



Molecular Characteristics and Distribution of Adult Human Corneal Immune Cell Types

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Background: The limbus is located at a 2-mm-wide area between the bulbar conjunctiva and the cornea and has been suggested to be the niche of corneal epithelial stem cells and immune cells. Like the skin and intestines, the cornea is also an important mucosal surface, and immune cells on the cornea play critical roles in immune surveillance to ensure barrier surface homeostasis and protection from various environmental damage and infections. Single-cell RNA sequencing (scRNA-seq) analysis of protein tyrosine phosphatase receptor type C positive (PTPRC⁺) hematopoietic cells from the corneal limbus could provide a single cell atlas of all the immune cell subsets.

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Li Y, Jeong J and Song W (2022) Molecular Characteristics and Distribution of Adult Human Corneal Immune Cell Types. Front. Immunol. 13:798346. doi: 10.3389/fimmu.2022.798346 **Methods:** We performed single-cell RNA sequencing to generate transcriptomic profile for 804 sort-purified hematopoietic cells from the corneal limbus of three healthy donors.

Results: Our analysis identified a primary transcriptomic pattern for multiple immune cell subtypes, including naive T cells, antiviral effector CD8⁺ T cells, and innate immune cells such as IDO1⁺ mature regulatory dendritic cells (mregDCs), macrophages, monocytes, and basophils in the human corneal limbus.

Conclusion: Overall, single-cell transcriptomic analysis of limbal immune cells suggested the possible contribution of these cells on the adaptive and innate immune response of the human cornea.

Keywords: single-cell transcriptome, corneal immune cells, MregDC, antiviral CD8+ T cells, chemotactic

INTRODUCTION

Ocular surface diseases represent a huge medical need and are a substantial burden to many families (1). Due to the limited treatments for these diseases, investigation of the immune system of the ocular surface is crucial. The ocular surface, which comprises the cornea, limbus conjunctiva, and tear film, plays a key role in the visual system. Among these structures, the cornea is an avascular and transparent anterior surface that, together with the lens focus, allows light to be transmitted to the retina for visual processing (2). Like other mucosal surfaces, the cornea is the surface between the inner tissue and the external environment. It is responsible for protecting the eyes against microbes through innate and adaptive immune systems. The cornea has five distinctive layers, and the corneal limbus is considered an important niche of epithelial cells and immune cells on the

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ocular surface (3). Generally, the physical barrier formed by corneal epithelial cells can prevent pathogens from invading, while the flow of tears along with blinking washes pathogens away. Most importantly, the immune cells on the human cornea provide crucial mucosal immune response to prevent infections and damage.

Human and animal studies have observed altered immune cell distributions or functions in particular eye diseases, such as dry eye and eye allergies (4-7). The ocular surface, like other mucosal tissues, can recruit a variety of immune cells to render protection and homeostatic regulation (8, 9). However, dysfunction of the immune cells on the ocular surface would cause disruption of the corneal epithelial barrier function and ocular surface homeostasis (7, 10, 11). Therefore, ocular surface immune profiling studies are important to understand ocular surface homeostasis and related diseases. So far, the immune cell types on the cornea of mice have been covered and investigated well. T cells, dendritic cells (DCs), macrophages, mast cells, natural killer cells, $\gamma\delta$ T cells, and innate lymphoid cells (ILCs) have been investigated on murine cornea (12). However, the present knowledge surrounding corneal immune cells has been primarily limited to murine data, while the composition of immune cells on the human cornea requires more investigation. Due to the rich distribution of capillaries and lymphatic vessels that serve as the entry and exit portals for various immune cells, the corneal limbus is home to immune cells that reside in both the central and peripheral corneal regions (13). In the present study, to better investigate the immune cell types on the human cornea, we performed single-cell RNA sequencing(scRNA-seq) to generate transcriptomic profile for sort-purifiedhematopoietic cells from the corneal limbus of three healthy donors. Unbiased analyses identified seven immune cell types, including innate and adaptive immune cell types. This transcriptomic map of healthy human corneal immune cells can be utilized to better understand the immune response and regulation of the human cornea and help lead toward potential cellular and immunotherapy approaches. Furthermore, transcriptomic information can provide functional insight into the mechanisms of diseases such as viral infections, wound repair, and autoimmune diseases like allergies.

MATERIALS AND METHODS

Human Samples and Single-Cell Isolation

The collection of human corneal tissue was approved by the Ethics Committee of Xiangya Hospital. Fresh corneal tissue was peeled gently from three healthy adults using surgical forceps under a stereoscope. To isolate the corneal limbus, the central cornea was carefully removed. After isolating the corneal limbus tissues, we dissociated the tissues and obtained single-cell suspensions based on a previous protocol (14). The corneas were briefly chopped in the media and then digested using collagenase A, dispase II, and DNAse I at 37°C for 20 min. The cell suspension was then sorted for live PTPRC⁺ cells using a FACSAria III cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) at 4°C into 1.5-ml DNA low-binding Eppendorf tubes

containing medium. Sorted purified samples were collected and pelleted for processing with 10X Genomics v2.

Genomics scRNA-seq and Data Analysis

Single cells suspended in phosphate-buffered saline (PBS) were loaded into a single-cell instrument from the 10X Genomics system. A barcoded cDNA library was constructed using Single-Cell 3' mRNA Kit (v2, 10X Genomics). On the Illumina NovaSeq 6000 platform, all libraries (paired-end) were sequenced after passing quality tests. The 10X Genomics single-cell transcriptome sequencing data were integrated from the pooled cells of three donors and processed with the Cell Ranger Single Cell software suite version 1.3 (https://support. 10xgenomics.com) as described previously (15). The output data were analyzed using the SeqGeq genomic tool version 9.0 (FlowJo, LLC, Ashland, OR, USA). Principal component analysis (PCA) reduction (15 dimensions) was performed, followed by an unbiased t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction. We then performed clustering with a k-means filtering of k = 57 to cluster the cells into seven populations based on the variability in the PCA. The PhenoGraph algorithm (16) was used to identify the distinct ILC progenitor clusters. The seven clusters identified by PhenoGraph were overlaid onto the t-SNE map. The Cluster Explorer plug-in was used to characterize the immunophenotype of each cluster. Mean cluster transcript expression plots and expression heatmaps of differentially expressed genes were acquired by conducting the Color Mapping program. Gene Ontology (GO) (molecular function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using Metascape (http://metascape.org) (17) and Reactome (https://reactome.org).

Flow Cytometry Analysis and Reagents

For the validation of individual markers, the cells were analyzed on a NovoCyte flow cytometer (Agilent Technologies, Santa Clara, CA, USA). Hematopoietic cells were isolated as PTPRC⁺ (anti-human CD45 and QA17A19) cells using a FACSAria III cell sorter (BD Biosciences). All the antibodies used in this study were from BioLegend (San Diego, CA, USA), unless specified otherwise: anti-human CD3 (UCHT1), anti-human CD8 (SK1), anti-human CD4 (A161A1), anti-human IFN- γ (MD-1), antihuman granzyme B (GB11), anti-human granzyme A (CB9), anti-human CD96 (NK92.39), anti-human CD74 (LN2), antihuman CCR7 (G043H7), anti-human IDO1 (V50-1886; BD Biosciences), anti-human HLA-A, HLA-B, and HLA-C (W6/ 32), anti-human HLA-DR (L243), anti-human HLA-DQ (HLADQ1), anti-human CD164 (67D2), anti-human IL-10 (JES3-19F1), anti-human CXCL16 (22-19-12), anti-human TGF-β1 (TW4-9E7; BD Biosciences), anti-human perforin 1 (DG9; antibodies-online Inc., Pottstown, PA, USA), antihuman IL-32 (373821; R&D Systems, Minneapolis, MN, USA), and anti-human CXCL2 (rabbit IgG polyclonal; biorbyt, Cambridge, UK). Anti-human CXCR6 monoclonal antibody and recombinant human CXCL16 were purchased from R&D System. For intracellular cytokine staining, freshly isolated cells were stained with surface markers and then reactivated for 4 h with 10 ng/ml phorbol myristate acetate (PMA) and 1 μ M ionomycin (Sigma, St. Louis, MO, USA) prior to fixation and permeabilization with cytokine staining. The data were then analyzed using FlowJo version 10 (TreeStar) and GraphPad Prism.

Chemotaxis Assay

Human corneal T-cell migration was evaluated using a 24-well Transwell plate (5.0- μ m pore size; Corning, Corning, NY, USA) as described previously (18). Freshly sorted purified corneal CD3⁺ T cells were washed once with RPMI 1640 medium and then placed in 100 μ l T-cell medium in the top chamber of the Transwell plate with or without the addition of 1 μ g/ml of antihuman CXCR6 antibody. The bottom chamber of the Transwell plate contained chemokine *CXCL16* (100 ng/ml) or the supernatant from the CD164⁺ corneal innate immune cell culture medium (600 μ l). After 90 min incubation at 37°C in a 5% CO₂ atmosphere, the top chamber was removed and the number of T cells that had migrated into the bottom chamber was counted using flow cytometry. Migration rate was determined by calculating the percentage of input cells that migrated into the lower chamber.

