

A comprehensive cell profiling reveals new cell subtypes and immune dysfunction in peritoneal fluid of endometriosis

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Research

Keywords: Endometriosis, Single-cell RNA-sequencing, Peritoneal fluid, Cell profiling, Immune dysfunction

Posted Date: October 14th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-89810/v1>

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Abstract

Background

Endometriosis is a refractory and recurrent disease and it affects nearly 10% of reproductive-aged women and 40% of infertile patients. The commonly accepted theory for endometriosis is retrograde menstruation where endometrial tissues invade into peritoneal cavity and fail to be cleared due to immune dysfunction. Therefore, the comprehensive understanding of immunologic microenvironment of peritoneal cavity deserves further investigation for the previous studies mainly focus on one or several immune cells.

Results

High-quality transcriptomes were from peritoneal fluid samples of patients with endometriosis and control, and firstly subjected to 10 × genomics single-cell RNA-sequencing. We acquired the single-cell transcriptomes of 10,280 cells from endometriosis sample and 7,250 cells from control sample with an average of approximately 63,000 reads per cell. A comprehensive map of overall cells in peritoneal fluid was first exhibited and it showed that immune cells predominated in peritoneal fluid. We unveiled the heterogeneity of immune cells and discovered new cell subtypes including TCR⁺ macrophages, proliferating macrophages and natural killer dendritic cells in peritoneal fluid, which was further verified by double immunofluorescence staining and flow cytometry. Pseudo-time analysis showed that the response of macrophages to the menstrual debris might follow the certain differentiation trajectory after endometrial tissues invaded into the peritoneal cavity. Our analyses also mirrored the dysfunctions of immune cells including decreased phagocytosis and cytotoxic activity and elevated pro-inflammatory and chemotactic effects in endometriosis.

Conclusion

TCR⁺ macrophages, proliferating macrophages and natural killer dendritic cells are firstly reported in peritoneal fluid. Our results also revealed that peritoneal cavity is an immune microenvironment and immune dysfunction happens in peritoneal fluid of endometriosis, which may be responsible for the residues of invaded menstrual debris. It provided a large-scale and high-dimensional characterization of peritoneal microenvironment and offered a useful resource for immunotherapy of endometriosis.

Background

Endometriosis is a chronic inflammatory disease and affects nearly 10% of reproductive-aged women and as high as 40% of infertile women [1]. It is clinically manifested with severe pelvic pain and reduced fertility and characterized by the presence and growth of endometrial tissue outside the uterus, which seriously reduces the life quality of patients and causes a heavy burden on the healthcare [2, 3]. The etiology is still incompletely understood and “retrograde menstruation” is one widely accepted theory that the reflux accounts for the accumulation of menstrual debris in peritoneal cavity [4, 5]. However,

retrograde menstruation occurs in almost all cycling women, while only a minority of them develops endometriosis, implying additional factors contribute to the development of endometriosis [6].

Immune cells contribute to scavenging menstrual debris in cycling women [7]. One of the possible causes of endometriosis is the defective immune response to the refluxed menstrual debris in peritoneal cavity, which determines the survival and implantation of ectopic endometrial cells and lesion formation [8]. Accumulated evidence over the past decade has suggested that development of endometriosis is accompanied with sustained peritoneal inflammation, including altered immune cell contents in peritoneal fluid and ectopic lesions, as well as changed immune cells cytotoxicity and activation [9]. Alterations in both innate and adaptive immunity contribute to the pathogenesis of endometriosis [9–11]. As the front line of innate immunity, macrophages comprise the largest immune cell population in peritoneal fluid of both healthy women and patients with endometriosis [12]. They are found to be increased and alternatively activated based on the M1/M2 (classically activated macrophages/alternatively activated macrophages) polarization paradigm in endometriosis [13]. In addition to macrophages, other immune cells also have been proposed to play important roles in the pathogenesis of endometriosis. Decreased cytotoxicity of natural killer (NK) cells had been reported in peritoneal fluid of endometriosis [14]. An increased proportion of regulatory T (Treg) cells in peritoneal fluid of women with endometriosis has been reported [15]. Dendritic cells (DCs), mast cells and B cells have been observed to be changed as well [16–18].

The previous studies mainly focus on one or several immune cells and are lack of comprehensive investigation, which leads to a failure to discover heterogeneous cell contents at an unbiased scale. Single-cell RNA-sequencing (scRNAseq) is an experimental approach to obtain an unbiased map of all mRNAs present in one cell. Recent advance in scRNAseq has the potential to resolve heterogeneous cell populations at an unprecedented scale [19]. It has been used to discriminate cell types in healthy tissues or tumors, to explore immune cell heterogeneity, and to reveal new types of immune cells [20, 21]. Since peritoneal fluid plays an important role in the pathogenesis of endometriosis, it deserves to conduct a study for the comprehensive and unbiased characterization of cell contents and scRNAseq of all the cells in peritoneal fluid seems to be a wonderful choice. Here, we found that the peritoneal microenvironment is mainly composed of different immune cells and we revealed that peritoneal fluid is an immune microenvironment. We further found that the immune cells in endometriosis were dysfunctional with decreased phagocytosis and cytotoxic activity and elevated pro-inflammatory and chemotactic effects. Importantly, our findings offer a useful resource for understanding pathology of endometriosis and potential immunotherapy of endometriosis.

Methods

Ethics and sample collection

This project was approved by the Ethics Committee of Women's Hospital, School of Medicine, Zhejiang University (IRB-20200003-R). Included patients supplied written informed consent for collection of

specimens and analyses of the derived genetic materials prior to their participation.

Peritoneal fluid samples were collected from 23 endometriosis patients and 13 non-endometriosis controls who were undergoing surgery at the hospital (details in Additional file 2: Table S1). Among them, cell suspensions from one endometriosis patient and one control patient with septate uterus were subjected to scRNAseq and the other 34 samples were applied for the validation using double immunofluorescence or flow cytometry. Samples were collected during laparoscopic surgery before any surgical procedure to avoid contamination from blood.

Cell preparation

Cells were pelleted from peritoneal fluid and washed three times in cold PBS by centrifugation at 1600 rpm for 5 min. Red blood cells were lysed using Ammonium-Chloride-Potassium Lysing Buffer (Gibco, USA) according to the manufacturer's instructions, then the suspension was washed in cold PBS for three times. Samples were next diluted with PBS containing 0.04% Bovine Serum Albumin (Sigma, USA) to the density of about 1×10^6 cells/mL. 10 μ L of this cell suspension was mixed with 10 μ L 0.4% trypan blue solution (Sigma, USA) and counted using an automated cell counter (Bio-rad, USA) to determine the density of live cells. Cell viability of samples used for single cell sequencing was 94% for endometriosis sample and 86% for control sample. Cells were maintained on ice whenever possible throughout the dissociation procedure, and the entire procedure was completed less than one hour.

scRNAseq using 10 × Genomics

The density of single cell suspension was counted and adjusted to 1,000 cells/ μ L. The cell suspension was loaded into Chromium microfluidic chips with 3' (v3) chemistry and barcoded with a 10 × Chromium Controller (10 × Genomics) in order to catch approximately 10,000 cells/chip position. The remaining procedures including reverse transcription and the library construction were performed according to the standard manufacturer's instructions. Single cell libraries were sequenced on NovaSeq with approximately 50,000 to 100,000 reads per cell. Single-cell analyses were performed using Cell Ranger 3.0 and Seurat unless mentioned specifically. For the quality control, low quality cells (< 3 cells/gene, < 200 genes/cell, > 6 500 genes/cell, > 5% hemoglobin genes and > 30% mitochondrial genes) were removed. The average gene detection, number of UMIs and the level of mitochondrial reads were similar between the two samples (Additional file 3: Table S2).

