Stress hormones are associated with inflammatory cytokines and attenuation of T-cell function in the ascites from patients with high grade serous ovarian cancer

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Mounting evidence suggests that chronic stress and subsequent distress can promote ovarian cancer progression. These altered psychological states have been linked to sustained release of stress hormones, activation of the β-adrenergic receptors in ovarian cancer cells, and induction of pro-tumoral signaling pathways. In addition, data suggest that chronic stress promotes an inflammatory landscape highlighted by increased infiltration of tumor-associated macrophages into the ovarian tumor microenvironment (TME). In ovarian cancer, ascites is a unique TME comprised of tumor, and immune cells, which secrete pro-tumoral cytokines and chemokines that modulate tumor-associated immunity. However, our knowledge about how stress hormones impact the ascites TME remains limited. We hypothesized that the ascites harbors measurable levels of stress hormones, and accumulation of these in the ascites generates a pro-tumorigenic, inflammatory, and immunosuppressive TME. We evaluated ascites samples from 49 patients with high grade serous ovarian cancer (HGSOC) and quantified cortisol and stress hormones metabolites, metanephrine (MN), and normetanephrine (NMN) in all samples. We also measured 38 individual cytokines in the ascites, including several pro-inflammatory cytokines, such as IL-6, which were positively correlated to MN or NMN levels of those samples. Conversely, we found cortisol levels were negatively correlated to several pro-inflammatory cytokines. As T-cells are integral to the TME and our analyses identified cytokines in the ascites known to modulate T-cell function, we characterized ascites-derived T-cells and assessed the impact of stress hormones on the T-cell phenotype. Our data show an altered CD4+ /CD8+ T-cell ratio and a heterogeneous expression of exhaustion markers in T-cells from the ascites, while ascites-derived CD8+ T-cells exposed to epinephrine had decreased co-expression CD38 and Granzyme B. To extend these findings to animal models, we subjected ovarian cancer-bearing mice to daily restraint stress, which resulted in increased tumor growth in two models. Congruent with our human analyses, we detected corticosterone, MN, and NMN in the ascites from tumor-bearing mice, and these stress hormones correlated with several inflammatory cytokines. Moreover, daily restraint stress leads to increased CD4+ PD-1+/CD8+ PD-1+ T-cell ratio in the ovarian tumor
## 1. Introduction

Ovarian cancer is one of the most lethal gynecologic malignancies and the fifth leading cause of cancer-related deaths among women in the United States (Siegel et al., 2022). The five-year survival rate remains below 50% for patients with high grade serous ovarian cancer (HGSOC) patients, the most frequently diagnosed type of epithelial ovarian cancer (Siegel et al., 2022). This low survival rate is partly attributed to a lack of screening tests leading to patients presenting with advanced disease. Platinum-based chemotherapy and surgery are mainstays of frontline treatment; however, most patients will recur and develop chemo-resistant disease (McCloskey et al., 2018; Mittica et al., 2016).

One clinical manifestation for some patients with advanced stage or recurrent ovarian cancer is the development of ascites in the peritoneal cavity (Huang et al., 2013). Patients who develop a large volume of ascites may require paracenteses over time to palliate symptoms, including distension, early satiety, bowel dysfunction, and pain (Ahmed & Stenvers, 2013). Several liters may be removed during paracentesis, and usually, the ascites is discarded. Treating the underlying cancer with cytoreductive surgery (at the time of diagnosis) and/or systemic therapy contributes to tumor shrinkage in most patients and can lead to a reduction in ascites burden over time (Chang et al., 2015). However, although therapeutic treatment of ovarian cancer does not typically focus on ascites alone, ascites has been associated with worse clinical outcomes (Ford et al., 2020). The ascites tumor microenvironment (TME) contains tumor, immune and mesothelial cells along with pro-inflammatory cytokines and soluble factors that influence tumor cell growth, inflammatory processes, and immunosuppression (Ahmed and Stenvers, 2013; Giuntoli et al., 2009). Further understanding of this often-discarded biological sample could identify cells and soluble factors which directly impact disease progression.

Another contributing factor associated with worse outcomes in patients with cancer is chronic stress and subsequent distress, often resulting from a newly diagnosed cancer and its treatment (Lutgendorf and Andersen, 2015). Chronic stress induces the activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary axis (HPA). The sustained release of stress hormones such as catecholamines (norepinephrine [NE] and epinephrine [Epi]) and cortisol in response to chronic stress are known to modulate immune cell populations, such as T-cells and macrophages in several solid tumors (Armaiz-Pena et al., 2013; Colon-Echevarria et al., 2019). In orthotopic mouse models of ovarian cancer, chronic stress leads to tumor-associated inflammatory processes and disease progression (Armaiz-Pena et al., 2015; Colon-Echevarria et al., 2020). In patients with cancer, behavioral disorders such as stress, depression, and anxiety can induce systemic and tumor-associated immunosuppression (Andersen et al., 1998; Blomberg et al., 2009; Lutgendorf et al., 2008; Segerstrom and Miller, 2004; Thornton et al., 2007). For example, high levels of perceived stress were associated with a decreased Th1/Th2 cytokine production ratio, a decline in T-cell proliferation, and decreased tumor-infiltrating lymphocyte activity (Andersen et al., 1998; Blomberg et al., 2009; Lutgendorf et al., 2008; Lutgendorf et al., 2005; Thornton et al., 2007). However, key questions remain as to whether or not ascites harbor stress hormones and if they can influence the ovarian immune TME.

