ONCOIMMUNOLOGY
2016, VOL. 5, NO. 8, e1192739 (14 pages)
http://dx.doi.org/10.1080/2162402X.2016.1192739

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ORIGINAL RESEARCH

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Human CD1c$^+$ DCs are critical cellular mediators of immune responses induced by immunogenic cell death

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ABSTRACT

Chemotherapeutics, including the platinum compounds oxaliplatin (OXP) and cisplatin (CDDP), are standard care of treatment for cancer. Although chemotherapy has long been considered immunosuppressive, evidence now suggests that certain cytotoxic agents can efficiently stimulate antitumor responses, through the induction of a form of apoptosis, called immunogenic cell death (ICD). ICD is characterized by exposure of calreticulin and heat shock proteins (HSPs), secretion of ATP and release of high-mobility group box 1 (HMGB1). Proper activation of the immune system relies on the integration of these signals by dendritic cells (DCs). Studies on the crucial role of DCs, in the context of ICD, have been performed using mouse models or human *in vitro*-generated monocyte-derived DCs (moDCs), which do not fully recapitulate the *in vivo* situation.

Here, we explore the effect of platinum-induced ICD on phenotype and function of human blood circulating DCs. Tumor cells were treated with OXP or CDDP and induction of ICD was investigated. We show that both platinum drugs triggered translocation of calreticulin and HSP70, as well as the release of ATP and HMGB1. Platinum treatment increased phagocytosis of tumor fragments by human blood DCs and enhanced phenotypic maturation of blood myeloid and plasmacytoid DCs. Moreover, upon interaction with platinum-treated tumor cells, CD1c$^+$ DCs efficiently stimulated allogeneic proliferation of T lymphocytes. Together, our observations indicate that platinum-treated tumor cells may exert an active stimulatory effect on human blood DCs. In particular, these data suggest that CD1c$^+$ DCs are critical mediators of immune responses induced by ICD.

Abbreviations: APC, antigen-presenting cell; ATP, adenosine triphosphate; CDDP, cis-diamino-dichloro-platinum (II) cisplatin; CRT, calreticulin; CTC, circulating tumor cell; DC, dendritic cell MHC; HMGB1, high-mobility group box 1; HSP, heat shock protein; ICD, immunogenic cell death; IL, interleukin; inflDC, inflammatory dendritic cell; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; moDC, monocyte-derived dendritic cell; OXP, oxaliplatin; PBL, peripheral blood leukocyte; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; TLR, toll-Like receptor

ARTICLE HISTORY
Received 13 January 2016
Revised 29 April 2016
Accepted 18 May 2016

KEYWORDS
CD1c$^+$ DCs; human dendritic cells; immune response; immunogenic cell death; platinum chemotherapy; T cell proliferation

Introduction

Platinum-based chemotherapy is currently approved as first-line treatment for several malignancies, including colon and testicular cancer.[1] Besides a direct cytotoxic effect on tumor cells, some chemotherapeutic compounds are now recognized to have beneficial effects on the immune system, which may contribute to their clinical effectiveness.[2-7] Accordingly, a number of anticancer drugs, including the platinum-based compound oxaliplatin (OXP), were shown to be more effective against tumors established in immunocompetent, as opposed to immunodeficient mice.[5] This was predicated on the ability of these drugs to induce a form of cell death that activated the immune system and promoted antitumor immune responses. As such, these agents are referred to as immunogenic cell death (ICD)-inducers.[8]

ICD-inducers cause severe cell stress, which activates distinct molecular pathways that can result in the induction of apoptosis.[4] Classically, ICD requires three molecular events, which may or may not be linked. First is the translocation of chaperone molecule calreticulin from the lumen of the endoplasmic reticulum (ER) to the cell surface (ecto-CRT).[9,10] Ecto-CRT serves as an "eat me" signal that marks tumor cells for engulfment by phagocytic cells.[9] Second is the active secretion of ATP into the extracellular environment, which acts as a

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Supplemental data for this article can be accessed on the publisher's website.
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chemoattractant for immune cells and directs the differentiation of myeloid precursors into inflammatory cells.[11,12] Thirdly, release of high-mobility group box 1 (HMGB1) into the extracellular milieu, which is required for optimal induction of T cell-mediated antitumor responses.[13] Other than the three "hallmarks" of ICD, the ER-resident chaperones, heat shock proteins (Hsp) Hsp70 and Hsp90, are exposed on the membrane of cells undergoing severe stress. These molecules are not included in the canonical definition of ICD, nevertheless they also act as danger signals and might contribute to the stimulation of antigen-presenting cells (APCs).[14]

Induction of a systemic tumor-specific immune response by ICD requires the recognition and integration of these separate signals (ecto-CRT, ATP, and HMGB1) into one command that drives T cell activation. Dendritic cells (DCs) are one of the main cell types that serve this role. They are the main APCs that link innate and adaptive immunity. DCs can encounter tumor cells in two ways. First, the chemoattractants ATP and HMGB1 induce intratumoral recruitment of DCs.[11] Secondly, tumor cells that have detached from the tumor bulk are likely to enter blood circulation, facilitating their spread to distant locations in the body.[15] In the blood stream, these circulating tumor cells (CTCs) can encounter blood-circulating DCs. Platinum-induced exposure of ICD-markers might contribute to make CTCs "visible" and more sensitive to DC recognition. Indeed, treatment with well-known ICD-inducers, anthracyclines, induced ecto-CRT expression on CTCs *in vivo*.[16] Chemotherapy treated-tumor cells are engulfed by immature DCs, which then undergo maturation. Mature DCs process and present tumor antigens to naive cytotoxic lymphocytes, prompting antitumor responses.[7,16] Accordingly, perturbation of key elements of adaptive immunity, such as *in vivo* depletion of DCs, or knockout of DC receptors, resulted in failure to prime an antitumor response in chemotherapy-treated mouse models.[5,13,17]

There are two major DC subsets circulating in human peripheral blood, myeloid DCs (mDCs), and plasmacytoid DCs (pDCs).[18] Classically, myeloid DCs are subdivided into $CD16^+$, $CD1c^+$, and $CD141^+$ DCs, based on the expression of specific surface molecules.[19] However, genome-wide expression profile analysis recently suggested that $CD16^+$ DCs may represent a particular subset of monocytes, with DC-like properties.[20] For simplicity, we will refer to them as $CD16^+$ DCs. Transcriptional, phenotypic, and functional studies highlight significant differences between human blood DCs, suggesting a biological specialization of these DC subsets.[21,22] Despite the great interest that ICD has gained in the past decade, the role of naturally occurring human DCs, especially for DCs that circulate in the blood, in this process is poorly understood, as most studies have been performed in murine models or with *in vitro* generated moDCs.[11,23] Here, we study induction of ICD in human tumor cells by two of the most widely used platinum compounds, OXP and cisplatin (CDDP), and how that affects human DC subsets. We report that, at clinically relevant concentrations, both compounds induced apoptosis of tumor cells, which was accompanied by the expression and release of ICD-associated molecules. Exposure of tumor cells to platinum drugs resulted in increased uptake of tumor fragments by naturally occurring blood DCs and stimulated DC maturation. Surprisingly, only $CD1c^+$ DCs were subsequently able to drive T cell proliferation.

Results

Cisplatin and oxaliplatin induce a form of cancer cell death consistent with ICD

Up till now most studies on induction of ICD by platinum compounds, OXP and CDDP were performed in mouse models and little is known about the ability of platinum compounds to induce ICD in human tumor cells.[5,9] We investigated the molecular hallmarks of platinum-induced cancer cell death *in vitro*. We treated human colon (Caco2), testicular (833KE and 2102EP), and melanoma (BLM) cell lines with increasing, clinically relevant doses of OXP or CDDP and studied cell death using Annexin V and DAPI staining. Both OXP and CDDP decreased viability of BLM cells in a time- and dose-dependent manner (Figs. 1A and B), as indicated by increased phosphatidylserine exposure and stronger nuclear DAPI staining. Similar cytotoxicity was observed for 833KE, 2102EP, and Caco-2 cells (data not shown). Given the chemosensitivity of testicular carcinoma cells, 833KE and 2102EP cell lines were treated with lower drug concentrations, compared to the other cell lines studied. Despite sharing similar mechanisms of action, OXP, but not CDDP, was previously described to trigger exposure of ecto-CRT on the murine colon cancer cell line CT26.[5] Surprisingly, we observed similar, if not stronger translocation of ecto-CRT after BLM and Caco-2 cells were exposed to CDDP compared to OXP (Figs. 1C–E; Fig. S1C). Analysis of expression kinetics on BLM cells showed that ecto-CRT was detected as early as 1 h after treatment and its exposure was dose- and concentration-dependent (Figs. S1B and C). On the other hand, OXP and CDDP had less marked effects on the translocation of ecto-CRT in 833KE, 2102EP and CT26 cell lines, at time and dosage tested (Figs. 1E and F; Fig. S1C and D).