Identification of cell clusters by UMAP analysis of scRNAseq datasets

To identify major cell types, we performed uniform manifold approximation and projection (UMAP) clustering using the Seurat package of R software. Highly variable genes were then generated for the perform of principal component analysis (PCA). The Seurat R package was used to identify significant clusters. To determine the cell types, we used a combination of marker genes identified from the literature and the web-based CellMarker databases (<http://biocc.hrbmu.edu.cn/CellMarker/>). The detailed marker genes were listed below: PTPRC/CD45 for leukocytes, CD68, CD14 and FCGR3A/CD16 for macrophages, ITGAX/CD11C, HLA-DRA and CD1C for DCs, CD3D, CD3G and CD3E for T cells, KLRD1, NKG7 and KLRB1

for NK cells, IGHM for plasma cells, CPA3 and GATA2 for mast cells, ITGAX, CD1C and KLRB1 for NKDCs, KRT8 for epithelial cells, SLC05A1 and NCCRP1 for stem cells. Heatmaps and violin plots were generated from R package using the default complete-linkage clustering algorithm.

Biological process enrichment analysis, pathways analysis and single cell trajectories

We used the web-based DAVID and KOBAS to performed biological process enrichment analysis with the differentially expressed genes in each cluster. Gene Set Enrichment Analysis (GSEA) was applied to identify a priori defined set of genes that show statistically significant differences in each cell types between endometriosis and control samples. We used the mean expression of genes in endometriosis and control samples as the input (Additional file 4: Table S3), and implied gene set of GO and KEGG pathway, which were corrected in Molecular Signatures Database. The Monocle package of R software was used to analysis single cell trajectories in macrophage subtypes in order to discover the developmental transitions of macrophages.

Double immunofluorescence staining

For the staining of membrane proteins, including CD14 (eBioscience, USA) and TCR C β 1 (Santa, USA), ascites cells were incubated with the indicated fluorochrome- or biotin-conjugated antibodies for 30 min at 4 °C. For the staining of KI67 (BD Biosciences, USA), the cells were firstly resuspended in 250 μ L BD Cytofix/Cytoperm solution at 4 °C for 20 min and then incubated with 20 μ L antibody for 30 min at 4 °C. The nuclear was stained by DAPI and the samples were analyzed with FV1000 confocal microscope.

Flow cytometry

Flow cytometry was conducted to measure the percentages of CD14⁺ TCR C β 1⁺ and CD14⁺ KI67⁺ cells in peritoneal fluid. For cell surface staining, the cell suspension was incubated with CD14 and TCR C β 1 antibodies. For CD14 and KI67 staining, the cell suspension was incubated with CD14 antibody at 4 °C for 30 min, and then incubated with the BD Cytofix/Cytoperm solution. After wash, the cells were incubated with 20 uL KI67 antibody for 30 min at 4 °C. The suspension was centrifuged, washed and re-suspended with 500 μ L PBS to detect the positive cells with Cytoflex S Flow Cytometer. The results were analyzed with CytExpert in percentage.

Statistical analysis

Data were presented as mean $\pm$ SD. Independent-sample t-test or Mann-Whitney U-test was applied when comparing two samples, and One-way ANOVA or Kruskal-Wallis was employed when comparing 3 or more samples. Statistical difference was considered to be significant at a value of $P < 0.05$ (*), highly significant at a value of $P < 0.01$ (**) and extremely significant when $P < 0.001$ (***). Differential gene expression testing was performed in Seurat as described in the scRNA-seq section.

Results

Single-cell expression atlas and cell types in peritoneal fluid indicated that peritoneal cavity was an immune microenvironment

To explore the cell profiling in peritoneal fluid, scRNAseq was performed (Fig. 1a). After initial quality control (Additional file 1: Fig. S1a), we acquired single-cell transcriptomes in a total of 10,280 cells from endometriosis sample and 7,250 cells from control sample with an average of approximately 63,000 reads per cell (Additional file 3: Table S2). Cell transcriptomes from the two samples were merged and analyzed together to gain power to detect rare cell types. To explore the intrinsic structure and potential functional subtypes of overall cells in peritoneal fluid, we applied PCA with variable genes across all cells and identified 19 clusters (Fig. 1b, Additional file 1: Fig. S1b and Additional file 5: Table S4). We then used well-known marker genes to define the identity of each cell cluster (see methods), such as co-expression of PTPRC/CD45, CD68, CD14 and FCGR3A/CD16 for macrophages (Fig. 1c)[22, 23]. Cluster 15 expressed marker genes for DCs (CD1C, ITGAX/CD11C) and NK cells (KLRB1) (Fig. 1d), and it mostly matched with natural killer dendritic cells (NKDCs), a rare intermediate cell type reported by Pillarisetty et al [24]. Eventually, we identified 9 main cell types (Fig. 1e), including macrophages (clusters 0, 1, 3, 6, 7, 9 and 11), T cells (clusters 4 and 5), DCs (clusters 2 and 14), NK cells (cluster 8), epithelial cells (cluster 12), mast cells (cluster 13 and 16), NKDCs (cluster 15), plasma cells (cluster 17) and stem cells (cluster 18). Otherwise, there was one cluster (cluster10) that we failed to match with any cell type because it lacked recognizable marker genes. Violin pictures and UMAP plots for each cell type further supported these cell types (Fig. 1f and Additional file 1: Fig. S1c). Eventually, we captured a comprehensive map of overall cells in peritoneal fluid (Fig. 1e, g and Additional file 6: Table S5). Immune cells predominated in peritoneal fluid of both endometriosis (96.5%) and control (95.5%), and macrophages were the main immune cells, followed by T cells, DCs, NK cells, mast cells, epithelial cells, NKDCs, plasma cells and stem cells, which indicated that peritoneal cavity was an immune microenvironment.

Distinct subtypes of macrophages revealed the heterogeneity of macrophages in peritoneal fluid

We identified 7 clusters representing different subtypes of macrophages (Fig. 2a). The proportions of each subtype of endometriosis and control groups were exhibited in Fig. 2b. Firstly, we investigated the M1/M2 polarization paradigm and found that one cell could express both M1 and M2 marker genes, such as the high expression of MARCO and S100A8 in cells of cluster 0 (Fig. 2c). Scatter plot further revealed that M1 gene signatures were correlated with M2 gene signatures and there was no significant shifting from M2 to M1 or from M1 to M2 (Fig. 2d). These findings supported the idea that macrophages in peritoneal fluid did not comport with the M1/M2 polarization model and the simplified view of M1/M2 model could not present the cell heterogeneity *in vivo* [25]. Therefore, we tried to explain the heterogeneity by functional enrichment of marker genes. Comparing the highly differentially expressed genes and function enrichments of each subtype (Fig. 2e and Additional file 7: Table S6), we found that scavenger receptors (MARCO and CD163) and complement receptors (C2, C1QA and C1QB) were highly expressed in cluster 0, indicating their phagocytic ability. VCAN was selectively expressed in cluster 1, which could promote the synthesis and secretion of inflammatory cytokines. In the meanwhile, genes related to proinflammatory cytokines, including LYZ, S100A8 and S100A9 were also highly expressed in cluster 1. In

contrast, genes related to adhesions and fibrosis such as SPP1 and CD9 were found at high levels in cluster 7. CCL2, CCL13, CCL18 and CXCL12 were highly expressed in cluster 3. Since CCL2 was the dominant chemokine gene for the migration of mononuclear phagocyte system and CCL13, CCL18 and CXCL12 were the critical chemokines, cluster 3 played an important role in the chemotactic function. Meanwhile, top differential genes also included APOE, APOC1 and LGMN in this cluster, showing their ability of plasma lipoprotein regulation. Genes involved in class II antigen presentation were present at highest level in cluster 6, showing their functions in antigen processing and presentation. Importantly, we found two new subtypes of macrophages which were not reported previously in peritoneal fluid. One was cluster 11 which expressed high levels of TCRs (TRBC1 and TRBC2). The critical components of the TCR signal transduction machinery were also expressed in cluster 11, such as CD3D, CD3E, LCK, ZAP70, LAT and FYN, which were mostly matched with TCR⁺ macrophages [26, 27]. The other one was cluster 9 where genes associated with cell proliferation, including MKI67, CDK1, UBE2C, BIRC5 and KIAA0101, were highly and selectively expressed, indicating that the macrophages were under the proliferating condition. Enriched GO analysis of each cluster supported these functions (Fig. 2e).

