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Characterization of Immunosuppressive Properties of Malignant Ascites in Ovarian Carcinoma

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Abstract

Objective: The main objective of the present study was to explore contact-independent immune suppressive mechanisms by humoral factors in malignant ascites from ovarian carcinoma that target effector T cells.

Methods: Flow cytometry was the main method used to detect T cell function as assessed by CFSE-proliferation rate, in the presence of different concentrations of ascites fluid. Cell-free ascites was sometimes pretreated using a biochemical approach. Different antibody inhibitors were used to reverse the ascites-induced inhibition of T cell proliferation.

Results: By culturing T cells in cell-free ascites we demonstrated malignant ascites fluid to be highly immunosuppressive both against autologous and allogeneic T cells (n=6, p<0,001). The inhibitory factor(s) in ascites did not appear to be secreted from the ovarian carcinoma (OC) cells, as transfer of culture media from tumor cells isolated from OC patients or the ovarian cancer cell-line SKOV-3 did not suppress effector T cell functions in vitro. A more detailed characterization demonstrated that the inhibition could not be reversed by targeting potential suppressive mechanisms like IL-6, IL-8, IL-10, CTLA-4, PD-1, B7-DC, B7-H1 or PI3K (n=5). Furthermore, we found the inhibitory factor(s) to be sensitive to proteases and denatured by heat and acetone (n=3).

Conclusion: In conclusion, our data indicate the presence of a yet unknown inhibitory protein factor(s) in malignant cell-free ascites, not secreted by tumor cells, but possibly by immune cells such as regulatory T cells (Tregs).

Keywords: Ovarian carcinoma; Malignancy; Ascites; Tumor microenvironment; Immune suppression; Regulatory T cells; Tumor immunity

Introduction

Ovarian carcinoma (OC) is the sixth most common malignant neoplasm among females and the leading cause of death from gynecological malignancies [1,2]. Regardless of cytoreductive surgery and chemotherapeutic regimens, the overall survival rate remains below 40% [3]. This is mainly due to inherent or acquired drug resistance and the fact that most tumors have developed to an advanced stage at the time of diagnosis [4]. Despite recent years' efforts to make advances in curative treatment, the relapse rate of OC remains disturbingly high, with a subsequent low overall survival rate [5].

Progressing OC spreads from the epithelium of the ovaries into the peritoneal cavity, and leads to production of malignant ascites [6]. The malignant ascitic fluid contains tumor cells in addition to inflammatory and mesothelial cells, thus representing a tumor microenvironment and battle zone that may foster anti-tumor immunity [7]. As the malignant cells extend into the peritoneal cavity, they remain confined in direct contact with the intraperitoneal fluid, both as free-floating tumor cells and embedded in the peritoneal lining [7].

The dynamic interaction between the host immunity and the developing tumor is striking in many malignancies. Activation of adaptive immune cells in response to a tumor may contribute to eradication of malignant cells. In line with this notion, an increased number of tumor-infiltrating T cells have been shown to be associated with a better clinical outcome in a number of different malignancies [8-10]. Furthermore, the type, density, and location of immune cells within the tumor have shown to be a better predictor of patient survival than the histopathological methods currently used to stage for example colorectal cancer [11]. Our recent studiy has revealed increased levels of activated regulatory T cells, known to be suppressive, in malignant ascites [12]. However, developing tumors employ different means to obstruct the action of the immune system by hindering immunological surveillance and providing immunologic escape.

Previous studies have shown that ascites in OC patients is immunosuppressive [13], although the exact mechanism has remained

elusive. During the 1980's, experiments showed that malignant ascites was inhibitory in both murine and human models, and many attempts were made to identify and isolate the suppressive factor(s) [13-20]. Earlier independent assessments have recognized the suppressive substance(s) as haptoglobin, carcinoembryonic antigen (CEA) and α-globulin [16,21,22]. Furthermore, the reported characteristics of the inhibiting factor(s) are heterogeneous; the factor(s) have been reported both as heat-stable and heat-sensitive, both high- and low molecular-weight, and to be partly resistant against trypsin or protease K treatment [13-15,17,20] using different techniques and readouts. More recent studies have applied contemporary methods such as multiplex cytokine array technology [23] and proteomic mass-spectrometry [24] to extensively analyze and characterize the components of ascites. Among the most widely recognized known immunosuppressive constituents of malignant ascites are interleukin 10 (IL-10), interleukin 6 (IL-6), and interleukin 8 (IL-8). Furthermore, regulatory T cells (Tregs) secrete prostaglandin E2 (PGE2) [25,26] and over-express cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) as part of their suppressive mechanisms, and their abundant presence in malignant ascites from OC patients correlates inversely with survival [27]. Another important signaling molecule in this regard is phosphoinositide 3-kinase-delta (PI3-K) which is a known mediator of the PGE2 pathway. Furthermore, B7 molecules and programmed cell death protein-1 (PD-1) are known to be present in malignant ascites and they are also potential and established targets for immune modulating therapies [28-31].

