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Original Research

Cisplatin increases immune activity of monocytes and cytotoxic T-cells in a murine model of epithelial ovarian cancer

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ABSTRACT

Epithelial ovarian cancer (EOC) is an immunologically active malignancy, but thus far immune therapy has had limited success in clinical trials. One barrier to implementation of efficacious immune therapies is a lack of knowledge of the effect of chemotherapy on the monocyte-derived component of the immune infiltrate within the tumor. We utilized the ID8 murine EOC model to investigate alterations within tumor ascites that occur following administration of platinum chemotherapy. Cisplatin treatment resulted in a significant increase in monocytes within the ascites of tumor bearing mice. We identified that $CD11b^+$ cells from the ascites of mice that have been treated with cisplatin elicits an increase in IFN-ɣ expression from $CD8^+$ T-cells compared to $CD11b^+$ cells from a mouse treated with vehicle control (604.0 pg/mL v. 4328.0 pg/mL; $p < .0001$). Splenocytes derived from tumor bearing mice released increase levels of IFN-ɣ after treatment with cisplatin when incubated with dendritic cells (DCs) and tumor antigen (62.0 v. 92.1 pg/mL; $p = .03$). Cisplatin induced an increase in T-cell and monocyte/macrophage activation markers (CD62L and CD301). Levels of IL-10, IL-6, and VEGF in the cell free ascites of mice treated with cisplatin decreased ($p > .05$). These results indicate that treatment with cisplatin leads to an increase of anti-tumor activity within the ascites related to alterations in the ascites monocytes. Further investigation of these findings in humans is necessary to identify how these cells behave in different patient subgroups and if there is a role for monocyte directed therapy in conjunction with T-cell directed therapy and/or chemotherapy.

Introduction

Epithelial ovarian cancer (EOC) remains the most deadly gynecologic malignancy in North America with a 5 year overall survival rate of 29% in women diagnosed with advanced stage disease [1]. Studies of human specimens of EOC have shown that this is an immunologically active cancer and that women with intra-tumoral cytotoxic T-cells have a more favorable prognosis [2–4]. Despite the prognostic relationship between the tumor and immune system, immunotherapies have thus far had limited efficacy in treating women with EOC [5,6]. Prior studies have demonstrated that many of the cell populations necessary to mount an anti-tumor immune response including antigen-presenting cells, neutrophils, and cytotoxic $CD8^+$T-cells can be found within the tumor and surrounding stroma, as well as within the peripheral blood of women with EOC, but that these cell populations are inhibited by the presence of tumor cells, immunosuppressive immune cells, and immunosuppressive cytokines within the ovarian cancer and ascites fluid [7–11]. This phenomenon suggests that the tumor alters the immune milieu within the tumor microenvironment (TME) resulting in protection from immune surveillance and attack. Studies have shown that this is accomplished at least in part by myeloid-derived suppressor cells (MDSC), including tumor associated macrophages and immunosuppressive neutrophils, as an intermediary between the tumor cells and effective T-cell mediated immune responses [7,12–14].

Importantly, while our understanding of the immune status of untreated EOC is broadening; little is known about the effects of chemotherapy on the immune cells within the TME. Patients enrolled in immune therapy clinical trials are often enrolled following or in conjunction with chemotherapy, but tumor characteristics are based on pre-treatment tumor specimens. Platinum based chemotherapy is the standard care for adjuvant treatment of EOC [15]. Interestingly, recent clinical studies of the immune cells within the TME of EOC patients have

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shown paradoxical changes to the T-cell population in the TME comparing pre and post chemotherapy specimens. Specifically, there is an increase in cytotoxic T-cells within the TME and shifts in immune related cytokines favoring an anti-tumor immune activation that coincides with an increase in check point marker expression [16–19]. Changes that occur with chemotherapy administration in the monocyte population within the TME is largely unknown. Given the role monocytes play in untreated tumors, we sought to further elucidate the changes that occur in the monocytes within the ascites when treated with chemotherapy and how those changes may lead to downstream effects on T-cells and the TME.

To address the gap in our understanding of the alterations seen in monocytes with chemotherapy we utilize the ID8 murine model of EOC to explore the alterations that occur in the monocytes, T-cells, and cytokines within the ascites following treatment with cisplatin chemotherapy. We have identified both phenotypic changes and alterations in the activity of cells in the TME as a result of chemotherapy that includes activation of monocytes to a pro-inflammatory state and tumor specific T-cell activation that occurs following cisplatin treatment. Our findings suggest that monocytes remain an integral component of the TME in response to chemotherapy and may be manipulated as a treatment strategy to improve patient outcomes.

Methods

Mice and cell lines

Five to six week old female C57BL/6 mice were purchased from Charles River (Wilmington, MA). CCR2 knock out (CCR2ko) mice were purchased from Jackson Laboratory (Bar Harbor, ME). OT-1 mice were generously provided by the lab of Dr. Mary Jo Turk (Geisel School of Medicine, Lebanon, NH). All animal experiments were approved by the Institutional Animal Care and Use Committee.