Tumor growth and disease progression are associated with the evasion of anti-tumor responses (Grywalska et al., 2019). Immune evasion by the tumor is achieved by activating immune checkpoint pathways to support self-tolerance and prevent autoimmunity (Grywalska et al., 2019). Immune checkpoint inhibitors, such as those targeting PD-1/PD-L1, exploit this interaction by competitively binding to their targets (either PD-1 or PD-L1) (Abdin et al., 2018; Marinelli et al., 2019), thus restoring the anti-tumor immunity and T-cell function. The incorporation of checkpoint blockade has significantly impacted outcomes for patients with many solid malignancies, including lung cancer and melanoma (Sharma et al., 2021). However, response rates for patients with epithelial ovarian cancer have been modest, and as a result, they are not routinely incorporated as the standard of care (Marinelli et al., 2019). Clinical trials evaluating the incorporation of checkpoint inhibitors in the frontline for epithelial ovarian cancer have terminated early or failed to meet the primary endpoint (Monk et al., 2021; Moore et al., 2021). Thus, there is an urgent need to uncover the underlying mechanisms that promote tumor-associated inflammation that could contribute to sub-optimal responses to checkpoint blockade and subsequent resistance.

This study aimed to establish the presence of stress hormones in the ascites from patients with HGSOC and their contribution to inflammation and T-cell response in the TME. To accomplish this goal, we measured stress hormone levels from ascites samples, which were then subjected to a comprehensive cytokine panel, and isolated ascites-derived T-cells. In addition, we utilized well-characterized syngeneic mouse models of ovarian cancer to study the contribution of chronic stress to these processes. Overall, our data suggest a role for ascites-derived stress hormones in inflammatory and immunosuppressive processes that could impact T-cell function and responses to immunotherapy.

## 2. Materials and methods

### 2.1. Study cohort and ascites sample processing

The study’s cohort consisted of patients diagnosed with primary and recurrent epithelial ovarian cancer (Table 1). Samples with histological subtypes other than high grade serous ovarian carcinoma (HGSOC) were excluded from this study. Ascites samples from patients with HGSOC were obtained after informed consent. Ascites were collected by paracentesis, and ascites not required for clinical care were centrifuged at 350 RPM for 10 min, and stored at −150 °C in aliquoted vials of 10–20 million cells per mL in 90% FBS/10% DMSO cryopreservation solution. In samples with heavy red blood cell (RBC) contamination, RBC lysis was performed using BD PharmLyse before freezing. All ascites sample collection and analysis protocols were approved by local institutional review boards at Duke Medical Center and Ponce Health Sciences University.

### Table 1

Clinical and pathological.

| Characteristics of Study Participants (n = 49) |   |
|---------------------------------------------|---|
| Age at diagnosis (years)                   | Median 64 Range 43–83 |
| Stage (FIGO)                               | II  2 (4.0%) III 35 (71.4%) IV 7 (14.3%) |
| Histologic subtype                         | High grade serous 49 (100%) |
| Recurrence status                          | Primary 25 (51%) Recurrent 22 (45%) |
| Chemotherapy sensitivity status            | Sensitive 40 (40%) Resistant 9 (18%) |

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Ahmed, A. N., Aquino-Acevedo, D., ... Zorn, J. (2021). This study aimed to establish the presence of stress hormones in the ascites from patients with HGSOC and their contribution to inflammation and T-cell response in the TME. To accomplish this goal, we measured stress hormone levels from ascites samples, which were then subjected to a comprehensive cytokine panel, and isolated ascites-derived T-cells. In addition, we utilized well-characterized syngeneic mouse models of ovarian cancer to study the contribution of chronic stress to these processes. Overall, our data suggest a role for ascites-derived stress hormones in inflammatory and immunosuppressive processes that could impact T-cell function and responses to immunotherapy.
2.2. Cytokine multiplex assay

Individual cytokine levels of ascites samples from patients with HGSOC or tumor-bearing mice were quantified using a human multiplex bead-based kit (Milliplex MAP human (MCYTOMAG-60K) containing 38 pro-inflammatory cytokines or a murine 32 pro-inflammatory cytokine panel (MCYTOMAG-70K cytokine/chemokine panel HCYTOMAG-60K; Millipore Sigma). Cytokine panels were utilized following the manufacturer’s established protocols. Samples were normalized by volume and assayed in technical duplicates. Data represent the average concentration of the technical duplicates for each cytokine per sample.

