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Article

The comparison of methods for isolating immune cells from the intestinal lamina propria of mice and the immune cell landscape in DSS-induced colitis in mice

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Abstract: Flow cytometry is a technology based on the detection of scattered light signals and fluorescent signals for multi-parameter, high-throughput, rapid analysis of individual cells or particles. It has broad application prospects in the biomedical field and is an indispensable technique, especially in immunological research. The preparation of a high-quality single-cell suspension is a key and often challenging step that directly affects the results of flow cytometry analysis. This study selected a primary tissue sample that is relatively difficult to prepare and commonly used in immunological research: The lamina propria of the mouse intestine. Three common enzymatic digestion methods were compared in terms of cell yield, viability, and immune cell markers. The results indicated that an enzymatic digestion method primarily using collagenase D is suitable for obtaining lymphocyte-derived immune cells from the mouse intestinal lamina propria, while enzymatic digestion methods primarily using Liberase™ and DNase I are more suitable for obtaining myeloid-derived immune cells from the same tissue. Additionally, by combining a multi-parameter staining scheme, the analysis of T cells, macrophages, and dendritic cells within the intestinal immune cells was achieved. Furthermore, immunolabeling analysis was conducted on the commonly used colitis model, which was induced by dextran sulfate sodium, providing a reliable detection method for accurately analyzing immune cell populations in the mouse intestinal lamina propria.

Keywords: flow cytometry; immune cell analysis; intestinal immune cells; colitis; inflammatory bowel diseases

1. Introduction

Studying intestinal immune responses is crucial for understanding both local and systemic immunity. In intestinal homeostasis, intestinal dendritic cells, macrophages, and T cells produce various cytokines that play significant roles in maintaining tissue homeostasis, inflammation, and inducing the resolution of inflammation [1–3]. T helper cell (TH) types Th1 and Th2 are involved in immune responses, while other T cell subsets, such as Th17 and regulatory T cells (Treg), may play roles in inflammatory bowel disease (IBD) and promote fibrosis in IBD [2,4–6]. Myeloid-derived cells in the intestinal lamina propria mainly include macrophages and conventional dendritic cells (cDCs), which are important in intestinal mucosal homeostasis, infection, and inflammation [1,7,8]. Macrophages play a critical role in intestinal homeostasis as they can distinguish between harmless

antigens and potential pathogens to maintain oral tolerance. Most homeostatic colonic macrophages are established through the infiltration of lymphocyte antigen 6C-high ($LY6C^{hi}$) monocytes, which express low levels of C-X3-C Motif Chemokine Receptor 1(CX3CR1) [9,10]. As $LY6C^{hi}$ monocytes enter the lamina propria, they gradually differentiate, first acquiring major histocompatibility complex II (MHCII), then losing LY6C expression, and subsequently upregulating F4/80, CD64, and CX3CR1 [3]. Lamina propria cDCs also express high levels of MHC-II and the surface maker CD11c [1]. Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of IBD. The dextran sulfate sodium (DSS) induced colitis model is particularly useful for studying the innate immune mechanisms of colitis [11]. Therefore, accurately understanding the state of intestinal immune cells is of great significance for studying intestinal diseases. The preparation of high-quality single-cell suspensions is both a prerequisite and a challenge for detecting intestinal immune cells, and different digestion methods have varying impacts on immune cell labeling. Thus, efficient and stable methods for preparing single-cell suspensions are very important.

The enzymes involved in digestion are diverse, with several common digestive enzymes including trypsin, collagenase, hyaluronidase, DNase I, neutral protease, elastase, and papain [12]. When isolating immune cells from the lamina propria of intestinal tissue, the main components of the digestive solution are collagenase and DNase I; however, some studies have added neutral protease to the digestive solution to enhance digestion efficiency.

The mechanisms by which these three enzymes act are different. Collagenase mainly hydrolyzes proline in the extracellular matrix to separate cells, having a digestive effect only on the extracellular matrix and causing minimal damage to the cells [13]. DNase I acts on the DNA released during cell separation, preventing cell aggregation caused by DNA, and does not harm cell integrity, though it is rarely used alone [14]. Neutral protease hydrolyzes peptide bonds provided by hydrophobic macromolecular amino acids such as leucine, phenylalanine, and tyrosine to achieve tissue digestion [15–18]. Using multiple enzymes in combination can facilitate the acquisition of immune cells from complex intestinal tissue, providing great potential and convenience for the analysis of gastrointestinal immune cells.

However, due to the softness and fragility of intestinal tissue compared to solid tissues, there are three main challenges during processing: 1) The cell yield is often insufficient, making it difficult to obtain a sufficient number for flow cytometry analysis; 2) the viability of the cells is often low, resulting in an inadequate number of target cell populations; 3) the absence of key immune markers leads to inaccuracies in subgroup analysis [19].

This study analyzes various key immune cell markers by comparing common enzymatic digestion methods for intestinal tissues, determining a reliable method for digesting the colon. It also identifies $CD4^+$ T cells, γδ T cells, as well as macrophage and DCs subsets in the context of DSS-induced colitis, providing a comprehensive immunological landscape of the intestine under DSS-induced colitis.

2. Materials and methods

2.1. Animals

Male mice (C57BL/6J background) were used for animal experiments. All mice were housed in pathogen-free animal rooms under controlled conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University and performed according to the guidelines of national regulations.

2.2. DSS induced colitis

Colitis induced by DSS was established by freely providing 2.5% DSS dissolved in autoclaved drinking water for 7 days to the mice [20].

2.3. Lymphocyte preparation

2.3.1. Digestive method (1) for processing colon tissue

Pre-digestion: Pre-digestion solution 1: For every 10 mL of phosphate-buffered saline (PBS), add ethylenediaminetetraacetic acid (EDTA, 200 uL) and 4-(2-hydroxyethyl) piperazine-1-ethane-sulfon (HEPES, 100 uL), we need 10 mL pre-digestion solution/sample. Cut appropriately sized segments of intestine (1.5 cm) and place them in 10 mL of pre-digestion solution 1, then incubate at 37 ℃, 100 r/min for 15–20 min, and repeat once. Rinse the pre-digested intestine segments twice with PBS, then use sterile surgical tweezers and scissors to cut the segments into 1 mm^2 pieces. Digestion: Place the pieces in 5 mL of RPMI-1640 containing 200 uL fetal bovine serum (FBS) and 0.5 mg/mL of collagenase D, and incubate at 37 ℃, 220 r/min for 20 min, we need 5 mL digestion solution/sample. After the initial 20 min, vortex the mixture and filter it through a 40 μm filter, repeating this process twice more. Adjust the volume to 35 mL with PBS, and centrifuge at 1000× *g*, 4 ℃ for 10 min.

2.3.2. Digestive method (2) for processing colon tissue

Pre-digestion: Pre-digestion solution 1: For every 10 mL of PBS, add 100 uL of DTT (1 mol/L) and 100 uL of HEPES (1 mol/L), we need 10 mL pre-digestion solution 1/sample; pre-digestion solution 2: For every 10 mL of PBS, add 625 uL of EDTA (0.5 mol/L) and 100 uL of HEPES (1 mol/L), we need 10 mL pre-digestion solution 2/sample. Cut appropriately sized segments of intestine (1.5–2 cm) and place them in 10 mL of pre-digestion solution 1, then incubate at 37 ℃, 220 r/min for 10 min, followed by vigorous manual shaking up and down for 2 min. Next, place the intestine segments in 10 mL of pre-digestion solution 2, incubate at 37 ℃, 220 r/min for 10 min, and again manually shake vigorously up and down for 2 min; repeat this process once more. Digestion: Preparation of the digestion solution: Mix RPMI-1640 containing 10% FBS with collagenase VIII (200–400 units/mL, the total units are calculated according to different batches and then added after dilution) and DNase I (0.15 mg/mL) and use it immediately, with 3 mL for each sample. Rinse the pre-digested intestine segments twice with PBS, then gently shake the segments in 5 mL of RPMI-1640 containing 500 uL FBS for 2 min. Subsequently, transfer them to a 6-well plate containing 3 mL of the digestion solution, and use sterile surgical

tweezers and scissors to cut the segments into pieces smaller than 0.5 cm, we need 3 mL digestion solution/sample. Incubate in a 37 ℃, 5% CO_2 cell culture incubator for 90 min for digestion. After digestion, transfer to a 15 mL centrifuge tube, add 7 mL of PBS to stop the digestion, and manually shake vigorously for 2 min. Filter the resulting cells through a 70 μm filter into a 50 mL centrifuge tube, adjust the volume to 35 mL with PBS, and centrifuge at 1000× *g*, 4 ℃ for 10 min.

