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LETTER TO THE JOURNAL

Ascites of patients with solid tumors shows distinct inflammatory patterns

Ascites formation in solid tumor patients is associated with an increased risk of death [1, 2]. The lack of pathophysiological insight limited the development of targeted treatment so far. With advances in immune modulating therapies, the inflammatory component of ascites moved into focus. Experimental approaches targeting immunologic dysregulation have shown only limited success [3, 4]. Therefore, we investigated inflammatory processes of ascites, taking the presence of tumor cells into account, while focusing on gastrointestinal tract malignancies due to their underrepresentation in literature.

A total of 63 patients were included in this study. Among these patients, 55 (87.3%) underwent paracentesis for ascites caused by advanced solid tumors, of which 30/63 (47.6%) patients had negative tumor cell cytology (paramalignant ascites) and 25/63 (39.7%) had positive tumor cell cytology (malignant ascites). Additionally, 8/63 (12.7%) patients with non-malignant ascites due to liver cirrhosis were included. Patients' characteristics are displayed in Supplementary Table S1.

To study differences in the inflammatory profiles based on tumor cells within ascites, nine cytokines (interleukin-6 [IL-6], IL-8, IL-10, IL-17, tumor necrosis factor-alpha [TNF-α], C-reactive protein [CRP], Eotaxin, vascular endothelial growth factor [VEGF], and the soluble programmed death ligand-1 [sPD-L1]) were measured in ascitic supernatant. Malignant ascites showed increased levels of IL-6, IL-8, VEGF, and sPD-L1, compared to paramalignant and non-malignant ascites, respectively (Figure 1A-D). In contrast, no differences in cytokine levels were observed between non-malignant and paramalignant ascites in all measured cytokines, indicating that the inflammatory composition of ascites correlates with the presence of tumor cells.

To correlate systemic inflammatory processes with local inflammation in ascites supernatant in patients with advanced solid tumors, we measured cytokine levels in serum samples obtained at the timepoint of paracentesis. Strong correlations between ascitic and serum cytokine levels were evident for IL-8 and IL-17 (Figure 1E-F). Additionally, strong correlation was evident for CRP levels (Figure 1G), while no or weak correlations were detected in other cytokines. CRP as well as levels sPD-L1were decreased in ascites compared to serum (Supplementary Table S2). Compared to serum, ascitic IL-6 was significantly elevated (Supplementary Table S2) without a correlation to systemic IL-6 levels ($\rho = 0.16$, $P > 0.05$), suggesting IL-6 signaling may be important for local inflammation.

To tie the link between soluble inflammatory markers and the cellular inflammatory compartment, we investigated correlations between cytokine levels and leukocyte as well as cell count for 19 overlapping samples. While no associations between leukocyte count and cytokine levels were observed, strong correlations between overall cell count in ascites and VEGF as well as sPD-L1 (Figure 1H-I) were evident.

DNA methylation analyses of 32 cellular samples of ascites by Infinium Methylation EPIC V2.0 microarray were performed to obtain further insight on cellular composition and inflammatory pathways of interest (Supplementary Materials and Methods). No difference in the methylation profile of samples according to TNF-α ($n = 19$) or IL-6 ($n = 17$) were observed. Methylation variation seemed to be driven by cytology, with tumor cell cytology positive and negative samples separating along the first dimension of the multidimensional scaling plot (Figure 1J). 37,494 CpG sites were differentially methylated between malignant and paramalignant ascites (FDR < 0.05). An enrichment of KEGG pathways for differentially methylated CpGs located on promotor revealed that hypermethylated probes were associated with Neuroactive ligand-receptor interaction (hsa04080) while hypomethylated CpGs were associated with Olfactory transduction (hsa04740, Supplementary Figure S1). Clustering of cellular ascites samples based on the top 20,000 differentially methylated CpGs, revealed three main clusters (cluster

List of abbreviations: CRP, C-reactive protein; GO, gene ontology; IL, interleukin; sPD-L1, soluble programmed death ligand 1; Treg, regulatory T cell; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