2.3. Enzyme-linked immunosorbent assay

Levels of cortisol (LOD: 0.2–10 ng/mL; R&D Systems, #KGE008B), corticosterone (LOD: 0.1–25 ng/mL; R&D Systems, #KGE009), and stress hormone metabolites, metanephrine (20-3,000 pg/mL), and normetanephrine (20-3,000 pg/mL; DLD Diagnostika GMBH, #EA612/92) in ascites samples were quantified by ELISA following each manufacturer’s protocol. Samples were assayed in technical duplicates, and data represent the average concentration of each stress hormone per sample.

2.4. Flow cytometry assay

To assess functional and inhibitory markers, 2 × 10⁶ ascites cells were plated in a 96-well plate in RPMI +10% FBS. Cells were initially stained with Zombie NIR (Biolegend) for 15 min at room temperature. Next, the cells were surface stained with 50 μL of a cocktail mix consisting of fluorescent conjugates for CD28 (CD28.2, Biolegend), CD45RA (HI100, Biolegend), TIM-3 (F38-2E2, Biolegend), PD-1 (EH12.2H7, Biolegend), CCR7 (G043H7, Biolegend), and Lag-3 (3D5223H, eBio-science), CD38 (HB-7, Biolegend), CTLA-4 (L3D10, Biolegend), ICOS (C398.4A, Biolegend) for 25 min at 4 °C. Following cell surface staining, cells were treated with cytofix/cytoperm (BD Biosciences) following the manufacturer’s recommendations. Intracellular staining was then performed for 30 min at 4 °C using fluorescent antibodies against Ki-67 (Ki-67, Biolegend), CD4 (RPA-T4, Biolegend), CD3 (SK7, BD), CD8 (SK1, BD). For hormone-related studies, Granzyme B (QA16A02, Biolegend) was included in the intracellular staining cocktail. Cells were fixed with 1% paraformaldehyde and acquired on a Fortessa flow cytometer (BD Biosciences).

2.5. Experimental design for animal studies

We utilized 8–12-week-old C57/BL6 female mice. Breeding pairs were acquired from Taconic Laboratories, bred, and housed at the Ponce Research Institute Animal Facility (70 °F and 50–55% humidity). All experiments were approved by the Institutional Animal Care and Use Committee at Ponce Health Sciences University. The investigator handled all mice one week before the start of each experiment. To induce stress in mice, we utilized a restraint stress model that consisted of physical restraint and limiting moving space for 2 h daily until the end of the experiment (Colon-Echevarria et al., 2020). While mice in the experimental group were subjected to restraint stress, mice in the control group were restricted from food and water access. Mice not subjected to restraint were used as controls. Three days after restraint stress began, mice were inoculated intraperitoneally with either 1 × 10⁶ ID8 or IG10 murine ovarian cancer cells. Eight weeks after inoculation or when any mouse became moribund, mice were necropsied, and the tumors and ascites were collected for cytokine, stress hormone measurements, and T-cell characterization. Mouse and tumor weights were also obtained.

2.6. Flow cytometry assay of mice tumors

Approximately 60 mg of each tumor sample was processed for FACS analyses. First, samples were rinsed with autoMACS Rinsing Solution (130-091-222, Miltenyi) and homogenized for two 60-s cycles on a gentleMACS Dissociator (Miltenyi). Next, samples were filtered utilizing a 30 μM separation filter and centrifuged at 4 °C and 1,200 RPM for 10 min. The resulting pellet was washed with 1 mL of Red Blood Lysis Buffer and centrifuged for 10 min at 4 °C at 1,200 RPM. The supernatant was discarded, and the pellet was resuspended with 200 μL PBS. Primary antibodies for CD4 (552051, BD Biosciences), CD8 (MCD0801, Bio-Analytical), and PD-1 (75781-958, VWR) were added for an hour. Samples were then centrifuged at 4 °C and 1,200 rpm for 7 min, and the pellet was collected and resuspended in 800 μL of 0.5 PFA. Cells were acquired on a FACS Melody (BD Biosciences), analyzed by density plots, and CD4+ and CD8+ populations were selected. The expression of PD-1 in CD4+ and CD8+ cells was determined. We utilized FlowJo to analyze the histograms and plots.