The murine ID8 ovarian tumor model is syngeneic with C57BL/6 mice [20]. Generation of ID8 murine ovarian cancer cells transduced with pFB-neoLuciferase (ID8-luc cells) were previously described [20,21]. ID8 cells transfected to express ovalbumin (ID8-ova) were kindly provided by Dr. Mary Jo Turk [22]. Intra-peritoneal tumor and ascites were induced by injecting 5×10^6 ID8-luc or ID8-ova cells intra-peritoneal (i.p.) into 5–6 week old mice. Following ID8-luc injection, mice were imaged for in vivo luciferase activity 4 weeks following inoculation. To image the mice, they were injected i.p. with 200 μL of 15 mg/mL D-Luciferin Potassium Salt (Gold Biotechnology, St. Louis, MI) in nine percent sodium chloride (Baxter, Deerfield, IL). Mice were then sedated with isoflurane gas. Images were obtained 10 min after Luciferin injection with the Xenogen VivoVision IVIS Bioluminescent and Fluorescent Imager (PerkinElmer, Waltham, MA). Cisplatin (SigmaAldrich, St. Louis, MO) therapy was initiated once mice expressed multiple sites of intra-peritoneal disease on imaging or following evidence of ascites in the ID8 ova model. Cisplatin was injected i.p. twice weekly at a dose of 2 mg/kg. Mice were treated for 3–4 weeks.

The bone marrow-derived dendritic cell (BMDC) culture protocol has been previously described [22]. In summary bone marrow-derived cells from OT-1 mice were suspended in DC media (RPMI 1640 medium, 10% heat-inactivated fetal/bovine serum, 100 units/ml penicillin/streptomycin, 50 mM B-mercaptoethanol, 10% conditioned supernatant from X63 cells secreting GM-CSF). Cells were plated in a 24-well tissue culture plate at 10^6 cells in one milliliter medium per plate. On days 2 and 4, the cells were washed and re-fed, and non-adherent cells removed. Semi-adherent cells were harvested on day six, which were phenotypically confirmed to be immature DCs by flow cytometry.

Tumor, splenocyte, and leukocyte isolation

Murine ascites was obtained from the peritoneal cavity of euthanized tumor bearing mice. To ensure consistent extraction, the peritoneal cavity was lavaged with 3cc normal saline. All obtainable peritoneal fluid was then harvested through an 18 g syringe and utilized for assays. Ascites was centrifuged and the cell free supernatant was collected for cytokine analysis. The cell pellet was treated with ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA) to lyse red blood cells. A single cell suspension of splenocytes was obtained with a 70 μM cell strainer (BD bioscience, San Jose, CA). The suspension was then treated with ACK lysis buffer and washed. $CD8^+$ T-cells and $CD11b^+$ leukocytes were isolated using bead isolation kits (Miltenyi Biotech, Auburn, CA) per manufacturer's protocol.

FACS

Tumor ascites was prepared as described above. Murine antibodies to Ly6g, Ly6c, CD11b, CD4, CD8, CD45, CD301 and CD62L were purchased from BioLegend (SanDiego, CA). Anti CD44 was purchased from Abcam (Cambridge, MA). 100 μL aliquots of single cell suspensions were prepared. Fc receptors were blocked by adding 1 μg purified anti-CD16/32 clone 2.4G2 per aliquot (Bioxcell, Lebanon, NH). Brilliant blue buffer (BD Biosciences, San Jose, CA) was also added to improve resolution of Brilliant Violet polymer dyes. Pre-titrated fluorophore antibodies were added, gently mixed, and incubated while shaking at room temperature for 30 min. Aliquots were then washed and the cell pellet was re-suspended in 200μL 2% paraformaldehyde fixative. Following fixation, the paraformaldehyde was washed out and the cell pellet was re-suspended in 200 μl PBS Ca/Mg free (SigmaAldrich, St. Louis, MO). Fluorescence acquisition was done on a Miltenyi MacsQuant 10 flow cytometer (Miltenyi Biotech) at optimized settings and flow rate. The flow data was analyzed utilizing FlowJo software (Ashland, OR). To gate on monocyte cells large cells were selected on forward and side scatter. This population was then further refined by eliminating cell doublets. The 11b/45 dual positive population was selected. From this subset the $Ly6C^+$ and $Ly6Gd^-$ population was identified as the cell population of interest. Similarly cells within the size range of monocytes that were $F4/80^+$ were selected and assessed for CD301. T-cells were identified by selecting the relevant subset on forward and side scatter. $CD8^+$ T-cells were considered cells that were $CD45^+/CD4^-/CD8^+$.

Luminex

Cell free ascites was collected as described above. Simultaneous determination of multiple cytokine concentrations was carried out by the Dart Lab Core Facility at the Norris Cotton Cancer Center (NCCC) on cell free ascites using the MILLIPLEX MAP Mouse/human Cytokine/Chemokine Magnetic Bead Panel— Premixed 32/41 Plex (EMD Millipore, Billerica, MA) on a Bio-Rad Bio-Plex Array Reader. Samples were diluted in cell culture medium to the dynamic range of each kit.

Interferon-gamma (IFN-ɣ) ELISA

To quantitatively measure induction of INF-ɣ, 1×10^5 $CD11b^+$ cells or cultured dendritic cells were combined with 1×10^6 $CD8^+$ T-cells or splenocytes. $CD11b^+$cells and $CD8^+$ T-cells with incubated with 50 nM PMA and 0.5 μM ionomycin (Sigma, St. Louis, MO). Cultured dendritic cells and splenocytes, from OT-1 mice were plated with 1 μg/mL SIINFEKL peptide. Three wells were plated for each sample. Cell-free supernatants were collected after 48 h of incubation and production of IFN-ɣ cytokines was quantitated by ELISA. DuoSet enzyme-linked immunosorbent assay (ELISA) kits for mouse INF-ɣ were acquired from R&D Systems (Minneapolis, MN). IFN-ɣ quantity was then derived from a standard curve of serial dilutions per the manufacturer's directions.