2.3.3. Digestive method (3) for processing colon tissue

Pre-digestion: Preparation of pre-digestion solution: 15 mL of PBS contains 300 uL of EDTA (0.5 mol/L), 150 uL of DTT (1 mol/L), and 300 uL of FBS, we need 15 mL pre-digestion solution/sample. Cut the colon into appropriately sized pieces (1.5–2 cm) and place them into a 50 mL centrifuge tube containing 15 mL of pre-digestion solution. Tilt the tube at a 45° angle and place it in a shaking incubator at 37 ℃, 250 r/min for 15 min. Remove the centrifuge tube and vortex it for 10 s. Repeat the pre-digestion process once more. Digestion: Preparation of digestion solution: Add 62.5 μL of Liberase™ (5 mg/mL) and 0.05 mg/mL of DNase I to 5 mL of RPMI-1640 medium containing 10% FBS, we need 5 mL digestion solution/sample. Rinse the intestine segments that underwent pre-digestion with PBS, then cut them into small pieces of about 0.5 cm and place them in a 15 mL centrifuge tube containing the digestion solution (adding 5 mL of digestion solution to each tube). Incubate on a horizontal shaker at 37 ℃, 250 r/min for 40–50 min, shaking vigorously every 15 min. After digestion, vortex the mixture vigorously for 10 s, and filter the resulting cells through a 70 μm filter into a 50 mL centrifuge tube. Then, adjust the volume with PBS to 20 mL and centrifuge at 1000× *g*, 4 ℃ for 10 min.

2.4. Density gradient centrifugation to obtain lamina propria immune cells

The single-cell suspensions obtained from digestion methods (1), (2), and (3) will be enriched for immune cells using Percoll density gradient centrifugation. After resuspending the cell pellet in 40% Percoll solution, it is layered on top of an 80% Percoll solution (to do this, tilt a 15 mL centrifuge tube containing 80% Percoll at a 45° angle, and slowly add the cell suspension along the wall of the tube to allow it to lay on top of the 80% Percoll). Centrifuge at 1000× *g* at room temperature for 22 min (set both acceleration and deceleration to 0). After centrifugation, first aspirate the upper layer of impurities, then carefully use a Pasteur pipette to collect the cells from the middle layer and transfer them into a 15 mL centrifuge tube. Adjust the volume to 14 mL with PBS and centrifuge at 1000× *g*, 4 ℃ for 10 min. Discard the supernatant and resuspend the cell pellet in 1 mL of PBS.

2.5. Cell stimulation and live-dead staining

The antibodies used for flow cytometry are listed in the key resources table. Immune cells isolated from the colon of mice were stimulated with medium containing 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich), 100 ng/mL ionomycin (Sigma Aldrich) and 2 uL/mL GolgiPlug (BD) for 6 h, in a 37 ℃, 5% CO_2 cell culture incubator. Following stimulation, cells were stained with 50

uL/tube LIVE/DEAD fixable dye Aqua (Dilution ratio: 2 uL/1000 uL, Thermo Fisher Scientific) 30 min to exclude dead cells. Add 500 uL/well of PBS to wash and centrifuge at 500× *g* for 5 min at 4 °C. Remove the supernatant, and add 50 uL/tube of Fc Blocker solution (Dilution ratio: 2 uL/1000 uL) and resuspend [19].

2.6. Cell surface maker staining

Add 50 uL/tube of surface antibody mixture (Dilution ratio: 1 uL/200 uL) and resuspend cells gently. Incubate at 4 °C for 30 min, add 500 uL/tube of PBS to wash and centrifuge at 500× *g* for 5 min. Finally, remove the supernatant from the tube.

2.7. Staining for cytokines as well as transcription factors

Use the FoxP3 transcription factor staining kit (eBioscience, FoxP3 Fixation/Permeabilization concentrate/FoxP3 Fixation/Permeabilization diluent: 1/3) to fix and permeate cells, and resuspend the cells with 200 uL/tube of the working solution and incubate for 20–30 min at room temperature. Centrifuge at 1000× *g* for 5 min and remove supernatant from tubes. Add 200 uL of Permeabilization buffer (10× Permeabilization buffer/Distilled water: 1/9) to each tube, and centrifuge at 1000× *g* for 5 min and remove supernatant. Finally, stained with 50 uL/tube cytokine and transcription factor antibodies (Dilution ratio: 1 uL/200 uL). All flow cytometry analyses were conducted on a CytFlex flow cytometer (BECKMAN COULTER) and analyzed using FlowJo software (BD) [19].

2.8. Flow cytometry cell gating strategy

T cell gating strategy: All flow cytometry date about $CD4^+$ T cells were gated on live, $CD45^+$, $TCR\beta^+$, $CD4^+$, $CD8^-$, and $CD19^-$ cells. γδT cells were gated on live, $CD45^+$, $TCR\beta^-$, $TCR\gamma\delta^+$ cells. Macrophage gating strategy: All flow cytometry data about macrophage cells were gated on live, $CD45^+$, $CD3e^-$, $TCR\beta^-$, $LY6G^-$, and $CD19^-$ cells. DCs gating strategy: All flow cytometry about DCs data were gated on live, $CD45^+$, $CD3e^-$, $TCR\beta^-$, $LY6G^-$, $CD19^-$ and $F4\backslash 80^-$ cells.

2.9. Data analysis

Statistical analyses were performed with Prism v.10.0.0 (GraphPad), FlowJo (BD). The statistical analysis between the two groups uses an unpaired Student's *t*-test. One-way ANOVA is used for statistical analysis of three groups and above. All data is presented as mean ± SD.

2.10. Drag and reagent

The drugs and reagents used in the experiment are shown in **Table 1**.

Table 1. Drag and reagent.

Reagents and drugs	Corporation
PMA	Sigma Aldrich
Ionomycin	MedChemExpress
Golgiplug	BD Biosciences
DSS	MP Biomedicals

Table 1. (*Continued*).

Reagents and drugs	Corporation
DTT	Millipore Sigma
EDTA 0.5 M pH 8.0	Biosharp
FBS	Hyclone
FBS	Biological Industries
Percoll	Sigma Aldrich
Rat anti-mouse CD16/CD32 (Mouse BD Fc BlockTM) (Clone ID 2.4G2)	BD Biosciences
Anti-mouse CD45 (Clone ID 30-F11) APC-700	BD Biosciences
Anti-mouse CD19 (Clone ID SJ25-C1) APC-CY7	Thermo Fisher Scientific
Anti-mouse TCRβ (Clone ID H57-597) FITC	BD Biosciences
Anti-mouse CD4 (Clone ID RM4-5) BB700	BD Biosciences
Anti-mouse F4\80 (Clone ID BM-8) FITC	Thermo Fisher Scientific
Anti-mouse CD8a (Clone ID 53-6.7) BV605	BioLegend
Anti-mouse FoxP3 (Clone ID FJK-16s) APC	Thermo Fisher Scientific
Anti-mouse CD64(Clone ID X54-5/7.1) ECD	BioLegend
Anti-mouse IFN-γ (Clone ID XMG1.2) BV421	BioLegend
Anti-mouse IL17A (Clone ID eBio17B7) BV786	Thermo Fisher Scientific
Anti-mouse TCRγδ (Clone ID GL3) BV650	BD Biosciences
Anti-mouse IL10 (Clone ID 1B1.3a) PE	BD Biosciences
Anti-mouse TNFα (Clone ID MP6-XT22) APC	BD Biosciences
Anti-mouse CD11c (Clone ID HL3) PC5.5	BD Biosciences
Anti-mouse CD11b (Clone ID M1/70) BV605	BD Biosciences
Anti-mouse CD103 (Clone ID NLDC-145) BV786	BD Biosciences
Anti-mouse MHC-II (Clone ID M5/114.15.2) APC750	BioLegend
Anti-mouse CX3CR1 (Clone ID Z8-50) BV421	BD Biosciences
Anti-mouse CD19 (Clone ID 1D3) PC-CY7	BD Biosciences
Anti-mouse CD3e (Clone ID 145-2C11) PC-CY7	BD Biosciences
Anti-mouse TCRβ (Clone ID H57-597) PC-CY7	BD Biosciences
Anti-mouse LY-6G (Clone ID 1A8) PC-CY7	BD Biosciences
eBioscience Foxp3/Transcription Factor Staining Buffer Kit	Thermo Fisher Scientific
LIVE/DEAD Fixable Aqua Dead Cell stain kit	Thermo Fisher Scientific