Pseudo-time analysis exhibited differentiation trajectory of macrophages in peritoneal fluid. To further investigate the differentiation trajectory of six clusters of macrophages (cluster 9 was excluded for the proliferating macrophages were not derived from monocytes), Monocle package of R software was applied for the analysis. The results showed that most cells from each cluster gathered based on the gene signatures and the six clusters formed into a relative process in pseudo-time. Specifically, it began with cluster 6 (antigen presentation), followed by cluster 1 (pro-inflammatory), and ended with cluster 3 (chemotaxis) and cluster 0 (phagocytosis) (Fig. 3a, b). Furthermore, Cluster 7 (adhesion and fibrosis) were presented in the whole period of the pseudo-time but highly enriched at the late period, which indicated that macrophages also had an ability for tissue repair in the whole development process and this ability was enhanced at the final stage of differentiation. As the newly discovered subtypes of macrophages, we discussed the functions of TCR⁺ macrophages below. Since retrograde menstruation was common in most women and macrophages were one of the main immune cells, the response of macrophages to the menstrual debris may follow the above differentiation trajectory after endometrial tissues invaded into the peritoneal cavity.

Two newly discovered subtypes of macrophages in peritoneal fluid

As above mentioned, we revealed two new subtypes of macrophages: TCR⁺ macrophages and proliferating macrophages. To confirm the existence of TCR⁺ macrophages in peritoneal fluid, double immunofluorescence staining of ascites cells was performed. We confirmed the marked existence of TCR Cβ1 (green) in CD14 (red) positive macrophages and the morphology of double positive cells looked as same as common macrophages and the size was bigger than that of T cells (Fig. 4a). Flow cytometry further confirmed that the percentage of TCR⁺ macrophages was elevated in endometriosis when compared to control (Fig. 4b), which was consistent with the results tested by scRNA-seq. Granzyme

(GZMK, GZMM, GZMH and GZMA) and immune mediators (CCL5, CCL4) were highly expressed (Additional file 7: Table S6) in this cluster, indicating their cytotoxic and chemotactic effects. GO terms further revealed that they were enriched in granulocyte activation, cytokine production and response to bacterium (Fig. 2e).

The majority of macrophages in peritoneal fluid were derived from monocytes, which were terminally differentiated and did not have the ability of proliferation. However, proliferating macrophages (cluster 9) were discovered and they were identified as tissue-resident macrophages [28]. We also verified the existence of this subtype of macrophages in peritoneal fluid using double immunofluorescence staining (Fig. 4c). Both scRNA-seq and flow cytometry revealed that the percentage of proliferating macrophages was decreased in endometriosis (Fig. 4d).

The dysfunction of macrophages in peritoneal fluid of endometriosis

In order to investigate if there was function deficiency of macrophages in peritoneal fluid of endometriosis, we applied GSEA to compare the differences between endometriosis and control samples. Cluster 0 which was the main subtype for phagocytosis had lower ability of phagocytosis in endometriosis. This dysfunction of phagocytosis could also be found in the other macrophage subtypes (Fig. 5a). For the subtypes with pro-inflammation (cluster 1), antigen presentation (cluster 6) and adhesion and fibrosis (cluster 7), GSEA showed that the corresponding functions were elevated in endometriosis (Fig. 5b). As to the two newly discovered subtypes of TCR⁺ macrophages and proliferating macrophages, GSEA also revealed the decreased ability of phagocytosis and elevated functions of pro-inflammation, antigen presentation and cell adhesion (Fig. 5c, d). These findings indicated that macrophages mainly had deficient ability for phagocytosis in endometriosis, which may be responsible for the incomplete clearance of refluxed menstrual debris.

The cytotoxic activity of NK cells was decreased while chemotactic effect was elevated in peritoneal fluid of endometriosis

Of all the cell types detected, the number of NK cells was increased most significantly in endometriosis sample (5.26%) when compared to the control sample (1.99%) (Additional file 6: Table S5). Comparing the differential genes, we found that this cluster was enriched in the KEGG pathway of natural killer cell mediated cytotoxicity (Fig. 6a), which further confirmed the identification of cluster 8. Then, we focused on the function of NK cells in peritoneal fluid of endometriosis. Go enrichment analysis and GSEA revealed that the cytotoxic activity of NK cells was decreased in endometriosis while the pro-inflammatory and chemotactic effects were elevated (Fig. 6b, c). The highly differential genes between the two groups showed that CCL3 and XCL1 were significantly elevated in endometriosis while cytotoxic molecules including GNLY, GZMB and GZMH were significantly down-regulated (Fig. 6d). These findings indicated that cytotoxic activity of NK cells was decreased while chemotactic effect was elevated in peritoneal fluid of endometriosis, which might play an important role in the pathology of endometriosis.

Two subtypes of DCs in peritoneal fluid

We identified two subtypes of DCs, cluster 2 and cluster 14 (Fig. 7a). Cluster 2 mapped closely to the well-established DC subtype of CD1C⁺ cDCs (Fig. 7b) [29]. FCER1A, CLEC10A, MRC1 and CD1E were also highly expressed in this cluster (Fig. 7c and Additional file 8: Table S7). As for cluster 14, it mapped most closely to THBD⁺ (CD141) cDCs. But this commonly used marker (THBD) was a poor discriminator for this cluster, being also expressed by cells captured in macrophages (Fig. 7b). As CLEC9A appeared to be a perfect discriminative marker gene for this cluster, we refer to this subtype as CLEC9A⁺ cDCs as previously reported [21]. In addition, XCR1 and DNASE1L3 were also highly and selectively expressed in this cluster. Both of the DCs subtypes had the abilities of antigen uptake, presentation and leukocyte activation (Fig. 7c). CD1C⁺ cDCs were the main subtype of DCs in peritoneal fluid, which occupied 93.3% of the total DCs. On the other hand, CLEC9A⁺ cDCs showed the special capacity to induce CD8⁺ CTL responses with the high expression of CLEC9A and XCR1, the well-known receptors to cross-present antigens to CD8⁺ T cells [30, 31].

NKDCs were firstly discovered in peritoneal fluid

As a rare cell type, NKDCs were not reported in peritoneal fluid previously, we investigated their differential marker genes and enriched functions. We found that NKDCs expressed high levels of DC markers (CD1C, ITGAX and CD1E) and MHC class II receptors (Additional file 5: Table S4), and also expressed NK cell markers (KLRB1, KLRD1 and NKG7) and T cell markers (CD3D, CD3E and CD3G) (Additional file 1: Fig. S2). Enriched GO analysis revealed that NKDCs had the abilities of antigen processing and presentation, T cell activation and response to IFN- γ , indicating that NKDCs had abilities of both DCs and NK cells (Fig. 7d). These findings were consistent with previous reports [24].

The dysfunction of T cells in peritoneal fluid of endometriosis

For T cells, we identified two subtypes (Fig. 7e). Cluster 4 mostly mapped the CD8⁺ T cells while cluster 5 mapped most closely to CD4⁺ T cells (Additional file 1: Fig. S3a). KLRD1, CCL4, CCL5, GZMH, GZMA and GNLY were highly expressed in CD8⁺ T cells, indicating their cytotoxic and effector functions. The native markers, including IL7R and LTB, were expressed at high levels in CD4⁺ T cells (Fig. 7f and Additional file 9: Table S8). We further investigated the markers for regulatory T cells which was reported by previous literatures that played an important role in endometriosis [15, 32]. Unfortunately, these markers including FOXP3, IL2RA and IKZF2 expressed with low levels and did not form a cluster (Additional file 1: Fig. S3b). We compared the functions of T cell between endometriosis and control sample as well. The number of T cells was elevated in endometriosis sample (Additional file 6: Table S5). However, the cytotoxic effect and chemotactic activity of T cells were dysfunctional in endometriosis (Additional file 1: Fig. S3c).