Statistical Analysis

For statistical analysis of normally distributed continuous variables between two groups, an unpaired Student's *t*-test was used. Significance between multiple groups was determined using one-way ANOVA. A *p*-value <0.05 was considered to be significant in this study. All data were presented as the mean \pm standard error of the mean (SEM).

Available Data

Processed data from scRNA-seq are available at the ArrayExpress database in the European Nucleotide Archive EMBL-EBI, with accession ID E-MTAB-11027.

RESULTS

Identification of Immune Cell Populations in the Corneal Limbus

To determine the transcriptome profiles of all immune cell subsets in the cornea, we decided to perform transcriptomic analysis on the sorted purified hematopoietic cells from the human corneal limbus. As our focus is primarily on immune cells, we used PTPRC, a marker for hematopoietic cells, to distinguish immune cells from other cell types such as epithelial and stromal cells in the cornea. We performed fluorescence-activated cell sorting (FACS) to isolate PTPRC⁺ hematopoietic cells from the corneal limbus after removal of the corneal endothelium and central cornea. Human adult corneas were excised from three healthy male donors (18, 50, and 78 years old). The collected cells were dissociated and subjected to the 10X Genomics platform for scRNA-seq (Figures 1A, B). Transcriptome profiling of 804 cells was performed after passing quality control. These cells were embedded, and seven major cell clusters were revealed using unsupervised *t*-SNE and unbiased clustering (Figure 1C). Clusters 1-7 were determined to comprise macrophages, naive T cells, double-negative (DN) T cells, CD8⁺ T cells, monocytes, basophils, and DCs, respectively, based on specific marker genes (Figure 1D). For instance, macrophages and monocytes were identified by CD68, while DCs were identified by the high expressions of the HLA subtypes and CD74. The percentage of each immune cell cluster was determined (Figure 1C). The data suggested that innate immune cells occupied around half of the clusters, while the other half was identified as T lymphocytes. Specifically, CD8⁺ T cells (19.1%) and macrophages (16.5%) were the predominant subsets in adaptive and innate immune cells, respectively. To gain more insight into the function of corneal immune cells, a total of 1,217 most differentially expressed genes were used to generate enriched ontology clusters with Metascape (Figures 1E, F). Two major clustering trees were visualized, and biological pathways such as response to cytokine, hormone, and lipid were associated with inflammatory response. Another putative biological function is cellular movement and development regulation. Overall, the genes expressed on the corneal immune cells were enriched in inflammatory response and cellular development. In addition, pathway analysis using Reactome also supported these differentially expressed genes being enriched in multiple immune response pathways such as cytokine regulation, MHC class II antigen presentation, and neutrophil degranulation (Supplementary Figures S1A, B).

Antiviral Effector CD8⁺ T Cells Are the Predominant T-Lymphocyte Subset

Three clusters of T lymphocytes were identified by the high expressions of specific T-lymphocyte markers: CD3E, CD3D, and LCK (Figure 2A). However, B lymphocytes were barely detected on the corneal limbus (Supplementary Figure S1C). Among the clusters, three T-lymphocyte subsets-naive T cells, DN T cells, and CD8⁺ T cells-were identified based on specific markers (Figures 2A-C). In line with a previous study (19), CD4 mRNA was hardly detected on the corneal limbus, according to our scRNA-seq data. However, it has been reported that the mRNA expression of CD4 did not match the protein expression (20), and detectable protein levels of CD4 were confirmed by flow cytometry (Figure 2D). Interestingly, the antiviral capacity of the CD8 T cell subset was identified based on the high expressions of the activation markers CD69 and Lag3 and the antiviral genes GZMA, GZMB, PRF1, IFNG, and IL32 (Figures 2B, C). Additionally, corneal limbal CD8⁺ T cells expressed the surface receptor CD96, which has been considered as a co-stimulatory receptor that enhances CD8⁺ T-cell activation in humans (21). We subsequently confirmed the expressions of IFN- γ , granzyme A, granzyme B, IL-32, perforin 1, and CD96 from the corneal limbal CD8⁺ T cells based on protein level by flow cytometry (Figure 2E). Therefore, we proposed that $CD8^+$ T cells specifically act as functional effectors in controlling viral spread on the cornea. Another important T-lymphocyte subset is naive T cells, which expressed CD28, CD27, and IL7R (Figures 2A, B). Furthermore, naive T cells also specifically expressed an



FIGURE 1 [Identification of immune cell types in the corneal imbus. (A) Schematic of the single-cell hive sequencing (schive-seq) worknow. The corneal imbus was gently digested to a single-cell suspension, enriched for hematopoietic cells by sorting PTPRC⁺ cells, and then scRNA-seq was performed. The data were then analyzed. (B) Gating strategy for sorting. Live CD45⁺ cells were selected. (C) Unbiased t-distributed stochastic neighbor embedding (t-SNE) clustering was used to determine the cell types and the frequency of the different immune cell types. (D) Heatmap of the specific marker genes for each cell type in scRNA-seq. (E) Network of enriched terms colored by cluster identity, where nodes that share the same cluster identity are typically close to each other. (F) Network of enriched terms colored by *p*-value, where terms containing more genes tend to have a more significant *p*-value.

important pioneer chromatin modifier, *BATF*, which is an essential transcriptional factor in regulating the differentiation of effector CD8⁺ T cells (22). Therefore, we suggested that corneal limbal BATF⁺ naive T cells could differentiate into antiviral effector CD8⁺ T cells. Unexpectedly, we also found a DN T-cell subset located in the corneal limbus that contained a few $\gamma\delta$ T cells (**Supplementary Figure S1C**), which requires

further investigation in the future. In summary, we proposed that corneal limbal CD8 T cells are the major subset of immune cells preventing the corneal tissue from becoming virally infected by producing cytokines and cytotoxic granules, including IFN- γ , granzyme A, granzyme B, IL-32, and perforin 1. Furthermore, effector CD8⁺ T cells are likely to be differentiated from BATF-expressing naive T cells.



FIGURE 2 | Antiviral effector CD8⁺ T cells are a predominant lymphocyte subset on the cornea. (A) Feature *t*-distributed stochastic neighbor embedding (*t*-SNE) plot showing the expressions of marker genes enriched on corneal T cells. (B) Heatmap of the top expressed genes in the corneal T-cell subsets. (C) Dot plot showing the expressions of genes of the different cytokines (*rows*) on each cluster (*columns*). The *color of each dot* represents the average log-scaled expression of each gene across all cells of a given cluster. The *size of the dot* represents the fraction of cells in the cluster in which transcripts for that gene were detected. (D) Expressions of CD8 and CD4 proteins on corneal CD3⁺ T cells by flow cytometry.

Anti-Inflammatory Macrophages and Monocytes Recruit Naive T Cells by Secreting *CXCL16*

Accordingly, four distinct clusters of innate immune cells-monocytes, macrophages, basophils, and DCs-were observed

and annotated through specific markers (**Figures 1D**, **3A**, and **Table 1**). Among these cell subsets, basophils were identified by the high expressions of *Lamp1/2* and *CD164*. Macrophages expressed specific genes such as *CD14*, *CD68*, and *FCGR2A/B*. Interestingly, various chemokines from the C–X–C motif ligand



FIGURE 3 | Immunoregulatory macrophages and monocytes recruit naive T cells by secreting CXCL16. (A) Heatmap of the top expressed genes in corneal macrophages, basophils, and monocytes. (B) Violin plot of the expressions of chemokine (C–X–C motif) ligands and chemokine (C–X–C motif) receptors in each cluster. (C) Expressions of IL-10, TGF- β , CXCL16, and CXCL12 on CD164⁺ corneal innate immune cells by flow cytometry. (D) Chemotactic activity of corneal CD3+ T cells to 100 ng/ml CXCL16 or the cell culture supernatant was determined with the Transwell migration system. (E) Pathway enrichment analysis of the differentially expressed genes in corneal macrophages, basophils, and monocytes. *Significant differences (P < 0.05, Student's t test) from control groups.)

family, including *CXCL1*, *CXLC2*, *CXCL3*, *CXCL5*, *CXCL8*, and *CXCL16*, were expressed on these innate immune cell types, especially monocytes and macrophages (**Figures 3A, B**). Among the CXCL chemokine family, CXCL16 is the ligand for CXCR6

which is also highly expressed on corneal naive T cells. It has been reported that *CXCL16* could induce the chemoattraction of CXCR6⁺ human skin T cells (23), so we were eager to investigate whether corneal innate immune cells could recruit naive T cells

TABLE 1 | Gene list of each cluster from Figure 3A.