In order to simulate the pharmacokinetics of platinum treatment, which is administered intravenously and remain in the body for a few hours,[24] we exposed cells to OXP or CDDP for 8 h, washed away the drug and cultured the cells for an additional 40 h under drug-free conditions. This short-term drug exposure to OXP or CDDP dose-dependently decreased viability of BLM cells and induced ecto-CRT expression (Figs. 1G and H), similar to long-term (48 h) treatment (Figs. 1B and C).

Next, we measured the expression of Hsp70, ATP, and HMGB1 on different tumor cell lines treated with platinum drugs. Both OXP and CDDP induced translocation of Hsp70 (ecto-Hsp70) to the cell surface of human BLM and 2102EP cells, as well as the murine CT26 cell line as observed using flow cytometry (Figs. 2A–C; Figs. S2A–C). Concurrent with increased ecto-HSP70, we observed increased secretion of ATP (Figs. 2D–F; Fig. S2D) and HMGB1 (Figs. 2G–I, Figs. S2E and F) in the supernatant of platinum-treated tumor cells. In all, we show that both OXP and CDDP cause apoptosis of human tumor cells, with the concomitant translocation of ecto-CRT and ecto-HSP and the extracellular release of ATP and HMGB1. These results suggest that both platinum compounds induce a form of cell death that fulfills the requirements for immunogenic apoptosis.

Human DCs preferentially phagocytose platinum-treated tumor cells in a CRT-dependent manner

We investigated whether platinum-induced ecto-CRT had an effect on the recognition and uptake of tumor cells by human blood

DCs.[9] First, we examined whether treatment with platinum drugs led to increased interaction between tumor cells and human blood DCs using confocal microscopy. For this experiment, the human melanoma cell line BLM was modified to stably express the fluorescent protein GFP (hereafter referred to as BLM-GFP). Untreated BLM and BLM-GFP showed no difference in surface expression of calreticulin (CRT). Furthermore, platinum-treatment induced comparable upregulation of CRT on both BLM and BLM-

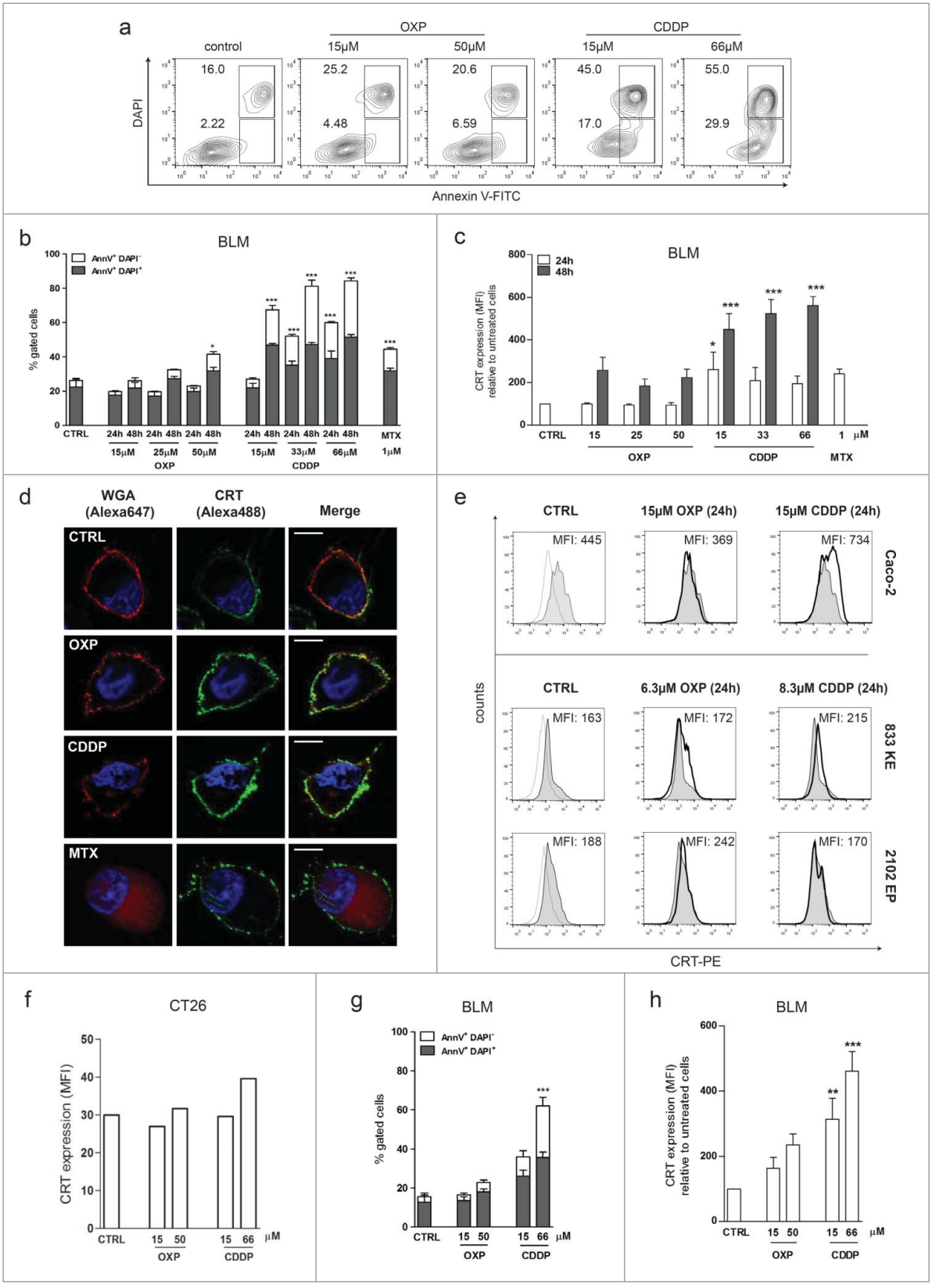

Figure 1. (For figure legend, see page 4).

GFP (Fig. S3B). Tumor cells were left untreated or treated with OXP or CDDP for 48 h and then co-cultured with CD1c$^+$ DCs for 3 h. We observed increased interaction between DCs and tumor cells treated with CDDP or OXP compared to untreated cells (Figs. 3A and B). Based on the fact that tumor cells are several orders of magnitude larger than blood DCs, we hypothesized that DCs, in contrast to macrophages, do not ingest whole tumor cells but only subcellular fragments of these cells. To evaluate whether fragments of platinum-treated cells are preferentially taken up by the three human DC subsets examined in this study, we established *in vitro* co-cultures of platinum-treated tumor cells and DCs. Tumor cells were exposed to OXP or CDDP. Concentration and duration of treatment with platinum drugs were specifically chosen for each cell line in order to maximize induction of ICD hallmarks, while maintaining cell viability at the start of the co-culture. Fluorescently labeled-tumor cells were co-cultured with DCs for 24 or 48 h. Uptake of untreated versus OXP- or CDDP-treated tumor cells was assessed by flow cytometry (Figs. 3C–F; Fig. S3). In order to distinguish between binding of tumor cells fragments to the cell membrane of DCs and active uptake, we performed co-culture experiments at 4°C vs. 37°C, respectively. As shown in Fig. 3C, DCs are capable of taking up (37°C) fragments of tumor cells. In contrast, there is a low level of binding (4°C) of tumor fragments to DCs, which did not increase upon treatment (Fig. 3C; Fig. S3C). Furthermore, while there was a considerable increase in the uptake of platinum treated cells between 24 and 48 h of co-culture, uptake of control cells was not markedly increased in time (Fig. 3D).

Distinct DC subsets have different capacities to phagocytose soluble and cell-associated tumor antigens.[22] We therefore tested the capacity of CD1c$^+$ DCs, CD16$^+$ DCs, and pDCs to take up OXP- or CDDP-treated tumor cells. Treatment of BLM cells with CDDP led to a significant increase in the uptake of tumor fragments by all DC subsets. Plasmacytoid DCs were the least efficient DC subset in engulfing tumor-derived particles, whereas CD1c$^+$ and CD16$^+$ DCs were more proficient (Fig. 3E). These results were consistent across different cell lines (833KE, 2102EP, and Caco2) used in the co-culture with CD1c$^+$ and CD16$^+$ DCs (Fig. 3F). OXP was less potent than CDDP in inducing uptake of BLM cells (Fig. 3E), in contrast to the other cell lines tested, for which OXP was slightly more potent than CDDP (Fig. 3F). These differences might be due to differences in drug uptake in the different cell lines.