Mast cells and other cell types in peritoneal fluid

There were also two subtypes for mast cells (Fig. 7g). Cluster 13 seemed to be the activated mast cells as TPSB2, TPSAB1 which encoding tryptase were highly and selectively in this cluster. KIT was also highly expressed in cluster 13, which was another activating marker for mast cells (Fig. 7h and Additional file 10: Table S9) [33]. Cluster 16 might be a transition state from basophils as they expressed high levels of CLC, MS4A3, IL3RA and GATA2 [34]. For plasma cells and other small number of cell types, we failed to conclude a significant functional difference during to the small number of cells.

Discussion

Previous studies that focused on the investigation of peritoneal fluid cell contents are mainly relied on flow cytometry or histological morphology [35, 36] while these techniques need prior knowledge and are limited to a small number of parameters. Here, we used scRNA-seq for the first time to investigate the cell contents and draw a comprehensive map of cell types in peritoneal fluid. We found that cells in peritoneal fluid were almost immune cells which are responsible for the clearance of refluxed menstrual debris and tissue defense. Macrophages are the largest immune population in peritoneal fluid followed by T cells and DCs, which is similar with previous studies [35, 36]. We also identified other groups of cells, including NK cells, mast cells, plasma cells and epithelial cells. Interestingly, we found an intermediate cell type which was named as NKDCs by Pillarisetty et al [24]. We also found new cell subtypes of macrophages and revealed their functions as well. Our study provides a fresh insight of peritoneal fluid cell contents and offers a useful resource for understanding menstruation and the pathology of endometriosis.

Our study consolidates and reinforces previous studies that immune dysfunction does existed in endometriosis [10]. As the first line of innate immunity, macrophages occupy the largest immune population in peritoneal fluid and have functional changes in endometriosis compared to control patients. The abilities of phagocytosis are defective in all seven subtypes which might lead to the incomplete clearance of refluxed menstrual debris and survival of endometrial cells. However, the pro-inflammatory, angiogenesis, adhesion and fibrosis effects are all elevated in endometriosis, which have been proved by previous studies [37]. The changed functions are also existed in other immune cells. NK cells are another main immune cell type to eliminate refluxed menstrual debris. Although the numbers of NK cells are elevated, the cytotoxic activity is found to be down-regulated in endometriosis. This decreased cytotoxic activity is also existed in T cells. These findings supported that immune dysfunction plays a central role in the development of endometriosis.

Therefore, we provide new insights into the peritoneal microenvironment in patients with advanced endometriosis and highlights several points of importance. Firstly, the peritoneal cavity is an immune microenvironment. The cells in peritoneal fluid are almost all immune cells which are responsible for the clearance of refluxed menstrual debris and tissue defense and infertility. Secondly, our results reveal that immune cells in peritoneal fluid, especially macrophages, are heterogeneous. Macrophages are the main immune cells in peritoneal fluid and did not comport with the M1/M2 polarization model. However, they might follow a certain differentiation trajectory after endometrial tissues invaded into the peritoneal cavity. Thirdly, the functions of immune cells in patients with endometriosis are defective. Generally

speaking, the phagocytic and toxic effects of the immune cells are reduced while the pro-inflammatory and chemotactic effects are elevated. This immune dysfunction might play a central role in the pathology of endometriosis.

The present study has several limitations. Firstly, selection bias was inevitable because of small numbers of cases, although we selected the patient of advanced endometriosis with severe dysmenorrhea and the control patient without pelvic abnormalities verified by laparoscopic surgery. Secondly, our results might only reveal the peritoneal microenvironment of early proliferative phase as the cell contents and functions might change in different stage of menstrual cycle. Therefore, well-designed larger scale studies are required.

Conclusion

Here, a comprehensive map of overall cells in peritoneal fluid was firstly exhibited by scRNAseq and then verified by double immunofluorescence staining and flow cytometry. We provide a large-scale and high-dimensional characterization of peritoneal microenvironment and firstly reported several novel cell subtypes including TCR + macrophages, proliferating macrophages and natural killer dendritic cells in peritoneal fluid. The results also consolidate that immune dysfunction does existed in endometriosis and offer a useful resource for immunotherapy of endometriosis.

Abbreviations

cDCs: conventional dendritic cells; DCs:dendritic cells; GSEA:Gene Set Enrichment Analysis; M1:classically activated macrophages; M2:alternatively activated macrophages; NK cells:natural killer cells; NKDCs:natural killer dendritic cells; PCA:principal component analysis; pDCs:plasmacytoid dendritic cells; scRNAseq:single-cell RNA-sequencing; TCR:T cell receptor; Treg:regulatory T cells; UMAP:uniform manifold approximation and projection; UMIs:unique molecule identifiers.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

ZG, WJ and XX conceived the project, designed the study, performed single-cell analysis and wrote paper. XP and ZL collect the clinical samples. GX isolated single cells. PY performed immunofluorescence staining. YQ and LT performed the flow cytometry. ZX conceived the project, designed the study and edited the manuscript with feedback from all authors. All authors read and approved the final manuscript.

Funding

This work was funded by National Key R&D Program of China (Grant number: 2017YFC1001202), National Natural Science Foundation of China (Grant numbers: 81974225, 81671429 and 81802591) and the Zhejiang Natural Science Foundation (LQ20H040007).

Availability of data and materials

All the data generated or analyzed during this study are included in this article and its supplementary files.

Ethics approval and consent to participate

This project was approved by the Ethics Committee of Women's Hospital, School of Medicine, Zhejiang University (IRB-20200003-R). All patients signed informed consent.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

The study was undertaken with the patient's consent

References

1. Brown J, Farquhar C. An overview of treatments for endometriosis. *JAMA*. 2008;313:296–97.
2. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN. Endometriosis. *Nat Rev Dis Primers*. 2018;4:9.
3. Simoens S, Dunselman G, Dirksen, et al. The burden of endometriosis: costs and quality of life of women with endometriosis and treated in referral centres. *Hum Reprod*. 2012;27:1292–9.
4. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol*. 1927;14:422–69.
5. Burney RO, Lathi RB. Menstrual bleeding from an endometriotic lesion. *Fertil Steril*. 2009;91:1926–7.
6. Patel BG, Lenk EE, Lebovic DI, Shu Y, Yu J, Taylor RN. Pathogenesis of endometriosis: interaction between endocrine and inflammatory pathways. *Best Pract Res Clin Obstet Gynaecol*. 2018;50:50–60.
7. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. *Front Immunol*. 2018;28:4–9.
8. Laux-Biehlmann A, d'Hooghe T, Zollner TM. Menstruation pulls the trigger for inflammation and pain in endometriosis. *Trends Pharmacol Sci*. 2015;36:270–6.
9. Symons LK, Miller JE, Kay VR, Marks RM, Liblik K, Koti M, et al. The immunopathophysiology of endometriosis. *Trends Mol Med*. 2018;24:748–62.

10. Guo SW, Miller J, Kay VR. Platelet-derived TGF- β 1 mediates the down-modulation of NKG2D expression and may be responsible for impaired natural killer (NK) cytotoxicity in women with endometriosis. *Hum Reprod.* 2016;31:1462–74.
11. Khan KN, Yamamoto K, Fujishita A, Muto H, Koshiba A, Kuroboshi, et al. Differential levels of regulatory T cells and T-helper-17 cells in women with early and advanced endometriosis. *J Clin Endocrinol Metab.* 2019;104:4715–29.
12. Hill JA, Faris HM, Schiff I, Anderson DJ. Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. *Fertil Steril.* 1988;50:216–22.
13. Bacci M, Capobianco A, Monno A, Cottone L, Puppo FD, Camisa B, et al. Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol.* 2009;175:547–56.
14. Sikora J, Mielczarek-Palacz A, Kondera-Anasz Z. Role of natural killer cell activity in the pathogenesis of endometriosis. *Curr Med Chem.* 2011;18:200–8.
15. Olkowska-Truchanowicz J, Bocian K, Maksym RB, Bialoszewska A, Wlodarczyk D, Baranowski W, et al. CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis. *Hum Reprod.* 2013;28:119–24.
16. Fainaru O, Adini A, Benny O, Adini I, Short S, Bazinet L, et al. Dendritic cells support angiogenesis and promote lesion growth in a murine model of endometriosis. *FASEB J.* 2008;22:522–9.
17. Li T, Wang J, Guo X, Yu Q, Ding S, Xu X, et al. Possible involvement of crosstalk between endometrial cells and mast cells in the development of endometriosis via CCL8/CCR1. *Biomed Pharmacother.* 2020. doi:10.1016/j.biopha.2020.110476.
18. Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update.* 2019;25:564–91.
19. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nat Rev Immunol.* 2018;12:35–45.
20. Stubbington MJT, Rozenblatt-Rosen O, Regev A, Teichmann SA. Single-cell transcriptomics to explore the immune system in health and disease. *Science.* 2017;358:58–63.
21. Villani A, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science.* 2017. doi:10.1126/science.aah4573.
22. Peng J, Sun BF, Chen CY, Zhou JY, Chen YS, Chen H, et al. Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. *Cell Res.* 2019;29:725–38.
23. Chung W, Eum HH, Lee HO, Lee KM, Lee HB, Kim KT, et al. Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nat Commun.* 2017. doi:10.1038/ncomms15081.

