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Distinct sub-populations of epithelial ovarian cancer cells can differentially induce macrophages and T regulatory cells towards a pro-tumor phenotype

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Abstract

Problem—The presence of immune infiltrates in the tumor does not always correlate with an anti-tumoral immune response. We previously identified two sub-populations of epithelial ovarian cancer (EOC) cells with differential cytokine profile. We hypothesize that these two sub-populations of EOC cells may differentially regulate the immune phenotype in the tumor microenvironment and therefore affect the immune response.

Method of Study—Macrophages derived from CD14⁺ monocytes and naive CD4⁺T cells were treated with conditioned media (CM) from two sub-populations of EOC cells. Differentiation markers and phagocytic activity was measured by western blot analysis and flow cytometry. Cytokine levels were quantified using xMAP technology.

Results—Type I EOC cells are able to enhance macrophages' capacity for tumor repair and renewal by enhancing expression of scavenger receptors and by promoting the secretion of cytokines associated with tissue repair. On the other hand, Type II EOC cells are able to create a tolerant microenvironment and prevent an immune response by inducing macrophages' to secrete IL-10 and by promoting the generation of T regs.

Conclusion—We demonstrate that each ovarian cancer cell sub-population can induce a unique phenotype of macrophages and T cells, both associated with tumor-supportive function.

Keywords

cancer stem cells; macrophages; ovarian cancer; T regulatory cells

Introduction

Epithelial ovarian cancer (EOC) is the most lethal of all gynecologic malignancies. Despite recent advances in the knowledge of ovarian cancer biology, the mortality rate has not improved in the past several years. Even with aggressive treatment modalities, which involve surgical debulking and combination chemotherapy, the 5-year survival is only 15%^{1, 2}. Therefore, additional treatment regimen is needed to complement currently available modalities.

The tumor microenvironment is composed of multiple cell types including cancer cells, fibroblast, and immune cells. It has been suggested that since solid tumors can not progress in the absence of a favorable environment, a defective immune response must already be in place upon the establishment of the tumor³. Therefore, a possible complementary approach to surgery and cytotoxic chemotherapy is to target the immune cells and re-educate them to elicit an anti-tumoral immune response. A better understanding of the complex cross talk that occurs in the tumor microenvironment is required for designing such treatment.

Ovarian cancer tumors are very heterogeneous. We have described at least two sub-populations of EOC cells with differential stemness potential, as well as inflammatory and cytokine profile⁴⁻⁶. CD44+/MyD88+ EOC stem cells (Type I EOC cells) are the tumor-initiating cells, express pluripotency markers, have a constitutively active NF- κ B pathway, and constitutively secrete the pro-inflammatory cytokines IL-6, IL-8, MCP-1, and GRO- α ^{6,7}. On the other hand, CD44-/MyD88- mature ovarian cancer cells (Type II EOC cells), which are derived from Type I EOC cells, lack stemness properties, represent the rapidly growing cell population, and do not constitutively secrete IL-6, IL-8, MCP-1, nor GRO- α ⁶.

Similarly for the immune cells, specifically for the monocyte/macrophage lineage, as well as the T cells, several subtypes have been described. Classically polarized macrophages (M1) function as effector cells against intracellular parasites and also tumor cells^{8,9}. On the other hand, the non-classical M2 phenotype is more associated with clean-up and repair^{10,11,12}. For the T cell lineage, CD4+ helper T cells and CD8+ cytotoxic T cells can elicit an immune response, whereas CD4+/FoxP3+ regulatory T cells (T regs) function as suppressors to limit an ongoing response or to create tolerance¹³.

Monocyte-derived macrophages are cells with a high level of plasticity and can differentiate depending on signals from the tissue microenvironment. The signals regulating the differentiation process influence the specific role of these macrophages within the tissue. Interestingly, the predominant form of macrophages found in solid tumors has tumor-supportive phenotype. In breast tumors, tumor-associated macrophages (TAMs) are able to support tumor growth and metastasis by producing cytokines and hormones, such as estrogen¹⁴. Similarly, in ovarian cancer, the majority of TAMs are of the M2 phenotype and their presence have been associated with tumor progression and chemoresistance¹⁵. Supporting the pro-tumoral role of macrophages, Tregs are capable of creating a tolerogenic microenvironment, which prevents anti-tumoral immune responses⁵.

