ORIGINAL ARTICLE

Distinct Subpopulations of Epithelial Ovarian Cancer Cells Can Differentially Induce Macrophages and T Regulatory Cells Toward a Pro-Tumor Phenotype

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Problem
Presence of immune infiltrates in the tumor does not always correlate with an anti-tumoral immune response. We previously identified two subpopulations of epithelial ovarian cancer (EOC) cells with differential cytokine profile. We hypothesize that these two subpopulations 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 from two subpopulations of EOC cells. Differentiation markers and phagocytic activity were 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 subpopulation can induce a unique phenotype of macrophages and T cells, both associated with tumor-supportive function.

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 because solid tumors cannot progress in the absence of a favorable environment, a defective immune response must already be in place upon the establishment of the tumor.[3] 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 subpopulations of EOC cells with differential stemness potential, as well as inflammatory and cytokine profile.[4–6] CD44+/MyD88+ EOC stem cells (type I EOC cells) are the tumor-initiating cells, express pluripotency markers, have a constitutively active NF-κB 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, or 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.[10–12] 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 tolerance.[13]

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 estrogen.[14] Similarly, in ovarian cancer, the majority of TAMs are of the M2 phenotype, and their presence has been associated with tumor progression and chemoresistance.[15] Supporting the pro-tumoral role of macrophages, Tregs are capable of creating a tolerogenic microenvironment, which prevents anti-tumoral immune responses.[5]

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 subpopulation 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, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA, USA).[6] Cancer cell–conditioned media (CM) was obtained by incubating 70% confluent cell cultures in RPMI 1640 supplemented with 1% FBS for 48 hr. Supernatants from these cultures were centrifuged for 15 min at 800 ***g*** and filtered using a 0.45-μm 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 macrophage-colony stimulating factor (M-CSF) (PeproTech, Inc., Rocky Hill, NJ, USA) for 6 day to obtain resting macrophages (Mθ). At the end of day 6, Mθ were either kept in M-CSF or cultured in 50% type I or type II EOC cell CM for another 6 day prior to characterization. For M2-positive control, Mθ were treated for 18 hr 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, BC, Canada) according to manufacturer's instructions. Purity of obtained cell population was verified by flow cytometry. One × 10^6

CD4+ T cells were seeded per well in a 24-well plate pre-coated with 10 μg/mL anti-CD3 and 1 μg/mL anti-CD28 (BD Bioscience, Billerica, MA, USA). 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 7 days prior to characterization. Fresh media and cytokines were added every 2 days. For experiments with TGF-β neutralizing antibody, CD4+ T cells were cultured in IL-2 plus 50% type II CM, plus 5 μg/mL anti-TGFβ (R&D Systems, Minneapolis, MN, USA).

Flow Cytometry

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

SDS–PAGE and Western Blot Analysis

Protein was extracted and measured as previously described.[18] Twenty micrograms of protein was used for SDS–PAGE, and western blots were performed as previously described[19] using goat anti-human SR-A1 (0.2 μg/mL; R&D Systems).

Cytokine Analysis

Levels of cytokines and chemokines were measured from CM or supernatants from Mθ 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 Green Fluorescen Protein (GFP)-Labeled Apoptotic Bodies and Phagocytosis Assay

Green fluorescen protein-labeled EOC cells were treated with 10 μg/mL Phenoxodiol[21] for 24 hr to induce apoptosis. The resulting GFP-labeled apoptotic bodies were collected by spinning culture supernatants for 15 min at 1500 rpm. Mθ obtained by M-CSF as described above were pre-educated with either type I or type II EOC cell CM for 24 hr prior to exposure to GFP-labeled apoptotic bodies. Levels of GFP-positive Mθ 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 type II EOC cells is their differential cytokine profile. Previously, we reported that although both cell types constitutively secrete macrophage inhibitory factor, only type I EOC cells constitutively secrete IL-6, IL-8, MCP-1, and GROα.[7,22] Conversely, vascular endothelial growth factor 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 I. Whereas type I EOC cells mostly secrete pro-inflammatory cytokines (IL-6, IL-8, MCP-1, GROα, and TNFα), type II EOC cells mostly

Table I Differential Cytokine/Chemokine Profile of CD44+/MyD88+ Type I EOC Cells and CD44−/MyD88− Type II EOC Cells