TABLE 1 | Continued

Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3
CCL3L1	CCL2	APOBEC3A	CD44	CTSZ	C11orf96
CCL20	CXCL5	KRT17	IL1A	KYNU	MMP1
CCL3	CD14	S100A2	ZEB2	OAZ1	AKR1C1
CCL4	TGFBI	KRT6A	OSBPL8	PID1	DSG1
CCL4L2	CTSD	PERP	DUSP4	MAFB	KLF5
IL1B	THBS1	TACSTD2	RCAN1	DUSP1	PHLDA2
EREG	FCER1G	KRT14	WTAP	SERF2	CLDN7
IL10	SPP1	KRT5	FCER1G	CYBA	JUP
CTSL	S100A9	CLDN4	GJB2		GSTP1
CXCL8	RNASE1	SFN	SLC7A11		SULT2B1
CXCL3	MI1G	SPRR2A	VIM		LDHA
CXCL2	PPBP	EMP1	GADD45B		SERPINB5
INHBA	S100A8	MMP3	PLA2G7		PPP1R14B
MMP9	TYROBP	ADIRF	MPC2		KRI13
CREGI	CISB	IVIAL2	SNAPC1		RPS8
GUS2	SERPINB2	KRI ID	IL1RN		INFRSF11B
30D2		CLDINI	FLNA		C40/73
AFUE TIMD1		DOF ACTC1	IIGB8		HMOXI
	EGALS I ETH1	ACTON	6755		CD9
PTGS2	CYCL3	SPRR1R	FUGRZA		IIGA2
C15orf49	ETI	5FH110 MM/D12			YBX3
ACSI 1	SI C11A1	SI PI	ILZNA MD7L1		SELENOM
IFR3	NAMPT	S100A14	DAR2		CEOrf122
ABCA1	MS4A7	ANXA1	DADZ KVNI I		BPS13
HSP90AA1	CD300E	CD24	MIR3945HG		SI C2A1
FNIP2	LAPTM5	LYPD3	SMS		
OGFRL1	HIF1A	KRT19	PEKER3		C19orf33
SOCS3	CD68	IGFBP3	DNAJB6		HMGA1
PDE4DIP	CAPG	GPRC5A	TNIP3		KRT12
PLAUR	DMXL2	CSTB	TNFAIP3		CD59
FTL	LCP1	LMO7	MMP14		KRT6B
CXCL1	AIF1	APOD	ZFYVE16		CAST
ATP2B1	MT1H	DSG3	IL3RA		SH3BGRL3
NAMPT	AKR1B1	FGFBP1	SNX10		DSTN
TNFRSF1B	GRN	PTGDS	MALAT1		PLS3
CD93	NPC2	ALDH1A3	EPB41L3		RPL5
BCL2A1	CXCL1	S100A10	CTNNB1		RPS6
CYP1B1	PLD3	TM4SF1	NRP1		HSP90B1
ANXA5	PLAUR	HSPA5	CTSH		IGFBP6
BASP1	BRI3	DSC2	GSTO1		NMB
ICAM1	C5AR1	SPRR2D	PILRA		RPS24
NFKBIA	S100A4	AKR1C2	MAP4K4		PLAU
IL24	LYZ	MT2A	PDPN		HSP90AB1
PSAP	FOS	ELF3	SERPINA1		GIPC1
ATPIJAJ	IVIT IF	STUDATO	KLF6		AQP3
E132 NDD			GRAIVIDTA		NACA
	FLINZ	5100A6	ASAH I		RPL3/A
DSF	SPI1	S100A0	EGRI		PRDX5
PDE4R	TUBB	EZR	IVIS4A7		13620
CD83	VCAN	ΔΝΙΧΔ2	10F4 SDD1		SDCBFZ
FARP5	PKM	RND3	MCL1		BPS5
SI C16A10	GAPDH	SERPINE2			RPL 7
PPP1R15A	LYN	TPT1	ARL4C		COI 17A1
NINJ1	ASAH1	DCN	TRAF1		RPL26
CTSZ	ATP6V1F	CAMK2N1	PTPN1		RPL7A
CD63	PILRA	ID1	SEMA6B		RPS18
SAMSN1	GPNMB	LGALS3	ATP6V1F		DST
STC1	TNFRSF1B	CRYAB	OTUD1		RPLP1
CTSB	RNF130	CD55	GK		RPL8
SMOX	AQP9	NDRG1	TLR2		HEBP2
PLEK	CEBPB	RPL24	PTPRE		GGCT
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ANP PATE CAUP PAPE PATE 8 PATE 9 PATE 8 PATE 9 PATE 9 MARCH PATE 9 PATE 9 CAUP PATE 9 PATE 9	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3
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Marges PR236 PR236 NMAP PM247 PR147 SMPC PR157 SELENCK SOTM PR167 SELENCK SOTM PR1702 PR180 SOTM PR171 SELENCK SOTM PR171 SELENCK SOTM PR171 SELENCK SOTM PR191 PR191 SOTM PR192 SOTM SOTM PR193 PR191 SOTM PR27 SOTM SOTM	PPIF		RPI 18			RPI 29
NAMA PAPER PAPER CNVC PAP 15 SED PANK SDSTMM PFP 1 NMMT SDSTMM PPP 12 NMMT SDSTM APAP 15 NMMT SDSTM APAP 12 NMT SDSTM APAP 12 NMT SDSTM APAP 12 NMT STMD PPP 12 NMT STMD PPP 12 PPP 12 STMD PPP 12 PPP 12 STMD PPP 12 PP 14 STMD PP 21 PP 21 STMD </td <td>MAP2K3</td> <td></td> <td>RPL36</td> <td></td> <td></td> <td>RPI 27</td>	MAP2K3		RPL36			RPI 27
CHC PH 15 SELENCK SOSTMM BF1 NMMT NMMT FAD AVAAT1 RAL23 SAPH AVAAT1 RAL23 CRLAR TMPSE72A AVAAT1 RAL23 SATT CEBPD RPS10 RPS10 SATT CEBPD RPS10 RPS10 SATA CEBPD RPS10 RPS10 SATA CEBPD RPS10 RPS10 SATA RPS10 RPS10 RPS10 SATA RPS10 RPS10 RPS10 SATA RPS21 RPS20 RPS20 UMPS10 RPS21 RPS21 RPS21 UMPS10 RPS21 RPS21 RPS21 UMPS10 RPS21 RPS21 RPS21 UMPS10 RPS21 RPS21 RPS21 RPS21 RPS21 RPS21 RPS21 UMPS10 RPS21 RPS21 RPS21 RPS21 RPS21 RPS21 RPS21 <t< td=""><td>NR4A2</td><td></td><td>RAP2B</td><td></td><td></td><td>RPL9</td></t<>	NR4A2		RAP2B			RPL9
SDCSMM BFT MMAT FMD1 PMPC/GB SDK ASPH AWA11 PH27A ASPH AWA11 PH27A SDK PMPSTSPA PH27A THBD PPST2 PMUST SUCMA PTMTS PM25A MMT PFST2 PMUST SUCMA PTMTS PMUST MMTSDHG PMOD PMVST MMTSDHG PMOD PMST MMTSDHG PMST PMST MMTSDHG PMST PMST MMTSDHG PMST PMST M	GYPC		RPL15			SELENOK
Photom Photog SK ASPH ANAATI RPL27A RPL23A CR AR NUMPSPTRA RPL23A RPL23A CR AR CEBPO RPS10S RPL24A SATT CEBPO RPS10 RPL34 SLGAA PTFN13 RPL34 RPL34 JUN ZFAST RPML28B RPL24 JUNA ZFAST RPL34 RPL34 JUN ZFAST RPL34 RPL34 JUNA ZFAST RPL34 RPL34 JUNA RPS3A RPL24 RPL34 JUNA RPS3A RPL24 RPS3A JUNA RPS3A RPL24 RPS3A JUNA RPS3A RPL24 RPS3A JUNASSIG RPS3A RPL24 RPL34 JUNASSIG RPS3A RPL24 RPS3A JUNASSIG RPS3A RPL34 RPL34 JUNASSIG RPS3A RPL34 RPL34 JUNASSIG SPL34 <td>SOSTM1</td> <td></td> <td>FIF1</td> <td></td> <td></td> <td>NNMT</td>	SOSTM1		FIF1			NNMT
ANDPY ANDPAT PRE/27A CPLAR TIMEP CA MCC160 THED HPST2A MCUC160 SAT1 CEEPD MCUC160 SLCSA3 PTPNT3 PRE24 JUN ZPAS1 PRAD3 MATISASC MRDD PAN ARSTB1 RPS2A PRAD3 MATISASC RPS2A PRAD3 LHFFL2 ITGA6 YMAD STR2A RPS2A PRAD3 MARS SPRP1 PRP2A MARS SPRP1 PRP2A <	FHD1		PPP1CB			SI K
CHUMB PHERFERA PHERS THOD PRS10 MUCIS SAT1 CEEPPO PHS10 SAT1 CEEPPO PHS10 SAT1 CEEPPO PHS10 SLCMAS PTPN13 PHL28 JUN ZEPAST PHL28 JUN PRS0A PHL28 JUN PRS0A PHL28 JUN PRS0A PH210 JUN PRS0A PH210 JUN PRS0A PH211 JUN PHS28 PHS14 JUN PHS21 PHS14 JUN PHS24 PHS14 JUN PHS14 PHS14 JUN PHS14 PHS14 JUN PHS14 </td <td>ASPH</td> <td></td> <td>ANXA11</td> <td></td> <td></td> <td>RPI 27A</td>	ASPH		ANXA11			RPI 27A
THEO MRS12 MALL MALL <t< td=""><td></td><td></td><td>TNERSE124</td><td></td><td></td><td>RPI 23</td></t<>			TNERSE124			RPI 23
SATT CFEPRO FP3 SECOND			RPS12			MUC16
Suchag PTPN17 PR24 WIN PXF0 PXF1 WIN1SNG PH00 PXF1 WIN1SNG PH00 PXF1 WIN1SNG PF02 PXF1 UHFF2 PXF1 PXF1 UHFF2 PXF1 PXF1 WIN1SN PF02 PXF2 WARXS PY703 PXF2 WARXS PY703 PXF2 PK01 PF923 PXF2 RP01 PXF2 PXF2 RP1 PXF2 PXF2 PXF4 PXF2 PXF2	SAT1		CERPD			RPS10
