Molecular mechanism study of the physiological and pathological role of myeloid cell receptor Clec7a in SOD1G93A ALS mouse model

A thesis submitted by Huilan Yan

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Advisor: Yongjie, Yang, Ph.D.

Abstract

Clec7a, also known as Dectin-1, is a pattern recognition receptor of the C-type lectin receptor (CLR) family that expressed on the myeloid cells. Although the main function of Clec7a previously studied is to defend fungi infection in peripheral macrophage and monocyte, transcriptomic analysis of patients' post-mortem motor cortex of amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by progressive degeneration of motor neurons in motor cortex and spinal cords, has found selective and consistent up-regulation of Clec7a mRNA and protein in diseased microglia. Currently, it remains unknown how Clec7a regulates microglia functions in vivo in physiological and ALS condition. By generating $Clec7a^{-/-}$, $SOD1G93A^{+}/Clec7a^{-/-}$ mice and examining the differentially expressed genes and their involved pathways in Clec7a deficiency with single-cell RNA sequencing, we aim to investigate the potential physiological and pathological role of Clec7a in regulation of microglia functions in vivo on a molecular level.

Our previous behavior and pathological findings reveal that Clec7a deficiency results in more activation of macrophage in sciatic nerve and worse early motor deficits in $SOD1G93A^{+}$ mice. However, in 120 days late-stage spinal cords of $SOD1^{+}$, $SOD1^{+}/Clec7a^{-/-}$ mice, single-cell mRNA sequencing and differentially expressed genes analysis of microglia shows that Clec7a deficiency has protective effects in late-stage disease by significantly and widely downregulating microglia-involved

inflammation pathways like NF-κB, IL1β, TNF signaling pathways. These results indicate the complex pathological role of Clec7a in ALS.

Additionally, Clec7a deficiency was mainly found to activate the microglia phagocytosis and mobility in physiological groups with increasing expression level of Rac3, Ccr2, Clec1a. This finding was consistent with observations of our previous apoptotic neuron injection study, which showed the enhanced microglia clearance of apoptotic neurons in Clec7a$^{-/-}$ group compared with WT group. These results prove its role as a phagocytotic checkpoint of microglia.

In summary, no Clec7a KO-specific clusters of microglia emerged in physiological and SOD1^{+} disease condition. The sequencing results reveal that Clec7a has pro-inflammatory effects on late stage SOD1G93A^{+} microglia, suggesting its detrimental role in CNS of SOD1G93A^{+} mice. Its main function of inhibiting microglia phagocytosis was observed in physiological condition.

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AD - Alzheimer's disease

ALS - Amyotrophic lateral sclerosis disease

Clec7a - C-type lectin domain family 7 member A

CLR - C-type lectin receptor

CNS - Central nervous system

PNS – Peripheral nervous system

IL-1β - Interleukin-1 beta

NFAT - Nuclear factor of activated T-cells

NF-κB - Nuclear factor-κB

ROS - Reactive Oxygen Species

Syk - Spleen Associated Tyrosine Kinase

Cst7- Cystatin F

Lpl - Lipoprotein lipase

DAM - Disease-associated microglia

SOD1G93A – $SOD1^{+}$

Scfd1- sec1 family domain containing 1

Tbk1 - TANK-binding kinase 1

Epha4 - Ephrin type-A receptor 4

Single-cell RNA sequencing - scRNA seq

PECAM1 - platelet and endothelial cell adhesion molecule 1

CD70 - CD70 molecule

Ccl9 - C-C motif chemokine ligand 9

EPHA2 - EPH receptor A2

EFNA2 - ephrin A2

Tbk1 - TANK Binding Kinase 1

Tlr3 - Toll Like Receptor 3

Tlr4 - Toll Like Receptor 4

Cd47 - Leukocyte Surface Antigen CD47

CLEC1A - C-type lectin domain family 1 member A

Klrk1 - killer cell lectin-like receptor subfamily K, member 1

NFAT5 - nuclear factor of activated T cells 5

BCL6 - BCL6 transcription repressor

EGR3 - early growth response 3

LRP2 - LDL receptor related protein 2

LCN2 - lipocalin 2

PRDM8 - PR/SET domain 8

MEF2B - myocyte enhancer factor 2B

IL1β - interlukine1β

ITGA11 - integrin subunit alpha 11

RAC3 - Rac family small GTPase 3

CCR2 - C-C motif chemokine receptor 2

CIITA - class II major histocompatibility complex transactivator

Chapter 1: Introduction

Clec7a, also known as Dectin-1, is a pattern recognition receptor of the C-type lectin receptor (CLR) family expressed on the myeloid cells, including monocytes, macrophages, microglia[1]. It has a low level of expression in healthy condition. Its most studied role is on regulating macrophage[2] and monocyte[3] antifungal function by recognizing cell wall component β-glucans and initiating proinflammatory immune responses against fungi. Upon binding to β-glucans, Clec7a can activates the Syk-dependent NFAT[4] and NF-κB pathways as well as the Syk-independent Raf-1 pathway[5], leading to the production of cytokines and chemokines[6]. It can also mediate antifungal responses through phagocytosis, reactive oxygen species (ROS) production, and inflammasome activation, which cleaves and activates inactive pro IL-1β to active IL-1β[7].

Interestingly, other than the fungi defection functions, Clec7a was also found involved in the disease progression of some neurodegenerative diseases like Alzheimer's disease (AD) and Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disorder characterized by progressive degeneration of motor neurons in motor cortex and spinal cords. According to the single-cell RNA sequencing studies in mouse models and human patients of AD and ALS[8, 9], selective and consistent up-regulation of Clec7a mRNA and protein was found in diseased associated microglia (DAM)[10]. This novel microglia subtype, DAM, was

first identified in AD[11]. It is activated sequentially in a Trem-2 independent manner[12] and a Trem-2 dependent manner[13], which includes upregulation of lipid metabolism and phagocytic genes such as Cst7 and Lpl in the AD mouse model[14]. The DAM subpopulation in ALS and AD is similar[10].

DAM in ALS is thought to be a status of activated microglial related to chronic neuroinflammation characterized by the upregulation of genes encoding cytokines, chemokines and inflammatory mediators. This chronic neuroinflammation may also affected by dysregulated glial crosstalk and contribute to neuronal damage and neurodegeneration in ALS[15]. Emerging evidence suggests that DAM may exhibit region-specific differences in ALS, reflecting the spatial and temporal heterogeneity of microglial activation in response to pathological insults[16]. However, the mechanism of the function changes of microglia in ALS is still unknown, thus understanding the molecular and functional heterogeneity of DAM in ALS is essential. By investigating how Clec7a, which upregulates dramatically in ALS microglia, regulates microglia functions in ALS condition may provide insights into disease progression and potential therapeutic targets.