We wondered whether uptake of tumor fragments was solely dependent on CRT expression and thus performed the uptake experiment in the presence of a CRT-blocking peptide.[25,26] An irrelevant peptide (derived from gp100) or a mouse isotype antibody were used as negative controls (Fig. 4A; Fig. S3D). As expected, the irrelevant peptide, as well as, the non-specific IgG control, did not affect phagocytic ability of CD1c$^+$ DCs. However, engagement of ecto-CRT with a CRT-blocking peptide completely abolished uptake of tumor cells, including those treated with platinum drugs. This observation confirmed the crucial role played by CRT in directing tumor cell fragment uptake. The most commonly described receptor for ecto-CRT is the low-density lipoprotein receptor-related protein 1 (LRP1), also known as CD91.[27] The expression of CD91 on distinct human blood circulating DC subsets has not been characterized before, thus we analyzed CD91 expression on CD1c$^+$DCs, CD16$^+$ DCs, and pDCs by flow cytometry. Monocytes (CD14$^+$) were previously shown to express CD91 and were used as a positive control (Fig. 4B).[28] Both CD1c$^+$ and CD16$^+$ DCs, as well as monocytes, clearly expressed the ecto-CRT receptor. On the other hand, and consistent with the low uptake of chemotherapeutically-induced tumor cell fragments, pDCs showed little-to-no expression of CD91. We observed a similar trend looking at the transcriptomes of these DC subsets (Fig. 4C). mRNA levels of CD91 were highest in CD16$^+$ and CD1c$^+$DCs, whereas pDCs showed the lowest expression. Subsequent blocking of CD91 on CD1c$^+$DCs, with a specific anti-CD91 blocking antibody, however, did not affect phagocytosis of tumor cells, despite its high expression levels (Fig. 4D). To complement this analysis, we also investigated the expression of alternative receptors for ecto-CRT. Both the scavenger receptor class-A (SR-A, also known as CD204) and the scavenger receptor expressed by endothelial cell-1 (SREC-I) were reported to bind ecto-CRT.[29,30] Transcriptomic analysis of SR-A and SREC-I revealed that these receptors are variably expressed on blood DC subsets, with CD1c$^+$ DCs again showing the highest expression (Fig. 4C). Taken together, our data demonstrate that blood DCs take up platinum-treated tumor cells more efficiently than untreated cells and this uptake is strictly dependent on CRT exposure. Additionally, while we observed uptake of tumor fragments by all DC subsets, they differentially express receptors capable of binding ecto-CRT.

Phagocytosis of platinum-treated tumor cells induces maturation of human DC subsets

Next, we investigated whether the CRT-mediated uptake of tumor cells fragments may lead to DC maturation. The transcriptomes of DCs for receptors that sense danger signals released by dying tumor cells were assessed. CD1c$^+$ DCs showed the highest mRNA expression levels of the P2RY2 (recognizing ATP), LOX-1 (HSPs), and RAGE (HMGB1) receptors; as well as, of the common receptors for HSPs and HMGB1 (TLR2) (Fig. 4C). Exceptions were P2RX7 (ATP) and

Figure 1. (see previous page) Sensitivity of tumor cell lines to platinum drugs and platinum-mediated exposure of ecto-CRT. (A, B) Frequency of apoptotic (Annexin V$^+$ DAPI$^-$) and secondary necrotic (Annexin V$^+$ DAPI$^+$) BLM cells after treatment with OXP or CDDP. Human melanoma BLM cells were cultured with platinum drugs or left untreated, for 24 or 48 h. Cells were stained with Annexin-V-FITC and DAPI and analyzed by flow cytometry. Data are presented as representative contour plot (A) or mean±SEM (at least $n = 2$, performed in duplicates) (B). (C, E, F) CRT exposure was assessed on Annexin V$^+$ DAPI$^-$ cells after treatment with OXP or CDDP by flow cytometry. BLM cells were treated as described above. Data are relative mean±SEM (at least $n = 3$, in duplicates) (C). Representative histograms show CRT expression (MFI) on human colon (Caco-2) and testicular (833KE and 2102EP) cancer cell lines following 24 h of treatment with OXP or CDDP. Caco-2 were treated with 15 μM of OXP or CDDP; 833KE and 2102EP were treated with 6.3 μM OXP or 8.3 μM CDDP. Isotype (gray line), control (gray filled histogram), treatment (black thick line) (E). Exposure of CRT on murine colon cancer CT26 cells was assessed after 24 h of treatment with 15 μM of OXP or CDDP. Data are means of duplicates of one representative experiment (F). (D) CRT expression was confirmed by confocal microscopy. BLM cells were stained with an anti-CRT antibody and the membrane marker, wheat germ agglutinin (WGA). Scale bar 10 μm. **(G, H)** Frequency of apoptotic vs. necrotic cells (F) and CRT exposure (G) on BLM cells, after short-term (8 h) drug exposure to OXP or CDDP, at indicated doses. Results are mean±SEM ($n = 3$ in duplicates). Significance was determined with One-way ANOVA, $^*p < 0.05$, $^{***}p < 0.001$, as compared to control (CTRL) cells.

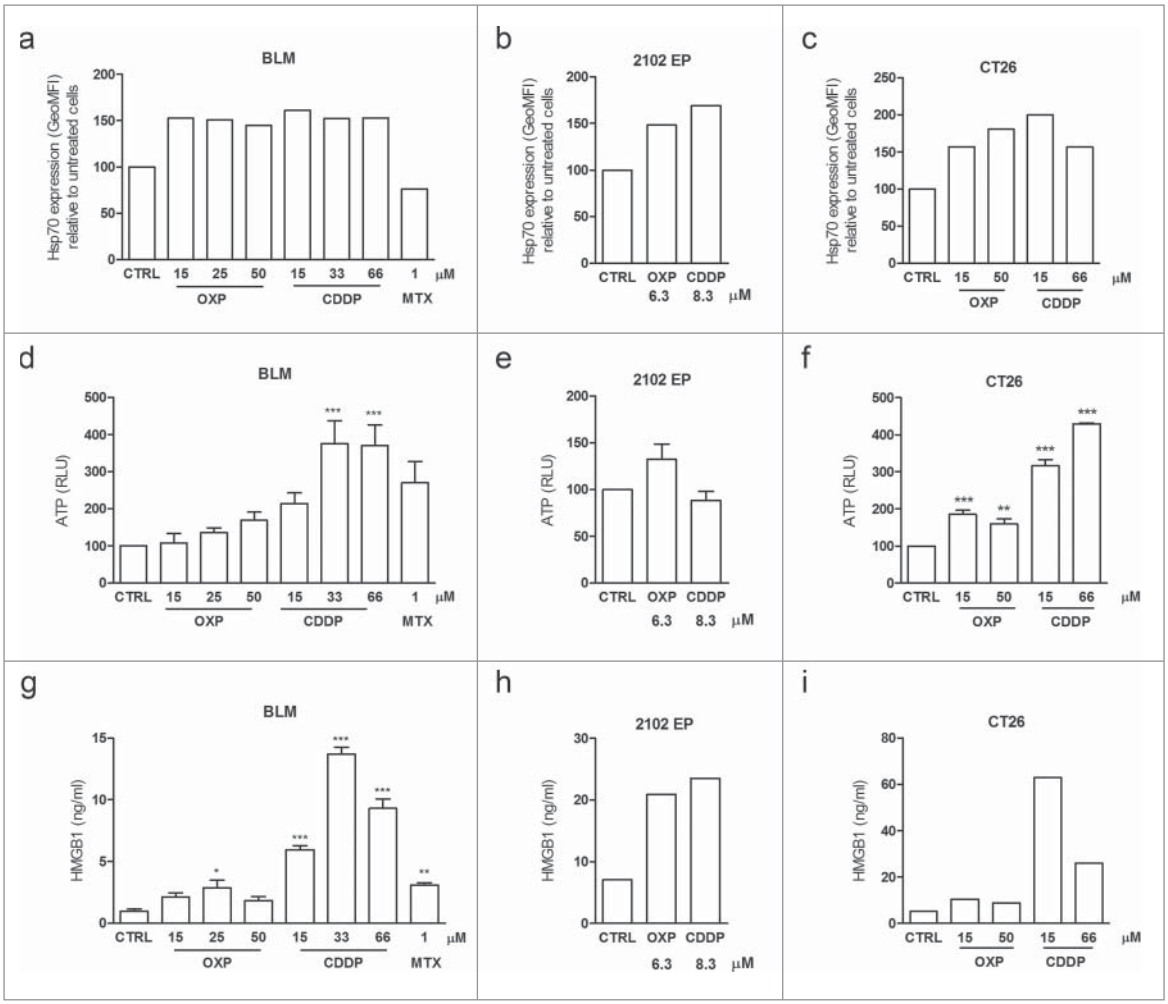

Figure 2. Platinum drugs induce release of immunogenic signals for DCs stimulation. (A, B, C) Surface expression of Hsp70 on BLM (A), 2102EP (B), and CT26 (C) cells, after platinum treatment, was assessed on Annexin V^+ $DAPI^-$ cells by flow cytometry. Cells were cultured with OXP or CDDP at indicated doses, for 24 (2102EP and CT26) or 48 h (BLM). Data are presented as mean ($n = 2$) (D, E, F). Extracellular ATP was measured by luciferase assay, in supernatants of cells cultured as described above. Results represent relative means±SEM (at least $n = 2$, done in duplicates). (G, H, I) Elisa detection of HMGB1 release in supernatant of cells cultured as described above. Data are means±SEM (at least $n = 2$). Significance was determined with One-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared to control (CTRL) cells.

TLR4 (HSPs and HMGB1), for which $CD16^+$ DCs had higher mRNA expression levels (Fig. 4C). On the other hand, pDCs showed the lowest expression of all these receptors on mRNA level (Fig. 4C).