3. Results

3.1. The impact of different digestive enzymes on cell yield, viability, and immune cell proportions

Obtaining high-quality single-cell suspensions is crucial for flow cytometry analysis. In this study, the digestion process of mouse colonic tissue is primarily divided into four steps: obtaining the desired intestinal segments for washing, using pre-digestion solutions to remove the epithelial cell layer and endothelial cell layer,

using digestive enzymes to digest the lamina propria, and using Percoll to enrich immune cells. Although there are several commonly used digestive enzymes in intestinal immune research, such as collagenase VIII, collagenase D, and Liberase™, there is limited literature comparing the differences between these enzymes and the types of immune cells they are suitable for. The digestion process has a significant impact on cell yield. To explore which digestion method yields more cells, we selected three different digestion methods to prepare samples. Method (1) uses a digestion solution primarily consisting of collagenase D and DNase I, method (2) uses a digestion solution primarily consisting of collagenase VIII and DNase I, and method (3) uses a digestion solution primarily consisting of Liberase™ and DNase I. We processed the colons of wild-type C57BL/6 mice according to the respective steps and diluted the resulting single-cell suspensions with 1 mL PBS before counting the cells using a flow cytometer. Finally, we compared the cell counts obtained from the three methods. The results showed no significant statistical difference in the number of cells obtained from the three methods (**Figure 1A**). During flow cytometry analysis, viability dyes are often added to distinguish the viability status of the cells, reducing the impact of dead cell autofluorescence on the detection and improving the accuracy of flow cytometry.

(See **Figure 1B–E**) Flow cytometry analysis revealed that all three digestion methods affected cell viability; the single-cell suspensions obtained using Liberase™ and collagenase D had a higher proportion of viable cells, while the single-cell suspensions obtained using collagenase VIII had a lower viability rate. Immune cells typically express CD45. Therefore, to assess the impact of different digestion methods on the proportion of immune cells, we measured the yield of immune cells under different digestion conditions. The results indicated that single-cell suspensions obtained using Liberase and collagenase D had a higher yield of immune cells, while those obtained using collagenase VIII had a lower yield of immune cells.

We found that the data on cell yield was quite dispersed; therefore, we calculated the coefficient of variation (supplementary information). We discovered that the method using collagenase VIII and Liberase™ had a higher coefficient of variation compared to the method using collagenase D. However, we observed that the viability of the samples and the proportion of immune cells within the samples were relatively uniform, with a smaller coefficient of variation. This suggests that there may be some individual differences among the mice we used.

From the above results, it can be observed that different digestion methods do not affect cell yield, but Liberase and collagenase D have a minimal impact on cell viability and yield a higher proportion of immune cells.

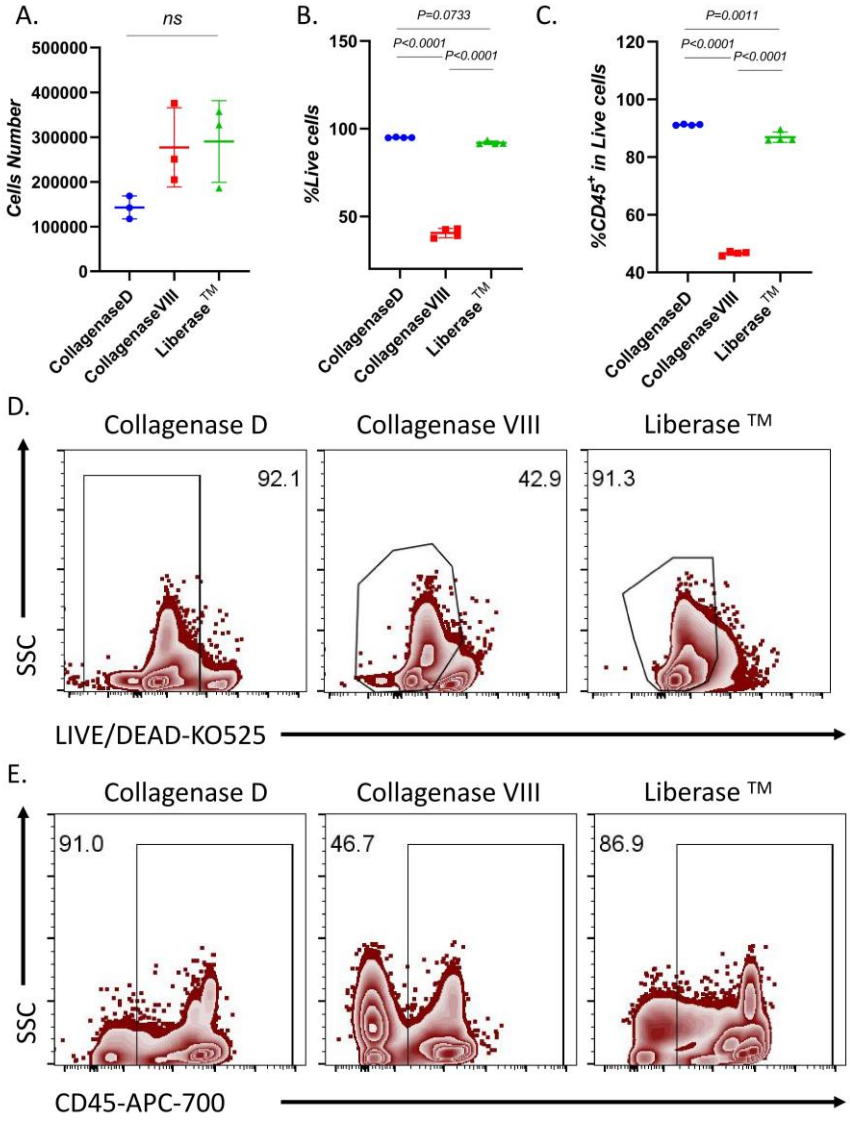

Figure 1. The cell yield and cell viability under different digestion methods were analyzed. **(A–C)** Cell yield, activity, and immune cell yield statistical analysis charts; **(D)** flow cytometry analysis chart of cell viability from single-cell suspensions obtained using three enzymatic digestion methods; **(E)** flow cytometry analysis chart of the proportion of immune cells in single-cell suspensions obtained using three enzymatic digestion methods ($n \geq 3$).

3.2. The impact of different digestive enzymes on lymphocyte surface markers

After analyzing the effects of different methods on cell yield, viability, and the proportion of immune cells, we will conduct surface marker analysis of different immune cell subpopulations. The binding of immune cell surface antigens to specific antibodies is of great significance for the classification and analysis of immune cells. First, we will compare the detection of surface markers on lymphocytes using the different methods. In this study, we focused on detecting important surface antigens of immune cells, primarily examining T cell-related markers TCR-β, CD4, and CD8(19); TCR-γδ marked a specific T cell population, γδ T cells, which despite their low proportion, play important roles in anti-infection and tumor cell recognition [21]; and the B cell marker CD19 [22]. To further analyze the expression of these

surface antigens, we incubated the single-cell suspensions obtained from the three digestion methods with fluorescent antibodies specific to major immune cell population surface markers at 4 ℃ for 30 min, followed by analysis using flow cytometry.