JUN ZAAST FAMP 200 MIRTSHOQ PROV PRAVA MIRTSHOQ PROSA PROVA AKTIPIT PRSAN PRUP LIFFL2 ITGAB YWHAQ CSP2PA PROSA PROSA MAROKS PROSA PROSA MAROKS PRS2B SODB MAROKS PRS2B SODB PROT PROSA PROSA MAROKS PRS2B SODB PROT SODB SERPINSB SERPINSB DEAPT MUC2S SERPINSB SODATS LEGALSS CSP2 CSTA PROST CATBAS MAUL KRTTS CALIB PRUS PRUST CALIB PRUS PRUST TALDOT PRUST SERCITS	SI C5A3		PTPN13			RPI.34
NIRTSR-G PHOD PANI NARTB1 PRSA PRIP2 LHFN2 ITGA6 YMHAD CSP2PA PR21 CMNS NNS PRS8 PRSM NNS PRS8 PRSM NNS PRSM SOGS NNS SOGS SPSM NNS SOGS SPSM SPSM SPSM SPSM ATRNOR SPSM SPSM SSEPRINES SPSM MCC22 SPAM1 SPCM MCC32 SPRME HOPX MCC32 SPMRE SPCM MCC32 SPRME SPCM SPGM CS2 SPCM SPGM CD104 MPL1 CAMB2 D015 MPC3 SPSM SPGM2 SPSM SPSM	. II IN		ZFAS1			FAM129R
#KH10************************************	MIR155HG		BHOD			RAN
Luffel 2ITG46YMP40CSP2PAPR2/8CM/8MANDSPR2/8PR2/8MANDSPR5/8SD03MANDSPR5/8SD03ILPL2PR5/7SD03PR01PR5/3PR28/1PR01PR5/3PR28/1PR01PR5/3PR28/1PR01PR5/3PR28/1PR01SD03/13CLALSLCSP2CSTAPR1/1CSP2CSTAPR1/3CR18PR1/1PR1/3CC18PR1/3CH2/9CC18PR1/3CH2/9CC18PR1/3PF1/3CC18PR1/3CH2/9CC18PR1/3PF1/3CC18PR1/3PF1/3CC18PR1/3PF1/3CC18PR1/3PF1/3CC18PR1/3PF1/3CC18PR1/3PF1/3CC18PR1/3PF1/3CC19PR1/3PF1/3CC19PR23PF1/3CD19PR23PF3/4CD201PR23PF1/3CD202PF3/5ACO2028/6CD204PF2/3PF3/5ACD202PF3/5ACD2028/6CD202PF3/5ACD2028/6CD202PF3/5ACD2028/6CD202PF3/5ACD2028/6CD202PF3/5ACD2028/6CD202PF3/5ACD2028/6CD202PF3/5ACD2028/6CD202PF1/3CL1/4PF3/5CD2028/6<	AKR1R1		RPS34			RPI P2
			ITGA6			MI/HAO
NNNE* PSSB PSSAK MARCKS PXV03 HBF3B IILPL2 APS7 S0D3 PD1 APS20 APS7 PD1 APS20 APS21 PD1 APS20 APS21 PD1 APS20 APS7 SEPAM1 S0D3 BEA1 SEPAM1 S0D413 MC22 CS72 CS7A PRUX1 SFARMS MAL MC22 CS74 APL53 PRUX1 SFARMS MAL MC41 CC18 RPL53 PRUX3 TADO1 RPL13 CHCH02 TADO1 RPL13 CHCH02 TAPS2 RACK1 PRS2 UB2D1 MMP10 MAC2 SPINT2 MARB RCH14 PRS2 SPS5 SDOEP RPC14 RPS54 QEPS3 ATPO142 RPL54 QEPS4 QEPS4 MMP10 VAC2 SPINT2 MB15	CSE2RA		RPI 21			CNN3
MARCKS PXDD HSDB MILPL2 PRST SODOB PD1 PRS23 SPRDE PD1 PRS23 SPRDE ATPSVDB SDC1 SERPT SERPNEBS DRAP1 MUC22 SERPNEBS SDC1 SERPT SERPNEBS DRAP1 MUC2 SERPNEBS DRAM1 STODATS DRAM1 STODATS LGALSL SERPNEBS DRAM1 MOC8 TMFARPS HOPX MM06 TMFARPS RPLPO PFDN2 CD103 MMH10 KRT16 LDD01 RPL11 CHC9 UB2D01 MM42 SPRN2 MAFB RPL34 SPRN2 LTAF RPL34 SPRN2 UB2D1 RPL37 ABIBQP MAFB RPL37 ABIBQP MAFB RPL34 SPRN2 LTAF RPL34 SPRN2 SDCBP RPL37 ABIBQP			DDS29			DDSAV
MILPL2 PR523 MILPL2 PR523 PR521 PD1 RP523 PR521 PR521 PD1 RP523 PR523 PR523 PD1 DRAP1 MUC22 PR533 PR010 SEPRINB9 DRAP1 MUC22 PR533 PR010 SSP2 CSTA PR010 PR011 MVC22 TRFARP HOPX PR011 MVC22 PR011 SCR1 PR011 MVC21 PR012 PR012 SCR1 RP103 MM21 KRT13 RSC11	MADOKS		EVVD2			H2E2D
Int D_L Int P223 RPS21 PLD RPS23 RPS21 ATPOVDB SDC1 MAC22 DRAW1 ST00A13 LGALSE CSF2 CSTA RPDX1 TMPAPB HOPX RPOX1 TMPAPB HOPX RP05 FH1 SCEL RP16 GRN183 MALL RRT18 CD133 MMP10 RP100 CD134 RP1-20 SPN172 MAPB RP135 SPC11 UB2D1 MMP2 SPN172 MAPB RP134 RP135 CEBPB RP141 RP526 CEBPB RP141 RP535 SDCPP RP527A RP535A SDCPP RP515A SDCBP GRAT3 LAMB3 SDCB GRAT3 LAMB3 SDCB GRAT3 LAMB3 CDD208B GRAT3 LAMB3 CDD208B GRAT3 GLAMB3 CD2008B			DD97			50D2
Ind Ind Ind Ind Ind Ind SERPINDB SCI SERPINDB SCI SERPINDB SERPINDB DRAP1 ILGALSL ILGALSL SERPINDB SCI MIC22 PRDXI SSP CSTA MPOX MYOS SFIND SCI MYOS MYOS FINH SCI MYOS MYOS FINH SCI MYOS RTTIS CCL18 RPLPO RTTIS RTTIS CCL000 MWP10 RTIS SERVI2 TALDO1 RPL13 SERVI2 RTIS CEB2D1 MMP2 SRVI2 RTSIA MAPB RTL31 RTSIA RESIA MAPB RPL37 ABISBP MMP10 VDAC2 RTSIA SDCPP RPS27A RTSIA CALMAR RPL34 CDDSPS GAPAT1 LLMM ARISP MMP10 RPL34 CDDSPS SDCPP RPS27A CDSPSS GAPAT3 LLMM ARISP MMP10 RPL34 CDDSPSS GAPAT3 LLMM ARISP MC1 RPL34 ARIF4 ATTSIA<			DC00			30D3
Alf POLOD SCI Alf PI MCC22 DRAM1 S100A13 LGALSI. CSF2 CSTA PRDX1 TIMFAIPB HOFX MYC62 CRIAM1 SCEL RPL60 GPR183 MALL KRT18 CCL18 RPLP0 PFDN2 CD109 MMP10 KRT10 TALDO1 RPL11 CHC4D2 TALDO1 RPL13 SECST TALS RPL35 SECST CD109 MMP10 KRT10 TALDO1 RPL11 CHC4D2 TALSC RPS134 RPS134 LIPAF RACK1 RPS24 CBCBP RPL41 RPS14 TMP1 RPL35 SECST0 SDCSP RPL36A CCC085B GAAP1 RPL36 LLMS TMP1 RPL36A CCC085B GAAP1 RPL36A CCC085B GAAP1 RPL36A CCC085B GLGAP1 RPL36A CCC085B <td>ATDEVOD</td> <td></td> <td>RP323</td> <td></td> <td></td> <td>RP321</td>	ATDEVOD		RP323			RP321
Januari Januari Januari Januari Januari CSF2 CSTA PRDX1 CSF2 CSTA MPDE ThFAIPB HOPX MPDE FIH SCEL RPL6 GRPRIS3 MALL RRT13 CC113 RPLP0 RRT13 CC113 RPL11 CHC602 TALDO1 RPL13 RRT10 TALDO1 RPL13 SPM12 TMFRS4 RPL33 SPM12 MAFB KRT15 SPC61G TMP1 RPL37 ABI3BP MAFB RPL31 ABI3BP MAFB RPL31 ABI3BP MAFB RPL31 ABI3BP MMF10 VDAC2 RPS25 CACPP RPS25 CACPPS CACPA RPS25 CACM2 CACA RPL4 AF0N MGLL RPL14 CACM2 GLA RPL4 AF0N MTX PPDF <td< td=""><td></td><td></td><td>5001</td><td></td><td></td><td>SERF I</td></td<>			5001			SERF I
Jmain STUGNIS LDSL20. CSF2 CSFA MPXI TNFAIPB HOFX MPXG TNFAIPB SCEL RPL6 GPR183 MALL RPT16 CD109 MMP10 RPL02 CD109 MMP10 RPL13 LDDO1 RPL11 CPCH202 NFR554 RPL35 RPL13 LB2D1 MMP2 SPINT2 MAFB RPC14 RPS14 LTAF RACK1 RPS14 TNP1 RPS37 ABIGBP MMP10 VDAC2 RPS14 SDCRP RPS27A CCD0268 GPA13 LAMB3 USP53 ATF01B2 RPL14 CALM2 GLA RPL4 CALM2 GLA RPL4 CALM2 GLA RPL4 CALM2 GLA RPL4 CALM2 GLA RPL36 RPC3 GLA RPL3 CALM2 GLM1			DRAF 1 \$100/12			10022
Odd ODFA PPUAN INFAUPB HOPX MP06 FTH1 SCEL RP16 ERH13 MALL RP111 CCL18 RPLP0 RP111 CD109 MMP10 RP113 TALDO1 RP.111 CHCH02 TALDO1 RP.113 CHCH02 UBE2D1 MMP2 SPIN12 MAFB RT15 SEC615 UTAF RACK1 RPS2 CEBPB RPL14 RPS14 SDCBP RPS25 CCD058 GGAP1 RPS25A CCD058 GGAP1 RPL10A API0 MGLL RPL14 CALM2 MGLA RPL14 CALM2 MGLA RPL14 CALM2 MGLA RPL19 LUM MTX PDPF EF1A1 MAS1 MAL CALM2 MATA PD15 CALM4 MATA RPL3 CALM4 MATA	ORAIVII		STUDATS			LGALSL