Previous studies of Clec7a mainly focused on the in-vitro studies of monocytes, macrophage functions on fungi defection, but the role of Clec7a in regulating microglia function in CNS in vivo remains unknown. Aiming to study the pathogenic role of Clec7a in ALS in vivo, SOD1G93A ($SOD1^{+}$) mouse model featuring

progressive disease course, motor neuron degeneration and global inflammatory responses in spinal cord was selected to simulate the human ALS progression. Around 15% of the familial ALS cases are due to genetic mutations in the Superoxide dismutase 1 gene (SOD1), a gene plays a role in protecting cells from oxidative stress by converting harmful superoxide radicals into oxygen and hydrogen peroxide. Aiming to investigate the potential changes of ALS progression with Clec7a deficiency, $SOD1^{+}$/ $Clec7a^{-/-}$ mice were designed and generated to compare with the $SOD1^{+}$ group. WT group and $Clec7a^{-/-}$ group of mice were set up as baseline and for exploring the physiological role of Clec7a in regulating microglia functions.

Behavior tests and pathology studies were previously conducted on the disease groups ($SOD1^{+}$, $SOD1^{+}$/ $Clec7a^{-/-}$) and physiological groups (WT, $Clec7a^{-/-}$) of mice to assess their early motor functions and the corresponding pathological changes in muscle and sciatic nerve (SN) in Clec7a KO condition. A significantly worse motor dysfunction was observed in $SOD1^{+}$/ $Clec7a^{-/-}$ mice compared to $SOD1^{+}$ mice at early stage. In SN, the immunostaining expression level of Clec7a was highly elevated in disease group, in correspondence with that of human patients. The pathology study showed significantly higher ratio of fully denervated neuromuscular junctions (NMJs) in gastrocnemius muscles and promoted early activation of SN macrophages with loss of Clec7a in disease group at early stage. In contrast, the Clec7a KO alone had no

effects on these phenotypes compared with WT group. These suggests a protective role of Clec7a plays in early motor phenotypes of $SOD1^{+}$ ALS mouse model.

After the phenotypes establishment and peripheral nervous system pathology study, we aimed to deeply explore the late-stage CNS microglia, which wasn't activated in early stage, in a molecular level in this study. Single-cell RNA sequencing (scRNA seq), a powerful next-generation sequencing technique widely used to examine gene expression patterns in individual cells[17], is utilized to provide insights into cell-to-cell variability, cell types, cell states, and cellular interactions in the spinal cords. By performing scRNA seq analysis on the 4 groups of mice spinal cords, the transcriptome profiles of the groups were obtained. Clustering was first done to find if there was Clec7a KO-specific clusters emerged. Then by conducting the gene enrichment analysis with IPA and GO, the functional changes of microglia were studied in both physiological and disease conditions.

The functional analysis reveals that Clec7a was involved in widely promoting neuroinflammation in microglia of $SOD1^{+}$ ALS model by regulating immune response pathways like NF-κB pathway, which suggests its potential detrimental effects. Together with our previous behavior and pathological study, which demonstrates its protective effects on early motor deficiency, these in-vivo results illustrate the complex pathological role of Clec7a plays in early and late stage of ALS, explaining the unchanged life span observed in Clec7a deficiency in this model.

In physiological group, DEG analysis of the scRNAseq data supports that Clec7a deficiency can activate the microglia phagocytosis and mobility with increasing expression level of phagocytosis-related genes like Rac3, Ccr2, Clec1a. This finding was consistent with the observations of our previous apoptotic neuron injection study, which showed the enhanced microglia clearance of apoptotic neurons in Clec7a$^{-/-}$ group compared with WT group. These results prove its role as a phagocytotic checkpoint of microglia.

In this study, we demonstrated the in-vivo evidence of how Clec7a regulates microglia functions in physiological and SOD1^{+}ALS condition. With the identification of the inflammation-related pathways downregulated in Clec7a deficiency in SOD1^{+} model, the understanding of how microglia involved in ALS disease progression was deepened in a molecular level. It may provide some insights into potential therapeutic targets for ALS.

Chapter 2: Materials and Methods

2.1. Animal preparation

P108-130 days male and female mice were generated from the Jackson lab purchased SOD1G93A mice and Clec7a$^{-/-}$ mice. Animals were kept 3-4 mice per ventilated cages, with free access to food and water, at the temperature of 21–23 °C, 50–60% humidity, under a 12 h/12 h day and night cycle. All experimental procedures on animals strictly followed the NIH Guide for the Care and Use of Laboratory Animals and the Guidelines for the Use of Animals in Neuroscience Research. Animal protocol (NIH2022-51) used in this study has been approved by Tufts University IACUC committee. Both male and female mice were used in all experiments.

2.2. Tissue dissociation

After anesthesia of control and experimental groups of mice, transcardial perfused mice with 20ml cold phosphate-buffered saline (PBS). Spines was cut off and spinal cord was flush out with cold PBS. 4cm spinal cord was placed on the glass petri dish with 1mlHanks' Balanced Salt Solution (HBSS, Gibco), and cut into small pieces using a scalpel. Then transferred pieces to 15ml tube, rinse the tube with 2ml HBSS. The mix was centrifuge at 250xg for 1min at 4℃ and aspirate the supernatant carefully. The enzyme used for tissue association was from the Neural tissue dissociation kit, (Miltenyi Biotec, #130-092-628). According to the kit instructions,

3900μl Enzyme mix 1 was prepared and pre-heated at 37℃ for 15mins before use. After adding the Enzyme mix 1, the tissue was incubated for 15 mins at 37° C water bath, the tube was gently inverted every 3-5mins to resuspend settled cells. 60μl of Enzyme Mix 2 was prepared per tissue and added to sample, inverted gently. Tissue was dissociated mechanically using fire-polished pasteur pipette by pipetting up and down 10 times slowly. Then it was incubated at 37℃ for 10mins in water bath, inverted every 3-5mins. This process was repeated for 3 times. The resulting cell suspension was filtered through a 70μm strainer and washed with 10ml of HBSS. Then the mixture was centrifuged at 300xg for 10mins at 4℃.

To remove the myelin, we resuspended cell pellets in 900μl 0.5% BSA PBS and added 100μl anti-myelin beads (Miltenyi Biotec) to it, shaking in cold room at 4℃ for 15mins. After washing it with 10ml PBS and centrifuged at 1400 rpm for 5mins, it was resuspended in 3.0 ml 0.5% BSA PBS. The material was decanted over LS column and let flow through completely, 1ml per time. The flow through was collected in a 15ml tube. Then 3ml of 0.5%BSA PBS was added to wash LS columns and collected in 15ml sample tube. Centrifuged the collected flow-through at 1400 rpm for 5mins. After resuspending cells in 100μl 0.5% BSA PBS, we counted cells with TC10 automated cell counter (BIO-RAD) to check viability with trypan blue.

2.3. Single-cell RNA sequencing.

After getting the single cell suspension for each sample, preparation of gel beads

in emulsion and construction of libraries were performed with Eppendorf Matercycler PCR thermocycle and Single-Cell Gene Expression Chemistry (10×Genomics), according to the Chromium Single-Cell 3′ Reagent Kits v3 user guide provided by the manufacturer. Next, sequencing was run in the rapid run flow cell and paired-end sequenced on an Illumina NovaSeq 6000 (Illumina, San Diego, CA 92122 USA).

