Systems and Comparative Analyses of Monocyte Dynamics Based Upon Single Cell Sequencing Data

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Abstract

Inflammatory diseases often involve complex and dynamic responses of monocytes, crucial cells of the innate immune system. Understanding these responses, particularly to lipopolysaccharide (LPS), a key inflammatory stimulus, is vital yet remains challenging due to their heterogeneity and plasticity. Upon analyzing available single-cell RNA sequencing data sets, we defined key patterns of monocyte inflammatory responses challenged with varying LPS dosages. We found that high-dose LPS induced the generation of exhausted monocytes with elevated expression of genes associated with pathogenic inflammation and immune suppression.. In contrast, super-low-dose LPS led to a state of low-grade inflammation, characterized by enhanced chemotaxis; immune-enhancement; and adhesion.. Pseudo-time analysis revealed a potential bifurcation of monocytes, starting from a proliferative, less-differentiated and premature state into either the exhausted state (under prolonged high dose LPS challenge) or the low-grade inflammatory state (under the prolonged super-low dose LPS treatment). Complementing our analyses with in vitro cultured murine monocytes, we observed similar exhaustion of monocytes collected from septic murine hearts published in an independent study. Furthermore, we analyzed publicly available scRNAseq datasets regarding monocytes from septic and severe COVID human patients and revealed a similar exhaustion phenotype as we documented in murine exhausted monocytes. In contrast, our analyses of newly published scRNAseq data regarding monocytes from chronic autoimmune patients reveal key distinct low-grade inflammation features. With translational potential, we analyzed the scRNAseq datasets of monocytes trained with 4-PBA, a potent anti-inflammatory compound, and observed that 4-PBA can effectively arrest monocytes in an anti-inflammatory state. Together, our comparative analyses reveal a systems landscape of monocyte memory dynamics

with distinct dosage and history of LPS challenges, and offer novel insights for potential therapeutic strategies for modulating both acute sepsis and chronic inflammatory diseases. Our studies also provide a foundation for guiding future mechanistic and translational studies regarding monocyte dynamics and their involvements in health and disease pathogenesis.

General Audience Abstract

Inflammation is the body's natural response to injury or infection. A key player in this process is a type of immune cell called monocyte. Monocytes are our body's first line of defense, rushing to the site of injury or infection. However, the way these cells respond can vary greatly, depending on the dosage and duration of external challenges.

In our research, we analyzed data collected through an advanced technique called single-cell RNA sequencing, in order to take a detailed look at how an individual monocyte responds to different amounts of LPS, a key substance found in most bacteria. We found that when exposed to a prolonged challenge of higher dose LPS, monocytes become exhausted with pathogenic inflammation and immune suppression, as seen in sepsis. However, when the LPS dose is low, these cells enter a state of low-grade inflammation, responsible for chronic inflammation as seen in autoimmune diseases, atherosclerosis and other chronic diseases.

We found that this paradigm of exhaustion and low-grade inflammation can be seen in data analyzed from either patients with severe infections such as sepsis or severe COVID-19, or patients with long-term autoimmune diseases.

In simpler terms, our study provides a detailed road map regarding how our body's first responders, namely the monocytes, react under different levels of threat, and how we might be able to guide their responses toward a beneficial direction. Understanding these processes more clearly may lead to new ways to treat a range of infectious or inflammatory diseases.

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Chapter 1

Overview of innate immune dynamics and involvement in disease

1.1 Immunity

Human beings encounter a vast array of microorganisms such as bacteria, viruses, fungi, and parasites regularly, which can potentially cause diseases and disrupt the normal functioning of our bodies. To counter these threats, the human body has developed a complex and sophisticated defense mechanism called the immune system.[1-2] The immune system comprises two primary categories of defense mechanisms, namely innate immunity and adaptive immunity, which work in coordination to detect and eliminate pathogens, thereby ensuring overall health and well-being.[1-3]

Innate immunity serves as the first line of defense against pathogens and is present from birth.[4-8] This system comprises physical barriers such as skin and mucous membranes, chemical mediators, and various immune cells that provide a rapid, nonspecific response to foreign invaders. The skin and mucous membranes act as physical barriers that prevent pathogens from entering the body. Mucous membranes also produce mucus that can trap pathogens and eliminate them through mechanisms such as coughing or sneezing.

Additionally, substances like lysozyme present in tears and saliva and acidic secretions in the stomach can destroy or inhibit the growth of pathogens.[9-10] Innate immunity involves several types of immune cells such as phagocytes, natural killer (NK) cells, and dendritic cells.[11-16] Phagocytes engulf and destroy pathogens, while NK cells directly kill virus-infected and cancerous cells. Dendritic cells capture and process antigens, which are then presented to adaptive immune cells.

Inflammation is a crucial aspect of the innate immune response, which is characterized by redness, swelling, heat, and pain.[17-22] Inflammation helps to recruit

immune cells to the site of infection and promotes the removal of pathogens and damaged tissue.

1.2 Inflammation and Disease

Inflammation serves as a critical biological response to various harmful stimuli, including pathogens, damaged cells, or irritants. It is an essential aspect of the immune system, functioning to eradicate the initial source of harm, remove damaged cells and tissues, and foster repair and regeneration in the impacted region. Inflammation is primarily classified into two categories: acute and chronic.[23-25] Acute inflammation is characterized by a rapid, immediate response to harmful stimuli, which is usually self-limiting and subsides once the stimulus is eliminated, and tissue repair takes place. Conversely, chronic inflammation involves a prolonged, unregulated response that can contribute to tissue damage and various illnesses.

Acute inflammation plays a vital role in preserving overall health, as it aids in fighting off pathogens and repairing injured tissue.[26-27] The acute inflammatory response consists of three primary stages: vascular alterations, cellular events, and the resolution of inflammation. Vascular changes encompass vasodilation and enhanced vascular permeability, leading to the hallmark signs of inflammation: warmth, redness, and swelling. Cellular events involve the recruitment and activation of leukocytes, predominantly neutrophils, which migrate to the site of injury or infection and eliminate pathogens and cellular debris through phagocytosis. Lastly, the resolution phase terminates the inflammatory response and promotes tissue repair and regeneration.

Acute inflammation and sepsis have a close relationship, as sepsis is a life-threatening condition that occurs due to an excessive systemic inflammatory response to an infection.[28-31] While acute inflammation is usually localized and advantageous, sepsis signifies a disordered and extreme inflammatory reaction that can cause extensive tissue damage and organ malfunction. During sepsis, the immune system experiences a paradigmatic situation that includes both proinflammatory and immune suppressive properties, leading to tissue damage and death of B and T cells. This conflicting response promotes bacterial growth, leading to an uncontrolled immune system response to infection. Consequently, vast amounts of pro-inflammatory cytokines and other mediators are released into the bloodstream, leading to a "cytokine storm." This storm can initiate detrimental effects such as systemic vasodilation, increased vascular permeability, coagulation abnormalities, and multiple organ dysfunction syndrome (MODS).

Gaining a deeper understanding of the mechanisms and interconnections between acute inflammation and sepsis is crucial in developing effective therapeutic interventions to modulate the inflammatory response and prevent further harm. Ongoing research into the cellular and molecular processes underpinning acute inflammation and sepsis can offer valuable insights into the development of innovative treatment approaches for these potentially life-threatening conditions.

Chronic inflammation is a persistent and dysregulated immune response that occurs when the body's natural defense mechanisms fail to effectively eliminate harmful stimuli, resulting in a prolonged state of inflammation. Unlike acute inflammation, which is typically a short-term, localized response to injury or infection, chronic inflammation can persist for months or even years, leading to tissue damage, organ dysfunction, and the development of various diseases. One such group of

disorders closely linked to chronic inflammation is autoimmune diseases, wherein the immune system mistakenly attacks the body's own healthy tissues and cells.

In chronic inflammation, monocytes can experience low-grade inflammation themselves. These monocytes play a critical role in the immune system, as they differentiate into macrophages and dendritic cells, both of which are essential for combating infections and initiating adaptive immune responses.

Autoimmune diseases encompass a diverse range of conditions characterized by abnormal immune responses targeting self-antigens, ultimately resulting in inflammation and damage to various organ systems.[32-36] Two such autoimmune diseases include Vogt-Koyanagi-Harada (VKH) disease and Behçet's disease.

Vogt-Koyanagi-Harada (VKH) disease is an uncommon autoimmune disorder that impacts various body systems, predominantly the eyes, auditory system, skin, and meninges (the membranes enveloping the brain and spinal cord).[37] This condition is marked by an inflammatory process involving the infiltration of immune cells, such as T cells and monocytes experiencing low-grade inflammation, into affected tissues. The exact cause of VKH disease remains elusive, but it is thought to result from a combination of genetic and environmental factors that trigger an autoimmune response against melanocyte-associated antigens. VKH disease is commonly associated with symptoms such as vision problems, hearing loss, skin changes, and neurological manifestations like headaches and meningitis. Treatment generally consists of corticosteroids and other immunosuppressive agents to manage inflammation and avert complications.

In contrast, Behçet's disease is a chronic multisystem inflammatory disorder that primarily targets blood vessels and is characterized by recurrent inflammation episodes

involving monocytes experiencing low-grade inflammation.[38] The exact cause of Behçet's disease remains unclear, but it is believed to stem from a combination of genetic predisposition and environmental triggers, such as infections, which can incite an abnormal immune response. Hallmark symptoms of Behçet's disease include recurrent oral and genital ulcers, skin lesions, and ocular inflammation, potentially leading to vision loss if left untreated. Treatment for Behçet's disease aims to manage symptoms and reduce inflammation using immunosuppressive drugs, corticosteroids, and other targeted therapies.

Understanding the intricate relationship between chronic inflammation, monocytes experiencing low-grade inflammation, and autoimmune diseases, such as VKH disease and Behçet's disease, is crucial for the development of targeted therapeutic strategies aimed at modulating the immune response and mitigating disease progression.

1.3 Monocyte Involvement in Inflammation

The human immune system is an intricate network of cells, tissues, and organs responsible for defending the body against harmful pathogens and maintaining overall health. It comprises two primary branches: the innate immune system and the adaptive immune system.

The innate immune system, which serves as the body's first line of defense against harmful substances and pathogens, provides a rapid, non-specific response to potential threats.

Monocytes, essential components of the innate immune system, play crucial roles in inflammation, immunity, and tissue repair. Originating from hematopoietic

progenitors in the bone marrow, monocytes enter the blood circulation and migrate to affected sites upon activation by various stimuli, such as infection or inflammation.[39-40] Subsequently, they differentiate into diverse subpopulations, including dendritic cells and macrophages, modulating the immune response by either promoting or suppressing inflammation. This review explores the distinct subpopulations of monocytes, their functions, and their roles in inflammation and immune response regulation.

Monocytes can be categorized into three subpopulations based on CD14 and CD16 cell surface marker expression: classical, intermediate, and non-classical monocytes.[41] Each subpopulation possesses unique properties and functions in orchestrating the immune response, with some overlap in their roles. Classical monocytes, characterized by high CD14 and low CD16 expression, are associated with pro-inflammatory responses. They express markers such as HLA-DR, cytokines (e.g., IL-1b, IL-6), chemokines (e.g., CCL3, CCL5), and the transcription factor NF-kB. However, classical monocytes can also exhibit immune suppressive properties, expressing PD-L1 and displaying compromised CD86 and HLA-DR expressions.

Intermediate monocytes, which express high levels of both CD14 and CD16, are thought to bridge the gap between classical and non-classical monocytes, possibly exhibiting a range of functions from both subpopulations.

Meanwhile, non-classical monocytes, marked by low CD14 and high CD16 expression, are vital for maintaining vascular homeostasis and suppressing pathogenic inflammation. These cells also contribute to blood vessel surveillance and patrolling and have been implicated in promoting tissue repair and angiogenesis.

From a molecular perspective, CD16 positively correlates with monocyte inflammation levels and is crucial for orchestrating the inflammatory response. In humans, CD16 serves as a key regulator of the TRIF-dependent TLR4 signaling pathway. TLRs, a family of pattern recognition receptors, detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to activate innate immune responses.[42-43] Tram specifically participates in the TLR4 and TLR3 signaling pathways, which recognize lipopolysaccharide (LPS) from Gram-negative bacteria and viral double-stranded RNA, respectively. Upon activation, TLR4 and TLR3 recruit Tram, which then interacts with other adaptor proteins, such as TRIF (TICAM1), to propagate the signal and ultimately activate transcription factors like IRF3, IRF7, and NF-κB. These transcription factors, in turn, induce type I interferon production and other pro-inflammatory cytokines, leading to the activation of innate immune cells, including monocytes.

CD38 is expressed on neutrophils and inflammatory monocytes, with inflammatory monocytes further upregulating CD38 during infection.[44] The absence of CD38 causes alterations in the migration patterns of both cell types to infection sites. Macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing. Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a key chemokine that regulates the migration and infiltration of monocytes/macrophages. Both CCL2 and its receptor CCR2 have been implicated in various diseases.[45]

Concurrently, co-regulator genes are expressed, such as CD80 and CD86 on monocytes, which are essential for activating lymphocytes and adaptive immunity.[46] Monocytes also express MHCII or HLA-DR genes in correlation with inflammation.[47]

During inflammation, monocytes play a critical role in orchestrating the immune response.[48] They secrete cytokines and chemokines, essential for recruiting and activating other immune cells, such as neutrophils and lymphocytes, to the site of infection or injury. Alongside this process, co-regulator genes are upregulated in immune cells to further fine-tune and coordinate the immune response. The dynamic interplay between cytokines, chemokines, and co-regulator genes is crucial for effectively eliminating pathogens and promoting tissue repair and regeneration in both acute and chronic inflammation.

