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Neutrophil Characteristics and Function During the progression of Liver Cirrhosis

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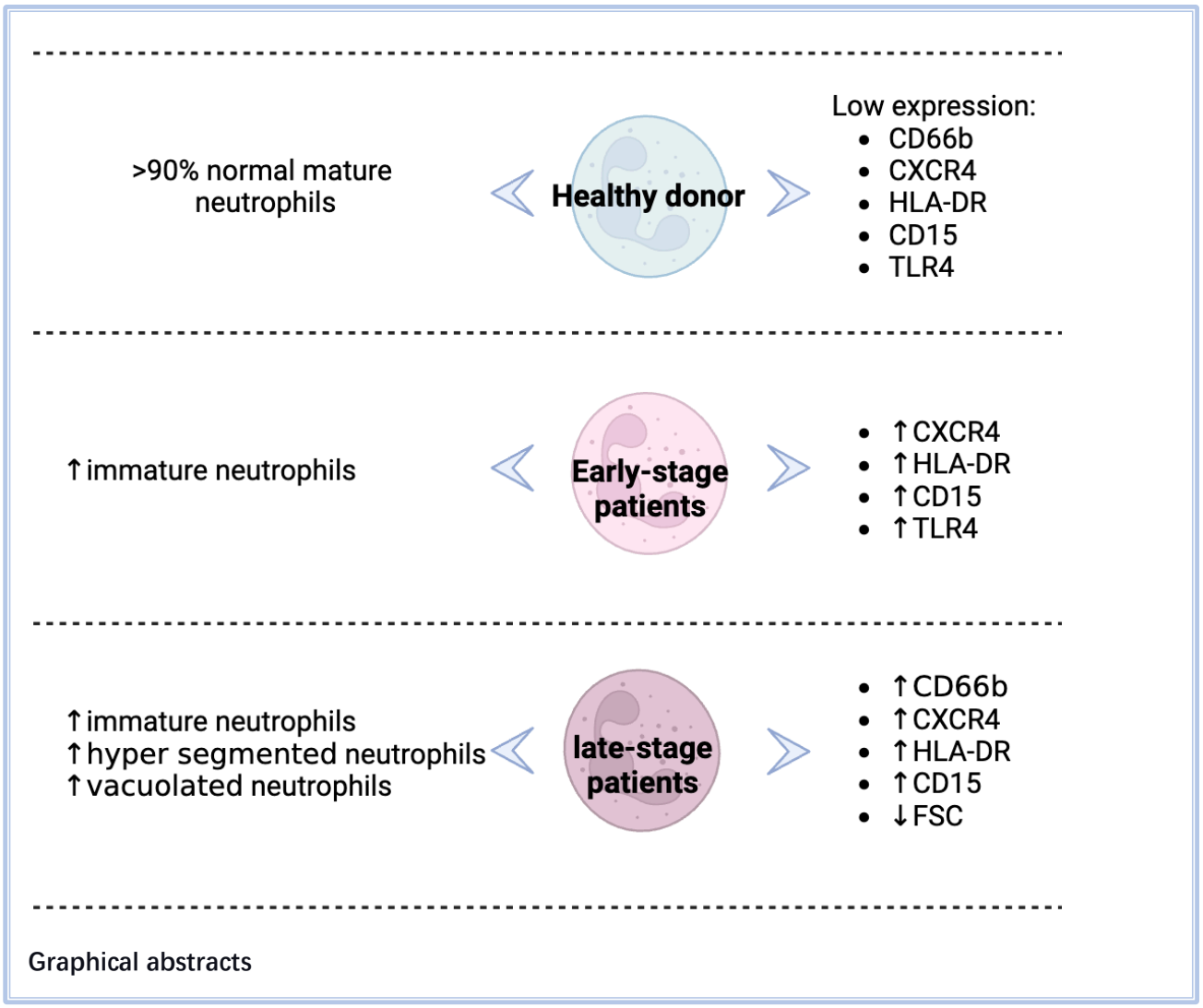
Abstract

Background: Neutrophils are essential components of the innate immune system and necessary for fighting against infections. Liver cirrhosis, the end-stage of chronic liver disease, often presents with neutrophil dysfunction. This study aimed to investigate the phenotypic, morphological, and functional changes affecting neutrophils in liver cirrhosis from early to advanced stages of the disease.

Methods: Whole blood samples were obtained from healthy volunteer and cirrhotic patients and evaluated for neutrophil surface marker expression, morphological characteristics, and mitochondrial content. Neutrophils were stimulated using formyl-methionyl-leucyl-phenylalanine (fMLF, or fMLP) to assess potential defects in activation. Statistical analyses were performed to assess differences between the study groups.

Results: CD66b expression was significantly increased in cirrhotic patients, particularly those in advanced stages, probably due to continuous bacterial infections. Individuals with liver cirrhosis showed greater HLA-DR, CD15, and TLR4 levels. Morphologically, both early and late-stage patients had more immature neutrophils, which suggested deregulation of neutrophil homeostasis. In addition, advanced cirrhosis patients had more hyper-segmented neutrophils, indicative of senescent neutrophils and a higher frequency of vacuolated neutrophils, suggestive of severe infections.

Conclusion: Our findings indicated variations in neutrophil marker expression, ageing patterns, and morphology across stages of liver cirrhosis. Neutrophil characteristics such as marker expression and morphological changes have the potential to serve as disease progression indicators. Our study, however, has limitations, such as a small sample size. More research is needed to determine the relationship between neutrophil dysfunction and the progression of liver cirrhosis.



Graphical abstracts

Introduction

1. Liver cirrhosis and Neutrophils

Cirrhosis of the liver is a complex multisystem disease, representing the end-stage of chronic liver disease (1). It primarily results from persistent liver injury, which severely impairs the normal function of the liver. Cirrhosis can emerge from various causes, with the most common troublemakers being viral infections, excessive alcohol consumption, and the accumulation of liver fat associated with features of the metabolic syndrome (1,2). All these factors can initiate inflammation, trigger liver fibrosis, and disrupt the conventional function and structural integrity of the liver (1). Cirrhosis has emerged as a leading cause of liver-related mortality worldwide, posing an escalating health burden and public health concern in recent years. For instance, in 2017, more than 160 million individuals globally were diagnosed with cirrhosis (3), contributing to over 1.32 million deaths among affected patients (4) in the same year.

Also, there are some primary aetiologies of liver cirrhosis. For example, Primary Biliary Cholangitis (PBC), known initially as primary biliary cirrhosis, is a chronic autoimmune liver disease that progresses slowly (5,6). This disease is distinguished by chronic cholestasis, which primarily affects the tiny bile ducts of the liver. Because bile ducts are required for bile transport, when they are damaged or destroyed in PBC, bile accumulates in the liver, causing liver cell injury, inflammation, and scarring (fibrosis), eventually leading to liver cirrhosis (5). Advanced PBC can cause spontaneous bacterial peritonitis, hepatoma development and other severe symptoms. At that time, liver transplantation was the only option (6). Unlike PBC, an autoimmune disease mainly impacting the bile ducts, non-alcoholic fatty liver disease (NAFLD) is characterised by excessive fat accumulation in the liver. This condition is typically diagnosed when triglyceride build-up within hepatocytes exceeds 5% of the weight of the liver. Furthermore, NAFLD can lead to liver inflammation and cellular damage. Key risk factors for NAFLD include obesity, type II diabetes, and metabolic syndrome, such as dyslipidaemia (7).

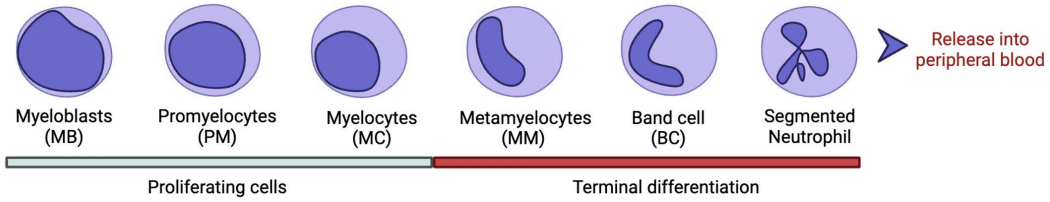
The liver is a central player in maintaining immune homeostasis within the human body (2). As a result, despite the fact that circulatory homeostasis is typically intact in patients with early cirrhosis (8), individuals with advanced hepatic cirrhosis constantly have a disruption in the immunological homeostasis of their liver (9). This disruption is commonly referred to as cirrhosis-associated immune dysfunction (CAID). Usually, it induces acquired alterations in innate and adaptive immunity, heightening the risk of various life-threatening complications, including immunodeficiency and systemic inflammation (1, 2,9). In the case of CAID, for example, bacteria are increasingly translocated from the gut, resulting in endotoxemia and higher cytokine production levels (10). These factors contribute to an excessive inflammatory response of the host and an increased vulnerability to systemic bacterial infections. Moreover, these

infections can progress to septic shock (11) and multiorgan dysfunction, resulting in more heightened mortality rates and frequent hospitalisations (2). Notably, because the degree of immune dysfunction appears to be directly related to the severity of liver injury (9), various novel investigations have been performed to explore the underlying connections and mechanisms between liver cirrhosis and the immune system.

Neutrophils, also known as neutrophilic granulocytes or polymorphonuclear leukocytes (PMNs) (1, 2), are the most abundant type of circulating white blood cells and serve as the frontline defenders of the immune system (13). During an immune response, neutrophils can rapidly be recruited from the blood to sites of infection or inflammation, where they can perform essential immunological functions (12). These functions include phagocytosis, the generation of reactive oxygen species (ROS), degranulation, the production of cytokines and chemokines, and the formation of neutrophil extracellular traps (NETs) (17,14). These remarkable capacities make neutrophils indispensable for triggering immediate immune responses against pathogens and tissue damage (2,12). However, early reports suggest that the circulating neutrophil activity and abilities in cirrhotic patients are impaired as the disease progresses (1), and this neutrophil dysfunction eventually impacts bacterial clearance, causing patients with liver cirrhosis to be susceptible to bacterial infections. Thus, understanding the functional mechanisms and characteristics of neutrophils in cirrhotic patients is critical for furthering our knowledge of the relationships between neutrophil dysfunction and the progression of liver cirrhosis.

2. Neutrophil Granulopoiesis

The generally accepted understanding of the neutrophil life cycle is that neutrophils initially differentiate from progenitor intermediates in the bone marrow before maturing into circulating blood neutrophils (13,15,16). Following this, macrophages play a crucial role in the clearance of aged neutrophils (15). Specifically, hematopoietic stem cells (HSCs) differentiate into myeloblasts (MB), the first cells of committed granulopoiesis. These myeloblasts then progress into promyelocytes (PM) and subsequently differentiate into myelocytes (MC), at which stage proliferation ceases. The subsequent stages of terminal differentiation and maturation include metamyelocytes (MM), which have the potential to differentiate into band cells (BC) and, finally, segmented neutrophilic cells (SC). Segmented neutrophils are mature neutrophils capable of being released into the peripheral blood circulation (15,16,17) (Figure. 1). Furthermore, various surface markers exhibit varying levels of expression throughout granulopoiesis (15,16,17). Figure. 1 provides detailed information on the expression levels of these distinct surface markers, which can aid in identifying and differentiating various phases of neutrophil maturation. However, extensive research has shown that inflammatory stimuli impact the expression of neutrophil surface molecules (18), and the frequency and duration of neutrophil activation influence infection outcomes (19).



	MB	PM	MC	MM	BC	Segmented Neutrophil
CD15	+	+++	+++	+++	+++	++
CD16	-	-	-	+	++	+++
CD10	-	-	-	-	-	++
CD66b	-	+++	+++	++	++	++
CD11b	-	-	++	++	++	++
CD11c	-	-	++	++	++	++
CD33	+++	+++	++	+	+	+
CD62L	++	++	++	++	++	++
CXCR2	+	+	+	+	++	++
CXCR4	++	++	++	++	+	+
HLA-DR	+	-	-	-	-	-

Figure 1: Neutrophil Granulopoiesis and Surface Marker Expression.
This figure depicts the stages of granulopoiesis, from myeloblasts to segmented neutrophils, and presents surface marker expression intensity at each stage. The expression of surface markers is represented as no expression (-), low expression (+), medium expression (++), or high expression (+++). This figure provides insights into marker expression during neutrophil development. Adapted from McKenna et al. (2021).

2.1 Emergency granulopoiesis

Granulopoiesis is tightly controlled in homeostasis, with limited fully differentiated neutrophils released from the bone marrow (13). However, granulopoiesis significantly increases during infection and other pathologies (13). Existing research offers valuable insights into the mechanisms underlying the increase in immature neutrophils during infections. Neutrophils play a pivotal role in the immune system, serving as frontline defenders against bacterial infections and responding to liver damage in cirrhosis patients by migrating from the bloodstream to sites of inflammation, where they actively engage in processes like phagocytosis and cytotoxicity (20). However, during infection or injury, there is an accelerated rate of neutrophil death and consumption, resulting in an increased demand for these immune cells (21). To counteract this considerable loss of neutrophil populations, the typical steady state granulopoiesis in the bone marrow transitions into the turn of emergency granulopoiesis (20,21) (Figure. 2). This involves a significant increase in neutrophil production and faster cellular turnover (21). However, it can induce a release of both immature and mature neutrophils from the

bone marrow into the peripheral circulation (21). That is why the presence of immature neutrophils can be one of the features of this urgent form of granulopoiesis.

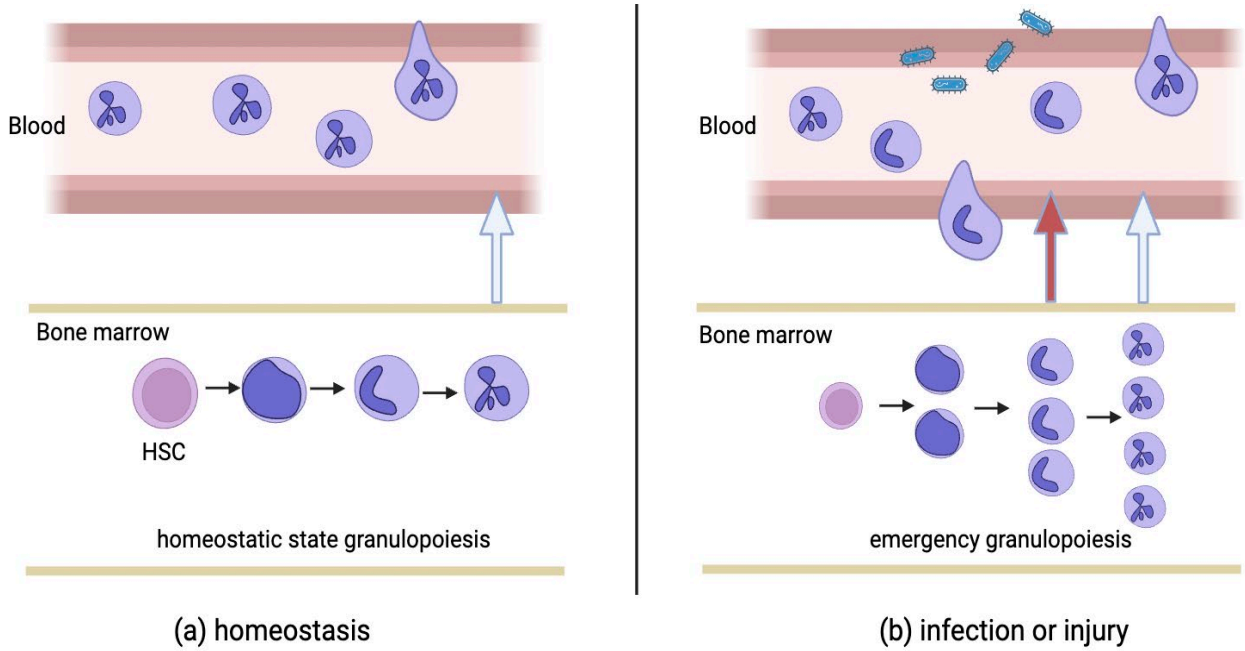


Figure 2: Granulopoiesis in Different States

(a) Granulopoiesis is finely regulated during homeostasis. Only fully mature neutrophils can be released to the circulation. (b) During severe bacterial infections or tissue injury, homeostatic granulopoiesis shifts to emergency granulopoiesis, characterized by increased neutrophil production within the bone marrow. This process includes the release of immature neutrophils into the circulation (highlighted by the red arrow) to compensate for neutrophil depletion and meet the urgent demand for neutrophils.