The creation of a pro-tumor immune profile in the tumor microenvironment would require a complex cross-talk between all the cellular components of the tumor. We hypothesize, that given their differential cytokine profile, the two subtypes of EOC cells may have a unique and specific effect on each type of immune cell. The objective of this study is to characterize the cross talk between immune cells and the two subtypes of EOC cells. We focused our attention on how Type I and Type II EOC cells can differentially regulate the phenotype of macrophages derived from CD14+ monocytes and of freshly isolated CD4+ naive T cells. Our data show that each ovarian cancer sub-population can induce a unique phenotype of macrophages and T cells, both associated with tumor-supportive function.

Materials and Methods

Cancer cell culture conditions and preparation of conditioned media

Type I and Type II EOC cells were isolated from human ovarian cancer tissues as previously described and cultured in RPMI 1640 (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA)⁶. Cancer cell conditioned-media (CM) was obtained by incubating 70% confluent cell cultures in RPMI

1640 supplemented with 1% FBS for 48h. Supernatants from these cultures were centrifuged for 15 mins at 1500 rpm and filtered using a 0.45 μ m filter to obtain cell-free CM.

Isolation and differentiation of CD14+ monocytes

Adherent CD14+ monocytes were isolated from concentrated buffy coat as previously described ¹⁶. After verification of purity by flow cytometry, CD14+ monocytes were cultured in RPMI 1640 with 5% FBS and 100 ng/ml M-CSF (PeproTech, Inc. Rocky Hill, NJ) for 6d to obtain resting macrophages (M ϕ). At the end of day 6, M ϕ were either kept in M-CSF or cultured in 50% Type I or II EOC cell CM for another 6d prior to characterization. For M2-positive control, M ϕ were treated for 18h with 20 ng/ml IL-4 ¹⁷.

Isolation and culture of CD4+ naive T cells

CD4+ T cells were isolated from peripheral blood mononuclear cells using EasySep Negative selection human naive CD4+ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) according to manufacturer's instructions. Purity of obtained cell population was verified by flow cytometry. One $\times 10^6$ CD4+ T cells were seeded per well in a 24-well plate pre-coated with 10 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 (BD Bioscience, Billerica, MA). CD4+ T cells were cultured in RPMI 1640 plus 10% FBS supplemented with 2 ng/ml IL-2 (PeproTech, Inc.) in the presence or absence of 10 ng/ml TGF- β (PeproTech, Inc.), or 50% Type I CM, or 50% Type II CM for 7d prior to characterization. Fresh media and cytokines were added every 2d. For experiments with TGF- β neutralizing antibody, CD4+ T cells were cultured in IL-2 plus 50% Type II CM, plus 5 μ g/ml anti-TGF β (R&D Systems, Minneapolis, MN).

Flow cytometry

Cells were collected and stained with mouse anti-human CD14-PE (1:10, eBioscience), mouse anti-human CD4-PE (1:10, eBioscience), or mouse anti-human HLA-DR-FITC (1:100; Beckman Coulter, Brea, CA) prior to fixation with 1% paraformaldehyde. For nuclear Foxp3 staining, we used the FITC-anti-human Foxp3 staining kit (eBioscience, San Diego, CA) according to manufacturer's instructions. Data was acquired using BD FACSCalibur and analyzed using FlowJo or CellQuest (BD Biosciences, San Jose, CA).

SDS-PAGE and western blot analysis

Protein was extracted and measured as previously described ¹⁸. Twenty μ g of protein was used for SDS-PAGE, and Western blots were performed as previously described ¹⁹ using goat anti-human SR-A1 (0.2 μ g/ml; R7D Systems).

Cytokine analysis

Levels of cytokines and chemokines were measured from CM or supernatants from M ϕ cultures using Bioplex Pro Cytokine Assay (Bio-Rad, Hercules, CA, USA). Data were acquired using the Bioplex system (Bio-rad) and analysis was performed using the Bioplex software as previously described ²⁰. Protein standards provided in the kit served as positive control.

Generation of GFP-labeled apoptotic bodies and phagocytosis assay

GFP-labeled EOC cells were treated with 10 μ g/ml Phenoxodiol ²¹ for 24h to induce apoptosis. The resulting GFP-labeled apoptotic bodies were collected by spinning culture supernatants for 15 mins at 1500 rpm. M ϕ obtained by M-CSF as described above were pre-educated with either Type I or II EOC cell CM for 24h prior to exposure to GFP-labeled apoptotic bodies. Levels of GFP-positive M ϕ were quantified by flow cytometry.