Statistics

All data was analyzed using Prism Graph Pad Software (San Diego, CA), with $p < 0.05$ considered statistically significant. Values were compared using a two-tailed Students *t*-test. All assays were at least replicated. Experiments had 4–6 mice per experimental group.

Results

Treatment with cisplatin induces an influx of monocytes and decreases cytotoxic T-cells

In order to determine the changes in the LygC$^+$/Ly6G$^-$ monocyte population and CD8$^+$ T-cell population that occur with chemotherapy, we employed the murine ID8 ovarian tumor model, which recapitulates critical characteristics of human epithelial ovarian cancer including a progressive accumulation of ascites, an immunosuppressive TME, and the recruitment of leukocytes including a substantial population of monocytic MDSC [14,20]. Tumor bearing C57BL/6 mice were treated with cisplatin or vehicle control twice weekly by i.p. injection. CCR2ko mice were used as a control for peritoneal monocyte recruitment [23, 24]. Monocytic cells were classified as CD45$^+$, 11b$^+$, Ly6c$^+$, and Ly6g$^-$ [25]. Tumor bearing wild type mice treated with cisplatin had an increase in the percentage of monocytes within their ascites compared to tumor bearing mice treated with vehicle control (Fig. 1A, $p < .0001$, CI 19.80–29.90). In mice treated with vehicle control, 9.35% ± 1.37 of CD45$^+$ ascites cells stained as monocytes. In mice treated with cisplatin, monocytes composed 34.2% ± 5.32 of the ascites cell population. CCR2 has previously been identified as key cytokine receptor responsible for the recruitment of monocytic cells to the ovarian cancer TME, and therefore we used CCR2ko (CCR2$^{-/-}$) mice, in parallel, as an internal control for specificity of cellular recruitment [14]. In CCR2ko mice treated with vehicle control only 1.84% ± 0.48 of the cells within the ascites were classified as monocytes, less than both the control and cisplatin groups ($p < .0001$). The percentage of CD45$^+$/CD8$^+$ T-cells within the ascites decrease from 7.44% ± 1.6 to 3.11% ± 1.08 ($p = .004$) in the setting of cisplatin treatment (Fig. 1B). These findings support that treatment with cisplatin leads to an influx of monocytes into the ascites of mice carrying ID8 EOC. In addition we show that the percentage of CD8$^+$ T-cells in the ascites decreases in the presence of cisplatin treatment.

In vivo cisplatin chemotherapy of ID8 ovarian cancer increases the ability of ascites-derived CD11b$^+$ cells to induce T-cell stimulation

To assess the function of the CD11b$^+$ cells within tumor ascites and the potential downstream effect of an increase in CD11b$^+$ monocytes, CD11b$^+$ cells from mice treated with vehicle control or cisplatin were isolated and tested for their quantitative ability to elicit a CD8$^+$ T-cell response. CD11b$^+$ cells were isolated from ID8 tumor ascites, and then combined with naive CD8$^+$ T-cells from non-tumor bearing mice and incubated for 48 h in the presence of ionomycin and PMA after which IFN-ɣ levels in the cell-free media was measured (Fig. 2). CD8$^+$ T-cells stimulated by tumor associated CD11b$^+$ cells derived from mice who were treated with vehicle control elicited 604.0 (±52.92) pg/mL of IFN-ɣ. In comparison, CD8$^+$T-cells stimulated by CD11b$^+$ cells from mice treated with cisplatin were elicited 4328.0 (±185.2) pg/mL of IFN-ɣ ($p < .0001$). Negative and positive experimental controls of un-stimulated T-cells and T-cells stimulated in the absence of CD11b$^+$ cells, respectively, are shown (Fig. 2). These findings demonstrate that not only does the concentration of CD11b$^+$ cells increase with cisplatin chemotherapy, but that it fundamentally alters the functional ability of these cells. Specifically, in vivo cisplatin treatment results in CD11b$^+$ cells within the TME that are able to induce significantly higher levels of IFN-ɣ from CD8$^+$T-cells compared to CD11b$^+$ cells from mice that have not undergone chemotherapy.

In vivo cisplatin treatment of ID8 tumor-bearing mice results in a pro-inflammatory tumor microenvironment

To identify how in vivo cisplatin treatment of ID8 tumors alters the inflammatory state of the TME, we analyzed cellular and inflammatory markers from ascites. Ascites was harvested from ID8 tumor bearing mice that had undergone either cisplatin or control treatment. Luminex was used to quantify the concentrations of IL-10, VEGF, and IL-6, cytokines associated with immune suppression, within the cell free ascites