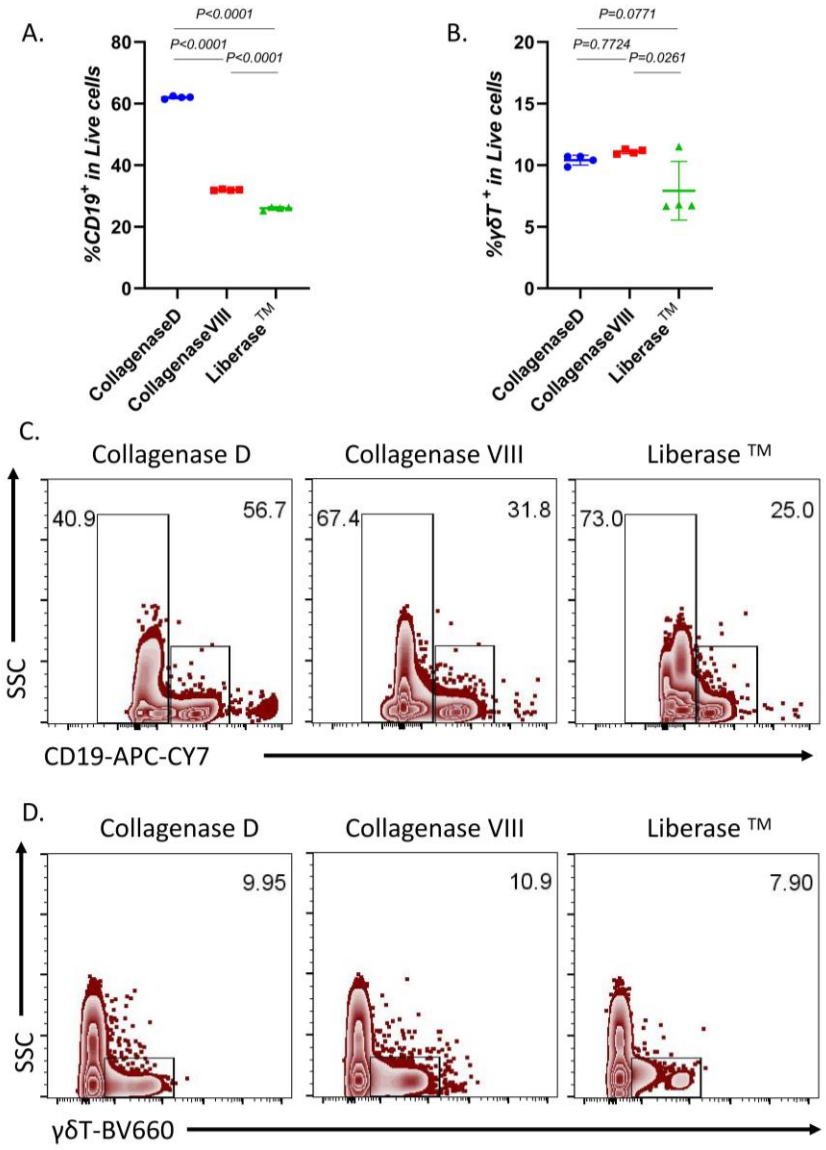

Figure 2. Detection results of B cell surface marker CD19 and γδ T cell surface marker TCRγδ. **(A)** statistical analysis chart of the yield of B cell surface marker CD19; **(B)** statistical analysis chart of the yield of γδ T cell surface marker TCRγδ; **(C)** flow cytometry analysis chart of the detection efficiency of CD19 in single-cell suspensions obtained using three enzymatic digestion methods; **(D)** flow cytometry analysis chart of the detection efficiency of TCRγδ in single-cell suspensions obtained using three enzymatic digestion methods (n = 3).

(See **Figure 2A–D**) The results indicated that collagenase D provided the best detection for B cells, while the detection of γδ T cells showed optimal results with both collagenase D and collagenase VIII. (See **Figure 3A–E**) TCR-β is a key marker for detecting $CD4^+$ and $CD8^+$ T cell subsets, and similar to previous results, collagenase D demonstrated the best detection efficacy. We then examined the two key T cell subsets, $CD4^+$ and $CD8^+$. The results showed that for CD8 detection, Liberase yielded the best results, while there were no significant statistical

differences among the three enzymes for $CD4^+$ T cell detection.

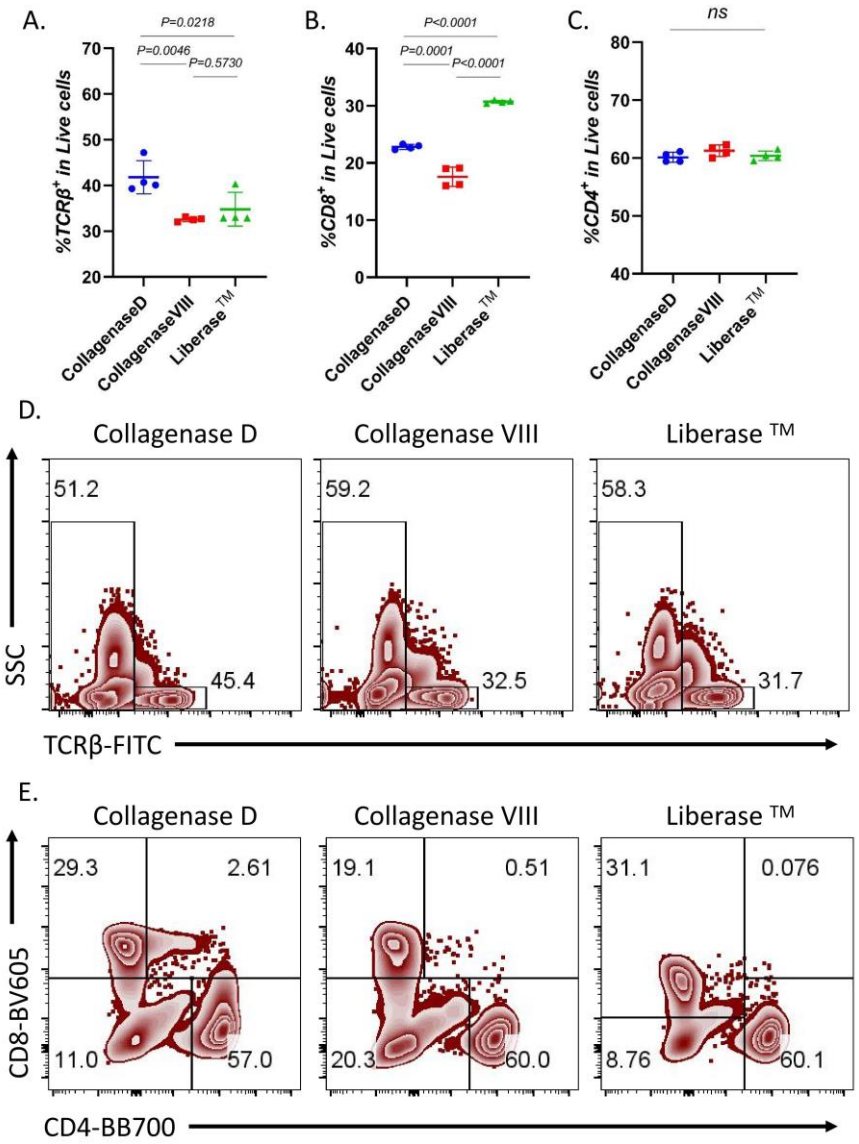

Figure 3. Compares the differences in $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells obtained from cell suspensions using three enzymatic digestion methods. **(A)** Statistical analysis chart of TCRβ detection results; **(B)** statistical analysis chart of CD8 detection results; **(C)** statistical analysis chart of CD4 detection results; **(D)** flow cytometry analysis chart of the detection efficiency of TCRβ in single-cell suspensions obtained using three enzymatic digestion methods; **(E)** flow cytometry analysis chart of the detection efficiency of CD4 and CD8 in single-cell suspensions obtained using three enzymatic digestion methods (n = 4).

In summary, collagenase D should be used for detecting B cells; either collagenase D or collagenase VIII can be selected for detecting γδ T cells, and for detecting the two key T cell subsets, $CD4^+$ and $CD8^+$, collagenase D is recommended based on its detection efficiency for TCR-β. From the above results, it can be observed that different digestion methods do not affect cell yield, but Liberase and collagenase D have a minimal impact on cell viability and yield a higher proportion of immune cells.