Type I EOC cells	Type II EOC cells
GM-CSF	GM-CSF
IFNγ	IFNγ
MIP-1α	MIP-1α
Rantes	Rantes
MIF	MIF
IL-6	n
IL-8	n
MCP-1	n
GROα	n
TNFα	n
n	IL-10
n	IL-12
n	TGFβ-1
n	TGFβ-2
n	TGFβ-3
n	G-CSF
n	VEGF

n, not detected; EOC, epithelial ovarian cancer; MIF, macrophage inhibitory factor; VEGF, vascular endothelial growth factor. Results shown are representative of those obtained from a panel of type I and type II EOC cell cultures.

secrete immunosuppressive IL-10 and the regulatory cytokines IL-12 and TGFβ. 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, Mθ were obtained *in vitro* by incubating freshly isolated CD14+ monocytes with M-CSF. Afterward, Mθ 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 Mθ controls, the Mθ 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 Mθ 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 Mθ cultured in type I versus type II CM.

To further characterize these macrophages, we determined whether there were any differences in their molecular phenotype by looking at the cell surface markers, scavenger receptor-A (SR-A) and HLA-DR. Mθ cultured with type I CM showed significant upregulation of SR-A compared to Mθ control and Mθ cultured with type II CM (Fig. 2a). There was, however, no significant difference in the level of HLA-DR between the groups (Fig. 2b).

We also characterized the cytokine profile of the Mθ cultured in different conditions. Type I and type II EOC cells generated distinct macrophage phenotypes with different cytokine profile. Fig. 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); Mθ cultured in both types of cancer CM secreted higher levels of GROα, IL-6, and IFN-γ compared to Mθ control (Fig. 3, panel ii); Mθ cultured in both types of cancer CM also secreted higher levels of MIP-1α, MIP-1β, and Rantes compared to control, but the levels of these cytokines are significantly higher in Mθ cultured in type I CM (Fig. 3, panel iii); finally, Mθ cultured in type II CM secreted higher levels of IL-10, IL-8, and Granulocyte colony-stimulating factor (Fig. 3, panel iv).

Because phenotypic differences were observed in Mθ cultured with either type I or type II CM, we then investigated whether there were functional differences. Mθ obtained from different culture

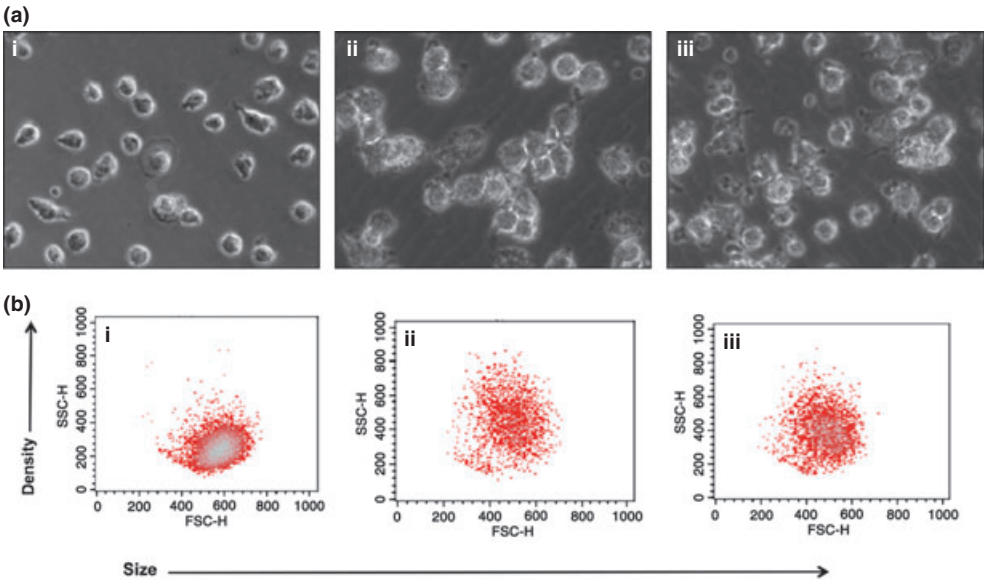

Fig. 1 Effect of cancer conditioned media (CM) on Mθ morphology. Mθ were cultured for 6 days in either M-CSF (i), type I CM (ii), or type II CM (iii), and morphology was assessed by microscopy (a) or flow cytometry (b). Results shown are representative of those obtained from a panel of type I and type II epithelial ovarian cancer cell cultures.