24. Pillarisetty VG, Katz SC, Bleier JI, Shah AB, Dematteo RP. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. *J Immunol.* 2005;174:2612–8.
25. Nahrendorf M, Swirski KF. Abandoning M1/M2 for a Network Model of Macrophage Function. *Circ Res.* 2016;119:414–7.
26. Beham AW, Puellmann K, Laird R, Fuchs T, Streich R, Breysach C, et al. A TNF-regulated recombinatorial macrophage immune receptor implicated in granuloma formation in tuberculosis. *PLoS Pathog.* 2011. doi:10.1371/journal.ppat.1002375.
27. Chávez- Galán L, Olleros M, Vesin D, Garcia I. Much more than M1 and M2 macrophages, there are also CD169⁺ and TCR⁺ macrophages. *Front Immunol.* 2015. doi:10.3389/fimmu.
28. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity.* 2013;38:792–804.
29. O’Keeffe M, Mok WH, Radford KJ. Human dendritic cell subsets and function in health and disease. *Cell Mol Life Sci.* 2015;72:4309–25.
30. Schreibelt G, Klinkenberg LJJ, Cruz LJ, Tacke PJ, Tel J, Kreutz M, et al. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3⁺ myeloid dendritic cells. *Blood.* 2012;8:2284–92.
31. Hartung E, Becker M, Bachem A, Reeg N, Jäkel A, Hutloff A, et al. Induction of potent CD8 T cell cytotoxicity by specific targeting of antigen to cross-presenting dendritic cells in vivo via murine or human XCR1. *J Immunol.* 2015;193:1069–79.
32. de Barros IBL, Malvezzi H, Gueuvoghlian-Silva BY, Piccinato CA, Rizzo LV, Podgaec S. What do we know about regulatory T cells and endometriosis? A systematic review. *J Reprod Immunol.* 2017;120:48–55.
33. Liang Y, Qiao L, Peng X, Cui Z, Yin Y, Liao H, et al. The chemokine receptor CCR1 is identified in mast cell-derived exosomes. *Am J Transl Res.* 2018;10:352–67.
34. Ohmori S, Moriguchi T, Noguchi Y, Ikeda M, Kobayashi K, Tomaru N, et al. GATA2 is critical for the maintenance of cellular identity in differentiated mast cells derived from mouse bone marrow. *Blood.* 2015;121:3306–15.
35. Oral E, Olive DL, Arici A. The peritoneal environment in endometriosis. *Hum Reprod Update.* 1996;5:385–98.
36. Guo M, Bafligil C, Tapmeier T, Hubbard C, Manek S, Shang C, et al. Mass cytometry analysis reveals a distinct immune environment in peritoneal fluid in endometriosis: a characterisation study. *BMC Med.* 2020;18:3.
37. Sun H, Li D, Yuan M, Li Q, Zhen Q, Li N, et al. Macrophages alternatively activated by endometriosis-exosomes contribute to the development of lesions in mice. *Mol Hum Reprod.* 2019;25:5–16.

Figures

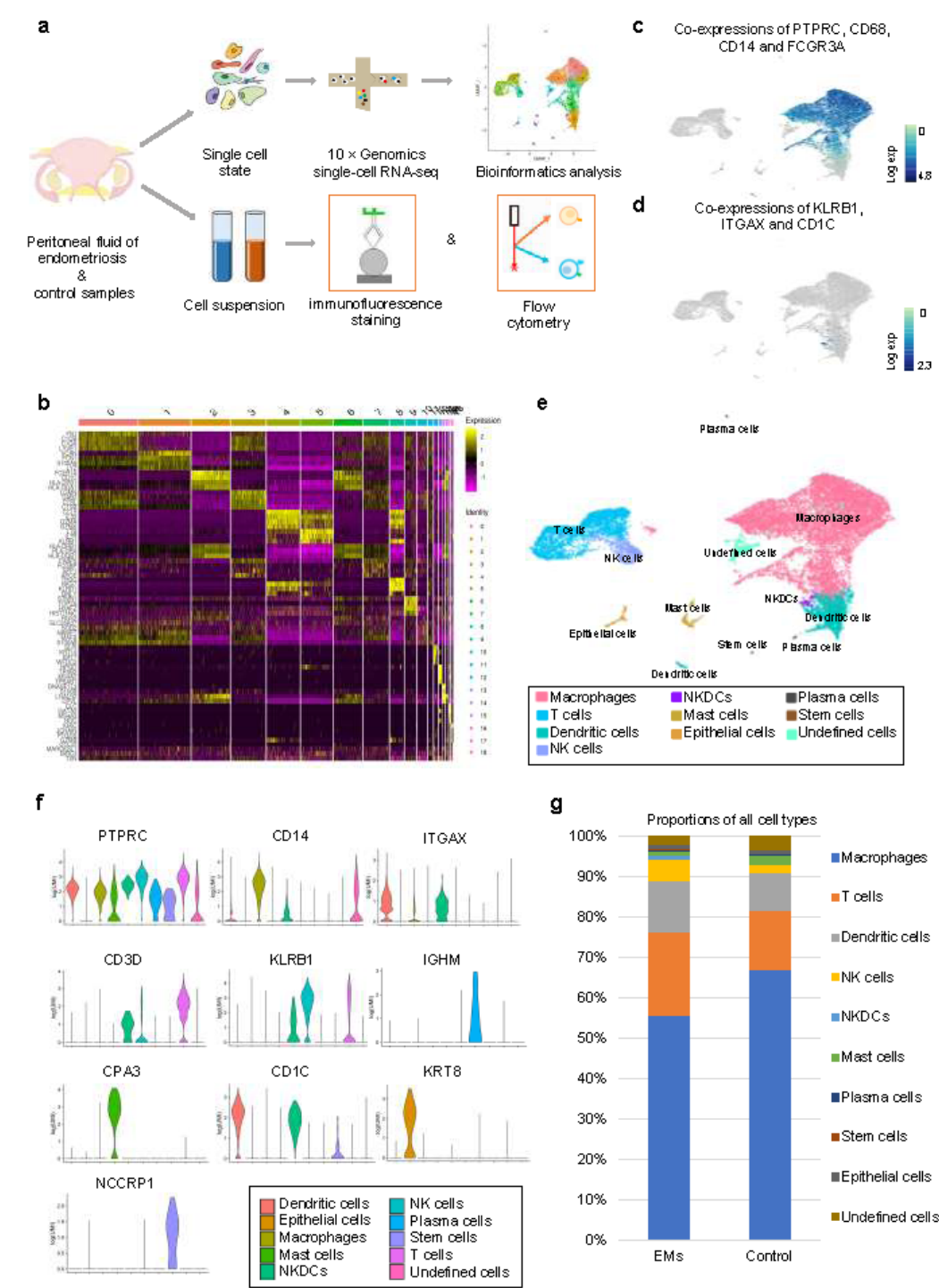


Figure 1

Cell profiling in peritoneal fluid delineated by single cell transcriptomic analysis. a The workflow that depicts the collection and processing of peritoneal fluid from endometriosis and control patients for single-cell RNA-sequencing and further study. b The heatmap exhibited that the differentially expressed

genes of 17,530 isolated cells gathered in 19 clusters. c Co-expressions of PTPRC/CD45, CD68, CD14 and FCGR3A/CD16 (macrophage makers) on the UMAP plot. d Co-expressions of KLRB1, ITGAX and CD11C on the UMAP plot. e 2D visualization of ten cell types on the UMAP plot. Each dot corresponds to one single cell. f Violin plots of specific marker genes in all cell types. g Bar plot of proportions of all cell types in the endometriosis and control samples. EMs: endometriosis; NK cells: natural killer cells; NKDCs: natural killer dendritic cells