3.3. The impact of different digestive enzymes on surface antigens of myeloid-derived immune cells

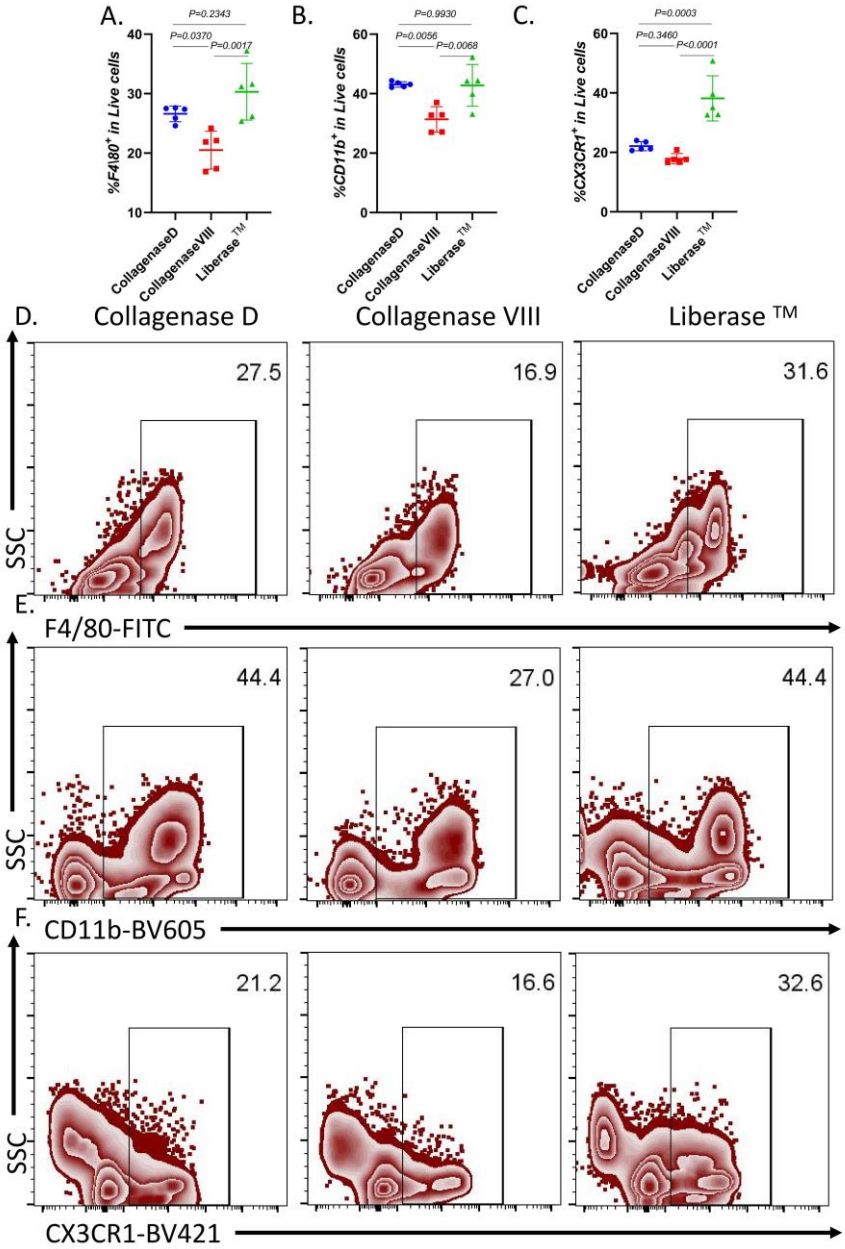

Figure 4. Compares the macrophage markers obtained from cell suspensions using three enzymatic digestion methods. **(A)** Statistical analysis chart of F4/80 detection results; **(B)** statistical analysis chart of CD11b detection results; **(C)** statistical analysis chart of CX3CR1 detection results; **(D)** flow cytometry analysis chart of the detection efficiency of F4/80 in single-cell suspensions obtained using three enzymatic digestion methods; **(E)** flow cytometry analysis chart of the detection efficiency of CD11b in single-cell suspensions obtained using three enzymatic digestion methods; **(F)** flow cytometry analysis chart of the detection efficiency of CX3CR1 in single-cell suspensions obtained using three enzymatic digestion methods (n = 5).

Myeloid-derived immune cells are also an important component of intestinal immunity, and the markers for myeloid-derived immune cells are more complex. To accurately analyze myeloid-derived immune cells, we similarly compared the markers for macrophages and dendritic cells using two different methods.

This study primarily focused on detecting macrophage and dendritic cell-related markers, including F4/80, CD11b, CD11c, CD103, MHC-II, and CX3CR1. F4/80 and CD11b are crucial for analyzing intestinal macrophages, with F4/80$^+$ and CD11b$^+$ commonly defined as macrophages [10,23].

(See **Figure 4A–F**) A comparison of the three digestion methods revealed that Liberase provided good preservation for these two markers. CX3CR1 is a key marker for distinguishing between intestinal macrophages (IMS) and resident macrophages (RMS) [10,23], and similar to the key macrophage markers F4/80$^+$ and CD11b$^+$, Liberase also exhibited the best detection efficacy.

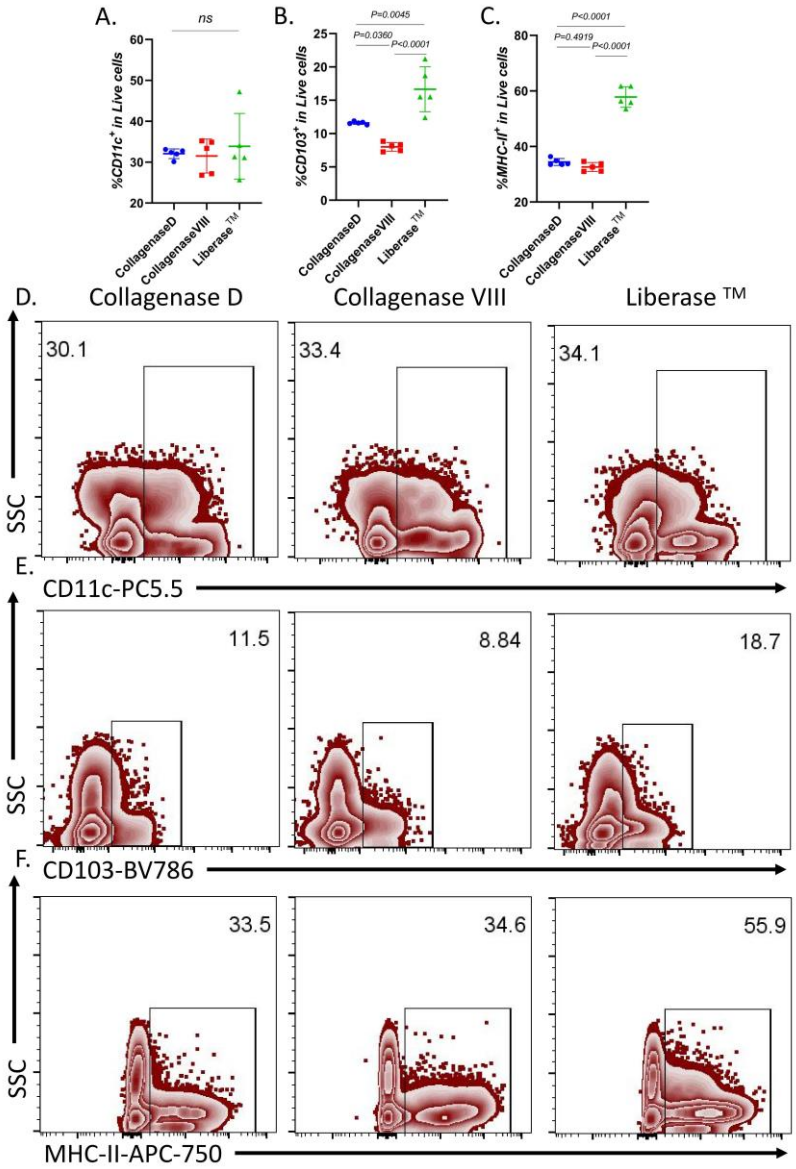

Figure 5. Compares the DCs markers obtained from cell suspensions using three enzymatic digestion methods. **(A)** Statistical analysis chart of CD11c detection results; **(B)** statistical analysis chart of CD103 detection results; **(C)** statistical analysis chart of MHC-II detection results; **(D)** flow cytometry analysis chart of the detection efficiency of CD11c in single-cell suspensions obtained using three enzymatic digestion methods; **(E)** flow cytometry analysis chart of the detection efficiency of CD103 in single-cell suspensions obtained using three enzymatic digestion methods; **(F)** flow cytometry analysis chart of the detection efficiency of MHC-II in single-cell suspensions obtained using three enzymatic digestion methods (n = 5).

(See **Figure 5A–F**) Next, we examined commonly used markers for identifying dendritic cells, which are typically defined by $CD11c^+$ and $MHC\text{-}II^+$ [24]. Additionally, CD103 and CD11b were used to differentiate between various dendritic cell subsets [7,25]. The detection results indicated that there were no significant differences in CD11c detection across the three digestion methods, while Liberase showed better results for detecting CD103 and MHC-II.

Overall, in contrast to lymphocyte detection, the optimal marker for myeloid-derived immune cells is Liberase rather than collagenase D.

3.4. Comparison of the landscape of lymphocyte populations in the lamina propria of DSS-induced colitis mice

After a comprehensive comparison of the surface markers of lymphocytes, macrophages, and DCs, we have selectively isolated intestinal lymphocytes and myeloid-derived immune cells using different methods, aiming to achieve optimal results in analyzing the various subpopulations of cells.

In addition, we will compare the differences in the intestinal immune landscape between mice with colitis and mice in a homeostatic state.

