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LETTER

Malignant ascites enhance γδ T cell cytotoxicity toward ovarian cancer *via* chemokine-mediated recruitment

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Ovarian cancer remains a leading cause of gynecological cancer mortality[1], and patients with advanced stage ovarian cancer frequently develop malignant ascites that foster immunosuppressive microenvironments and therapeutic resistance[2,3]. Although ascites have traditionally been considered detrimental, we report a paradoxical role in which they enhance the cytotoxicity of γδ T cells—a unique T cell subset that can be allogenically transferred for cancer treatment[4,5]—toward ovarian cancer.

Malignant ascites collected from patients with ovarian cancer enhanced the cytotoxicity of γδ T cells toward both ovarian cancer cell lines and primary cancer cells (**Figure 1A, B, Figure S1A-C**). Because malignant ascites are complex mixtures (containing both proteins and small molecules)[6,7], we sought to determine the functional component. Only the small molecule component significantly enhanced the cytotoxicity of γδ T cells toward ovarian cancer cells (**Figure 1C, Figure S1D, E**). Pretreatment of either cancer cells or γδ T cells with ascites before cytotoxicity assays indicated that the ascites acted on cancer cells, given that the cytotoxicity of γδ T cells was enhanced only with pretreatment (**Figure 1D**).

We performed transcriptome sequencing on ovarian cancer cells before and after ascites treatment to reveal the underlying mechanisms. A volcano plot indicated critical transcriptional changes after ascites treatment (**Figure 1E**), including significant changes in *Cxcl8*, *Cxcl2*, *Cxcl1*, and *Ccl2*. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and gene set enrichment analysis also revealed enrichment in signaling pathways including the TNF signaling pathway and cytokine related signaling pathways (**Figure S2A, B**). Given the critical roles of cytokines in anti-tumor immunity[8,9], and the positive correlations between expression of some of these cytokines/chemokines and survival of patients with ovarian cancer (**Figure S2C**), we focused on CXCL8 (IL-8), CXCL2, CXCL1, and CCL2. We hypothesized that the upregulation of these cytokines/chemokines might enhance γδ T cell-mediated cytotoxicity with ascites treatment.

An enzyme-linked immunosorbent assay (ELISA) was first established to verify the cytokine/chemokine secretion in ovarian cancer cells after ascites treatment. Most ascites from patients promoted secretion of CXCL8 and/or CXCL2 (**Figure 1F, G, Figure S3A, B**), but not CCL2 and CXCL1 (**Figure S3C, D**). Chemokines recruit certain immune cells to inflammation sites *via* the chemokine receptors expressed on immune cells. We therefore hypothesized that the enhanced γδ T cell cytotoxicity toward cancer cells after ascites treatment might result from enhanced γδ T cell recruitment to cancer cells through upregulated secretion of CXCL8 and