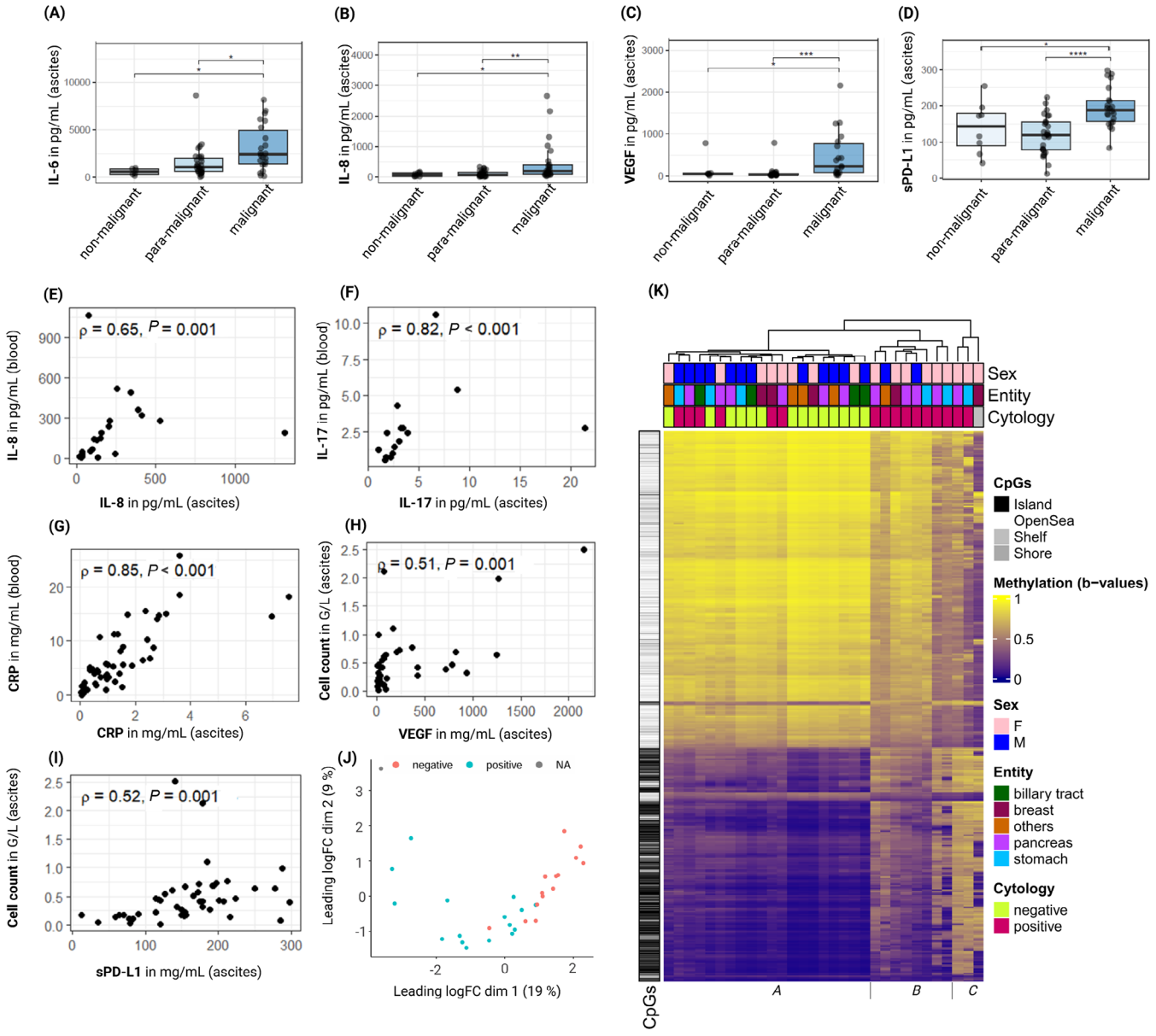

FIGURE 1 Differences in ascitic inflammation profiles according to subtype. (A-D) Comparison of cytokine levels between patients with non-malignant, paramalignant, and malignant ascites, elevated levels of cytokines (A, IL-6 [$n = 56$]; B, IL-8 [$n = 63$]; C, VEGF [$n = 51$]; D, sPD-L1 [$n = 57$]) were found in malignant ascites compared to non-malignant and paramalignant ascites. (E-G) Correlation between cytokine levels in serum and ascites of patients with solid tumors, strong correlations were observed between ascitic and systemic levels in (E, IL-8 [$n = 23$]; F, IL-17 [$n = 16$]; G, CRP [$n = 24$]; tested using Spearmans ρ, $n = 24$). (H-I) Correlation between cytokine levels and overall cell count in ascites of patients with solid tumors, strong correlations were observed between cell count and VEGF ($n = 50$) as well as sPD-L1 levels ($n = 56$, tested using Spearmans ρ). (J-K) DNA methylation landscape in ascites. Multidimensional scaling plot of 20,000 most variable CpG sites (J). Samples are colored based on cytology status. supervised clustering according to cytology of the top 20,000 differentially methylated CPG sites of ascites bulk DNA derived from the cellular ascites compartment of solid tumor patients (K). Three distinct methylation profile clusters were identified (cluster A-C). Cytokine levels in pg/mL, CRP in mg/dL, cell count in G/L. Figure created with Biorender. All P values were corrected according to Bonferroni-Holm. *P value < 0.05; **P value < 0.01; ***P value < 0.001; ****P value < 0.0001; $n = 32$. Abbreviations: CRP, C-reactive protein; IL, interleukin; sPD-L1, soluble programmed death ligand 1; VEGF, vascular endothelial growth factor.