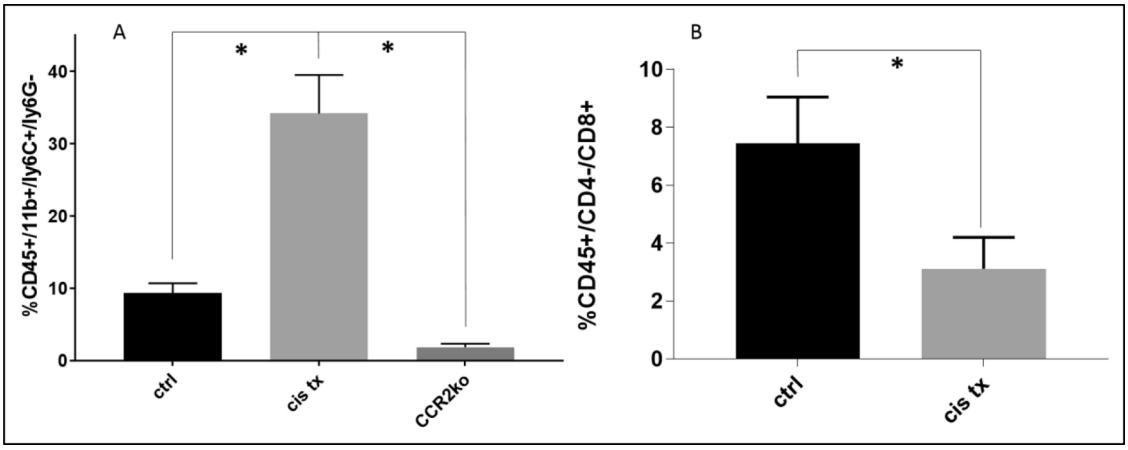

Fig. 1. In vivo cisplatin treatment of murine ID8 ovarian tumors results in increased Ly6C$^+$monocyte recruitment to the tumor microenvironment and a decrease in CD8$^+$ T-cells. In order to determine the changes in the Ly6c+ monocyte and cytotoxic T-cell population that occurred with chemotherapy, tumor bearing C57BL/6 (4–6 mice per group) were treated with cisplatin or vehicle control. CCR2ko mice were used as a control. In Fig. 1A monocytes were classified as CD45+, 11b+, Ly6c+, and Ly6g-. We found that in wild type C57BL/6 mice that did not receive cisplatin the percentage cells that stained as monocytes was 9.35% ± 1.37. In wild type C57BL/6 that were treated with cisplatin monocytes composed 34.2% ± 5.32 of the cell population. The difference between these groups is statistically significant ($p < .0001$, CI 19.80–29.90). In CCR2 knock out mice treated with vehicle control only 1.84% ± 0.48 stained as monocytes. This is significant less than both the control and cisplatin groups ($p < .0001$). In Fig. 1B CD8$^+$ T-cells showed a relative decrease in the ascites following cisplatin therapy from 7.44% ± 1.6 to 3.11% ± 1.08 ($p = .004$). These findings indicate that treatment with cisplatin leads to an influx of Ly6c$^+$ monocytes and a decrease of CD8$^+$ T-cells into the malignant ascites of mice carrying ID8 epithelial ovarian cancer. Additionally, CCR2 is a key component in the recruitment of the Ly6c+ cell population into the ascites. (*=$p <$ .05; ctrl=control group; *CIS* tx=group treated with cisplatin.

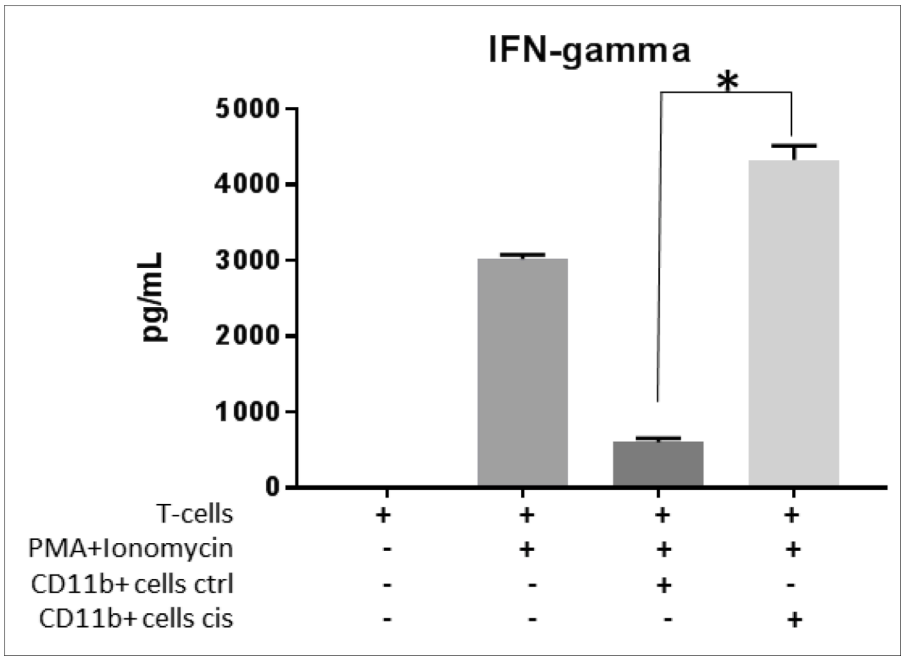

Fig. 2. CD11b$^+$ cells from the ascites of mice treated with cisplatin activate CD8$^+$ T-cell more effectively than CD11b$^+$ cells from untreated mice. In order to identify the mechanistic nature of CD11b+ cells recruited to the ascites we isolated this cell population from the ascites of tumor bearing mice treated with vehicle control or cisplatin (4–6 mice per group). The CD11b+ cells were then combined with CD8+ T-cells of non-tumor bearing mice and incubated for 48 h after which IFN-ɣ levels in the cell free media was measured. The first two columns represent positive and negative controls respectively. The combination of CD11b cells from tumor bearing mice that were treated with vehicle control and T-cells elicited 604.0 (±52.92) pg/mL of IFN-ɣ. When CD11b cells from tumor bearing mice treated with cisplatin were combined with T-cells they elicited 4328.0 (±185.2) pg/mL of IFN-ɣ. CD11b+ cells in mice receiving chemotherapy induced significantly higher levels of IFN-ɣ from T-cells compared to CD11b+ cells from mice treated with vehicle control (*=$p < .0001$).