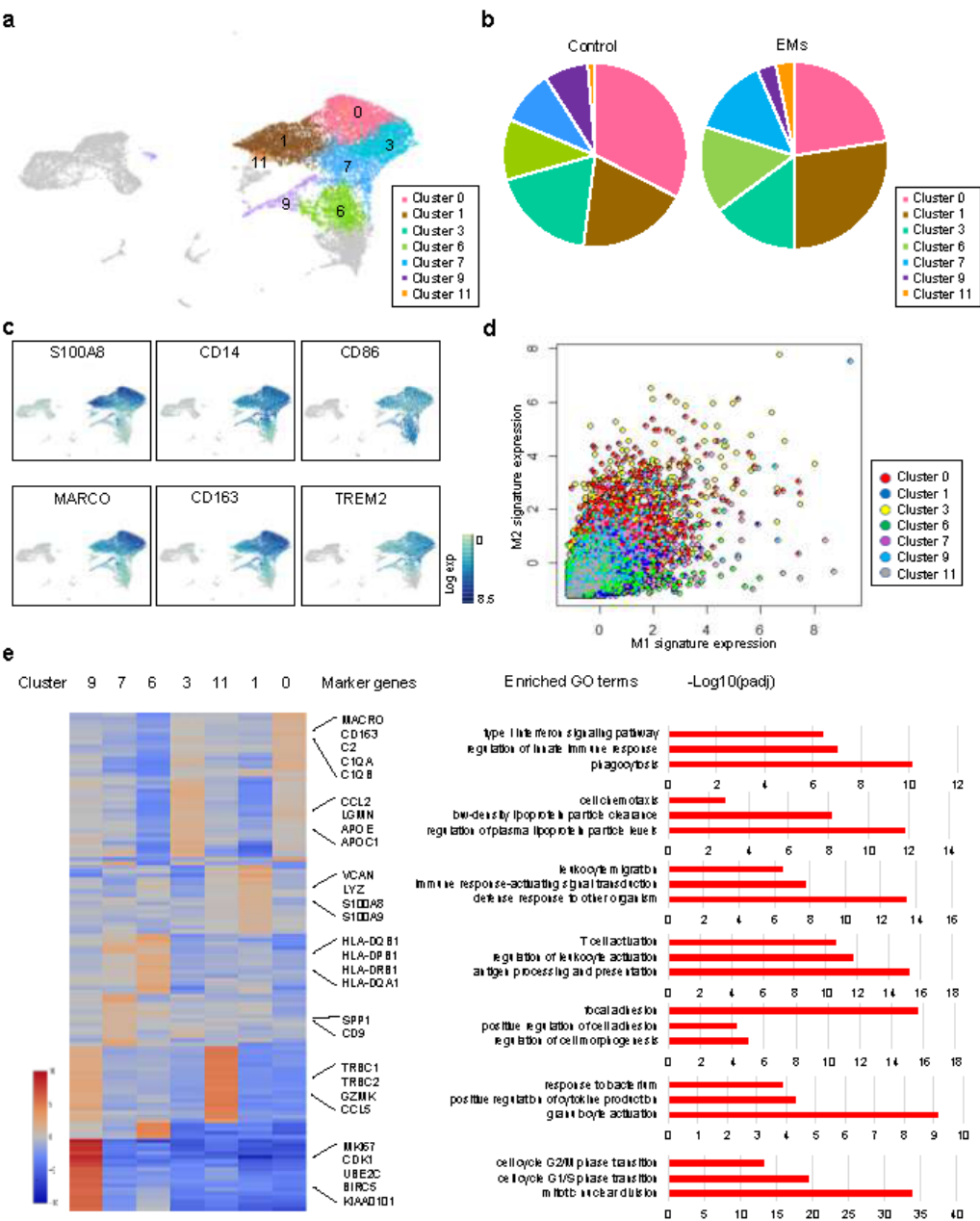


Figure 2

The heterogeneity of macrophages in peritoneal fluid. a 2D visualization of seven clusters of macrophages on the UMAP plot. b Pie charts of proportions of seven macrophage subtypes in endometriosis and control samples. c Distributions of M1 (S100A8, CD14 and CD86) and M2 (MARCO, CD163 and TREM2) marker genes on the UMAP plots. d Scatter plot of normalized gene expression of M1 and M2 signatures per cell. Each dot corresponds to one single cell. e Heatmap, marker genes and enriched GO terms of seven subtypes of macrophages. EMs: endometriosis; M1: classically activated macrophages; M2: alternatively activated macrophages

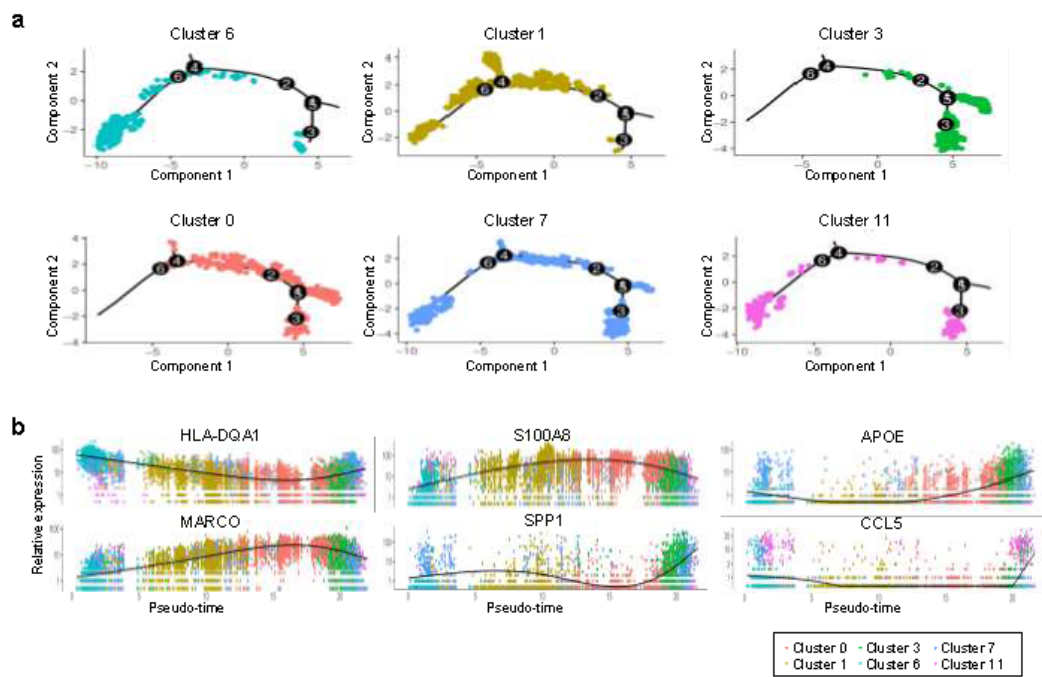


Figure 3

The differentiation trajectory of macrophages in peritoneal fluid by Pseudo-time analysis. a Pseudo-time of six subtypes of macrophages in peritoneal fluid inferred by Monocle package of R software. Each dot corresponds to one single cell. b Expressions of differential marker genes across six subtypes of macrophages, ordered by Monocle analysis in pseudo-time.