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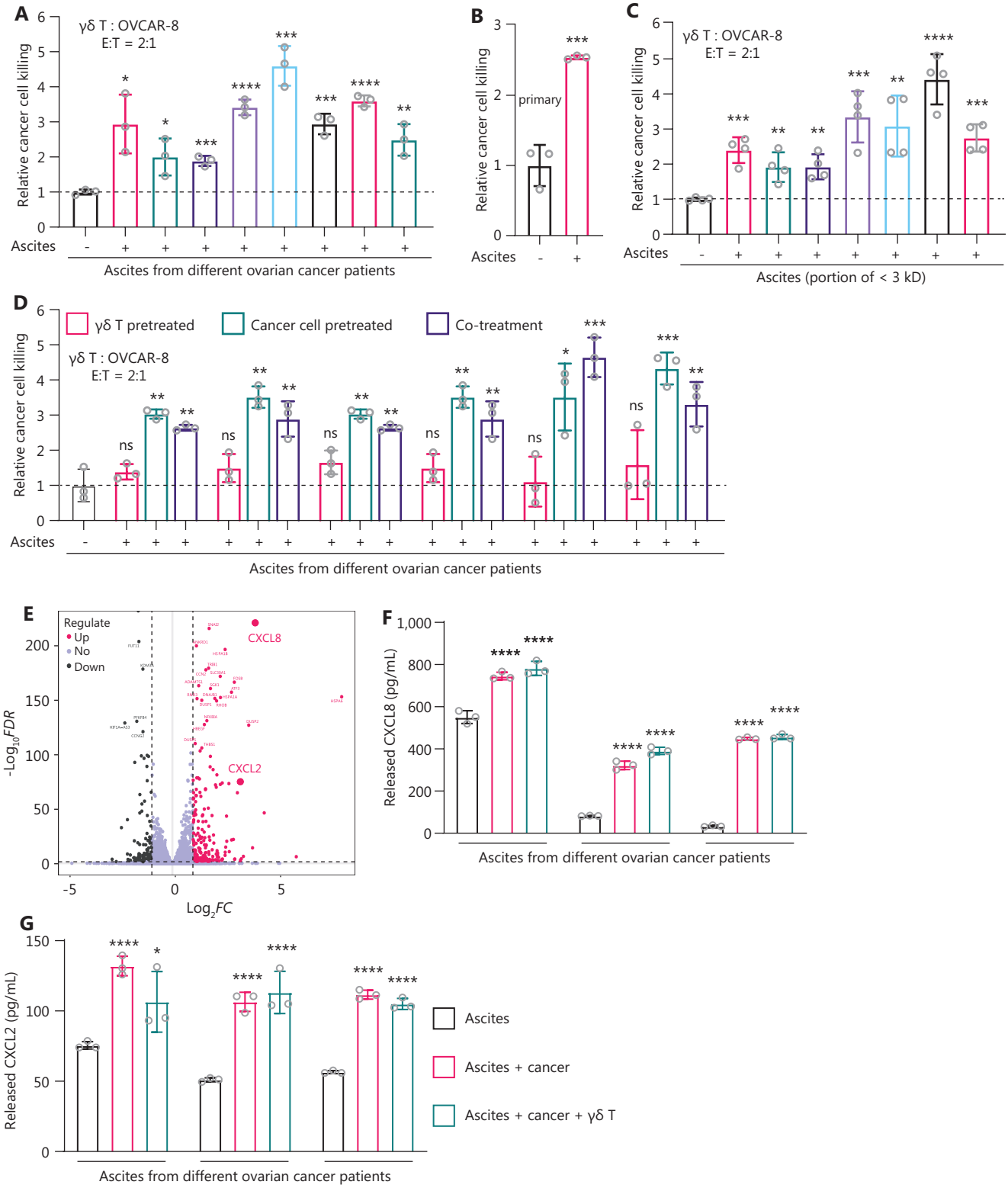


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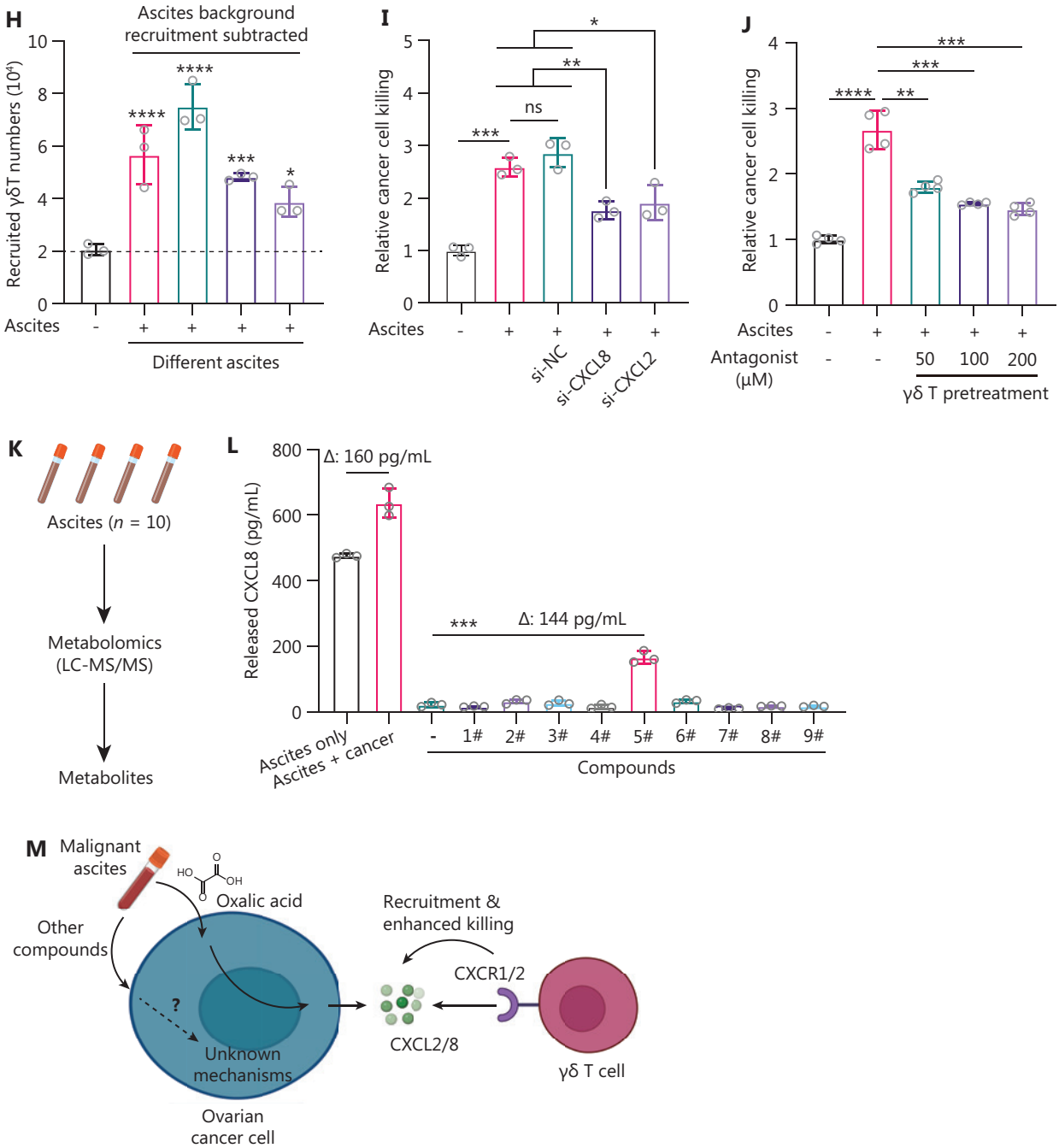