INPARS NOXA M105 FH1 SCEL RPL6 RPL60 GRR183 MALL KRT18 KRT18 C0109 MMP10 KRT10 PEDN2 C0109 MMP10 KRT10 CPC00 TALDO1 RPL11 CPC00 CPC00 TALDO1 RPL11 CPC00 CPC00 MAFB KRT15 SEC610 SPN72 MAFB KRT15 SEC610 SPN72 MAFB KRT15 SEC610 SPN72 CEBPB RPL41 RPS14 RPS14 TNP1 RPL37 ABI38P MMP19 VDAC2 RPL14 CC0263B GPA13 USPS3 SDCBP RP257A RPS16A GPC083B USPS3 ATPOVIB2 RPL10A AFDN GPDN2 MGLL RPL14 CALM2 AFDN MGLL RPL14 CALM2 AFDN MGLL RPL13 CL1A RPS3 GLA			LODY			PRDXT
FINI SCEL PPL0 PPL0 GR1183 MALL KRT18 KRT18 C0L18 RPLP0 KRT10 KRT10 TALDO1 RPL11 OHCHD2 TALDO1 RPL13 RPL13 UBE2D1 MMP2 SPINT2 MARB KRT16 SEC61G UTAF RACK1 RPS3 CEBPB RPL41 RPS34 MMP19 VDAC2 RPS15A SDCBP RPS27A RPS55 IOGAP1 RPL35A CCD085B IOGAP1 RPL35A CCD085B IOGAP1 RPL36A USP53 ITPEVTB2 RPL14 CALM2 GLA RPL14 CALM2 MTX PPDF EFE1A1 THS1 MAL KL4 PD3 GUX1 RPT3 MTX PPDF EFE1A1 THS1 MAL CLMA RCC RPL3 CDT1 RCAC RPL3 </td <td></td> <td></td> <td>HOPX</td> <td></td> <td></td> <td>IVI Y OO</td>			HOPX			IVI Y OO
GHT 163 MALL N113 CD103 MPL0 HFDN2 CD109 MMP10 KRT10 TLDO1 APL11 OHCH02 TMFRSF4 RPL35 RPL13A UBE2D1 MMP2 SPIN72 MAFB KRT15 SEC616 LTAF RACK1 RP52 CEBPB RPL37 ABB8P MMP19 VDAC2 RPS15A SDCBP RPS27A RPS25 ICGAP1 RPL36A COC068B GPA13 LAMB3 USP53 ICGAP1 RPL16A AFDN GLA RPL16 LUM MGLL RPL16 LUM GLA RPL16 LUM MTX PPDPF EEF1A1 THSIS1 MAL KL44 MTK RPL10A ACTM GCC RPL13 CLM GCC RPL13 CLM GCC RPL13 CLM RPG5C			SCEL			KPL0
OLL16 MMP10 FRUNC CD109 MMP10 KRT10 TALDO1 RPL11 CHCH02 TALDO1 RPL13 RPL132 UBE2D1 MMP2 SPINT2 MAFB KRT15 SE081G LTAF RACK1 RPS14 TINPS RACK1 RPS14 LTAF RACK1 RPS14 LTAF RACK1 RPS14 MP19 VDAC2 RPS15A SDCBP RPS27A RPS26G MAFB LAMB3 USP53 SDCBP RPS27A RPS26G GAT3 LAMB3 USP53 ATPEV12 RPL10A ARF4 MGLL RPL14 CALM2 GLA RPL13 LUM MTX PPDPF EEF1A1 MTX PPDPF EEF1A1 RCC RPL30 CLTA GUL ZFAND5 EEF182 LPXN RPL22 CD41 RPL3<	GPR 103		IVIALL			NRT 10
Dulty MMP10 AFI 10 CD103 RPL11 CHOHD2 TMFRSF4 RPL35 RPL134 UBE2D1 MMP2 SPL072 MAFB KRT15 SEC61G LTAF RACK1 RPS14 TMFRSF4 RPS14 RPS14 TMFR RACK1 RPS14 TMFR RPL37 ABI8BP MMP19 VDAC2 RPS164 SDCBP RPS27A RPS26 ICGAP1 RPL35A CC0C88B GPAT3 LAMB3 USPS2A ATPOVIB2 RPL10A AFP4 ATPEV4 RPL14 CALM2 GLA RPL14 CLM MGLL RPL13 CLM GLA RPL13 CLM GCC RPL30 RL14 MTX PDPF EF141 MTX RPL30 RL14 MTX RPL30 RL14 MTX RPL30 RL14 MTNX <td>CCL18</td> <td></td> <td>RPLPU</td> <td></td> <td></td> <td>PFDN2</td>	CCL18		RPLPU			PFDN2
IALLOI HPL11 CHCH2 IARDOI HPL13A RPL13A UBE2D1 MMP2 SPINT2 MAFB KRT15 SEC61G LITAF RACK1 RPS2 CEBPB RPL41 ABBBP MMP19 VDAC2 RPS15A SDOBP RPS27A RPS25S IGGAP1 RPL13A USPS3 ATREV182 RPL10A ATEN MGLL RPL16A ARF4 ATRP34 GLMAB3 USPS3 GLA RPL19 LUM MGLL RPL19 LUM MGL RPL19 LUM MTX PDDPF EEF1A1 TRSS1 MAL KLF4 PDIA3 GUK1 PTX1 RCCC RPL30 RC11A RCC RPL30 RC11A RCC RPL30 RC11A RCC RPL30 RC11A RCC RPL30 RC1A RPL3	CD109		IVIIVIP10			KRITU
INPLS3 PPLS3 PPLS3 MAFB KRT15 SPINT2 MAFB KRT15 SPC616 LITAF PACK1 PRS1 CEBPB PPL41 PRS14 TMIP1 PRL37 ABBBP MMP19 VDAC2 PRPS15A SDCBP PS27A PRS25 DCAP1 PPL35A CCDC55B DCAP1 PPL36A CCDC55B DCAP1 PPL44 AFPN MGLL PPL44 AFPN MGLL PPL4 AFPN MGL PPL4 AFPN MGL PPL4 AFF4 ATPSB4 PPL4 AFF4 THS1 MAL KLF4 PDA3 GUK1 CLTA FF5 PL30 PTX1 GLA PPL3 CLTA LPNN PPL3 CLTA LPNN PPL3 CDH1 GLUL ZFAND5 EFF182 LPN PPL3<	TALDUT TNEDOF 4		RPLII			CHCHD2
UBEED/I IMMP2 SFINI2 SFINI2 MAFB KRT15 SEC616 UTAF RACK1 RPS2 CEBPB RPL41 RPS14 TINP1 RPL37 ABI3BP MMP19 VDAC2 RPS15A SDCBP RPS27A RPS25 GGAP1 RPL35A CCDC55B GPA73 LAMB3 USPS3 ATP6V1B2 RPL14 CALM2 GLA RPL14 CALM2 GLA RPL4 AFP4 MTX PDDFF EEF1A1 THS51 MAL KLF4 PDA3 GUK1 PTX1 RGCC RPL13 CLTA EF5 RPL30 RAC1 CRMM FOSL RPS3 GLUL ZFAND5 EEF182 LPXN RPL39 MT-ND4L RPL39 RPL39 RPC31 GLUL ZFAND5 EEF182 LPXN RPL39 RPC33 <			RPL35			RPL I JA
MARB NR115 SEC016 ITAF RACK1 RPS14 TNIP1 RPL37 RPS15 MMP19 VDAC2 RPS15 SDCBP RPS27A RPS25 GGAP1 RPL35A CCD065B GPAT3 LAMB3 USP53 ATPSV182 RPL10A AFPN MGLL RPL14 CALM2 GLA RPL19 LUM GLA RPL19 LUM GLA RPL19 LUM MTX PDPF EEF1A1 THS51 MAL KLF4 PDIA3 GUK1 PTX1 RGCC RPL13 CLTA EF5 RPL30 RC1 CRIM1 FOSL1 MC1 CRIM1 RPL3 CDH1 NSTABP DCG3 RPS15 GLUL ZFAND5 EEF182 LPXN RPL33 CDH1 RPL34 RPS35 COL642 RPL3 R	UBEZDI		IVIIVIP2			SPIN12
LIAF PROC PROV PROV PROV PROV PROV PROV PROV PROV	IVIAFB		NRI ID			SECOIG
CEB*B FrFL41 FrFS14 MMP19 VDAC2 RPS15A SDCBP RPS27A RPS25 GGAP1 RPL35A CCDC6SB GPAT3 LAMB3 USP53 ATFOV1B2 RPL10A AFDN MGLL RPL14 CALM2 GLA RPL14 AFF4 ATFSV1B2 RPL14 CALM2 GLA RPL14 CALM2 GLA RPL4 AFF4 ATFB84 RPL19 LUM MTX PPDFF EEF1A1 TBS1 MAL KLF4 PDI3 GUK1 PTX1 RGCC RPL30 CLTA EF5 RPL30 RC1 CRM1 FOSL1 ACTN4 INNSIABP DSC3 RPL32 GUL ZFAND5 EEF1B2 LPXN RPL32 COL6A2 RPL32 RPL34 RPS15 LPXN RPL34 RPS11 RPL35			RACK I			RP32
Inviri Inviri ABJ3P MMP19 VDAC2 PR515A SDCBP RPS27A RP525 IQGAP1 RPL35A COD085B GPAT3 LAMB3 USP53 ATP6V1B2 RPL10A AFDN MGLL RPL4 AFDN GIA RPL4 AFF4 ATP884 RPL19 LUM MTX PPDPF EEF1A1 MAL PPDFF EEF1A1 PDIA3 GUK1 PITX1 RGCC RPL30 CLTA EIF5 RPL30 RC1 CRIM1 FOSL1 ACTN4 INS1ABP DSC3 RPS3 GUL ZPAND5 EEF1B2 LPXN RPL32 CDH1 RPL32 RPS15 COL6A2 RPL32 RPL34 RPS11 RPL32 RPS15 COL6A2 RPL34 RPS14 RPS11 RPL34 RPS11 RPS11 RPL3			RPL41			RP314
Minutring VDA/22 PRS15A SDCBP RPS27A RPS25 IQGAP1 RPL35A CCDC85B GPA73 LAMB3 USP53 ATP6V1B2 RPL14 CALM2 GLA RPL14 CALM2 MTX PDPF EEF1A1 THBS1 MAL KLF4 PDIA3 GUK1 PT11 RGCC RPL13 CITA EIF5 RPL30 RPC3 GLUL ZFAND5 EEF1B2 LPXN RPL32 CDH1 RPL32 CDL6A2 RPL32 RPL32 RPL32 NEAT1 RPL32 RPL32 NEAT1 RPL32			NDAC2			ADIODE DD015A