2,4,6-trinitrobenzenesulfonic acid (TNBS), oxazolone, and acute and chronic DSS colitis are the most widely used chemically induced models of intestinal inflammation [20]. In the first two models, colitis is induced by rectal administration of the reagents TNBS/oxazolone; in the DSS model, mice are given drinking water containing DSS for several days [20]. DSS modeling can be divided into acute DSS models and chronic DSS models. The acute model commonly uses 2%–5% DSS for continuous induction over 7 days; the chronic model typically uses a lower dose of DSS for 7 days followed by 14 days of normal drinking water, cycling through three such cycles. The acute DSS colitis model is particularly useful for studying the role of innate immune mechanisms in the pathogenesis of colitis, but there is relatively little literature providing a comprehensive report on the immune cell populations in the DSS model. For the C57BL/6 strain, drinking water containing 2% DSS for 7 days can induce severe colitis, but the mortality rate is low [20]. Therefore, in this study, we used the acute DSS model with C57BL/6 mice, administering 2% DSS for 7 days and then providing normal drinking water on the 8th day, followed by a 12-hour fasting period before euthanizing the mice for tissue collection.

By comparing three methods for obtaining immune cells from the lamina propria of mouse colon through enzymatic digestion, we found that using collagenase D was more suitable for subsequent multiparameter flow cytometry analysis of lymphocyte subpopulations. Thus, we used collagenase D to extract immune cells from the mouse colon, and we analyzed the obtained immune cells for T lymphocytes as well as related transcription factors and cytokines. The specialized T cell population γδT cells has a non-redundant role in anti-infection functions; effector T cells (Teff) produce pro-inflammatory factors such as IFN-γ and IL-17A, which are the main T cell populations in patients with IBD. In non-inflammatory states, the function of pro-inflammatory T cells in the colon is suppressed by thymic and peripherally generated natural and induced regulatory T cells (Tregs), which typically express the transcription factor FoxP3. Therefore, we primarily detected

γδT cells; IFN-γ$^+$, IL-17A$^+$, and Foxp$^+$CD4$^+$ T cells. We found that following DSS modeling, γδT cells were significantly increased compared to the normal drinking water group (**Figure 6A,B**). Interestingly, we did not observe a decrease in the Foxp$^+$CD4$^+$ T cell population in the DSS model group, but rather a significant increase (**Figure 6C,D**). To observe changes in the two key pro-inflammatory populations TH1 and TH17 T cells, we marked the cytokines IFN-γ and IL-17A, indicating that both IFN-γ and IL-17A were significantly elevated in DSS-induced colitis with statistical significance (**Figure 6E–H**).

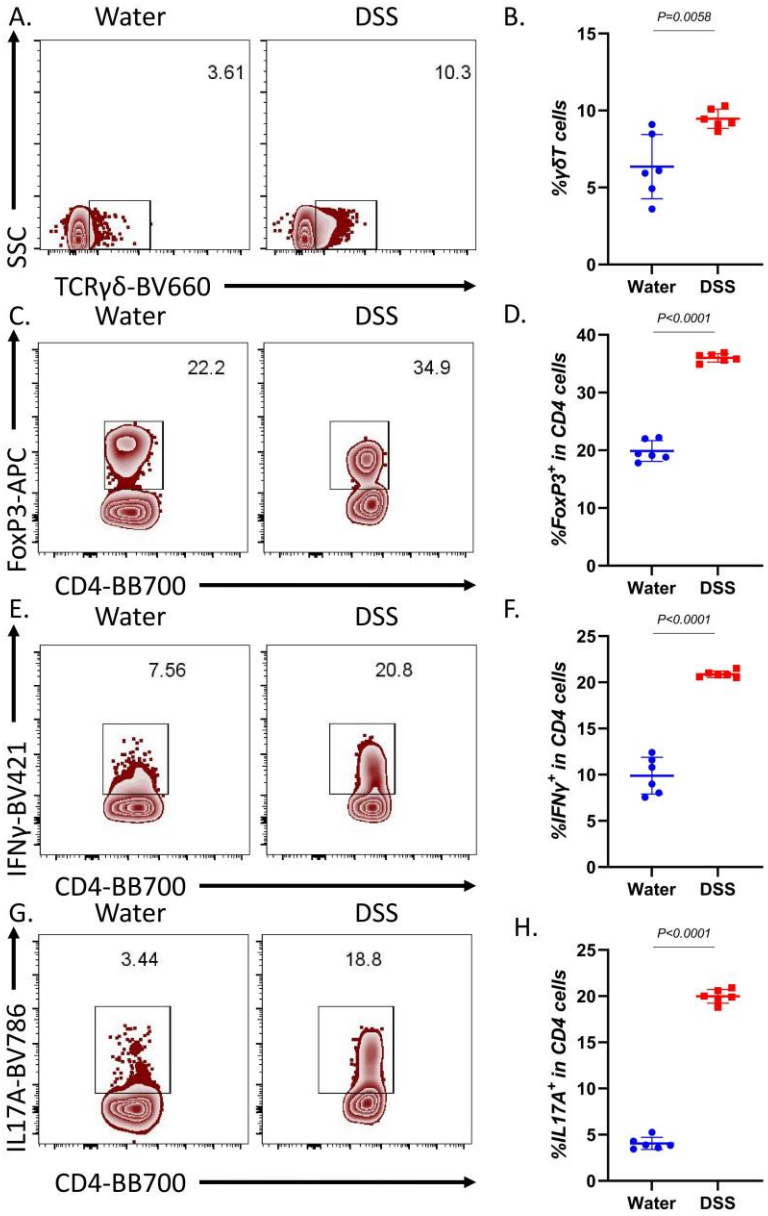

Figure 6. Immunological landscape related to T lymphocytes after DSS-induced colitis in mice. **(A)** Flow cytometry analysis chart of γδ T cells; **(B)** statistical analysis chart of γδ T cells; **(C)** flow cytometry analysis chart of Tregs; **(D)** statistical analysis chart of Tregs; **(E)** flow cytometry analysis chart of TH1 cytokines; **(F)** statistical analysis chart of TH1 cytokines; **(G)** flow cytometry analysis chart of TH17 cytokines; **(H)** statistical analysis chart of TH17 cytokines (n = 6).