from mice treated with vehicle control or cisplatin (Fig. 3A,3B, AND 3C respectively) [13,26]. Within the ascites, mice treated with cisplatin had lower levels of IL-10 (8.80 ± 7.84 pg/mL) VEGF (10.68 ± 10.77) and IL-6 (51.21 ± 39.44) compared to those treated with vehicle control (53.41 ± 37.85 pg/mL IL-10 ($p > .05$); 1035 ± 1140 pg/mL VEGF; 102.9 ± 8.089 IL-6 ($p > .05$)). These differences were not statistically significant, but suggest a trend toward a more immunologically active TME with administration of cisplatin. To determine if this pro-inflammatory shift in the TME is reflected in the cellular phenotypes, FACS analyses were performed on single-cell suspensions of cells derived from the ascites. F4/80$^+$ cells from mice treated with cisplatin had lower levels of CD301 (MFI = 13,495 ± 6208), a marker of inhibitory macrophages, compared to mice treated with vehicle control (20,115 ± 5229; $p > .05$) (Fig. 3D). In order to further characterize the T-cells recruited to the

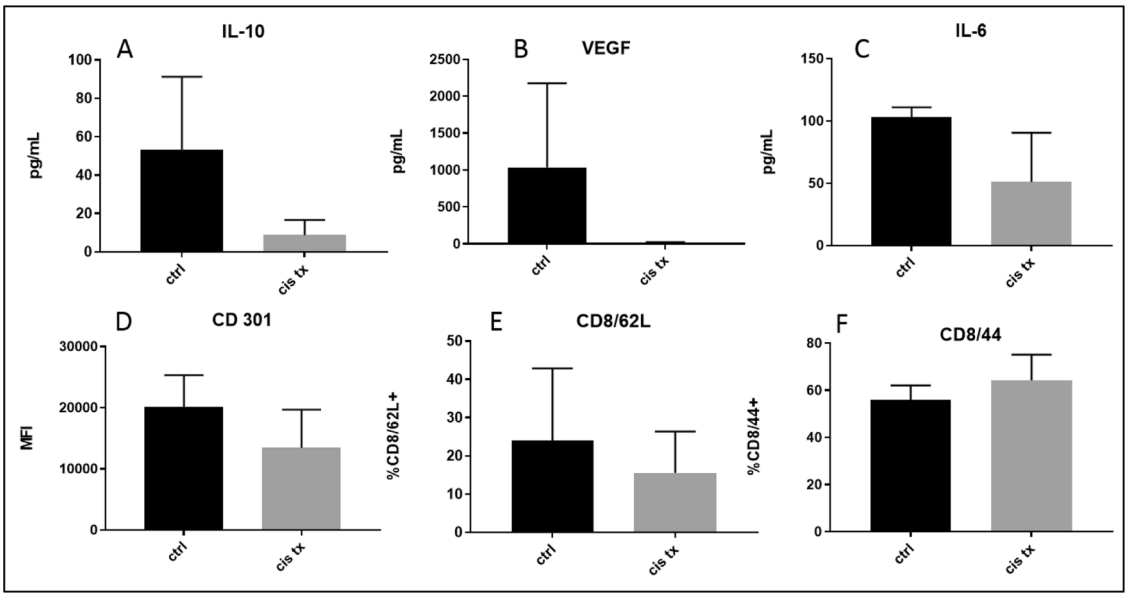

Fig. 3. Cytokines and cell markers associated with immune cell activation and burnout indicate an increased in ascites immune activation following cisplatin treatment. Luminex was used to quantify the immunosuppressive cytokines IL-10, VEGF, and IL-6 within the cell free ascites in mice treated with vehicle control or cisplatin (Fig. 3A,3B, and 3C respectively) and FACS was used to assess markers of macrophage activation (Fig. 3D), T-cell activation (Fig. 3E), and T-cell burn out (Fig. 3F). Mice treated with cisplatin had lower levels of IL-10 (8.80 ± 7.84 pg/mL), VEGF (10.68 ± 10.77), and IL-6 (51.21 ± 39.44) in the cell free ascites compared to those treated with vehicle control (53.41 ± 37.85 pg/mL IL-10; 1035 ± 1140 pg/mL VEGF; 102.9 ± 8.089 IL-6). These differences were non-significant, but suggest a trend toward a more immunologically active TME with administration of cisplatin. Although non-significant, mice treated with cisplatin had lower levels of CD301 (13,495 ± 6208) compared to mice treated with vehicle control (20,115 ± 5229) measured by MFI. Although non-significant, expression of CD44, a marker of T-cell activation, increased with cisplatin therapy (55.9 ± 6.17 v 64.21 ± 10.99) measured by MFI. Additionally, The MFI of CD62L, a marker of CD8 T-cell burn out, decreased in the ascites of mice treated with cisplatin (24.11 ± 18.75 v 15.60 ± 10.75). Although the difference between the treated and untreated groups did not reach statistical significance, these assays show a trend towards a more immunologically active TME following administration of chemotherapy.

tumor ascites we performed FACS analyses for CD 44 or CD 62 L on ascites-derived CD8+ T cells. The expression of CD44, a marker of T-cell activation, increased with cisplatin therapy (55.9 ± 6.17 v 64.21 ± 10.99; $p > .05$) (Fig. 3E). Additionally, expression of CD62L, a marker that is down regulated following T cell activation, decreased with cisplatin treatment (24.11 ± 18.75 v 15.60 ± 10.75; $p > .05$) (Fig. 3F). Although these differences did not reach statistical significance, they bolster our other findings indicating that cisplatin induces a pro-inflammatory state within tumor ascites that enables infiltrating $CD8^+$ T cell activation.