2.2 Morphological changes during granulopoiesis

The nuclear morphology of neutrophils has long served as a cornerstone parameter in haematology, offering a means to identify and distinguish between various maturity stages of neutrophils (22). A closer examination of granulopoiesis, the process of generating neutrophils, reveals the most remarkable transformations occurring within their nuclei (refer to Figure. 1). For example, myeloblasts are the earliest and most immature neutrophils. Myeloblasts are characterised by large, spherical nuclei containing few nucleoli (17). As these immune cells progress along their developmental trajectory, promyelocytes and myelocytes emerge, devoid of nucleoli and displaying heightened chromatin condensation compared to myeloblasts. The hallmark of a metamyelocyte, the subsequent stage of neutrophil maturation, is an indented nucleus. The journey continues as band cells take shape, featuring nuclei reminiscent of a horseshoe, as their nuclei are usually U or S shape, eventually constricting to form nuclear lobes (refer to Figure. 1). Finally, mature segmented neutrophils, readily identifiable by their nuclei sporting three to four lobes, complete

the developmental cycle (17). Beyond these established stages, neutrophils can occasionally undergo hyper segmentation, a phenomenon marked by neutrophils bearing six or more lobes. Hyper segmented neutrophils may be observed in specific conditions, such as megaloblastic anaemia or certain systemic inflammatory disorders (23). Moreover, it is worth noting that neutrophils may exhibit vacuolisation in the blood —a degenerative condition highly indicative of infection. The extent of vacuolisation often corresponds with the severity of the disease in the patient (24).

3. Neutrophil cell markers

In neutrophil research, marker expression plays a crucial role in distinguishing different populations of these immune cells. For instance, while there is still no standard agreement on the phenotypic flow cytometry markers for identifying human neutrophils, CD15+CD16+CD14- is a commonly employed combination to differentiate these populations (17). In detail, as a differentiation marker, CD15 is present in all neutrophil subpopulations, and CD16, a phagocytic capacity marker, permits to distinguish CD16-positive neutrophils from CD16-negative eosinophils. Although some studies have noted very low-level CD14 expression in neutrophils, the expression of CD14, typically associated with macrophages and monocytes, can help to select CD14-negative or low-expression neutrophils.

3.1 Neutrophil plasticity

Indeed, neutrophils possess remarkable plasticity that enables them to make diverse responses to various stimuli. This flexibility results in significant phenotypic diversity among neutrophils, with variations in surface marker expression serving as indicators of distinct subsets and specialised functions within the neutrophil population (17,25). Therefore, beyond its role in distinguishing neutrophil populations, marker expression is also essential in displaying the subpopulation, complex dynamics of neutrophil behaviour and their distinct functional responses across different physiological and pathological conditions (17,25). Consequently, exploring alterations in neutrophil marker expression within the context of liver cirrhosis takes on significant importance. The following section introduces some markers with special relevance to neutrophils.

3.2 Maturity and immaturity markers of neutrophils

CD33, HLA-DR, CD10, and CD15 serve as valuable markers, offering insights into the various stages of neutrophil maturation (refer to Figure. 1). HLA-DR, also known as major histocompatibility complex class II (MHC II), plays an essential role in antigen presentation to CD4+ T cells (17). This molecule is exclusively expressed in the earliest neutrophil stage, the myeloblast (as indicated in Figure. 1) (17). However, as neutrophils mature, HLA-DR expression becomes typically absent in circulating neutrophils. Notably, mature neutrophils in circulation can occasionally express HLA-DR, but this upregulation is usually associated with neutrophil activation, phagocytosis, or specific inflammatory diseases (13,26). For example, HLA-DR can be found on

tissue neutrophils in the synovial fluid of rheumatoid arthritis patients (26).

Moving on to CD15 (Lewis X), this carbohydrate antigen is expressed in human neutrophils and helps distinguish them from other myeloid cells (17, 29, 67). CD15 is crucial for several vital neutrophil functions, including degranulation and respiratory burst (67, 68). Additionally, it facilitates cell-to-cell communication. For instance, as a high-affinity carbohydrate ligand of DC-SIGN, CD15 can enhance neutrophil-dendritic cell (DC) contact (27). This critical intercellular interaction promotes dendritic cell maturation, enabling DCs to trigger significant T cell proliferation and type 1 T helper cell polarization (27). This relationship highlights a unique mechanistic link between innate and adaptive immunity. CD15 is more commonly observed in immature neutrophils and can be a marker of neutrophil maturity (Figure. 1).

On the other hand, CD10, also known as the common acute lymphoblastic leukaemia antigen (CALLA), neurolysin, or neutral endopeptidase (NEP), is a phenotypic marker exclusively found on segmented mature neutrophils (28, 29). CD10 is absent on band neutrophils or less mature neutrophil precursors (28). This unique expression pattern can underscore the importance of CD10 as a marker for distinguishing and identifying mature neutrophils from their immature counterparts, aiding researchers in characterizing these distinct cell populations (29).

3.3 Ageing markers of neutrophils

Once neutrophils mature, they exit the bone marrow and enter the circulation. However, under homeostatic conditions, most mature neutrophils typically reside within the hematopoietic compartment of the bone marrow (30). Only a tiny fraction, approximately 2%, of mature neutrophils are released into the bloodstream and tissues (12,30). The regulation of neutrophil migration and release from the bone marrow commonly involves two crucial chemokine receptors, CXC chemokine receptors 2 and 4 (CXCR2 and CXCR4) (12).

In detail, the bone marrow stromal cells produce the CXCR4 ligand, CXCL12, which contributes to the retention of CXCR4-expressing neutrophils within the bone marrow (Figure. 3). Nevertheless, neutrophils can be rapidly released from the bone marrow reserve in response to inflammatory stimuli, increasing circulating neutrophil counts. Various chemokines, including CXCL2 and CXCL8 (also known as IL-8), trigger this release, which can be produced by endothelial cells outside the bone marrow. As previously mentioned, freshly matured neutrophils express a high level of CXCR2 on their surface, and both chemokines are ligands for CXCR2. Therefore, releasing CXCL2 and CXCL8 in the infection site can stimulate the rapid mobilization of fresh CXCR2 high mature neutrophils from the bone marrow (30). Then, senescent neutrophils in the circulation upregulate CXCR4 expression, preceded by enhanced binding of annexin V—a marker of neutrophil apoptosis (31). This phenomenon is particularly notable in aged neutrophils when they need to be cleared from circulation, enabling them to return to the bone marrow for final clearance (12). Finally, Figure. 3

shows the CXCR2 and CXCR4 retention, release, and return mechanisms.

CXCR2 and CXCR4 not only serve dual roles as crucial chemokine receptors and age-related indicators for neutrophils. They can also be considered markers of neutrophil maturation. While CXCR4 is not commonly employed as a neutrophil maturation marker, CXCR4 signals are expressed at higher levels in immature neutrophils (17) than their mature counterparts. Early research indicates that CXCR2 surface expression is lower in immature neutrophils, specifically before the band stage (32).

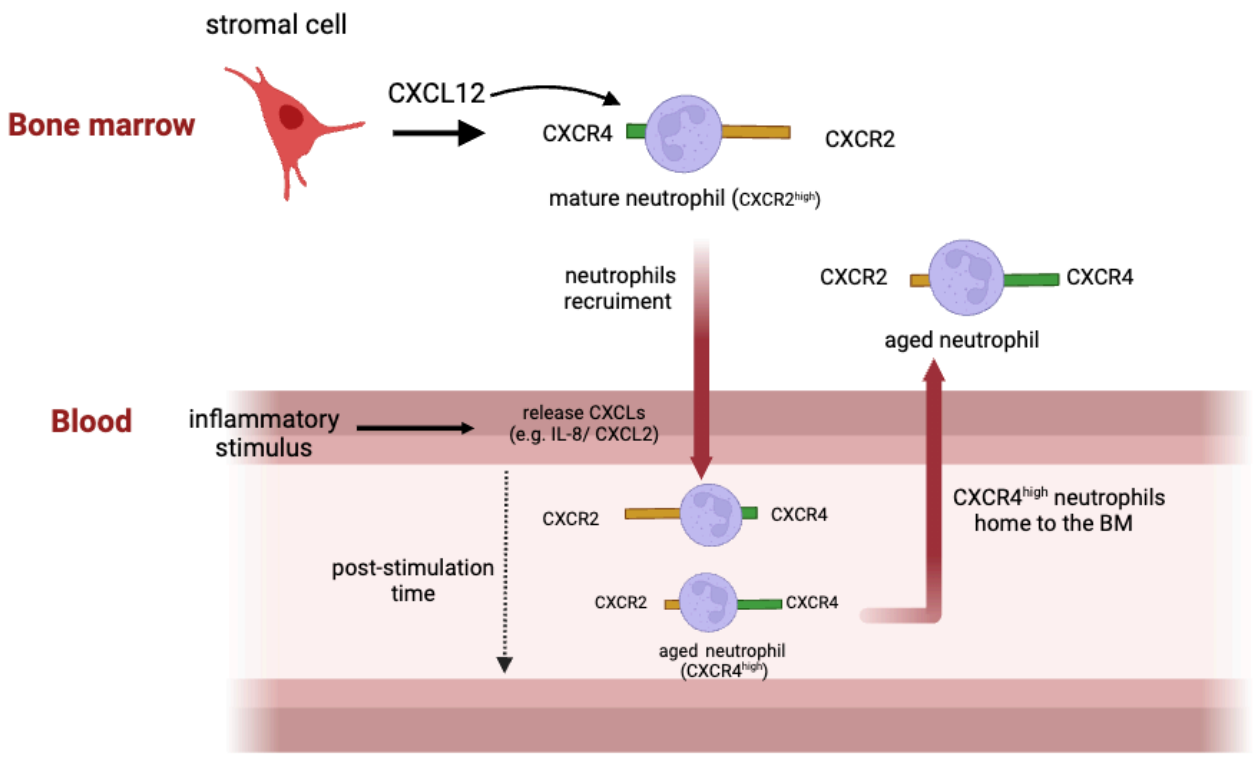


Figure.3: The CXCL12/CXCR4 Neutrophil Retention Axis

This illustration elucidates the role of the CXCL12/CXCR4 neutrophil retention axis in regulating the release and return of neutrophils to the bone marrow. In a healthy state, most mature neutrophils are stored in the bone marrow, primarily due to the production of CXCL12 by stromal cells and CXCR4 on neutrophils themselves.

However, during periods of inflammation, the release of CXCR2 ligands, such as CXCL2, can trigger the release of mature neutrophils from the bone marrow into circulation. As these neutrophils circulate and become stimulated, they age and subsequently increase their expression of CXCR4. This heightened CXCR4 expression facilitates their return to the bone marrow, where they are eventually cleared from circulation. This process plays a vital role in maintaining neutrophil homeostasis and enables an efficient response to inflammatory challenges.

3.4 Activation markers of neutrophils

CD11b and CD66b are widely recognised as neutrophil activation markers, and their expression levels typically increase following neutrophil stimulation. These markers

are essential indicators of neutrophil activation and play significant roles in their functions.

CD11b, also known as integrin alpha-M or macrophage-1 antigen, is a prominent member of the integrin family, responsible for mediating essential processes such as phagocyte attachment (18), neutrophil migration (33), endothelial surface adhesion (33, 34), and ingestion of complement-opsonised particles (35, 36). Neutrophil migration largely relies on transitioning from rolling along the endothelium to firmly attaching to it, a process facilitated by CD11b (37). Moreover, CD11b can initiate intracellular signals that enable cells to respond to physiological stimuli by increasing the production of reactive oxygen species (ROS) (38). Typically, neutrophils exhibit low levels of CD11b on their surface without stimulation, but their expression can be rapidly upregulated in response to various stimuli, including TNF-alpha and IL-8 (38, 39). Consequently, CD11b functions as both a sensor and effector of neutrophils during inflammation, rendering it a sensitive indicator of neutrophil activity (39).

CD66b (aka CEACAM8, CGM6, and NCA-95) belongs to the immunoglobulin superfamily and is a single-chain glycosylphosphatidylinositol-anchored protein exclusively expressed on the human granulocytes, particularly neutrophils (18,40). Functionally, CD66b plays a crucial role in granulocyte activation, contributing to neutrophil degranulation (41), endothelial cell adhesion (34, 42), and the formation of ROS (42). These functions collectively enhance pathogen clearance. Commonly, neutrophils express minimal levels of CD66b under steady-state conditions. However, exposure to inflammatory agonists in vitro can rapidly upregulate the expression of CD66b (43). This up-regulation is part of the neutrophil response to inflammatory stimuli, enabling them to mount a more robust defence against pathogens and contribute to the immune response.

Other neutrophil activation markers, such as CD62L, act differently than CD11b or CD66b. Specifically, the expression levels of CD62L, also known as L-selectin, typically decrease after neutrophil activation. CD62L also plays a vital role in various neutrophil functions, including phagocytosis, chemotaxis, and migration (44). One essential function of CD62L is facilitating the rolling of neutrophils along the vessel wall. However, when neutrophils are stimulated, CD62L undergoes shedding through proteolytic cleavage near the cell surface. This shedding process enhances neutrophil adhesion to the endothelium lining of blood vessels (45,46). This phenomenon is referred to as "L-selectin shedding". It is a strategic mechanism that strengthens neutrophil adherence and aids their migration through the endothelium to reach the site of inflammation.

Additionally, CXCR2 can also serve as an activation marker. Stillie et al. extensively reviewed the mechanism of CXCR2 internalisation, demonstrating that CXCR2 can be internalised via clathrin-coated vesicles upon ligand binding. Subsequently, it is compartmentalised for degradation, ultimately resulting in decreased CXCR2

expression after neutrophil activation (47). Usually, formyl-methionyl-leucyl-phenylalanine (fMLF, often referred to as fMLP) plays a pivotal role as an activator for polymorphonuclear cells (48, 49), helping to initiate a cascade of cellular reactions such as the generation of reactive oxygen species (ROS), Neutrophil extracellular traps (NET) production, and phagocytosis. This phenomenon is commonly referred to as "neutrophil activation" (48). Previous in vitro studies have further supported the rapid desensitisation and internalisation of CXCR2 on neutrophils following fMLP stimulation (50,51).