In sepsis, however, the immune system faces a unique challenge. The intense acute inflammation leads to a surge in cytokine and chemokine production by monocytes, which should ideally enhance the immune response.[49-50] Paradoxically, this overwhelming response is accompanied by a dysregulation of co-regulator genes, leading to immune suppression. This conflicting profile creates a favorable environment for bacterial growth and exacerbates the inflammatory response, ultimately resulting in tissue damage.

The excessive inflammation observed in sepsis can culminate in a cytokine storm—a massive release of pro-inflammatory cytokines into the bloodstream. This phenomenon can have disastrous consequences for the host, including widespread tissue damage, multi-organ failure, and even death.

Understanding the complex interplay between monocytes, cytokines, chemokines, and co-regulator genes during sepsis is essential for developing targeted therapies that can modulate the immune response, mitigate tissue damage, and improve patient outcomes.

1.4 Monocyte Activation Dynamics

The monocytes experience a smooth, gradual differentiation towards macrophage. Once in the tissues, monocytes can differentiate into macrophages, which are highly versatile and plastic cells capable of adopting different phenotypes depending on the local micro-environment. Two main polarization states have been widely described: the classically activated M1 macrophages and the alternatively activated M2 macrophages.[51-54] These distinct subsets exhibit unique functional properties and play contrasting roles in inflammation and tissue homeostasis.

M1 macrophages, also known as classically activated macrophages, are primarily induced by pro-inflammatory signals, such as interferon-gamma (IFN-γ) and lipopolysaccharide (LPS). M1 cells are characterized by their potent microbicidal and tumoricidal activities, as well as their ability to produce high levels of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). They also generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) to combat pathogens and facilitate antigen presentation. These cells are crucial for initiating and maintaining acute inflammatory responses, helping to eliminate pathogens and damaged cells. However, uncontrolled activation can contribute to tissue damage and chronic inflammation if not properly resolved.

M2 macrophages, also referred to as alternatively activated macrophages, arise in response to anti-inflammatory signals, such as interleukin-4 (IL-4) and interleukin-13 (IL-13). M2 cells display a wide range of functions, including immunosuppression, tissue repair, and resolution of inflammation. They produce anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), which help to dampen the inflammatory response and promote tissue remodeling. As

well as angiogenesis, fibrosis, and the clearance of apoptotic cells. While M2 cells are essential for resolving inflammation and promoting tissue repair, their excessive activation or dysregulation may contribute to the development of fibrotic diseases, tumor progression, and chronic infections.

The coexistence of M1 and M2 macrophages in the same tissue or inflammatory environment adds another layer of complexity to the immune response, creating a dynamic and heterogeneous landscape. The presence of both macrophage subsets at the site of inflammation or injury suggests that they work in concert to fine-tune the immune response and maintain tissue homeostasis. This interplay between M1 and M2 macrophages is essential for a balanced and effective immune response; however, it also poses challenges for researchers investigating the role of these cells in various disease contexts.

In addition to the M1/M2 paradigm, monocytes can display a range of functional states depending on the nature and intensity of the stimuli they encounter. For example, under high-dose LPS stimulation, monocytes may enter a state of exhaustion, characterized by impaired cytokine production, reduced phagocytic activity, and altered expression of cell surface markers. Monocyte exhaustion has been implicated in the pathogenesis of various diseases, including sepsis, COVID-19, and autoimmune disorders. On the other hand, low-dose LPS stimulation can induce monocytes to enter a state of low-grade inflammation, which is often observed in common inflammatory conditions.

The simultaneous presence of M1 and M2 macrophages, as well as the varying functional states of monocytes depending on the stimulus, complicates the assessment of their contributions to disease progression and resolution.

However, the current paradigm of M1 and M2 is artificial and can not fully recapitulate the complexity of in vivo monocyte dynamics. Monocytes are not only responsive to distinct cytokines (e.g. M1 response to IFNgamma or M2 response to IL-4), they also exhibit highly complex and dynamic adaptation to varying dosages of external stimulations. Studies from our group and others recently identified that monocytes can be differentially programmed into either a low-grade inflammatory state or an exhausted state when challenged with different dosages of LPS. [55-56] Complementary to these in vitro studies, recent single cell-RNA sequencing-based studies of monocytes collected from both murine sepsis and human septic patients revealed similar patterns of monocyte exhaustion dynamics. [57-58]

Capitalizing on these studies, we aim to further elucidate the complex dynamics of monocyte subsets and their functional states in various disease contexts by re-analyzing the publically available datasets based on single-cell RNA sequencing (scRNA-seq) technology. This cutting-edge approach enables the dissection of cellular heterogeneity at an unprecedented resolution, revealing unique cell subsets, gene expression profiles, and functional properties that were previously obscured by the intricacies of mixed monocyte populations. Our comparative analyses enable the elucidation of dynamic bifurcation of monocytes into either the low-grade inflammatory state under chronic inflammatory conditions or the exhausted state under septic conditions.

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Chapter 2

Experimental Methods

2.1 Cell preparation and information gathering

Cell preparation and LPS dose (high dose 100ng/ml, low dose 100pg/ml) were described as we reported.[1-3]

The experimental data used in this study were obtained either from publicly available databases or previously published in our own laboratory. Murine data were described in our previous publication and deposited under the accession number GSE190856. Human data were obtained from recently published sources, including the Single Cell Portal SCP548, Array Express E-MTAB-9357, GSE148020, and GSE198616. Murine monocytes harvested from septic heart were as reported.[4-8]

2.2 Data Analysis

10x raw sequencing data provided, and processed and aligned using Cellranger 3.1.0. Following the alignment, the Seurat package was employed for further analysis.[9-12] The raw data underwent normalization via the 'LogNormalize' function, and variable genes were determined using the 'FindVariableFeatures' function with default parameters. Principal component analysis (PCA) was carried out on the variable genes, and the top 10-20 principal components were utilized for clustering analysis. The 'FindClusters' function facilitated cell clustering based on gene expression profiles at a resolution of approximately 0.1. Cluster visualization was achieved using Uniform Manifold Approximation and Projection (UMAP) alongside the default clustering provided by Seurat.[13-16]

Differential Expression Gene (DEG) analysis was conducted using Seurat's 'FindMarkers' function and visualized through 'heatmap' or 'dotplot' methods. Genes

displayed in heatmaps were further analyzed for Gene Ontology (GO), while dot plot analyses examined representative genes defining the fundamental characteristics of distinct memory monocyte subsets.[17]

2.3 scRNAvelo analyses

RNA velocity is a valuable metric for assessing the rate of change in gene expression, offering insights into the future state of individual cells and their developmental trajectories. This measurement can be particularly helpful in addressing questions related to monocyte differentiation and fate determination. To obtain RNA velocity estimates, the aligned data is processed using Velocyto, a Python library that quantifies transcriptional dynamics within single cells by comparing the ratios of pre-mRNA to mRNA.[18-19]

Building upon the Velocyto approach, ScVelo (single-cell velocity) is designed to provide more accurate and scalable RNA velocity estimates by employing an ordinary differential equation (ODE) based model. In ScVelo, the concentrations of pre-mRNA and mRNA are considered within the ODE model, allowing the single-cell data to be fitted accordingly. This approach not only organizes RNA expression into a consistent velocity framework but also significantly improves accuracy and robustly addresses issues related to poor data quality. Through ScVelo, researchers can gain a deeper understanding of monocyte differentiation processes and the cellular fates involved.

2.4 SCENIC

To gain further insights into the entire RNA expression profile, the data was also analyzed using SCENIC (Single-Cell rEgulatory Network Inference and Clustering), a computational method tailored for the analysis of single-cell RNA sequencing (scRNA-seq) data.[20-21] SCENIC specializes in reconstructing gene regulatory networks and identifying cell types or states based on the activity of transcription factors and their target genes.

In the scRNA-seq data, SCENIC identifies co-expressed gene sets or modules that consist of genes with highly correlated expression levels across individual cells, suggesting that they are co-regulated by common transcription factors. For each co-expression module, SCENIC predicts the potential transcription factors responsible for regulating the genes within the module, using a binding motif library to assess the targeting gene regulatory regions. Following this, SCENIC infers a gene regulatory network by connecting the transcription factors to their predicted target genes based on the co-expression and motif enrichment analysis.

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Chapter 3

Comparative analyses of monocyte dynamics

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3.1 Key features of monocyte exhaustion in vitro

As previously stated, monocytes may adopt an exhausted state followed by prolonged intense stimulations, such as those encountered during sepsis or COVID-19 infection. Understanding how monocytes become exhausted is essential for developing strategies to modulate immune responses and improve patient outcomes.

In cases of intense stimulation, existing literature indicates that monocytes may enter a state of exhaustion, characterized by a loss of immune functions, increased inflammation, and potential harm to the host.[1-3] This phenomenon is of particular interest, as it has been associated with poor clinical outcomes in various pathological conditions. Therefore, it is crucial to unravel the underlying mechanisms governing monocyte exhaustion and the factors contributing to this phenomenon.

In this section, we sought to gain a deeper understanding of monocyte exhaustion by employing single-cell RNA sequencing (scRNA-seq) to investigate the changes in monocyte subpopulations and their gene expression profiles upon exposure to intense stimulations. To achieve this, we utilized an excessive amount of lipopolysaccharide (LPS) derived from E. coli as a stimulant, simulating conditions that might lead to exhaustion. This experimental approach allowed us to capture the dynamic responses of monocytes to high-dose challenges, providing valuable insights into their activation and differentiation patterns.

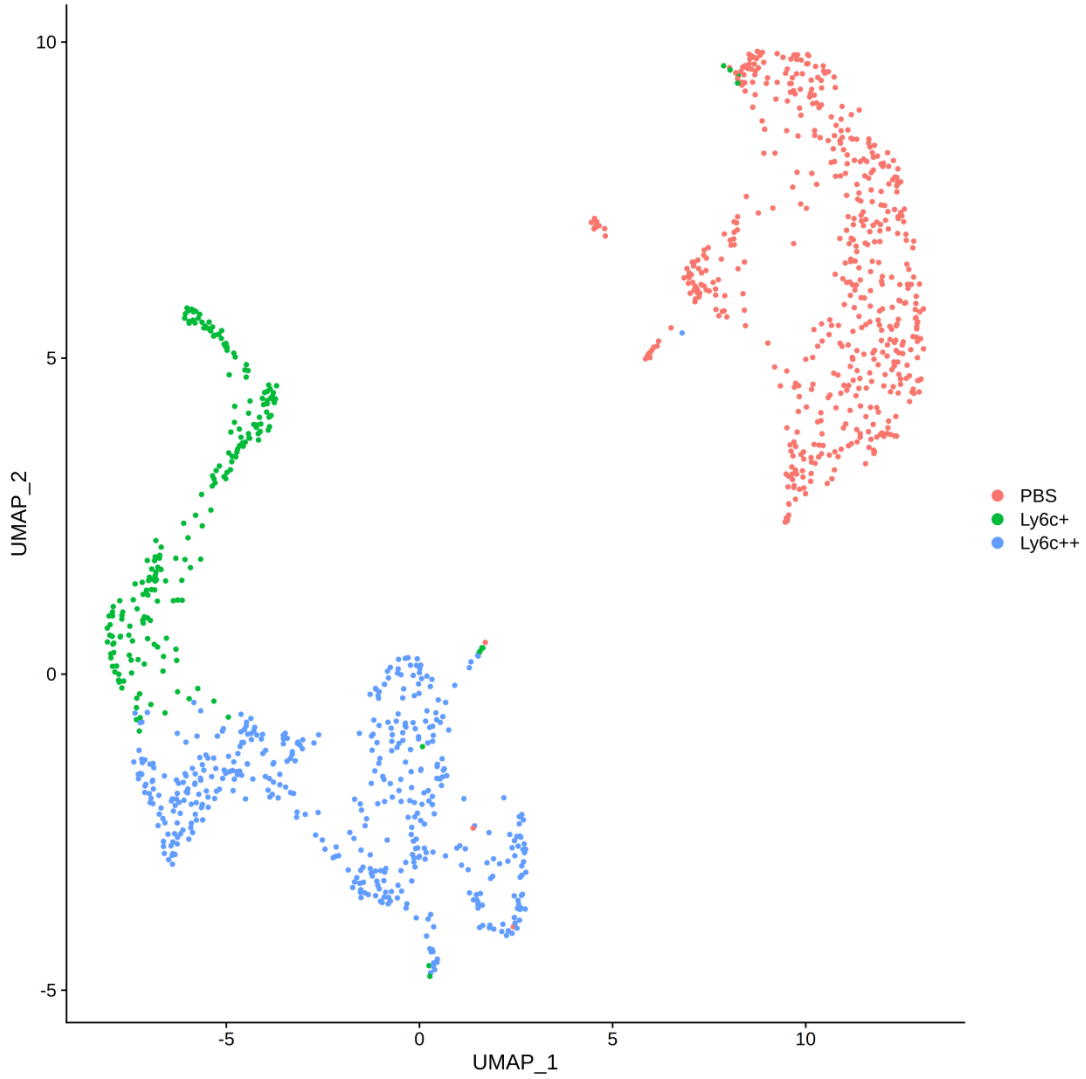


Figure 3-1-1 This UMAP representation displays the distribution of monocytes stimulated by high dose LPS in comparison to PBS control monocytes. The visualization distinctly identifies three clusters, namely the PBS control group, and two subpopulations of stimulated monocytes characterized by Ly6c+ and Ly6c++ expression.