In vivo cisplatin treatment results in increased tumor-specific T-cell responses

Finally, we extended the phenotypic inflammatory status of the TME following cisplatin treatment to functionally assess the impact of this treatment, and the resultant TME, on T-cell activity. The T-cell response to antigen presenting cells is a key step in determining the status, and impact, of the immune environment. In order to identify the effect of cisplatin treatment on T-cell responses to tumor-specific antigens, we used the ID8-ova tumor model in which ID8 cells ectopically express such that SIINFEKL is a pre-determined, tumor-specific antigen. Groups of mice were subsequently treated with cisplatin or a control. To quantitatively assess and compare the relative responses, splenocytes from tumor bearing OT-1 mice were combined with cultured dendritic cells pulsed with the $CD8^+$ T-cell epitope of ova, SIINFEKL, and co-cultured for 48 h. SIINFEKL is a known epitope of $CD8^+$ T-cells therefore we expected alterations in signal to be driven by $CD8^+$ T-cells [22]. IFN-ɣ levels in the cell free media was assessed by ELISA as a quantitative marker of activity. IFN-ɣ elicited from wild type splenocytes were utilized as a negative control. Ova-pulsed dendritic cells were able to induce a higher tumor specific T-cell response, measured via IFN-ɣ, from CD8+ T-cells derived from tumor bearing mice treated with cisplatin (94.08pg/mL±45.26) compared to mice treated with vehicle control (62.03 pg/mL ± 34.4) ($p = .03$). As a negative control, splenocytes from non-tumor bearing mice, and therefore not expressing the tumor-specific ova antigen, expressed 32.83 pg/mL ± 10.10 of IFN-ɣ. This was significantly less than mice treated with cisplatin ($p = .03$), but not significantly less than untreated mice ($p = .17$) (Fig. 4). These findings indicate that the T-cells recruited to the tumor have increased anti-tumor activity potential following chemotherapy treatment. In conjunction with the other data presented here, these findings strongly support that chemotherapy facilitates de-repression of the anti-tumor immune environment and tumor-specific CD8+ *T* cell response.

Discussion

Development and utilization of efficacious immune therapies in EOC requires understanding the immune status of a patient's tumor and how the immune milieu changes with the various stages of cancer treatment. Many ovarian cancers have immune infiltrates present at the time of diagnosis with the cells favoring a tumor-protective environment. Current research supports that the immune balance within tumors of untreated patients is primarily driven by the tumor cells [7–11]. The changes that take place with respect to the immune infiltrate as a tumor goes through chemotherapy, regression, and then eventual recurrence are not as well understood, but are key to the design and implementation of effective immune therapies. Furthermore, studies in humans on the effect of chemotherapy on the intra-tumoral immune environment suggest that even within the same histologic subgroup, the immune changes within the tumor can be drastically different between patients [16,17].

Thus far, studies of the effect of chemotherapy on tumor immune infiltrate have focused on the T-cell populations within the TME and PD-1 expression. These studies have shown that in patients with a favorable prognosis, chemotherapy treatment corresponds to an increase in the ratio of cytotoxic T-cells within the TME. In both human and murine data there is an accompanying increase in PD-1 or PDL-1 expression without therapeutic benefit to PD-1/PDL-1 inhibitor co-treatment [16,17,19,27]. However, the alterations and subsequent role of the monocyte population within the TME during treatment with platinum is not well understood. To help address this question, we present our study of the immune environment in a murine model of epithelial ovarian cancer after treatment with a clinically-relevant platinum agent. Our findings on the phenotype and functional changes of the cytokine and cellular infiltrate within the ascites demonstrate a compelling shift to an anti-tumor immune environment that includes an influx of $Ly6C^+$ monocytes and an increase in the ability of $CD11b^+$ monocytic cells within the TME to stimulate $CD8^+$ T-cells with specificity for tumor antigen. Prior studies have suggested that intra-tumoral monocytes are one of the key mediators of the suppressive nature of the anti-tumor immune activity in treatment naïve patients [7,12–14]. Our data supports the continued importance of monocytes as a driver of the immune milieu during treatment with chemotherapy.

Our findings add to the emerging literature demonstrating that macrophage directed immune therapies may be efficacious in the treatment of EOC. Specifically, a monocyte inhibitor may be most efficacious in treatment-naïve patients or in patients who fail to convert to a M1 phenotype with chemotherapy. Additionally, there may be a group