2.4. Raw sequencing data analysis

Original Illumina data was converted to fastq files using the Cell Ranger v7.1 (10xGenomics). Sequencing results were mapped to a mouse genome dataset acquired from the 10x Genomics website and quantified. The total number of cells identified by the Cell Ranger was 120,000 cells. Data analysis was performed in R using Seurat v5. To filter out empty droplets, low-quality cells, and possible multiplets, cells with < 200 transcripts were excluded from the analysis. Cells of poor quality, which had >5% of transcripts coming from mitochondrial genes, were excluded from the downstream analysis.

Data from 14 samples were integrated using Seurat v5. Gene expression level measurements for each cell were normalized by the total number of counts in the cell, multiplied by a scale factor of 10,000 and the normalized values were natural log(ln) -transformed using log1p with "LogNormalize" method. The average expression level was divided by the total cell number.

Data dimensionality reduction was performed using a principal component analysis (PCA), and the first 12 principal components were used in the downstream analyses. For each condition separately, the expression profiles were then clustered using an unsupervised, graph-based approach with the resolution parameter set to 0.5. Clustering results were visualized using two-dimensional UMAP. To facilitate identification of cell types, marker genes that were tested in the previous single-cell RNA sequencing, were examined for their expression level in every cluster and then the clusters were annotated to various cell types. The microglia lineage cell clusters in four groups were identified and further analyzed.

2.5. Differential Expression Analysis

Differentially expressed genes between the microglia of Clec7a$^{-/-}$ vs. WT group, SOD1+/Clec7a$^{-/-}$ vs. SOD1+ group. AggregateExpression was used to sum together gene counts of all the cells from the same sample for each cell type. This results in one gene expression profile per sample and cell type. DEG analysis using DESeq2 was performed on the sample level to get the gene fold change and their p-value. Genes were considered differentially expressed if the absolute value of their log2fold-change was greater than 1 (p-value < 0.05). Ingenuity pathway analysis (IPA) was performed in the QIAGENA IPA software. By inputting the DEG names and their fold change, analysis of

functions or canonical pathways changed was obtained. Molecular analysis was also done to categorize the genes involved by types. A biological process Gene Ontology (GO) analysis was conducted for upregulated and downregulated DEGs in microglia of disease and physiological groups, using the GO Enrichment Analysis (https://geneontology.org/).

2.6. Collaboration

Raw data analysis was conducted by Haichao Wei in University of Texas Health, Houston. Other methods were performed by Huilan Yan.

Chapter 3 Results

3.1. Single-cell RNA sequencing of WT, Clec7a$^{-/-}$, SOD1^{+}, SOD1^{+}/Clec7a$^{-/-}$ mice shows diverse cell types but no Clec7a KO -specific cell population.

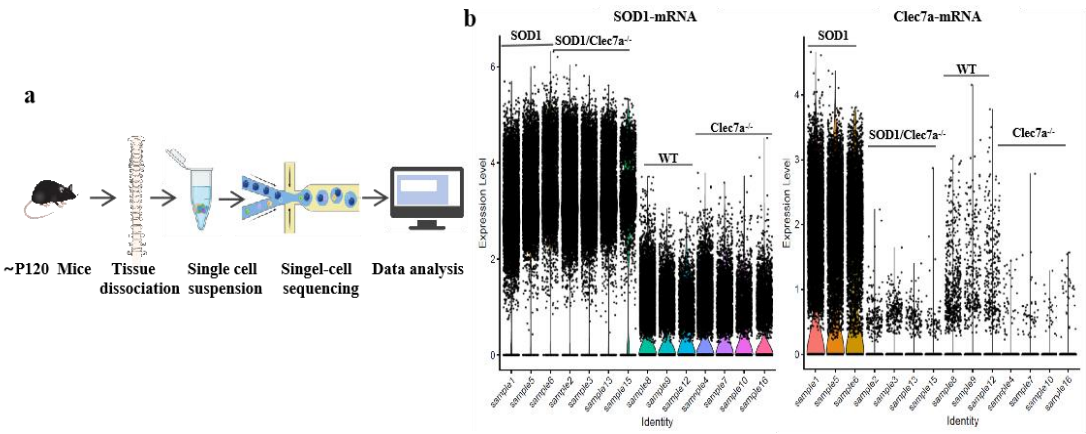
In order to investigate the potent cell-type distribution and transcriptome profile change in Clec7a KO condition, spinal cords from 14 mice (WT and SOD1+ group: 3 mice/group; Clec7a$^{-/-}$ and SOD1+/Clec7a$^{-/-}$ group: 4 mice/ group) were used to perform the single-cell RNA seq and obtain the mRNA level data [18](Fig 3.1a). The mice were sacrificed at around P115, when the time point corresponds to a symptomatic stage of ALS.

First, the expression level of SOD1+ and Clec7a was detected to confirm the establishment of the 4 groups. In SOD1^{+} and SOD1^{+}/Clec7a$^{-/-}$ group, SOD1+ expression level was highly elevated compared to physiological groups. In both of the Clec7a KO group, merely no cells had expression level bigger than 1. In WT group, a smaller number of cells expressed Clec7a and the overall mRNA level was low whereas in SOD1^{+} group, Clec7a expression level was globally and highly elevated, coordinating with the clinical findings (Fig 3.1b).

After quality control and normalization for correcting technical variations, the integrated datasets composing of around 120,000 cells and 18131 genes were represented by the two-dimensional Uniform Manifold Approximation and Projection

(UMAP). The cells were divided into 12 clusters in each group and no Clec7a KO-specific clusters were present (Figure 3.2c). Distribution of clusters was similar within both physiological and disease groups. Cluster 0 ratio was high elevated in disease groups.

To characterize the cell identity of the clusters, various cell markers ware applied. The cell identities were inferred by matching the significantly overexpressed genes in each cluster (Figure3.1d). Analysis of the distribution of these cell types per genotype revealed that all major cell types were represented by each genotype. Major cell types identified include microglia, endothelia cells, astrocytes, oligodendrocyte progenitor cells, pericytes, neurons, fibroblasts. The proportion of each cell type was similar compared with that of Clec7a KO condition in both physiological and disease groups (Figure 3.1e). According to the microglia markers (Cd68, Aif1, Plprc, Itagam) expression level, cluster 0, 5, 8, 10, 11 were identified as microglia-like cells. In disease groups, the microglia proportion was highly increased by 25% compared with the homeostasis groups.