Upon analyzing the distribution of monocytes into clusters using UMAP, we identified three distinct clusters. The resting, or PBS cluster, was located in the right upper part of the figure, while the two stimulated clusters were found at the left bottom. We then generated a heatmap using selected genes and conducted Gene Ontology (GO) analysis to characterize these clusters. Our findings revealed that both clusters of Ly6c++ d and Ly6c+ exhibit compelling features of exhaustion, ase represented by reduced differentiation, pathogenic inflammation and immune suppression.

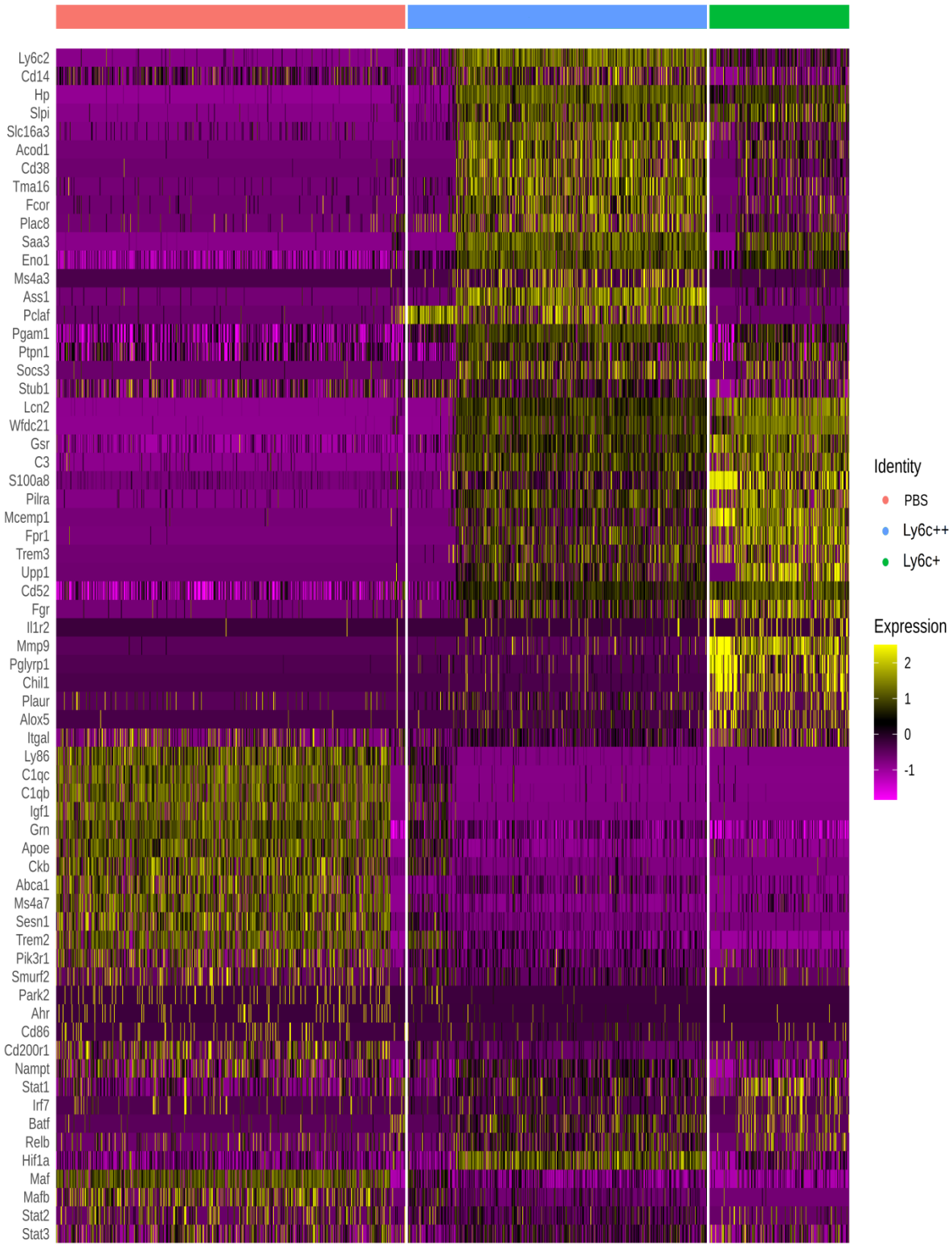


Figure 3-1-2 The heatmap presented here showcases the expression patterns of gene markers across the three identified clusters: PBS control, Ly6c+, and Ly6c++. The color gradient represents gene

expression levels, with darker shades indicating higher expression. This visualization aids in distinguishing the unique molecular signatures of each cluster.

Under high-dose LPS stimulation, monocytes exhibited increased expression of various genes involved in pathogenic inflammation, such as Cd38, Plac8, S100a8, Ptpn1, Lcn2, Ms4a3, Il1r2, Plaur, and Alox5.[4-15] Cd38 is a multifunctional enzyme that plays a role in cellular signaling, calcium regulation, leukocyte adhesion, migration, and activation during inflammation. Plac8, a protein associated with immune cell activation and cytokine production, contributes to macrophage activation and inflammatory responses. S100a8, a member of the S100 calcium-binding protein family, is involved in immune processes such as leukocyte recruitment, activation, and pro-inflammatory cytokine production. Ptpn1, a tyrosine phosphatase, regulates immune cell signaling pathways linked to inflammation and cytokine production. Lcn2, an acute-phase protein, is involved in the innate immune response by binding to bacterial siderophores, limiting iron availability, inhibiting bacterial growth, and regulating neutrophil activation and pro-inflammatory cytokine production. Ms4a3, a member of the membrane-spanning 4-domain family, is implicated in monocyte activation and inflammation through signal transduction. Il1r2 functions as a decoy receptor that modulates the inflammatory response by binding to pro-inflammatory cytokines and inhibiting their activity. Plaur contributes to extracellular matrix remodeling and cell migration during inflammation, as well as leukocyte recruitment and activation at sites of inflammation. Alox5, an enzyme that catalyzes the production of leukotrienes, is involved in inflammation and immune cell recruitment.

STAT1, an inflammation transcription factor, was highly correlated with these genes and is a key transcription factor involved in the regulation of pathogenic inflammatory responses.[16-17] STAT1 mediates the cellular response to cytokines,

such as interferons (IFNs), interleukins (ILs), and growth factors, and plays a critical role in the induction of genes that promote pathogenic inflammation.

Although both Ly6c++ and Ly6c+ clusters demonstrated pro-inflammatory and exhausted properties, there were differences in their gene expression profiles. Ly6c+ was more associated with apoptosis and immune suppression, as seen with Alox5, Trem3, Plaur, Il1r2, S100a8, Mmp9, and Itgal, which were less apparent in Ly6c++. These genes are involved in the regulation of apoptosis in various cell types, contributing to processes such as immune suppression, and modulating the activation of various signaling pathways that control cell survival and death.

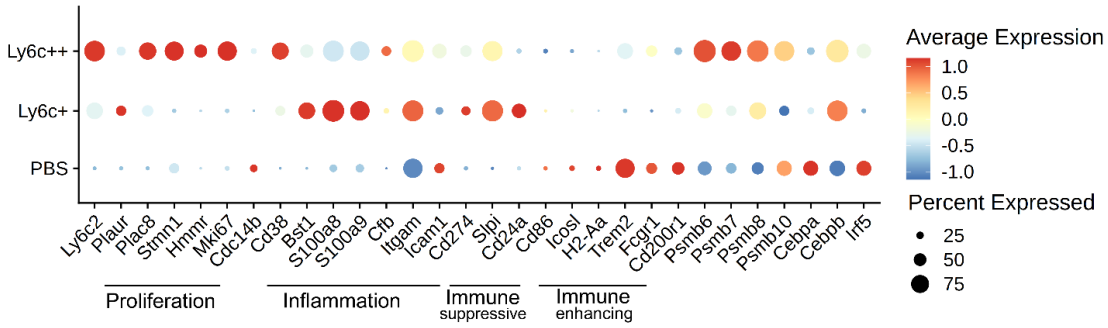


Figure 3-1-3 This dot plot presents the expression of selected gene markers specifically associated with monocyte exhaustion across different clusters.This visual representation allows for the evaluation and comparison of exhaustion traits among the various monocyte populations.

In vitro exhausted monocytes, which include both Ly6C+ and Ly6C++ groups, exhibit increased expression of genes involved in proliferation, such as Plac8, Plaur, Stmn1, Hmmr, and Mki67, and reduced expression of Cdc14b. These monocytes also show elevated levels of genes associated with pathogenic inflammation, including Cd38, Bst1, Itgam, and Icam1, and immune suppression, such as Cd274, Slpi, CD24a, and reduced levels of Cd86, Icosl, and H2-Aa.

Among the genes involved in cell proliferation, STMN1 interacts with microtubules and serves as an indicator of mitosis, while MKI67 condenses in the

nucleus during mitosis.[18-19] PLAC8 and PLAUR are also known to play roles in cell proliferation.[20-21] On the other hand, CDC14B has recently been shown to promote cell differentiation and prevent excessive proliferation. In the context of immune-suppressive genes, CD274 (PD-L1) protein effectively suppresses T cell functions, while SLPI and CD24A proteins participate in anti-inflammatory immune suppression.[22-24] In contrast, CD86, ICOSL, and H2-Aa proteins contribute to enhancing adaptive immune functions.[25-27]

Regarding pathogenic inflammatory genes, both CD38 and BST1 proteins result in the depletion of cellular metabolic fuel NAD and the generation of inflammatory secondary mediators.[28-29] CD11B and ICAM1 proteins are involved in the inflammatory adhesion of monocytes to vasculature. Furthermore, as less-differentiated monocytes, in vitro exhausted monocytes lose phagocytic receptors Trem2, Fcgr1, and immune modulator Cd200r1.[30-34] Collectively, in vitro exhausted monocytes display altered gene expression profiles characterized by reduced differentiation, enhanced pathogenic inflammation, and immune suppression.

To gain further mechanistic insights into their generation, we examined key signaling components and transcription factors modulating monocyte behaviors. Proteasomes are known to be elevated in activated monocytes, and we observed an increase in proteasome components such as Psmb6 and Psmb10 in exhausted monocytes. SOCS3 protein is known to be elevated in "tolerant" monocytes with reduced expression of immune-enhancing mediators, and we observed increased Socs3 in exhausted monocytes.

In terms of transcription factors, C/EBPα and C/EBPβ proteins are differentially involved in monocyte differentiation and activation.[35-36] C/EBPα facilitates cell

differentiation, while C/EBPβ promotes inflammatory activation. C/EBPα is also involved in suppressing the generation of myeloid-derived suppressor cells. We observed that C/ebpα levels are reduced in exhausted monocytes, correlating with reduced differentiation. In contrast, C/ebpβ levels are elevated in exhausted monocytes, corresponding to increased pathogenic inflammation. IRF5 protein is a key transcription factor promoting the expression of immune-enhancing genes such as Cd86, H2-Aa, and Cd40.[37] Exhausted monocytes display reduced Irf5 levels, which correspond to decreased expression of Cd86 and H2-Aa.

3.2 Key features of monocyte exhaustion in vivo

To determine the scope of our study, we compared our findings to those reported in other research. One independent study published in Nature Metabolism provided single cell profiling of infiltrating macrophages and monocytes in murine septic heart tissues.[38] The authors primarily focused on interpreting the alterations of tissue macrophages in a time-dependent manner following the initial insult of cecal ligation and puncture, with an initial depletion of TREM2 positive macrophages and rapid restoration of TREM2 macrophages 7-21 days after the initial sepsis.