A-C, Figure 1K). Cluster B and C show a similar signature of hypermethylated CpGs in CpG Islands and consist of malignant ascites samples. Cluster A represents paramalignant ascites samples, with some cytology positive samples showing a similar signature.

As immune cell-type proportions may influence observed differences in DNA methylation, we inferred the cellular compositions by deconvolution. However, no deconvolution tools are optimized for ascites analysis, so results should be interpreted cautiously. In line with this, only weak to medium correlation of the estimated cell fraction by deconvolution with cell counts from cytology analysis was observed for overlapping samples (n = 21, neutrophils [Pearson R = 0.55, P = 0.01], T and B cell [Pearson R = 0.67, $P < 0.001$], monocytes [Pearson R = 0.27, $P > 0.05$). In alignment with cytology, we observed a higher cancer cell fraction according to the deconvolution in malignant versus paramalignant ascites samples (FDR adjusted $P < 0.001$). Next, we compared estimated cell fractions between methylation clusters based on differentially methylated CpGs between malignant and paramalignant ascites (Figure 1K). No significant differences for immune cell types were observed, while a significantly higher cancer cell fraction was found in samples present in cluster B+C compared to cluster A (FDR adjusted $P <$ 0.001). When correlating estimated immune cell fractions with cytokine levels for overlapping samples (n = 17-19), we observed significant, albeit weak correlations (NK cells and IL-8, Pearson R = 0.44, P = 0.047; NK cells and IL-6, Pearson R = -0.48, P = 0.036; CD4 cells and IL-8, Pearson R = 0.46, P = 0.036; CD4 cells and TNF-a, R = 0.46, P = 0.046; CD8 cells and IL8, Pearson R = 0.52, P = 0.016).