2.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism (Version 9.3.1) and R/Studio. Patient characteristics were summarized using descriptive statistics, including median and range for continuous measures and proportions and frequencies for categorical measures. T-test or Mann-Whitney tests were performed for parametric or non-parametric values, respectively, as determined by Shapiro-Wilk normality tests. The association between continuous variables and primary or recurrent status and sensitive or recurrent status were assessed using Mann-Whitney tests. Data are presented as mean ± standard error of the mean (SEM). The Spearman correlation coefficient was used to determine the correlation between ascites levels of individual cytokines and stress hormones. Heatmaps of cytokine expression were generated using the R Bioconductor package ComplexHeatmap (Gu et al., 2016). Co-expression patterns among cytokines to identify modules of correlated genes were identified through weighted correlation network analysis implemented in the R package WGCNA (Langfelder and Horvath, 2008). Groups of co-expressed genes were further related to stress hormones levels and clinical variables of interest using Spearman and Pearson correlations for continuous and dichotomous variables, respectively. All results are presented as two-tailed P-values, and P < 0.05 was considered statistically significant.

3. Results

3.1. Study cohort description

All ascites samples utilized in this study were collected from patients diagnosed with primary or recurrent high grade serous ovarian cancer (HGSOC). Samples were obtained at the time of surgery or during a therapeutic/diagnostic paracentesis, both performed as part of the standard of care. The mean age of patients was 64 years (SD = 10.81). The majority of patients were advanced stage, FIGO Stage III-IV (n = 42). Forty-five percent (n = 22) had ascites removed in the recurrent setting, and 51% (n = 25) had ascites preserved at the time the participant was undergoing primary treatment with surgery and/or chemotherapy. Nine participants (18%) had platinum-resistant disease, whereas 40 (82%) were sensitive to platinum-based chemotherapy (Table 1).

3.2. Stress hormones are detectable in the ascites from HGSOC patients

First, we sought to determine if stress hormones are present and measurable in the ascites from patients with HGSOC (n = 49). We measured cortisol, normetanephrine (NMN), and metanephrine (MN) in these samples, as NMN and MN have been described as stable
metabolites of NE and Epi, respectively. (Peaston and Weinkove, 2004). Our data show that NMN, MN, and cortisol were detectable in these samples (average concentration among cohort: NMN 152.96 pg/mL [range: 0–422.35 pg/mL], MN 224.67 pg/mL [range: 0–887.12 pg/mL], and cortisol 81.68 ng/mL [range: 20.44–164.38 ng/mL] (Fig. 1A). These values are comparable, albeit slightly higher than published values found in blood samples (Levine et al., 2007; Peaston and Weinkove, 2004).

During chronic stress and subsequent distress, both SNS and HPA are activated, leading to the concurrent release of stress hormones. Here, we investigated if the expression levels of cortisol, NMN, and MN correlated with each other in ascites samples. Our data show that NMN and MN were correlated with each other in these samples, while this relationship was not observed with cortisol ($R = 0.29; P < 0.047$; Fig. 1B–D). Even though we did not assess the mental health status in our cohort, our data show that stress hormones are detectable in ascites samples from patients with HGSOC and could play a key role in the modulation of immune-related pathways and disease progression.

3.3. Stress hormones are associated with inflammatory and immunosuppressive cytokines in the ascites from HGSOC patients

Stress hormones can modulate immune function and directly regulate cytokine expression in ovarian cancer (Colon-Echevarria et al., 2019). We detected biologically significant levels of stress hormones in ascites from patients with HGSOC; therefore, we sought to assess the impact on the immune microenvironment. To characterize the interplay between stress hormones and cytokine levels in the ascites samples, we utilized a cytokine kit to quantify 38 cytokines. We subsequently correlated each individual cytokine with each stress hormone (Supplementary Table 1). We found NMN levels were significantly correlated with increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), macrophage-derived chemokine (MDC), monocyte chemoattractant protein-1 (MCP-1), interleukin-7 (IL-7) and growth-regulated protein (GRO) (Fig. 2A). Several of these cytokines have been shown to be induced by stress hormones and promote tumor-associated inflammation and progression (Armaiz-Pena et al., 2015; Nilsson et al., 2007; Thaker et al., 2006). Additionally, MN correlated with increased levels of monocyte chemotactic protein-3 (MCP-3), macrophage inflammatory protein-1 alpha (MIP-1α), macrophage-derived chemokine (MDC), and macrophage inflammatory protein-1 beta (MIP-1β) (Fig. 2B). On the other hand, elevated cortisol was correlated to decreased interferon-gamma (IFNγ), FMS-like tyrosine kinase 3 ligand (FLT-3L), interferon gamma-induced protein 10 (IP-10), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1 beta (MIP-1β) and interleukin-2 (IL-2) (Fig. 2C). Fig. 2E and F include heatmaps depicting the expression of significantly modulated cytokines by stress hormones among the study cohort. Several of these cytokines have been associated with immunosuppressive processes that lead to immune evasion and progression (Lippitz, 2013). When taken together, these data support a role for stress hormones as potential modulators of inflammation and immunosuppression in the ascites from HGSOC patients.