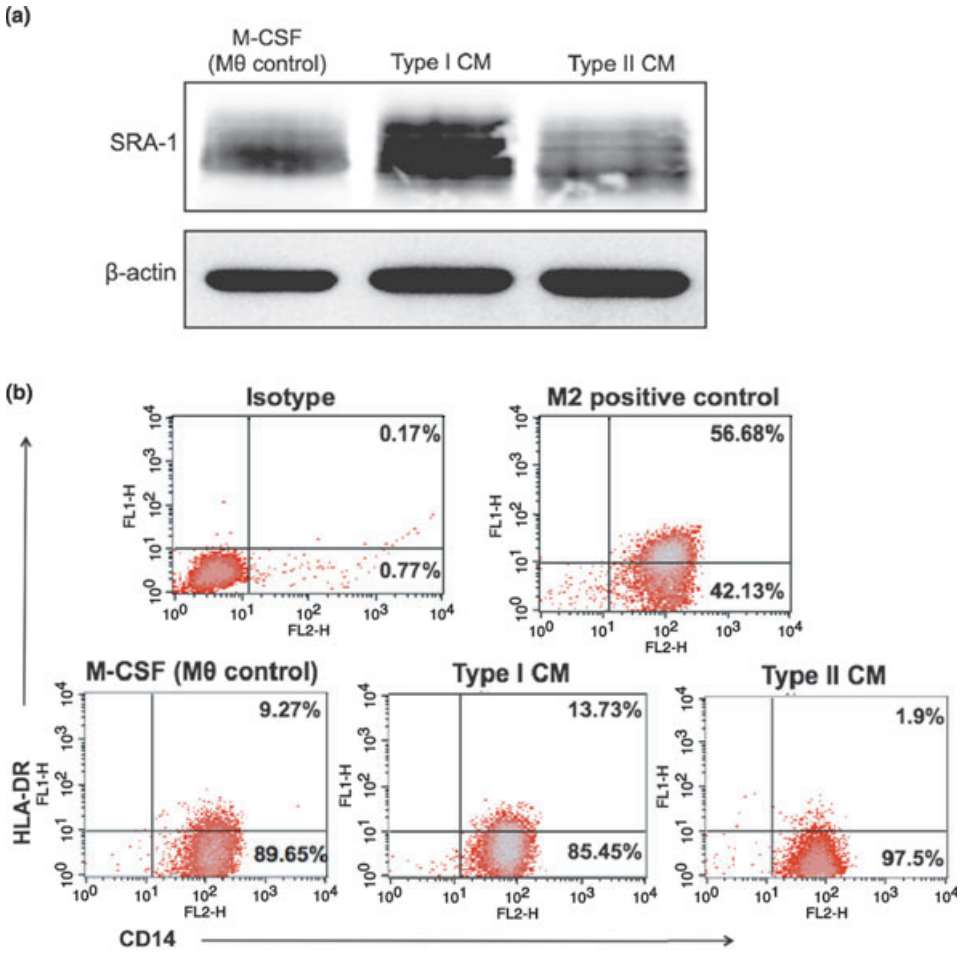

Fig. 2 Effect of cancer conditioned media (CM) on levels of SR-A1 and HLA-DR on Mθ. Mθ were cultured for 6 days in either M-CSF, type I CM, or type II CM. (a) Levels of SR-A1 were determined by western blot analysis, and (b) HLA-DR was quantified by flow cytometry. Results shown are representative of those obtained from a panel of type I and type II epithelial ovarian cancer cell cultures.

conditions were exposed to equivalent amounts of GFP-labeled apoptotic bodies. Flow cytometry analysis showed that Mθ pre-educated with type I CM exhibited enhanced phagocytic activity as evidenced by higher mean fluorescence intensity levels compared to Mθ pre-educated with type II CM (Fig. 4).

Taken together, these data suggest that type I EOC cells may enhance the phagocytic activity of Mθ and its capacity for repair, via upregulation of SR-A and repair-associated cytokines such as MIP-1α, MIP-1β, 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 subpopulations 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, were 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).