Following co-culture with platinum-treated tumor cells, we measured surface expression of several markers on DCs, involved in antigen presentation and co-stimulation (Figs. 5 and 6). As a positive control, DCs were stimulated with TLR ligands (poly I:C (pI:C) or R848). Co-culture of DCs with platinum-treated BLM cells induced a significant upregulation of co-stimulatory molecules, CD80 and CD86. Although pDCs were less efficient in taking up tumor fragments (Fig. 3E), upregulation of co-stimulatory molecules (CD80 and CD86) was more prominent in pDCs than $CD1c^+$ or $CD16^+$ DCs (Fig. 5A), despite the lower expression of receptors recognizing the dying cell fragments on these cells. In addition, we analyzed the expression of major histocompatibility complex (MHC) classes I and II, required for antigen presentation to T cells. Platinum-treatment only moderately influenced MHC I and II expression on DC subsets (Fig. 5B). We observed similar effects of platinum treatment for 2102EP (Figs. 6A and B) and 833KE (Figs. 6C and D) cells co-cultured with $CD1c^+$ or $CD16^+$ DCs (Fig. S4).

In addition to phenotypical maturation, we tested cytokine secretion in the supernatant of tumor-DC co-cultured overnight upon induction of ICD (Figs. 5C and D). $CD1c^+$ DCs markedly increased the production of the proinflammatory cytokine, TNF-α, as well as the anti-inflammatory cytokine, IL-10, in response to interaction with both OXP- and CDDP-treated BLM cells. On the other hand, $CD16^+$ DCs and pDCs showed no significant response to platinum-treated tumor cells. Furthermore, we could not detect any IL-2, IL-4, IL-5, IL-12, TNF-β, and IFNγ (data not shown).

Platinum-treated tumor cells stimulate human $CD1c^+$ DCs to induce a T cell response

Co-culture of DCs with different platinum-treated tumor cell lines (BLM, 2102 EP, and 833KE) led to uptake of tumor cells and subsequent maturation of DC. Next, we investigated the ability of these mature DCs to stimulate allogeneic T cell proliferation. Proliferation of $CD3^+$ T cells was quantified by measuring CFSE dilution in a mixed lymphocyte reaction (MLR) (Fig. 7A, Fig. S5). As shown in Fig. 7b, $CD16^+$ DCs were not able to induce significant T cell proliferation in any of the

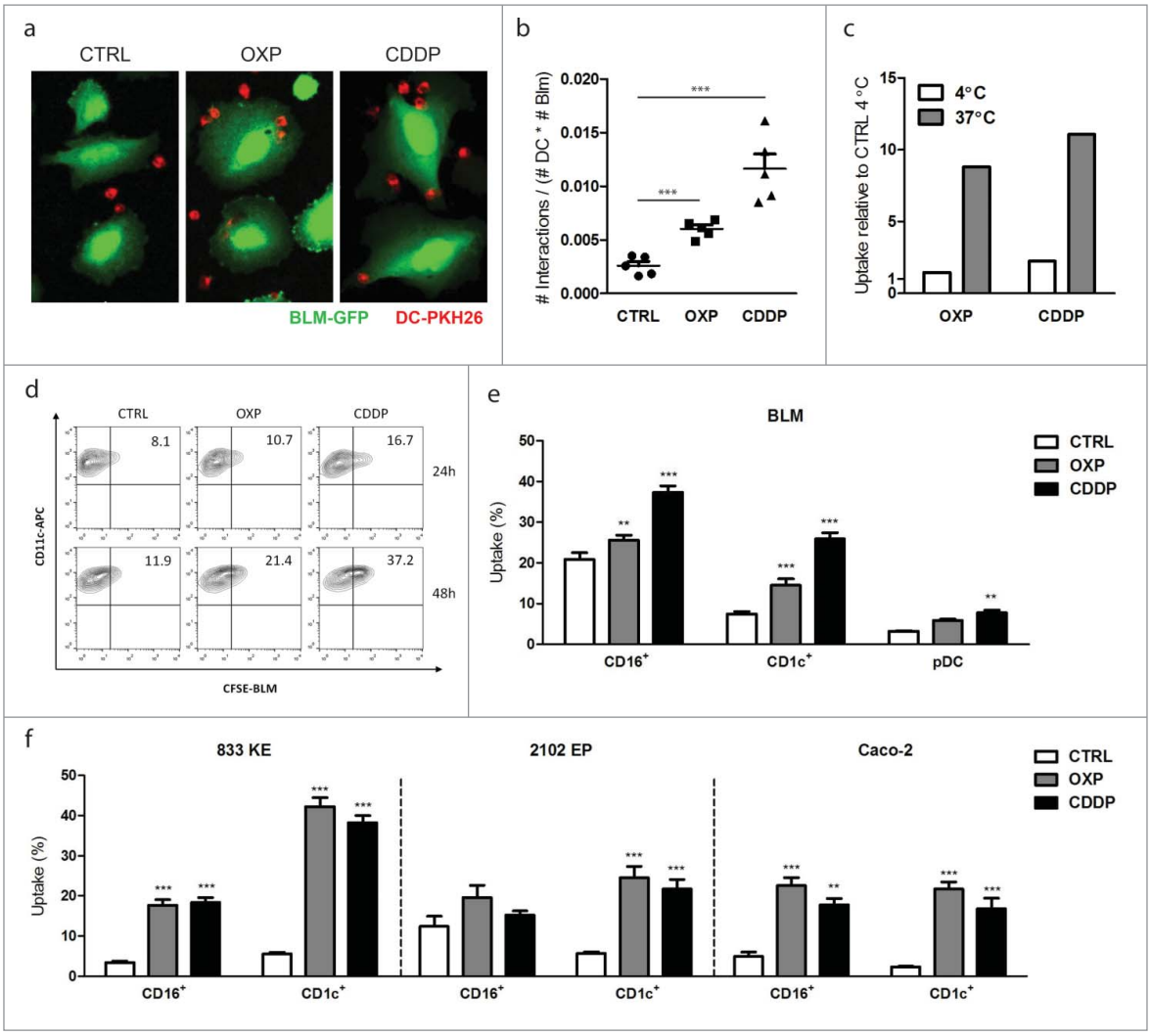

Figure 3. Platinum-treatment increases phagocytosis of tumor cells by human DC subsets. (A, B) BLM-GFP cells were treated with 15 μM OXP or CDDP for 48 h and co-cultured with CD1c$^+$ DCs (pre-labeled with PKH26) for 3 h. Representative confocal image (A) and quantification (B) of BLM-DC interactions. A total of five images per sample were taken and the number of interactions was normalized over the number of DC and BLM cells present in the image. Data represent mean±SEM of one representative experiment. (C) Percentage of uptake (48 h co-culture, 37°C, gray bar) of 15 μM OXP or CDDP treated-BLM cells by CD1c$^+$ DCs versus binding (4°C, white bar). Values are relative to binding of control (CTRL) BLM cells and show mean ($n = 2$). (D) Representative contour plot of control (CTRL) or platinum treated-BLM cells uptake by CD1c$^+$ DCs upon 24 or 48 h of co-culture. (E, F) Percentage of phagocytosis of BLM (E), 833 KE, 2102 EP, Caco2 (F) human tumor cell lines by different DC subsets (CD16$^+$, CD1c$^+$, and pDCs). BLM and Caco-2 were treated with 15 μM of OXP or CDDP for 48 h; 833KE and 2102EP were treated with 6.3 μM OXP or 8.3 μM CDDP for 24 h. CTRL (white bar) or treated tumor cells (gray or black bars) were co-cultured with DCs for 48 h and extend of uptake was assessed by flow cytometry. The graph shows the mean±SEM (at least $n = 2$, in duplicate). Significance was determined by Two-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared to CTRL cells.

conditions tested. On the other hand CD1c$^+$ DCs co-cultured with OXP- or CDDP-treated tumor cells, showed a significant increase in T cell proliferation compared to untreated cells. Plasmacytoid DCs were able to induce allogeneic T cell proliferation but there was no difference between pDCs co-cultured with untreated cells or platinum-treated cells. Altogether these results seem to suggest that CD1c$^+$ DCs are most effectively activated by tumor cells undergoing ICD and might have the potency to drive subsequent immune responses.

Discussion

The human immune system plays a fundamental role in tumor recognition and control.[31] Recent advances, including the discovery of ICD and its contribution to clinical efficacy, suggest that durable clinical responses to chemotherapy require the presence of a functional immune system.[3,32,33] DCs are the key cells in this scenario, as they are required to kick-start effective adaptive immune responses.[34-36]

Here, we study the function of the three most abundant human blood circulating DCs (CD1c$^+$ DCs, CD16$^+$ DCs, and pDCs) in ICD induced by two of the most used platinum drugs, OXP and cisplatin (CDDP). We observed that, contrary to previous reports, both drugs were able to induce expression of the three hallmarks of ICD: CRT exposure, secretion of ATP, and release of HMGB1. Furthermore, we show that all three DC subsets preferentially take up fragments derived from platinum-treated tumor cells and subsequently undergo phenotypical maturation.