Next, we evaluated if clinical factors such as primary HGSOC, recurrent HGSOC, platinum-sensitivity, or resistance altered measurable cytokines and stress hormone levels within the ascites. First, informed by clinicopathological information, samples were divided into those from primary (N = 25) or recurrent disease (N = 22). These two groups showed no significant differences in stress hormone levels or notable

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**Fig. 1. Stress Hormones levels in the ascites from HGSOC patients.** (A) Mean concentration of cortisol, normetanephrine, and metanephrine in ascites samples (N = 49). Relationships between stress hormones levels measured in ascites obtained from patients with HGSOC as determined by Spearman correlation analyses between levels of (B) normetanephrine and metanephrine ($R = 0.29; P = 0.047$), (C) metanephrine and cortisol ($R = 0.04; P = 0.80$) and (D) normetanephrine and cortisol ($R = 0.14; P = 0.36$).
cytokine expression patterns. However, we observed decreased levels of IL-2 ($P = 0.04$) in ascites samples from patients with recurrent disease (Supplementary Figure 1A). We also dichotomised ascites samples into groups from platinum-sensitive ($N = 40$) or platinum-resistant ($N = 9$) disease. In the platinum-resistant disease group, several pro-inflammatory cytokines, such as Eotaxin ($P = 0.0005$), FLT-3L ($P = 0.015$), and IL-7 ($P = 0.004$) were increased in the ascites (Supplementary Figure 1B).

Next, we identified four significant clusters of co-expressed cytokines in our cohort (visualized by the brown, yellow, blue, and teal colors; Fig. 3A). Then, we assessed if the identified cytokine clusters were associated with stress hormone levels and clinical variables. These analyses show that increased NMN-associated IL-6 and IL-7 levels (blue cluster) were enriched in these populations, while no enrichment could be seen with MN- and cortisol-associated cytokines (Fig. 3A and B). Further analyses revealed that the IL-6/IL-7 cluster (blue) correlated

Fig. 3. Weighted Gene Co-expression Network Analysis (WGCNA). (A) Dendrogram of the 37 cytokine genes analyzed as determined by average linkage hierarchical clustering. Each gene is assigned to a module based on the branches of the clustering tree. The significant modules are designated by color code (brown, yellow, teal, and blue). The genes that do not belong to a module are represented by grey. (B) Module-trait relationships illustrating the correlations between each color-coded gene expression module and stress hormones levels or clinical characteristics. Each row corresponds to a module and the columns are clinical traits. The values in the cells are ‘Pearson r ($P$ value)’. Colors indicate the direction of correlation (red is positive and blue is negative). The color intensity represents the strength of the correlation (darker red or blue indicate a higher R value). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
with resistant disease, while the MIP-1β/MIP-1α cluster (yellow) correlated with MN levels (Fig. 3B). Taken together, these data suggest that stress hormones are associated with inflammatory and immunosuppressive cytokines in the ascites from patients with HGSOC.

3.4. Ascites-derived T-cells exhibit a heterogeneous pattern of activation and inhibitory receptors

We evaluated if pro-inflammatory and immunosuppressive cytokines could impact the immune TME within the ascites. We performed bioinformatic analyses that utilized the STRING platform to identify immune-related signaling pathways driven by cytokine/stress hormone interactions (Szklarczyk et al., 2021). These analyses identified potential interactive networks between cytokines modulated by stress hormones (Fig. 4A). Next, we interrogated potential functional cytokine pathways using the GO biological processes database to identify immune cell types differentially regulated in response to changes in stress hormone-induced cytokines. The top four hits from these analyses involved cytokine-mediated signaling, leukocyte chemotaxis, function, and cell surface receptor regulation (Fig. 4B). These bioinformatic analyses, the data presented linking stress hormones, and several of these cytokines’ known roles in T-cell biology led us to characterize T-cells from the ascites and assess the impact of stress hormones on their functionality.