Figure 1 Malignant ascites enhance γδ T cell cytotoxicity toward ovarian cancer *via* chemokine-mediated recruitment. A. Malignant ascites enhance γδ T cell cytotoxicity toward the ovarian cancer cell line OVCAR-8 *in vitro*. B. Malignant ascites enhance γδ T cell cytotoxicity toward primary ovarian cancer cells derived from one patient *in vitro*. C. Low molecular weight components of the malignant ascites enhance γδ T cell cytotoxicity toward an ovarian cancer cell line *in vitro*. D. Malignant ascites act on cancer cells by enhancing γδ T cell cytotoxicity. E. Volcano plot showing transcriptional changes in the ovarian cancer cell line OVCAR-8 after treatment with malignant ascites for 3 h. F. ELISA validation of CXCL8 secretion by ovarian cancer cells treated with malignant ascites from multiple patients for 3 h. G. ELISA validation of CXCL2 secretion from ovarian cancer cells treated with malignant ascites from multiple patients for 3 h. H. Recruitment of γδ T cells by cell culture medium supernatants from ovarian cancer cells treated with ascites from multiple patients. The background recruitment of the ascites was subtracted. I. Knockdown of CXCL8 and CXCL2 attenuates ascites' enhancement of γδ T cell cytotoxicity toward ovarian cancer cells. J. Pretreatment of γδ T

cells with CXCR1/2 antagonist attenuates ascites' enhancement of γδ T cell cytotoxicity toward ovarian cancer cells. K. Schematic of the metabolomics-based strategy for identifying target metabolite molecules. L. ELISA validation of CXCL8 secretion by ovarian cancer cells treated with the 9 identified compounds for 3 h. M. Proposed mechanism of ascites derived compounds influencing the cytotoxicity of γδ T cells toward cancer cells. Oxalic acid enhances γδ T cell cytotoxicity *via* promoting CXCL2 and CXCL8 chemokine secretion by cancer cells that recruit γδ T cells, whereas the mechanisms for the other compounds remain to be determined. In all experiments, malignant ascites from multiple patients with ovarian cancer were tested and yielded similar results. Data are presented as mean ± SD. n = 3 or 4. ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

CXCL2 chemokines by cancer cells. To verify this hypothesis, we conducted a Transwell-based γδ T cell recruitment assay (**Figure S4A**). Treatment of the cancer cells with all 4 tested ascites samples, or recombinant CXCL2 and CXCL8 proteins, enhanced γδ T cell recruitment (**Figure 1H, Figure S4B, C**), thus suggesting that the enhanced γδ T cell cytotoxicity was due to the proximity between γδ T and cancer cells. To further confirm the key roles of the chemokines CXCL2/8 and their corresponding receptors, CXCR1/2, we knocked down either *Cxcl8* or *Cxcl2* in cancer cells by using siRNAs (**Figure S5**) or pre-blocked CXCR1/2 through CXCR1/2 antagonist treatment in γδ T cells[10] (**Figure S6**). Both experimental settings significantly decreased the enhancement in γδ T cell cytotoxicity after ascites treatment (**Figure 1I, J**). On the basis of these data, we propose a mechanism in which malignant ascites treatment first induces secretion of CXCL2 and CXCL8 chemokines by ovarian cancer cells. Subsequently, concentration gradients around the cancer cells recruit γδ T cells to cancer cells *via* the chemokine receptors CXCR1/2, which are expressed on γδ T cells and promote cytotoxicity toward ovarian cancer cells (**Figure S7**).