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IAGAPI IPL3SA COLDASD GPAT3 LAMB3 USPS3 ATP6V1B2 RPL10A AFDN MGLL RPL14 CALM2 GLA RPL14 CALM2 GLA RPL4 AFF4 ATP8B4 RPL19 LUM MTix PPDPF EEF1A1 THBS1 MAL KLF4 PDA3 GUK1 PITX1 RGCC RPL13 CLTA EIF5 RPL30 CLTA CRM1 FOSL1 ACTIV4 INNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPL3 MT-NPAL RPS15 COL6A2 RPL12 CYCS RPL32 ILAN RPS1 RPL33 MT-NPAL RPS1 RPL32 RPL3 RPIC3 RPL32 RPL3 RPC3 RPL33 RPS3 RPS4 RPL34	SDCBP		RPS27A			RPS25
GrA13 Control OSP-93 MGLL RPL10A AFDN MGLL RPL14 CALM2 GLA RPL4 ARF4 ATP89/TB2 RPL19 LUM GLA RPL4 ARF4 ATP89/TB2 RPL19 LUM GLA RPL19 LUM ATP89/TB2 RPL19 LUM MGL PDPF EEF1A1 THBS1 MAL KLF4 PDA3 GUK1 PITX1 RGCC RPL30 RAC1 CRIM1 FOSL1 ACTN4 INSTABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL3 MT-ND4L RPS15 COL6A2 RPL32 RPL32 RPL3 RPGX3 RPL32 RPL39 RIOK3 IL6 RPS11 RPS4 NPM1 CAPN2 RPS4 NPM1 CAPN2 LAMC2 (Continued)	IQGAP I		RPL30A			
APPOT ID2 PPL 104 APD/M GLA RPL14 CALM2 GLA RPL4 ARF4 ATP8B4 RPL19 LUM MT1X PPDPF EEF1A1 THBS1 MAL KLF4 PDA3 GUK1 RPL3 RGCC RPL3 CLTA EF5 RPL30 RAC1 CRIM1 FOSL1 ACTN4 INS1ABP DSC3 RF13 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPL32 RPL12 CVCS RPL12 CVCS RPL32 IL6 RPL34 RPS31 NPM1 RPL3A RPS31 IL6 RPL3A RPS31 RPL12A RPS3 CAPN2 RPL13A RPS3 CAPN2 RPL3A NPM1 CAPN2 RPL3A RPL3A CAPN2 RPL12A CAPN2 RPL13A RPS3 CAPN2 RPL13A COTIONED CAPN2 RPL3A CAPN2 RPL3A CAPN2 RPL3A CAPN2 COTIONED COTIONED	ATREVIDO		LAIVIDS			03-33
MalL HrL14 CALM2 GLA RPL4 ARF4 ATP8B4 RPL19 LUM MT1X PPDPF EEF1A1 THBS1 MAL KLF4 PDJA3 GUK1 PITX1 RGCC RPL13 CLTA EIF5 RPL30 RAC1 CRIM1 FOSL1 ACTN4 INNS1ABP DSC3 RPS13 GLUL ZFAND5 EEF1B2 LPXN RPL2 CDH1 RPL3 MT-ND4L RPS15 COL6A2 RPL12 CYCS RPL32 NEAT1 RPL3 RIOK3 IL6 RPS14 NPM1 CAPN2 RPL3A CAPN2 RPL3A CAPN2 RPL3A RPS1A RPL3A RPS1A CYCS RPL32 RPL3A RPS4 CAPN2 RPS4 CAPN2 RPL3A RPL3A	ATPOVIB2		RPLIUA DDL14			AFDN
GLA HPL4 AH74 MTX RPL19 LUM MTX PPDPF EEF1A1 THBS1 MAL KLF4 PDIA3 GUK1 PTX1 RGCC RPL30 CLTA EIF5 RPL30 RAC1 CRM1 FOSL1 ACTN4 INNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL3 MT-ND4L RPS15 COL6A2 CYCS RPL32 RPL39 RICK3 IL6 RPS11 RPS3 RPL18A NPM1 CAPN2 NPM1 RPL33 RPS3 IL6 RPS11 RPS3 RPL34 RPS4 CAPN2 RPL23A NPM1 CAPN2 NPM1 CAPN2 LAMC2 (Continued) (Continued) (Continued)	NGLL		RPL14			
Al Pada4 HPL19 LUM Al Pada4 PPL19 EEF1A1 THS1 MAL KLF4 PDIA3 GUK1 PTX1 RGCC RPL13 CLTA EIF5 RPL30 RAC1 CRM1 FOSL1 ACTN4 IVNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPS15 COL6A2 RPL12 CYCS RPL32 RPL33 IL6 RPS11 RPL18A RPS4 NPM1 CAPN2 (Continued) COntinued	ATDOD 4					
MITA PPDP EEF IAT MAL KLF4 PDIA3 GUK1 PTX1 RGCC RPL33 CLTA EIF5 RPL30 RAC1 CRM1 FOSL1 ACTN4 INNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF182 LPXN RPL22 CDH1 RPL3 MT-ND4L RPS15 COL6A2 RPL39 RFS15 L6 RPS11 RPL18A RPS1 NPM1 CAPN2 RPL3A LAMC2	ATPOD4		RPLIS			
INBS1 IMAL NLP4 PDIA3 GUK1 PITX1 RGCC RPL13 CLTA EIF5 RPL30 RAC1 CRIM1 FOSL1 ACTN4 INNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPS15 COL6A2 RPL32 NEAT1 RPL32 NEAT1 RPL39 IL6 RPL18A RPS1 RPL18A RPS3 IL6 RPS1 RPL18A RPS4 NPM1 CAPN2 LAMC2 ILAC2			PPDPF			EEFIAI
FDIA3 GON FITAT RGCC RPL13 CLTA RGCC RPL30 CLTA EIF5 RPL30 AC1 CRIM1 FOSL1 ACTN4 INNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPL3 MT-ND4L RPS15 RPL32 RPL32 CYCS RPL39 RIOK3 RPS11 RPL18A RPS3 RPS11 RPL18A RPS4 CAPN2 VPM1 Continued COLTA (Continued) (Continued) (Continued)			IVIAL			
HGCCHFL 13OL 1AEIF5RPL30RACTN4CRIM1FOSL1ACTN4INNS1ABPDSC3RPS3GLULZFAND5EEF1B2LPXNRPL22CDH1RPL3MT-ND4LRPS15COL6A2RPL32RPL32NEAT1RPL39IL6RPS11RPL18ARPS3NPM1CAPN2(Continued)(Continued)	PDIA3		GUK I			PIIXI
EIF5 HPL30 HAC1 CRIM1 FOSL1 ACTN4 INNS1ABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPS15 COL6A2 RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPS14 RPS12 VNM1 CAPN2 (Continued) (Continued)	RGCC		RPL13			CLIA
CHIMIN FOSL I ACTIN4 INNS IABP DSC3 RPS3 GLUL ZFAND5 EEF1B2 LPXN RPL2 CDH1 RPL3 MT-ND4L RPS15 COL6A2 RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPS18 RPS12 VNM1 CAPN2 (Continued) (Continued)	EIF5		RPL30			RACT
INNSTREP DSC3 HPS3 GLUL ZFAND5 EEF1B2 LPXN RPL22 CDH1 RPL3 MT-ND4L RPS15 COL6A2 RPL12 CYCS RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPL39 RIOK3 IL6 RPS11 RPL18A RPSA NPM1 CAPN2 IL6 RPS1 RPL3A CAPN2 IL6 RPS1 COL6A2 (Continued) (Continued)			FUSLI			ACTIV4
GLUL ZFANDS EEF 182 LPXN RPL22 CDH1 RPL3 MT-ND4L RPS15 COL6A2 RPL12 CYCS RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPL18A RPS11 RPS11 RPS11 RPS14 CAPN2 LAMC2 (Continued) (Continued)	IVINSTABP		DSC3			RPS3
LPAN HPL22 CDH1 RPL3 MT-ND4L RPS15 COL6A2 RPL12 CYCS RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPL18A RPS11 RPL18A RPS1 RPL3A CAPN2 LAMC2 (Continued) (Continued)	GLUL		ZFANDO			EEF I B2
HPL3 MI-hD4L RPS15 COL6A2 RPL12 CYCS RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPL18A RPSA NPM1 CAPN2 (Continued) (Continued)	LPXN		RPL22			
NPS13 COL0A2 RPL12 CYCS RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPL18A RPSA NPM1 CAPN2 (Continued) (Continued)			nrlj dreie			IVII-IND4L
HPL12 CYCS RPL32 NEAT1 RPL39 RIOK3 IL6 RPS11 RPL18A RPSA NPM1 CAPN2 RPL23A LAMC2			MP313			OULBA2
HPL32 NEA11 RPL39 RIOK3 IL6 RPS11 RPL18A RPSA NPM1 CAPN2 RPL23A LAMC2			KPL12			CYCS
HPL39 RIOK3 IL6 RPS11 RPL18A RPSA NPM1 CAPN2 CAPN2 LAMC2 (Continued) (Continued)			KPL32			NEA11
IL6 RPS11 RPL18A RPSA NPM1 CAPN2 RPL23A LAMC2 (Continued)			KPL39			RIOK3
HH-L18A RPSA NPM1 CAPN2 RPL23A LAMC2 (Continued) (Continued)			IL6			RPS11
NPM1 CAPN2 RPL23A LAMC2 (Continued) (Continued)			RPL18A			RPSA
			NPM1			CAPN2
(Continued) (Continued			KPL23A			LAMC2
			(Continued)			(Continued)