Results

Type I and Type II EOC cells have a differential cytokine profile

One of the observed differences in the molecular phenotype of Type I and II EOC cells is their differential cytokine profile. Previously, we reported that although both cell types constitutively secrete MIF, only Type I EOC cells, constitutively secrete IL-6, IL-8, MCP-1, and GRO α ^{7, 22}. Conversely, VEGF was only observed in Type II cultures ^{6, 7, 23}. Further characterization of the cytokine/chemokine profile showed additional differences, which is summarized in Table 1. Whereas Type I EOC cells mostly secrete pro-inflammatory cytokines (IL-6, IL-8, MCP-1, GRO α , TNF α), Type II EOC cells secrete more of the immunosuppressive IL-10 and the regulatory cytokines IL-12 and TGF β . These suggest that the two types of EOC cells would have a different effect on the regulation of immune response.

Differential effects of ovarian cancer cells on macrophages

Two major immune cell populations have been described in ovarian cancer, tumor infiltrating macrophages and T regs . We first focused our attention on the possible differential effect of Type I and Type II EOC cells on macrophages. Thus, M ϕ were obtained *in vitro* by incubating freshly isolated CD14⁺ monocytes with M-CSF. Afterwards, M ϕ were cultured in either 50% Type I CM, 50% Type II CM, or as control, maintained in M-CSF media. Morphological assessment showed that compared to M ϕ controls, the M ϕ exposed to CM from Type I and Type II EOC cells were bigger and had a more granular cytoplasm (Fig. 1A). This observation was confirmed by flow cytometry analysis, which showed a shift in both FSC and SSC when M ϕ were cultured in CM from the two subtypes of EOC cells compared to control (Fig. 1B). Interestingly, there seemed to be no morphologic difference between M ϕ cultured in Type I versus Type II CM.

To further characterize these macrophages, we determined if there were any difference in their molecular phenotype by looking at the cell surface markers, scavenger receptor- A (SR-A) and HLA-DR. M ϕ cultured with Type I CM showed significant up regulation of SR-A compared to M ϕ control and M ϕ cultured with Type II CM (Fig. 2A). There was however, no significant difference in the level of HLA-DR among the groups (Fig. 2B).

We also characterized the cytokine profile of the M ϕ cultured in the different conditions. Type I and Type II EOC cells generated distinct macrophage phenotypes with different cytokine profile. Figure 3 groups the cytokines according to the trend observed. There was no significant difference between the levels of MCP-1 secreted among the groups (Fig. 3, panel i); M ϕ cultured in both types of cancer CM secreted higher levels of GRO α , IL-6, and IFN- γ compared to M ϕ control (Fig. 3, panel ii); M ϕ cultured in both types of cancer CM also secreted higher levels of MIP-1 α , MIP-1 β , and Rantes compared to control, but the levels of these cytokines are significantly higher in M ϕ cultured in Type I CM (Fig. 3, panel iii); finally, M ϕ cultured in Type II CM secreted higher levels of IL-10, IL-8, and GCSF (Fig. 3, panel iv).

Since phenotypic differences were observed in M ϕ cultured with either Type I or II CM, we then investigated whether there were functional differences. M ϕ obtained from the different culture conditions were exposed to equivalent amounts of GFP-labeled apoptotic bodies. Flow cytometry analysis showed that M ϕ pre-educated with Type I CM exhibited enhanced phagocytic activity as evidenced by higher MFI levels compared to M ϕ pre-educated with Type II CM (Fig. 4).

Taken together, these data suggest that Type I EOC cells may enhance the phagocytic activity of M ϕ and its capacity for repair, via up-regulation of SR-A and repair-associated

cytokines such as MIP-1 α , MIP-1 β , and Rantes, whereas Type II EOC cells may promote tolerance through IL-10 and G-CSF.

Differential effect on naive CD4⁺ T cells

As mentioned above, the ovarian cancer microenvironment is characterized by high number of T regs, which may prevent an anti-tumoral response^{5, 24}. We hypothesized that the high number of T regs present in the tumor is a result of secreted factors generated by the cancer cells. Our next objective is to determine whether the two sub-populations of EOC cells can promote T reg differentiation. Thus, naive CD4⁺ T cells were cultured in either Type I or Type II CM and levels of FoxP3, which is the master regulator of T reg activation and function, was quantified. Our results showed that secreted factors from Type II EOC cells are able to significantly increase the number of FoxP3⁺ cells (24.8% and 71.6% for IL-2 control and Type II CM, respectively). This effect was not observed in naive CD4⁺ T cells cultured in Type I CM (24.8% and 39.4% for IL-2 control and Type I CM, respectively) (Fig. 5A).