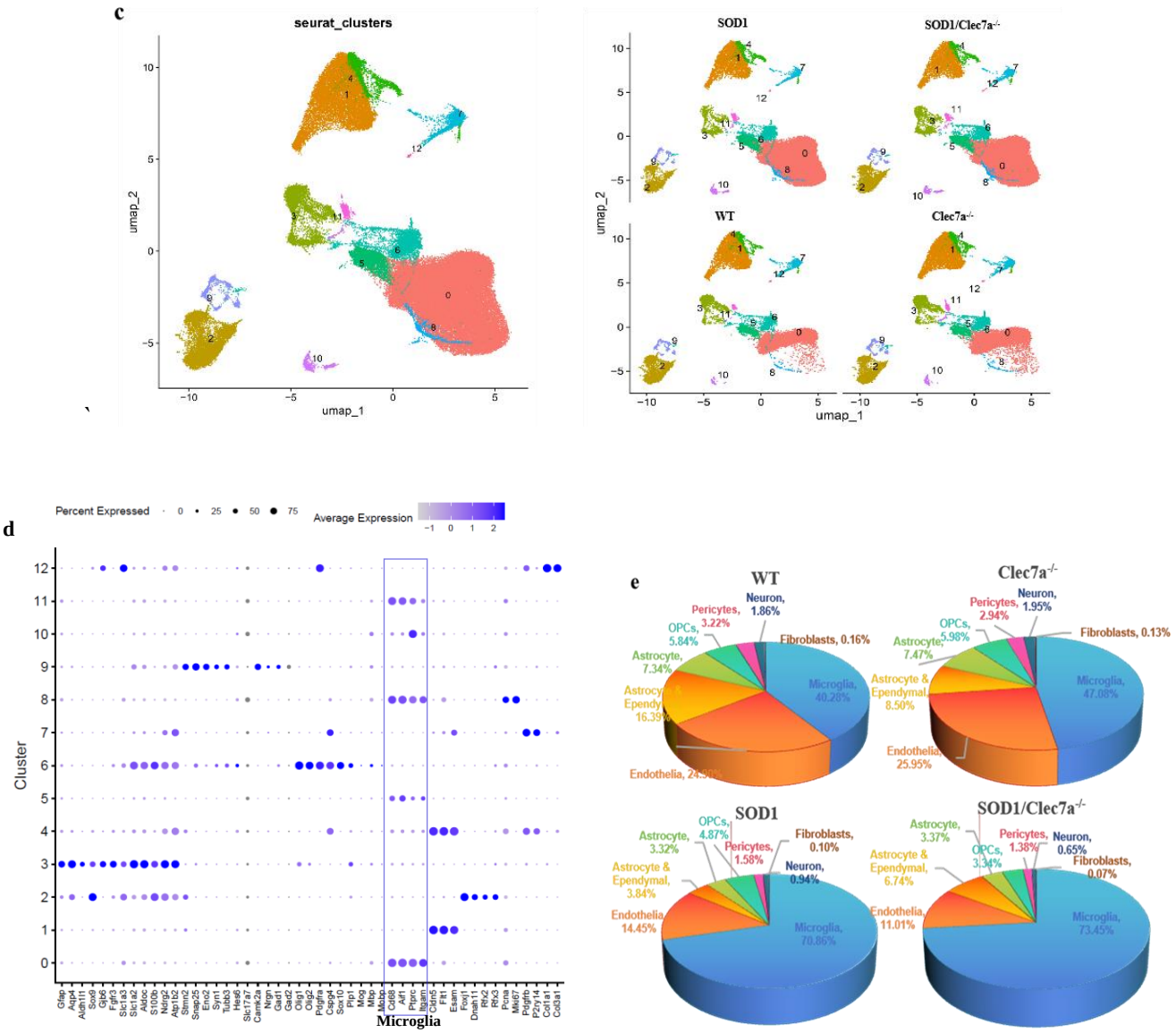


Figure 3.1. Single-cell RNA sequencing of WT, Clec7a$^{-/-}$, SOD1^{+}, SOD1^{+}/Clec7a$^{-/-}$ mice shows diverse cell types but no Clec7a KO -specific cell population.

a, Scheme of the experimental workflow.

b, Expression level of SOD1^{+}, Clec7a mRNA in each sample, grouped by genotype. Expression level was featured total counts transformed by log1p and averaged by cell number per sample.

c, UMAP plot of clusters of WT, Clec7a$^{-/-}$, SOD1^{+} and SOD1^{+}/Clec7a$^{-/-}$ group of mice. Each dot represents a single cell.

d, Dot plot showing expression of selected cell markers for identified cell types. Microglia markers are highlighted.

e, Pie charts demonstrating distribution of the identified cell types in different genotypes.

3.2. Clec7a deficiency results in distinct microglia transcriptome profile in disease and physiological groups but no Clec7a-KO specific microglia subpopuation.

To investigate how Clec7a specifically affects the microglia functions, we selected the Cells in cluster 0, 5, 8, 10, 11, which were identified as microglia lineage cells, to further analyze their subpopulations. UMAP of around 58,000 cells was plot by group. The distributions of 11 subclusters were similar within $SOD1^+$ disease group and physiological group. No Clec7a-deficiency specific subcluster was observed (figure 3.2a). The biggest change was the proportion of cluster 0 decreasing 50% in $SOD1^+$ groups and cluster 1, 2 showed around 30% increase in disease groups compared to physiological groups.

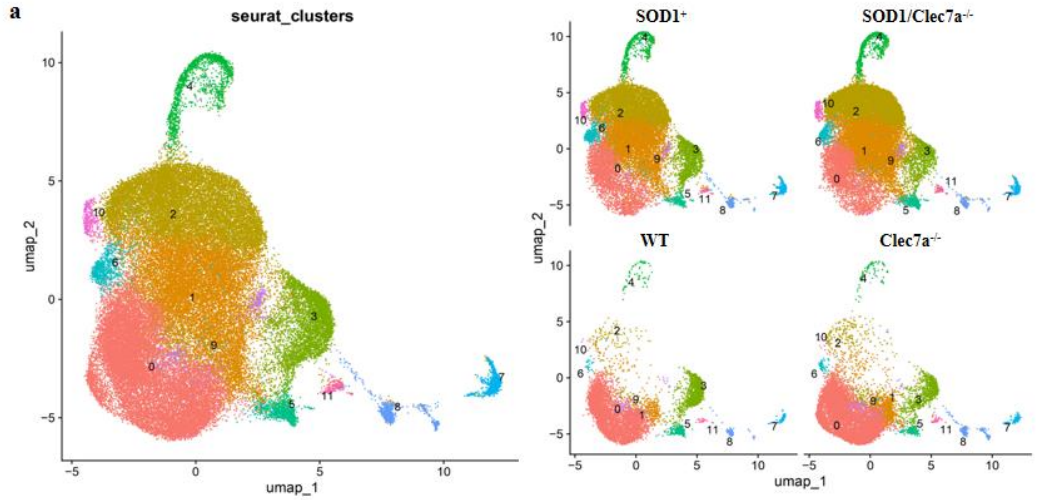
To further identify the subpopulations of microglia and investigate the immune cells infiltration, dot plot was made to illustrate the expression level of the selected cell markers in each cluster (figure3.2b). Cluster 0, which had the largest proportion, was identified as homeostatic microglia. Cluster 1 and 2, which had high level of activated microglia genes were assumed as activated microglia. Cluster 6 and 9 showed similar level of homeostatic and activated microglia genes and according to their location in the UMAP, they were considered transitional status microglia, showing a dynamic process from homeostatic to activated. Cluster 7 and 8 were away from the clusters of microglia, and their marker