Additionally, neutrophilic granulocytes can upregulate the expression of the Fcγ receptor, also known as the CD64 antigen (52, 53), upon activation. CD64 is recognised as a high-affinity receptor for the Fcγ portion of the IgG heavy chain, capable of binding both monomeric IgG1 and IgG3 and aggregated IgG (54). This activation level differs from the upregulation of Mac1 or the shedding of CD62L. Typically, CD64 is predominantly expressed by monocytes/macrophages under steady-state conditions. However, during infections, it is robustly upregulated on neutrophils (55). The presence of this FcγRI receptor is significant, as it plays a pivotal role in the process of phagocytosis against various bacteria and microorganisms. Consequently, the expression of CD64 is generally indicative of an active physiological role, mainly when neutrophils are functioning as phagocytes (52).

3.5 Atypical marker of neutrophils

Toll-like receptors are expressed at lower levels in neutrophils than in monocytes. However, members of the TLR family, particularly Toll-like receptor 4 (TLR4), play critical roles in neutrophil cellular activities such as the formation of reactive oxygen species (ROS) and cytokine synthesis (56,57).

CD74 is not a typical surface marker found on neutrophils but is usually present on professional antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells (59). However, it is a class II-associated invariant chain (Ii) of the MHC, and neutrophils can occasionally increase MHC-II expression. The most critical role of CD74 involves the formation of a complex with the α and β chains of MHC class II within the endoplasmic reticulum (ER) (60). This complex serves the crucial purpose of safeguarding the peptide-binding groove of MHC class II molecules in the ER environment, as it effectively prevents the premature binding of antigenic peptides within this cellular compartment. Then, this complex moves to endosomal compartments where exogenous antigenic proteins undergo processing. In these compartments, proteases can help cleave and release CD74 from MHC class II molecules. This enzymatic cleavage event leads to the formation of MHC class II dimers, which are now primed to bind antigenic peptides. This antigen-peptide-loaded complex subsequently travels to the surface of antigen-presenting cells, where it can present antigens to CD4+ T cells and contribute to the initiation of the adaptive immune response (60,61).

Furthermore, neutrophils can have numerous important markers in immunological function and health. For example, Beta-2 microglobulin (β2M), a component of MHC class I antigen presentation (58), is crucial for antigen presentation and contributes to various biological processes, including mucosal immunity, tumour surveillance, and immunological tolerance. Deviations in β2M levels can signify underlying health conditions, from inflammatory disorders to malignancies (62). Finally, we look at CD11c, also known as αX, a cell surface protein that forms the CD11c/CD18 complex (63), which is involved in cell adhesion, signalling, and interactions. While previously associated with dendritic cells, a current study has revealed that it is highly expressed in neutrophils, regulating their migration, survival, and proliferation (63).

3.6 Intracellular marker of neutrophils

Approximately 45% of the proteins found in neutrophil cytoplasm consist of S100A8 and S100A9, which have the capability to form non-covalently bound heterodimers (64,65). In detail, S100A9 is a cytoplasmic protein typically present in immune cells such as neutrophils and macrophages. However, when released extracellularly, these heterodimers take on the role of damage-associated molecular patterns (DAMPs) by interacting with TLR4. They can play a pivotal role in inflammatory processes (65), as their contribution lies in the regulation of inflammatory cytokines, reactive oxygen species (ROS), and nitric oxide (NO). Significantly, during the early stages of bacterial infection-induced sepsis, there is a substantial increase in the expression of S100A9 (64). Due to their fundamental role in inflammatory conditions, S100A8/A9 serves as a valuable biomarker for various inflammatory disorders, including rheumatic diseases, fever syndromes, and vasculitis (65). For a comprehensive overview of the neutrophil markers under investigation, please refer to Table. 1, which provides essential details and information.

Marker	Position	Protein name	Marker type	Reference
CD14	Cell surface	Cluster of differentiation 14	myeloid cell differentiation marker	17,66
HLA-DR	Cell surface	MHC class II	Antigen presenting cells marker	26
TLR4	Cell surface	Toll-like receptor 4	Pattern Recognition Receptor	56
CD15	Cell surface	Lewis X, X-hapten	Neutrophil differentiation marker, Neutrophil lineage marker	17,29,67
CD62L	Cell surface	L-selectin	Neutrophil activation marker	44,45
CD16	Cell surface	FcγRIIIa	Phagocytosis marker	29
CD11b	Cell surface	Integrin alpha-M, Macrophage-1 antigen	Phagocytosis marker, Activation marker	18,33
CXCR4	Cell surface	C-X-C chemokine receptor type 4	Neutrophil aging marker Neutrophils retention in/ home to bone marrow	12,30,31
CXCR2	Cell surface	CXC chemokine receptor 2	Neutrophil aging marker	69,70
CD66b	Cell surface	Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8)	Granulocyte activation marker, Neutrophil lineage marker	18,40
CD33	Cell surface	Sialic acid binding Ig-like lectin 3, Gp 67	Differentiation marker	71,72
CD10	Cell surface	Common acute lymphoblastic leukemia antigen (CALLA), Neutral endopeptidase (NEP)	Differentiation marker	28,29
CD64	Cell surface	Cluster of Differentiation 64 FcγRI	Neutrophil activation marker	53,73
CD11c	Cell surface	Integrin alpha X	Migration marker	73
CD74	Cell surface	HLA-DR antigens-associated invariant chain	Transport cofactor for MHC class II	74
β2-microglobulin	Cell surface	A component of MHC class I	Tumour marker	75
S100A9	Intracellular	calprotectin	Neutrophil extracellular traps release marker	64

Table 1. Detail of granulocytes markers investigated in this research.

4. Mitochondria in Neutrophils

Mitochondria are commonly referred to as the powerhouses of eukaryotic cells due to their central role in efficiently generating adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (76). As one of the most essential membrane-bound organelles, mitochondria are indispensable for cellular energy production and maintenance. Over the past decade, mitochondria have become increasingly evident that these organelles are pivotal in regulating cell death, as they serve as crucial signalling hubs orchestrating various aspects of cell death processes (77). In the context of neutrophils, investigations have revealed the complex functions of mitochondrial involvement. For example, studies conducted by Fossati et al. demonstrated the presence of mitochondrial networks within neutrophils, extending throughout the cytoplasm and detectable by using specific dyes (78). This discovery has shed light on the multifaceted roles of mitochondria in neutrophil biology. Also, neutrophil mitochondria participate in various critical functions, including NET formation, adhesion and migration, respiratory burst, neutrophil development, and more (76) (Figure. 4). As a result, alterations in mitochondrial levels appear capable of influencing neutrophil cellular functions and efficacy. Also, immature and mature neutrophils contain different mitochondrial levels. A study dating back to 1971, conducted by Bainton et al., delved into the development of neutrophils in human bone marrow. They observed abundant mitochondria in myeloblasts, the earliest form of immature neutrophils. However, they also reported that as these neutrophils matured, the level of mitochondria progressively decreased. From then on, the lower mitochondrial levels are considered characteristic of mature neutrophils (79,80).

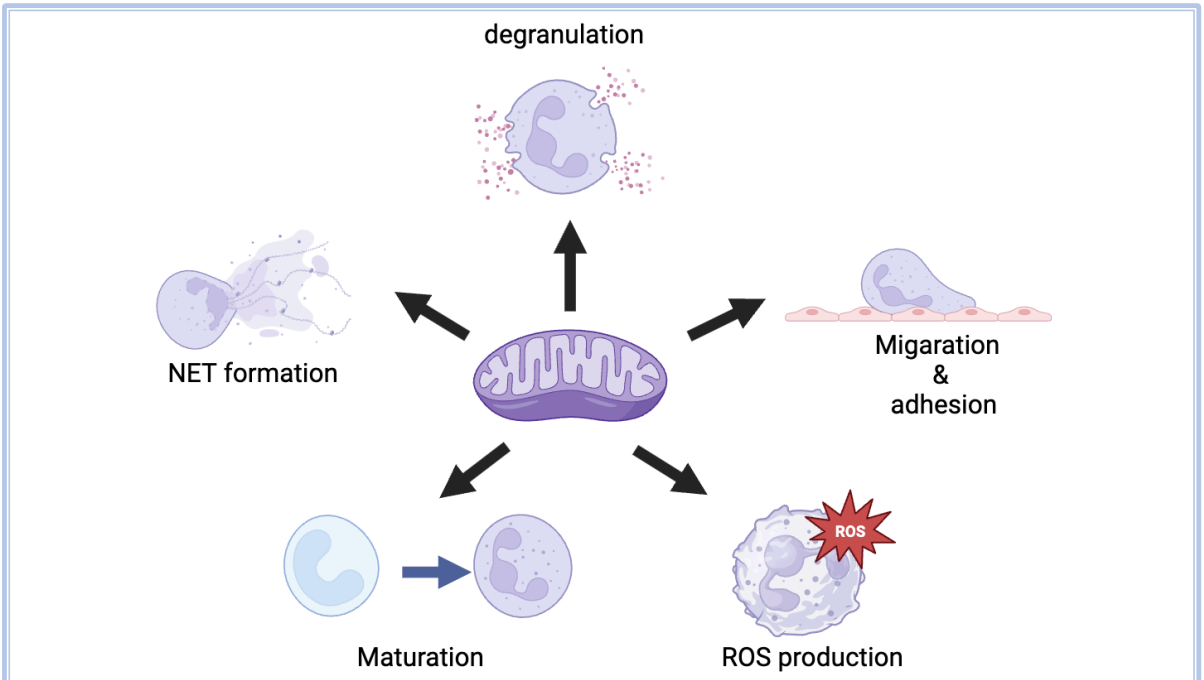


Figure 4: Mitochondrial Participation in Various Neutrophil Functions.

To comprehensively investigate these aspects, we adopted a multifaceted approach. We carefully selected a range of typical and atypical neutrophil markers, subsequently using specific antibodies for staining. Then, we meticulously quantified the expression levels of those markers across individuals representing diverse health conditions by the multicolour flow cytometry, and both isolated neutrophils and blood samples were analysed to ensure a thorough examination. Then, the cytospin was utilised to generate cell slides to assess neutrophil morphology for further distinguishing morphological distinctions in liver cirrhosis background. Additionally, we used intracellular mitochondrial indicators to quantify mitochondrial levels within neutrophils obtained from various donors precisely. The multifaceted approaches above provided a comprehensive understanding of neutrophil features and their behaviour as liver cirrhosis progresses. These insights offer valuable opportunities to enhance our understanding of neutrophil dynamics in chronic liver illnesses.

Method and Material

Method

a. Ethics statement

The Biomarker Discovery in Liver Disease (BOLD) is a prospective cohort study designed to recruit participants of both genders above 18 years of age with a clinical diagnosis of liver disease but without viral origins. Specific exclusion criteria have been established, including individuals with active or prior hepatocellular carcinoma (HCC), a history of portal vein thrombosis, previous organ transplantation, as well as those lacking the capacity to provide consent or consent from their relatives or welfare guardians (reference number 22/EE/0044). These criteria are in place to ensure that the participants in this research are suitable for the investigation and to maintain ethical standards throughout the study.

b. Sample Collection

The BioResource is run by NHS Lothian, which operates under the ethical approval of a Research Ethics Committee (15/ES/0094). Its primary function is collecting and preserving biospecimens, including small tissue samples, cells, and various body fluid samples. These biospecimens are systematically accumulated to establish a valuable resource for research and educational purposes. Furthermore, any usage of these collected samples is contingent upon approval by a scientific review committee, ensuring that all research applications meet rigorous scientific standards and ethical guidelines. Additionally, the collected samples are surplus, which means they are acquired concurrently with clinical procedures or blood sampling, avoiding additional burdens on the participants. Specifically, the BioResource liver study aims to gather blood samples during the liver transplant assessment week and liver tissue samples during the transplant operation, contributing to valuable research about disease and transplantation.

c. Liver cirrhosis patients and control samples

This study encompassed diverse participants, including liver cirrhosis patients and healthy donors who were recruited strictly following ethical guidelines. Healthy donors were recruited through the blood donor registers under the Centre for Inflammation Research at the University of Edinburgh, following Ethics Reference 21-EMREC-041. On the other hand, patient blood samples were collected from individuals recruited from outpatient clinics in NHS Lothian. These patients were regularly monitored at 6-month intervals to track the development of complications or disease progression. Extensive clinical phenotyping and immune profiling were conducted to establish

correlations with disease stage, and the collected samples were screened for the presence of biomarkers of liver disease.

d. Classification of Stages of Liver Cirrhosis

The traditional Child-Pugh classification system was used to determine the severity of liver failure in cirrhotic patients (146), with individuals classified into Class A to C based on their overall status. In our study, individuals with early-stage liver cirrhosis were classified as Child-Pugh Class A, whereas those with late-stage cirrhosis were classified as Child-Pugh Class B or C.

e. Isolation of polymorphonuclear leukocytes (PMN) from Human Peripheral Blood

e.1 Blood Collection and Processing

Trained phlebotomists collected whole blood in ethylenediaminetetraacetic acid (EDTA)-containing tubes. The blood samples were promptly transported to the laboratory and processed within 4 hours. All centrifugation steps were carried out at room temperature unless otherwise specified.

e.2 Polymorphonuclear cells Isolation

Lymphoprep (STEMCELL Technologies, 07801), a density gradient medium, was pipetted carefully into the SepMateTM-50 tube (STEMCELL Technologies, 85450) via the central hole of the SepMateTM-50 tube insert. Human peripheral blood from EDTA tubes was diluted with an equal volume of phosphate-buffered saline (PBS) and 2% fetal bovine serum (FBS) (STEMCELL Technologies, 07905). The diluted sample was then pipetted into the SepMateTM tube. After centrifuging whole blood at 1200 x g for 12 minutes at room temperature with the brake engaged, the top layer enriched with mononuclear cells was extracted for a separate study. Cell pellets were aspirated into fresh tubes, and leukocytes and erythrocytes were separated through 6% dextran sedimentation in saline pre-warmed to 37°C and sedimented for 20 to 30 minutes at room temperature. The upper layer rich in leukocytes were transferred to a new 50-ml tube, topped up to 50 with pre-warmed saline, and centrifuged at 350xg for 6 minutes at room temperature. Polymorphonuclear cells and peripheral blood mononuclear cells (PBMC) in the pellets were then separated through a discontinuous Percoll gradient (81%, 70%, and 55%), followed by a 20-minute centrifugation at 720 x g. Isolated polymorphonuclear cells were washed twice in cation-free Dulbecco's phosphate-buffered saline (DPBS-/-) for 6 minutes at 230 x g. The separated cells were subsequently resuspended in appropriate Dulbecco's phosphate-buffered saline with calcium and magnesium-enriched DPBS (DPBS+/+).

e.3 Percoll Gradients

Percoll solution (Cytiva, 7-0891-01) was rendered isotonic with 10x PBS (ThermoFisher, 70011044). Separate Percoll gradients of 81%, 70%, and 55% cation-

free Dulbecco's phosphate-buffered saline (DPBS-/-) (ThermoFisher, 14190094) were prepared. A gradient was formed by layering 3 mL of 81% Percoll solution at the bottom of an empty 15 mL tube, followed by adding 3 mL of 70% Percoll solution as an overlayer. After dextran sedimentation, the top layer enriched with leukocytes was collected and centrifuged for 6 minutes at 350 x g. The pellet was gently placed atop after resuspension with 3 mL of 55% Percoll solution (see Figure. 5a).

e.4 Gradient interface

Following a 20-minute centrifugation of the gradient, mononuclear cells could be collected from the 55/70 interface, while granulocytes could be collected from the 70/81 interface. Erythrocytes settled at the bottom of the tube (see Figure. 5b).