Differentiation of naive CD4⁺ T cells to T regs involves TGF β signaling^{25, 26}. Since we observed elevated levels of the three isoforms of TGF β in Type II CM, we hypothesized that the generation of T regs resulted from this occurrence. Thus, to determine the role of TGF β in Type II EOC cell-induced T reg differentiation, naive CD4⁺ T cells were incubated with Type II CM in the presence of a TGF β neutralizing antibody. Figure 5B shows that anti-TGF β can prevent Type II EOC cell-induced upregulation of FoxP3 in CD4⁺ T-cells (41.15% and 27.85% for Type II CM alone and Type II CM plus anti-TGF β , respectively). These data suggest that by secreting TGF β , Type II EOC cells preferentially push naive CD4⁺ T cells to the T reg phenotype.

Discussion

In this study we demonstrate that EOC cells can regulate the differentiation of immune cells. Each sub-population presents a unique cytokine profile, which determines the type of immune cell that can be affected. Interestingly, in spite of the differences, the final outcome is the promotion of a pro-tumor microenvironment. Type I EOC cells are able to enhance macrophages' capacity for tumor repair and renewal by increasing the expression of scavenger receptors and enhancing phagocytic activity, as well as promoting the secretion of cytokines associated with tissue repair. Type II EOC cells are able to create a tolerant microenvironment and prevent an immune response by inducing M ϕ to secrete IL-10 and by promoting the generation of T regs.

Studies that looked into the role of immune infiltrates in patient survival have not always been in concordance. Several studies showed that the magnitude of infiltrating immune cells correlate with better prognosis^{27, 28}. However, other reports claimed that immune cell density is associated with cancer invasiveness and therefore poor prognosis²⁹. Given the plasticity of immune cells and the heterogeneity of the tumor microenvironment, it is possible that the specific phenotype of immune cells in the tumor site would predict survival more than just their mere presence. The profile of cytokines/chemokines in the tumor site depends not only on the phenotype of cells that make up the tumor microenvironment (i.e. neoplastic cells, immune infiltrates, and supporting stroma) but also on their interaction with each other. In this study we demonstrate another level of complexity on tumor-immune interactions. We demonstrate that the sub-populations of EOC cells making up the heterogeneous tumor can differentially affect the process of immune cell differentiation and ultimately its function.

Recently we described the identification and characterization of at least two sub-populations of EOC cells in ovarian tumors. Type I EOC cells have the characteristics of cancer stem cells and are characterized by constitutive NF- κ B activity and constitutive production of pro-inflammatory cytokines^{7, 30}. In contrast, Type II EOC cells present characteristics of terminally differentiated cancer cells such as rapid proliferation and chemosensitivity. These two types of EOC cells express different cellular surface markers, which complicates the process of identifying a common cancer antigen that can be exploited to elicit an anti-tumoral immune response. Along with these antigenic differences, we also found major differences on the types of cytokines that these cancer cells secrete. It was therefore not surprising that the two sub-types of EOC cells exhibited different effects on immune cells.

As part of the innate immune response, monocytes derived from CD34+ myeloid progenitor cells in the bone marrow enter tissues and differentiate into M ϕ . *In vitro*, it has been demonstrated that exposure to IFN- γ with or without LPS, TNF, or GM-CSF can classically polarize M ϕ to the M1 phenotype, which is a potent mediator of immune response against intracellular parasites and even transformed cells^{31, 32}. M1 macrophages are IL-12^{high}, IL-23^{high}, IL-10^{low}, and secrete reactive oxygen intermediates as well as inflammatory cytokines such as TNF and IL-6. On the other hand, M ϕ exposed to either IL-4, IL-13, or IL-10, are polarized to the M2 phenotype^{12, 15, 33, 34}. M2 macrophages are IL-12^{low}, IL-23^{low}, and acquire high levels of scavenger receptors. Transcriptional profiling of human monocyte-macrophage differentiation and polarization showed that under homeostatic conditions, there is a default shift towards the M2 phenotype^{10, 11}.

Previous studies showed that in the tumor microenvironment, M ϕ are preferentially polarized to the M2 phenotype, which has immunosuppressive properties and therefore promote tumor growth¹². Immunostaining of 40 ovarian tumors for markers related to M2 macrophages showed that almost all tumor-infiltrating macrophages are of the M2 phenotype¹⁵. Moreover, co-culture of human macrophages with several ovarian cancer cell lines was associated with the polarization to the M2 phenotype^{35, 36}. We show in this study that Type I EOC cells are able to induce M ϕ to differentiate into a specific phenotype characterized by expression of SR-A1 and upregulated secretion of MIP-1 α , MIP-1 β , and Rantes. In contrast, Type II EOC cells are able to induce M ϕ to secrete significantly higher levels of IL-8, IL-10, and G-CSF.

