplatin impaired the suppressive activity of splenic M-MDSCs.[30] We found that the diminished suppressive activity in oxaliplatin-treated M-MDSCs coincided with decreased STAT3-COX2 signaling as indicated by impaired COX-2 expression. However, cisplatin also blocked suppressive function but did not clearly lower COX-2 expression in melanoma-induced M-MDSCs *in vitro* as it did in M-MDSCs *in vivo*. This suggests that M-MDSC-targeting effects by platinum compounds are not limited to STAT3-COX-2 inhibition but likely also affects other pathways through which M-MDSCs exert their suppressive functions, such as production of NO.[7] In this regard, platinum drugs have different effects on multiple STAT-regulated pathways including STAT1, STAT5, or STAT6, which are also involved in M-MDSC suppressive function.[13] Future studies are needed to establish which additional signaling pathways are inhibited by platinum drugs in human M-MDSCs and how these contribute to the impaired immunosuppressive function.

To validate the effect of platinum-based chemotherapeutics on M-MDSCs *in vivo*, we identified that patients with HNSCC are the most suitable patient population because these are, unlike most tumor types, treated with platinum drugs as single chemotherapeutic modality. M-MDSC frequency and suppressive function were determined in blood samples of patients undergoing cisplatin-based chemo-radiotherapy. We showed for the first time that chemo-radiotherapy with cisplatin as the sole chemotherapeutic agent reduces the frequency and suppressive activity of M-MDSCs in cancer patients. So far, in mice both MDSC-depleting and -promoting effects of platinum drugs have been described. This discrepancy is likely due to differences in drug concentrations, treatment intervals, MDSC localization, timing of MDSC sampling and tumor models.[31] Nevertheless, studies that examined the functionality of MDSCs showed that a single dose of 7.5 mg/kg cisplatin inhibited the frequencies and suppressive activity of intratumoral and splenic MDSCs, but not peripheral blood-borne MDSCs from B16 melanoma-bearing mice.[32] A second study, using a murine CT26 colon cancer model, demonstrated that one intraperitoneal injection of 10 mg/kg oxaliplatin depleted MDSCs and in particular M-MDSCs in the spleen but not in the tumor. These studies suggest that assessment 2–3 days after platinum treatment is too early to detect systemic changes in the myeloid compartment or that single doses are insufficient in inducing these systemic effects.

In our study, cisplatin-treated patients underwent a 6 week course of radiotherapy concomitant to the chemotherapy. In this regard, it was previously shown that a direct immunological effect on myeloid cells by radiotherapy is unlikely as evidenced by unchanged STAT phosphorylation in monocyte-derived dendritic cells after *in vitro* irradiation.[22] Moreover, the patients were given a single dose of 8 mg dexamethasone for anti-emetic purpose prior to each cisplatin administration in order to tolerate clinical doses. As dexamethasone is also widely used as immunosuppressant this has been a relevant concern in recent chemo-immunotherapy studies. There, it was observed that anti-emetic doses of dexamethasone did not constrain immune responses in mice and in melanoma patients, including anti-tumor immunity induced by immunotherapy.[33–35] In accordance, we found that the frequency of monocytes in cisplatin-treated patients remained unaltered, which argues in favor of minimal immunological interference by dexamethasone in the monocytic compartment.

Our *in vitro* observations clearly demonstrate an immunostimulatory effect of platinum drugs via the direct inhibition of M-MDSCs. This was complemented by the drastically decreased expression of COX-2 and ARG1 observed in M-MDSCs *in vivo* after only two rounds of cisplatin treatment, indicative of a direct effect on STAT3 signaling. Furthermore, *in vivo*, M-MDSCs are possibly affected by additional indirect effects of platinum drugs. For example, reduced tumor burden as result of the platinum-based chemo-radiotherapy could indirectly contribute to M-MDSC depletion by affecting tumor-derived signals in patients.[28] This supports the

administration of platinum drugs to overcome M-MDSC-mediated resistance to immunotherapy in order to improve clinical benefit for patients.

A recent clinical study of HPV+ oropharyngeal cancer patients undergoing radical therapy consisting of mixed strategies including platinum-based chemo-radiotherapy demonstrated that patients had decreased HPV-specific T cell responses 6 to 16 weeks post-treatment.[36] This suggests that the potential to elicit anti-tumor immunity in HNSCC patients after such a period post-radical therapy is unfavorable for immunotherapies. The importance of timing when combining chemo- with immunotherapy was also evidenced in a recent clinical trial where administering cisplatin 1–2 hours prior to DC-based cancer vaccines had no added benefit over DC vaccination alone in melanoma patients.[33] However, when an HPV cancer vaccine was administered 2 weeks after the second round of carboplatin treatment combined with alkaloid chemotherapeutic paclitaxel, it resulted in unusually strong T cell responses in cervical cancer patients, which remained during the course of the chemotherapy.[35] It was shown that the cancer-induced expansion of the myeloid compartment including MDSC frequencies were normalized during this window starting between the second and third round of chemotherapy. In line, our observations also show that the suppressive activity of M-MDSC in HNSCC patients was decreased between the second and third round of cisplatin treatment and that this effect lasted until at least 2 weeks after the sixth round. Combined, these data emphasize the contribution of M-MDSC-mediated immunosuppression to the refractoriness of immunotherapy and the potential that platinum-based chemotherapy has to overcome this obstruction. Whether two rounds of chemotherapy prior to immunotherapy is sufficient to achieve optimal T cell responses or what time point in the now proposed window is most effective to start immunotherapeutic intervention still needs to be investigated. Lastly, our *in vitro* data suggest that M-MDSC-targeting effects by cisplatin and oxaliplatin could already be achieved by sub-clinical doses in patients. This could potentially translate into lowering chemotherapeutic doses strictly needed for combined chemo-immunotherapy, which in turn could alleviate therapy burden for cancer patients.

In conclusion, we have shown that platinum-based drugs impair the suppressive activity of M-MDSCs both *in vitro* and in cancer patients, likely by the inhibition of STAT3 and its downstream targets including COX-2 and ARG-1. Due to the broad relevance of M-MDSCs in cancer these novel findings support the application of platinum-based combined chemo-immunotherapeutic strategies across several tumor types. Additional studies will have to demonstrate whether the combination of platinum drugs and properly timed immunotherapy results in added clinical benefit.

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Disclosure statement

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G.F.W., N.H., T.A.M., Y.M., S.V.H. and I.J.M.V. designed and performed experiments and analyzed the data. M.B. and S.HB. provided clinical patient information and contributed to data interpretation. G.F.W. and N.H. were responsible for writing the manuscript. R.K.´s laboratory provided the model for *in vitro* induction of MDSCs and established the melanoma tumor line. S.K.H., R.K., C.M.L.H., G.FG., S.V.H., and I.J.M.V substantially revised the manuscript.

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