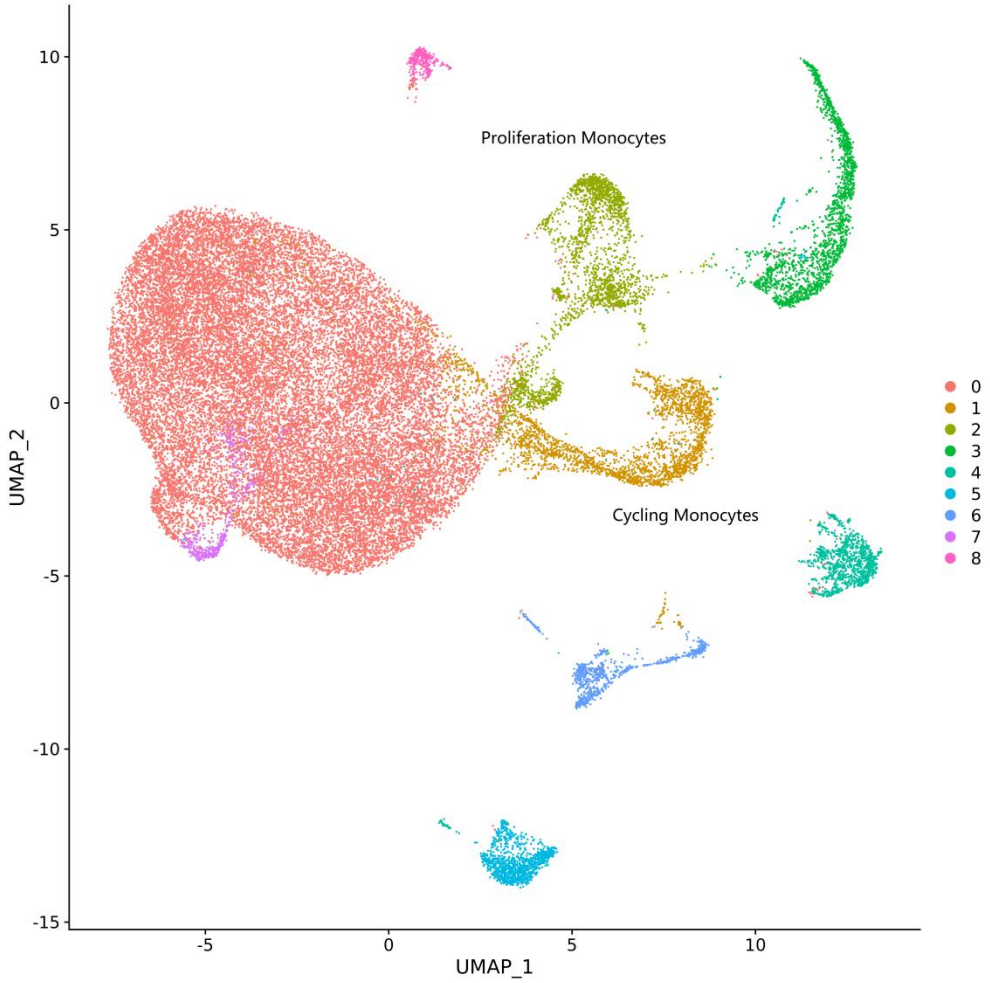


Figure 3-2-1 This UMAP (Uniform Manifold Approximation and Projection) visualization illustrates the distribution of mouse cells over different time points, including the baseline (day 0, negative control), and 3, 7, and 21 days following the onset of septic shock. The map prominently features clusters of cycling and proliferating monocytes, providing a temporal and spatial representation of the dynamic shifts in monocyte populations under septic shock conditions over time. This helps elucidate the evolution of immune responses during the progression of severe infection.

Although the authors did not provide further details, we noticed a sustained expansion of infiltrating monocytes within the septic heart that lasted throughout the 21-day observation period and never returned to baseline. There was also an expansion of a highly proliferative "cycling cell" population that persisted throughout the 21-day post-sepsis period.

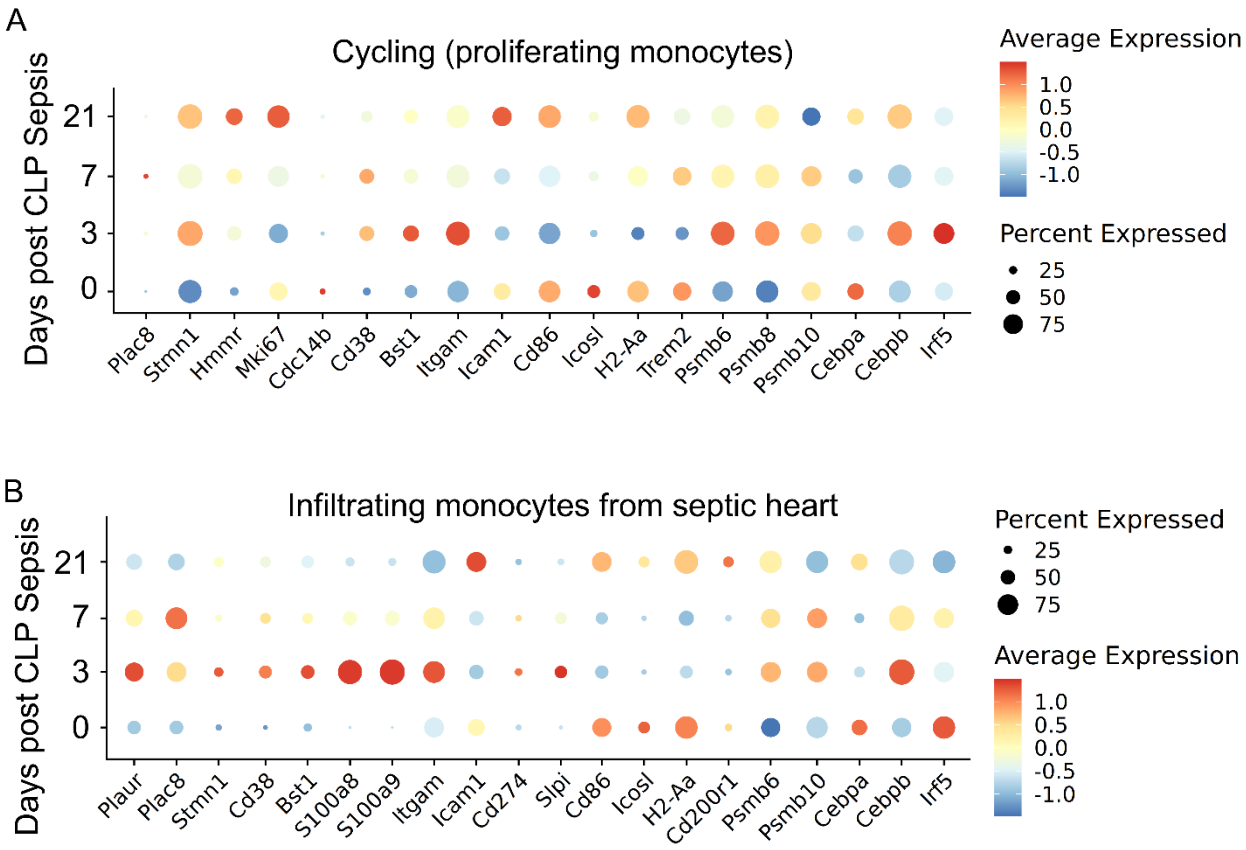


Figure 3-2-2 Analyses of monocyte memory dynamics collected from septic animals. scRNAseq data collected from mice subjected to cecal ligation and puncture were used for focused analyses of either cycling myeloid cells (A) or infiltrating monocytes (B) in the heart tissue. Representative genes were captured in the dot plot.

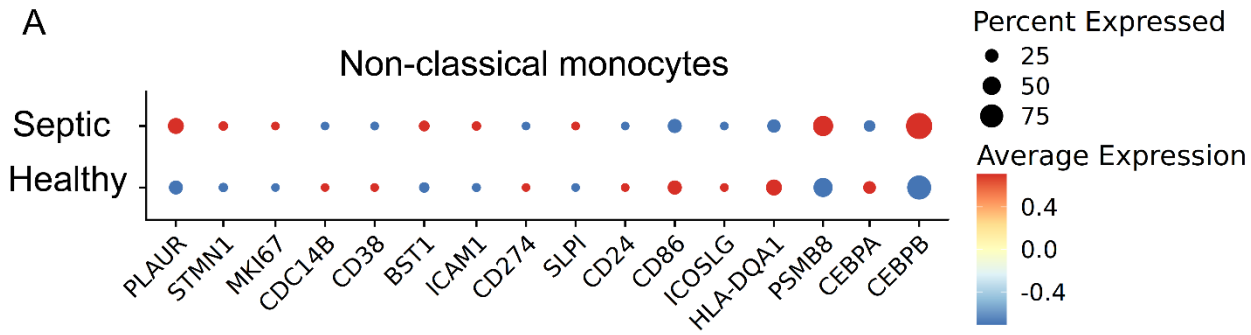
We then analyzed the monocyte gene expression profiles over time following the onset of murine sepsis. We observed that at day 3 post-sepsis, infiltrating monocytes within the heart underwent significant exhaustion, reflected in pathogenic inflammation (increased Cd38, Bst1, Itgam) and immune suppression (decreased Cd86 and increased Cd274), as well as reduced differentiation and enhanced proliferative potential (increased Stmn1). Many of these key features persisted throughout the 21-day period. Mechanistically, we observed similar activation of C/ebpβ and reduction of C/ebpα as well as Irf5. Collectively, our systems analyses reveal highly conserved exhaustion profiles of monocytes from human septic patients, murine experimental sepsis, and in

vitro exhausted monocytes. Similar profiles of exhaustion can also be seen in the cycling monocytes.

3.3 Key features of monocyte exhaustion in human

Using recently available scRNAseq data of human septic monocytes, we conducted comparative analyses of key genes representing monocyte exhaustion between murine and human systems.[39] Human monocytes can be divided into classical, intermediate, and non-classical subpopulations based on the expression levels of CD14 and FCGR3A (CD16) markers.[40-41] We analyzed the relative expression of previously described genes identified in exhausted murine monocytes among human monocyte subsets from healthy and septic patients.

As reported independently, septic patients exhibit a reduction of non-classical monocytes and an expansion of intermediate and classical monocytes. We then separately compared gene expression profiles based on the scRNAseq clusters of each monocyte subset.



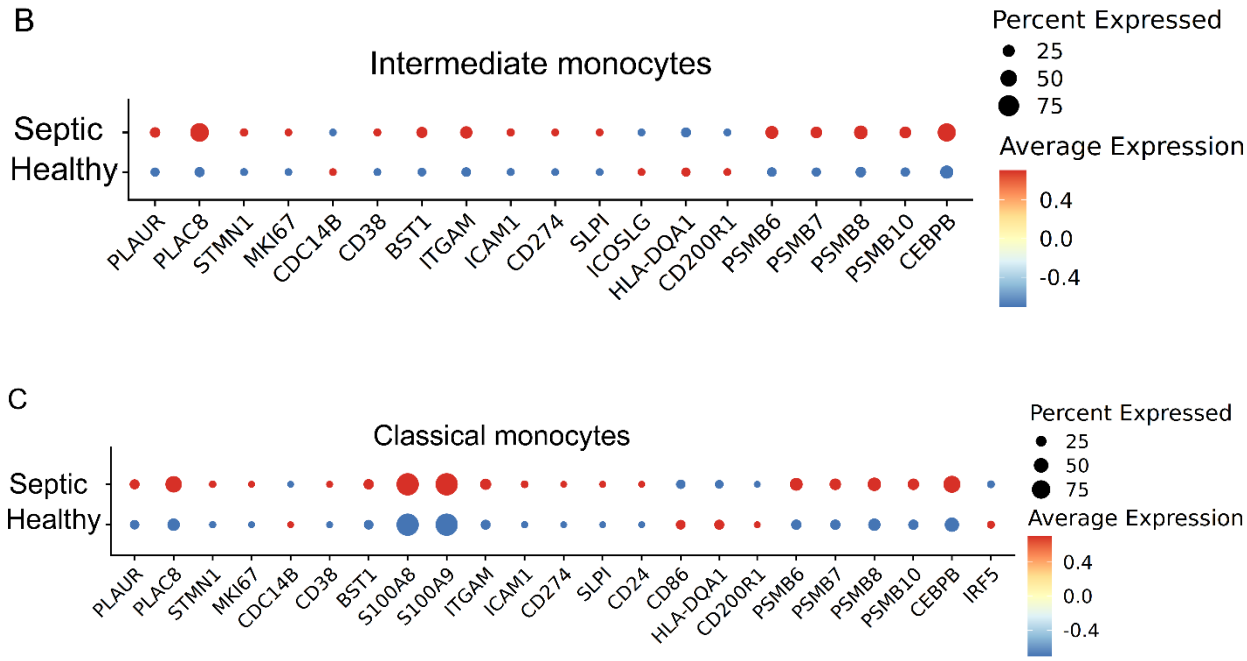


Figure 3-3-1 Capturing of key exhaustion maker genes from each subset of monocytes from septic patients. A. Dot plot analyses capturing selected genes comparing the non-classical subset of monocytes from healthy or septic patient blood. B. Dot plot analyses capturing selected genes comparing the intermediate subset of monocytes from healthy or septic patient blood. C. Dot plot analyses capturing selected genes comparing the classical subset of monocytes from healthy or septic patient blood.