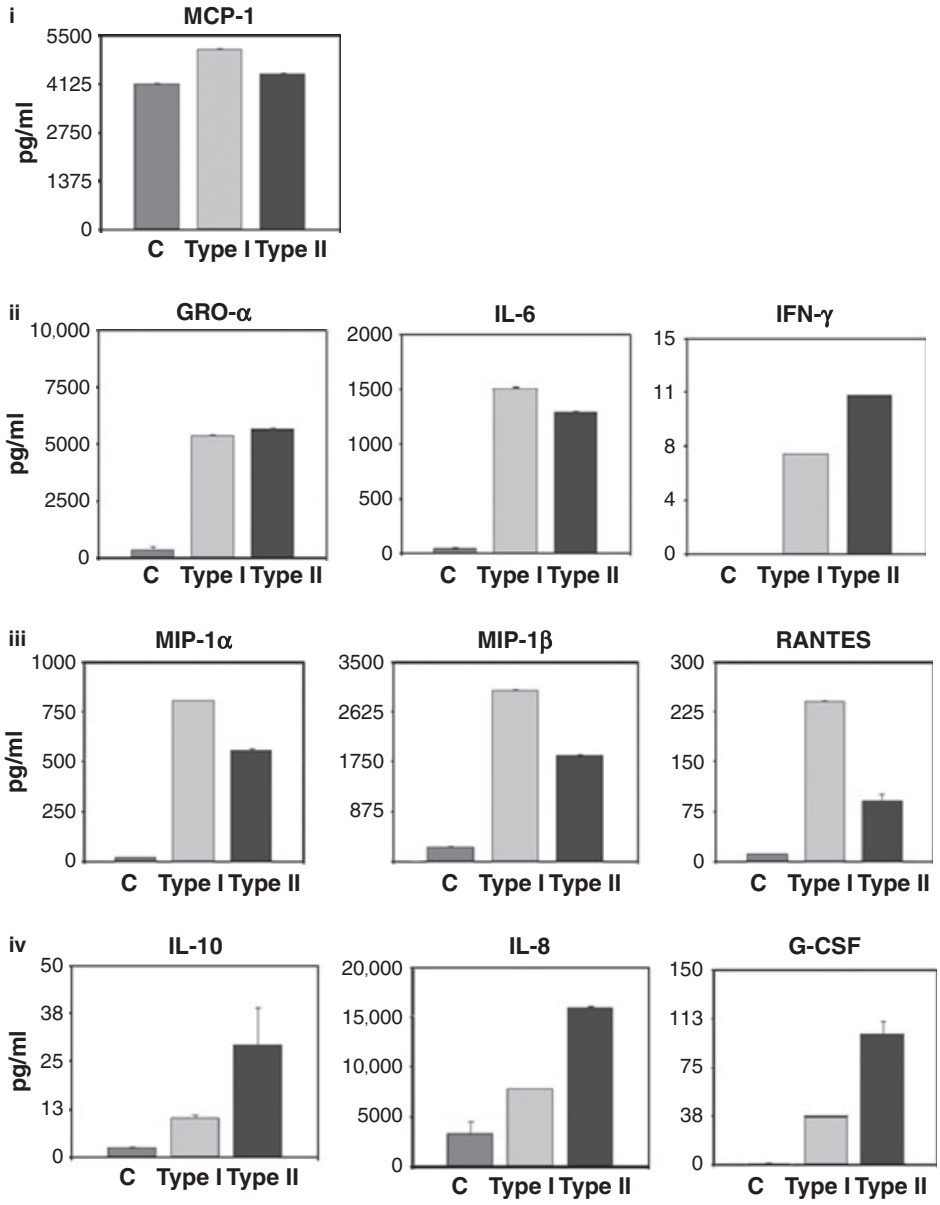

Fig. 3 Cytokine profile of Mθ after culturing in cancer conditioned media (CM). Mθ were cultured for 6 days in either M-CSF (C), type I CM, or type II CM and levels of cytokines/chemokines measured in cell-free supernatants as described in the Materials and methods section. Detected levels in cancer CM were subtracted to obtain levels secreted by Mθ. Results shown are representative of those obtained from a panel of type I and type II epithelial ovarian cancer cell cultures.

