Selective oxidative protection leads to tissue topological changes orchestrated by macrophage during ulcerative colitis

Juan Du

First Affiliated Hospital of Zhejiang University School of Medicine

Junlei Zhang

First Affiliated Hospital of Zhejiang University School of Medicine

Xiaocang Cao

Tianjin Medical University General Hospital, Tianjin Medical University

Xun Wang

First Affiliated Hospital of Zhejiang University School of Medicine

Jinyuan Song

First Affiliated Hospital of Zhejiang University School of Medicine

Lin Wang

Jie Zhang

Department of Gastroenterology, the First Affiliated Hospital, College of Medicine, Zhejiang University

Jianghui Tang

First Affiliated Hospital of Zhejiang University School of Medicine

Jiangchao Wu

First Affiliated Hospital Zhejiang University

Yongtao Ji

First Affiliated Hospital of Zhejiang University School of Medicine

Yaxing Zhao

First Affiliated Hospital of Zhejiang University School of Medicine

Fei Cheng

The First Affiliated Hospital, School of Medicine, Zhejiang University

Jun Li

First Affiliated Hospital of Zhejiang University School of Medicine

Irene Soncin

Nanyang Technological University

Vincent Tano

Lee Kong Chian School of Medicine, Nanyang Technological University

Guang Li

Beijing Chaoyang Hospital, Capital Medical University, Chaoyang District

Xinjuan Liu

Beijing Chaoyang Hospital, Capital Medical University, Chaoyang District

Jianpeng Sheng (✉ shengjp@zju.edu.cn)

First Affiliated Hospital of Zhejiang University School of Medicine

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Abstract

Background and Aims: Ulcerative colitis(UC) is a chronic inflammatory bowel disorder with highly cellular heterogeneity. Mass cytometry(Cy-TOF) and single-cell RNA sequencing (scRNA-seq) have revealed cellular heterogeneity of UC. However, comprehensive elucidation of tissue topological changes within the UC ecosystem is still missing. And we aimed to illustrate compositional and spatial changes of the UC ecosystem.

Methods: Imaging mass cytometry (IMC) and scRNA-seq were applied to depict the single-cell landscape of colon ecosystem.

Results: We noticed tissue topological changes featured with macrophage disappearance reaction (MDR) in UC region. MDR only occurred for CD163⁺ tissue-resident macrophages. We found reactive oxygen species (ROS) level were higher in UC region but ROS scavenging enzyme SOD1/2 were barely detected in resident macrophages, resulting selective oxidative protection for inflammatory macrophages and resident macrophage disappearance reaction. Furthermore, inflammatory macrophages replaced resident macrophages during UC, which played a key role in forming the inflammatory cellular network by producing TNF- α and IL-1 β .

Conclusions: Our study dissected the microenvironment of UC lesions at single-cell resolution while preserving its architecture, based on which, we discovered the mechanism of MDR in UC region and resident macrophage specific MDR resulted in infiltration of inflammatory macrophage, which formed the cytokine producing network within the local cellular neighborhood.

Introduction

Ulcerative colitis (UC) is a chronic idiopathic inflammatory bowel disorder of the colon featuring continuous mucosal inflammation, affecting millions of people worldwide, and its incidence has increased over recent years ¹. Tissue damage in UC patients is mainly driven by a dysregulated immune response. Various types of immune cells, such as monocytes, macrophages, and T cells, play a crucial role in UC pathogenesis ².

Macrophage in the lesion is important for UC development and treatment. We and others have shown that macrophages in the intestinal space consist of resident and infiltrating inhabitants ³, and resident macrophages are believed to suppress the inflammation during UC ⁴ while infiltrating monocytes give rise to proinflammatory effector cells ⁵.

Pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), are critical for disease progression as well ⁶. And TNF- α neutralizing antibody treatment for UC has become favorable in the past decade ⁷ due to its successful application in the induction and maintenance of UC remission. Macrophage was reported to be involved in the TNF- α mediated UC ^{8,9}. However, many

patients are still resistant to anti-TNF- α therapy ¹⁰. Thus, comprehensive elucidation of macrophage population change during UC is important for understanding and intervention of UC.

The macrophage disappearance reaction (MDR) was first noticed in response to certain stimuli such as inflammation and infection ¹¹, but its mechanism was only illustrated partially in a recent report ¹² that peritoneal macrophage disappeared after bacterial infection based on factor V dependent coagulation process. However, if MDR happens in UC and whether same coagulation process occurred during UC progression was yet not understood.