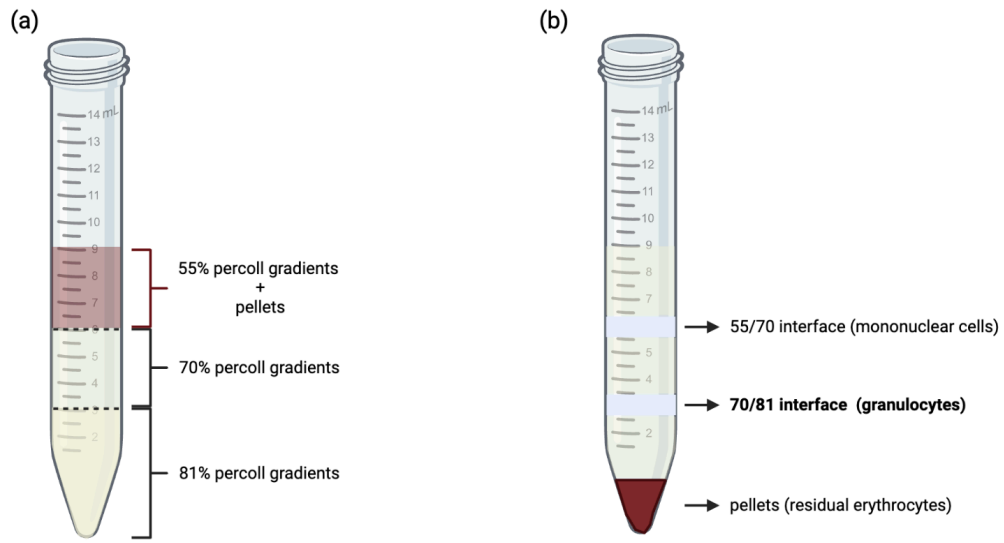


Figure 5: Percoll Density Gradient

(a) Percoll Discontinuous Gradients. The bottom layer consists of 3 mL of 81% Percoll, middle layer contains 70% Percoll, and 55% Percoll layer is carefully mixed with 3 mL of cell pellets, which are then resuspended in the 55% Percoll layer.

(b)Different Cell Layers after Percoll Density Gradient Centrifugation. Mononuclear cells can be extracted from the 55/70 interface, and granulocytes are obtained from the 70/81 interface. Finally, residual erythrocytes are pelleted at the bottom.

f. Cytospin and analysis of neutrophil morphology

Isolated neutrophils were immobilised onto glass slides for microscopic analysis of neutrophil morphology. Neutrophils were resuspended in DPBS (+/+) at a concentration of $5.0x10^6$ cells/ml. The glass slides, filter cards, and cytology funnel were assembled and placed in the cytospin centrifuge, ensuring balance. 150 µL of

the resuspended solution ($0.75x10^6$ neutrophils) was added to the funnel and cytocentrifuged at 300 rpm for 3 minutes at room temperature. Subsequently, the slides were immersed in 100% methanol (ThermoFisher, M/4000/17) and stained with Kwik-Diff dye (ThermoFisher, 9990706 and 9990707) before being air-dried for 1 hour. The slides were then mounted using mounting media (Sigma, 06522) and left to air-dry overnight at room temperature. Glass slides were visualised using a Leica DMi1 microscope with 400X magnification. Each sample was subjected to random image capture, with five images obtained per sample. The following morphological parameters were then analysed: Number of Granulocytes, Number of Neutrophils, Number of Eosinophils, Number of Neutrophil Lobes (categorized as normal, immature, or hyper-segmented), Presence of Vacuolation in Cells and Average Count of the Mentioned Parameters per Sample.

g. Flow-cytometric staining:

g.1 Staining on whole blood.

A fresh 100 µL whole blood sample was incubated with fluorochrome-conjugated anti-human antibodies for 20 minutes on ice in the dark. See the reagent table for antibodies. For red blood cell lysis, stained blood samples were treated with FACS lysing buffer (BD, 349202, according to manufacturer instruction) for 8 minutes at room temperature. Subsequently, neutrophils were pelleted by centrifugation at 300 x g for 5 minutes at 4 °C and washed in 1X FACS buffer (1X FACS buffer was prepared by diluting 10X FACS buffer in 1X PBS at a 1:10 ratio). Cells were then fixed overnight in 100µL of fixation buffer (ThermoFisher, 00-5223-56 and 00-5123-43). The following day, the neutrophils were resuspended in 1XFACS buffer, ready for flow cytometry analysis (BD, 6L LSRFortessa).

g.2 Staining on isolated neutrophils.

Isolated neutrophils were treated for 20 minutes on ice with fluorochrome-conjugated anti-human antibodies in the absence of light, as detailed in the reagent table. Subsequently, the neutrophils underwent two washes in 1XFACS buffer at 300 x g for 5 minutes at 4 °C to eliminate unbound antibodies. The neutrophils were then fixed overnight in 200 µL of fixation buffer (ThermoFisher, 00-5223-56 and 00-5123-43). The following day, the neutrophils were resuspended in 1XFACS buffer and prepared for flow cytometry analysis (BD 6L LSRFortessa).

g.3 Mitochondrial staining.

Neutrophils were stained with MitoSpyTM Red CMXRos (ThermoFisher, 424801) to assess the mitochondrial content of neutrophils using flow-cytometry. The reagent was dissolved in DMSO to establish a 1 mM stock solution. Subsequently, purified neutrophils were incubated with 50 nM Red CMXRos in 37 °C water bath for 30 minutes. Cells then underwent centrifugation at 300 x g for 5 minutes prior to staining with surface markers CD14 and CD16 on ice. Mitochondrial fluorescence intensity was then quantified using flow cytometry (BD, 6L LSRFortessa).

g.4 Intracellular staining.

A 100 µL whole blood sample was incubated on ice for 20 minutes with anti-human antibodies in the dark. Table.2 lists the antibodies. Following that, the stained blood samples were treated for 8 minutes at room temperature with FACS lysing buffer (BD, 349202, diluted 1/10 in water according to the manufacturer's recommendations). After centrifugation at 300 x g for 5 minutes at 4 °C, neutrophils were pelleted and treated in fixation buffer for 1 hour in the dark at room temperature. -. Next, the samples were treated with 1x Permeabilization buffer (ThermoFisher, 00-8333-56) and centrifuged. The pellets were then suspended overnight for labelling with antibodies detecting intracellular epitopes (details in Table.2). At this point, the control group received an identical amount of permeabilisation buffer. The next day, neutrophils were washed in permeabilisation buffer and resuspended in 1XFACS buffer in preparation for flow cytometry analysis (BD, 5L LSRFortessa).

h. Stimulation

Purified neutrophils were suspended in DPBS (+/+) at a concentration of $5.0x10^6$ cells/ml. The neutrophils were then activated using 100 nM fMLP and incubated in a 37°C water bath for 30 minutes, with gentle flicking every 15 minutes. Following 30 minutes of fMLF stimulation, neutrophils were pelleted by centrifugation at 300 x g for 5 minutes at 4°C and then stained with antibodies. Details were described in method e.2.

i. Compensation Setting

i.1 Beads

For optimization of fluorescence compensation settings in multi-colour flow cytometric analysis, we employed UltraCompeBeadsTM Compensation Beads (Thermofisher, 01-2222-42) on the flow cytometers (5L LSRFortessa and 6L LSRFortessa). These beads were stained with appropriate fluorochrome-labelled anti-human antibodies for 20 minutes on ice. Following staining, the beads were washed with 1xPBS buffer and resuspended for subsequent flow cytometric analysis. For the samples, our compensation process involved both neutrophils and whole blood samples.

i.2 Neutrophils

Neutrophils were isolated from blood samples obtained from healthy human volunteers. Cell concentrations were adjusted to $5x10^6$ cells/mL. We added fluorochrome-conjugated antibodies targeting various surface markers (Table.2) at the specified concentrations and allowed them to incubate with the isolated neutrophils for 20 minutes on ice. After undergoing two washes with 1xFACS buffer, the cells were resuspended in preparation for flow cytometric analysis.

i.3 Blood

Whole blood samples from healthy donors were stained using fluorochrome-conjugated antibodies for 20 minutes on ice (Table.2). Subsequently, an 8-minute

lysing step was performed using the FACS lysing buffer. The lysed whole blood was then washed and resuspended in 1xFACS buffer, making it ready for flow cytometric analysis.

Flow cytometry	Fluorophore	Antibody	Sample		
			beads	neutrophils	Whole blood
6L LSRFortessa	BV421	CD14	√	√	√
	FITC	HLA-DR	√	√	√
	PE	TLR4	√	√	√
	PEDazzle 594	CD15	√	√	√
	PerCPcy55	CD62L	√	√	√
	PE/Cy7	CD16	√	√	√
	AF700	CD11b	√	√	√
	BV605	CXCR4	√	√	√
	APC AF 647	CXCR2	√	√	√
	APC-Cy7	CD66b	√	√	√
	BV650	CD33	√	√	√
	BV711	CD10	√	√	√
5L LSRFortessa	BV421	CD64	√		√
	FITC	S100A9	√		√
	BV650	CD11c	√		√
	PE	CD14	√		√
	PEDazzle 594	CD15	√		√
	APC	CD74	√		√
	AF700	CD11b	√		√
	APC H7	B2M	√		√

Table 2. Flow Cytometry Experiment Details

j. Gating strategy

-For whole blood: To analyse the expression levels of markers on neutrophil surfaces within whole blood and isolated neutrophils, we employed the FlowJo analysis software (Becton Dickinson, UK) to generate FACS plots. Initial identification of granulocytes (Granulocytes Gating) within the sample was achieved by setting a gate based on scatter characteristics (SSC-A vs. FSC-A). Within the granulocyte population, we isolated individual cells (Single Cell Gating) using a gate based on forward scatter height (FSC-H) against forward scatter area (FSC-A). We further refined our analysis gating on CD14-CD16+ neutrophils (Neutrophil Gating) (Figure. 6a).

-For purified neutrophils: The first step in identifying granulocytes was to set a gate based on scatter characteristics (SSC-A vs. FSC-A). Single cells (Single Cell Gating) were selected from the granulocyte population using a gate established by forward scatter height (FSC-H) vs. forward scatter area (FSC-A). Neutrophil we then gated as

CD14-CD16+ population (Figure. 6b).

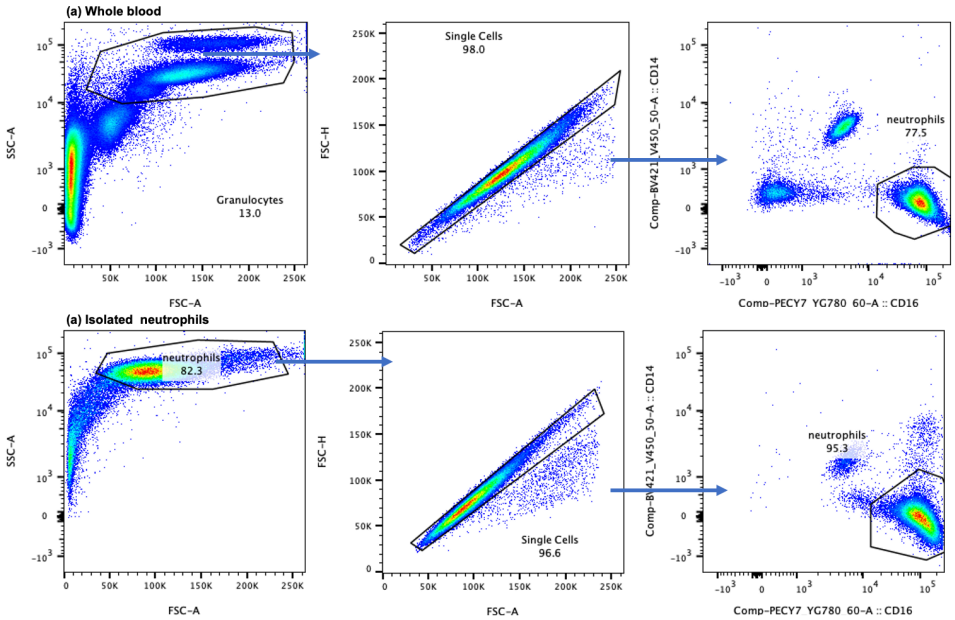


Figure.6. Gating Strategy:
Gating for whole blood samples (a), and gating for isolated neutrophils (b).

Data Analysis

Statistical analyses were performed using GraphPad Prism version 9 (GraphPad, USA). Before analysis, outliers within each sample were identified by the ROUT method and removed. Normality and lognormality tests were conducted to ensure data distribution adherence. Experiment results were subjected to analysis of variance (ANOVA), unpaired or paired T-tests as deemed appropriate. In our representation of results, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significance in our experiments was defined by P values lower than 0.05.

Material

All reagents with lowest possible endotoxin level.

