Single-cell transcriptional profiling of murine conjunctival immune cells reveals distinct populations expressing homeostatic and regulatory genes

Jehan Alam, Ghasem Yazdanpanah, Rinki Ratnapriya, Nicholas Borcherding, Cintia S. de Paiva, DeQuan Li and Stephen C. Pflugfelder

Immune cells in the exposed conjunctiva mucosa defend against environmental and microbial stresses. Expression profiling by single-cell RNA sequencing was performed to identify conjunctival immune cell populations expressing homeostatic and regulatory genes. Fourteen distinct clusters were identified, including myeloid cells (neutrophils, monocytes, macrophages), dendritic cells (DC), and lymphoid cells (B, T, γδT, ILC2, and NK) lineages. Novel neutrophil (lipocalin (Lcn2) high and low), and MHCIIlo macrophage (MP) clusters were identified. More than half of the cells map to myeloid and dendritic cell populations with differential expression profiles that include genes with homeostatic and regulatory functions: Serpinb2 (MHCIIlo macrophage), Apoe (monocyte), Cd209a (macrophage), Cst3 (cDC1), and Il4i1 in migratory DC (mDC). ILC2 expresses the goblet cell trophic factor IL-13. Suppressed inflammatory and activated anti-inflammatory/ regulatory pathways were observed in certain myeloid and DC populations. Confocal immunolocalization of identity markers showed mDC (CCR7, FASCIN1) located on or within the conjunctival epithelium. Monocyte, macrophage, cDC1 and IL-13/IL-5+ ILC2 were located below the conjunctival epithelium and goblet cells. This study found distinct immune cell populations in the conjunctiva and identified cells expressing genes with known homeostatic and immunoregulatory functions.

Mucosal Immunology (2022) 15:620–628; https://doi.org/10.1038/s41385-022-00507-w

INTRODUCTION

The conjunctiva is a unique mucosal tissue that is exposed to the environment, produces, and secretes tear-stabilizing goblet cell mucins, and provides immune surveillance for the clear avascular cornea. A variety of immune cells including, myeloid, dendritic, natural killer (NK), B cells, and conventional and unconventional T cells, have been identified in the conjunctiva by immunostaining or flow cytometry using antibodies to conventional lineage markers. Certain antigen lineage markers are recognized to lack specificity. For example, the integrin CD11b is expressed by a variety of cells, including neutrophils, eosinophils, monocytes, macrophages, dendritic cells, and NK cells. Because the conjunctival immune system is subjected to danger signals from bacterial, fungal, parasitic, and viral pathogens, as well as desiccating and osmotic stress, regulation of the immune response is of paramount importance to prevent loss of conjunctival goblet cells and exuberant sight-threatening corneal inflammation and proteolysis. Certain cytokines and pathways have been found to have homeostatic functions in the conjunctiva. The cytokine IL-13 was found to be a trophic factor for the conjunctival goblet cells and secreted into tears and produced by the conjunctival goblet cells suppress the production of inflammatory mediators. Nevertheless, there is much to discover regarding which conjunctival cell populations produce homeostatic and regulatory factors and how these cells interact.

This study used single-cell transcriptional profiling of murine conjunctival immune cells to identify distinct populations expressing homeostatic and regulatory genes and specific identity markers based on gene expression profiles.

RESULTS

Single-cell transcriptome profiles reveal heterogeneous immune cell populations in the C57BL/6 mouse conjunctiva

We performed droplet-based single-cell RNA sequencing (scRNA-seq) as an unbiased approach to evaluate immune cell types in the mouse conjunctiva. We constructed a scRNA-seq library from CD45+ immune cells sorted from the conjunctiva of normal C57BL/6 J (B6) mice (n = 8 biological replicates) and obtained a transcriptomic profile of 11,165 cells using the x10 Genomics platform (Fig. 1a). The scRNA-seq data analysis was performed using Seurat V4.1.0. After quality assessment (Fig. S1a), filtering standard pre-processing, and doublet exclusion (Fig. S1a), a total of 8909 cells with 2500 variable features were analyzed. Graph-based clustering using Seurat divided the cells into 14 clusters (Fig. 1b) that were identified based on the expression of signature marker genes listed in Table S1. The identified clusters include
neutrophil, monocyte, macrophage, mast cell, natural killer cell, T cell, group 2 innate lymphoid cells (ILC-2), γδ-T cell, B cell, conventional dendritic cells 1 (cDC1) and migratory dendritic cell (mDC) lineages (Fig. 1b). The number of cells and percentage of the whole population in each cluster are shown in Fig. 1c. Myeloid cells (monocytes and macrophages) in clusters 0, 3, and 4 comprise approximately half (52.82%) of the total cells. Cluster 0 contains the largest percentage of cells (34.59%) that express both
Conventional flow cytometry cell surface markers are found on a variety of cells with distinct gene expression profiles