As part of the adaptive immune response, naive CD4+ T cells can polarize either into Th1 or Th2 cells to elicit an immune response. Later on, it was shown that as a regulatory and feedback mechanism, naive CD4+ T cells can also differentiate into T regs, which can suppress an ongoing immune response. In ovarian cancer, analysis of 104 tumors showed that the presence of CD4+CD25+FoxP3+ T regs are associated with reduced survival^{24, 37}. Moreover, expression of FoxP3 was shown to be a negative prognostic factor³⁸ and intra-epithelial CD8+/T reg ratio was shown to be the strongest predictive factor in patients with ovarian cancer²⁷. In this report, we identified the Type II population as one of the primary mediator of T regs generation in ovarian cancer.

It should be noted that data presented were obtained by culturing the immune cells in cancer CM, and therefore do not reflect any possible additive effect of cell-cell interaction. Moreover, it should also be noted that ovarian tumors are comprised of a dynamic mixture of Type I and Type II EOC cells. Our unpublished data showed that the percentage Type I EOC cells in ovarian tumors can range between 10% to 80%. Therefore, further studies are required to determine how the Type I/Type II ratio can affect the status of the immune system in the tumor microenvironment. Nevertheless, the data presented provide a baseline characterization based on these two subtypes of epithelial ovarian cancer cells. Data

presented may aid in the development of new approaches to enhance tumor rejection and prevent immune-induced tumor progression.

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Figure 1. Effect of cancer CM on M0 morphology. M0 were cultured for 6d in either M-CSF (i), Type I CM (ii), or Type II CM (iii), and morphology assessed by microscopy (A) or flow cytometry (B). Results shown are representative of those obtained from a panel of Type I and II EOC cell cultures.

Figure 2.

Effect of cancer CM on levels of SR-A1 and HLA-DR on M0. M0 were cultured for 6d in either M-CSF, Type I CM, or Type II CM. (A) Levels of SR-A1 were determined by western blot analysis and (B) HLA-DR was quantified by flow cytometry. Results shown are representative of those obtained from a panel of Type I and II EOC cell cultures.

Figure 3.

Cytokine profile of M0 after culturing in cancer CM. M0 were cultured for 6d in either M-CSF (C), Type I CM, or Type II CM and levels of cytokines/chemokines measured in cell-free supernatants as described in the Materials and Methods section. Detected levels in cancer CM was subtracted to obtain levels secreted by M0. Results shown are representative of those obtained from a panel of Type I and II EOC cell cultures.

Figure 4.

Effect of cancer CM on phagocytic activity of M0. M0 were pre-educated with Type I or Type II CM for 24h then loaded with GFP-labeled apoptotic bodies. GFP-fluorescence was quantified by flow cytometry.

Figure 5.
Differential effect of Type I and Type II CM on Foxp3 expression in CD4⁺ naive T cells.
(A) CD4⁺ naive T cells were cultured in the different conditions for 6 days and nuclear

Foxp3 evaluated by flow cytometry; (B) CD4⁺ naive T cells were cultured in Type II CM with or without TGF β inhibitor and nuclear Foxp3 measured by flow cytometry. Results shown are representative of those obtained from a panel of Type I and II EOC cell cultures.

Figure 6.

Proposed model for the role of ovarian cancer cell sub-populations in M0 and CD4+ naive T cells. In M0, Type I EOC cells enhance the capacity for repair while Type II EOC cells induce a tolerant phenotype. In the T cells, Type II EOC cells induce differentiation to Tregs.

Table 1

Differential cytokine/chemokine profile of CD44+/MyD88+ Type I EOC cells and CD44-/MyD88- Type II EOC cells. *n*, not detected. Results shown are representative of those obtained from a panel of Type I and II EOC cell cultures.

Type I EOC cells	Type II EOC cells
GM-CSF	GM-CSF
IFN γ	IFN γ
MIP-1 α	MIP-1 α
Rantes	Rantes
MIF	MIF
IL-6	N
IL-8	N
MCP-1	N
GRO α	N
TNF α	N
n	IL-10
n	IL-12
n	TGF β -1
n	TGF β -2
n	TGF β -3
n	G-CSF
n	VEGF