REAGENT OR RESOURCE	DESIGNATION	SOURCE	Clone	IDENTIFIER	ADDITIONAL INFORMATION
Antibody	Brilliant Violet 421™ anti-human CD14 Antibody	BioLegend	63D3	Cat #: 367144 RRID: AB_2810580	Concentration: 1:40
	FITC anti-human HLA-DR	BioLegend	L243	Cat #: 307604 RRID: AB_314682	Concentration: 1:40
	BD Pharmingen™ PE Mouse Anti-Human TLR4 (CD284)	BD	TF901	Cat #: 564215 RRID: AB_2738674	Concentration: 1:40
	PE/Dazzle™ 594 anti-human CD15 (SSEA-1) Antibody	BioLegend	W6D3	Cat #: 323038 RRID: AB_2564103	Concentration: 1:40
	PerCP/Cyanine5.5 anti-human CD62L Antibody	BioLegend	DREG-56	Cat #: 304824 RRID: AB_2239105	Concentration: 1:40
	PE/Cyanine7 anti-human CD16 Antibody	BioLegend	3G8	Cat #: 302016 RRID: AB_314216	Concentration: 1:100
	Alexa Fluor® 700 anti-mouse/human CD11b Antibody	BioLegend	M1/70	Cat #: 101222 RRID: AB_493705	Concentration: 1:500
	Brilliant Violet 605™ anti-human CD184 (CXCR4) Antibody	BioLegend	12G5	Cat #:306522 RRID: AB_2563631	Concentration: 1:40
	Alexa Fluor® 647 anti-human CD182 (CXCR2) Antibody	BioLegend	5E8/CXCR2	Cat #:320714 RRID: AB_492940	Concentration: 1:40
	APC/Cyanine7 anti-human CD66b Antibody	BioLegend	G10F5	Cat #:305126 RRID: AB_2750184	Concentration: 1:40
	Brilliant Violet 650™ anti-human CD33 Antibody	BioLegend	WM53	Cat #:303430 RRID: AB_2650934	Concentration: 1:40
	Brilliant Violet 711™ anti-human CD10 Antibody	BioLegend	HI10a	Cat #: 312226 RRID: AB_2565876	Concentration: 1:40
	MRP14 Antibody, anti-human, REAfinity™	Miltenyi	REA859	Cat #:130-114-515	Concentration: 1:50
	APC/Cyanine7 anti-human β2-microglobulin Antibody	BioLegend	A17082A	Cat #: 395717 RRID: AB_2904417	Concentration: 1:50
	Brilliant Violet 650TM anti-human CD11c	BioLegend	Bu15	Cat #: 337238 RRID: AB_2721552	Concentration: 1:50

	Antibody				
	APC anti-human CD74 (Cytoplasmic) Antibody	BioLegend	Pin.1	Cat #: 357606 RRID: AB_2716217	Concentration: 1:50
	Brilliant Violet 421™ anti-human CD64 Antibody	BioLegend	10.1	Cat #: 305020 RRID: AB_2561828	Concentration: 1:50
	PE anti-human CD14	BioLegend	HCD14	Cat #: 325606 RRID: AB_830679;	Concentration: 1:50
REAGENT	FACS™ Lysing Solution	BD	N/A	Cat #: 349202	Concentration: 1:10 (in H_2O)
	Lymphoprep™	STEMCELL	N/A	Cat #: 07851	N/A
	DPX Mountant for histology slide mounting medium	SIGMA	N/A	Cat #: 06522	N/A
	Ethylenediaminetetraacetic acid disodium salt solution	Sigma-Aldrich	N/A	Cat #: E7889	N/A
	Bovine Serum Albumin	Sigma-Aldrich	N/A	Cat #: A7906	N/A
	eBioscience™ Fixation/Permeabilization Diluent	ThermoFisher	N/A	Cat #: 00-5223-56	1:3(Permeabilization : Diluent)
	eBioscience™Fixation/Permeabilization Concentrate	ThermoFisher	N/A	Cat #: 00-5123-43	1:3(Permeabilization : Diluent)
	UltraCompeBeads™ Compensation Beads	ThermoFisher	N/A	Cat #: 01-2222-42	N/A
	MitoSpy™ Red CMXRos	BioLegend	N/A	Cat #: 424801	1:200 (PBS+/+)
	DPBS,no calcium,no magnesium	ThermoFisher	N/A	Cat #: 14190094	N/A
	DPBS, calcium, magnesium	ThermoFisher	N/A	Cat #: 14040091	supplemented with 1 g/L glucose and 4 mM sodium bicarbonate (PBS++)
	PBS (10X), pH 7.4	ThermoFisher	N/A	Cat #: 70011044	
	D-(+)-Glucose solution	Sigma-Aldrich	N/A	Cat #: G8769	1.1 ml to 500 ml of PBS++
	Sodium bicarbonate solution	Sigma-Aldrich	N/A	Cat #: S8761	2.24 ml to 500 ml of PBS ++
	NaCl 0.9% Sodium Chloride (Saline)	Baxter	N/A	Cat #: TRF7124	N/A
	Percoll 1L	Cytiva	N/A	Cat #:17-0891-01	N/A
	Methanol	ThermoFisher	N/A	Cat #:M/4000/17	N/A

	Kwik-Diff™ Reagent 2, Eosin	ThermoFisher	N/A	Cat #: 9990706	N/A
	Kwik-Diff™ Reagent 3, Methylene Blue	ThermoFisher	N/A	Cat #: 9990707	N/A
	Relyon Virkon Tablets	Virkon	N/A	Cat #: 57804632	N/A
	Permeabilization Buffer (10X)	ThermoFisher	N/A	Cat #: 00-8333-56	1:10 dilution
	Dulbecco's Phosphate Buffered Saline with 2% Fetal Bovine Serum	STEMCELL	N/A	Cat #: 07905	N/A
Software and Algorithms	ImageJ	NIH	N/A	Schneider, C.A., et al, 2012.	
	Prism	GraphPad	N/A	RRID:SCR_002798	
	FlowJo™ v10.9	BD Life Sciences	N/A	RRID: SCR_008520 https://www.flowjo.com/solutions/flowjo	
	R	The R Foundation		https://www.r-project.org	
other	SepMate™-50	STEMCELL	N/A	Cat # :85450	

Results

Chapter 1. Neutrophil Marker Expression During Liver Cirrhosis Progression

Assessing neutrophil markers in healthy individuals and cirrhotic patients is a pivotal approach to understanding the interplay between neutrophils and the development of liver cirrhosis. This chapter will specifically focus on evaluating the expression of surface markers and delve into the complex behaviour of neutrophils upon stimulation.

1.1 Neutrophils from early and advanced cirrhosis patients show normal activation pattern after stimulation with fMLP

We used fMLP to study the activation of isolated neutrophils in patients with early and advanced cirrhosis. Neutrophils were incubated for 30 minutes with fMLP before analysing cell surface marker expression. Activation of neutrophils is known to lead to increased expression of CD11b (39), CD66b (43), CD10 (17,81) and CXCR4 (12), and decreased expression of CD62L (45,46), CXCR2 (47,50,51), and the decrease of forward scatter (FSC) of neutrophils (82). Our results showed, as expected, that following incubation with fMLP, neutrophils from healthy control showed increased expression of CD11b (Figure. 7a), CD66b (Figure. 7b), CD10 (Figure. 7c), and CXCR4 (Figure. 7d) and decreased expression of CD62L (Figure. 7e), and CXCR2 (Figure. 7f), they also showed lower FSC value than the unstimulated groups (Figure. 7g).

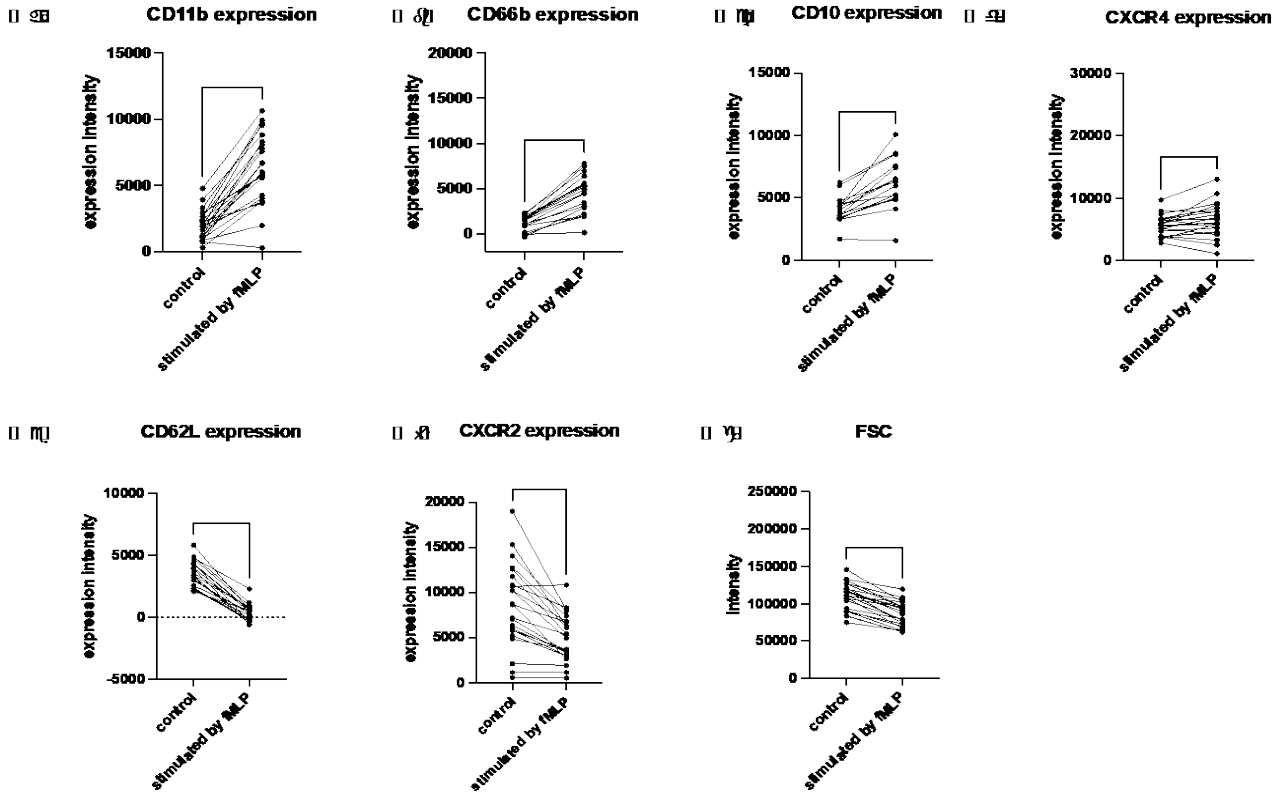


Figure 7: Impact of fMLP Stimulation on Typical Neutrophil Activation Marker Expression (healthy donor)

This figure elucidates alterations in the expression of typical neutrophil activation markers, including (a) CD11b, (b) CD66b, (c) CD10, (d) CXCR4, (e) CD62L, and (f) CXCR2. Also, the (g) changes in neutrophil forward scatter (FSC) before and after fMLP stimulation in neutrophils isolated from healthy donors. The sample size for healthy donors was 24. Data normality and logicality have been rigorously examined, with any outliers appropriately addressed.

Statistical significance is depicted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Two dependent experiments and has been analysed by paired t-test.

It was interesting that neutrophils from early and advanced cirrhosis patients displayed a similar response (Figure. 8a-c, and e-g). It was worth noting a particular exception: while CXCR4 expression typically decreases upon activation, this trend was not observed in isolated neutrophils from late-stage liver cirrhosis patients (Figure. 8d). Moreover, we also observed an increased trend in CD15 expression after fMLP stimulation (Figure. 9a-c), which was observed in neutrophils from healthy individuals and patients with liver cirrhosis. However, CD15 is not considered a typical activation marker.

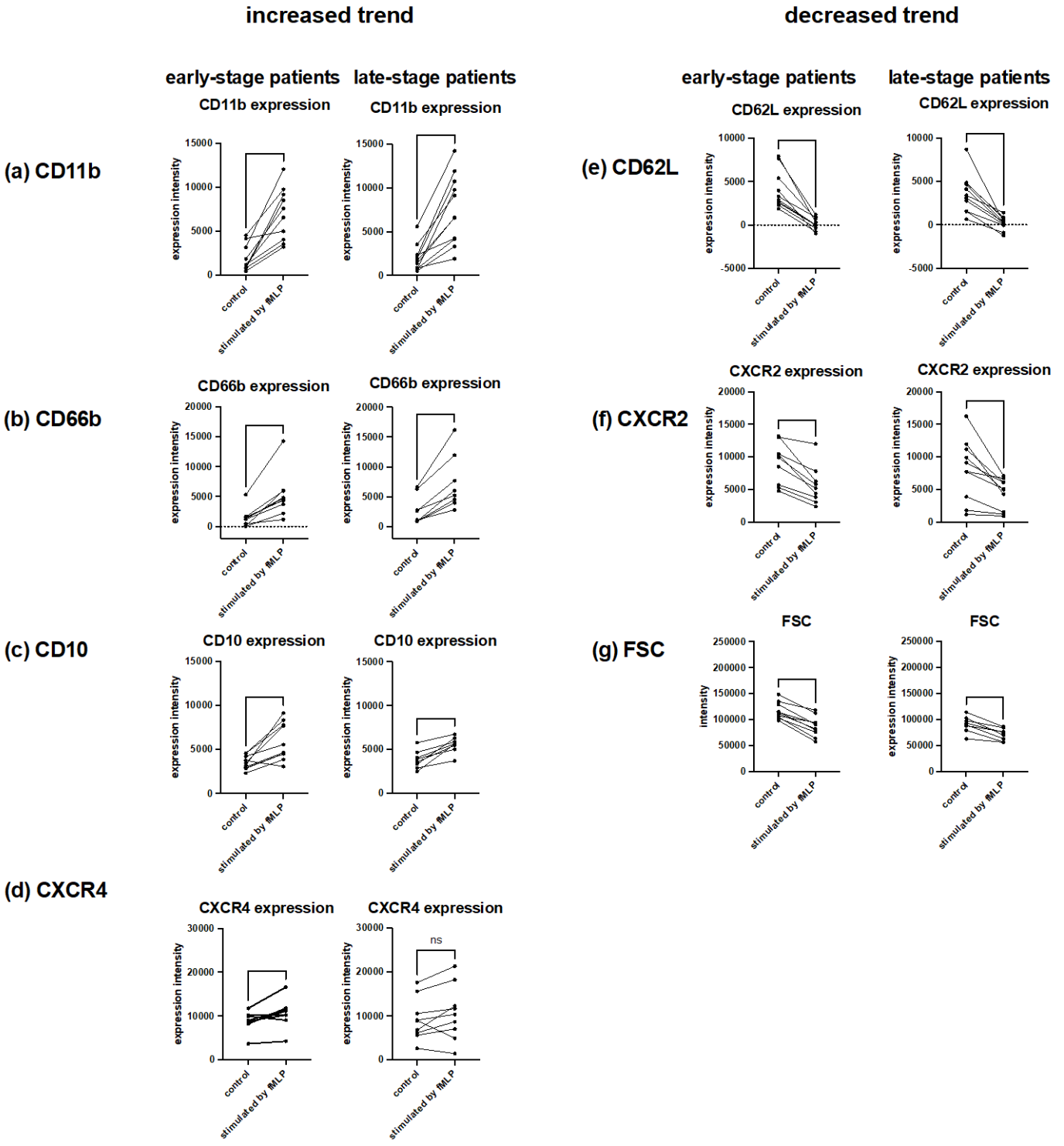


Figure 8: Impact of fMLP Stimulation on Typical Neutrophil Activation Marker Expression in Liver Cirrhosis Patients

This figure provides insights into the alterations observed in the expression of typical neutrophil activation markers, specifically those markers that exhibit an increased trend following fMLP stimulation: (a) CD11b, (b) CD66b, (c) CD10, (d) CXCR4, and markers that display a decreased trend:(e) CD62L, (f) CXCR2, along with (g) FSC, and display their expression level before and after fMLP stimulation in neutrophils isolated from groups of liver cirrhosis patients. Our sample size comprised n=11 for both early and late-stage patients. Stringent assessments were meticulously conducted to ensure data normality and logicality, with any outliers promptly addressed.

Statistical significance is elegantly represented as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Both experiments were dependent and have been analyzed by paired t-test.