High-dimensional single-cell analytics such as mass cytometry (Cy-TOF) and single-cell RNA sequencing (scRNA-seq) have revealed cellular heterogeneity in UC ^{13,14,15} and have identified many important cellular subsets promoting or attenuating UC, such as CD14⁺ macrophage ¹⁶, IL-17⁺ CD8⁺ T cells ¹³, and CD26⁺ CD8⁺ T cells ¹⁷. However, the tissue topology, such as spatial distribution and neighborhood relations of different cells, is lost during both Cy-TOF and scRNA-seq analysis, which rely on dissociated cells. And the spatial immune network is yet to be defined. In contrast to scRNA-seq and CyTOF analyses, imaging mass cytometry (IMC) enables spatial resolved phenotyping of the UC ecosystem.

Here, we aimed to dissect the tissue UC ecosystem while preserving its architecture so that we might understand how tissue topology changes during UC progression. Using a combination of single-cell transcriptomics and proteomics method, we resolved both cellular and spatial heterogeneity of the UC microenvironment at single-cell resolution. Additionally, we noticed macrophage disappearance reaction (MDR) in UC region, but only occurred for CD163⁺ tissue-resident macrophages and IL-1 β ⁺ inflammatory macrophage replaced CD163⁺ tissue-resident macrophage, which played a key role in forming the inflammatory cytokine network of TNF- α and IL-1 β . We found MDR in UC was not based on factor V dependent coagulation process but due to uneven expression of ROS scavenging enzyme SOD1/2 resulted oxidative stress. Our study might shed light on the potential macrophage-based immunotherapy for UC.

Materials And Methods

Ethics statement

The study followed the Declaration of Helsinki principles and was approved by the Medical Ethics Committee of the First Affiliated Hospital, Zhejiang University, Hangzhou, China. Samples were obtained from all patients by surgical resection or biopsies with informed consent. Animal experiments were approved by the Animal Care and Medical Ethics Committee of the First Affiliated Hospital, Zhejiang University.

Animal Studies

C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (China). All mice were bred in an SPF facility under the regulation of the Institutional Animal Care & Use Committee

(IACUC).

DSS-Induced Colitis Model

Male mice at 4-6 weeks of age were randomly divided into two groups which were oral administration with 1.5 % or 3% (w/v) Dextran sulfate sodium (DSS; Sigma, 42867) dissolved in drinking water respectively for one week, followed by normal water for a four-week recovery phase. Changes of bodyweight were monitored every day to identify the successful of mice model throughout the experimental period. After five weeks, all mice were sacrificed and the entire colon were collected for immunohistochemistry analysis and Fluorescence-activated cell sorting (FACS) analysis.

IMC analysis

Paraffin-embedded and formalin-fixed tissues from UC patients by biopsies were cut into 4 μ m sections followed by heating at 68°C for 1 hour. Dewaxing in xylene was performed twice for 10 min each. Sections were rehydrated sequentially in 95%, 85%, and 75% ethanol for 5 min each, followed by 100°C heat-mediated antigen retrieval for 30 min. After cooling naturally, sections were washed twice for 5 min each with PBS-TB (PBS with 0.5% Tween-20 [93773, Sigma-Aldrich] and 1% Bovine Serum Albumin [SRE0098, Sigma-Aldrich]). Then, sections were blocked with SuperBlock (37515, Thermo Fisher Scientific) for 30 min. Following another three PBS-TB washes, sections were incubated with an antibody cocktail at 4°C overnight. The antibodies were mostly labeled with metals using the Maxpar® X8 Antibody Labeling Kit (Fluidigm). Briefly, an X8 polymer was linked to the reduced antibody, and a specific metal was loaded onto the polymer. The final product panel is presented in Supplementary Table S1. After antibody incubation overnight at 4°C, three additional PBS-TB washes were performed. The sections were incubated with a 1.25 μ M Intercalator-Ir solution (201192B, Fluidigm) in PBS-TB for 30 min at RT to label nuclei, followed by two PBS-TB washes and one final ddH₂O wash. An imaging mass cytometer (Fluidigm, Hyperion) was employed to scan the prepared sections to generate the multiplexed images. For segmenting image data into single-cell data, we used CellProfiler software (Whitehead Institute for Biomedical Research and MIT's CSAIL) to acquire mask files, which were put into histoCAT software for t-SNE, PhenoGraph, and cell neighborhood analyses.

Cell neighborhood analysis

The primary neighbors of itself and secondary neighbors of its primary neighbors was calculated by cell neighborhood analyses as previously reported¹⁸. Cellular composition was analyzed for each cell neighborhood. In order to cluster cell neighborhoods into numerous meta-cell neighborhood clusters¹⁸ PhenoGraph was then applied to this cellular composition matrix.