The aim of the present study was to further characterize the suppressive effect of cell-free ascites fluid from OC patients using modern techniques such as carboxyfluorescein succinimidyl ester (CFSE)-proliferation assays. By distinguishing inhibitors of effector T cell function, our intent was to identify any humoral factor(s) in the tumor microenvironment which may inhibit the clinically important anti-tumor immune responses mediated through T cells.

Material and Methods

Patients and clinical study control

The study was approved by the Regional Ethical Committee (REC) and written informed consents were obtained from all patients. Ascites fluid and blood samples were acquired in a prospective, nonselective fashion from patients referred for primary surgery to the Haukeland University Hospital, Bergen, Norway and Oslo University Hospital, Radium Hospital, Oslo, Norway. Only patients with advanced adenocarcinoma (International Federation of Gynecology and Obstetrics (FIGO) stage III-IV) were included. Buffy coats from healthy blood donors (Oslo University Hospital, Blood Bank) were used as controls, to acquire peripheral blood mononuclear cells (PBMC).

Reagents and antibodies

Unless otherwise stated, cells were cultured in Roswell Park Memorial Institute (RPMI)1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (TCS Cellworks), 100 U/ML penicillin/streptomycin, 1 mM sodium pyruvate and 1:100 nonessential amino acids (referred to as complete medium).

The following antibodies were used for flow cytometry: CD3 allophycocyanin (APC), CD 4 peridinin-chlorophyll-protein (PerCP), CD3 PerCP (BD BioSciences Pharmingen, New Jersey, USA). Inhibitors used in the present study were against CTLA-4, PD-1, B7-homolog 1 (B7-H1), B7-DC (eBiosciences), PGE2 (Cayman Chemicals), IL-10, IL-8, IL-6 (R&D Systems) and PI3K-delta (IC87114; Sigma Aldrich). Cells were stained for analysis of cell surface markers, according to the manufacturer's instructions, and they were washed twice in phosphate-buffered saline (PBS) containing 2% FCS prior to data acquisition.

Isolation of cells (from blood and ascites)

The ascites was filtered through a 40 μM mesh to remove tissue remains and centrifuged at 350×g in room temperature for 10 min. The cell-free supernatant was separated and frozen in Nunc™ CryoTube vials at -80°C, until being used. The cells were washed twice in phosphate-buffered saline (PBS) before being directly fixed in Buffer A (Human FoxP3 Buffer Set, BD Biosciences) and stored at -80°C until analysis.

Human peripheral blood CD3+ T cells were purified by negative selection using a RosetteSep enrichment kit (StemCell Tecnologies), followed by Isopaque-Ficoll (Lymphoprep Nycomed Pharma AS, Oslo) gradient centrifugation, or by Isopaque-Ficoll gradient centrifugation alone (PBMC). The cells were routinely analyzed by flow cytometry and the purity of the PBMC and CD3+ T cell populations was consistently over 98%.

Culture of adhesive cells from malignant ascites and SKOV-3 cell-lines

The cell pellet isolated from ascites containing OC cells (based on EpCam staining by flow cytometry) was plated and incubated in Dulbecco's modified eagles medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% FCS and 100 U/ML penicillin/streptomycin, for 72 hours at 37°C and 5% CO_2. The supernatant from these cultures was isolated by centrifugation at 300×g in room temperature for 10 min.

SKOV-3 ovarian cancer cell-lines were cultured a similar manner, in complete medium for 72 hours at 37°C and 5% carbon dioxide (CO_2). The supernatant from cultures was isolated by centrifugation at 300×g in room temperature for 10 min.

Pre-treatment of cell-free ascites fluid

Ascites was heated by incubation in a Techne Dri-Block® DB-2D at 70°C for 30 min or at 100°C for 10 min. Ascites was proteolysed by incubating ascites supernatant with 0.25% trypsin for 4 hours at 37°C. The reaction was stopped by adding heat-inactivated FCS. Alternatively, protein digestion in the ascites fluid was accomplished by incubating ascites with 200 μg/ml Proteinase-K for 60 min at 37°C, and inactivation at 100°C for 15 min.