Our lab and others have previously identified heterogeneous conjunctival immune cell populations with flow cytometry or immunostaining using antibodies to conventional cell surface antigens. scRNA-seq identifies the diverse conjunctival immune cell populations with higher resolution. The sequencing data shows that genes encoding conventional myeloid cell markers, including CD11b, CD11c, and Ccr2 are expressed by several cell types, including non-myceloid cells and lack specificity for any particular cell type (Fig. 2a). In contrast, Fig. 2b demonstrates that many of the markers used to identify the conjunctival cell clusters show high expression levels primarily in that cluster; however, there is overlap for some markers (i.e. Mark3sl1, Cd74, H2-Ab1) between myeloid and dendritic cells and Jund transcription factor is expressed by all clusters.

DISCUSSION

Functions of the conjunctival immune cells include pathogen defense, trophic support of the mucin secreting goblet cells, wound healing, and production of regulatory factors to minimize tissue-damaging inflammation. Characterization of conjunctival immune cells in the past has primarily relied on immunostaining or flow cytometry using antibodies to conventional lineage markers. Immune cells, including monocyte derived macrophages and DCs, macrophages and T cells were also identified by our study that used scRNA-seq to profile immune cells at a deeper level. To the best of our knowledge, this is the first report using this technology to profile gene expression in conjunctival immune cells. Collin, Queen, Zert, Bojic, Dorgau, Mouye and colleagues reported single cell transcriptional profiles of cells isolated from the human limbus. Five immune cell types (monocyte derived macrophage/dendritic cells, 2 types of macrophages and...
2 CD8 T cells) were identified. Top differentially expressed genes by these cells aligned with similar cell types we found in the conjunctiva except for the CD81 cluster. We identified 14 cell clusters and most clusters mapped to the cell types with the highest identity match in the CIPR database. The lowest identity match was for cluster 0 that we designated macrophage MHCIIlo. Alox15, an eosinophil-associated marker, and Adgre (F4/80), a gene expressed by macrophages and eosinophils were among the top differentially expressed genes in this study.
Fig. 3  Cell-cell interaction, enriched pathways and expression of retinoid pathway genes. a Circle network plots showing number (left) and weights/strengths of cell-cell interactions generated with cellchat.; b Relative enrichment based on pathway analysis generated with Qiagen IPA; c Violin plots of RXRα and Aldh1a2 expression in cell clusters.
Fig. 4 Cluster specific expression (left) and confocal immunolocalization of cluster-specific factors (in parenthesis) in conjunctival whole mounts. 

- a) Ccr7 and Fscn1 (mDC); b) MHCI and CST3 (cDC1); c) APOE (monocyte) and CSF1R (CD115) (monocytes); d) CD209a and MHCI (macrophage); e) SERPINB2 and ADGRE (F4/80) (macrophage MHCI-); f) IL-13 and IL-5 (ILC2). Scale bar = 100 μm. Biological replicates (n = 3) and technical replicates (n = 2).
cluster; however, eosinophils were not observed by histochemical staining in the conjunctiva. Expression of some traditional cell antigen markers was found in several clusters, indicating their relative lack of specificity. The expression profiles revealed genes, for example, Serpinb2 (macrophage MHCIIlow), Apoe (monocyte), ILS for ILC2 and Fascln1 (mDC), that can be used for cell identification and/or provide insight into the function of these cells.