Fluorescence-activated cell sorting (FACS) analysis

Tissue obtained by biopsies or mice colon were digested in culture medium supplemented with 0.6 mg/mL collagenase IV (17104019, Gibco) and 0.01 mg/mL DNase I (11284932001, Merck) in a 170-rpm

constant temperature shaker (Eppendorf) at 37°C for 1 hour. Digested tissues were passed through a 0.4 µm cell strainer (352340, BD) to acquire single-cell suspensions before centrifuging at 300 g for 5 min and resuspending in 35% Percoll (P4937, Sigma). After another centrifugation at 500 g for 5 min, cells were resuspended in 10 mL of blood lysis buffer (555899, BD) for 10 min at RT to eliminate red blood cells. Cell suspensions were centrifuged at 300 g for 5 min and then incubated with 2.5 µg/mL Fc blocker (156604 mouse; 422302 human, BioLegend) on ice for 15 min. The suspensions were further incubated with fluorochrome-labeled antibodies at 4°C for 30 min. The antibody panel is listed in Supplementary Table S1. The samples were washed and resuspended in PBS and supplemented with 2% FBS for analysis on a five-laser flow cytometer (BD Bioscience, Fortessa). The data were analyzed with FlowJo software (TreeStar).

Single cell RNAseq analysis

Single cells were dissociated from the gut samples obtained by biopsies through the method mentioned in the FACS analysis. Briefly, the single cells were loaded into Chromium microfluidic chips with v3 chemistry, then barcoded with a 10× Chromium Controller (10X Genomics, Pleasanton, CA, USA). RNA from the barcoded cells was subsequently reverse-transcribed and sequencing libraries were constructed with reagents from a Chromium Single Cell v3 reagent kit (10X Genomics) according to the manufacturer's instructions. Library sequencing was performed at Novogene Co., Ltd (Tianjin Novogene Technology Co., Tianjin, China) with Illumina HiSeq 2000 according to the manufacturer's instructions (Illumina, San Diego, USA).

Statistical analysis

Raw data obtained from FACS and histoCAT were copied into GraphPad software. Statistical tests were selected based on the appropriate assumptions for the data distribution and variability characteristics. Sample data were analyzed by a two-tailed Student's *t*-test to identify statistically significant differences between the two groups. One-way ANOVA with the Bonferroni post-test was used to identify differences among three or more groups. The data are represented as the means ± SEMs. A *p*-value < 0.05 indicated statistical significance.

Results

Spatial resolved portrait of UC ecosystem

In order to dissect the spatial heterogeneity of the UC microenvironment while preserving the architecture of the UC ecosystem, we established a 39-marker IMC panel for UC ecosystem (Figure 1A,B), based on published protocols for intestinal tissue and cancer^{19,20,21,22}. Our 39-marker IMC panel covered markers for epithelial, endothelial, stromal cells, and various immune cells; the cytokines IL-1β, TNF-α, and IL-6; the proliferation marker Ki-67; and the apoptotic marker cleaved caspase-3 (Supplementary Table S1), which allowed highest level of multiplexing of IMC so far.

We first analyzed colon tissues of healthy donors, which revealed typical structural markers, such as Collagen I and CD31, immunological markers, such as CD3 and CD20 (Figure 1B), and cytokines, such as TNF- α and IL-1 β after staining for the 39-marker panel (Figure 1B).

Distinct histological features, including intestinal crypts revealed by Pan-keratin staining (cyan) of epithelial cells and smooth muscle layer revealed by α SMA staining (yellow) of smooth muscle cells, were consistent with those determined by hematoxylin and eosin (H&E) staining but were resolved in greater detail (Figure 1C).

We detected major cell types, including CD31⁺ endothelial cells, α SMA⁺ smooth muscle cells, and Collagen I deposition in the extracellular matrix (Figure 1C). Immune cell types detected included B cells (CD20⁺), T cells (CD3⁺). Notably, T cells and B cells were highly enriched in the intraepithelial space and gland surrounding area (Figure 1D). Resident macrophages (CD163⁺ CD68⁺ CD16⁺ CD11b^{low}), and inflammatory macrophages (CD163⁻ CD68⁺ CD16⁻ CD11b⁺)²² could be detected mainly underneath the epithelial cells (Figure 1E).

Furthermore, to characterize the cellular heterogeneity of the UC microenvironment while preserving gross structural features, formalin-fixed, paraffin-embedded (FFPE) human tissue samples from 9 healthy donors and 23 UC patients with different Mayo grading scores (Supplementary Table S2) were processed and stained with 39 metal-conjugated antibodies, before scanning by IMC. The scanned images were subjected to cell segmentation using the established bioinformatic tool, histoCAT, developed by Schapiro et al.²³ (Figure 1A). The expression of markers in each cell was quantified, and 26 cell clusters were identified by PhenoGraph (Figure 1F), which were then annotated based on the expression of key markers.