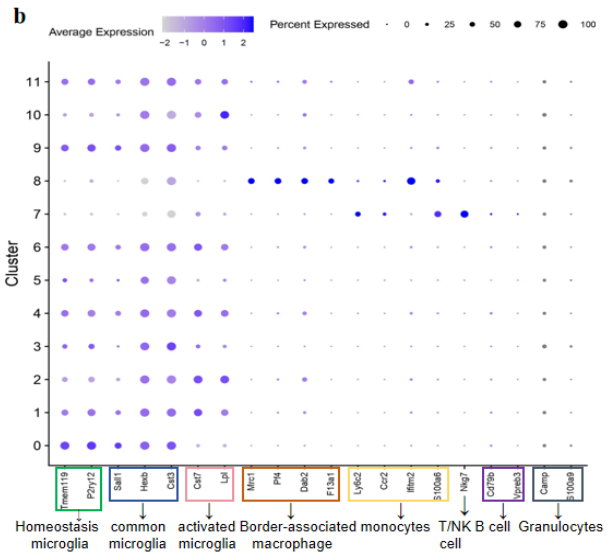
genes showed they were peripheral monocytes and border associated macrophage (BAM) respectively. There were no granulocytes and B cells detected. Cluster 5 decreased half in Clec7a$^{-/-}$ group compared with WT group while it showed increase in Clec7a deficiency disease group. Cluster 3 decreased 50% in both disease and physiological groups but it only had common microglia genes expression, further identification needs to be done.

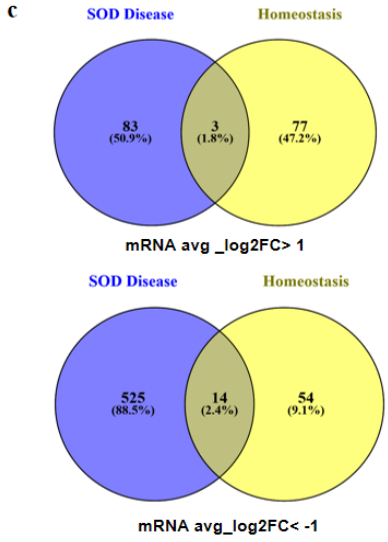
Microglia genes were considered differentially expressed if the absolute value of the log2 fold change of the Clec7a condition relative to the WT or SOD1^{+} group was greater than 1.0 (p-value < 0.05). We found that in disease group, 525 genes were downregulated and 83 genes were upregulated in microglia; in physiological group, 53 genes were downregulated and 77 genes were upregulated. The Venn plot showed there were very small proportion of overlapping genes upregulated or downregulated in both conditions, indicating distinct mRNA profiles with Clec7a knockout in disease group and physiological group (figure 3.2c).

Additionally, we examined the expression level of some human ALS risk genes. Atxn3, Tbk1, Scfd1, Epha4 were found significantly downregulated in SOD1+/Clec7a$^{-/-}$ group compared with SOD1+ group but not in physiological groups. Tbk1 is a IκB kinase involved in the regulation of type I interferons and NF-κB signal transduction, enhancing the

induction of interferon-stimulated microglia and phagocytotic microglia[19]. Epha4 is an ephrin receptor subfamily, mediating proinflammatory phenotypes[20]. The protein encoded by SCFD1 plays a role in regulating the fusion of intracellular transport vesicles with their target membranes, involved in neurotransmitter release in neurons. Downregulations of the risk genes show that Clec7a KO may have protective effects in ALS disease condition.







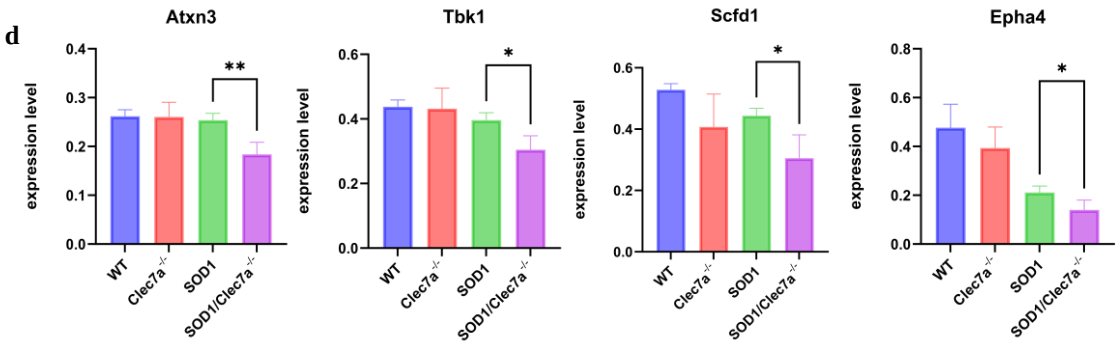


Figure 3.2. Clec7a deficiency in SOD1^{+} disease groups and physiological groups results in distinct microglia transcriptome profile but no Clec7a-KO specific microglia subpopulation emerges.

a. UMAP plot of subclusters of microglia in WT, Clec7a$^{-/-}$, SOD1^{+} and SOD1^{+}/Clec7a$^{-/-}$ group of mice. Each dot represents a single cell.

b. Dot plot of expression level of cell markers for different microglia lineage cells.

c. Venn diagrams for the numbers of differentially expressed genes in disease groups and physiological groups. Upregulated genes in Clec7a deficiency: log2FC> 1; downregulated genes in Clce7a deficiency: log2FC< -1.

d. ALS related risk genes expression level in disease groups and physiological groups.

3.3. DEGs analysis shows that Clec7a KO can induce the activation of phagocytosis and enhancement of cell mobility in physiological group.

The transcriptome profile change was first analyzed in Clec7a$^{-/-}$ vs. WT group. The differential expressed genes (DEGs) were selected with a log2FC >1(p-value < 0.05). Ingenuity pathway analysis was conducted by inputting the gene list and their fold change and p-value. The network summary illustrates the most changed pathways were upregulated (figure 3.3a). Among these pathways, activation and recruitment of phagocytes were significantly upregulated, which was consistent with our previous

apoptotic neuron injection experiment. The molecular study and pathology study both showed that the Clec7a depletion can activate phagocytosis, confirming its role as a checkpoint for microglia phagocytosis. Cell mobility was also observed upregulated to facilitate the phagocytotic activity.

Next, the specific biological processes were listed with the order of log(p-value). As shown in figure 3.3b, phagosome formation had a very significant increase with a log(p-value) of -3.3. Cell mobility related pathways like actin nucleation, which is a critical step in the regulation of actin cytoskeleton dynamics, and Rho-GTPase pathway were largely upregulated. Additionally, inflammation responses like interferon gamma pathway and NK pathway were observed increased. But the phagocytosis and mobility promotion took the dominant in all changes.

After specifying the potentially altered pathways in Clec7a KO condition, we focused on the molecules most changed in the microglia phagocytosis and mobility functions. The log2FC of these DEGs were present on the heatmap respectively (figure 3.3c). Rac3 is a member of the Rho family of small GTPases, playing a crucial role in the regulation of the actin cytoskeleton, which facilitates the mobility. Clec4d, another member of the C-type lectin receptors, functions as an immunoreceptor that recognizes various carbohydrate ligands, present on the surface of pathogens or host cells. Saa3 is one of the members in serum amyloid A protein family. The upregulation of Saa3 expression is part of the host defense mechanism to combat

pathogens. It can also activate Toll-like receptors (TLRs) receptors on immune cells. Ccr2 also significantly upregulated to mediate the migration and recruitment of immune cells. Ccr2 activates intracellular signaling pathways that promote cytoskeletal rearrangements and cell motility, leading to the directional migration of immune cells.