In addition, ascites was precipitated with acetone that was kept at -20°C overnight, and added in a 4:1 ratio to the ascites. The fluid was vortexed and incubated at -20°C over-night, then centrifuged at 13.000×g for 15 min. The supernatant was discarded, and the protein pellet dissolved in PBS. Lastly, we included a combined pre-treatment with heat-inactivation at 70°C for 30 min, followed by acetone precipitation, adding the supernatant to ascites fluid.

Proliferation assays

For proliferation assays, the purified cells were resuspended in RPMI 1640 and labelled with 4 μM CFSE for 10 min at 37°C. Labelling was quenched with complete medium and the cells were washed twice.

Purified and CFSE-labelled cells were diluted to 1×10^6 cells/ml in complete medium and different concentrations of cell-free ascites, and stimulated (T cell activation/Expansion kit; Miltenyi Biotec) at a bead to cell ratio of 1:2. Cells were then cultured for 96 hours at 37°C and 5% CO_2.

After 96 hours stimulation, cells were stained for analysis of cell surface markers, according to the manufacturer's instructions. Cells were washed twice in PBS containing 2% FCS and cell division was assessed by CFSE dilution. The FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) was used for data acquisition and FlowJo 8.8.6 for analysis of the data.

Statistical analysis

Data are presented as mean ± SD and were analysed by two-sided Mann-Whitney test using Excel 2010 (Microsoft Office) and Graphpad Prism software (version 6; Graphpad Software, La Jolla, CA, USA).

Results

Suppressive activity of cell-free ascites from ovarian carcinoma patients

To analyze the effect of ascites from patients with OC on T cell immune responses, we examined the ability of cell-free ascites to suppress in vitro proliferative responses of T cells. As shown in Figures 1a and 1b, ascites from OC patients was able to suppress autologous T cell proliferation in a concentration-dependent manner as analyzed by CFSE-proliferation assay of anti-CD3/anti-CD2/anti-CD28 activated PBMC. Adding ascites to the PBMC at a 1:50 dilution reduced T cell proliferation by 30%, whereas dilutions of 1:10 and less reduced T cell proliferation by 50%. To further examine the suppressive activity of ascites, we performed a similar assay using CFSE-labelled PBMC from healthy blood donors, showing that ovarian cancer-derived ascites also inhibited allogeneic T cell proliferation in a concentration-dependent manner (n=6, p<0.005; Figures 1c and 1d). These results show that ascites from patients with OC is highly immunosuppressive both against autologous and allogeneic T cells and may contain soluble immunosuppressive factor(s) (Figure 1).