CD45⁻ Pan-keratin⁺ cells indicated epithelial cells (Supplementary Figure S1A), endothelial cells were defined by CD31 and Vimentin staining (Supplementary Figure S1B), smooth muscle cells were identified by α SMA⁺ cells (Supplementary Figure S1C), and CD68⁺CD163⁺CD11b^{low} and CD68⁺CD163⁻CD11b⁺ cells were defined as resident and inflammatory macrophages, respectively (Supplementary Figure S1D), which was consistent with scRNA data (Supplementary Figure S2). T cells and B cells were concentrated in the intraepithelial space (Figure 1D), thus, T cell, B cell, and epithelial markers overlapped due to the resolution limit of IMC (Supplementary Figure S1E). However, T cells and B cells can be separated from epithelial cells based on CD45 expression (Supplementary Figure S1E). Overall, the T cell, B cell, and NK cell markers showed co-staining in clusters 6, 7, 9, 18, and 22, which were defined as lymphocytes (Figure 1F). FoxP3⁺ CD25⁺ cells were defined as regulatory T cells (Supplementary Figure S1F). In total, the 26 cell clusters included 7 from epithelial cells, 6 from endothelial cells, 1 from smooth muscle cells, 11 from immune cells, and 1 from unstained cells. Their frequencies in different patients are shown in Figure 1G.

In summary, tissue architecture, major stromal cell types, diverse immune cell populations, and cytokine production in the human colon microenvironment could be observed with our 39-marker panel by IMC

and our IMC results revealed a detailed landscape of UC ecosystem, including epithelial cells, stromal cells, and various immune cells.

MDR occurs only for resident macrophage during UC progression

Next, we wanted to characterize cellular changes during UC progression. Firstly, 9 healthy donors and 23 UC patients' samples were separated into 3 major classes based on their cellular frequencies (Figure 2A,B). All samples from the 9 healthy donors were grouped together (green) whereas, samples from UC patients were separated into mild (blue, Mayo grade 1) and severe groups (red, Mayo grade 2-4), Mayo grade was determined by the patients' colonoscopy (Figure 2C).

Further analysis showed that epithelial cells decreased from healthy donors to severe UC patients (Figure 2D; clusters 4 and 23), perhaps due to increased tissue damage, which was also confirmed by flow cytometry analysis of colon biospecimen (Figure 2E). While lymphocytes increased (Figure 2F; clusters 6, 18, and 22), indicating increased lymphocyte infiltration due to inflammation. Since the resolution of IMC for lymphocytes in UC region was poor, we also performed flow cytometry analysis for UC samples, and we noticed heavy infiltration of T cells and B cells, especially for B cell and CD4⁺ T cells (Figure 2G).

Furthermore, we also noticed that the macrophage disappearance reaction occurred only for resident macrophage during UC progression (Figure 2H; cluster 11, 14, and 24). While inflammatory macrophages (Figure 2J; cluster 25) increased when UC developed, suggesting a population switch occurred in the macrophage system. Macrophage disappearance reaction (MDR) was confirmed in UC patients' samples by FACS. CD68⁺ CD11b⁻ resident macrophages almost disappeared in UC patients while CD68⁺CD11b⁺CCR2⁺HLA-DR⁺ inflammatory macrophages increased significantly (Figure 2K).

Both IMC and FACS data revealed a change in cellular components with UC progression, especially for the macrophage system, where inflammatory macrophages replaced resident macrophages.

Macrophage replacement kinetics during UC

To analyze the macrophage replacement kinetics during UC, we used the canonical mouse DSS model (Figure 3A) ^{4, 17, 24}. We found that CD11b⁺ F4/80^{hi} Fraction I cells represented colon tissue-resident macrophages and CD11b⁺ F4/80^{int} Fraction II cells consisted of Ly6c⁺ MHC II⁻ monocytes (P1), Ly6c⁺ MHC II⁺ inflammatory macrophages (P2), Ly6c⁻ MHC II⁺ infiltrating macrophages (P3), and Ly6c⁻ MHC II⁻ eosinophils (P4), as described previously ³ (Figure 3B).