The most decreased gene, Clec1a, acts as a checkpoint in myeloid cell immunity, limiting the phagocytotic activity in physiological condition[21]. Its downregulation with Pecam (CD31), one of the ‘don’t eat me’ signals inhibiting phagocytosis by interacting with specific receptors on phagocytes, demonstrates the loss of checkpoint and thus increase of phagocytotic activity.

In summary, the Clec7a deficiency in physiology activates the microglia migration and phagocytosis by upregulating genes on actin skeleton, immune cell recruitment, pathogen recognition and downregulating the ‘don’t eat me’ signals.

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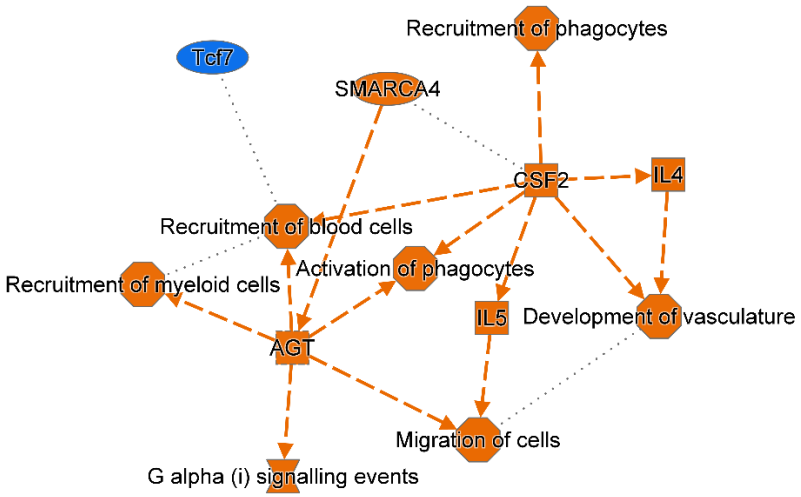


Ingenuity pathway analysis

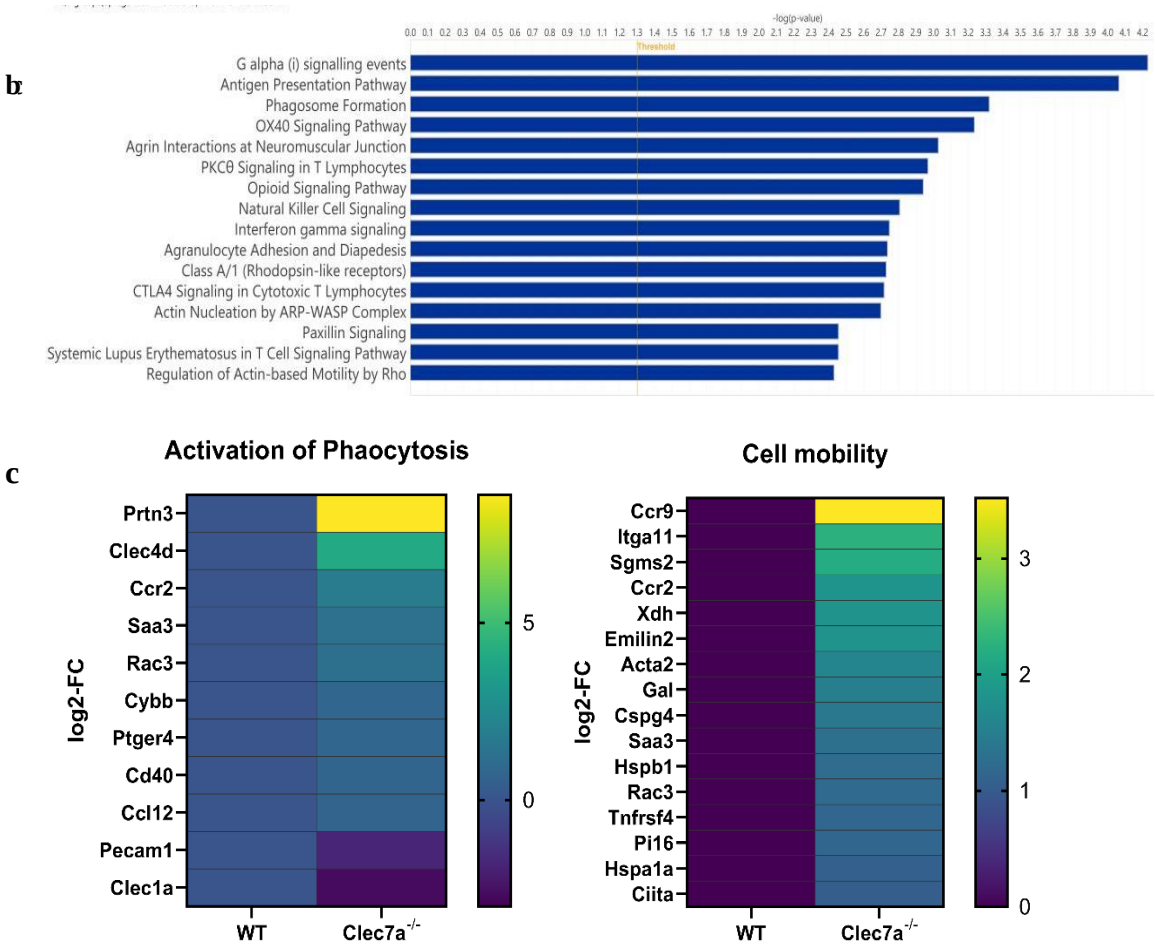


Figure 3.3. Functional analysis of differentially expressed genes (DEG) in microglia of Clec7a$^{-/-}$ group with WT group shows the activation of phagocytosis and enhancement of cell mobility.

a. Summary of ingenuity pathway analysis (IPA) of DEG in Clec7a-/- group. Blue: inhibition; Orange: activation. This plot was generated with QIAZHEN IPA platform.

b. Top 15 canonical pathways clustered from the DEGs using IPA.

c. Heatmap of the expression level change (log2 fold change) of the DEG involved in activation of phagocytosis and cell mobility compared with WT group.

3.4. Functional analysis of DEGs in $SOD1^{+}/Clec7a^{-/-}$ microglia reveals that loss of Clec7a downregulates inflammation pathways in $SOD1^{+}$ disease condition.

In disease groups, the global inflammation caused by SOD1mutation created a much more complicated environment compared with homeostasis groups. 525 gens and 83 genes respectively found downregulated or upregulated in Clec7a deficiency with a log2FC >1 or <-1 ($p<0.05$) were analyzed in IPA. As shown in figure 3.4a, most pathways were downregulated compared with $SOD1^{+}$ group. Among them many were immune response-related pathways like IFNG, TNF, IL1β, VEGFA signaling pathways. The neuroinflammation signaling pathway was also found decreased.