Ascites formation in patients with solid tumors remains a clinical challenge. Existing clinical trials were designed without biomarkers and potential biological subtypes in ascites were not acknowledged [5, 6]. Our data suggests that distinct inflammatory subtypes of ascites in cancer patients exist on cellular and cytokine levels, as inflammatory profiles of patients with negative tumor cytology resembled non-malignant ascites. Given their low inflammation levels, cancer patients without tumor cells within ascites might profit from treatment established in non-malignant ascites patients like diuretic treatment [7]. Importantly, malignant ascites, defined by the presence of tumor cells within ascites, presented with increased inflammation compared to paramalignant and non-malignant ascites. We certainly have to acknowledge the limitation, that only one liter of ascites was processed per patient, leaving the theoretical possibility of single tumor cells in patients with paramalignant ascites. However, given the differing inflammatory profiles according to the observed tumor cell cytology, local inflammatory processes differ according to the amount /presence of tumor cells and serve as a potential therapeutic target particular in patients with positive cancer cytology. As the inflammatory profile of ascites was mainly independent of the systemic profile, the immunological effects might be locally specific. Local IL-6 was directly linked to paradox immunosuppressive effects in malignant ascites [8]. Adding to the heterogeneity of soluble inflammatory profiles, DNA methylation of cellular ascites samples revealed distinct profiles according to cytology. Three distinct methylation clusters were identified, where clustering seemed to be driven by the local presence of tumor cells. Pathway analysis suggested differences in Olfactory transduction and Neuroactive ligand-receptor interaction, both linked to immunosuppression [9]. Although the heterogeneity of our cohort needs to be acknowledged, previous studies in other entities as ovarian cancer are rare. Given the impaired clinical efficacy of bevacizumab in ascites treatment of patients with other primary tumors than ovarian cancer, the identified inflammatory targets are of interest for further investigation. Given the particular inflammatory profile of malignant ascites in our study, future research should focus on IL-6, IL-8 and immune checkpoints. Still, these first findings need to be evaluated in subsequent studies with bigger subgroups, to further assess their pathophysiologic relevance in ascites formation and potential clinical implications.

AUTHOR CONTRIBUTIONS

Conceptualization: Julia M. Berger, Birgit Fendl, Barbara Niederdorfer, and Anna S. Berghoff. **Data curation:** Julia M. Berger, Martin Korpan, Carina Zierfuss, Katharina Syböck, Erwin Tomasich, Andreas Kienzle, Maria Koenig, Markus Kleinberger, Lynn Gottmann, Birgit Fendl, Cihan Ay, Johannes Pammer, Catharina Müller, Rudolf Öhler, Lorenz Balcar, Thomas Reiberger, Elisabeth S. Bergen, and Barbara Niederdorfer.Formal analysis: Julia M. Berger, Barbara Niederdorfer, and Anna S. Berghoff. **Funding acquisition:** Julia M. Berger, Matthias Preusser, and Anna S. Berghoff. Investigation: Julia M. Berger, Barbara Niederdorfer, and Anna S. Berghoff. **Methodology:** Julia M. Berger, Carina Zierfuss, Barbara Niederdorfer, and Anna S. Berghoff. **Project administration:** Julia M. Berger and Martin Korpan. **Resources:** Thomas Reiberger, Matthias Preusser, and Anna S. Berghoff. **Software:** Julia M. Berger and Barbara Niederdorfer. **Supervision:** Anna S. Berghoff. **Validation:** Julia M. Berger, Barbara Niederdorfer, and Anna S. Berghoff. **Visualization:** Julia M. Berger and Barbara Niederdorfer. **Writing: original draft:** Julia M. Berger, Barbara Niederdorfer, and Anna S. Berghoff. **Writing: review & editing:** Julia M. Berger, Martin Korpan, Carina Zierfuss, Katharina Syböck, Erwin Tomasich, Andreas Kienzle, Maria Koenig, Markus Kleinberger, Lynn Gottmann, Birgit Fendl, Cihan

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CONFLICT OF INTEREST STATEMENT

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All patients were treated according to best clinical practice and to current treatment guidelines throughout their whole clinical course of disease at our tertiary care center. This study was approved by the Ethics Committee of the Medical University of Vienna (vote number 2100 of 2022) and performed according to the Declaration of Helsinki and its Amendments. Consent to participate in this study was waived.

DATA AVAILABILITY STATEMENT

Data of this study is available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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