Additionally, we performed a time point analysis for resident and inflammatory macrophages in an acute DSS model. 1.5% and 3% DSS treatments represented mild and severe UC (Figure 3C). Similar macrophage replacement kinetics were observed for both the mild and severe DSS models, where

resident macrophages reduced to a minimum level around day 9 before returning to a normal level around day 28 (Figures 3 C, D, and E). Simultaneously, Ly6c⁺ MHC II⁺ inflammatory macrophages reached a maximum around day 9 and returned to a basal level around day 14, while Ly6c⁺ MHC II⁻ monocytes reached the basal level slightly later at around day 21 (Figure 3C, D, and E). Ly6c⁻ MHC II⁺ infiltrating macrophages and CD11b⁺ F4/80⁻ neutrophils showed different recruitment kinetics during mild and severe UC. Ly6c⁻ MHC II⁺ infiltrating macrophages decreased to the minimum level around day 9, similar to CD11b⁺ F4/80^{hi} resident macrophages, and replenishment of Ly6c⁻ MHC II⁺ infiltrating macrophages was completed around day 35 for mild UC but took longer for severe UC (Figures 3C, D, and E). Neutrophil recruitment reached a maximum between days 7 to 14, which was only resolved around day 28 for mild UC and much later for severe UC (Figures 3C, D, and E). It is well known that monocyte recruitment is dependent on the chemokine CCR2, thus we wanted to try if we could disrupt the inflammation network formation through CCR2 knockout. However, the Ly6c⁺ MHC II⁺ macrophage infiltration into the inflammatory region was not affected (Figure 3F), which was probably due to complementary roles of other chemokines like CCR5.

Overall, our dynamic analysis of macrophage replacement using the mouse DSS model showed that inflammatory macrophages could replace resident macrophages during DSS treatment and return to a basal level when inflammation was resolved. In addition, CCR2 knockout was not enough to block the mouse inflammatory macrophage infiltration.

scRNA-seq analysis confirms MDR of the UC ecosystem

To confirm the IMC pathological landscape of the UC microenvironment and illustrate the mechanism of MDR, scRNA-seq analysis was performed on four healthy donors' colon, four UC patients' self-control and corresponding lesion samples (Figure 4A). 52716 single cells were captured with high sequencing quality and we found 15 major cell clusters (Figure. 4B), which was annotated based on their specific markers (Figure 4C), such as EpCAM⁺ epithelial cells (Supplementary Figure. S2), DCN⁺ smooth muscle cells²⁵, CD31⁺ endothelial cells and rare Gnat3⁺ Tuft cell (Supplementary Figure S2A)²⁶. In addition, various immune cell subsets were identified, including CD4⁺, CD8⁺ T cells, FoxP3⁺ regulatory T cells (Treg) and IL7R⁺ memory T cells (Figure. 4B and Supplementary Figure S2B). B cells could also be separated into IgG producing plasma cells, CD83⁺ activated B cells²⁷ and Lymphotoxin β⁺ B cells (Supplementary Figure S2B)²⁸. Furthermore, macrophages were classified into CD68⁺ CD163⁺ CD11b^{low} resident macrophage and CD68⁺ CD11b⁺ IL-1β⁺ inflammatory macrophages. CD11b⁺ inflammatory macrophage expressed higher level of CCR2, which was central chemokine for macrophage recruitment (Supplementary Figure S2B).

We also observed decreased epithelial cells with heavy infiltration of T cells and B cells, including T cells, CD83⁺ activated B cells and plasma cells, in the UC region compared to the control region (Figure 4D).

And Treg cells were also elevated in UC region (Figure 4D), indicating a negative feedback for the inflammation process in the UC region.

In addition, consistent with the results showed in Figure 2J, the scRNA-seq analysis showed that resident macrophage decreased, while inflammatory macrophages increased in UC region (Figure 4D), indicating the MDR occurred only in the resident macrophage subset¹¹. Overall, our scRNA-seq landscape covers the major ICC TME cellular components and depicts the dynamic changes of the intestinal ecosystem, such as the MDR occurred in the UC region, which was specific to resident macrophage, confirming the IMC results.

Uneven expression of ROS scavenging enzyme SOD1/2 resulted ROS induced resident macrophage apoptosis

Next, we tried to illustrate the mechanism for resident macrophage disappearance during UC progression. First, we tested if MDR observed in UC region also involved the Factor V dependent coagulation process, as previously reported¹². However, Factor V was not expressed by resident macrophages or other cells (Figure 5A). In order to explore the mechanism by which resulted in MDR, we analyzed the differentially expressed gene (DEG) between resident macrophage and infiltrating macrophage due to the opposite cellular dynamics between these two macrophage populations observed during UC. Among the DEGs we found differentially expressed of SOD2 which participate in the process of oxidative stress (Figure 5B). Furthermore, we found ROS scavenging enzyme SOD1/2 expression was uneven. SOD1 was generally expressed by many cells except resident macrophage and SOD2 was highly expressed specifically only in inflammatory macrophages (Figure 5C). As a result, ROS level was quite high in resident macrophage compared with inflammatory macrophage, detected by FACS compatible ROS probes (Figure 5D).