3.5. Comparison of changes in macrophage subpopulations in the lamina propria of DSS-induced colitis mice

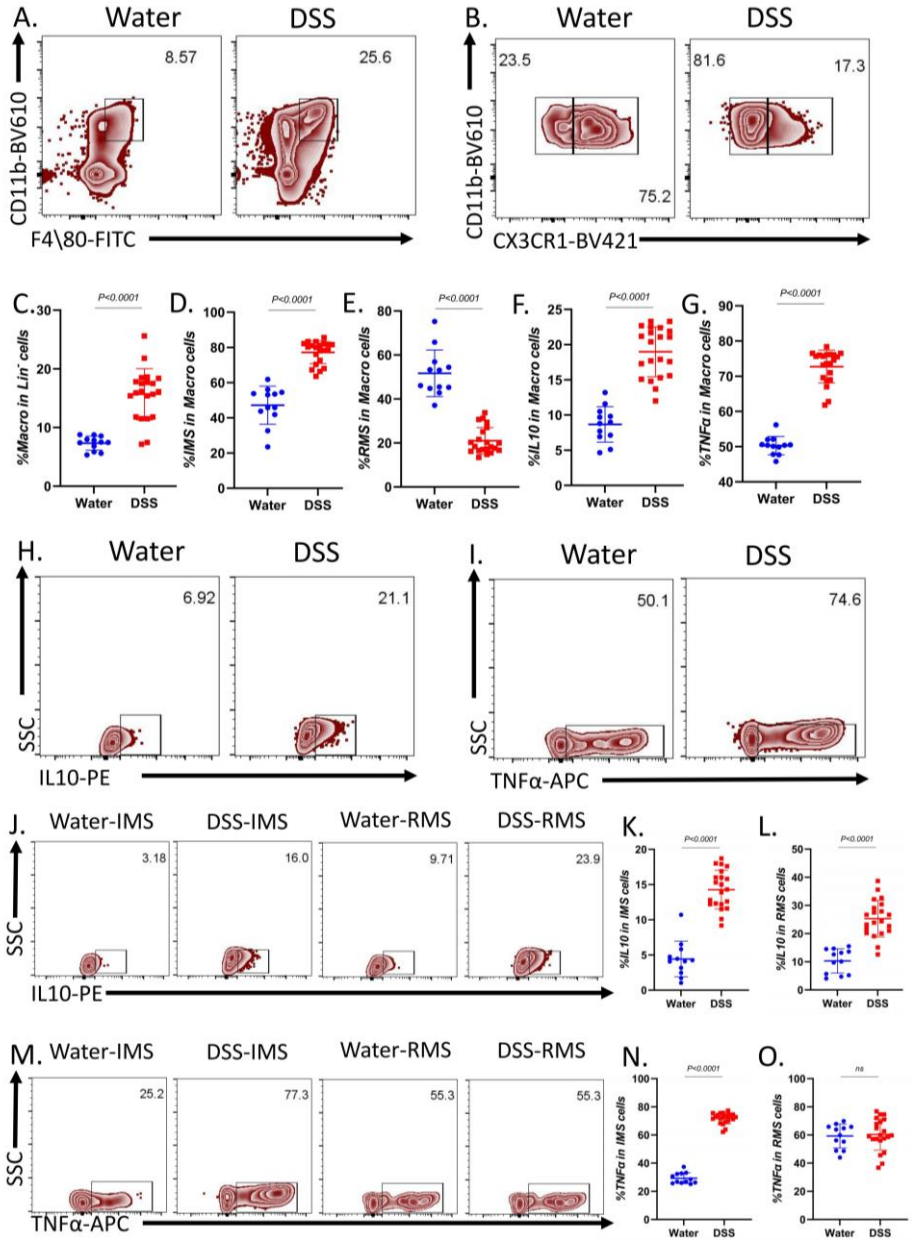

Figure 7. Immunological landscape related to macrophages after DSS-induced colitis in mice. **(A)** Flow cytometry analysis chart of the overall proportion of macrophages; **(B)** flow cytometry analysis chart of IMS and RMS; **(C)** statistical analysis chart of the overall proportion of macrophages; **(D)** statistical analysis chart of the proportion of IMS; **(E)** statistical analysis chart of the proportion of RMS; **(F)** statistical analysis chart of IL-10 secreted by all macrophages; **(G)** statistical analysis chart of TNF secreted by all macrophages; **(H)** flow cytometry analysis chart of IL-10 secreted by all macrophages; **(I)** flow cytometry analysis chart of TNF secreted by all macrophages; **(J)** flow cytometry analysis chart of IL-10 secretion by IMS and RMS before and after modeling; **(K)** statistical analysis chart of IL-10 secretion by IMS before and after modeling; **(L)** statistical analysis chart of IL-10 secretion by RMS before and after modeling; **(M)** flow cytometry analysis chart of TNFα secretion by IMS and RMS before and after modeling; **(N)** statistical analysis chart of TNFα secretion by IMS before and after modeling; **(O)** statistical analysis chart of TNFα secretion by RMS before and after modeling ($n \geq 12$).

The immune cell status and composition in the intestinal environment are very complex, and myeloid-derived immune cells also play important roles. Therefore, assessing the secretion of macrophages and their cytokines is essential. Macrophages play a critical role in preventing excessive immune responses. During tissue damage, the phagocytosis of dead cells triggers a functional transition of macrophages to an anti-inflammatory transcriptional program, similar to an alternative macrophage activation pattern, producing cytokines, growth factors, and lipids that are essential for restoring homeostasis [3].

(See **Figure 7A–O**) Therefore, we used $F4/80^+$ and $CD11b^+$ to label the macrophage populations. The results showed that, compared to the control group, there was a significant increase in the total number of macrophages in the DSS-induced colitis model. Next, we used CXC3R1 to distinguish two key macrophage subpopulations, identifying the levels of IMS (inflammatory macrophages, IMs) and RMS (resident macrophages, RMs). We found that the level of IMS was significantly elevated in the DSS group compared to the control group, while RMS was significantly decreased in the DSS group compared to the control group. We also measured the secretion of two key cytokines, IL-10 and TNFα, from IMS and RMS. First, we assessed the cytokine secretion levels of all macrophage populations and found that the overall ability of macrophages to secrete IL-10 and TNFα was significantly enhanced after DSS modeling. To further understand the cytokine secretion of different macrophage subpopulations, we analyzed the expression levels of cytokines in IMS and RMS. We found that under homeostatic conditions, RMS was in a predominantly activated state, with significantly higher secretion of IL-10 and TNFα compared to IMS. Following DSS modeling, the secretion levels of IL-10 from both IMS and RMS were significantly elevated compared to homeostatic conditions; however, TNFα secretion was significantly increased in IMS, while there was no significant difference in TNFα secretion in RMS. In summary, under homeostatic conditions, intestinal macrophages are predominantly driven by RMS, whereas in DSS-induced colitis, they are predominantly driven by IMS.

3.6. Comparison of changes in DCs subpopulations in the lamina propria of DSS-induced colitis mice

DCs are potent and multifunctional antigen-presenting cells, and their migratory ability is crucial for initiating protective pro-inflammatory and tolerogenic immune responses.

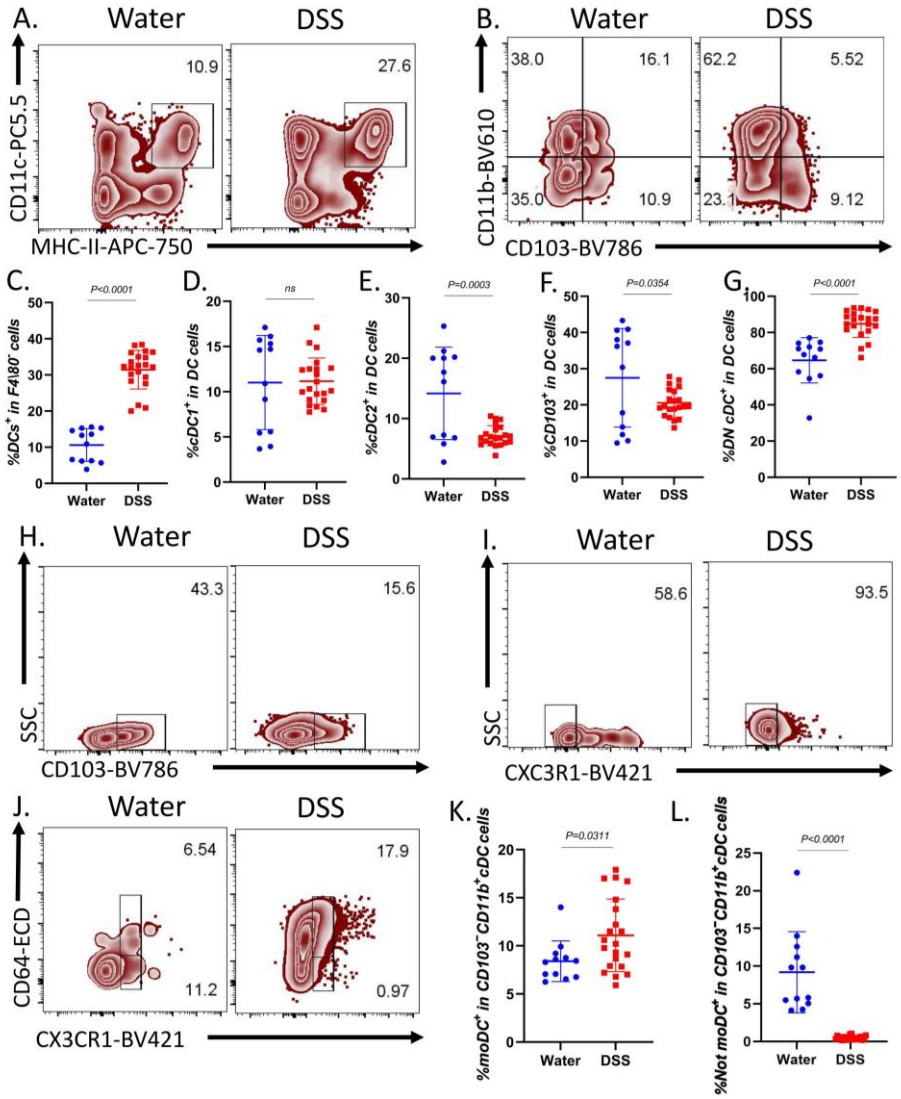

Figure 8. Immunological landscape related to DCs after DSS-induced colitis in mice. **(A)** Flow cytometry analysis chart of the overall proportion of DCs; **(B)** flow cytometry analysis chart of cDC1 and cDC2; **(C)** statistical analysis chart of the overall proportion of DCs; **(D)** statistical analysis chart of the proportion of cDC1; **(E)** statistical analysis chart of the proportion of cDC2; **(F)** statistical analysis chart of $CD103^+$ DCs; **(G)** statistical analysis chart of DN cDCs; **(H)** flow cytometry analysis chart of $CD103^+$ DCs; **(I)** flow cytometry analysis chart of DN cDCs; **(J)** flow cytometry analysis chart of moDCs and non-monocyte-derived DCs before and after modeling; **(K)** statistical analysis chart of moDCs before and after modeling; **(L)** statistical analysis chart of non-monocyte-derived DCs before and after modeling ($n \geq 12$).