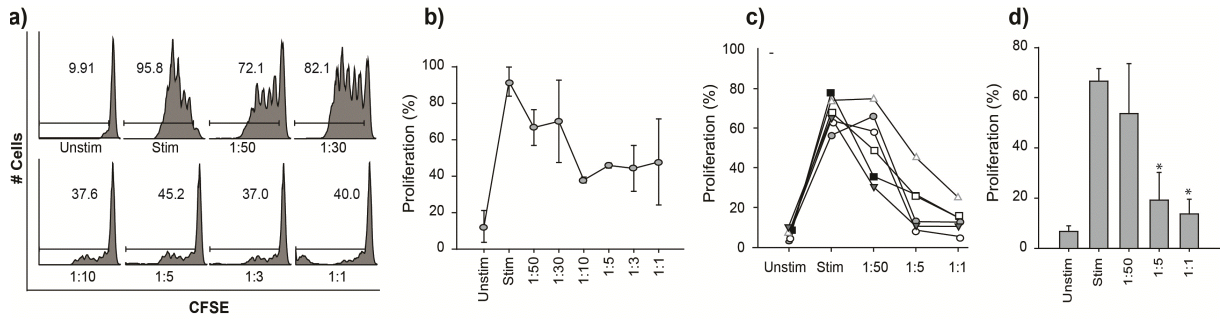

Figure 1: Cell-free ascites from ovarian carcinoma patients inhibit proliferation of T cells in a concentration-dependent manner. a) PBMCs were stimulated for 72 hours with anti-CD3/anti-CD2/anti-CD28-coated beads in presence of different concentrations of cell-free ascites. Cells were stained with CFSE for assessment of T cell proliferation. Flow cytometry histograms of CFSE fluorescence intensity as measure of proliferation of autologous PBMC at different dilutions of cell-free ascites (1:50, 1:30, 1:10, 1:5, and 1:3, 1:1) are shown for two patients. (b) Average proliferation rate of autologous PBMC in the presence of indicated dilutions of cell-free ascites as in A (mean ± half range; n=2). (c) Individual proliferation rates of CD3/CD2/CD28-stimulated allogeneic PBMC from 6 different donors in the presence of indicated dilutions of cell-free ascites (CFSE assay as described above). (d) Amalgamated data from C showing average proliferation rate (CFSE-assay; mean ± standard deviation; n=6) at three dilutions of ascites (1:50, 1:5*, 1:1*), *p<0.005 determined by two-tailed Mann-Whitney test.

Ovarian cancer tumor cells do not appear to secrete the inhibiting factor(s)

To further investigate the suppressive properties of OC ascites, we next compared the immune inhibitory effect of cell-free ascites from an OC patient with i) that of cell-free supernatant produced by adherent cells isolated from the same ascites cultured in vitro and ii) cell-free supernatant produced by ovarian cancer cell-line SKOV-3. As shown in Figure 2, ascites from the OC patient again suppressed allogeneic T cell activity in a concentration-dependent manner. However, neither the supernatant produced by the cancer cells from the same ascites, nor that from SKOV-3 cell cultures, attenuated or augmented the allogeneic T cell proliferation (Figure 2).

Inhibition of immunosuppressive cytokines known to be present in ascites did not reverse the immune suppression

The immunosuppressive properties of ascites supernatant on autologous CD3+ T cells proliferation was not reversed in the presence of inhibitors to PI3K and blocking antibodies against CTLA-4, PD-1, B7-H1, B7-DC, IL-10, PGE-2, IL-8 and IL-6 (Figure 3). However, there was a tendency that anti-PGE2 to some extent increased the proliferation, although not significantly (Figure 3).

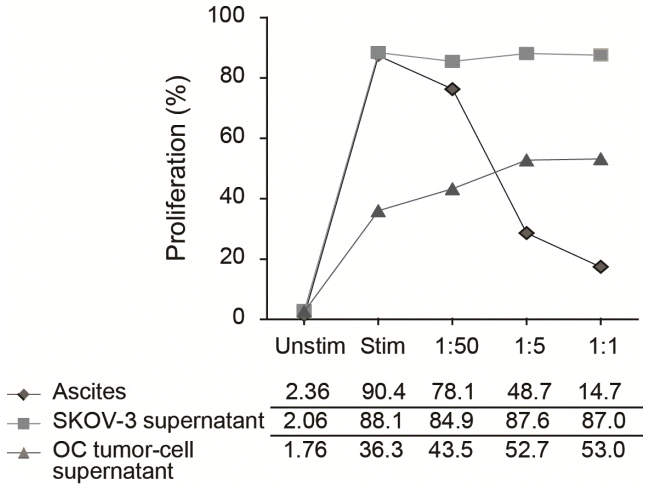

Figure 2: Cell-free culture supernatant from ovarian carcinoma tumor cells and SKOV-3 cell-lines do not inhibit proliferation of T cells in a concentration-dependent manner. Average proliferation rates of allogeneic CD3+ T cells (n=3) at different dilution of cell-free ascites, cell-free SKOV-3 culture supernatant or cell-free ovarian cancer culture supernatant analyzed as in 1A).

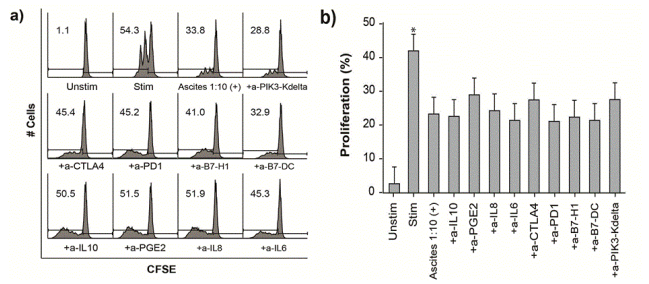

Figure 3: Inhibitors against molecules known to be present in malignant ascites do not reverse ascites-induced T cell suppression. (a) CD3+ T cells from a blood bank donor was stimulated with anti-CD3/anti-CD2/anti-CD28-coated beads in the presence of cell-free ascites (1:10 dilution) with addition of inhibitor against PI3-K, and the following blocking antibodies: anti-CTLA-4, anti-PD-1, anti-B7-H1, anti-B7-DC, anti-IL-10, anti-PGE-2, anti-IL-8 and anti-IL-6. (b) Amalgamated data from A showing average proliferation rate (CFSE-assay; mean ± standard deviation; n = 5) in the presence of ascites and different inhibitors; *p<0.05 determined by two-tailed Mann-Whitney test.