Although the first compounds discovered to initiate an immunogenic form of apoptosis were structurally similar and all belonged to the class of the anthracyclines, the list of ICD inducers has since been expanded.[5,37-40] Previously, Tesniere et al. found that the platinum drug CDDP fails to initiate ICD by itself, despite

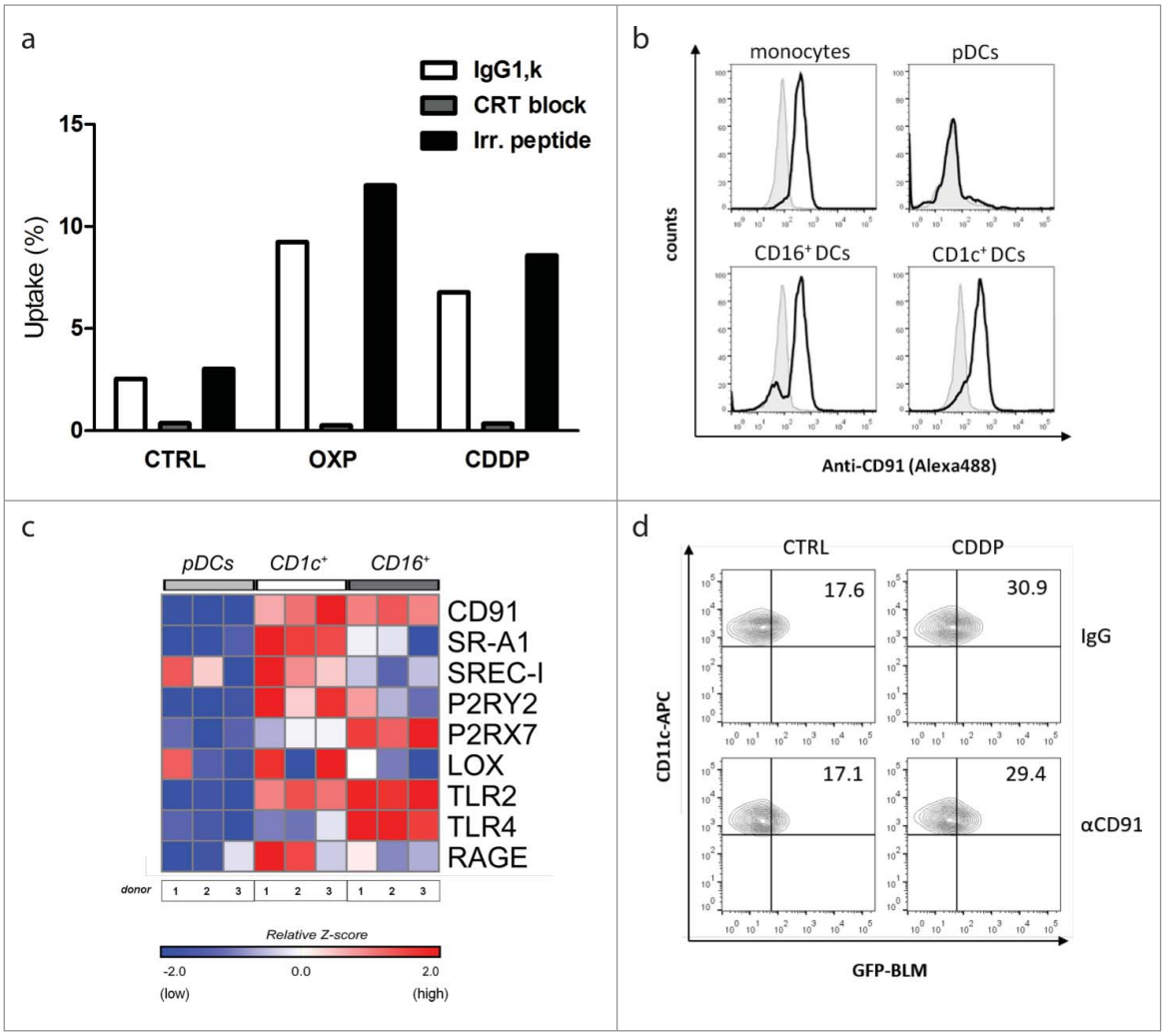

Figure 4. Human DCs take up platinum-treated tumor cells in a CRT-dependent manner. (**A**) Percentage of uptake of 15 μM OXP or CDDP treated-BLM cells by CD1c$^+$ DCs. Control or treated tumor cells were co-cultured with DCs in the presence of isotype (white bar), CRT blocking peptide (gray bar) or irrelevant tumor antigen (gp100) peptide (black bar) for 2 h. Extent of uptake was assessed by flow cytometry. Data show means of duplicates of one representative experiment. (B) Representative histograms showing CD91 expression on human monocytes (CD14$^+$) and DCs (CD16$^+$, CD1c$^+$, and pDCs). Isotype (gray filled histogram), anti-CD91 (black thick line). (C) Heatmap of relative mRNA expression levels of genes in human DC subsets. Heatmap shows the normalized expression of genes (Z-scores) in CD16$^+$, CD1c$^+$, and pDCs. Data are represented from three healthy donors. (D) Representative contour plot of CTRL or CDDP (15 μM, 48 h) treated-BLM cells uptake by CD1c$^+$ DCs upon functional blocking of CD91 on DCs. BLM cells and DCs were co-cultured overnight in the presence of isotype control or CD91-blocking antibody. Percentage of phagocytosis was assessed by flow cytometry.

its similarity in structure and function to the ICD-inducer OXP.[4,5] Nonetheless, CDDP was converted into an ICD inducer by exogenous co-administration of Cxcl2 (ortholog of the human pro-inflammatory cytokine/chemokine IL-8).[41] Here, we show that CDDP, as well as OXP, could upregulate exposure of ecto-CRT and induce release of ATP and HMGB1 in human tumor cells of distinct origins. Our observation that the human melanoma cell line BLM secretes high amounts of IL-8 (data not shown) could be a possible explanation for the discrepancy between our study and the results of Tesniere et al. This suggests that different cell lines may have distinct intrinsic potentials to emit immunogenic signals. In apparent accordance with this notion, UV irradiation has been described to induce ICD in murine models, yet it failed to do so in human cancer cell lines tested in another study.[23,40] In addition to OXP and CDDP, widely used in clinical practice, other platinum drugs, such as carboplatin, might be relevant in the context of ICD. These platinum analogs differ in their toxicity profiles and efficacy in distinct malignancies, therefore understanding their potential as ICD inducers might be useful for improving therapeutic outcomes.[42]

In addition to the three ICD hallmarks described above, surface translocation of the heat shock protein Hsp70 could expand the general definition of ICD.[23,39] We here report that both OXP and CDDP were able to upregulate expression of Hsp70 in various human tumor cell lines.

Treatment of human tumor cells with OXP or CDDP increased uptake of tumor fragments by all three human DC subsets. As previously reported,[22] CD1c$^+$ and CD16$^+$ DCs efficiently phagocytosed cell fragments, whereas pDCs showed lower uptake capacity. This uptake was dependent on ecto-CRT, however blocking of the CRT receptor did not abrogate tumor cell fragment uptake, suggesting that other receptors might be involved. The multi-ligand scavenger receptors SR-A (CD204) and scavenger receptor expressed by endothelial cell-1 (SREC-I), have been proposed to be involved in ecto-CRT binding.[29,30] Our transcriptome analysis of blood DC subsets