TABLE 1 | Continued

Cluster 1	Cluster 2	Cluster 3
		RPS19
		CST3
		RPL38
		IGFBP4
		EPS8L1
		EEFZ TNEAIDE
		SI C2543
		TMSB10
		PKP1
		RPL28
		FAM83A
		LMAN1
		POLR1D
		MYL12B
		COX7A1
		CLMP
		ECM1
		GOLGA4
		KABTIA
		IGERP2
		PPI
		RPS9
		RPS26
		TGM1
		RPS29
		YWHAE
		PRNP
		CCND1
		SLC39A14
		RPL36AL
		RABITHPT
		TPIVI4 DTNIA
		SOX4
		PDLIM4
		ADGRF1
		ACTB
		NUPR1
		UBC
		SET
		YWHAZ
		BZW1
		HES1
		HNRNPA1
		HERPUD1
		COX7A2
		NIT-CYB
		5114 RPI 264
		NCI
		FALL
		PHI DA3
		SE3B6
		PAX6
		ERO1A
		ARF6
		FST
		TUBB4B

TABLE 1	Continued

ΙΔΡ1Ι 1
PIA PL10
EACAM6
5

by generating CXCL16. Additionally, all three innate immune cells highly expressed CD164 mRNA, which is generally expressed by granulocytes, based on the scRNA-seq data (Figure 1D). We also confirmed the production of CXCL16 from corneal limbal CD164⁺ cells by flow cytometry (**Figure 3C**). To conduct an in vitro chemotaxis assay, we placed the recombinant CXCL16 or supernatant from the CD164⁺ corneal limbal immune cell culture medium in the lower chambers and the sorted purified corneal CD3⁺ T cells in the presence of isotype or anti-CXCR6 antibody in the upper chambers. The chemotaxis data (Figure 3D) suggested that both recombinant CXCL16 and the cell culture supernatant can attract T cells from the upper chamber. In addition, blocking CXCR6 using antibodies could significantly inhibit the chemoattraction of T cells toward the recombinant CXCL16 or the cell culture supernatant in the lower chamber. Therefore, the data indicated that corneal limbal innate immune cells can potentially recruit naive T cells into the corneal limbus and generate antiviral effector CD8⁺ T cells downstream. Furthermore, the generation of anti-inflammatory cytokines such as IL-10 and TGF- β from monocytes and macrophages indicated that they play an immunoregulatory role in the immune response of the human corneal limbus (Figures 2C and **3A**, **B**). Wound healing of the cornea is a multistep process with four overlapping but distinct stages: hemostasis, inflammation, proliferation, and remodeling. Corneal limbal macrophages and monocytes produced the chemokines CXCL1, CXCL2, and CXCL8, which are important for the inflammation and proliferation stages of wound healing (Figures 3B, C). Furthermore, both macrophages and monocytes could respond to wounding based on the KEGG pathway and GO annotation analyses (Figure 3E). It also suggested that corneal macrophages, monocytes, and basophils are capable of the degranulation and regulation of cytokine production. Thus, besides attracting naive T cells, cornea limbal innate immune cells also have the potential to suppress the immune response and aid in wound healing.