In UC region, epithelium barrier is usually disrupted and commensal bacterial invades the intestinal space, which results bacterial infection defense system including ROS generation²⁹. To mimic this scenario, THP-1 cells were first induced to M0 cell with PMA³⁰ and then cocultured with bacterial component LPS. LPS induced ROS generation (Figure 5E), as previously reported³¹. Enhanced activation of caspase-3 was observed, upon LPS exposure (Figure 5F), which resulted macrophage apoptosis (Figure 5G), consistent with an earlier report on macrophage³².

In general, we found general elevated oxidative phosphorylation and ROS level in the UC region compared with healthy control and self-control. Meanwhile, ROS scavenging enzyme SOD1/2 were highly expressed only in inflammatory macrophage but not resident macrophage, which resulted ROS induced resident macrophage apoptosis as previously reported³³.

Cellular neighborhood changes during UC

It is widely accepted that the changes of macrophages can further influence the nearby immune system in situ³⁴. Hence, it is critical to identify the local changes of the inflammation network following the switch of macrophages. Therefore, we performed the regional cellular neighborhood analysis. Cell neighborhood was identified for each cell, primary neighbors within 4 μm of the target cells and the secondary neighbors of the primary neighbors were defined as interaction partners of the target population (Figure 6A)^{19,22}. In order to figure out different cellular neighborhood function units, cellular neighborhoods were annotated according to the major cellular clusters (Figure 6B). Then based on the CN composition, Voronoi plot was used for each IMC image to visualize whether the defined CN function units were associated with the inflammation caused by UC (Fig 6C) as previously reported³⁵. As shown in figure 6C, the topology map of IMC represented by Voronoi plot with various CN indicated by different colour was aligned nicely with IMC image. We found lymphocyte-enriched CN and infiltrating macrophage centered CN were highest in severe UC patients. Besides, we found epithelial-enriched CN and resident MF-enriched CN were lowest in severe UC patients (Figure 6D).

Thus, CN changes during UC was consistent with the cellular cluster changes.

Infiltrating macrophages recruitment forms the inflammatory cytokine network within the cellular neighborhood

Next, we intend to explore to the mechanism and results of the CN changes. We noticed profound cytokine production in UC patients based on both IMC and scRNA-seq results. IMC showed that TNF- α was produced mainly by lymphocytes (Figure 7A), and scRNA-seq helped to identify the T cells, CD83⁺ activated B cells and inflammatory macrophages as major TNF- α producers (Figure 7A). In addition, our IMC and scRNA-seq data showed that IL-1 β was produced mainly by infiltrating macrophages (Figure 7B).

Furthermore, cell-cell interaction analysis using scRNA-seq data was implemented based on the published tool, NicheNET, by Browaeys et al³⁶, which models intercellular communication by linking ligands to target genes. Inflammatory macrophages were shown to induce T cells and B cells to produce TNF- α through IL-1 β , based on NicheNET analysis (Figure 7C).

Consistent with the scRNA-seq analysis, neighbors of resident macrophage expressed less TNF- α , while neighbors of inflammatory macrophages expressed more TNF- α . As shown in figure 7D, to prove the crucial role of the infiltrating macrophage in formation of inflammation network, we sorted the Ly6c⁺MHC II⁺ macrophage from DSS treated mouse and co-cultured with the lymphocytes from the draining mesenteric lymph nodes (Figure 7E). And we found co-culture with Ly6c⁺MHC II⁺ macrophage could induce both T cells and B cells to secrete TNF- α (Figure 7E).

In summary, by combining IMC and scRNA-seq data, we suggested that during UC, bacteria invasion caused ROS production led to resident macrophage specific MDR and infiltration of inflammatory

macrophage, which formed the inflammatory CN with lymphocytes and orchestrated the inflammatory cytokine network (Figure 7F).

Discussion

The UC microenvironment is composed of epithelial cells, immune cells, and stromal cells. Recently, scRNA-seq has helped resolve the heterogeneity of the UC microenvironment among different patients at the transcriptional level^{16, 17, 37}. However, single-cell spatial heterogeneity of the UC microenvironment remains to be deciphered. Here, we combined scRNA-seq analysis and IMC with 39 protein markers to analyze UC heterogeneity in great detail, while preserving the architecture of the microenvironment. We generated the first single-cell pathology landscape collection of human UC. Furthermore, we discovered that MDR occurred only for resident macrophages and inflammatory macrophages replaced resident macrophages, forming the cytokine network of TNF- α and IL-1 β with T cells and CD83⁺ B cells. And resident macrophage apoptosis was due to selective oxidative stress exposure.