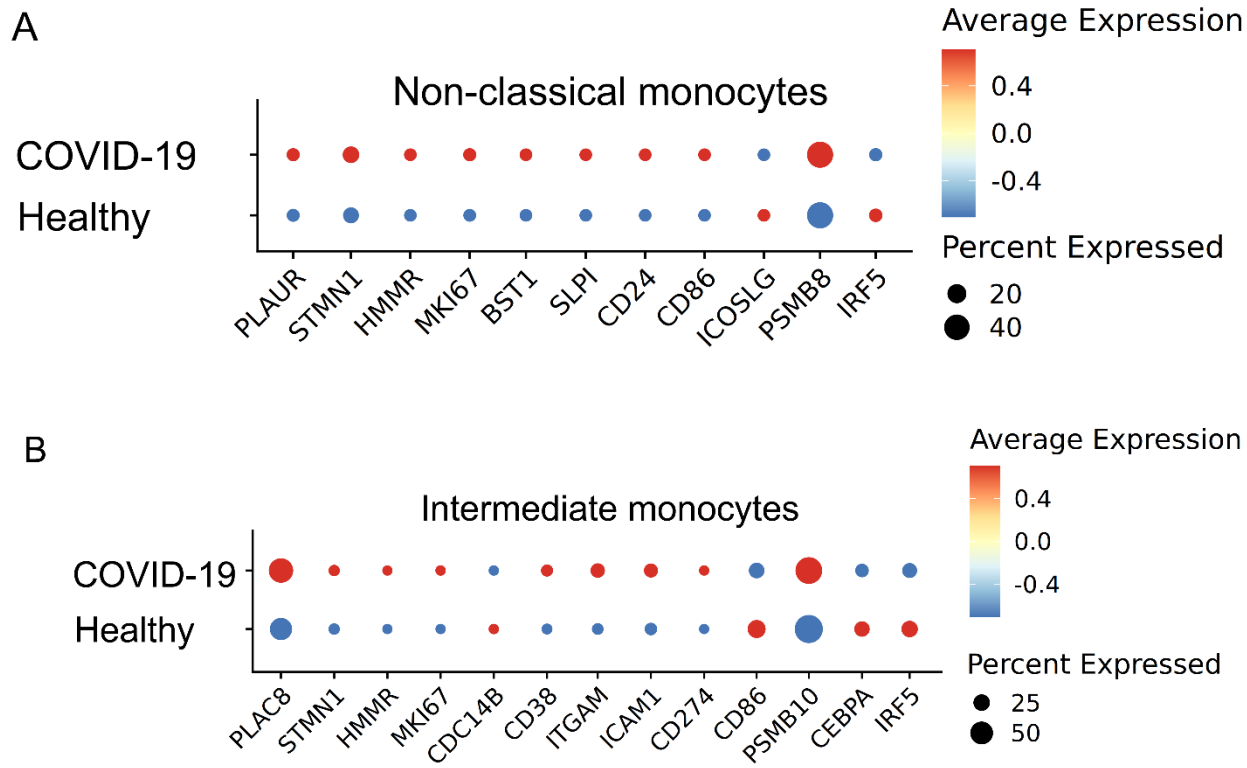
The less-differentiated signatures (elevated STMN1, MKI67, and reduced CDC14B) are observed to be elevated in septic non-classical monocytes, suggesting the conserved characteristic of reduced differentiation and proliferative potential of septic monocytes. The immune-enhancing genes such as CD86, ICOSL, and HLA-DQA1 are all reduced, suggesting the development of immune suppression. Key transcription factor C/EBPα involved in differentiation, as well as the transcription factor IRF5 involved in immune-enhancing gene expression, were both reduced in septic non-classical monocytes.

Among the expanded intermediate and classical subsets, septic monocytes also exhibit elevated levels of proliferative genes (STMN1, MKI67). Furthermore, additional pathogenic inflammatory genes are elevated, including CD38, BST1, ICAM1, and ITGAM, as well as the immune suppression gene CD274 (PD-L1). Mechanistically, proteasome components such as PSMB6 and 10, as well as the

pathogenic inflammatory transcription factor C/EBPβ, were all elevated in the intermediate and classical subsets of septic monocytes compared to healthy subsets. Together, our targeted analyses confirm that human septic monocytes share the cardinal features of experimentally exhausted monocytes with reduced differentiation, pathogenic inflammation, and immune suppression, key attributes leading to compromised host defense and multi-organ injuries.

There is growing interest in COVID-related immune alterations, which may share similarities with human sepsis.[42] We therefore further investigated whether key exhaustion features may also be present in monocytes obtained from COVID patients.

As reported through the scRNAseq data, COVID patients similarly experience a reduction of the non-classical population and an expansion of the classical monocyte population. We specifically compared the classical monocytes among healthy and COVID patients.



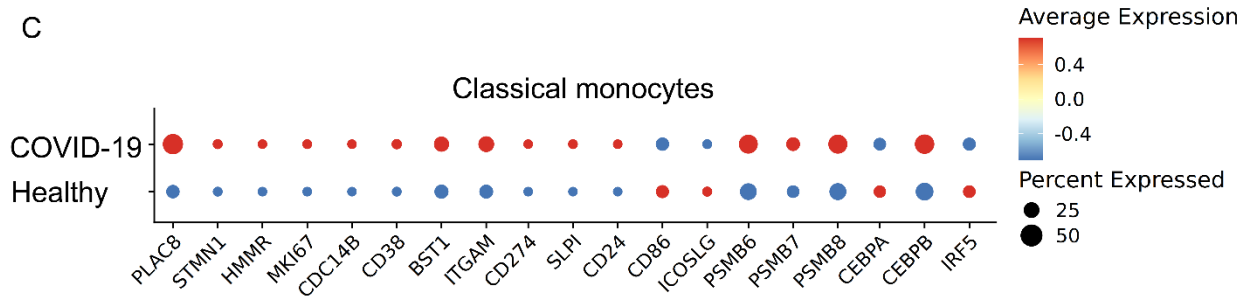


Figure 3-3-2 Capturing of key exhaustion maker genes from each subset of monocytes from COVID-19 patients. A. Dot plot analyses capturing selected genes comparing the non-classical subset of monocytes from healthy or COVID-19 patient blood. B. Dot plot analyses capturing selected genes comparing the intermediate subset of monocytes from healthy or COVID-19 patient blood. C. Dot plot analyses capturing selected genes comparing the classical subset of monocytes from healthy or COVID-19 patient blood.

We observed similar profiles of enhanced proliferative potential (elevated STMN1 and MKI67), pathogenic inflammation (elevated CD38, BST1, ITGAM), and immune suppression (elevated PD-L1, SLPI, CD24 and reduced ICOSL). Mechanistically, we observed elevated proteasome components and increased C/EBPβ in COVID patient monocytes. Our findings confirm that key signatures of monocyte exhaustion are conserved in patients with systemic inflammation caused by COVID-19 infection.

3.4 Key features of monocyte low-grade inflammation in vitro

Monocytes can encounter low dose stimulations in various contexts, which can significantly impact their behavior and function in immune responses and inflammatory processes. It is essential to understand monocyte subpopulations' roles under low dose stimulation to elucidate their roles in immune responses.

In this study, we employed single-cell RNA sequencing (scRNA-seq) to investigate the changes in monocyte subpopulations and their gene expression profiles upon low dose stimulation. We utilized lipopolysaccharide (LPS) from E. coli as a stimulant to

simulate this common situation, which allowed us to capture the dynamic responses of monocytes to low dose challenges.

Figure 3-4-1 This UMAP visualization displays the different clusters of monocytes following stimulation with low-dose LPS. Several distinct clusters emerge, including the PBS control group and three additional clusters, labeled as L1, L2, and L3. This UMAP representation provides a graphical overview of the diverse cellular responses elicited under low-dose LPS stimulation, highlighting the heterogeneity within the monocyte population.

Upon stimulation with low dose LPS, our scRNA-seq analysis identified distinct monocyte clusters, each exhibiting a unique gene expression profile. We observed four major clusters: L1, L2, L3, and PBS. The L1 and L2 clusters represented the majority of the monocyte population and displayed characteristics of classical activated and proinflammatory monocytes. In contrast, the L3 cluster consisted of proliferating monocytes with no strong indications of inflammation. The PBS cluster, serving as the control group, displayed an overall low expression of genes, indicative of a resting monocyte state.

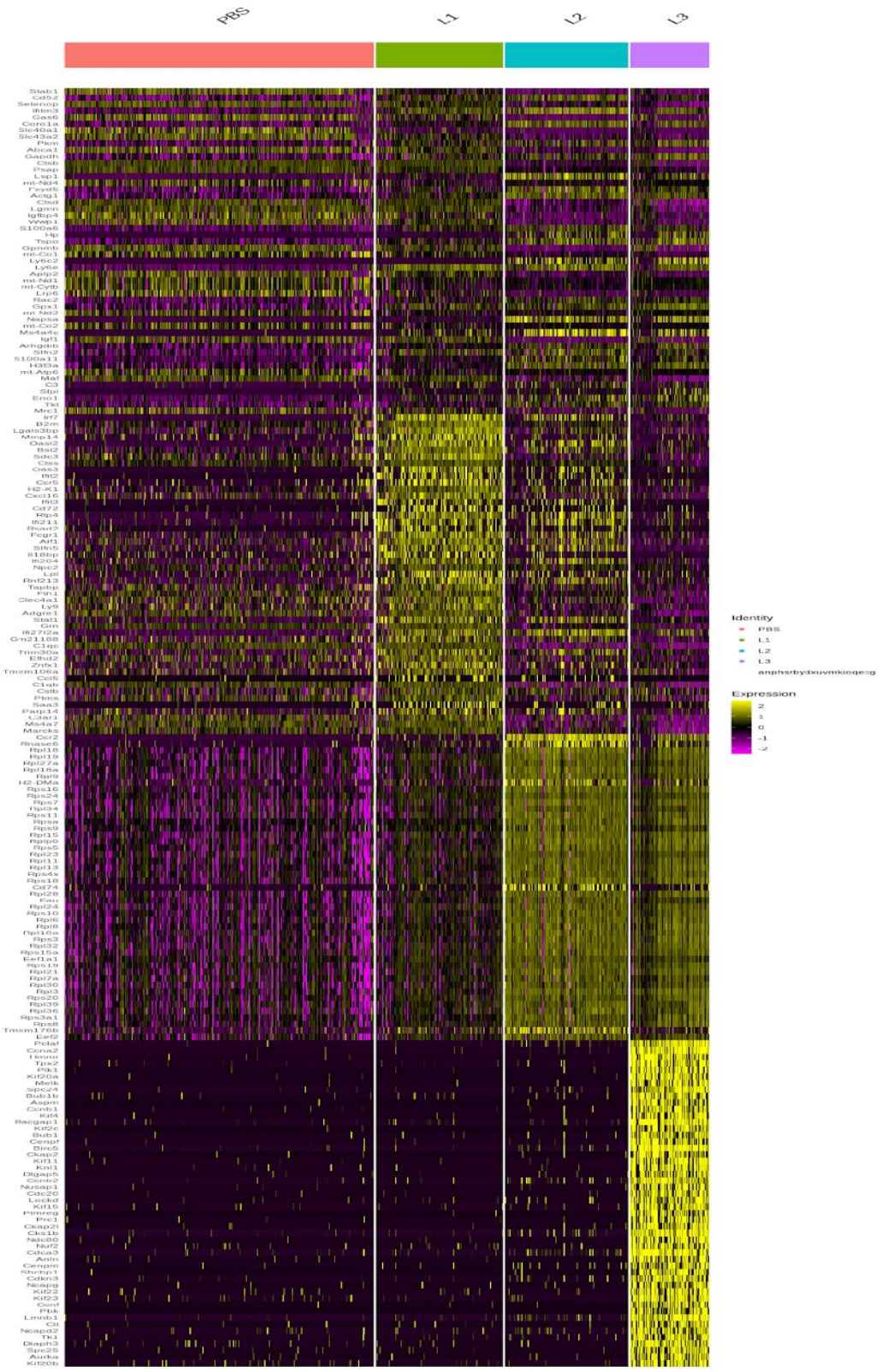


Figure 3-4-2 This heatmap depicts the expression of gene markers identified by the Seurat algorithm in monocytes stimulated with a low dose of LPS. The color intensity corresponds to the level of gene expression, providing a visual representation of the molecular response to low-dose LPS stimulation across the various gene markers. This data helps to elucidate the molecular mechanisms underlying monocyte response to different inflammatory stimuli.

The PBS cluster served as a control group for resting monocytes, exhibiting an overall low expression of genes. Comparing the gene expression profiles of the L1, L2, and L3 clusters with the PBS cluster revealed a clear distinction in the activation state and functional roles of these monocyte subsets. The L1 and L2 clusters displayed elevated expression of inflammation markers, while the L3 cluster showed a proliferation-associated gene expression profile. These findings highlight the diverse functional states and roles of monocytes in response to different conditions and stimulations.

A detailed gene expression analysis of the L1 and L2 clusters revealed several shared features. Both clusters displayed elevated expression of genes associated with inflammation, such as Irf7 (interferon regulatory factor 7), which plays a critical role in the regulation of interferon-dependent immune responses; Cxcl16 (C-X-C motif chemokine ligand 16), a chemokine involved in the recruitment and activation of immune cells; Ifi204 (interferon-inducible protein 204), which participates in the innate immune response and regulation of cell proliferation; Ms4a7 (membrane-spanning 4-domains subfamily A member 7), a protein involved in immune system processes and signaling; Stat1 (signal transducer and activator of transcription 1), a transcription factor that mediates cellular responses to interferons and other cytokines; and Ccr5 (C-C chemokine receptor type 5), a receptor for various inflammatory chemokines that plays a role in the recruitment and activation of immune cells.[43-48] These genes serve as markers for classical monocytes and macrophages, confirming the proinflammatory nature of these clusters.

However, a key difference between the L1 and L2 clusters was observed in the expression of ribosomal protein genes. The L2 cluster exhibited high expression of Rps

and Rpl family genes, suggesting an active role of ribosome in the production of cytokines and chemokines in this cluster.