We next sought to identify the key components in the malignant ascites responsible for enhancing γδ T cell-mediated cytotoxicity toward ovarian cancer cells. We performed untargeted metabolomics to identify key small molecule metabolites (**Figure 1K**). Small molecule components from various ascites were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and the detected ions were mapped to human metabolite databases to identify potential metabolites in the ascites. Two analysis strategies were used to discover the key metabolites driving the enhancement of γδ T cell cytotoxicity toward cancer cells (**Figure S8A**). In the first strategy, the ascites samples were divided into 2 groups according to their enhancement efficacy (i.e., high or low enhancement of γδ T cell cytotoxicity) in a screening set and a validation set. The mass ion peak area, representing the relative concentration of one compound, was the key parameter affecting enhancement of γδ T cell cytotoxicity. Metabolites that were both screened and validated in the screening and validation sets were considered potential key metabolites. Through volcano plot analysis, we identified 8 potential compounds (**Figure S8B, C**). In cytotoxicity assays, treatment with all 8 compounds enhanced γδ T cell cytotoxicity toward ovarian cancer (**Figure S8D**). In the second analysis strategy, using metabolomics data, we conducted direct correlation analysis between metabolite concentration and γδ T cell cytotoxicity (**Figure S8A**). One compound showed high correlation efficiency (**Figure S8E, F**) and also enhanced γδ T cell cytotoxicity toward ovarian cancer cells (**Figure S8G**). In validation experiments in which ovarian cancer cells were treated with the 9 identified compounds, only compound 5 (oxalic acid) induced enhanced the secretion of both CXCL2 (**Figure S9**) and CXCL8 (**Figure 1L**) to levels comparable to the ascites induced enhancement. Because malignant ascites are complex mixtures, they might potentially enhance γδ T cell cytotoxicity through multiple mechanisms including the induction of the CXCL2 and CXCL8 chemokines that recruit the γδ T cells. This finding was also consistent with the observation that CXCL8 secretion levels after various ascites treatments did not strictly correlate with γδ T cytotoxicity (**Figure S10**). Herein, we propose a mechanism in which compound 5 (oxalic acid) enhances γδ T cell cytotoxicity through the chemokine-chemokine receptor pathway, whereas the mechanisms of the other 8 compounds remain to be investigated (**Figure 1M**).

To better demonstrate the importance of the unexpected role of malignant ascites, we further validated ascites function in chimeric antigen receptor T (CAR-T) cells, which have revolutionized cancer treatment. We sought to determine whether ascites might enhance CAR-γδ T cell cytotoxicity. We constructed "CAR-T like" anti-PD-L1 antibody-γδ T cell conjugates (γδ T-ACC) through chemical engineering to simulate the CAR-γδ T cells and mesothelin targeting MSLN-CAR-γδ T cells (**Figure S10A-D**). *In vitro* experiments revealed that, as expected, malignant ascites from multiple patients with

ovarian cancer enhanced the killing of ovarian cancer cells by "CAR-T like" γδ T-ACC and MSLN-CAR-γδ T cells (**Figure S10E, F**).

Overall, our work revealed another unexpected favorable function of malignant ascites from patients with ovarian cancer, in which malignant ascites enhance the killing of cancer cells by γδ T cells *via* a mechanism involving chemokine-mediated γδ T cell recruitment. This work may guide future fundamental and translational studies on adoptive γδ T cell transfer for the treatment of ovarian cancer with malignant ascites formation, which is present in 90% of patients with advanced stage ovarian cancer.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Long Chen, Jian Lin, Yinglin Zhou, Hongyan Guo.
Performed the experiments: Zhanqun Yang, Ying Liu, Mengzhu Zheng, Hui Li, Ruoyao Cui.
Collected clinical samples: Pan Wang, Tianhui He.
Analyzed the data and drafted the manuscript: Zhanqun Yang, Long Chen.
Revised the manuscript: Long Chen, Jian Lin, Yinglin Zhou, Hongyan Guo.

Data availability statement

The data are available from the corresponding author on reasonable request.

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