We showed that the MDR occurred in both human and mouse UC for CD163⁺ and CD11b⁺F4/80^{hi} resident macrophages, respectively. In addition, resident macrophages only returned to normal levels when inflammation was resolved in the mouse DSS model. Thus, chronic inflammation might prevent the replenishment of resident macrophages, the major immune suppressive cells, in UC lesions of patients. We also noticed that IL-1 β ⁺ inflammatory macrophages predominated over resident macrophages in the DSS mouse model and formed the inflammatory cytokine network with T cells and B cells. Our findings were consistent with a previous report, which showed that CD14⁺ macrophages in Crohn's disease consisted of IL-1 β producing CD163^{-/dim} cells¹⁶. We believed such macrophage replacement dynamics was originally adopted as the combinatory bacterial defense mechanism that ROS was elevated as the first defense of the invading bacteria. At the same time, immune suppressive resident macrophage was wipe out purposely due to their sensitivity to ROS and replaced by inflammatory macrophage which was resistant to ROS based on high level of SOD1/2. However, if the inflammation process was not resolved properly, persisted loss of resident macrophage and infiltration of inflammatory macrophage would exacerbate UC.

The resolution of IMC was limited for the regions with high density of cells. However, single-cell proteomics and transcriptomics analysis did complement each other. For example, when T cell and B cell markers were overlapped in areas of high cell density, scRNA-seq could resolve T cells and B cells effectively. Also, scRNA-seq was unable to determine the location of cytokine production while IMC revealed that TNF- α producing lymphocytes were surrounding the inflammatory macrophage but not resident macrophages.

In summary, we not only provide novel spatial insights into the ecosystem but also identify oxidative stress as the key player responsible for the UC macrophage subset balance during UC. And future immunomodulation therapeutic application could be developed to modulate the CN based on our tissue topological analysis presented here.

Declarations

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Declaration of interests

The authors declare no competing interests.

Data availability statement

The data underlying this article are available in BioProject titled as Ulcerative colitis(UC) scRNA-seq, and can be accessed with PRJCA005436/CRP002957.

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Figures

Figure 1

Representative mass cytometry images from a healthy donor sample using the 39-marker panel. A, The workflow of IMC. Patients' UC and self-control samples and healthy donor's samples were analyzed by IMC. B, Single color staining of the indicated marker above each plot. C, Pan-cytokeratin (cyan), αSMA (yellow), collagen I (blue), and CD31 (red) were used to portray the structure of colonic tissue (left image). H&E staining (right image). Scale bars 100 μm. D, Pan-cytokeratin (cyan), CD20 (yellow), and CD3 (red) staining of colonic tissue structure. Scale bars 100 μm. E, CD68 (cyan), CD163 (yellow), and CD11b (red) staining of colonic tissue structure. Scale bars 100 μm. F, Cell segmentation results showing 26 cell types. G, Heatmap showing gene expression profiles across various genes and cell types.

(magenta) highlight the distribution of T cells and B cells in colonic tissue. Arrow 1 (magenta) and arrow 2 (yellow) indicate the T cells and B cells, respectively. Scale bars 100 μ m. E, CD68 (red), CD163 (blue) and CD11b (green) distinguish resident macrophages and inflammatory macrophages. Due to the overlap of colors, we used arrow 3 (plum) to highlight the resident macrophages (CD68+CD163+CD11b-) and arrow 4 (orange) to point out the inflammatory macrophages (CD68+CD163-CD11b+). Scale bars 100 μ m. F, t-SNE plots were based on the single-cell data extracted from IMC images. 26 clusters of cells in ulcerative colitis samples were defined according their markers. G, The heat map showing the z-scored mean marker expression of 26 clusters with their frequency distribution pattern across different patients shown in box plot.

Figure 2

Intestinal ecosystem changes during UC progression. A and B 32 patient and donors' samples were separated into 3 major classes: severe patients (red), mild patients (blue), healthy patients (green) based on their cluster frequency. C, The images of 3 classes of UC samples determined by Mayo Clinic Endoscopic Subscore and normal donors. D and E, The distribution of epithelial cell 2 and 3 based on IMC results in C. And verification by FACS analysis of live samples, as shown in E. Statistic results are

shown in the right panel of E. F and G, The distribution of lymphocyte 1, 3 and 4 based on IMC results shown in F and verification by FACS analysis of live samples, as shown in G. H, The distribution of resident macrophage1, CD11C+ resident macrophage1, and resident macrophage2 based on IMC results showed in H. J, The distribution of inflammatory macrophages based on IMC results showed J. And verification by FACS analysis of live samples, as shown in K. Statistic results are shown in the right panel of K.