Differentiation of naive CD4+ T cells to T regs involves TGFβ signaling.[25,26] As we observed elevated levels of the three isoforms of TGFβ in type II CM, we hypothesized that the generation of T regs resulted from this occurrence. Thus, to determine the role of TGFβ in type II EOC cell–induced T reg differentiation, naive CD4+ T cells were incubated with type II CM in the presence of a TGFβ-neutralizing antibody. Fig. 5b shows that anti-TGFβ 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-TGFβ, respectively). These data suggest that by secreting TGFβ, type II EOC cells preferentially push naive CD4+ T cells to the T reg phenotype.

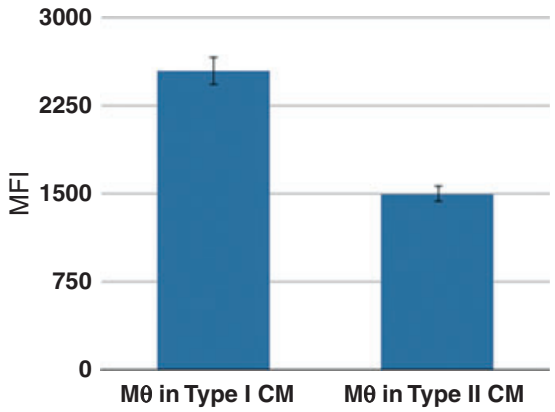

Fig. 4 Effect of cancer conditioned media (CM) on phagocytic activity of Mθ. Mθ were pre-educated with type I or type II CM for 24 hr then loaded with GFP-labeled apoptotic bodies. GFP fluorescence was quantified by flow cytometry.

Discussion

In this study, we demonstrate that EOC cells can regulate the differentiation of immune cells. Each subpopulation 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 Mθ to secrete IL-10 and by promoting the generation of T regs (Fig. 6).

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 prognosis.[29] 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 subpopulations 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 subpopulations of EOC cells in ovarian tumors. Type I EOC cells have the characteristics of cancer stem cells and are characterized by constitutive NF-kB 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 subtypes 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 Mθ. *In vitro*, it has been demonstrated that exposure to IFN-γ with or without LPS, TNF, or GM-CSF can classically polarize Mθ 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}, and IL-10^{low} and secrete reactive oxygen intermediates as well as inflammatory cytokines such as TNF and IL-6. On the other hand, Mθ 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} and 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, Mθ are preferentially polarized to the M2 phenotype, which has immunosuppressive properties and therefore promote tumor growth.[12] Immunostaining of 40 ovarian tumors for markers related to M2 macrophages showed that almost all