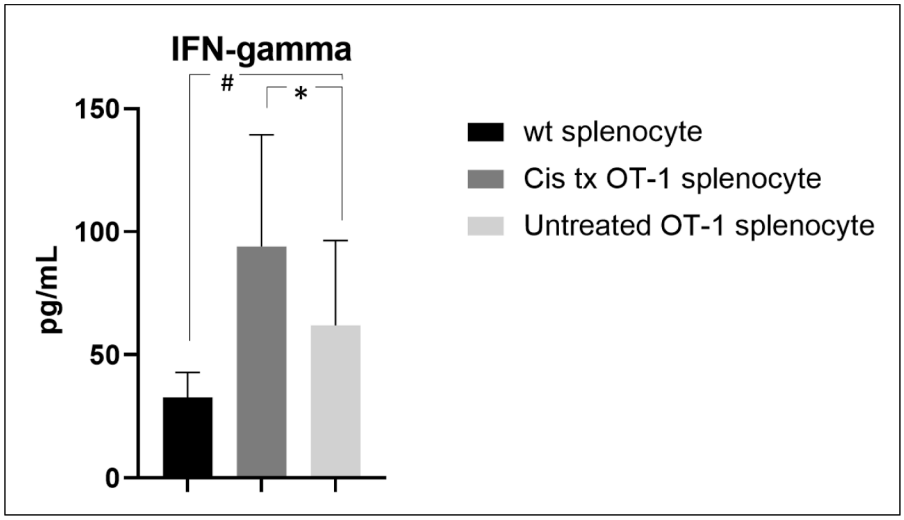

Fig. 4. Splenocytes from tumor bearing mice treated with cisplatin released more IFN-ɣ in response to tumor specific antigens when compared to untreated tumor bearing mice. In order to identify differences in the tumor specific activation potential of T-cells recruited to the ascites we combined splenocytes from OT-1 mice bearing ID8-ova tumors treated with either cisplatin or vehicle control (4–6 mice per group) with cultured dendritic cells pulsed with SIINFEKL. Cells were incubated for 48 h after which IFN-ɣ levels in the cell free media was assessed. In this experiment wild type splenocytes served as baseline control. Dendritic cells were able to induce a higher response, measured via IFN-ɣ, in splenocytes from mice treated with cisplatin (94.08±45.26) compared to mice treated with vehicle control (62.03 ± 34.4) ((*)$p = .03$). The difference between negative control wild type splenocytes (32.83±10.1) and untreated OT-1 splenocytes was not statistically significant ((#) $p = .17$). These findings indicate that the splenocytes recruited to the tumor have more anti-tumor activity potential following chemotherapy treatment.

of patients who benefit from further disinhibition of the macrophages present within their tumor. Cisplatin was chosen to conduct these studies because it is a standard of care adjuvant chemotherapy agent and has efficacy when injected into the peritoneum [15]. However, it is noteworthy that alternative chemotherapies may affect the host immune response in different ways. Prior studies have shown that chemotherapies with different mechanisms of action such as Gemcitabine or Liposomal Doxorubicin alter the intra-tumoral immune infiltrates in different ways. Importantly, some chemotherapies induce immune-mediated tumor cell death while others do not [28,29]. Thus, not only do we need to take the patient's tumor into account, but also the chemotherapy being used when designing immune treatments.