First, we characterized activation and immune checkpoint markers on ascites-derived T-cells from seven ovarian ascites samples from our previously described cohort. Cells isolated from ascites were analyzed using a 14-color immune checkpoint flow cytometry panel that assesses T-cell function. These analyses showed that the ascites-derived CD4+ and CD8+ T-cells expressed a heterogeneous pattern of activation and inhibitory receptor expression. (Fig. 4C, D). Notably, epinephrine reduced the expression of markers associated with T-cell function, as demonstrated by the decreased expression of activation and immune checkpoint markers on T-cells from patients with ovarian cancer (n = 4) stimulated with αCD3 and αCD28 for seven days in the presence and absence of epinephrine. Flow cytometry analysis of Granzyme B and CD38 co-expression was performed on ascites mononuclear cells. Gated CD4 and CD8 T-cells are shown. (D) Ascites derived T-cells from patients with ovarian cancer (n=4) stimulated with αCD3 and αCD28 for seven days in the presence and absence of epinephrine. Flow cytometry analysis of Granzyme B and CD38 co-expression (P = 0.0042). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. Ascites-derived T-cells exhibit a heterogeneous activation and inhibitory receptors pattern, while epinephrine reduces the expression of markers associated with T-cell function. (A) Protein-protein interaction network between cytokines positively correlated with stress hormones/metabolites. (B) Bioinformatic analyses utilizing the GO biological processes based on the protein-protein interaction of stress hormone correlated cytokines. (C) Cellular expression of activation and immune checkpoint markers on T-cells isolated from ovarian ascites. 14-color polychromatic flow cytometry analysis was performed on ascites mononuclear cells. Gated CD4 and CD8 T-cells are shown. (D) Ascites derived T-cells from patients with ovarian cancer (n=4) stimulated with αCD3 and αCD28 for seven days in the presence and absence of epinephrine. Flow cytometry analysis of Granzyme B and CD38 co-expression. Quantification from individual patients showing the frequency of Granzyme B and CD38 co-expression (P = 0.0042). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
inhibitory receptors (Fig. 4C). We also observed elevated CTLA-4, LAG-3, PD-1, and TIM-3 inhibitory receptors within T-cells derived from ascites of patients with HGSOC.

3.5. Epinephrine reduces the expression of markers associated with T-cell function

As our data showed that several cytokines, such as IFNγ, were correlated with increased stress hormones in the ascites, we sought to assess if epinephrine could modulate T-cell function ex vivo. For these experiments, ascites-derived CD8⁺ T-cells from ovarian cancer patients (n = 4) were stimulated with αCD3 and αCD28 for seven days in the presence or absence of 10 μM epinephrine. Flow cytometry analysis of Granzyme B and CD38 co-expression (markers for activation and functional feasibility) suggests that epinephrine reduced CD38 and Granzyme B co-expression in CD8⁺ T-cells (Fig. 4D; P = 0.0042), suggesting that decreased T-cell function and cytotoxicity. These results support a role for stress hormones as suppressors of T-cell activity in the ascites from patients with HGSOC.

3.6. Daily restraint stress leads to increased tumor growth and inflammatory cytokines in syngeneic mouse models of ovarian cancer

To further assess the biological significance and the effect of chronic stress on inflammatory processes and ovarian tumor growth, we used a well-characterized restraint stress method on syngeneic (IG10 and ID8 murine cell lines) tumor-bearing mice to replicate the physiological effects of chronic stress (Fig. 5A). Moreover, these models produce significant amounts of ascites. Compared to their unstressed controls, restraint-stressed tumor-bearing mice (IG10 and ID8) had significantly increased tumor weight (ID8: 1.78-fold change, P = 0.0068; IG10: 1.31-fold change, P = 0.008; Fig. 5B). These data suggest an association between psychosocial factors and disease progression.

Informed by our data in humans that suggest that stress hormones in the ascites are associated with pro-tumoral cytokines, we sought to measure these in ascites from ovarian cancer mouse models. We collected ascites samples from the control and restraint stressed ID8 model groups and assessed cytokines using a panel of 32 chemokines/cytokines and stress hormones by ELISA (NMN, MN, and corticosterone as it is the main glucocorticoid in rodents). Stress hormones were quantifiable within the mouse ascites (average concentration for corticosterone: 778.62 pg/mL, NMN: 449.26 pg/mL, and MN: 100.65 pg/mL), but no statistically significant differences were observed between the control and restraint stressed groups (Fig. 5C and D). This observation may be due to samples being collected at the end of the restraint stress regimen when the HPA and SNS might be completely dysregulated. Next, we investigated whether protumoral cytokines correlated to stress hormones in ascites from tumor-bearing mice. Our data identified several cytokines and inflammatory factors such as IL-6 (R = −0.72; P = 0.004), VEGF (R = 0.71; P = 0.005), KC (R = −0.73; P = 0.003), IL-3 (R = −0.68; P = 0.002), MIG (R = −0.64; P = 0.01), and M-CSF (R = 0.60; P = 0.02).

Fig. 5. Daily restraint stress promotes tumor growth and inflammatory cytokines in the ascites from ovarian tumor-bearing mice. (A) Experimental in vivo design of daily restraint stress of ID8 and IG10 tumor-bearing mice. (B) Mann-Whitney analyses show that daily restraint stress increased tumor growth in ID8 (control group SEM: 0.0423, stress group SEM: 0.04879) and IG10 (control group SEM: 0.02244, stress group SEM: 0.02760). (C-D) Concentration of corticosterone, normetanephrine, and metanephrine in ascites samples from ID8 tumor-bearing mice. (E) Spearman correlation analyses show that corticosterone levels were correlated with VEGF (R = 0.71; P = 0.005) and MIG (R = −0.63; P = 0.02) while metanephrine levels were correlated to M-CSF (R = 0.60; P = 0.02).
3.7. Daily restraint stress leads to increased CD4⁺PD-1⁺/CD8⁺PD-1⁺ T-cell ratio in the ovarian tumor microenvironment