Identification of the properties of an inhibiting factor(s) using a biochemical approach

In order to further map properties of the inhibiting factor(s), we subjected cell-free ascites from ovarian cancer patients to different treatments (Figures 4a and 4b). The pre-treated ascites (n=3) was next added at different dilutions to CFSE-labelled allogeneic PBMC, and flow cytometry was used to investigate the effect on T cell proliferation.

As shown in Figure 4a, suppressive function of the ascites was fully or partially lost when ascites protein factors were precipitated by acetone (precipitate examined), ascites subjected to proteinase K or trypsin proteolysis or denatured at 70°C or 100°C. When activated by anti-CD3/anti-CD2/anti-CD28-coated beads, 70% of the cells in PBMC proliferated (T cell fraction of PBMC). However, cell-free ascites suppressed this to 30%. The inhibitory effect of ascites was most efficiently alleviated by acetone precipitation (proteins and supernatant examined) and proteinase K proteolysis, reversing proliferation back to 60% and 66%. Heat treatment partly abolished suppression, with a proliferation rate of 54%. Trypsination at 1:5 dilutions relieved the suppression from 30% to 46%.

A further investigation uncovered that the combined treatment of heat denaturation at 70°C, followed by acetone precipitation (supernatant examined), completely reversed inhibition (Figure 4b). At three different dilutions (1:50, 1:5 and 1:1) the treated ascites produced resulted in a proliferation equal to that of the activated PBMC control (Figure 4).

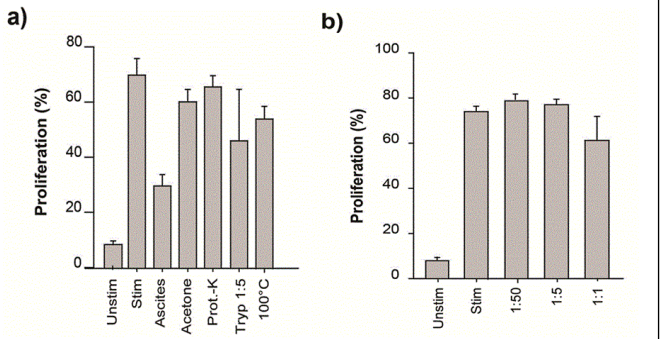

Figure 4: Characterization of the inhibiting factor(s) in ascites from ovarian cancer patients. (a) Ascites from three patients were subjected to different treatments in an attempt to map out the properties of the inhibiting factor(s). Ascites was precipitated with acetone (precipitate examined), incubated with proteinase-K or trypsin (1:50 and 1:5 dilution) or heat-inactivated at 70°C for 30 min or at 100°C for 10 min. The treated ascites (different rows) was next added at three groups of dilutions (1:50, 1:5 and 1:1) to anti-CD3/anti-CD2/anti-CD28 activated allogeneic PBMC, which were subsequently analyzed in a CFSE proliferation assay. Amalgamated data from the 1:5 dilution of ascites (mean ± SD, n=3), presented as a histogram. (b) Ascites from three patients were subjected to heat-treatment at 70°C followed by acetone precipitation (supernatant examined). The heat-denatured, acetone supernatant was then added at three different dilutions (1:50, 1:5 and 1:1) to anti-CD3/anti-CD2/anti-CD28 activated allogeneic PBMC, which was subsequently analyzed in a CFSE proliferation assay. Data (mean ± SD, n=3) presented as a histogram.

Discussion

Patients with advanced stage of OC have poor survival. The degree of suppression of anti-tumor immune activity plays an important role in this context. This study on malignant ascites from OC patients confirms and extends on earlier observations regarding its immunosuppressive properties [16,19]. Inhibitory activity in cell-free ascites was observed against both autologous and allogeneic T cells in a

concentration-dependent manner. This suggests that the ascites-induced T cell suppression is mediated by a soluble factor, and not by contact-dependent inhibition as observed for some Treg subsets [32]. Furthermore, our results show that the ascites-mediated suppression is acting both on self and non-self T cells, indicating that it is a global and not antigen-dependent inhibition.