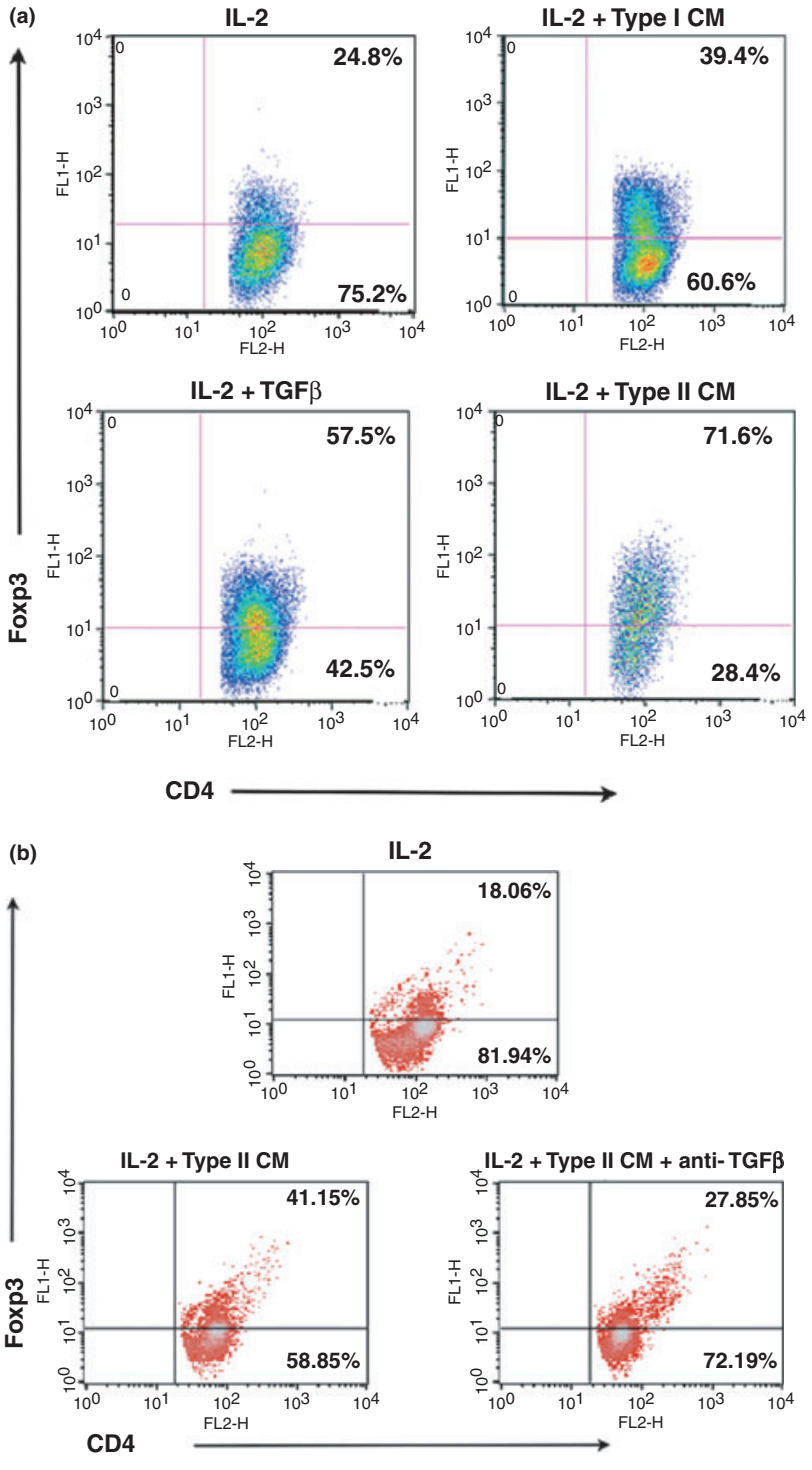

Fig. 5 Differential effect of type I and type II conditioned media (CM) on Foxp3 expression in CD4+ naive T cells. (a) CD4+ naive T cells were cultured in different conditions for 6 days and nuclear Foxp3 evaluated by flow cytometry; (b) CD4+ naive T cells were cultured in type II CM with or without TGFβ inhibitor and nuclear Foxp3 measured by flow cytometry. Results shown are representative of those obtained from a panel of type I and type II epithelial ovarian cancer cell cultures.

tumor-infiltrating macrophages are of the M2 phenotype.[15] 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 Mθ to differentiate into a

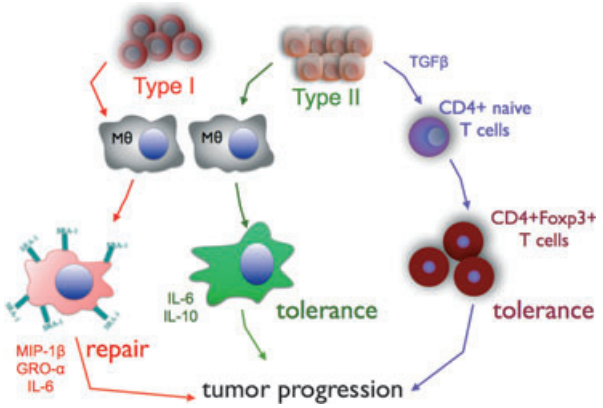

Fig. 6 Proposed model for the role of ovarian cancer cell subpopulations in MΘ and CD4+ naive T cells. In MΘ, type I epithelial ovarian cancer (EOC) cells enhance the capacity for repair, while type II EOC cells induce a tolerant phenotype. In the T cells, type II EOC cells induce differentiation to Tregs.

specific phenotype characterized by the expression of SR-A1 and upregulated secretion of MIP-1α, MIP-1β, and Rantes. In contrast, type II EOC cells are able to induce MΘ 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 into either 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 factor,[38] and intra-epithelial CD8+/T reg ratio was shown to be the strongest predictive factor in patients with ovarian cancer.[27] In this report, we identified the type II population as one of the primary mediator of generation of T regs 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 and 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 EOC cells. Data presented may aid in the development of new approaches to enhance tumor rejection and prevent immune-induced tumor progression.

Acknowledgments

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