To determine if daily restraint stress could modulate T-cell populations and function in the tumor microenvironment, we characterized CD4⁺ and CD8⁺ T-cell subtypes and the expression of PD-1 by flow cytometry on snap-frozen tumor samples from ID8 tumor-bearing mice that were subjected to daily restraint stress. As shown in Fig. 6A, lymphocyte selection criteria were established by density plots. Next, we determined the CD4⁺, CD8⁺ and PD-1⁺ expression distribution in the ID8 tumor samples (Fig. 6B). Our analyses show that daily restraint did not significantly modulate PD-1-expressing CD4⁺ and CD8⁺ cells in the ovarian tumor microenvironment (Fig. 6C and D). On the other hand, our data indicate that daily restraint stress leads to an increased ratio of PD-1 expressing CD4⁺ and CD8⁺ T-cells (P = 0.036) in the ovarian tumor microenvironment (Fig. 6E). These data suggest that daily restraint stress could enhance immunosuppression of T-cells by upregulating PD-1-dependent processes.

4. Discussion

Sustained activation of the SNS and HPA leads to the release of catecholamines, norepinephrine and epinephrine, and cortisol, respectively. These stress hormones are secreted into the blood or locally delivered into the tumor microenvironment, which promotes disease progression (Cole et al., 2015; Faulkner et al., 2019). However, the presence and role of these hormones (and their metabolites) within the ascites from ovarian cancer patients has not been well established. The main findings of this study are that stress hormones are present in the ascites from patients with HGSOC and animal models of ovarian cancer, and these are associated with inflammatory and immunosuppressive cytokines, as well as decreased function of ascites-derived T-cells. Specifically, normetanephrine and metanephrine metabolites correlated with increased pro-inflammatory factors, VEGF, IL-6, and MCP-1, MCP-3, among others. Moreover, our weighted correlation network analysis indicated that some of these cytokines were co-expressed in the ascites. For example, we identified an IL-6/IL-7 co-expression cluster that has been shown to play a key role in the transition from innate to adaptive immunity, while IL-6 plays a key role in stress...
hormone-mediated tumor growth (Gagnon et al., 2008; Nilsson et al., 2007). On the other hand, cortisol negatively correlated with IFN-γ, IP-10, GM-CSF, IL-2, and FLT3L. Among these, IP-10, also known as interferon gamma-induced protein 10, mediates immune responses by activating and recruiting T-cells, among other leukocytes (Bronger et al., 2016), which further reinforces the importance of dissecting the role of ascites-derived stress hormones in pro-tumoral processes and attenuating anti-tumoral immune responses. Given the rich immune composition of the ascites (Ahmed and Stenvers, 2013; Ford et al., 2020; Giuntoli et al., 2009), our data highlight the usefulness of asces in assessing immune-related processes, cytokines, and T-cell function in the context of ovarian cancer and response to therapy.

Our study identified pro-inflammatory cytokines, T-cell surface markers, and leukocyte chemotaxis regulation as possible stress hormone-regulated processes in the ascites from HGSOC patients. We, and others (Dai et al., 2020; Le et al., 2016; Voorhees et al., 2013), have shown that cytokines such as IL-6, VEGF, and MCP-1 positively correlate with epinephrine or norepinephrine. The prognostic value of these cytokines has been established in several cancers, including ovarian cancer (Browning et al., 2018; Dalal et al., 2018; Fahmi et al., 2021). Studies have shown that stress hormones can induce IL-6 secretion (Nilsson et al., 2007) and result in the activation of the JAK/STAT3 pathway (Landen et al., 2007), which promotes tumor proliferation (Kolomeyevskaya et al., 2015) and resistance to chemotherapy (Browning et al., 2018). Similarly, VEGF and MCP-3 (CCL8) promote cancer progression by inducing angiogenesis, tumor invasion, and metastasis (Fahmi et al., 2021) (Hao et al., 2020). Specifically, VEGF has been shown to be secreted in response to stress hormone release by chronic stress (Thaker et al., 2006). We identified several inflammatory or immunosuppressive cytokines in our ovarian cancer animal models, including VEGF, KC, IL-3, MIG, M-CSF, IL-3, LIX, and IL-12. These cytokines were correlated with stress hormones/metabolite levels. VEGF is a well-known biological mediator for cell survival, as well as vascular permeability and blood vessel growth (Dalal et al., 2018). Moreover, VEGF mediates angiogenesis, which allows tumor cells to access nutrients and oxygen, essential components for tumor growth and development, and is associated with increased secretion of other pro-tumoral cytokines, such as MCP-1, IL-6, and M-CSF (Horikawa et al., 2020; Lien et al., 2020; Nilsson et al., 2005). Similarly, studies show that overexpression of M-CSF may lead to increased recruitment of tumor-associated macrophages (TAMs), which are known to play a key role in tumor cell survival and proliferation (Sidorkiewicz et al., 2019).