Another interesting finding was that the OC cells did not secrete the suppressive, soluble factor(s) as evident from parallel experiments with supernatant from cultures of ascites-derived tumor cells and SKOV-3 cell-lines. This may indicate that this humoral tumor immune-suppresion is mediated by a factor(s) secreted by cells other than tumor cells suspended in ascites. One prevalent hypothesis is that the presence of Tregs in ascites accounts for immune inhibition. Tregs are abundant in malignant ascites, and they are known to inhibit T cell function and activity [33]. The suppressive function of Tregs has been shown to be a combination of humoral factors and direct cell-to-cell contact [32,34,35]. The abundance of Tregs in OC ascites, and their known suppressive function, suggests that Tregs may secrete suppressive factor(s) in ascites. Alternatively, such an immunosuppressive factor may originate from the solid tumor itself, the surrounding tumor periphery or the mesothelial lining of the peritoneal cavity.

Our attempt to reverse the ascites supernatant induced T cell inhibition through blocking of known immune modulating substances known to be present in ascites, including IL-6, IL-8, IL-10, PGE2, PI3K, CTLA-4, PD-1, B7-H1, B7-DC, were unfortunately to no avail, as none of the blocking antibodies contributed to a significant reversal of ascites-induced inhibition. However, blocking of PGE2 showed some tendency to reversal, which is interesting since secretion of these molecules are part of the Treg suppressive repertoire. It is possible that several molecules working synergistically confer the inhibitory effect, so that a combined blocking must be attempted to accomplish a significant attenuation of ascites-induced immunosuppression.

In further efforts to distinguish the immune inhibitory constituent(s), we discovered that it was heat-sensitive. Heating at 70°C for 30 min and 100°C for 10 min, reversed inhibition almost completely, and indicates that the suppressive factor is denatured by heat. This is in contrast to previous studies that revealed that the suppressive factor is heat stable [13,14,20]. However, Sheid and Boyce [15] found that the suppressive factor was heat-stable at 70°C for 10 min, but unstable at 100°C for 15 min, more in line with our own findings.

Further characterization also revealed that the factor is cleaved by proteinase K and trypsin. After treatment with the two proteases, suppression of T cell proliferation was reversed completely (proteinase K) and partially (trypsin). This was repeated in 3 patients, showing similar results. These findings are in accordance with previous findings that showed that the inhibitory substance(s) was partially resistant to prolonged proteinase K and trypsin treatment [36]. On the contrary, Medoff et al. demonstrated that suppressive activity was not affected by proteolytic enzyme digestion, suggesting that it was most likely not a peptide [37].

Finally, our findings show that the suppressive factor is not active after acetone precipitation and re-suspension of the precipitate. We also examined the supernatant after acetone precipitation, but could not find a suppressive activity. In contrast, Medoff et al. found that the suppressive factor remained soluble in 5% trichloroacetic acid (TCA) after purification, showing a 95-fold increase in specific activity.

Importantly, earlier reports have looked at the immunosuppressive properties of malignant ascites in inhibiting cell lysis and proliferation of peripheral blood mononuclear cells to phytohemagglutinin, while we have here looked at immune regulation of effector T cell proliferation by CFSE-dilution assays, which is the standard method to assess Treg and T cell function. This may in itself account for differences both with respect to the active inhibitory factor as well as to the sensitivity of the immune function read-out system.

Ovarian cancer patients with advanced disease are initially highly responsive to surgery and platinum- and taxane-based chemotherapy, but the majority succumbs to recurrent disease that is resistant to further treatment [38]. Despite efforts over the past decade to cure OC, the advances in treatment has done little do reduce the overall survival rate, and established therapies fail to induce a cure at diagnosis [39]. Novel therapies mainly focus on immunoediting, specifically targeting immune modulating factors such as Tregs. In our study, we looked closer at a potential suppressive, soluble factor in malignant ascites. Through characterization of the properties of the inhibiting factor(s) we hope to come one step closer to recognizing it. Identification of such an inhibitory factor(s) may again create a potential for immune modulating treatment in advanced ovarian carcinoma.

Conflict of Interest

The study sponsors had no pertinent role in any aspect of data handling or submission of manuscript. No financial or personal conflict of interest is reported by any co-authors.

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