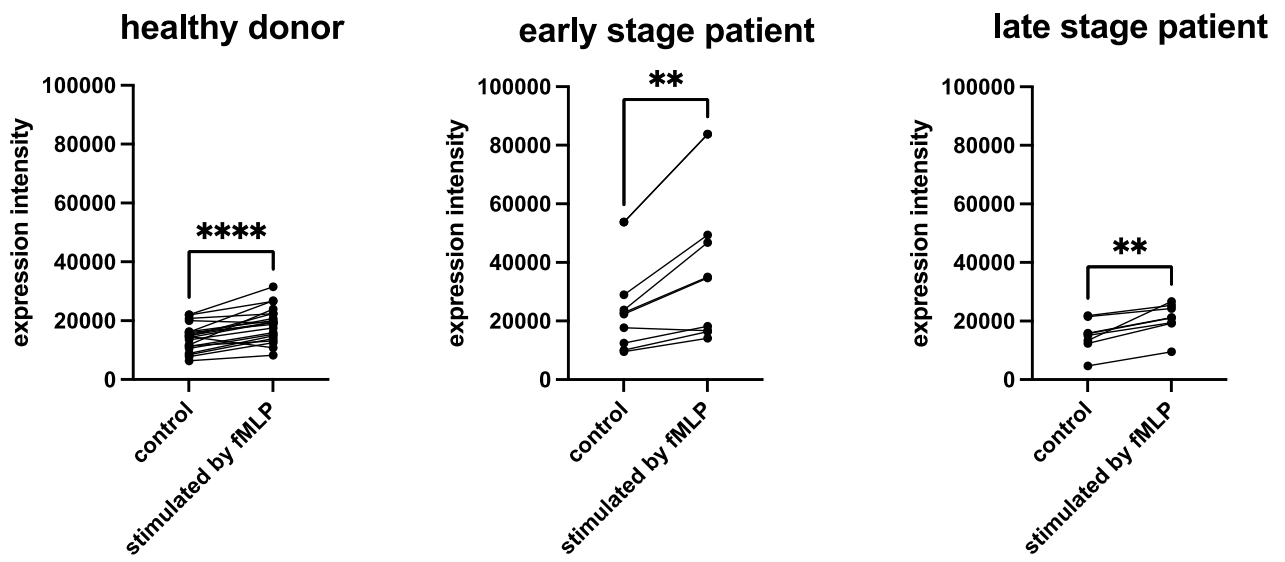


Figure 9: Impact of fMLP Stimulation on CD15 Expression across Different Health Status

This figure provides insights into the alterations observed in CD15 expression levels before and after fMLP stimulation in neutrophils isolated from groups of both healthy individuals and liver cirrhosis patients. Our sample size included n = 21 pairs for healthy individuals, 10 pairs for early-stage patients, and n = 8 for late-stage patients. Rigorous assessments were meticulously conducted to ensure data normality and logicality, with any outliers promptly addressed.

Statistical significance was elegantly represented as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Both experiments were dependent and have been analysed by paired t-test.

In this study, we also assessed the expression of additional surface markers on neutrophils, as detailed in Table. 1, including CD33, TLR4, HLA-DR, and others (Figure. S1)**.** However, there were no statistically significant differences in the expression levels of these markers before and after the fMLP challenge. It is established that fMLP stimulation can effectively induce neutrophil activation, resulting in altered expression levels of multiple surface markers, indicative of changes in various facets of neutrophil immunological reactivity and dynamics. However, more than a simplistic comparison of expression level changes associated with stimulation is required to provide a comprehensive understanding of neutrophils in the context of liver cirrhosis progression. Consequently, the subsequent sections will employ a longitudinal approach to compare the expression patterns of distinct surface markers in healthy donors and patients, enabling a more intricate exploration of the evolving dynamics of neutrophils throughout the progression of liver cirrhosis.

1.2 Changes in level of expression of CD66b, CXCR4, CD15, and HLA-DR in patients with early and advanced cirrhosis

In our previous section, we observed that neutrophils, whether purified from the blood of healthy individuals or liver cirrhosis patients, exhibited seemingly similar responses to the inflammatory signal, fMLP. Using flow cytometry, we comprehensively examined marker expression levels on unstimulated neutrophils extracted from healthy donors and patients at various stages of this disease.

Our initial observation highlighted increased expression of CD66b (Figure. 10a) on unstimulated neutrophils of patients with advanced cirrhosis compared to those in the early stages of liver disease. Furthermore, there was a progressive increase in the expression of CXCR4 (Figure. 10b) and HLA-DR (Figure. 10c) on neutrophils with the advancement of cirrhosis. Additionally, neutrophils in cases with advanced disease still exhibited the highest levels compared to other groups, and this increase was statistically significant compared to healthy donors. Regarding CD15 and TLR4 expression (Figure. 10d-e), we observed an increase in the early-stage groups, and the expression levels of these markers were also significantly different from those in healthy donors. Additionally, there was a notable decrease in forward scatter (FSC) on unstimulated neutrophils extracted from advanced patients (Figure. 10f), and this decline was statistically significant compared to healthy and early-stage individuals.

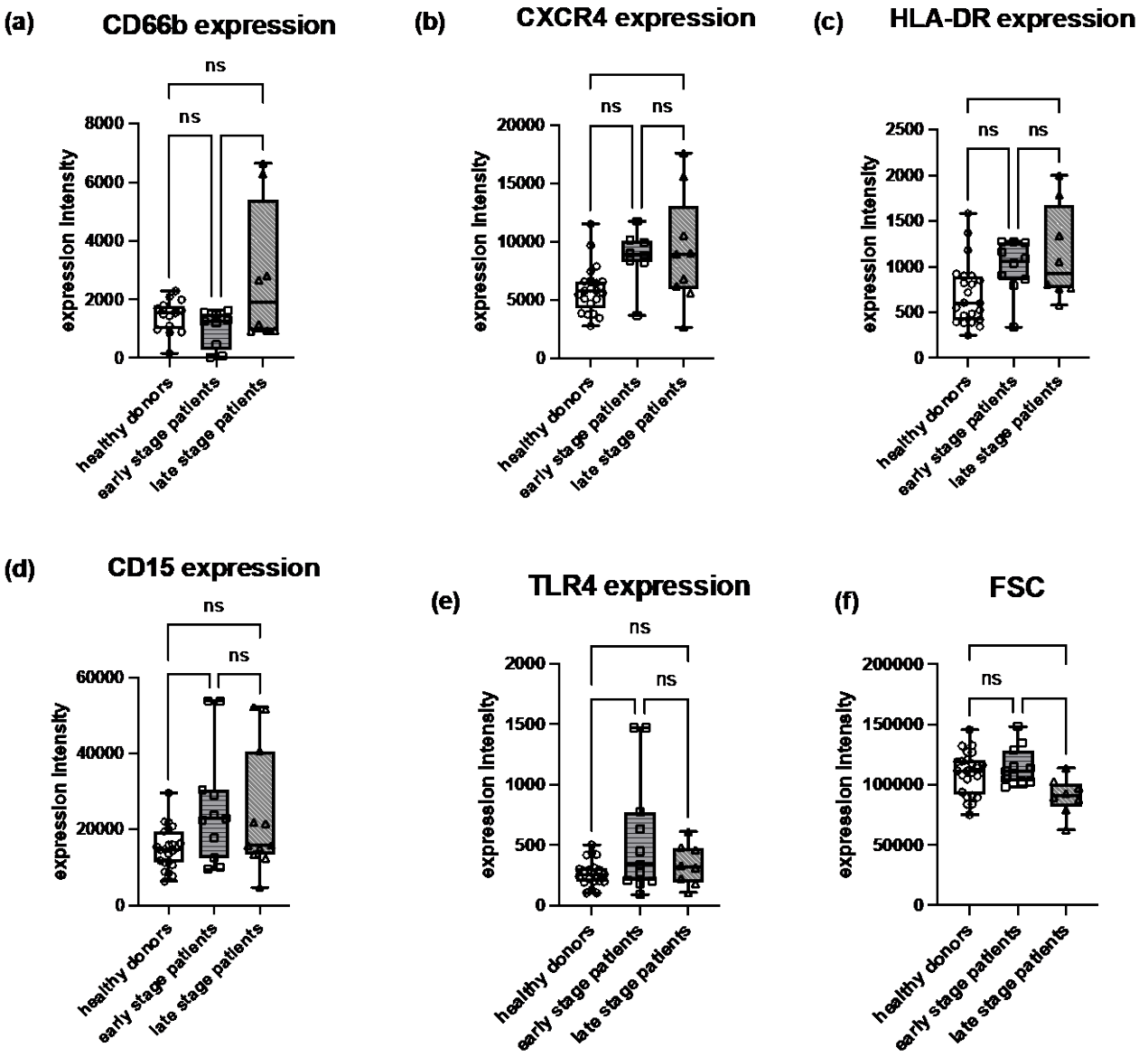


Figure 10: Unstimulated Neutrophil Surface Marker Expression

This figure illustrates the expression levels of various surface markers on neutrophils obtained from healthy donors and individuals with early to late-stage liver cirrhosis. These markers include CD66b (a), CXCR4 (b), HLA-DR (c), CD15 (d), and TLR4 (e). Additionally, it presents the forward scatter (FSC) values (f) for unstimulated purified neutrophils across various health statuses.

Sample sizes for each marker are as follows: CD66b (n = 15 healthy control donors, 9 early-stage patients, and 8 late-stage patients), CXCR4 (n = 21 healthy control donors, 9 early-stage patients, and 8 late-stage patients), HLA-DR (n = 22 healthy control donors, 10 early-stage patients, and 8 late-stage patients), CD15 (n = 22 healthy control donors, 11 early-stage patients, and 11 late-stage patients), TLR4 (n = 19 healthy control donors, 11 early-stage patients, and 8 late-stage patients), and FSC (n = 24 healthy control donors, 11 early-stage patients, and 8 late-stage patients). Data normality and logicality have been tested, and outliers have been removed.

Statistical significance is denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data represents the outcomes of independent experiments and has been analyzed using the one-way ANOVA test.

1.3 Fold changes of marker expression in responses to inflammatory challenges

Subsequently, we analysed the fold change in the expression of various surface markers before and after fMLP stimulation. Notably, we observed a considerably higher change in CD11b expression in the early-stage patient group compared to healthy donors and advanced-stage patients (Figure. 11a). This difference suggests that following fMLP stimulation, the increase in CD11b expression in early-stage neutrophils is more pronounced than in neutrophils from the other groups. Furthermore, we found the highest fold change in Forward Scatter (FSC) in the late-stage individuals, indicating the most significant size changes in neutrophils within that population after stimulation (Figure. 11b).

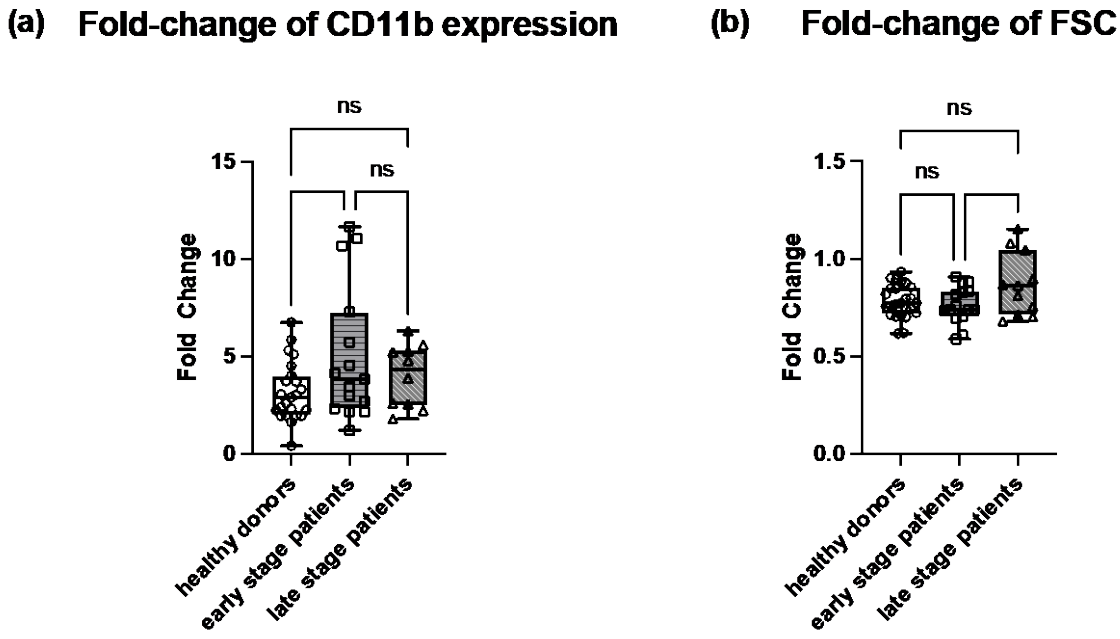


Figure 11: Fold Change in Surface Marker Expression before and after fMLP stimulation

This figure illustrates the fold change in CD11b (a) expression, as well as the forward scatter on neutrophils (b), before and after isolated neutrophils were stimulated by fMLP in different groups.

The sample sizes for each marker are as follows: CD11b (n = 23 healthy control donors, 15 early-stage patients, and 10 late-stage patients), and forward scatter (n = 25 healthy control donors, 15 early-stage patients, and 11 late-stage patients). Data normality and logicality have been tested, and outliers have been removed.

Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data represents the outcomes of independent experiments and has been analysed using the one-way ANOVA test.

Chapter 2. Neutrophil Morphology in Liver Cirrhosis Progression

Neutrophil morphology includes characteristics such as the cell shape and nuclear structure all of which impact the function of neutrophils inside the immune system. This chapter starts with a detailed look into neutrophil morphology in the context of liver cirrhosis progression.

2.1 Morphological Diversity and Subpopulations of Neutrophils

Eosinophils and neutrophils, categorised as granulocytes prevalent in human peripheral blood and tissues, share fundamental attributes, most notably their diminutive, round-shaped structures (83). However, they are readily identifiable by their different structures of cell nuclei. For example, neutrophils frequently exhibit 3 to 4 lobes, and most circulating neutrophils have three. (Figure. 12 a). In striking contrast, eosinophils are spherical immune cells and can be distinguished by a bilobed nucleus and substantial acidophilic cytoplasmic granules (Figure. 12 b).

Regarding neutrophils, different stages of neutrophil development are typically associated with distinct nuclear morphologies. For example, the hallmark of a metamyelocyte is an indented nucleus, and band cells were also immature neutrophils featuring nuclei reminiscent of a horseshoe, as their nuclei are usually U or S shape, eventually constricting to form nuclear lobes (Figure. 12 c). Finally, mature segmented neutrophils are readily identifiable by their nuclei sporting three to five lobes (Figure. 12a) (17). Beyond these stages, neutrophils can occasionally undergo hyper segmentation, a phenomenon marked by neutrophils bearing six or more lobes (Figure. 12 d). Hyper-segmented neutrophils have been observed in specific conditions, such as megaloblastic anaemia or certain systemic inflammatory disorders (23).

Moreover, it is worth noting that neutrophils may exhibit vacuolisation in the blood (Figure. 12e) —a degenerative condition highly indicative of infection or poisoning. Vacuolation is characterised by small vacuoles or translucent areas within the cytoplasm of the neutrophils. It is also important to note that neutrophil vacuolation can indicate various underlying health conditions, including infections or inflammation (24,84). The extent of vacuolisation often corresponds with the severity of the disease in the patient (24).

To examine neutrophil morphology, we prepared cytospins. Stained slides were then examined by light microscopy. Figure 12 offers a visual representation of the diverse morphologies observed. Subsequently, we counted several morphological parameters, including the number of granulocytes, number of neutrophils, number of eosinophils, number of neutrophil lobes (classified as regular, immature, or hyper-segmented), and the presence of vacuolation in cells. We then calculated the average percentage of these morphological parameters and compared them across groups with varying

health statuses.