In our study, VEGF and M-CSF were found to be increased in relation to stress hormones, corticosterone, and metanephrine, respectively. On the other hand, MIG, which further increases the production of IFNγ, important for cytotoxic CD8 T-cell differentiation (Li et al., 2020), was found to be decreased in relation to corticosterone levels. These results further validate a role for stress hormones and inflammatory cytokines that promote cancer progression, as well as the reduction of cytokines essential for anti-tumor responses. Work from our group and others has shown that these cytokines play key roles in adrenergic-stimulated ovarian cancer growth (Armaiz-Pena et al., 2015; Nilsson et al., 2007; Thaker et al., 2006). Similarly, LIX (CXCL5) and IL-12p40, although not significant, positively correlate with normetanephrine levels, and promotes proliferation in solid tumors (Zhang et al., 2020) and immunosuppression (Marks et al., 2017), respectively. These changes support the rationale that stress hormones drive inflammatory processes, which promote tumor growth and generate optimal conditions for tumor immune evasion.

One of the key observations of this study is that the accumulation of stress hormones, or their metabolites, in the ascites is associated with pro-inflammatory cytokines and attenuation of T-cell functions in the ascites from patients with HGSOC. Work from others has shown that chronic stress induces sustained activation of the SNS and HPA, which influences physiological responses across tumor-infiltrating lymphocytes (Morey et al., 2015). Specifically, this process leads to the redistribution of T-cells, CD8+ T-cells suppression, increased T-reg activity (Schmidt et al., 2016), and disease progression (Frick et al., 2009). Our data extend these concepts by showing that ascites-derived CD8+ T-cells exhibit decreased CD38 and Granzyme B co-expression upon epinephrine stimulation, suggesting a direct immunosuppressive effect of stress hormones on ascites-derived T-cell function. Furthermore, our study reveals heterogeneous expression of T-cell exhaustion markers in the ascites from HGSOC patients. More specifically, elevated concentrations of inhibitory receptors CTLA-4, LAG-3, PD-1, and TIM-3 within ascites are consistent with a highly immune-suppressed tumor microenvironment (Imai et al., 2018). The data presented here is congruent with previous literature and presents a new role for ascites-derived stress hormones in promoting inflammation and dampening T-cell responses in the ascites from HGSOC patients.

Currently, response rates to checkpoint blockade in patients with ovarian cancer are modest, and most patients will progress or recur (Monk et al., 2021; Moore et al., 2021; Yang et al., 2020). Mounting evidence points towards the enriched co-expression of multiple inhibitory molecules in tumor-associated T-cells, such as LAG-3, PD-1, and CTLA-4, for the lack of an effective response to immune checkpoint inhibitors (Barber and Matei, 2021). Our study is congruent with this observation and supports the idea that stress hormones could influence this process by promoting an inflamed TME that attenuates T-cell activity. Thus, a better understanding of the relationship between stress hormones, pro-tumoral cytokines, and T-cell function in ascites is needed to identify reliable predictive biomarkers.

A potential limitation of our study is that clinical data regarding granularity into whether patients were platinum-refractory or platinum-resistant, as well as treatment timelines, including specific regimens, and psychological assessments, were unavailable for our cohort. Also, we acknowledge that there is little overlap between the mouse and human ascites data. These discrepancies might be related to inherent variability between human and murine immune and behavioral responses that could impact these same processes in ascites samples and the heterogeneous nature of biological samples, which may lead to translational limitations. We also note that no significant differences in stress hormone and metabolites levels were observed between the control and restraint-stressed groups. This observation may be due to samples being collected at the end of the restraint stress regimen when the HPA and SNS might be completely dysregulated or resulting from differences in how stress hormones are accumulated and metabolized in the ascites.

We acknowledge that further research is warranted to continue addressing whether the interaction between stress hormones and the immune TME can influence the effectiveness of immunotherapies that relies on T-cell function. Our work highlights a potential relationship between stress hormones, inflammatory cytokines, and T-cell-mediated responses in the ascites from patients with HGSOC. These data provide the rationale for assessing if blocking stress hormone-mediated signaling pathways could be exploited to enhance responses to immune targeting regimens in patients with HGSOC.

Declaration of competing interest

RAP has served on advisory boards for Myriad Genetics and Natera. SLG has served on advisory boards for Arcus, AstraZeneca, Elevar Therapeutics, GSK, Novartis, and Immunogen. SLG has received research funding from AstraZeneca, Clovis, Genentech, Iovance, Tesaro/ GSK, Compugen, and Tempe. SLG holds a patent that Duke University licensed to Sermonix. No other potential conflict of interest to disclose.

Data availability

Data will be made available on request.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2022.100558.

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