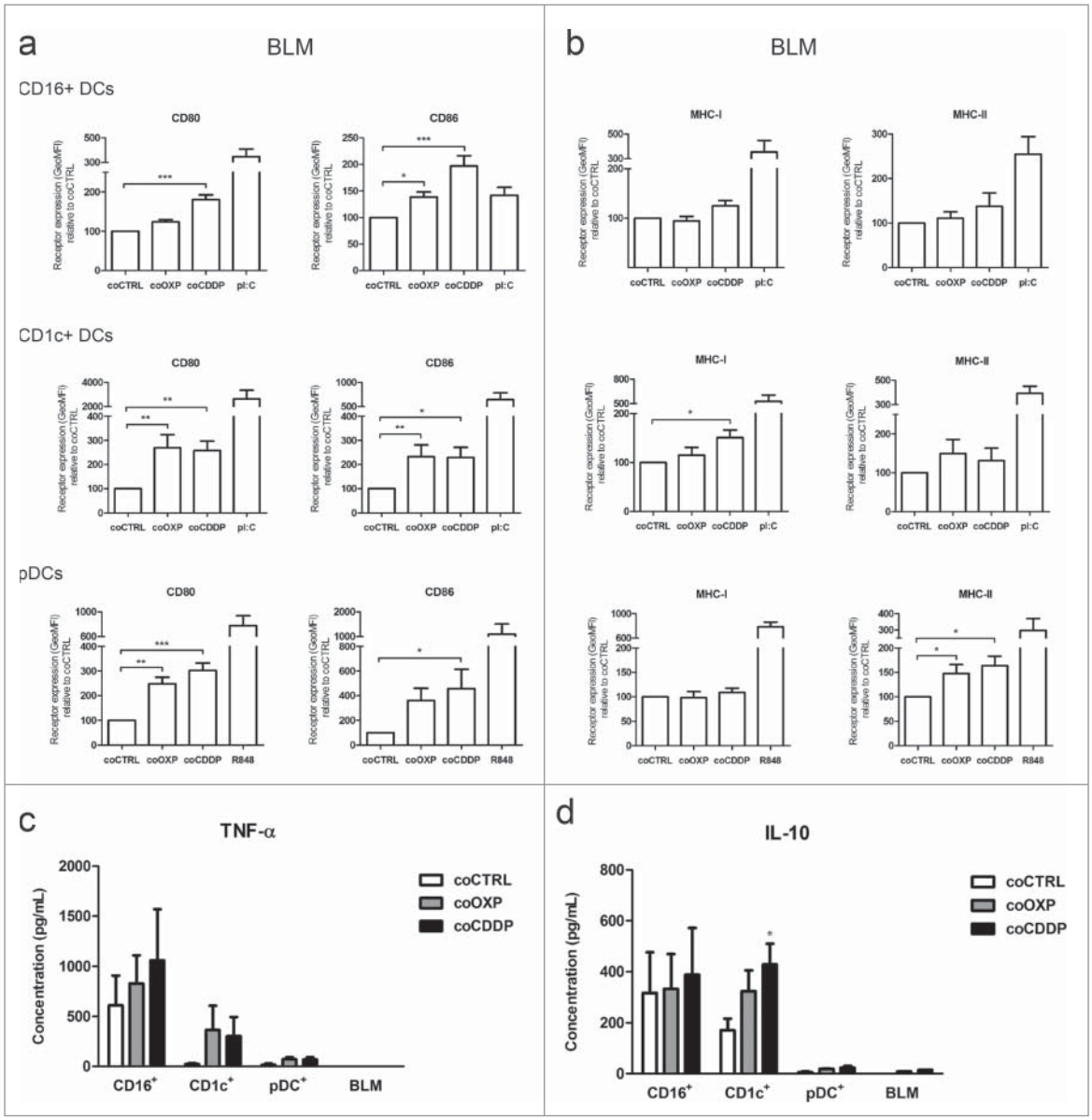

Figure 5. Phagocytosis of platinum-treated BLM cells induces maturation of human DC subsets. (A, B) The expression of maturation markers on DC subsets ($CD16^+$, $CD1c^+$, and pDCs) following 48 h of co-culture with control or platinum treated BLM cells. DC stimulated with TLR ligands (4 μg/mL R848 for pDCs and 2 μg/mL poli(I:C) for $CD16^+$ and $CD1c^+$) were used as positive controls. The expression levels were determined by flow cytometry and depicted as GeoMFI values, relative to those of the co-culture with control tumor cells (coCTRL). The graphs show the mean±SEM ($n = 7$). Significance was determined by One-way ANOVA, $^*p < .05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C, D) TNF-α and IL-10 production was analyzed in supernatants of overnight co-cultured pretreated-BLM and DCs by a multiplex FlowCytomix kit. Data are mean±SEM ($n = 5$). Significance was determined by 2-tailed *t*-test, $^*p < .05$.

confirms that SR-A and SREC-I are expressed on distinct human DCs.[43] The presence of these alternative receptors might explain why blocking of CD91 did not diminish the uptake. Furthermore, we cannot exclude the possibility that there may be more receptors involved in ecto-CRT binding and that these have yet to be described. More research is required to reveal which receptor is responsible for CRT-mediated uptake on human DC-subsets.

The human DC population is characterized by high degree of heterogeneity that reflects their phenotypical and functional properties, as well as their location in the body.[44] Consequently, DCs can interact with tumor cells in multiple ways. Immature DCs populate peripheral tissues and organs, where they are committed to (tumor) antigen capture.[44] In addition, myeloid and plasmacytoid DC subsets are found circulating in the blood, where they may encounter CTCs that have detached from the primary tumor and entered into the bloodstream.[18] Furthermore, DCs can home to and infiltrate tumors. As an example, inflammatory dendritic cells (inflDCS), were described as a distinct subset of DCs originating from *in situ* differentiation of monocytes recruited to the site of inflammation.[45] In mice, monocytes were recruited into the tumor bed within 12 h following mitoxantrone treatment, and differentiated into inflDCs. Addition of a neutralizing antibody against CD11b abrogated activation of tumor-specific $CD8^+$ T cells, indicating that DCs play a central role in ICD-mediated initiation of antitumor responses.[11] In this perspective, distinct DC subsets might be relevant as cellular mediators of ICD. Among blood DC populations, $CD141^+$ DCs are also equipped with the potent ability to cross-prime cytotoxic T lymphocytes.[22]

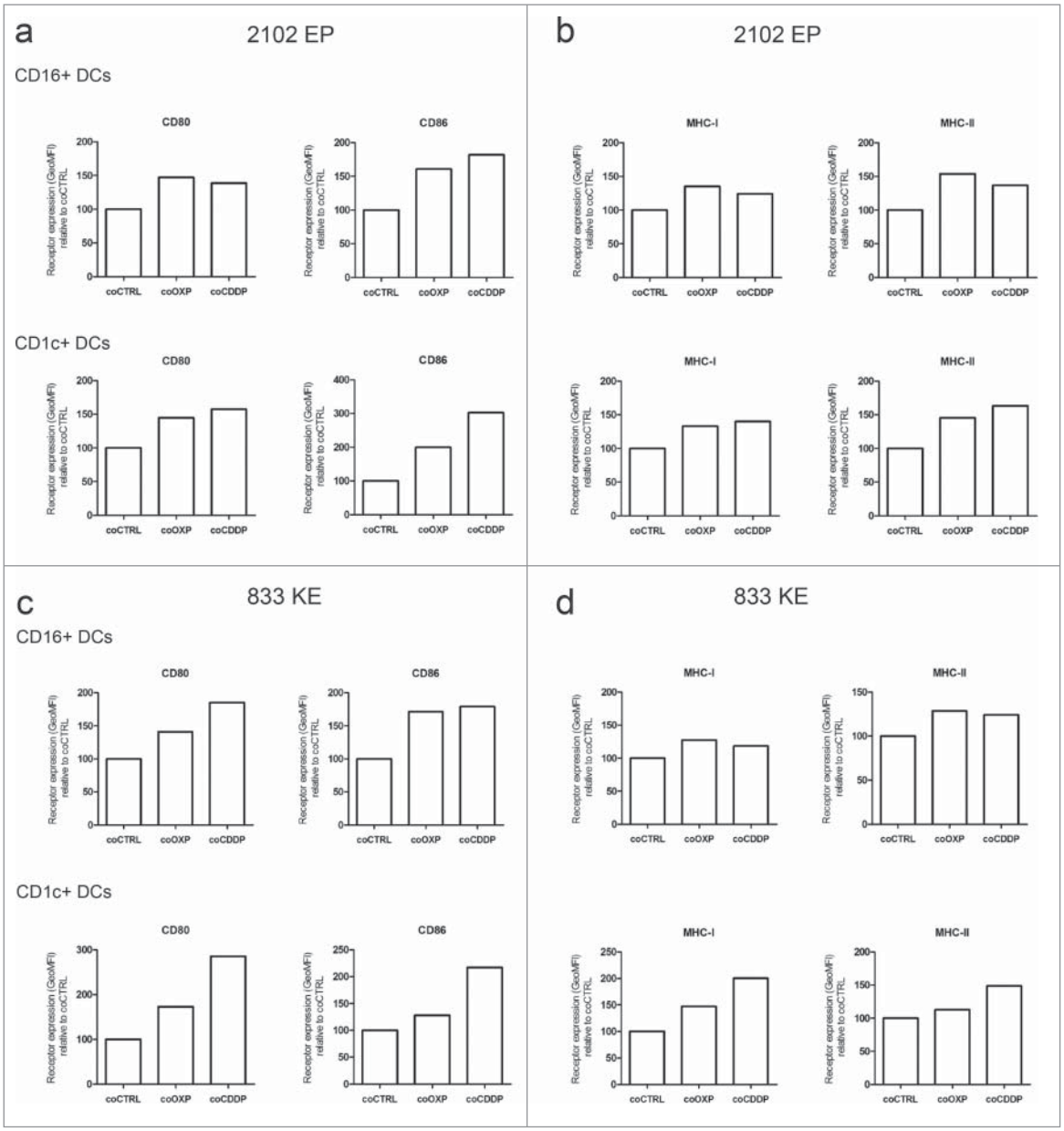

Figure 6. Phagocytosis of platinum-treated testicular cancer cells induces maturation of human DC subsets. The expression of maturation markers on DC subsets ($CD16^+$, $CD1c^+$, and pDCs) following 48 h of co-culture with control or platinum treated 2102EP (A, B) or 833KE (C, D) cells. The expression levels were determined by flow cytometry and depicted as GeoMFI values, relative to those of the co-culture with control tumor cells (coCTRL). The graphs show the mean ($n = 2$).

However, their scarce frequency represents a major hurdle in investigating their role in many aspects of DC biology.