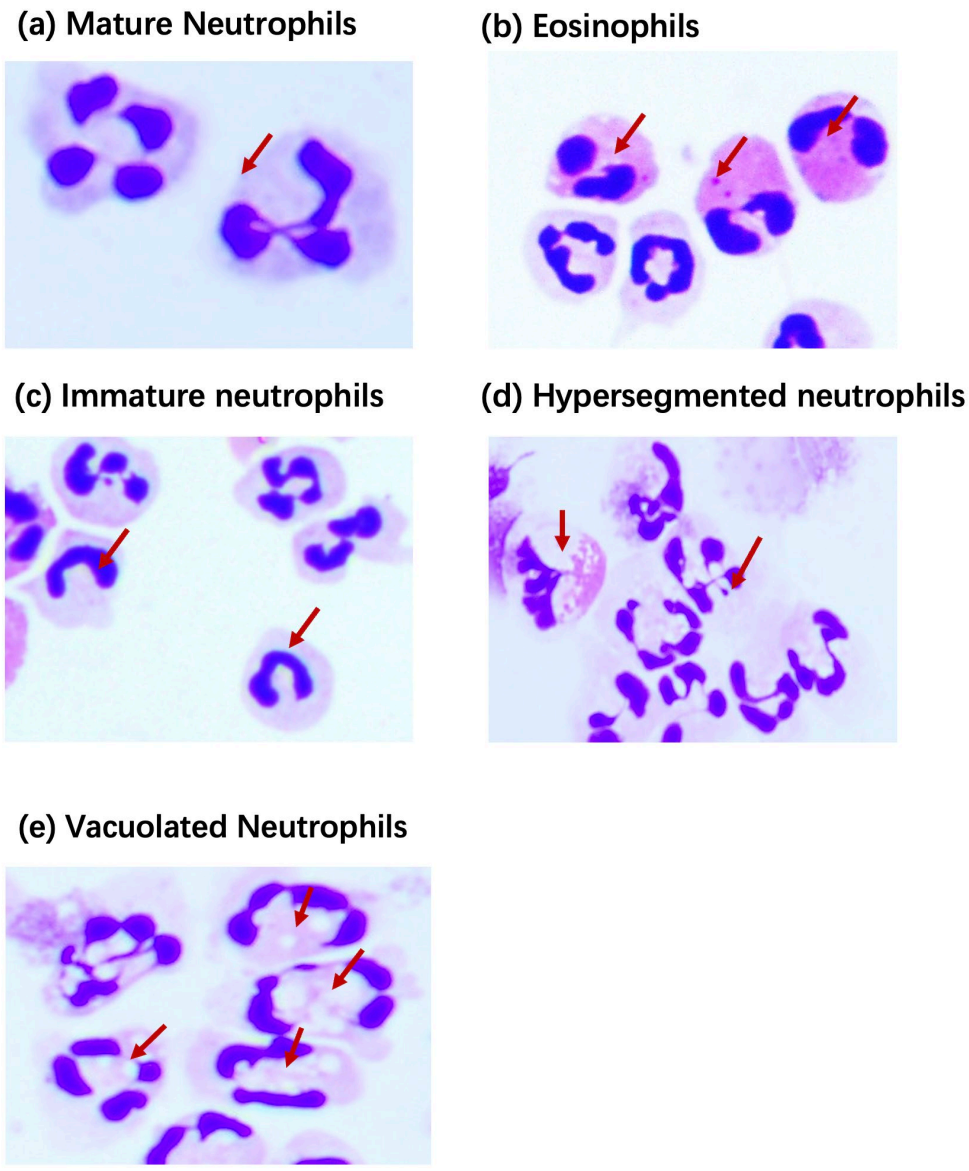


Figure 12. Neutrophil and Eosinophil Morphology.
(a) Mature neutrophils, indicated by red arrows, typically feature 3-5 lobes. (b) Eosinophils (red arrow pointed) typically display two lobes and appear pink. (c) Immature neutrophils (red arrow pointed) exhibit unsegmented "C" or "S" shaped nuclei. (d) Hyper-segmented neutrophils (red arrow pointed) present with more than 5 lobes. (e)Vacuolated neutrophils (red arrow pointed) demonstrate vacuoles within their structure.

2.2 Reduced Neutrophil Proportions in Normal Mature Neutrophils in Liver Cirrhosis Patients

Initially, we evaluated the proportion of neutrophils within the isolated granulocyte population in each group and conducted statistical comparisons. Our findings revealed a significant decrease in the proportion of neutrophils within the granulocyte population in advanced-stage patients than in healthy or early-stage groups, suggesting these individuals were neutropenic. However, there was no noticeable difference in the number of neutrophils between healthy individuals and those in the early stages of liver cirrhosis (Figure. 13 a). Following our macroscopic analysis of neutrophil proportions in granulocytes within each group, we delved deeper to investigate whether the progression of liver cirrhosis had discernible effects on neutrophil morphology, including the number and shape of their nuclear lobes. The initial focus was assessing the proportion of normal mature segmented neutrophils in groups representing different health conditions.

Our findings (Figure. 13 b) revealed that more than 90% of neutrophils in healthy donors exhibited the characteristic mature lobulated morphology with a standard number of lobes. However, compared to healthy individuals, both the early and late-stage patient groups displayed a significantly lower proportion of neutrophils with this typical mature morphology (Figure. 13 b). This decrease suggests neutrophils in liver cirrhosis patients might exhibit differences in maturity, as evidenced by their distinct nuclear morphologies compared to mature cells (Figure. 1 granulopoiesis). This observation underscores the need for further investigation into the functional implications of these morphological differences in neutrophils within the context of liver cirrhosis.

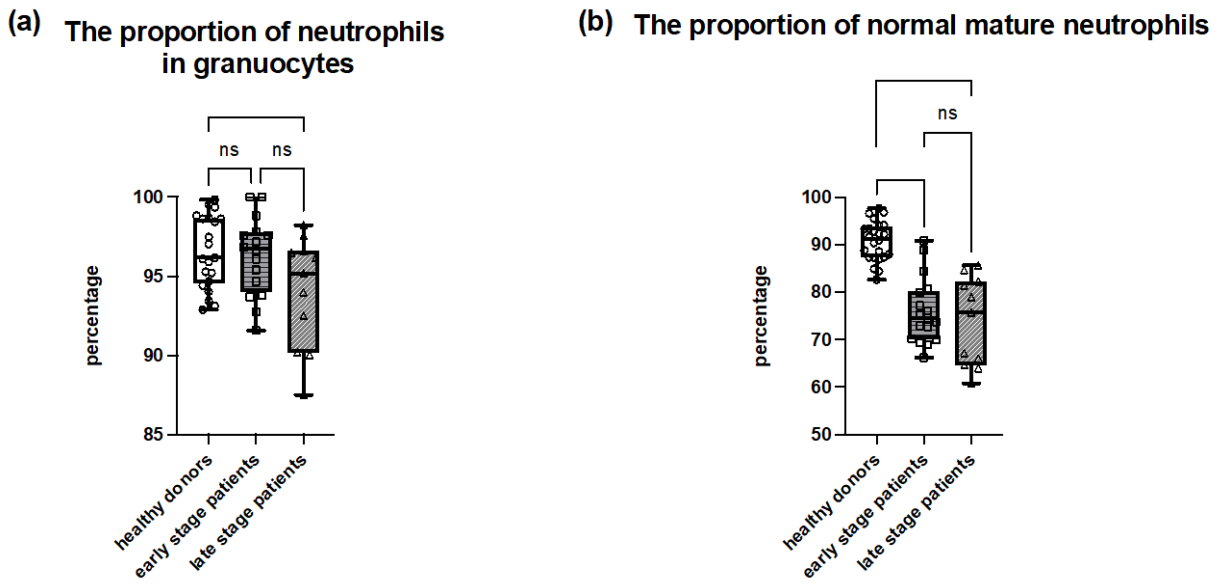


Figure 13: Neutrophil Morphologies Across Different Health Status Groups

This figure illustrates the percentages of neutrophil populations within isolated granulocytes (n = 21 healthy control donors, 16 early-stage patients, and 11 late-stage patients) (a) and the normal mature neutrophils (n = 24 healthy control donors, 17 early-stage patients, and 11 late-stage patients) (b) among donor groups ranging from healthy individuals to those with early and late-stage liver cirrhosis. Data normality and logicality have been tested, and outliers have been removed.

Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data represents the outcomes of independent experiments and has been analyzed using the One-way ANOVA Test, providing insights into the distribution of neutrophil morphologies across different health status groups.

2.3 Increased percentage of immature and abnormal neutrophils in patients with liver cirrhosis

Compared to healthy individuals, a significant increase in band neutrophils (immature neutrophils) was observed in both early-stage and late-stage patient groups (Figure. 14a).

Furthermore, we identified that late-stage patients exhibited a significantly higher percentage of hyper segmented neutrophils than healthy individuals or those in the early stages of the disease (Figure. 14b). Specifically, less than 5% of neutrophils in healthy individuals or those in the early stages of the disease displayed hyper segmentation. In contrast, late-stage patients showed an increase, with approximately 10% of neutrophils exhibiting hyper segmentation.

In addition to the previously mentioned neutrophil morphologies, our examination of cytospin slides also revealed the presence of vacuolation in some neutrophils. The

percentage of vacuolated neutrophils was significantly increased in advanced patients (Figure. 14c). These morphological changes in neutrophils further emphasize the complexity of their behaviour in liver cirrhosis. Further investigations are needed to elucidate the functional implications of these morphological variations in the context of liver cirrhosis.

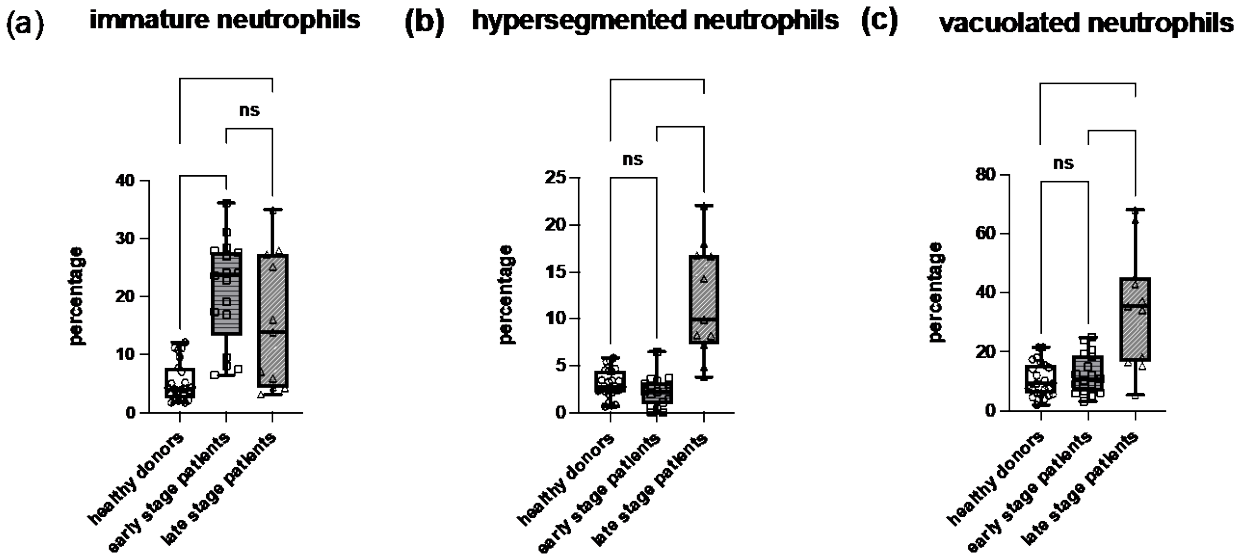


Figure 14: Distribution of Neutrophil Morphologies Across Different Health Status Groups

This figure illustrates the percentages of immature neutrophils (n = 23 healthy control donors, 17 early-stage patients, and 11 late-stage patients) (a), hyper segmented neutrophils (n = 23 healthy control donors, 17 early-stage patients, and 11 late-stage patients) (b), and vacuolated neutrophils (n = 23 healthy control donors, 17 early-stage patients, and 11 late-stage patients) (c) among donor groups ranging from healthy individuals to those with early and late-stage liver cirrhosis. Data normality and logicality have been tested, and outliers have been removed.

Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data represents the outcomes of independent experiments and has been analyzed using the One-way ANOVA Test, providing insights into the distribution of neutrophil morphologies across different health status groups.

2.4 Neutrophils from patients with early liver cirrhosis contain more mitochondria

To determine whether the progression of cirrhosis affected neutrophil mitochondrial levels, we systematically analysed the difference in mitochondrial levels across persons with varying health statuses. Despite failing to achieve statistical significance in our comparison of data from three distinct groups, including healthy individuals and early to late-stage cirrhosis patients, we observed an increase in early cirrhosis patients (Figure. 15a). Then, we performed unpaired T-tests comparing healthy individuals to those in the early stages of liver cirrhosis. We obtained statistically

significant results, indicating that neutrophils from early-stage patients exhibited higher mitochondria levels.

Figure. 15 b represents the outcomes of a confocal experiment by my colleague, Jiaqi Shen. In his experiment, mitochondria were stained red using the Mitospy stain, while neutrophil nuclei were stained in blue with Hoechst. His results show that the Mitospy stain effectively labelled the mitochondria, confirming their presence in the neutrophils.

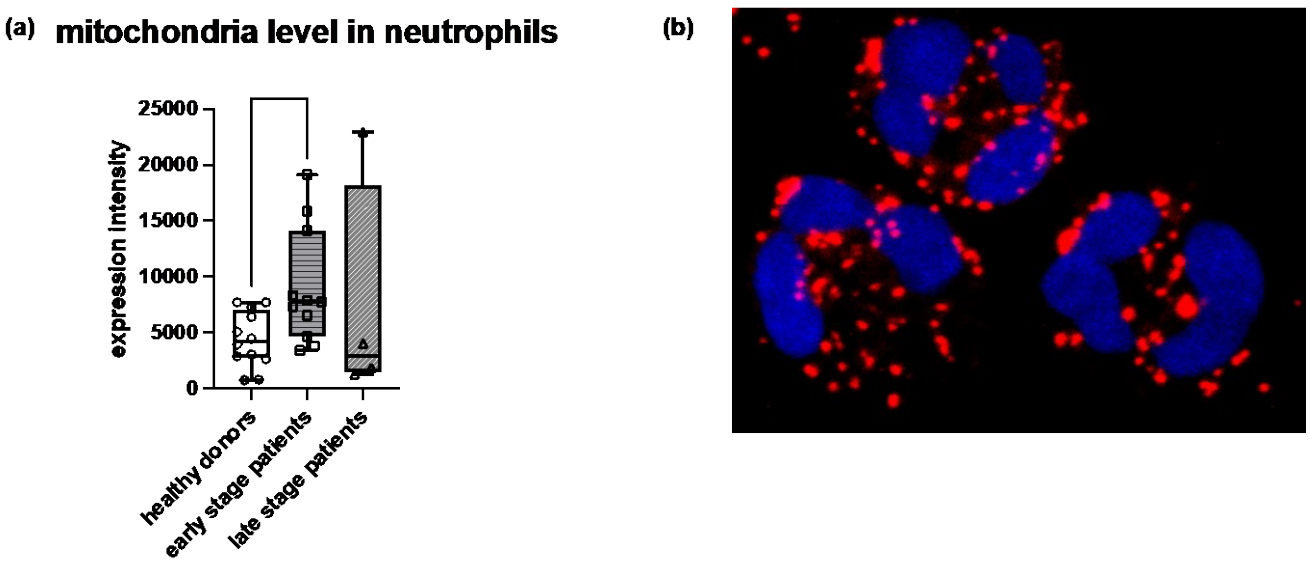


Figure 15: Mitochondria Level in Neutrophils Across Different Health Statuses

(a) Neutrophil mitochondrial levels were assessed in three different groups, including healthy control donors and individuals at different disease stages (n = 12 healthy controls, 11 early-stage patients, and 4 late-stage patients). Data normality and logicality have been verified, with outliers removed. The analysis was employed the unpaired T-Test, indicating statistical significance with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. These data, derived from independent experiments.