Corneal Limbal Dendritic Cells Are IDO1⁺ mregDCs

DCs comprise the unique cell type among the corneal innate immune cells. Although other immune cells such as macrophages and monocytes could express the major HLA subtypes, such as *HLA-A/B/C/E*, *HLA-DRA*, and *HLA-DRB1*, corneal limbal DCs expressed various other subtypes, including *HLA-DPB1*, *DPA1*, *DQB1*, *DQA1/2*, and *DMA* (Figures 4A, B). Therefore, it is suggested that DCs function as the major antigen-presenting cells connecting the innate and adaptive immune

(Continued)

FIF2S2



FIGURE 4 | IDO1⁺ mature regulatory dendritic cells (mregDCs) are the major antigen-presenting cells on the cornea. (A) Dot plot showing the expressions of genes of the HLA subtypes (*rows*) in each cluster (*columns*). The *color of each dot* represents the average log-scaled expression of each gene across all cells of a given cluster. The *size of the dot* represents the fraction of cells in the cluster in which transcripts for that gene were detected. (B) Heatmap of the top expressed genes in corneal dendritic cells. (C) Pathway enrichment analysis of the differentially expressed genes in corneal dendritic cells. (D) The *t*-distributed stochastic neighbor embedding (*t*-SNE) plot showing comparative expressions of mregDC-specific signature genes from our data and those of Nakamizo et al. (20).

systems on the human cornea. The KEGG pathway and GO annotation analyses were performed by using Metascape and showed that corneal DCs participate in cytokine signaling in the immune system and regulate leukocyte activation (**Figure 4C**).

Surprisingly, we found that the corneal limbal DCs are mregDCs, which displayed high expressions of the markers *LAMP3* and *BIRC3* (24) (**Figure 4B** and **Supplementary Figure S2A**). This new subset of DCs has recently been identified in human skin

mucosal tissues. To better compare the mregDCs between the corneal limbus and the skin, we reanalyzed other publicly available skin datasets (GEO accession no. GSE176509) (24) and identified skin mregDCs using the same methods of t-SNE analysis and clustering. We found that, except for LAMP3 and BIRC3, both corneal and skin mregDCs highly expressed CCR7, CD74, ID2, GPR183, CCL22, IL411, CD40, and TXN (Figure 4D and Supplementary Figure S2). In addition, corneal limbal mregDCs also expressed high levels of the pro-inflammatory cytokines IL1B, IL-15, and IL-23A, which are associated with mucosal inflammation (24). MregDCs have also been found to be involved in cytokine signaling and regulation of leukocyte activation (Figures 2C and 4C, D). The analysis indicated that mregDCs potentially play a pro-inflammatory role in the immune response of the cornea. Interestingly, consistent with recent papers (25), we also found that the mregDCs in both the skin and corneal limbus specifically expressed indoleamine-2,3dioxygenase (IDO1), a counter-regulatory and tolerogenic molecule (Figure 4D). Although there were several subsets of DCs identified in the skin, only one corneal limbal DC subset was detected on the cornea limbus, and this DC subset belongs to mregDCs. Furthermore, corneal limbal mregDCs do not only act as antigen-presenting cells but also participate in regulating immune tolerance by coordinating with other innate immune cells.

DISCUSSION

On the ocular surface, the cornea is a unique and highly specialized tissue that is avascular and transparent in order to allow light to be transmitted for vision. Recent studies have identified cell types in the cornea using scRNA-seq; however, they primarily paid attention to epithelial and stromal cells, the major cell types in the cornea (19, 25, 26). In this study, we concentrated on the immune cell types and observed seven distinct immune cell clusters that differed in function. Overall, we reported a primary scRNA-seq analysis of the human corneal innate and adaptive immune cell types, including antiviral effector CD8⁺ T cells, naive T cells, and innate immune cells such as IDO1⁺ mregDCs, macrophages, monocytes, and basophils, providing a detailed map of corneal immune cell function. It should be noted that, because aging could lead to changes in the immune system, such as the distribution and number of immune cells (27, 28), despite using similar numbers of cells from each donor, the wide donor-to-donor age variation could affect the results to a certain extent. We identified subtypespecific transcriptional factors and surface markers for the different immune cell types. Through flow cytometry, but not scRNA-seq, a few CD4⁺ T cells were detected on the human cornea in a steady state. In addition, human corneal naive T cells highly expressed BATF, which has been proven to be crucial for the differentiation of effector CD8⁺ T cells in a steady state (22) and during viral infection (29). Therefore, BATF is likely to be an important regulator for the differentiation of naive T cells into antiviral effector CD8⁺ T cells in the human cornea. The costimulatory receptor CD96 and the activation markers Lag3 and CD69 were highly expressed on CD8⁺ T cells (21, 30, 31). Furthermore, several antiviral cytokines and cytotoxic granules, such as IFN- γ , granzyme A, granzyme B, perforin 1, and IL-32, were produced by corneal CD8⁺ T cells, which further confirmed that CD8⁺ T cells in the human cornea have the ability to fight against viruses even at a steady state. Therefore, we believe that CD8⁺ T cells are a significant immune cell type that prevents virus infection in the human cornea, which is consistent with a previous finding on mouse corneas (32). Furthermore, it has been proven that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was detected on the human cornea (33, 34). As is known, CD8⁺ T cells are crucial to the prevention of SARS-CoV-2 infection (35), so it can be considered that corneal CD8⁺ T cells may also contribute to protecting the eyes from SARS-CoV-2 infection. We also proved that, from in vitro studies, CXCR6-expressing naive T cells are attracted by the specific chemokine CXCL16 produced by corneal innate immune cell types, including macrophages, DCs, and monocytes. This result indicated that CXCL16 is not only required for the colon's immune system (36) but is also important in maintaining the immune response of the cornea. Because we did not detect regulatory T cells in the cornea, we were eager to search for other immunoregulatory immune cell types. Interestingly, sequencing and flow cytometry data indicated that macrophages and monocytes are dominant cell types that produce IL-10 and TGF- β , which are known antiinflammatory cytokines that suppress the immune response. Meanwhile, they also express various chemokine genes such as CCL3, CCL20, CCL4, CC3L1, CCL4L2, CXCL2, CXCL8, and CXCL5. We believe that these innate immune cells can recruit and regulate distinct cell types by generating chemokines. Analyses of KEGG pathway and GO annotation also indicated that macrophages, monocytes, and basophils could degranulate and be involved in wound healing. Another interesting innate immune cell type comprise DCs, the major antigen-presenting cells in the cornea. DCs displayed their ability to present antigens by highly expressing various HLA subtype genes such as HLA-DQA, HLA-DPA, and HLA-DPB. Recently, researchers have identified a small subset of skin DCs called mregDCs with high expressions of BIRC3, LAMP3, IL15, CD40, and CCR7, and this subset has been thought to be associated with wound healing and the exacerbation of atopic dermatitis (24, 37, 38). It is surprising to find that corneal DCs also belong to mregDCs, with high expressions of the unique markers BIRC3 and LAMP3. Furthermore, we compared the corneal and skin mregDCs by reanalyzing the data from public scRNA-seq and unexpectedly found that there are high levels of similarity between the mregDCs from these two mucosal tissues. The three major pro-inflammatory cytokine genes expressed on corneal DCs were IL1B, IL-15, and IL-23A. Consistently, all three cytokines were produced by skin DCs and considered to be associated with atopic dermatitis and psoriasis. Additionally, GO enrichment analysis of the upregulated genes in DCs suggested that corneal DCs participate in cytokine signaling and regulation of leukocyte activation. Consequently, we suggested that the cytokines IL-1 β ,

IL-15, and IL-23a produced by corneal mregDCs could be crucial to ocular inflammation. Intriguingly, both corneal and skin mregDCs also expressed IDO1, which is a heme-containing enzyme that can suppress T-cell response. Additionally, IDO1-expressing DCs may provide an immunoregulatory network by promoting the development of regulatory T cells (39). Therefore, DCs could play a crucial role in maintaining and regulating a dynamic balance between pro- and anti-inflammatory signals.

In summary, the goal of this study was to provide crucial information regarding all the immune cell types located in the adult human cornea limbus. Identification of the different corneal immune cell types using transcriptomic analysis can help in understanding the eye immune network. In addition, this study revealed the genes/pathways of immune cells that could lead to improvements in immunotherapies for corneal disease and wound repair of the corneal limbus.

DATA AVAILABILITY STATEMENT

The processed data of single-cell RNA-seq are available at the ArrayExpress database in European Nucleotide Archive EMBL-EBI with accession ID E-MTAB-11027.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Xiangya Hospital. The patients/participants provided written informed consent to participate in this study.

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AUTHOR CONTRIBUTIONS

WS conceptualized, acquired funding, and supervised this study. YL performed sample collection, single-cell dissociation, and library preparation. Data were processed, curated, and visualized by YL. YL, JJ and WS drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.798346/full#supplementary-material

Supplementary Figure S1 | Supporting data for cluster analysis. (A) Overview of relevant pathways involved in the immune system obtained from Reactome.
(B) Most relevant pathways sorted by p-value for differentially expressed genes.
(C) Feature t-SNE plot showing expression of other important marker genes.

Supplementary Figure S2 | The expression of DCs signature genes and chemokine receptors. (A) Violin plot of expression of DCs signature genes in each cluster. (B) Violin plot of expression of CC chemokine receptor genes in each cluster.

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