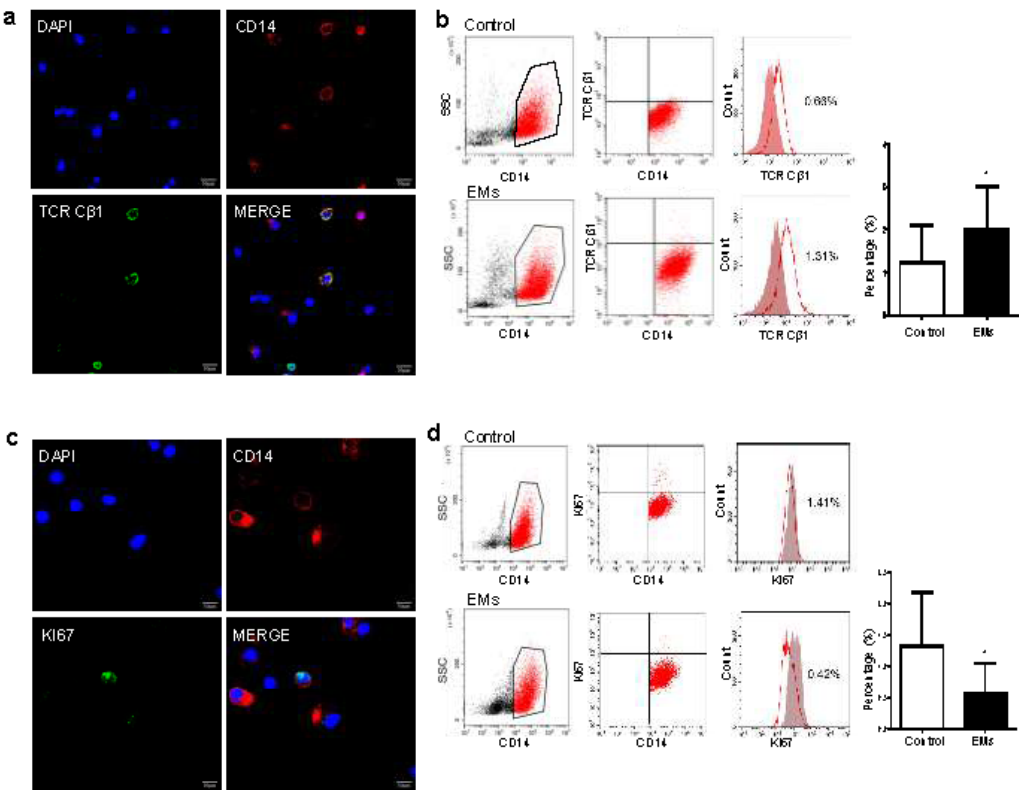


Figure 4

Two newly discovered subtypes of macrophages in peritoneal fluid. a Double immunofluorescence staining for CD14 (red) and TCR C β 1 (green) antigens of ascites cell. Original magnification 1,000 $\times$, scale bars = 10 μ m. b The rate of TCR C β 1 positive cells in isolated CD14 positive peritoneal macrophages tested by flow cytometric analysis. Bar plot shows the percentage of TCR C β 1 positive macrophages in CD14 positive peritoneal macrophages isolated from the endometriosis (n = 13) and control samples (n = 9), p = 0.0206. c Double immunofluorescence staining for CD14 (red) and KI67 (green) antigens of ascites cell. Original magnification 1,000 $\times$, scale bars = 10 μ m. d The rate of KI67 positive cells in isolated CD14 positive peritoneal macrophages was tested by flow cytometric analysis. Bar plot shows the percentage of KI67 positive macrophages in CD14 positive peritoneal macrophages isolated from the endometriosis (n = 11) and control samples (n = 9), p = 0.0229. EMs: endometriosis

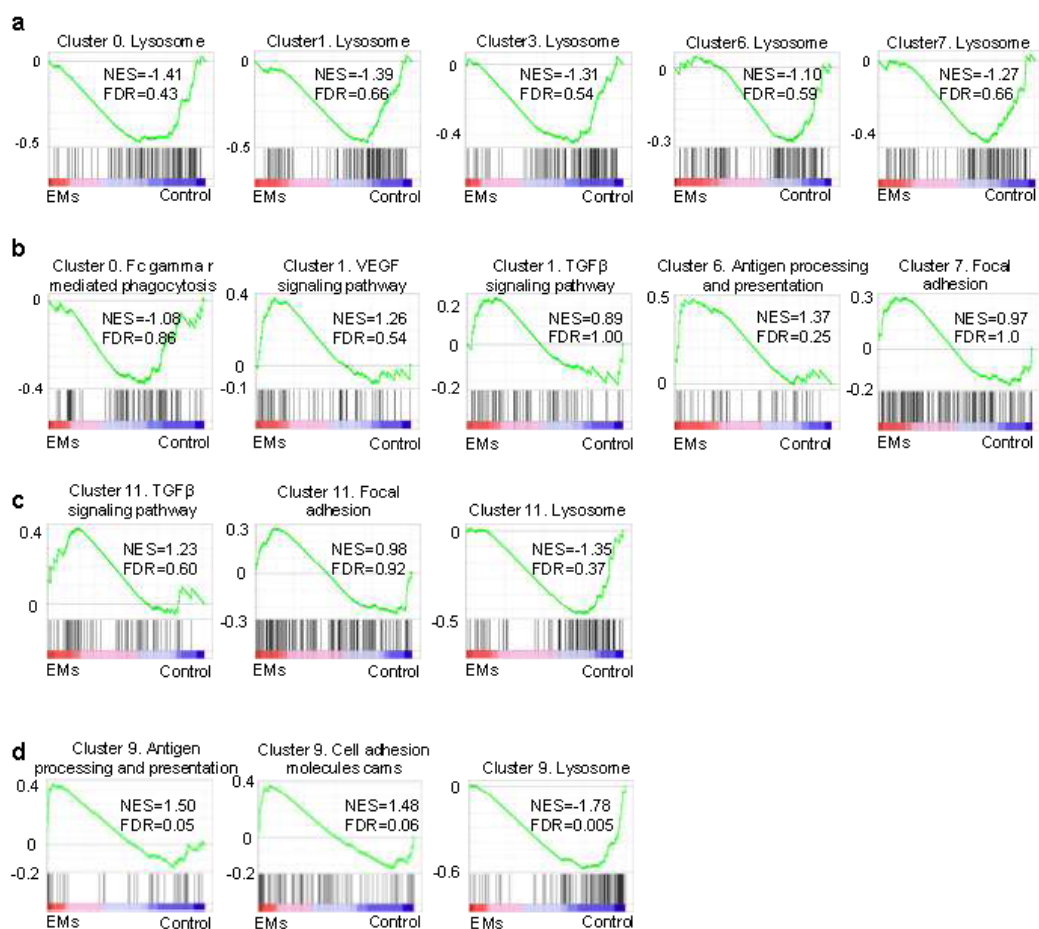


Figure 5

The dysfunction of macrophages in peritoneal fluid of endometriosis. a GSEA of Lysosome pathway in different clusters of macrophages in endometriosis and control samples. b GSEA of corresponding KEGG pathways in different clusters of macrophages in endometriosis and control samples. c GSEA of Lysosome, Focal adhesion and TGFβ signaling pathways of TCR+ macrophages in endometriosis and control samples. d GSEA of Lysosome, Cell adhesion molecules cams and Antigen processing and

presentation pathways of proliferating macrophages in endometriosis and control samples. EMs: endometriosis; NES: normalized enrichment score; FDR: false discovery rate; GSEA: Gene Set Enrichment Analysis