Biological processes analysis was conducted with gene ontology study to specify the top 20 canonical pathways in a sequence of log (p-value), as shown in figure 3.4b. The most significantly changed pathways were innate immune response, cellular response to cytokine stimuli, inflammatory response, interferon gamma induction and NF-κB signal transduction. Cell adhesion, mobility and proteolysis were also observed altered. The IPA and GO analysis both demonstrated the microglia's significant and wide reduction of inflammation in Clec7a deficiency in $SOD1^{+}$ model.

To further study the molecules involved in these immune responses, most changed genes were selected and categorized into different types. Their expression level in $SOD1^{+}/Clec7a^{-/-}$ group relative to that in SOD1 were presented by heatmap (figure3.4c). In cytokines and chemokines, Cd70 is a cell surface protein belonging to

the tumor necrosis factor (TNF) superfamily. It functions as a ligand for the CD27 receptor and CD70-CD27 signaling plays important roles in regulating immune responses, including T cell activation. Il7 and Il-1β are signaling molecules that play a central role in the initiation and amplification of inflammatory responses by inducing the production of other pro-inflammatory cytokines, chemokines, and adhesion molecules. Epha2 and Epha4 are in ephrin receptor subfamily, regulating proinflammatory responses. Epha4 is one of the ALS risk genes. Tbk1 is another ALS risk gene involved in type1 interferon and NF-κB signal transduction. Cd47, one of the 'don't eat me' signals, was significantly downregulated in Clec7a KO disease condition, may indicate an activation of phagocytosis pathway.

In receptor and transporter group, Lcn2 was upregulated largely. It is an anti-inflammatory regulator in macrophage activation by an activation of NFκB -STAT3 loop. Its upregulation was consistent with the clec7a KO anti-inflammatory effects. Ccr9 regulating immune cell trafficking, which is important for immune surveillance also increased. In those decreased genes, Klrd1 and Klrk1 are both important receptors involved in the recognition and elimination of target cells by NK cells and other cytotoxic lymphocytes. Their functions are tightly regulated to prevent excessive immune activation and maintain immune homeostasis. Orl1, also known as clec8a, is a lipoprotein receptor amplifies pro-inflammatory response.

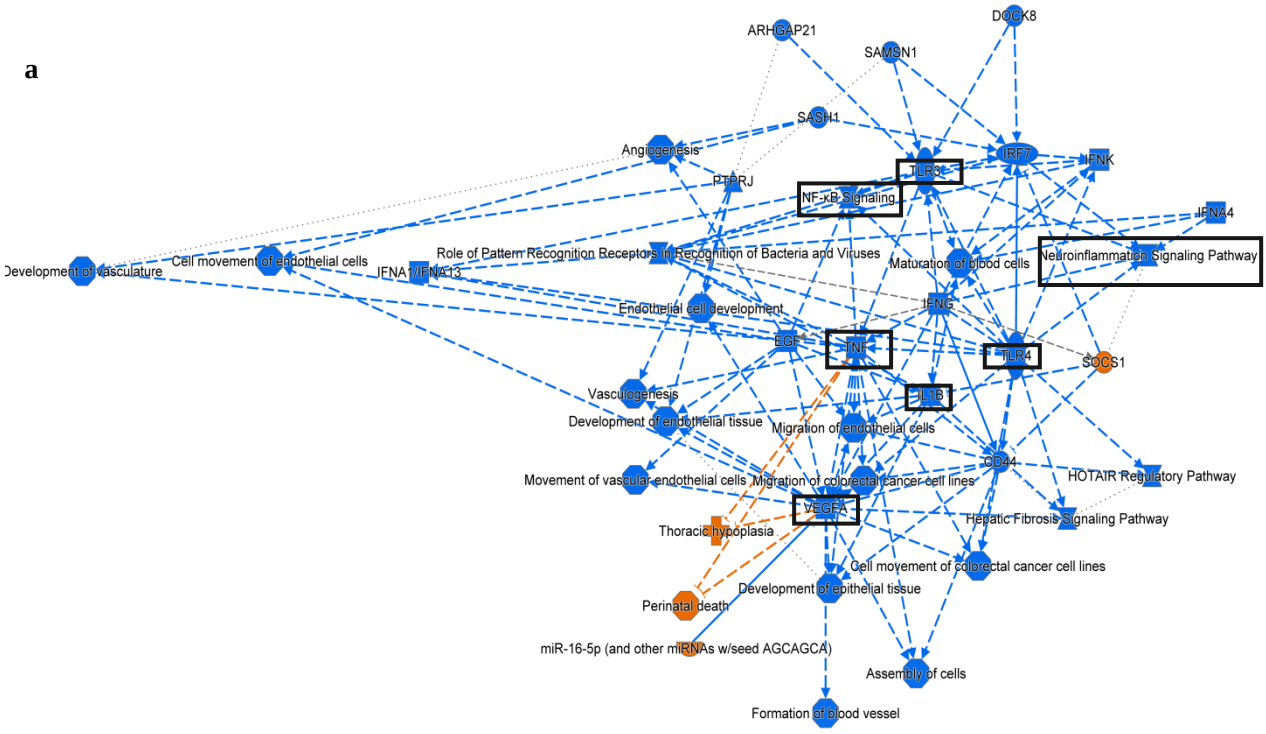
In the transcription regulator group, the most reduced gene Egr3 is a zinc finger transcription factor that can modulate the activation, differentiation, and function of immune cells. Dysregulation of Egr3 expression or activity has been implicated in various neurological disorders like AD. Nfat5 is the downstream of Clec7a, regulating the activation of NLRP3 inflammasome. It decreased significantly when Clec7a was depleted. Tl3 and Tl4 are regulators of Toll-like receptor family which plays fundamental role in pathogen recognition and activation of innate immunity. Bcl6 is a transcription suppressor highly expressed in ALS. It is a master regulator of the germinal center reaction, where it controls the balance between B cell proliferation, differentiation and apoptosis.

The upregulated Mef2b is involved in the development and function of immune cells and is a microglia M0 transcription factor. It can regulate the expression of genes involved in immune cell activation, cytokine production. Dysregulation of Mef2b have been observed in ALS patients. Mef2b signaling pathways may play a role in motor neuron degeneration and disease progression. Prdm8 was also upregulated . Identified genetic variants of Prdm8 are found associated with late-onset AD. Its transcriptional regulatory activity may influence various cellular processes critical for neural development and function.