Mapped clusters include myeloid cells (neutrophil, monocyte, macrophage), two DC clusters (cDC1, mDC), and lymphoid cells (B, T, γδT, ILC2, and NK). Novel lipocalin2 (Lcn2) high and low neutrophil, and Retnla31 macrophage subclusters were identified. Myeloid and dendritic cells were the predominant populations comprising 54.12% of the isolated cells. These cells express genes with known homeostatic (protease inhibitors) and regulatory functions (anti-inflammatory and tolerance induction). Among these, Serpinb2 (macrophage MHCIIlow), also known as plasminogen activator inhibitor, suppresses activation of plasmin, which in turn can promote ocular surface inflammation by activating complement and metalloproteases and generating fibrin degradation products. Cst3 (cystatin C) expressed by cDC1 inhibits activation of CTSS, a protease involved in MHCII-restricted antigen presentation30,31. Reduced levels of Cst3 in the tears has been implicated as a cause for the increased tear CTSS activity that is associated with disease severity in Sjögren syndrome associated keratoconjunctivitis sicca32. Apoe the top differentially expressed gene by the monocyte cluster, has been found to suppress macrophage recruitment and activation, independent of its role in lipid transport33,34. It also functions in scavenging apoptotic cell bodies, an activity that also suppresses inflammation35. Cd209a (DC-sign), the top differentially expressed gene in the macrophage cluster is associated with the generation of immune tolerance36. Expression of IL-4 signature genes (IL4i1, IL4ra) in the mDC cluster is characteristic of regulatory DCs that promote immune tolerance and suppress generation of IFN-γ producing T cells22. cDC2 has been previously identified in the conjunctiva using cell surface markers32, but was not identified as a distinct cluster based on a unique transcriptional profile. cDC2 is a heterogenous cell population that shares markers with monocyte derived DCs and macrophages, particularly at inflamed mucosa33. It is possible cDC2 is included in macrophage cluster that shows strong correlation (94–100%) with CD11b+ DCs and macrophages in the CIPR database, and may be identified as a unique cluster if a larger starting cell population is used.

The number and strength of cell-cell interactions were highest for myeloid and dendritic cells that suggests a close interaction between monocytes, macrophages, cDC1 and mDC for an effective innate immune response. Consistent with the gene expression profiles, pathway analysis shows suppressed inflammatory (IL-1, TLR) and activated anti-inflammatory/ regulatory pathways (RXR, PPAR, vitamin D receptor nuclear receptor signaling) in certain myeloid and DC populations. These findings are consistent with the need to promote immune tolerance and suppress potentially sight threatening inflammation on the ocular surface. We have found RXRα nuclear receptor is expressed by most conjunctival myeloid cells and that the natural ligand 9-cisRA has potent immunosuppressive activity on stimulated monocytes7,31. There is increasing recognition that vitamin D signaling through its receptor, a heterodimeric partner of RXR also promotes immune tolerance32,33.

A surprise finding among the lymphoid cell subsets was that ILC2 had significantly higher expression of the goblet cell trophic factor IL-13 than the other clusters. The physiological importance of IL-13 has been reported to stimulate goblet cell proliferation and mucus production in vivo and in cultured cells9.9. The physiological importance of IL-13 on goblet cell maintenance in the conjunctiva is reinforced by reports that antibody neutralization of IL-4Ra, the receptor component shared by IL-4 and IL-13 in patients with atopic dermatitis can cause conjunctival inflammation, dry eye and goblet cell loss34,35. We had previously attributed IL-13 production in the conjunctiva to NKT cells; however, it is now recognized that markers used to detect NK cells are also expressed by ILCs36. As expected, IL-17 was among the top DEG in γδ T cells. IL-17 has been implicated in the pathogenesis of corneal epithelial barrier disruption and lymphangiogenesis in ocular surface inflammatory disease16,17,38.

We performed confocal microscopy to immunolocalize cells producing homeostatic and regulatory markers and the findings are summarized in Fig. 5. We found mDC located on the surface of the conjunctiva where they are directly exposed to the environment and pathogens. Both were MHCII+ , and CCR7+ mDCs were noted to align with LYVE-1+ lymphatic vessels in the superficial stroma. CST3+ cDC1 were located within or below the conjunctival epithelium. Monocytes and macrophages were in the stroma below the epithelium and CD209a+ macrophages were noted to touch processes of the overlying MHCII+ dendritic cells. IL-5/IL-13+ ILC2 were located below the conjunctival epithelium and goblet cells where they can provide trophic support for these cells.