(See **Figure 8A–L**) We defined dendritic cells using $CD11c^+$ and MHC-II. First, we assessed the overall proportion of dendritic cells, and found that the number of dendritic cells was significantly increased after DSS-induced colitis. Subsequently, we identified five key subpopulations of dendritic cells: $CD103^+$ $CD11b^-$ (cDC1) [26], $CD103^+$ $CD11b^+$ (cDC2) [27], $CD103^-$ $CD11b^-$ $CX3CR1^-$ (DN cDC) [28], $CD103^-$ $CD11b^+$ $CD64^+$ CX3CR1mid (moDC) [29], and $CD103^-$ $CD11b^+$ CX3CR1mid cDC (not monocyte-derived DC) [7].There was no statistically significant difference in cDC1 between colitis and homeostatic conditions; cDC2 is crucial for responding to bacterial-derived antigens in the gut, and the number of cDC2 in DSS-induced colitis was significantly decreased compared to the control

group. $CD103^+$ dendritic cells are a key population for inducing Tregs, and their numbers were significantly reduced in the DSS group compared to the control group. DN cDC can promote the differentiation of TH17 cells, and the proportion of DN cDC was significantly increased in the DSS group. MoDC can promote IL-12 production during infection, inducing Th1 cell differentiation and promoting colitis through the production of pro-inflammatory cytokines, and the number of moDC was significantly increased in the DSS group compared to the control group. Additionally, the not monocyte-derived DC population was significantly decreased in the DSS group compared to the control group. In DSS-induced colitis, there were significant changes in dendritic cell subpopulations, with pro-inflammatory populations showing a clear advantage. Due to the certain discrete trends in dendritic cell subpopulations under homeostasis, as colitis occurs in mice, there is a noticeable trend of concentration in these subpopulations. We conducted a calculation of the coefficient of variation, which suggests that there are differences in dendritic cell subpopulations in mice under homeostatic conditions (supplementary information). However, with the progression of inflammation, there is a significant expansion of pro-inflammatory dendritic cell subpopulations, and this change is relatively stable.

4. Discussion

Enzymatic digestion is a common method for preparing single-cell suspensions. The three digestion methods selected in this study all contain EDTA, DTT, and DNase I. The primary roles of EDTA and DTT are to dissociate intestinal epithelial cells, allowing collagenase to more efficiently dissociate the lamina propria and obtain a single-cell suspension; DNase I degrades the DNA exposed by dead cells during the tissue dissociation process, preventing cell aggregation and adhesion [19]. By comparing the cell viability of these three methods, we found that collagenase D and Liberase performed exceptionally well. This may be because collagenase VIII is a crude collagenase, which is a mixture composed of collagenase and several other different enzymes. Crude collagenase contains clostripain, which can be activated by DTT and has cytotoxic effects. In addition, crude collagenase may exhibit significant batch effects, as using the same unit of collagenase from different batches to dissociate tissues could result in considerable differences. This may also be one of the key factors affecting cell viability and marker expression. For more detailed information, please refer to the tissue dissociation guidelines on the Merck official website: Types of collagenase, dispase, and liberase [13,18,30–38]. Liberase™ contains high-purity collagenase I and collagenase II, which reduces the effects of bacterial toxins present in some enzymes on cell viability; however, its content of a certain concentration of thermolysin may be one reason for the lower viability compared to Method 1 [31,32,39,40]. HEPES can enhance the viability of immune cells, perhaps due to the fact that it is not added during digestion, resulting in the exposure of cells in the lamina propria to the pre-digestion solution and reduced cell activity [41,42]. Additionally, the lower shaking speed used with collagenase D may better preserve cell viability. In contrast, collagenase VIII is a crude collagenase that may contain relatively high levels of cytotoxins and non-specific neutral proteases, which could significantly affect cell viability. The lower cell yield obtained with

Method 1 compared to the other two methods may be due to the lower content of neutral proteases and trypsin; neutral proteases and trypsin might improve cell yield but at the cost of cell viability [37].

Furthermore, flow cytometry for analyzing cell subpopulations and functions relies on the specific binding of antigens to antibodies, so the loss of surface antigens on cells can significantly impact detection. For the surface markers of lymphocytes, the three methods exhibited considerable differences, with collagenase D showing the best detection results. Therefore, we recommend using collagenase D when analyzing lymphocyte-related indicators. On the other hand, Liberase provides better protection for myeloid-derived markers, so we recommend using Liberase for processing tissues when analyzing myeloid-related cell populations.

Animal models of intestinal inflammation are essential for understanding the pathogenesis of IBD [20]. However, existing studies still lack comprehensive analyses of intestinal immune status. Therefore, we conducted an analysis of immune cells in the lamina propria of DSS-induced colitis mice. We found that the specialized T cell population, γδT cells [2,4,5,11], was significantly increased, and the secretion of pro-inflammatory cytokines IFN-γ and IL-17A by the major pro-inflammatory T cell population was elevated [43–47]. Additionally, Tregs also showed expansion, indicating that during the tissue damage caused by DSS in normal mice, the major pro-inflammatory T cell populations were activated, and Tregs exhibited a compensatory increase to balance the inflammatory state in the gut.

DCs are core participants in the immune system, bridging innate and adaptive immunity. Most DC-driven immune responses, whether maintaining tissue tolerance or promoting immunity to pathogens, require their migration to specific sites [1,48]. Dendritic cells showed significant expansion in DSS-induced colitis, with notable changes in the main subpopulations, including a marked increase in moDC and DNDC. The increase in moDC promotes IL-12 production in the lamina propria during inflammation, leading to TH1 cell polarization, and through the production of pro-inflammatory cytokines, it promotes colitis [23,49–51]. The increase in DNDC can activate TH17 responses in vitro, which may be one reason for the large production of cytokines by pro-inflammatory T cells [7,28]. In contrast, cDC2 was significantly decreased in the DSS group; cDC2 is the primary cell type for presenting antigens from intestinal bacteria, and its absence can enhance inflammatory responses in the gut.

The number of macrophages also exhibited changes similar to those of dendritic cells, with the macrophage population in DSS-induced colitis being dominated by IMS, which secreted large amounts of TNF-α and IL-10. Additionally, compared to the control group, the RMS in the DSS group showed varying degrees of activation, producing more TNF-α and IL-10. Furthermore, the overall levels of TNF-α and IL-10 secreted by macrophages were significantly elevated in the DSS group. Thus, it is evident that DSS-induced colitis is characterized by significant activation in both $CD4^+$ T cell populations and myeloid cell populations, with a certain compensatory effect.

In addition, we found that compared to mice under homeostasis, the flow cytometry data from the intestines of colitis mice exhibited a more pronounced trend

of concentration. After calculating the coefficient of variation, we discovered that, in general, the flow cytometry data from colitis mice had a smaller coefficient of variation, and these smaller coefficients were concentrated within the pro-inflammatory immune cell populations. This further indicates that the intestinal immune landscape in colitis mice displays a very strong pro-inflammatory pattern in both adaptive and innate immunity.

In summary, this study provides guidance for the processing of flow cytometry tissue samples and offers a comprehensive understanding of the immune status in DSS-induced colitis, providing insights for the preparation of immune cells and the analysis of intestinal immune cell subpopulations under homeostatic or inflammatory conditions.

5. Conclusion

The intestinal system is the largest mucosal system in the body, and the complex environment of the gut gives rise to its diverse immune functions. As research progresses, it has become evident that various immune cells play important roles in intestinal immunity and the maintenance of homeostasis. Therefore, obtaining high-quality single-cell suspensions from intestinal tissues is a crucial prerequisite for using flow cytometry to detect intestinal immune cells. This study selected three common digestion methods to compare the yield, viability, and the impact on important immune cell surface markers obtained by different methods. Ultimately, suitable methods for tissue processing were chosen. Additionally, the subpopulation analysis of T cells, macrophages, and dendritic cells, as well as the analysis of important cytokines and transcription factors in DSS-induced colitis, were conducted.

Author contributions: Conceptualization, BZ and SZ; methodology, BZ; software, BZ; investigation, JX; writing—original draft preparation, BZ; writing—review and editing, BZ; supervision, JX. All authors have read and agreed to the published version of the manuscript.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki. The animal study protocol was approved by The Institutional Animal Care and Use Committee of Sichuan University (protocol code 20241128012).

Conflict of interest: The authors declare no conflict of interest.

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