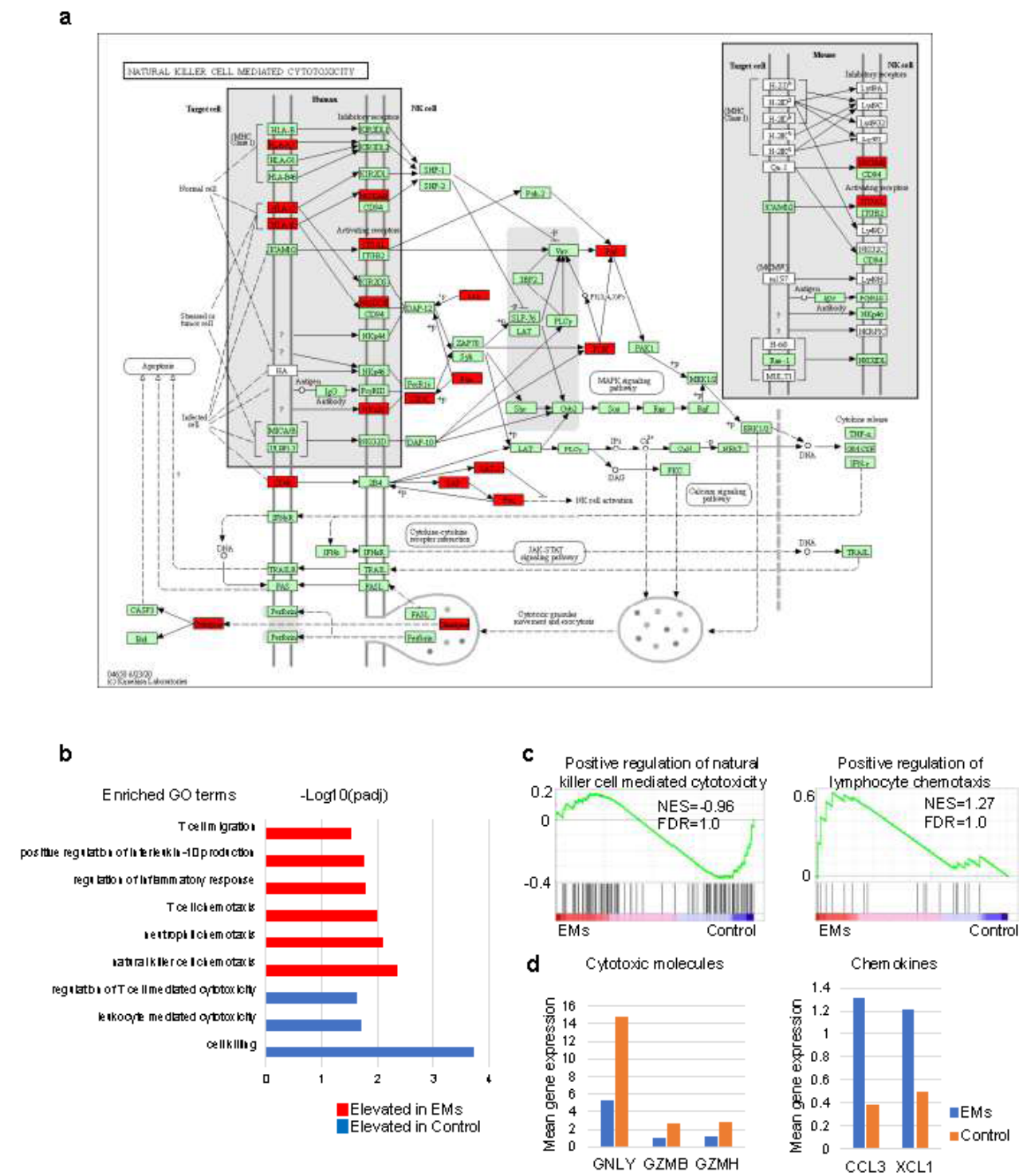


Figure 6

Dysfunction of NK cells in endometriosis. a Enriched KEGG pathway of natural killer cell mediated cytotoxicity of cluster 8. Red presents differentially expressed gene. b Enriched GO terms of natural killer

cells. c Gene Set Enrichment Analysis of positive regulation of natural killer cell mediated cytotoxicity pathway and positive regulation of lymphocyte chemotaxis pathway of natural killer cells in endometriosis and control samples. d Mean gene expressions of GNLY, GZMB, GZMH, CCL3 and XCL1 of natural killer cells in endometriosis and control samples. EMs: endometriosis; NES: normalized enrichment score; FDR: false discovery rate

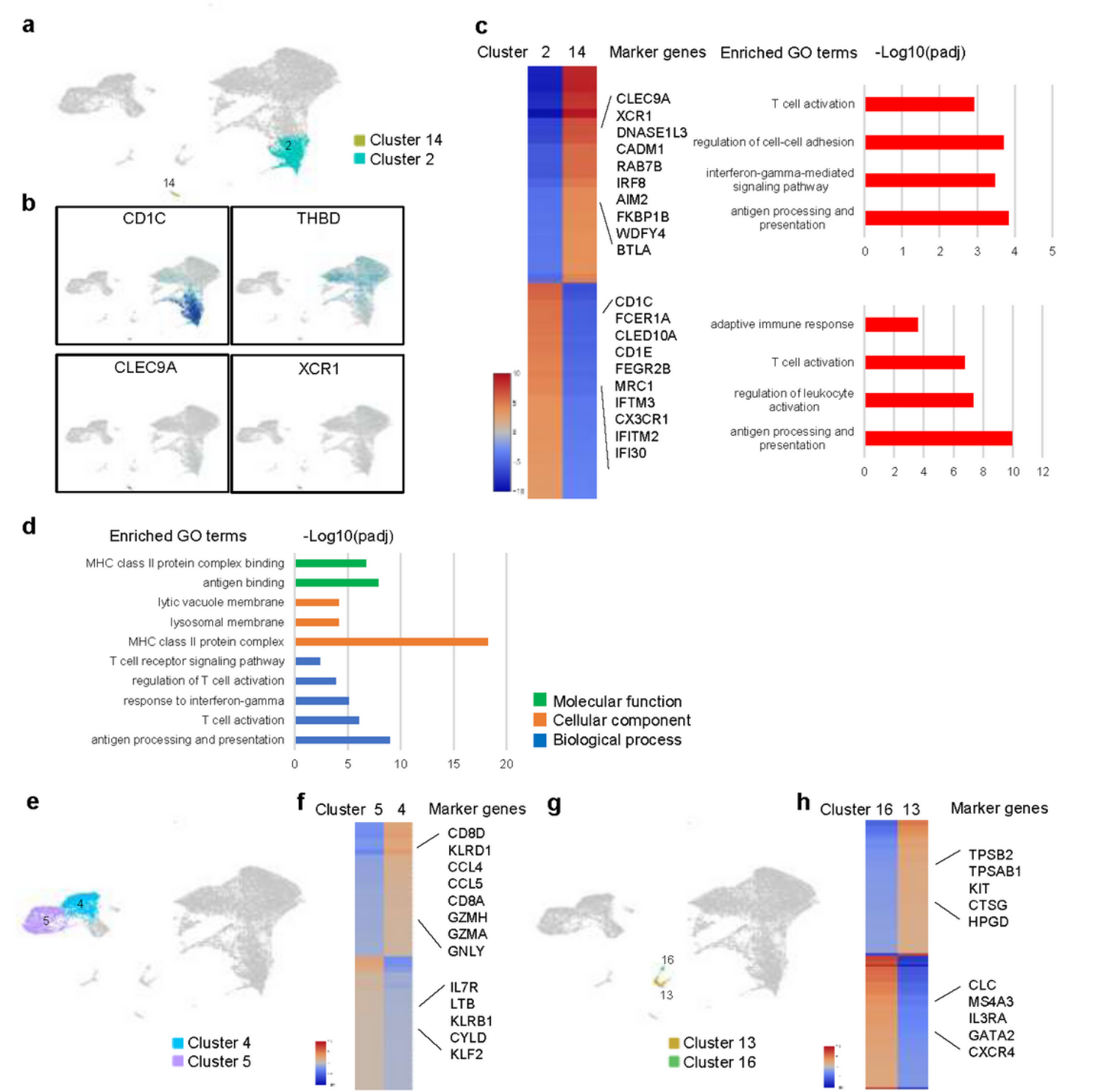


Figure 7

Dendritic cells, natural killer dendritic cells, T cells and mast cells in peritoneal fluid. a, e, g 2D visualization of clusters of dendritic cells (a), T cells (e) and mast cells (g) on the UMAP plots. Each dot corresponds to one single cell. b Distributions of CD1C, THBD, CLEC9A and XCR1 on the UMAP plots. c Heatmap, marker genes and enriched GO terms of dendritic cells. d Enriched GO terms of natural killer dendritic cells. f, g Heatmap and marker genes of T cells (f) and mast cells (h).

Supplementary Files

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