Figure 3

Dynamics of myeloid cell changes during UC. A, DSS treatment schedule. Mice were treated with 1.5% or 3% DSS in drinking water, followed by a four-week chase period with normal drinking water. Colon tissue was isolated and analyzed at indicated time points. B, Gating strategy for different myeloid cells in normal colon tissue at day 0. CD11b⁺ F4/80^{hi} Fraction I cells were separated into MHC II⁻ and MHC II⁺ subsets of resident macrophages. CD11b⁺ F4/80^{int} Fraction II cells were separated into P1 (Ly6c⁺ MHC II⁻ monocytes), P2 (Ly6c⁺ MHC II⁺ inflammatory macrophage), P3 (Ly6c⁻ MHC II⁺ infiltrating macrophage), and P4 (Ly6c⁻ MHC II⁻ eosinophil). CD11b⁺ F4/80⁻ Fraction I cells were neutrophils. C, D and E, Representative FACS plots, subset percentages and absolute numbers for the different myeloid cells shown in Figure 5B during different time points of the DSS model.

Figure 4

A single-cell transcriptomic and proteomic atlas of the immune microenvironment in UC. A, The workflow of scRNA-seq. Patients' UC and self-control samples and healthy donor's samples were analyzed by scRNA-seq. B, t-distributed stochastic neighbor embedding (t-SNE) plots based on the scRNA-seq data and 15 cell clusters of 12 samples were defined. C, The heat map showing the z-scored mean marker

expression of 15 clusters. Color scale indicated normalized marker intensity. D, The charts reveals the distributions of epithelial cell and various immune cells among healthy donor, self-control and UC region.

Figure 5

The mechanism of resident macrophage disappearance reaction A. The UMAP plot of Factor V expression based on scRNA-seq data. B. The differentially expressed gene (DEG) between resident macrophage and infiltrating macrophage. C. The UMAP plot of SOD1 and SOD2 expression in

macrophages revealed by scRNA-seq analysis. Resident macrophage and inflammatory macrophage were highlighted with blue and red dot line. D. The different ROS production between two kinds of macrophages was measured using FACS compatible ROS probes based on UC patient sample. E, F and G. THP-1 cells were first induced to M0 state and then cocultured with LPS. ROS level was measured by immunofluorescence compatible probes in E. Activation of Caspase-3 was checked by western blot in F. Apoptosis was analyzed by Annexin V/PI staining and statistical results were shown in G.

Figure 6

Cellular neighborhood changes during UC. A, Interaction partner detection model used to identify neighborhood cells within 4 μm of the central cell. B. Eighteen major types of CN were annotated by the major cell type within CN and clustered by the scaled frequency of each type in CN. C. The topology of IMC image was represented by Voronoi plot with various CN indicated by different colour. Representative IMC and Voronoi plot for normal region and inflammation region were shown. D. Comparison of lymphocyte-enriched CN , infiltrating macrophage centered CN, epithelial-enriched CN, and resident MF-enriched CN among different tissues. E. Comparison of lymphocyte-enriched CN , infiltrating macrophage centered CN, epithelial-enriched CN, and resident MF-enriched CN among different tissues. F. Comparison of lymphocyte-enriched CN , infiltrating macrophage centered CN, epithelial-enriched CN, and resident MF-enriched CN among different tissues.

Figure 7

Infiltrating macrophages recruitment forms the inflammatory cytokine network within the cellular neighborhood. A, TNF- α expression pattern shown by t-SNE plots based on IMC (left panel) and scRNA-seq (right panel) analysis. Epithelial cells are highlighted with black dotted lines, lymphocytes with green dotted lines, resident macrophages with blue dotted lines and inflammatory macrophages with red dotted lines. B, IL-1 β expression pattern shown by t-SNE plot based on IMC (left panel) and scRNA-seq (right panel) analysis. C, Circos plot showing the cell-cell interactions between inflammatory macrophages and T cells (left panel) and inflammatory macrophages and B cells (right panel). Cell-cell interactions were performed by NicheNET. Macrophages (sender, outer ring)'s ligands engaged with receptors on T cells and B cells (receiver, outer ring) and induced target genes (inner ring). Different colors of T cells and B cells indicate different subsets within T cells and B cells. D, TNF- α expression pattern of the interaction partners of resident macrophages (the left panel) and inflammatory macrophages (the right panel). E, Ly6c⁺ MHC II⁺ inflammatory macrophages were sorted from colon in DSS treated mouse and cultured with lymphocytes from MLN. Representative plots and statistics results are shown for TNF- α production from CD3⁺ T cells and CD19⁺ B cells after the co-cultivation. F, The abstract diagram interprets the mechanism of resident macrophage disappearance effect. ROS was elevated as the first defense of the invading bacteria. At the same time, immune suppressive resident macrophage was wipe out purposely due to their sensitivity to ROS and replaced by inflammatory macrophage which was resistant to ROS based on high level of SOD1/2. Furthermore, inflammatory macrophages played a key role in forming the inflammatory cellular network by producing TNF- α and IL-1 β .

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