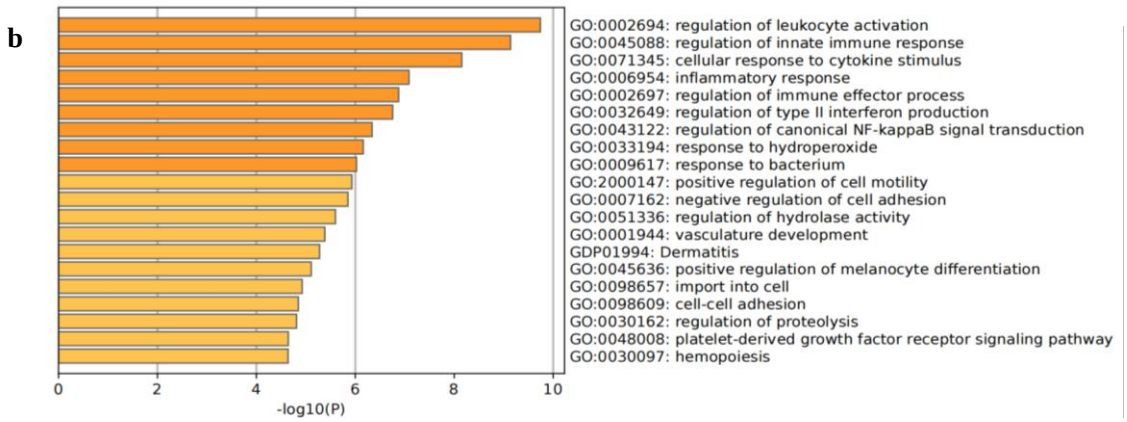
In summary, the Clec7a deficiency plays an anti-inflammatory role in microglia of ALS model, indicating it has pro-inflammatory effects in disease condition. The

affected immune pathways are wide including NF-κB, IL1β, TNF pathways. Gene expression level related to immune cell activation, cytokine release was decreased. Some ALS risk genes were also found downregulated in SOD1^{+}/Clec7a$^{-/-}$ group.

a



b



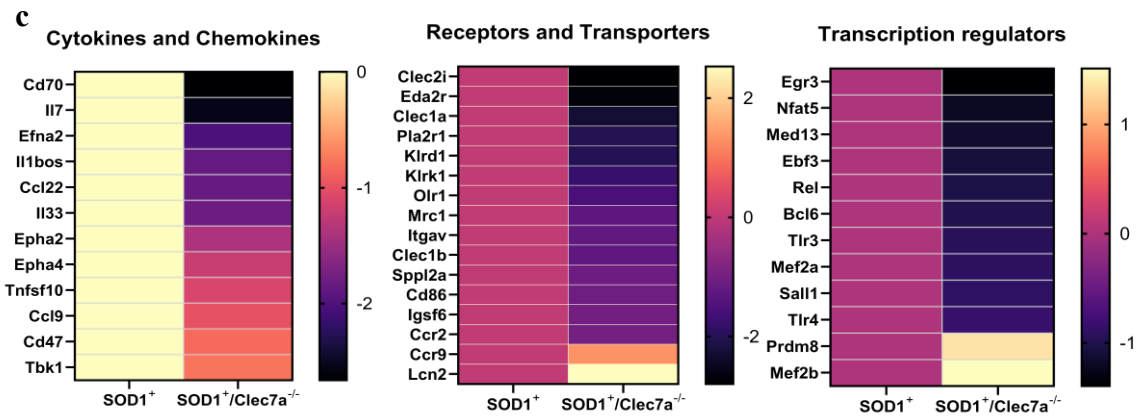


Figure 3.4. Functional analysis of DEGs shows that Clec7a deficiency downregulates inflammation in SOD1^{+} disease condition.

a. Summary of ingenuity pathway analysis (IPA) of DEG in SOD1+/Clec7a$^{-/-}$ group. Blue: inhibition; Orange: activation. This plot was generated with QIAZHEN IPA platform.

b. Gene Ontology (GO) analysis of the top 20 canonical pathways clustered from the DEGs. P-value is shown as -log10(P).

c. Heatmap of the expression level change (log2 fold change) of the downregulated neuroinflammation-related genes in SOD1+/Clec7a$^{-/-}$group compared with SOD1+ group.

2.6. Collaboration

Figure 3.1b, c, d and figure 3.2a, b were generated by Haichao Wei in University of

Texas Health, Houston.

Other results were got by Huilan Yan.

Chapter 4: Discussion

With single-cell RNA sequencing of late-stage spinal cords from 4 experimental groups, we got altered microglia transcriptome profile with Clec7a deficiency in both disease and physiological groups, indicating how Clec7a regulating microglia functions respectively. In disease condition, the results from our previous behavior and pathological studies suggested that Clec7a plays different role in peripheral nervous system and central nervous system and in early stage and late stage of disease. In late stage of CNS, knockout of Clec7a had anti-inflammatory effects, indicating its detrimental regulations. But in the early-stage PNS, Clec7a deficiency causes early motor deficits of $SOD1^{+}$ mice, suggesting a protective role of Clec7a in maintaining early motor function and delaying disease progression. In physiological groups, the apoptotic neurons injection experiments and the sequencing results support each other, showing that the Clec7a deficiency would increase the microglia phagocytosis by upregulating related genes on actin cytoskeleton, immune cell recruitment and pathogen recognition and downregulating the ‘don’t eat me’ signals.

Our findings give in vivo pathological and molecular evidence on the Clec7a regulation of microglia immune response and phagocytosis, deepening the understanding of the role of microglia in ALS. It may provide some insights into potential therapeutic targets for ALS.

The limitation of the study is that the DEGs analysis was conducted on all the microglia lineage cells which may lead to a reduced difference between a specific subpopulation in different groups. To specify the different pathways changed in Clec7a deficiency, we need to examine the level changes in each subtype of microglia to confirm the real fold change and p-value in the most involved clusters. Also, the detected total counts of featured genes should be considered to determine if they play a relative important role in biological processes.

The upcoming research will focus on elucidating altered pathways by examining changes in protein expression levels through immunostaining spinal cord slides from four experimental groups of mice because the sequencing results are mRNA expression level, not the protein level changes. The primary emphasis of this study will be on investigating direct microglial functional changes. However, considering the transcriptome profiles, upregulation of cell-cell communications was observed in the Clec7a KO condition. Therefore, additional downstream analyses, such as ligand-receptor analysis and cell trajectory analysis, will be conducted to explore how interactions between astrocytes, neurons, and microglia are altered under Clec7a-depleted conditions. This approach aims to achieve a more comprehensive understanding of the pathological mechanisms underlying ALS.

To specifically investigate how Clec7a deficiency affects microglial functions, we have developed a novel mouse model, $SOD1^{+}/CX3CR1^{\Delta/\Delta}/Clec7a^{f/f}$ mice. This model allows for the conditional depletion of Clec7a exclusively in microglia, as opposed to a global knockout we used currently. By comparing the lifespan and phenotype of these mice, we aim to elucidate the specific role of the Clec7a receptor in the context of ALS.

In summary, the single-cell sequencing analysis reveals Clec7a involved in promoting inflammation in microglia of $SOD1^{+}$ ALS model by regulating immune response pathways like NF-κB pathway and IFNG pathway, which suggests its potential detrimental role. Together with our previous behavior and pathological study, which demonstrates its protective effects on early motor deficiency, these in-vivo results illustrate the complex pathological role of Clec7a in early and late stage of ALS, explaining the unchanged life span in Clec7a deficiency. The understanding of how microglia involved in ALS disease progression was deepened in a molecular level. It may provide some insights into potential therapeutic targets for ALS.

Chapter 5: Bibliography

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