The L3 cluster, consisting of proliferating monocytes, displayed a distinct gene expression profile compared to the L1 and L2 clusters. Although overexpression of RPS (ribosomal protein S) and PRL (ribosomal protein L) genes was observed in L3, this phenomenon may have resulted from impurities originating from the L2 cluster. The L3 cluster also exhibited increased expression of Cenpf (centromere protein F), Cenpm (centromere protein M), and Kif (kinesin family) genes, which are involved in cellular proliferation.[49-51]

Cenpf is a key component of the kinetochore, playing a crucial role in chromosome segregation during cell division. Cenpm is another centromere-associated protein, involved in the assembly of functional kinetochores and chromosome segregation during mitosis. Kif genes encode kinesin motor proteins, which are essential for intracellular transport and cell division, specifically in the organization and dynamics of the mitotic spindle.

The ribosome activity in the L3 cluster might be attributed to the increased demand for proliferation to replenish the monocyte population upon stimulation. The upregulation of these genes highlights the importance of cellular proliferation in the L3 cluster, as these cells work to maintain and expand the monocyte population in response to external stimuli. The gene profile of the L3 cluster suggests that it represents a non-classical monocyte subset. Non-classical monocytes play a crucial role in maintaining vascular homeostasis, suppressing pathogenic inflammation, and promoting tissue repair and angiogenesis.

The scRNA-seq analysis of monocytes under low dose stimulation revealed distinct monocyte clusters and their unique gene expression profiles. The identified clusters displayed diverse functional states and roles in response to different conditions and stimulations.

The impact of Tram deficiency on monocyte subpopulations and their response to low-dose LPS stimulation was assessed through scRNA-seq analysis.

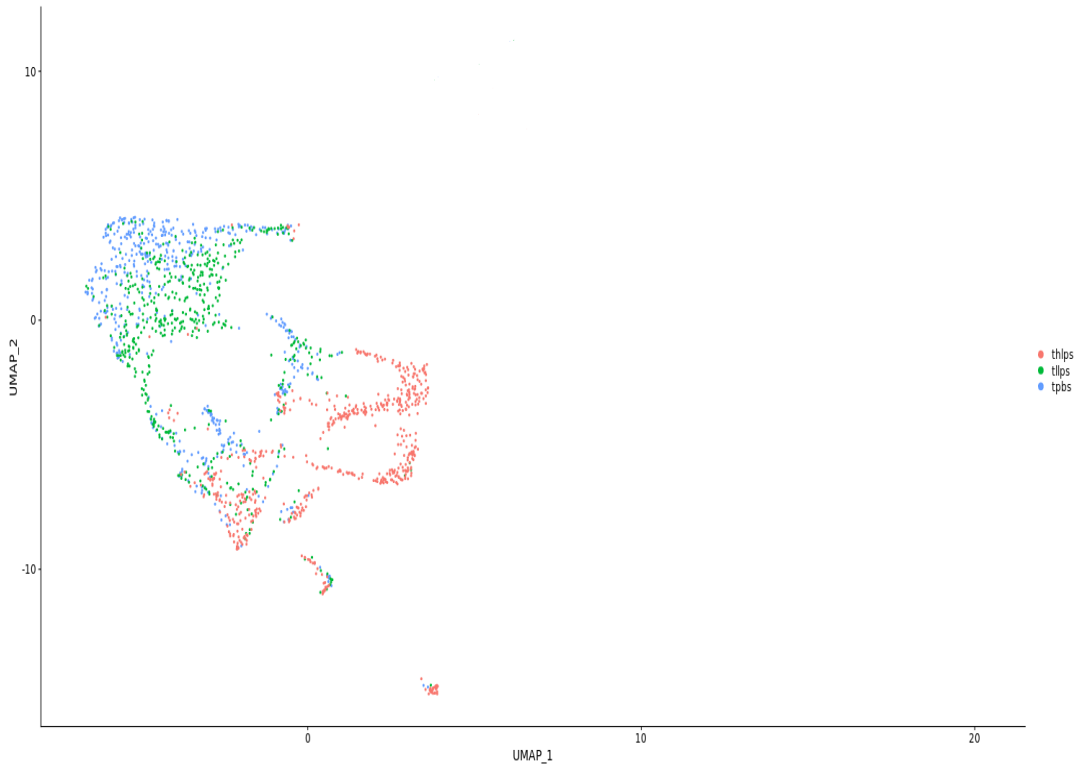


Figure 3-4-3 This UMAP visualization presents a comparative analysis of Tram-/- monocytes under different conditions. The clusters are color-coded to represent distinct stimulations: red for high-dose LPS stimulation, green for low-dose, and blue for PBS control cells. Interestingly, the resting cells gravitate towards the PBS side, while the L3 cluster is situated on the opposite end. Notably, due to the deletion of Tram, the L1 and L2 clusters that are usually present in normal monocytes are absent in this case. The visualization underscores the significant role of Tram in monocyte response to LPS stimulation.

The absence of Tram in monocytes resulted in a notable alteration of the subpopulation distribution, with the elimination of L1 and L2 clusters, which predominantly consist of classical monocytes, and an increase in the proportion of the L3 cluster. This observation underscores the critical role of Tram in monocyte immune

responses and suggests that knocking out Tram could potentially mitigate pathogenic inflammation.

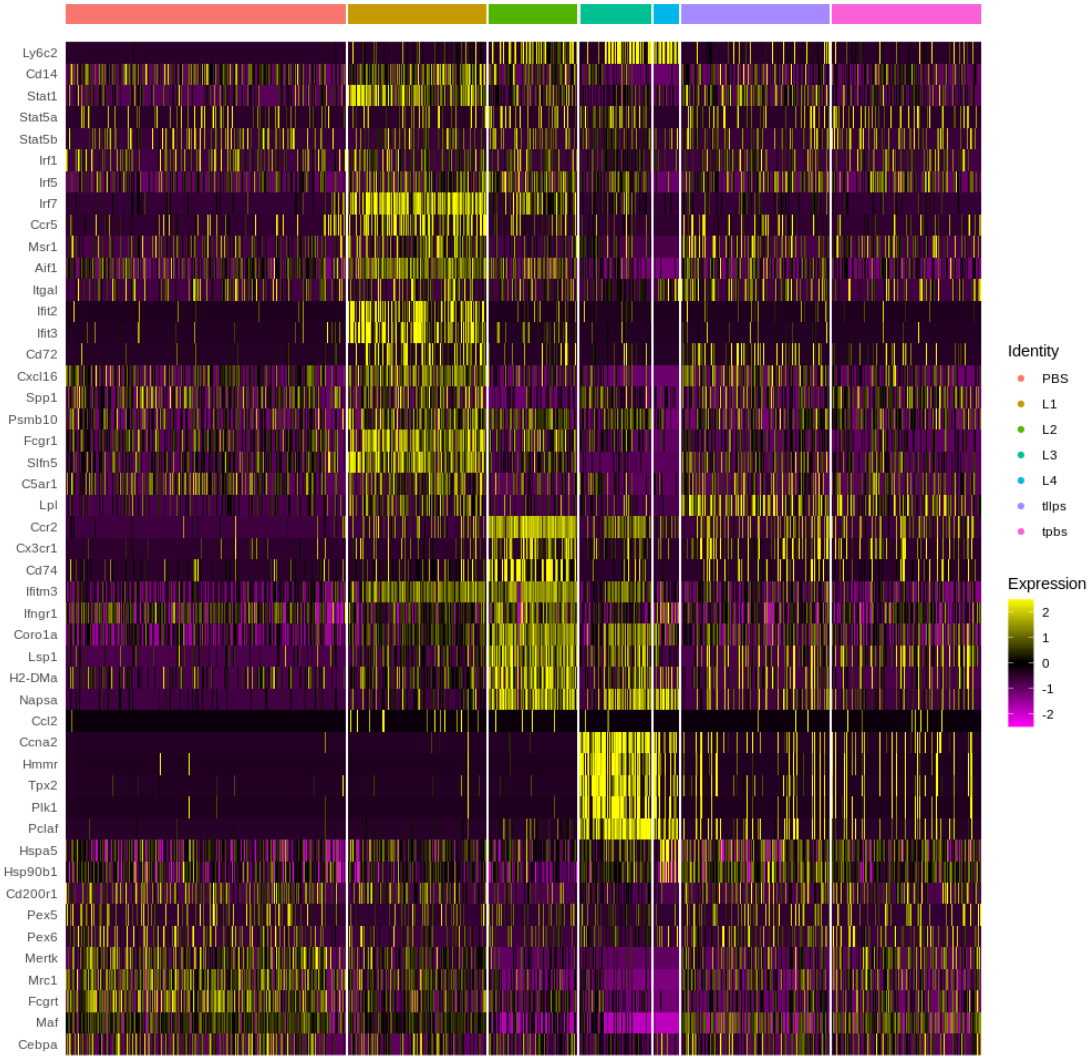


Figure 3-4-4 This heatmap presents a comparative analysis of gene expression profiles across monocytes exposed to low-dose LPS and Tram-/- monocytes. Interestingly, Tram-/- monocytes appear to exhibit a gene expression profile closely resembling that of PBS control monocytes, suggesting the critical role of Tram in mediating monocyte response to LPS stimulation. The similarities and differences highlighted in this heatmap provide valuable insights into the molecular mechanisms governing monocyte activation and response.

Differential gene expression (DEG) analysis revealed that L1 and L2 clusters are characterized by Ly6C+ and Ly6C++ markers, consistent with classical monocytes. Compared to unstimulated cells, both clusters expressed high levels of inflammatory mediators, such as Ccr2 (C-C chemokine receptor type 2), which plays a role in

recruiting inflammatory cells, Ccr5 (C-C chemokine receptor type 5), involved in immune cell activation and migration, Spp1 (secreted phosphoprotein 1, also known as osteopontin), regulating immune cell adhesion and migration, Cd72 (cluster of differentiation 72), associated with B-cell activation and differentiation regulation, and Cd74 (cluster of differentiation 74), involved in antigen processing and presentation.[52-56]

Signature inflammatory transcription factors were highly expressed in both clusters, including Stat1 (signal transducer and activator of transcription 1), mediating cellular responses to interferons and cytokines, Stat5 (signal transducer and activator of transcription 5), involved in lymphocyte development and activation, Irf1 (interferon regulatory factor 1), playing a role in interferon-induced immune responses, Irf5 (interferon regulatory factor 5), implicated in proinflammatory cytokine production activation, Irf7 (interferon regulatory factor 7), crucial for type I interferon-dependent immune response regulation, and Aif1 (allograft inflammatory factor 1), participating in macrophage activation and immune response modulation.[57-62]

Moreover, interferon-stimulated genes, such as Ifit2, Ifit3, Ifitm3, and Ifngr1, were highly expressed in both clusters, indicating similar low-grade inflammatory features. Notably, there were differences in the expression pattern between the two subsets: Ly6C+ L1 monocytes showed higher expression of C5ar1, Ccr5, Cd72, Spp1, and Aif1, while Ly6C++ L2 monocytes displayed higher expression of Cd74, Ccr2, and Ly6c.

Low-dose LPS treatment suppressed the expression of anti-inflammatory mediators (Cd200r) and pexophagy (Pex5). Furthermore, scRNA-seq identified an additional unique Ly6C++ monocyte subset (L3). Compared with the classical inflammatory CD14+Ly6C++ monocytes, the L3 cells were less inflammatory (CD14-)

and exhibited a proliferative profile based on Gene Ontology (GO) gene enrichment analysis. This pattern suggests a potential non-exhaustive and non-pathogenic mode of action for L3 cells.

DEG analysis of Tram-/- monocytes demonstrated that LPS stimulation failed to polarize the cells, with repeated experiments showing that stimulated cells were similar to resting PBS cells. Compared to the wild-type (WT) inflammatory clusters, The absence of Tram in monocytes led to the elimination of L1 and L2 clusters, which were predominantly classical monocytes, and an increase in the proportion of the L3 cluster. Altogether, the Tram knockout provided a clearer picture of monocyte response under low-dose stimulation. However, the clinical application of gene suppression as a therapeutic strategy may be limited by potential issues related to clinical stages.

The endoplasmic reticulum (ER), an organelle crucial for protein synthesis and folding, can become overwhelmed and dysfunctional due to various cellular stressors, leading to an accumulation of misfolded or unfolded proteins. This condition, termed ER stress, has been implicated in enhancing inflammatory responses and contributing to the progression of various inflammatory diseases. The underlying mechanism, while not fully delineated, has been associated with the potential modulation of 4PBA, a known inhibitor of ER stress. Thus, 4PBA application could potentially alleviate ER stress, leading to a subsequent attenuation of the inflammatory response.