There are several shortcomings of this study. This study investigated conjunctival immune cells only in the C57BL/6 strain.
that was chosen because it is the most frequently used strain for dry eye models, and it is commonly used for studying other infectious and inflammatory diseases, including microbial keratitis. Cluster 0 could be mapped to several cell types, including eosinophils, granulocytes and MHCII<sup>low</sup> macrophages. It wasn’t possible to perform immunostaining for highly expressed factors in all cell clusters. Therefore, we concentrated on myeloid cells because they collectively comprised the greatest percentage of cells and because we have previously found that these cells are conditioned by tear and goblet-cell-derived factors<sup>3,39</sup>. We also did not investigate the function of top differentially expressed genes, but these findings provide direction for future studies investigating the role of these genes in conjunctival immunity.

This study provides valuable information that can be used for more specific cell identification and cell-specific gene expression profiles that can be compared with those in ocular surface inflammatory diseases, such as dry eye where there is recruitment and activation of immune cells. It provides biomarkers that can be used to determine factors, such as diet and microbiome, that maintain the production of the homeostatic and regulatory factors by the conjunctival immune cells.

**MATERIALS AND METHODS**

**Animals**

The animal protocol for this study was designed according to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (BCM). Female C57BL/6 J (B6) (n = 8) mice aged 6–8 weeks were purchased from Jackson Laboratories (Bar Harbor, ME), and allowed to house in a non-stressed (NS) environment at 50–75% relative humidity before the experiment. The age of the mouse at the time of the experiment was 16 weeks.

**Flow Cytometry**

Conjunctivae were excised, chopped with scissors into tiny pieces, and incubated with 0.1% type IV Collagenase for 1 hour to yield single-cell suspensions. Samples were incubated with anti-CD16/32 (2.4G2, Catalog no. 553141, BD Pharmingen<sup>®</sup>, San Diego, CA), for 5 minutes at room temperature and subsequently stained with anti-CD45 (clone 30-F11, Catalog no. 103138, BioLegend) and with an infra-red fluorescent viability dye (Catalog no. L10119, Life Technologies, Grand Island, NY). The gating strategy was as follows: lymphocytes were identified by forward -scatter area (FSC-A) and side scatter area (SSC-A) gates, followed by two singlets gates (FSC-A vs. FSC-W and SSC-A vs. SSC-W) followed by live/dead identification using the infra-red fluorescent viability dye. The CD45<sup>+</sup> cells were sorted using the Aria-II cell sorter at the Baylor College of Medicine cytometry and cell sorting core.

**Library preparation**

Single-cell gene expression libraries were prepared using the Chromium Single Cell Gene Expression 3v3.1 kit (×10 Genomics) at the Single Cell Genomics Core at Baylor College of Medicine. In brief, single cells, reverse transcription (RT) reagents, Gel Beads containing barcoded oligonucleotides, and oil were loaded on a Chromium controller (×10 Genomics) to generate single-cell Gel Beads-In-Emsulations (GEMs) where full-length cDNA was synthesized and barcoded for each cell subclone. Subsequently the GEMs are broken and cDNA from every single cell is pooled. Following cleanup using Dynabeads MyOne Silane Beads, cDNA is amplified by PCR. The amplified product is fragmented to optimal size before end-repair, A-tailing, and adaptor ligation. The final library was generated by amplification.

**Sequencing of 10X GEM 3’v3.1 single cell libraries**

The BCM Genomic and RNA Profiling (GARP) Core initially conducted sample quality checks using the NanoDrop spectrophotometer and Agilent Bioanalyzer 2100. To quantitate the adapter-ligated library and confirm successful P5 and P7 adapter incorporations, the Applied Biosystems ViA7 Real-Time PCR System and a KAPA Illumina/Universal Library Quantification Kit (p/n KK4824) was used. The GARP core sequenced the libraries on the NovaSeq 6000 Sequencing System using the S2 v1.0 Flowcell as follows. Cluster Generation by Exclusion Amplification (ExAMP): Using the concentration from the ViA7 TM qPCR machine above, 150 pM of the equimolar pooled library was loaded onto one lane of the NovaSeq S2 v1.0 flowcell (Illumina p/n 20012,860) following the XP Workflow protocol (Illumina kit p/n 20021,664) and amplified by exclusion amplification onto a nanowell-designed, patterned flowcell using the Illumina NovaSeq 6000 sequencing instrument. PhiX Control v3 adapter-ligated library (Illumina p/n FC-110-3001) was spiked-in at 1% by weight to ensure balanced diversity and to monitor clustering and sequencing performance. The libraries were sequenced according to the 10X Genomics protocol, 28 cycles for Reads 1, 10 cycles each for the 17 and 15 reads, and 90 cycles for Read 2. An average of 251 million read pairs per sample was sequenced. FastQ file generation was executed using bcl2fastq and QC reports were generated using CellRanger v5.0.1 by the BCM Multisomics Core.