Conclusion

Given the complexity and redundancy within the immune system it will likely be necessary to target tumor cells, monocytes, T-cells, and cytokines at various time points and in various combinations to effectively leverage the immune system against EOCs. Our findings utilizing cisplatin chemotherapy support prior data showing that the immune infiltrate within a tumor shifts with chemotherapy and this shift is significant enough that it needs to be accounted for when implementing immunotherapies. Additionally, our data support consideration of monocytes as well as T-cells and cytokines as targets for immune therapies. Given the diversity of immune responses to chemotherapy within a single histologic subtype of EOC, additional human studies are needed to elucidate the combination of tumor characteristics, chemotherapy, and immune therapy that will result in targeted cancer cell death.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] American Cancer Society, Cancer Facts & Figures 2020, American Cancer Society, Atlanta, 2020.
[2] E. Sato, S.H. Olson, J. Ahn, et al., Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer, Proc. Natl. Acad. Sci. USA 102 (51) (2005 Dec 2005) 18538–18543.
[3] T.J. Curiel, G. Coukos, L. Zou, et al., Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival, Nat. Med. 10 (9) (Sep 2004) 942–949.
[4] L. Zhang, J.R. Conejo-Garcia, D. Katsaros, et al., Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer, N. Engl. J. Med. 348 (3) (Jan 2003) 203–213.
[5] J. Hamanishi, M. Mandai, T. Ikeda, et al., Safety and Antitumor Activity of Anti-PD-1 Antibody, Nivolumab, in patients with platinum-resistant Ovarian Cancer, J. Clin. Oncol. 33 (34) (Dec 2015) 4015–4022.
[6] C.L. Schwab, D.P. English, D.M. Roque, et al., Past, present and future targets for immunotherapy in ovarian cancer, Immunotherapy 6 (12) (2014) 1279–1293.
[7] A.B. Alvero, M.K. Montagna, V. Craveiro, et al., Distinct subpopulations of epithelial ovarian cancer cells can differentially induce macrophages and T regulatory cells toward a pro-tumor phenotype, Am. J. Reprod. Immunol. 67 (3) (Mar 2012) 256–265.
[8] A. Fialova, S. Partlova, L. Sojka, et al., Dynamics of T-cell infiltration during the course of ovarian cancer: the gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T-cells, Int. J. Cancer 132 (5) (Mar 2013) 1070–1079.
[9] T. Hagemann, J. Wilson, F. Burke, et al., Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype, J. Immunol. 176 (8) (Apr 15 2006) 5023–5032.
[10] J. Surówka, I. Wertel, K. Okła, et al., Influence of ovarian cancer type I and type II microenvironment on the phenotype and function of monocyte-derived dendritic cells, Clin. Transl. Oncol. 19 (12) (2017 Dec) 1489–1497.
[11] F. Ghiringhelli, P.E. Puig, S. Roux, et al., Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation, J. Exp. Med. 202 (7) (2005 Oct 3) 919–929.
[12] D. Duluc, Y. Delneste, F. Tan, et al., Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells, Blood 110 (13) (Dec 2007) 4319–4330.
[13] I. Kryczek, S. Wei, G. Zhu, et al., Relationship between B7-H4, regulatory T cells, and patient outcome in human ovarian carcinoma, Cancer Res. 67 (18) (Sep 2007) 8900–8905.
[14] K.M. Hart, S.P. Bak, A. Alonso, B. Berwin, Phenotypic and functional delineation of murine CX(3)CR1 monocyte-derived cells in ovarian cancer, Neoplasia 11 (6) (Jun 2009) 564–573.
[15] National Comprehensive Cancer Network. Ovarian Cancer (Version 2.2020. https://www.nccn.org/professionals/physician_gls/pdf/ovarian_blocks.pdf. Accessed March 10.2021.
[16] S. Böhm, A. Montfort, O.M. Pearce, et al., Neo-adjuvant chemotherapy modulates the immune microenvironment in metastases of tubo-ovarian high-grade serous carcinoma, Clin. Cancer Res. 22 (12) (Jun 2016) 3025–3036.
[17] C.S. Lo, S. Sanii, D.R. Kroeger, et al., Neo-adjuvant Chemotherapy of Ovarian Cancer Results in Three Patterns of Tumor Infiltrating Lymphocyte Response with Distinct Implications for Immunotherapy, Clin. Cancer Res. 23 (4) (Feb 2017) 925–934.
[18] C.L. Chang, Y.T. Hsu, C.C. Wu, et al., Dose-dense chemotherapy improves mechanisms of antitumor immune response, Cancer Res. 73 (1) (2013 Jan 1) 119–127, https://doi.org/10.1158/0008-5472.CAN-12-2225. Epub 2012 Oct 29.
[19] S.J.L. Mesnage, A. Auguste, C. Genestie, et al., Neo-adjuvant chemotherapy (NACT) increases immune infiltration and programmed death-ligand 1 (PD-L1) expression in epithelial ovarian cancer (EOC), Ann. Oncol. 28 (3) (2017 Mar 1) 651–657.
[20] K.F. Roby, C.C. Taylor, J.P. Sweetwood, et al., Development of a syngeneic mouse model for events related to ovarian cancer, Carcinogenesis 21 (4) (2000) 585–591.
[21] E. Huarte, J.R. Cubillos-Ruiz, Y.C. Nesbeth, et al., Depletion of dendritic cells delays ovarian cancer progression by boosting antitumor immunity, Cancer Res. 68 (18) (2008) 7684–7691.
[22] M.A. Barber, T. Zhang, B.A. Gagne, Sentman Cl. NK cells negatively regulate antigen presentation and tumor-specific CTLs in a syngeneic lymphoma model, J. Immunol. 178 (10) (2007 May 15) 6140–6147.
[23] L. Boring, J. Gosling, S.W. Chensue, et al., Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice, J. Clin. Invest. 100 (10) (1997) 2552–2561.
[24] K.M. Hart, S.P. Bak, A. Alonso, B. Berwin, Phenotypic and functional delineation of murine CX(3)CR1 monocyte-derived cells in ovarian cancer, Neoplasia 11 (6) (2009) 564–573.
[25] R.S. Soong, R.K. Anchoori, B. Yang, et al., RPN13/ADRM1 inhibitor reverses immunosuppression by myeloid-derived suppressor cells, Oncotarget 7 (42) (2016 Oct 18) 68489–68502, https://doi.org/10.18632/oncotarget.12095. PMID: 27655678; PMCID: PMC5340091.
[26] A.C. Ziogas, N.G. Gavalas, M. Tsiatas, O. Tsitsilonis, E. Politi, E. Terpos, A. Rodolakis, G. Vlahos, N. Thomakos, D. Haidopoulos, A. Antsaklis, M. A. Dimopoulos, A. Bamias, VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2, Int. J. Cancer 130 (4) (2012 Feb 15) 857–864.
[27] E. Amiel, S. Nicholson-Dykstra, J.J. Walters, H. Higgs, B. Berwin, Scavenger receptor-a functions in phagocytosis of E. coli by bone marrow dendritic cells, Exp. Cell Res. 313 (7) (2007) 1438–1448.
[28] J. Peng, J. Hamanishi, N. Matsumura, K. Abiko, K. Murat, T. Baba, K. Yamaguchi, N. Horikawa, Y. Hosoe, S.K. Murphy, I. Konishi, M. Mandai, Chemotherapy induces programmed cell death-Ligand 1 overexpression via the Nuclear Factor-κB to foster an immunosuppressive tumor microenvironment in Ovarian Cancer, Cancer Res. 75 (23) (2015 Dec 1) 5034–5045.
[29] M. Michaud, I. Martins, A.Q. Sukkurwala, S. Adjemian, Y. Ma, P. Pellegatti, S. Shen, O. Kepp, M. Scoazec, G. Mignot, S. Rello-Varona, M. Tailler, L. Menger, E. Vacchelli, L. Galluzzi, F. Ghiringhelli, F. di Virgilio, L. Zitvogel, G. Kroemer, Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice, Science 334 (6062) (2011 Dec 16) 1573–1577.