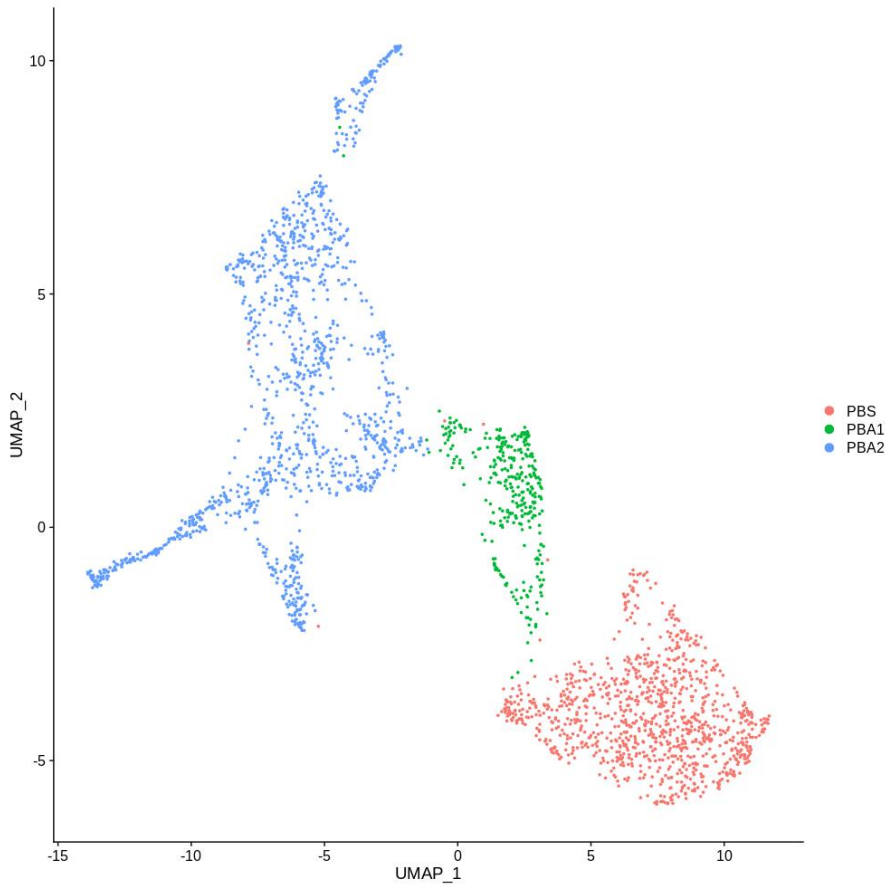


Figure 3-4-5 This UMAP representation showcases monocytes treated with PBA, segregated into three distinct clusters: a PBS control cluster representing resting monocytes, a smaller PBA1 cluster, and a larger PBA2 cluster. The relative positions and distributions of these clusters provide insights into the varying responses of monocytes to PBA treatment, with the PBA1 and PBA2 clusters indicating distinct cellular states induced by the intervention.

Upon 4PBA administration, we observed a bifurcation of monocytes into two distinct clusters: PBA1 and PBA2. PBA2, housing the majority of stimulated monocytes, was positioned farther from the control (PBS) cluster, while PBA1, albeit housing fewer monocytes, was closely situated to the control cluster.

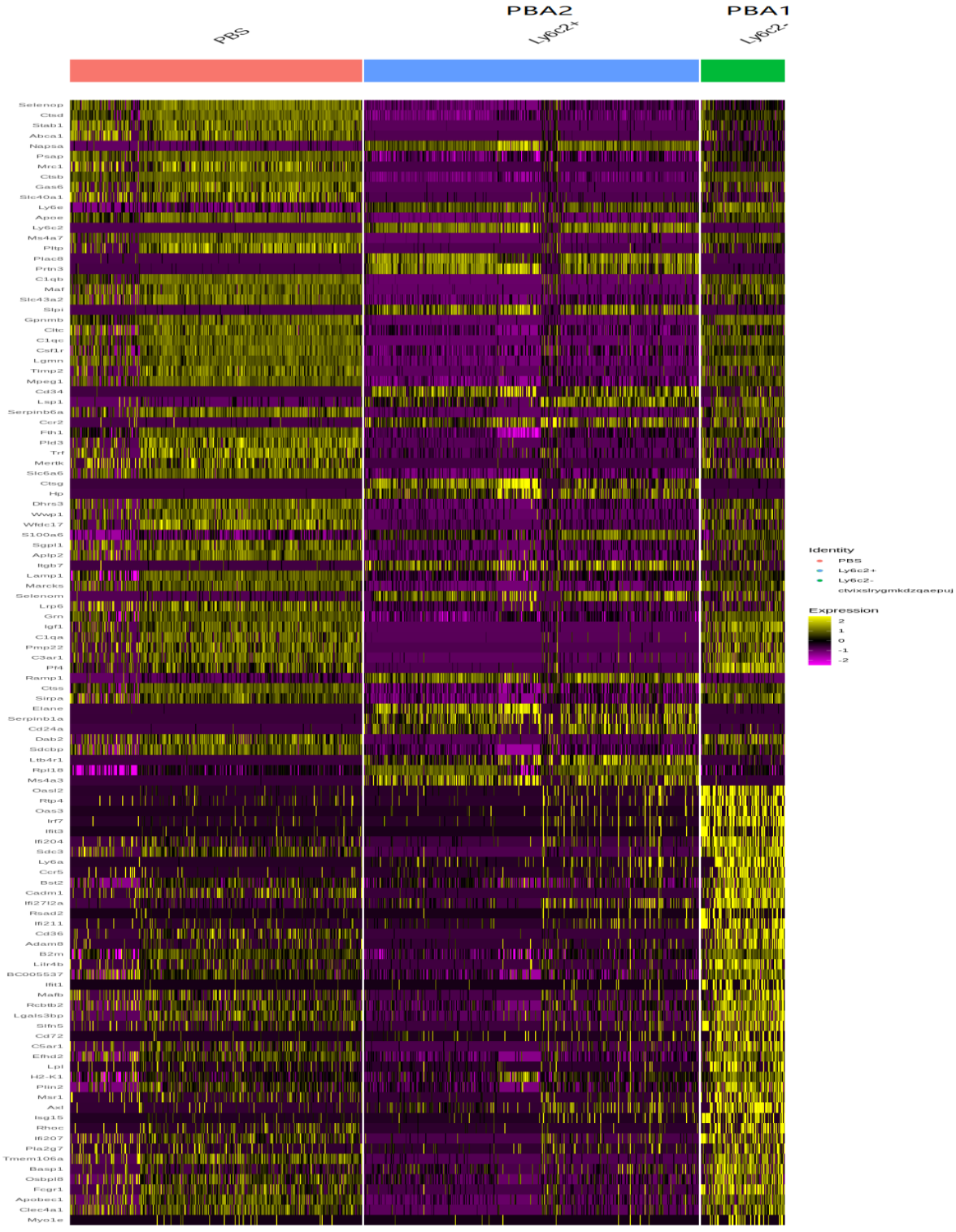


Figure 3-4-6 The heatmap presents the expression of gene markers in monocytes treated with PBA, as identified through the Seurat algorithm. The color gradient indicates the expression level of each gene, illustrating the molecular changes induced by PBA treatment. This data aids in understanding the impact of PBA on monocyte gene expression and potential anti-inflammatory mechanisms.

PBA1 was characterized by elevated expression of inflammation-associated genes such as Ly6a and Ly6e, genes encoding lymphocyte antigens; Irf7, a transcription factor playing a crucial role in interferon-induced immune responses; Mmp8, a matrix metalloproteinase involved in extracellular matrix remodeling; and S100a6 and S100a11, members of the S100 protein family involved in intracellular calcium signaling. Also noteworthy was the expression of Ccr5, a chemokine receptor involved in immune cell migration; Ifitm3, a protein linked to immune cell activation; Stat2, a signal transducer and activator of transcription; Pf4, a platelet factor involved in wound healing and inflammation; Cenpa, a centromere protein essential for cell division; and Klf6, a transcription factor involved in cell proliferation and differentiation.[63-73] These genes are typically associated with classical monocytes or macrophages, implicating the cells of the PBA1 cluster as such. Interestingly, 4PBA treatment resulted in a reduction in classical clusters compared to the previous LPS stimulation experiments, indicating a dampening of the inflammatory response.

Conversely, PBA2 exhibited upregulated expression of Csf1r, a cell surface receptor critical for monocyte and macrophage production, and Hmgb2, a chromatin-associated protein involved in DNA repair and apoptosis.[74-75] However, Plac8, a protein associated with cell proliferation; Mertk, a receptor tyrosine kinase involved in phagocytosis; and Apoe, a major component of lipoproteins, were downregulated. Furthermore, this cluster showed an anti-apoptotic profile, evidenced by the overexpression of Cd34, a cell surface glycoprotein involved in cell-cell adhesion; Grn, a secretory protein implicated in cell proliferation and tissue repair; and Cd24a, a cell adhesion molecule, along with the suppression of Apoe expression.[76-81] Such gene expression patterns suggest that PBA2 resembles the proliferative cluster. The significant shift towards this proliferative cluster post-4PBA treatment suggests that

4PBA promotes proliferation while concurrently reducing the number of inflammatory monocytes.

3.5 Key features of monocyte low-grade inflammation in autoimmune

We then turned our attention to chronic low-grade inflammatory diseases, and examined whether monocytes from humans with chronic diseases may have distinct alteration features of monocytes. This is based on our data that monocytes with prolonged challenges with low-grade inflammatory signals bifurcate into a distinct low-grade inflammatory state, reflected in pathogenic inflammation and immune-enhancing characteristics.

To test this, we examined recently published scRNAseq data collected from human patients with Vogt-Koyanagi-Harada (VKH) disease, which is a systemic autoimmune disorder characterized by a dysregulated immune response resulting from altered monocyte phenotype and function.[82] Patients with VKH disease exhibit hyperactivity of monocytes and elevated secretion of pro-inflammatory cytokines, which are implicated in the observed inflammation in affected tissues. While the exact cause of VKH disease remains unknown, it is believed to result from a combination of genetic and environmental factors.

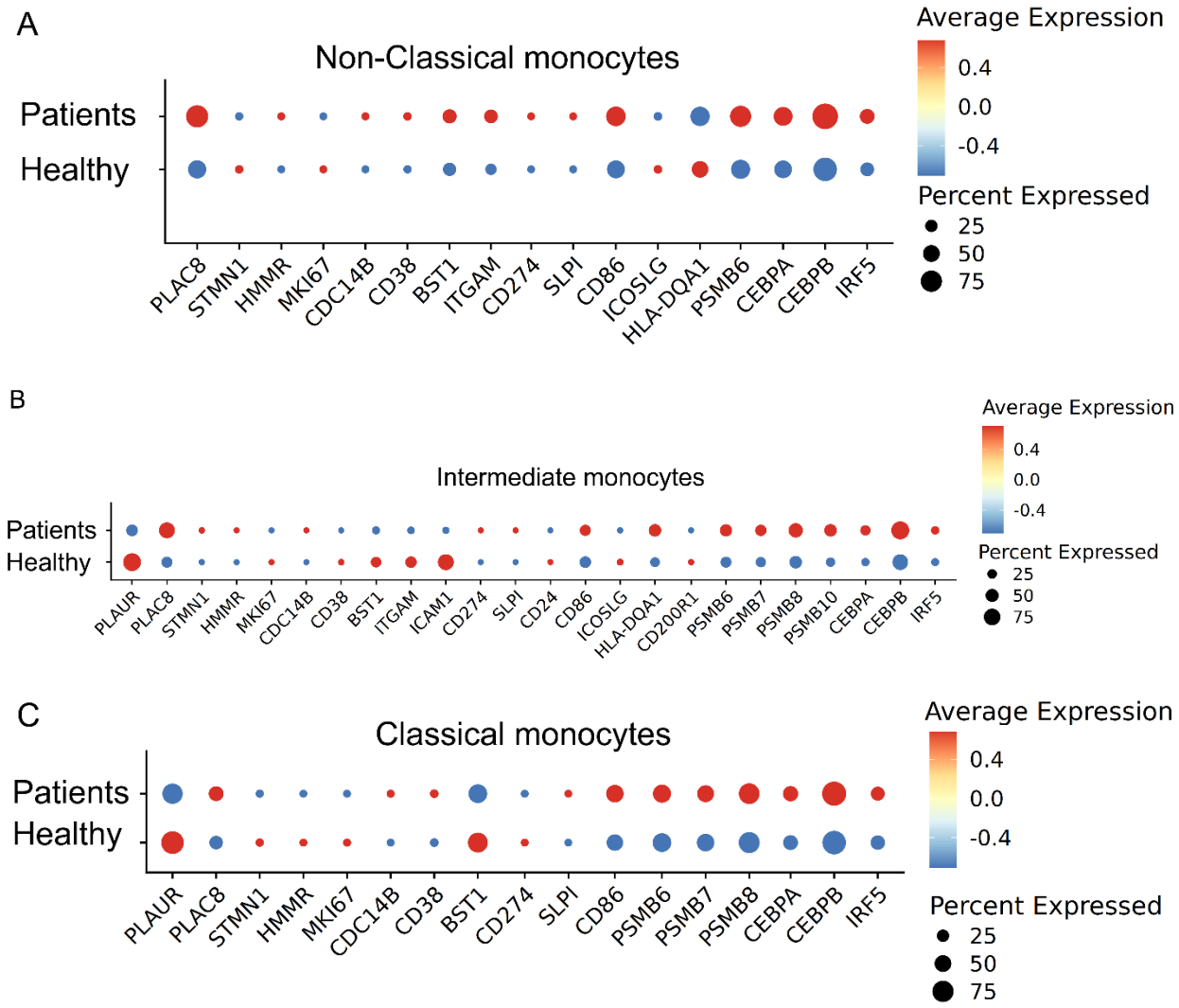


Figure 3-5-1 Capturing of key exhaustion maker genes from each subset of monocytes from VKH patients. A. Dot plot analyses capturing selected genes comparing the non-classical subset of monocytes from healthy or VKH patient blood. B. Dot plot analyses capturing selected genes comparing the intermediate subset of monocytes from healthy or VKH patient blood. C. Dot plot analyses capturing selected genes comparing the classical subset of monocytes from healthy or VKH patient blood.