**Bioinformatic analysis of scRNA-seq data**

Raw sequence reads in the FASTQ format were aligned to the mouse reference genome using Cell Ranger Count v6.0.1 pipeline (https://cloud.10xgenomics.com) with the default settings for alignment, barcode assignment, and UMI counting of the raw sequencing data with genome reference Mouse (mm10) 2020-A. The resulting gene expression matrix was subjected to preprocessing following the guideline provided by Seurat v4.1.0.1. Briefly, single cells with fewer than 200 genes and greater than 2500 genes were filtered to remove empty droplets and probable doubles, respectively. We also removed the genes that were expressed in less than 3 cells in our data. In addition, we removed cells that had more than 5% mitochondrial reads. Next, we employ a global-scaling normalization method using the Seurat function “LogNormalize” that normalizes the feature expression.

**Clustering, visualization and cell annotation**

First, we used the “FindVariableFeatures” function to identify a set of 2500 genes that are highly variable that were used for downstream analysis such as dimensionality reduction and clustering. We then performed Principal Components Analysis (PCA) to construct a linear dimensionality reduction of the dataset that contain most of the complexity of the dataset. The cells were clustered in a graph-based approach within PCA space, and then non-linear dimensionality reductions were applied using UMAP for further visualization purposes. Finally, differential expression was performed using the “FindMarkers” function in Seurat to find cluster-specific marker genes. A heatmap of the top 10 expressing genes of each cluster was prepared. We then used top 20 DEG markers to assign annotation to each cluster using the Cluster Identity Predictor (CIPR) web-based tool (https://aekiz.shinyapps.io/CIPR/).

**Cell-cell interaction and gene pathway analysis**

Cell-cell interactions were analyzed by CellChat (http://www.cellchat.org/). Gene pathway analysis in each cluster was performed with Qiagen IPA (Germantown, MD).

**Whole-mount Immunofluorescence Staining and confocal microscopy**

The conjunctival tissue samples were dissected from female C57BL/6 J mice (age 16 weeks) and fixed in 100% methanol for 20 minutes at –20°C followed by washing with Hanks’ buffered saline solution (HBSS) for 3 × 5 min with gentle shaking at room temperature (RT). Tissues were permeabilized with 0.4% Triton X-100 in HBSS for 30 minutes at RT and gentle shaking, 20% goat serum (Sigma, USA) diluted in HBSS was used for 1 hour blocking at RT. Subsequently, the conjunctival tissue samples were incubated with primary antibodies (Table S2) diluted in 5% goat serum in HBSS at the mentioned concentrations overnight at 4 °C with gentle shaking at dark. The samples were then washed with 0.4% Triton X-100 for 3 × 6 min at RT with gentle shaking, followed by incubation with secondary antibodies (Supplemental Table 1) diluted in 5% goat serum/HBSS for 1 hour at RT with gentle shaking and light protection. The samples were then washed for 3 × 10 min with 0.4% Triton X-100 in HBSS, and then counterstained with wheat germ agglutinin, Alexa Fluor 647 Conjugate (1:200 in HBSS, ThermoFisher, Cat No. W32466) for visualization of conjunctival goblet cells, and Hoechst (1:500 in HBSS) for nuclei staining (30 min at RT and dark with gentle shaking). The samples were washed 3 × 5 min with HBSS, mounted on slides, and flattened with coverslips. Immunofluorescence staining in whole-mount conjunctival tissue samples was visualized using laser scanning Nikon confocal microscope (Nikon A1 RMP, Nikon, Melville, NY, USA) and 0.5 μm Z-step. The captured images were processed using NIH Elements Advanced Research (AR) software version 4.20 (Nikon).