Stimulation of an effective T cell response is determined by several critical steps. These include (tumor) antigen uptake, processing, and (cross)-presentation, as well as phenotypical and functional maturation of DCs.[46,47] Murine DCs were reported to upregulate maturation-associated markers upon phagocytosis of bortezomib-treated colon cancer cells.[48] Similar effects were observed for human moDCs that were co-cultured with Idarubicin- or Bortezomib-treated tumor cell lines.[23,39] Accordingly, we showed that interaction of OXP- or CDDP-treated tumor cells with blood DC subsets induced phenotypical maturation of DCs, observed as upregulation of co-stimulatory molecules (CD80 and CD86). In line with previous observations,[22] although pDCs were less able to take up antigens from their environment than other subsets, they efficiently matured and induced high levels of co-stimulation. Despite strong phagocytic ability and induction of maturation, $CD16^+$ DCs appeared to be the least efficient inducers of T cell responses, probably due to their minimal cross-presenting capacity.[22] This seems to be in accordance with the hypothesis that $CD16^+$ DCs may share some biological functions with DCs, yet they are more similar to monocytes, based on the comparison of their gene expression profiles.[20] The most effective response was observed for $CD1c^+$ DCs. Notably, $CD1c^+$ DCs were the only DC subset that, in response to interaction with platinum-treated tumor cells, secreted TNF-α and IL-10, cytokines typically induced upon exposure to different maturation stimuli.[49,50] This myeloid DC population was capable of engulfing platinum-treated tumor cells, responding to

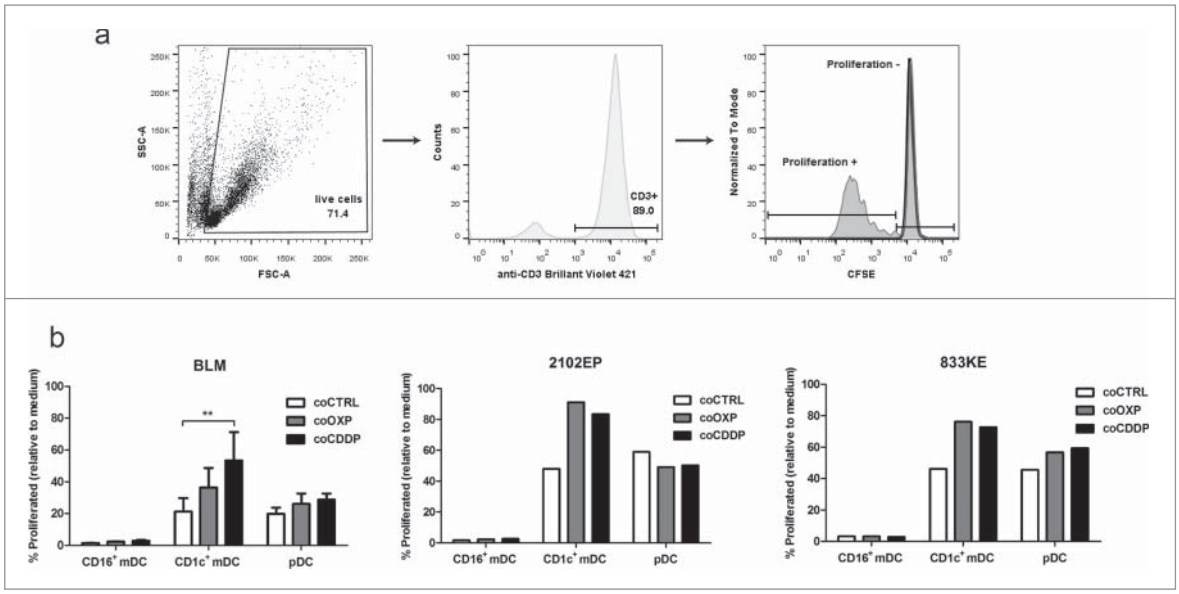

Figure 7. Human CD1c$^+$ DCs mediate T cell activation against chemotherapy treated-tumor cells. The ability of CD16$^+$, CD1c$^+$, and pDCs to induce T cell proliferation upon co-culture with control (white), OXP (gray), or CDDP (black) treated-tumor cells was determined in a mixed lymphocyte reaction. FACS sorted DCs were co-cultured with allogeneic CFSE-labeled peripheral blood lymphocytes (PBLs) for 5 d. (A) PBL proliferation was measured as the percentage of CD3$^+$ cells showing CFSE dilution and expressed relative to CD3$^+$ cells cultured in medium. (B) Percentage of proliferated CD3$^+$ cells. Data are mean±SEM of at least triplicates of $n = 3$ (BLM) or one representative (833KE and 2102EP) experiments. Significance was determined by Two-way ANOVA, $^{**}p < 0.01$.

activatory signals and inducing T cell proliferation. In support of the functional observations, our transcriptomic analysis revealed that CD1c$^+$ DCs had higher expression of DC receptors sensing danger signals released by dying tumor cells, on mRNA levels as compared to the other two DC subsets. Appropriately, deficiency or loss-of-function mutations of genes encoding these receptors, was shown to compromise the efficacy of anticancer chemotherapy, stressing their crucial role in ICD.[5,13,17]

In summary, we have investigated for the first time the role of the three most abundant human blood DC populations (CD1c$^+$ DCs, CD16$^+$ DCs, and pDCs) in the context of ICD and show that only CD1c$^+$ DCs were capable of inducing allogeneic T cell response *in vitro*. Moreover, we expanded the list of ICD inducers, showing that—similarly to OXP—CDDP induces a form of tumor cell death consistent with ICD. Together, these observations point toward an active stimulatory effect of platinum-treated tumor cells on DCs that naturally occur in the human body and may contribute to the translation of current knowledge on ICD into clinical settings.

Materials and methods

Cell culture, transduction, and stable cell line

Cells were cultured at 37°C, 5% CO_2. Human testicular carcinoma (2102EP and 833KE) and murine colon carcinoma (CT26) cell lines were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific, catalog# 42401–018), supplemented with 10% fetal calf serum (FCS, Greiner bio-one), and Ultra-glutamine (Lonza, catalog# BE17-605E/U1). Human melanoma (BLM and BLM-GFP) and colorectal adenocarcinoma (Caco-2) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, 31966–021), with 5% or 20% FCS, respectively. The Lenti6/Block-iT-shScramble (GFP) vector was a kind gift of Prof. Peter Friedl (RIMLS, The Netherlands). The specific scramble shRNA sequence has no homology to any known mammalian gene. BLM cells were infected with lentiviral vector and (10 μg/mL) polybrene and incubated at 37°C, 5% CO_2, overnight. Then medium was refreshed and cells were analyzed after 72 h of treatment. Stable cell line was selected with 5 g/mL blasticidin.

Cell death induction and quantification

Tumor cells were treated with OXP (lot# R01730), cisplatin (cis-diamminedichloroplatinum(II), CDDP, lot# PR00202) (both Accord) or mitoxantrone (MTX, Sandoz, lot# D50333A) to induce cell death. Cell death was assessed by double staining with annexin V fluorescein isothiocyanate-FITC (AnnV-FITC, BD Bioscience, catalog# C34554) and DAPI (Sigma-Aldrich, catalog# 32670). Briefly, cells were collected, washed with PBS and incubated with AnnV-FITC (1:40), $CaCl_2$ (1M, 1:666) in PBS on ice, in the dark for 15 min. An equal volume of DAPI in PBS (final concentration 1:2000) was added immediately prior measurement by flow cytometry.

Flow cytometric analysis of ecto-CRT and ecto-HSP70

6×10^4 tumor cells were let adhere in 24-well plates and treated with OXP or CDDP for 24 or 48 h. Subsequently, cells were harvested with cold TEN buffer (40 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 7.8), transferred to a 96-well plate and washed twice with PBA. After incubation with a Fc-receptor blocking buffer (2% human or murine serum in PBS, 15 min at 4°C) cells were stained for 30 min at 4°C with a primary antibody in PBA, followed by extensive washing and incubation with an Alexa488-conjugated monoclonal secondary antibody

(Goat-anti-Mouse IgG1, Life Technologies, catalo#A-21121) in PBA for 20 min at 4°C. Isotype-matched IgG antibodies were used as control. Surface expression of CRT (anti-CRT, clone ab2907, Abcam, catalog# ab2907; anti-CRT-PE, clone FMC 75, Enzo Life Sciences, catalog# ADI-SPA-601PE-F) and Hsp70 (anti-HSP70, clone C92F3A-5, Enzo Life Sciences, catalog# ADI-SPA-810-D) was analyzed by flow cytometry (Table S1).

Detection of ATP and HMGB1 release

Tumor cells (6×10^4) were adhered in 24-well plates in 1ml of heat-inactivated complete medium and treated with OXP or CDDP for 24 or 48 h. Supernatants were collected, dying floating cells removed by centrifugation and supernatants frozen immediately. ATP secretion was measured with the ENLITEN ATP Assay (Promega, catalog# FF2000) according to the manufacturer's protocol. HMGB1 release was assessed by enzyme-linked immunosorbent assay (ELISA, IBL International, catalog# ADI-SPA-810-D) according to manufacturer's instructions.