We clustered scRNAseq data into three clusters based on CD14 and CD16 expression levels into the non-classical, intermediate, and classical subsets. We then examined representative genes in the categories of pathogenic inflammation, proliferative potential and immune modulation. In common with the septic patient monocytes, VHK patient monocytes also have the elevated pathogenic inflammation feature with increased expression of CD38, BST1, ITGAM. However, in contrast to septic monocytes, we observed that VKH patient monocytes do not exhibit consensus feature of proliferation, suggesting the emergency hematopoiesis and reduced

differentiation is not a striking feature of this disease. Furthermore, unlike the septic monocytes, VKH patient monocytes express elevated immune-enhancing genes such as CD86, suggesting the development of the low-grade immune-enhancing phenotype in these patients. Mechanistically, these patient monocytes exhibit an overall elevation of C/EBPα, C/EBPβ and IRF5. Our analyses reveal that chronic auto-immune types of diseases exhibit distinct features of monocyte reprogramming, with enhanced pathogenic inflammation and immune enhancing characteristics, resembling low-grade inflammatory monocytes we identified in the murine system.

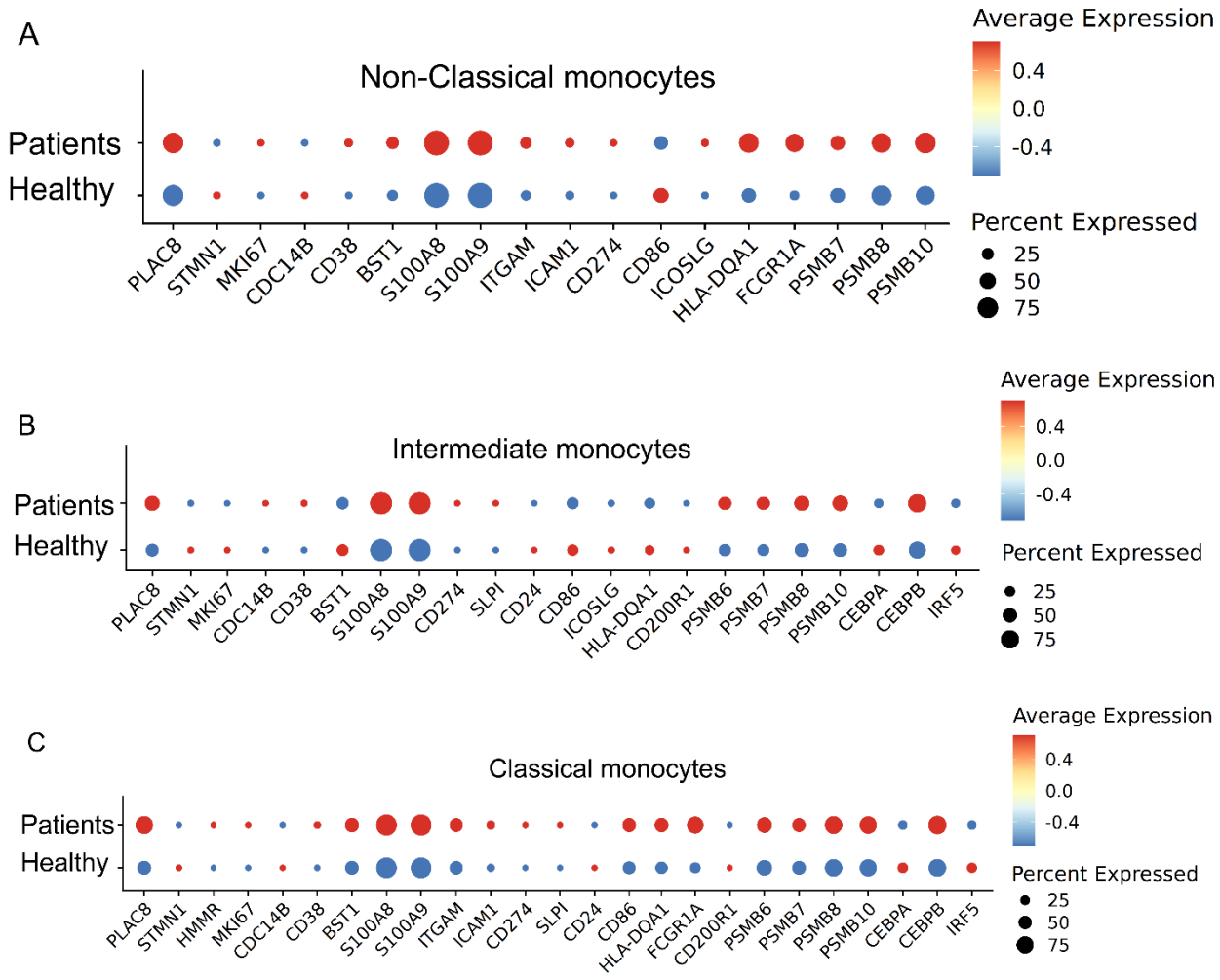


Figure 3-5-2 Capturing of key exhaustion maker genes from each subset of monocytes from BD patients. A. Dot plot analyses capturing selected genes comparing the non-classical subset of monocytes from healthy or BD patient blood. B. Dot plot analyses capturing selected genes comparing the intermediate subset of monocytes from healthy or BD patient blood. C. Dot plot analyses capturing selected genes comparing the classical subset of monocytes from healthy or BD patient blood.

We further examined another form of human auto-immune disease. Behcet's disease (BD) is a more severe chronic systemic inflammatory disorder that often presents with recurrent oral/genital ulceration and skin lesions, and in severe cases, can result in multi-organ malfunctions leading to significant morbidity and mortality.[83] Based on recently published scRNAseq data of monocytes collected from BD patients, we similarly grouped BD samples into non-classical; intermediate; and classical clusters, and examined key signatures we identified in this manuscript. Similar to VHK patients and distinct from septic or COVID-19 patients, the non-classical and intermediate BD patient monocytes do not have a proliferative signature. The pathogenic inflammatory feature, however, is evident from BD patient monocytes with elevated levels of CD38, BST1, ITGAM. In contrast to the VHK patients, the intermediate and classical monocytes from BD patients showed higher expression of pathogenic inflammatory genes, as well as reduced immune-enhancing genes, similar to septic monocytes. The "hybrid nature" of semi-exhausted monocytes from the BD patients may correlate with the severe multi-organ dysfunctions.

3.6 RNA velocity analysis

One of the advantages in analyzing single cell data is the potential to perform pseudo-time analyses for their ontogeny and trajectory. We then performed scVelo analyses of monocytes with prolonged challenges of either super-low dose or high dose LPS, or an anti-inflammatory agent 4-PBA together with super-low dose LPS. [84-85]

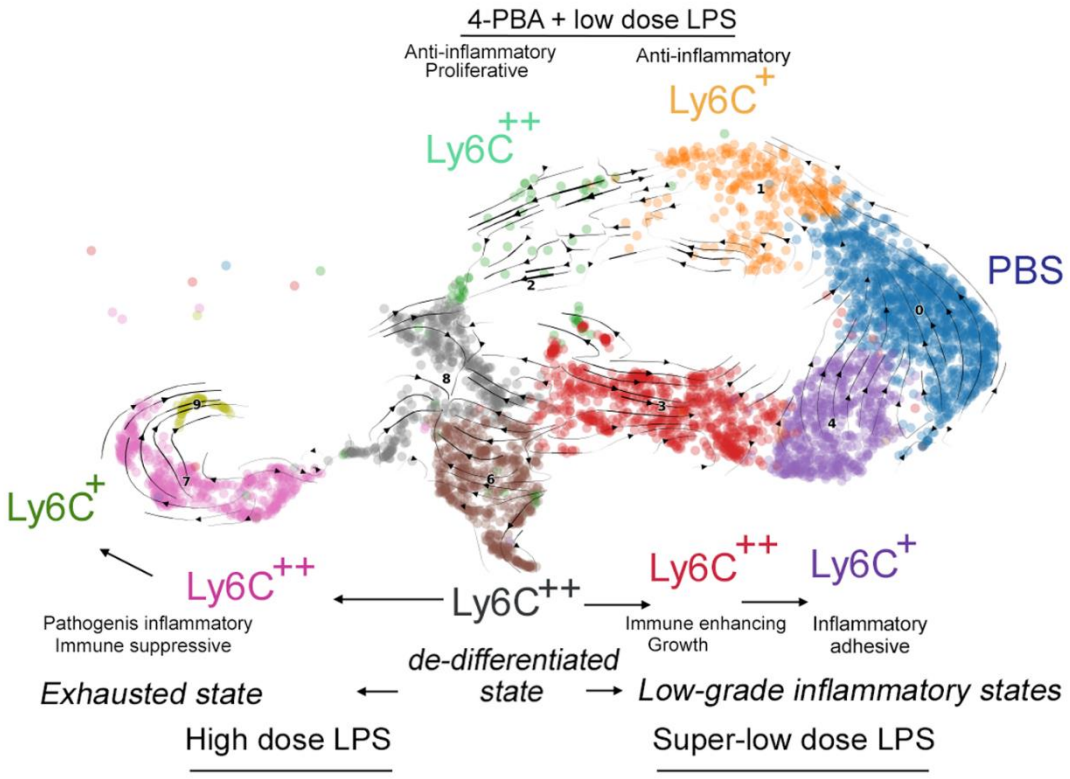


Figure 3-6-1 Analyses of monocyte memory dynamics in vitro. scRNAseq data collected from monocytes treated with 5-day challenges of high dose LPS, super-low dose LPS, or 4-PBA plus super-low dose LPS were clustered together and used for the scVelo analyses, revealing their dynamics pseudo-time trajectory.

Our analyses reveal that cells initially prompted into the proliferative, less differentiated state following either super-low or high dose LPS. Subsequently, monocytes track into the exhausted Ly6C++ monocytes and gradually into the Ly6C+ exhausted monocytes with lower proliferating potential.

In contrast, monocytes challenged with prolonged signals of super-low dose LPS bifurcate into a totally different direction. Our analyses reveal that low-dose LPS treated monocytes first move into the Ly6C++ monocytes with growth-promoting and immune-enhancing state, and then into the Ly6C+ chemotactic and adhesive low-grade inflammatory state.

In our previous experimental studies, we applied anti-inflammatory mediator 4-PBA and reported that 4-PBA blocked the generation of inflammatory monocytes induced by super-low dose LPS. We superimposed 4-PBA plus low-dose LPS scRNAseq onto the scVelo map, and further validated that 4-PBA arrests low-dose LPS treated monocytes into the proliferating or anti-inflammatory state.

3.7 Reference

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Chapter 4

Discussion and Future Directions

In this work, we seek to shed light on the implications and mechanisms of monocyte responses to varying dosages of lipopolysaccharide (LPS), a potent endotoxin that induces inflammation. Our comparative analysis, conducted across human and murine systems, unveils several fundamental principles of monocyte dynamics.

High doses of LPS are known to elicit a robust inflammatory response, characterized by a significant influx of monocytes into affected tissues. In our study, this was mirrored by the identification of two distinct monocyte clusters with exhaustion features in our single-cell RNA sequencing analysis. Both clusters exhibited elevated expression of genes relating to pathogenic inflammation and immune suppression, indicative of an exhaustive state in sepsis leading to multiorgan injuries and compromised host defense toward secondary infections.

In contrast, low-dose LPS exposure induced a distinct low-grade inflammatory monocyte response, marked by reduced differentiation, enhanced pathogenic inflammation, growth, chemotaxis and adhesion. This indicates that the initial de-differentiation process is a common response for monocytes facing endotoxin challenges of varying signal strength. Interestingly, super-low dose LPS nudges monocytes into low-grade inflammatory states, sequentially transitioning from the initial de-differentiated state to an immune-enhancing, pro-growth state, and finally to a mature state with inflammatory and adhesive properties.

Our pseudo-time analyses further suggest that monocytes initially halt differentiation and gain proliferative characteristics in response to a challenge. This observation correlates with the previously identified “emergency hematopoiesis” during sepsis, and indicates a bifurcation, with monocytes either entering an exhausted

state when faced with severe challenges or transitioning into a low-grade immune-enhancing state when the challenges are mild.

At the translational level, we found that blood monocytes from septic and severe COVID patients share overlapping features of exhaustion, while monocytes from chronic autoimmune patients present distinct features of low-grade inflammation. This highlights the plasticity and adaptability of the immune system and underscores the importance of context and intensity of signals in shaping immune responses.

This detailed understanding of differential responses to varying LPS dosages lays a solid foundation for future research. Critical questions remain, such as the molecular mechanisms that underlie the switch between robust immune activation and immune suppression, and the causes and implications of monocyte exhaustion. Future research directions should aim to address these questions, as further understanding of these mechanisms may potentially lead to the development of novel therapeutic strategies for sepsis and other inflammatory conditions involving monocyte dysfunction.