Isolation of human blood immune cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteers (Sanquin), after written informed consent and according to institutional guidelines, and purified via centrifugation over a ficoll density gradient (Axis-Shield) in SepMate tubes (Stemcell technologies). Isolation of human blood DC subsets was achieved by a sequence of negative and positive selection steps using magnetic beads (Human $CD16^+$ Monocyte Isolation Kit, catalog# 130-091-765; Human $CD1c^+$ (BDCA-1) Dendritic Cell Isolation Kit, catalog# 130-090-506; Human $CD304^+$ (BDCA-4/Neuropilin-1) MicroBead Kit, catalog# 130-090-512; all Miltenyi Biotec). DC purity was assessed by staining with primary labeled antibodies (see also Table S1): anti-CD1c-PE (Miltenyi biotec, clone AD5-8E7, catalog# 130-090-508), anti-CD11c-APC (Miltenyi biotec, clone MJ4-27G12, catalog# 130-092-412), anti-CD20-APC (eBioscience, clone 2H7, catalog# 17-0209), anti-CD15-FITC (Miltenyi biotec, clone VIMC6, catalog# 130-081-101), anti-CD56-PE (IQ Products, clone MOC-1, catalog# IQP-114R), anti-CD123-APC (Miltenyi biotec, clone AC145, catalog# 130-090-901) and anti-CD303-PE (Miltenyi biotec, clone AC144, catalog# 130-090-511). Purity levels higher than 98% were achieved, determined by flow cytometry. Peripheral blood leukocytes (PBLs) were isolated from PBMCs by depletion of monocytes via adherence to plastic culture flasks (1 h at 37°C). Floating cells (PBLs) were collected and re-suspended in X-VIVO-15 medium (Lonza, catalog# BE04-418Q) supplemented with 2% human serum (HS, Sanquin).

Microarray data

For the analysis publically available affymetrix, CEL files containing expression data of resting human pDCs, $CD1c^+$, and $CD16^+$ were downloaded from ArrayExpress (accession: E-TABM-34). Data have been published by others.[51] Raw files were processed in the R programming environment and intensity values across the different datasets were normalized using the RMA normalization function of the affy package.[52] Specific annotation packages for human were used to map array probe identifiers to corresponding species-specific gene symbols. In case of redundant probes, the probes with highest summed intensity of all samples were considered. The normalized log2 transformed data sets was z-scored (setting the data to a mean = 0 and a variance = 1) and heat maps were generated using freely availably GeneE program (http://www.broadinstitute.org/).

Immunofluorescence

For evaluation of CRT expression, BLM cells were cultured on glass coverslips and treated overnight with 50 μM OXP, 66 μM CDDP, 1 μM MTX or left untreated. Cells were washed, fixed in 0.25% paraformaldehyde (PFA) in PBS for 5 min at RT. Cells were blocked and subsequently incubated with wheat germ agglutinin-biotinylated (WGA) for 45 min at 4°C. After extensive wash, cells were stained with primary anti-CRT antibody (Abcam, clone ab2907, Table S1) in cold confocal laser scanning microscope (CLSM) buffer (3% bovine serum albumin, 50 mM Glycine in PBS) for 30 min at RT, followed by incubation with secondary Alexa conjugates in cold CLSM blocking buffer, for 20 min at RT. Cells were then fixed in 4% PFA/PBS for 20 min at RT. Nuclei were stained with DAPI (1:3000) in CLSM for 5 min at RT. Samples were imaged with an Olympus FV1000 Confocal Laser Scanning Microscope. Images were analyzed using the open source-imaging platform, Fiji (imageJ 64 Bit for Windows).

Co-cultures and uptake assays

Tumor cells were stained with 2 μM of the fluorescent probe 5 (6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Invitrogen, Thermo Fisher Scientific, catalog# C34554), according to manufacturer's instructions. Fluorescently–labeled (CFSE or GFP) tumor cells were seeded in T75 flasks (Corning), adhered and treated with OXP or CDDP. After treatment, tumor cells (5 × 105 cells, target) and DCs (5 × 105 cells, effector) were co-cultured at a 1:1 ratio in Falcon tubes (BD Falcon) in X-VIVO-15 supplemented with 2% HS (final concentration 1 × 106/mL). Co-cultures were stained with an APC-labeled primary antibody recognizing a specific DC surface marker (CD11c for $CD1c^+$ and $CD16^+$ DCs, CD123 for pDCs; see also Table S1) and analyzed by flow cytometry. Extent of phagocytosis was determined as the percentage of double positive events (i.e., $CD11c^+$ or $CD123^+$-APC/GFP^+ or $CFSE^+$). For blocking experiments, tumor cells were pre-incubated (30 min at 4°C) with blocking agents or negative controls: CRT blocking peptide (20 μg/mL, MBL International, catalog# JM-3077BP-50), irrelevant gp100 peptide272-300 (20 μg/mL, JPT), anti-CD91 (20 μg/mL, Thermo Fisher Scientific, clone 8G1, catalog# MA1-27198), or IgG1, k (20 μg/mL, Biolegend, clone MG1-45). Extra volume blocking agents or matched controls were added to co-cultures at same final concentration.

Flow cytometric analysis of DC maturation

Phenotypical assessment of DC maturation after 48 h of co-culture with tumor cells was performed by flow cytometry. Briefly,

cells were washed in PBA, incubated with Fc-receptor blocking buffer (2% HS in PBS, 15 min at 4°C) and subsequently stained with primary antibodies in PBA (30 min at 4°C). Monoclonal directly labeled anti-human antibodies used were (Table S1): anti-CD11c-APC (Miltenyi biotec, clone MJ4-27G12, catalog# 130-092-412), anti-CD123-APC (Miltenyi biotec, clone AC145, catalog# 130-090-901), anti-CD80-PECy7 (BD PharMingen, clone L307.4, catalog# 561135), anti-CD86-PECy7 (BD PharMingen, clone 2331, catalog# 561128), anti-HLA-ABC-PE (BD PharMingen, clone G46-2.6, catalog# 555553), and anti-HLA-DR-PE (BD PharMingen, clone G46-6, catalog# 555812). Appropriate isotype controls were included. Geometric mean fluorescence intensity (GeoMFI) of maturation markers was assessed on $CD11c^+$ (for $CD1c^+$ and $CD16^+$ DCs) or $CD123^+$ (for pDCs) populations. As a positive control, DCs were stimulated with poly I:C ($CD1c^+$ and $CD16^+$ DCs, 2 μg/mL, Enzo Life Science, catalog# ALX-746-021-M005) or R848 (pDCs, 4 μg/mL, Enzo Life Science, catalog# ALX-420-038-M025). To improve *in vitro* pDC viability, IL-3 (10 ng/mL, Cellgenix, catalog# 1002-050) was added to culture medium.

Cytokine quantification

Supernatants from tumor cell-DC co-cultures (18 h) were collected, dying floating cells removed by centrifugation and supernatants frozen immediately for detection of secreted cytokines. Human Th1/Th2 cytokines (IFNγ, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), TNF-α, and TNF-β) were quantified with a multiplex FlowCytomix kit (eBioscience, BMS810FF) according to the manufacturer's instructions.

DC sorting and mixed lymphocyte reaction (MLR)

Tumor-DC co-cultures were established as described above. After wash in cold wash buffer (PBS/0.1% BSA/5 mM EDTA) and incubation in Fc-receptor blocking buffer (2% HS in wash buffer, 15 min at 4°C), cells were stained with sterile primary antibodies in wash buffer (20 min at 4°C). The following antibodies were used (Table S1): anti-HLA-DR-PECy7 for all subsets (BD Biosciences, clone L243, catalog# 335813), anti-CD1c-PE for $CD1c^+$ DCs (Miltenyi biotec, clone AD5-8E7, catalog# 130-090-508), anti-CD16-APC for $CD16^+$ DCs (Miltenyi biotec, clone VEP13, catalog# 130-098-101), and anti-CD304-PE for pDCs (Miltenyi biotec, clone AD5-17F6, catalog# 130-090-533). Sorting of DCs was performed using a Fluorescence Activated Cell Sorter Aria (FACSAria, BD Bioscience), based on FSC/SSC properties and positivity for DC markers (Fig. S5). Allogeneic PBLs were stained with 5 μM CFSE (Invitrogen), according to manufacturer's instructions and added to the sorted DCs at a ratio of 5:1 (Lymphocytes:DCs), for an additional period of 5 d, in 2% HS X-VIVO-15. After 5 d, co-cultures were stained with a primary anti-CD3-BV421 antibody (BD Horizon, clone SK7, catalog# 563798) and analyzed by flow cytometry. The percentage of proliferating T cells ($CD3^+$) was determined by assessing CFSE dilution in the fraction of $CD3^+$ cells.

Statistical analysis

Unless otherwise indicated, experiments were performed at least three times, yielding comparable results. Data were analyzed by means of Prism v. 5.03 (GraphPad Software). Statistical significance was assessed by One-way Anova, followed by a Dunnett's post-test or Two-way Anova, followed by a Bonferroni's post-test, as appropriate. p values <0.05 were considered as statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by a grant from the Dutch Cancer Society and Alpe deHuZes foundation to S.V.H. (KUN2013-5958) and Dutch Cancer Society grant (KUN2009-4402). CGF received an NWO Spinoza award and ERC Adv Grant PATHFINDER (269019). IJMdV received NWO Vici Grant 918.14.655.

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