(b) The confocal image in this section, created by Jiaqi, displays mitochondria stained red with Mitospy and neutrophil nuclei stained blue with Hoechst.

Chapter 3. Factors that can Influence on Neutrophil Markers and Morphology

3.1 Variations in marker expression across distinct fundamental aetiologies

Cirrhosis of the liver can result from a variety of chronic liver diseases, conditions, and risk factors. Chronic viral hepatitis, alcoholism, non-alcoholic fatty liver disease (NAFLD), primary biliary cholangitis (PBC), and numerous other conditions are among them. It is critical to note that our study focused on patients with NAFLD and PBC. As a result, this section aims to investigate whether distinct types of liver diseases linked with cirrhosis can induce differences in neutrophil characteristics.

Possible variances in the age and maturity of neutrophils may exist among early-stage liver cirrhosis patients with NAFLD and PBC as the driving factors. For example, the expression of CXCR4 and CXCR2, markers linked with neutrophil ageing, showed significant changes between these two groups. Patients with NAFLD-related early liver cirrhosis had lower levels of neutrophil CXCR2 (Figure. 16a) expression but higher CXCR4 signals (Figure. 16b). These findings indicated that neutrophils circulating in NAFLD patients were likely to be more senescent than in PBC-derived patients. Moreover, compared to NAFLD patients, individuals with early cirrhosis caused by PBC displayed significantly higher CD10 and CD15 expression (Figure. 16c-d). CD10 and CD15 are markers associated with neutrophil differentiation, suggesting the potential changes in the ability of neutrophil differentiation in different primary aetiology. Due to the limited data collected from PBC patients in the cytospin experiment, our current study could not evaluate whether the NAFLD or PBC-associated liver cirrhosis may create different morphological modifications to neutrophils.

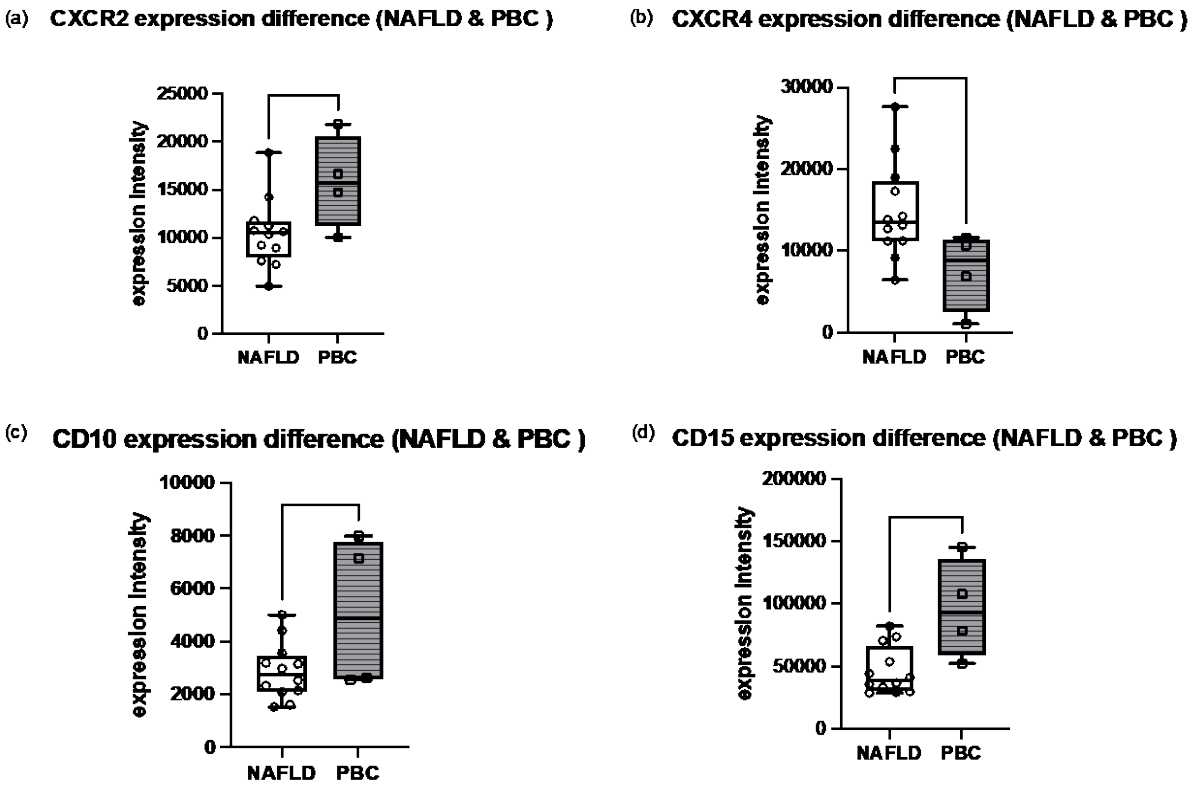


Figure 16: Neutrophil Marker Expression in Patients with Early-Stage Liver Cirrhosis due to NAFLD or PBC

This figure illustrates the expression levels of neutrophil markers in patients with early-stage liver cirrhosis, specifically those with either NAFLD (Non-Alcoholic Fatty Liver Disease) or PBC (Primary Biliary Cholangitis) etiology. The analyzed markers include CXCR2 expression (a), CXCR4 expression (b), CD10 expression (c), and CD15 expression (d). The sample size comprises 12 patients with NAFLD-induced liver cirrhosis and 4 patients with PBC-induced liver cirrhosis. Data normality and logicality have been validated, with outliers removed.

Statistical significance is denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The data represents the results obtained from independent experiments and has undergone analysis using the Unpaired T Test.

3.2 Changes in marker expression and morphology of neutrophils with increasing age

It is well-established that ageing can boost susceptibility to inflammation and infections. Numerous prior studies have also demonstrated age-related functional deficiencies in neutrophils, including impaired recruitment and killing capabilities, which could contribute to the increased vulnerability to infections in older individuals. We must emphasize the significant age difference evident within our study groups, from healthy donors and cirrhosis patients (Figure. S2). Patients with early cirrhosis had an average age of approximately 63, while those with advanced cirrhosis averaged around 61 years. In contrast, our healthy donor group displayed notably younger individuals, with

an average age of about 32. This substantial age distinction drives a crucial question: Could the age differential between healthy donors and cirrhosis patients introduce variables unrelated to cirrhosis into our findings? To comprehensively address these concerns, the following section will investigate neutrophil expression profiles and cell morphology across distinct age groups to mitigate potential age-related influences.

We separated healthy volunteers into two age groups: those aged 20 to 30 and those over 40. The expression of typical neutrophil markers was then examined within these two groups. Age considerably impacted the expression of CD10, CD15, and CD16 (Figure. 17 a-c). All these markers were statistically significantly higher in the younger age groups (between 20 and 30).

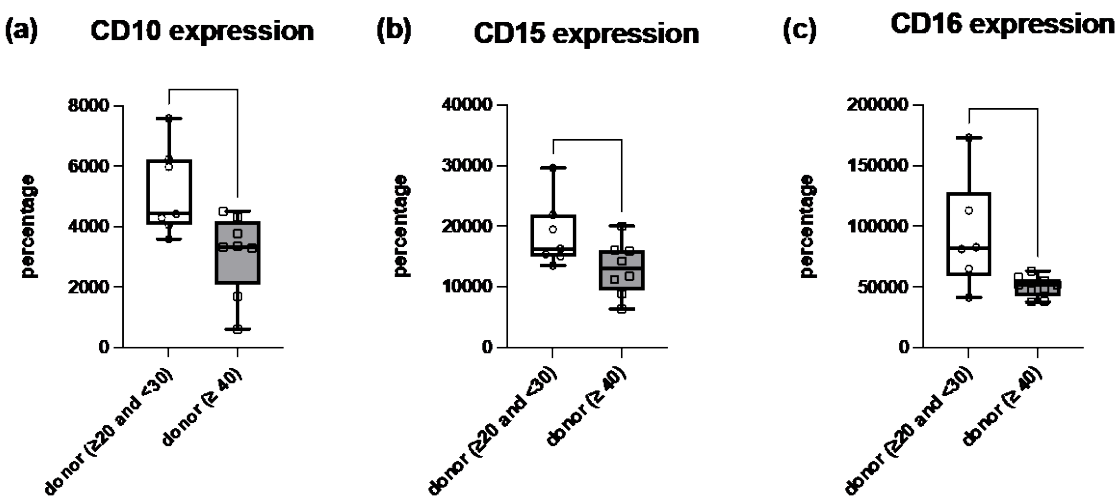


Figure 17: Expression of Neutrophil Markers in Healthy Donors of Various Ages

This figure presents the expression levels of three crucial neutrophil markers: CD10 (a), CD15 (b), and CD16, in healthy donors from different age groups. The sample size includes 7 donors aged between ⩾20 and <30 years and 8 donors aged ⩾40 years. Data normality and logicality have been verified, with outliers removed.

Statistical significance is denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

We subsequently investigated the different proportions of neutrophil morphologies in healthy individuals in these two age groups. Our analyses showed no statistically significant differences (Figure. 18 a-d), suggesting that the advancement in age has no major impact on neutrophil morphology. Nevertheless, the limited size of our dataset introduces the potential for error associated with our result. Future investigations employing larger sample sizes will establish this hypothesis.

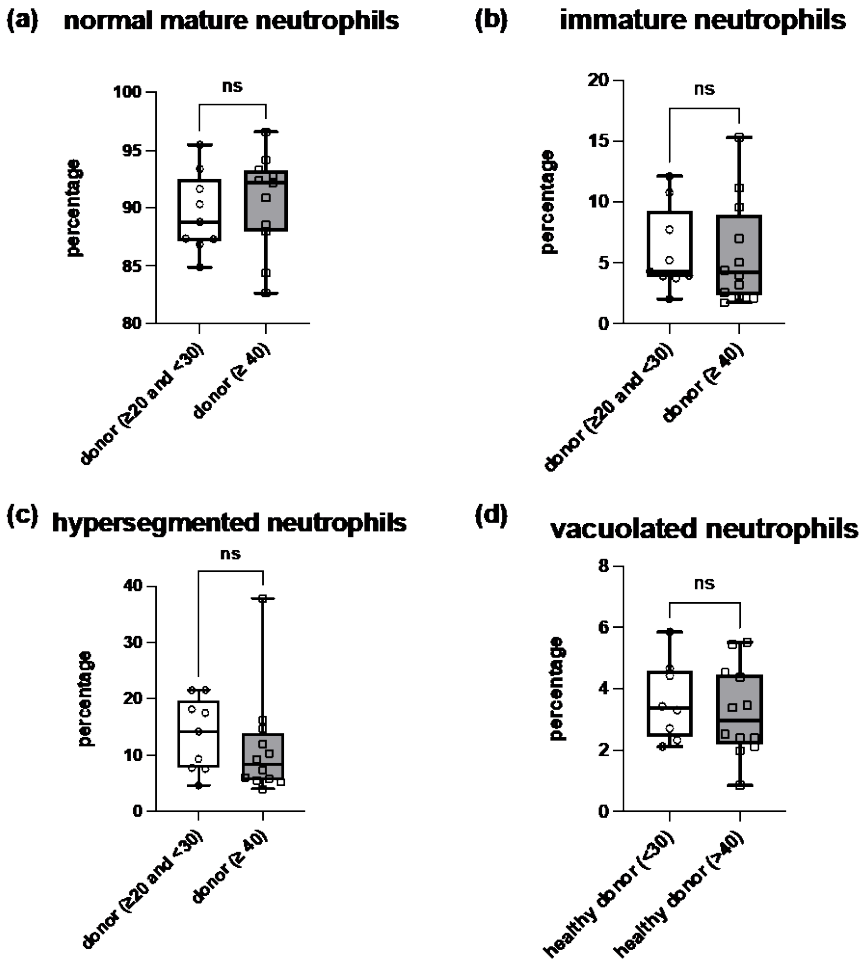


Figure 18: Neutrophil Morphologies in Healthy Donors of Various Ages

This figure presents the proportions of neutrophil populations with different morphologies, including normal mature (a), immature (b), hyper segmented (c), and vacuolated (d) neutrophils, in healthy donors across varying age groups. The sample size includes 9 donors aged between ⩾20 and <30 years and 11 donors aged ⩾40 years. Data normality and logicality have been confirmed, with outliers removed.

Statistical significance is denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data represents the results obtained from independent experiments and has been analyzed using the Unpaired T Test.

Discussion

Liver cirrhosis is a disease with increasing global concern, bringing substantial burdens on medical systems worldwide. As the end-stage of chronic liver disease, it gives rise to various complications, including infections and systemic inflammation. These consequences disrupt the immune system homeostasis and cause patients with cirrhosis to be more vulnerable to pathogens, notably bacteria. In this complex background, neutrophils, pivotal participants in the innate immune system, are the frontline in the battle against invading pathogens and tissue damage. The relentless progression of this disease induces neutrophil dysfunction in cirrhosis patients. Thus, we investigated neutrophil behaviour and features in the context of liver cirrhosis progression by analysing neutrophil properties such as shape, surface markers, and mitochondrial contents and attempted to shed light on several critical aspects of the intricate connections between neutrophil characteristics and cirrhosis pathophysiology.

1. The expression pattern of CD11b, CD66b, CD10, CD15 CXCR2, and CD62L after fMLP stimulation

A study by Thomas et al. nearly a decade ago observed that CD11b and CD66b expression on neutrophils increased in response to various stimuli, including fMLP (34). Other typical activation markers, such as CD62L and CXCR2, typically exhibit a declining trend after neutrophil activation. Specifically, CD62L undergoes a "CD62L shedding" process, while CXCR2 can be internalised for degradation. Furthermore, previous in vitro investigations have indicated a rapid desensitisation and internalisation process of CXCR2 on neutrophils following fMLP stimulation (50, 51).
Furthermore, CD10 can hydrolyse peptide bonds on the N-terminal site of hydrophobic amino acids, including Phe, Leu, Ile, Val, Tyr, Ala, and Trp (86). Consequently, physiologically active peptides such as fMLP can serve as substrates for CD10 (81, 85,86). A prior study published in 1992 demonstrated that adult polymorphonuclear neutrophil leukocytes can enhance their CD10 expression in response to fMLP stimulation (81). However, the significant increase in CD10 expression levels in healthy individuals and those with liver cirrhosis is not solely associated with fMLP. It may also correlate with the increases in CD11b or CD66b mentioned earlier. For example, a recent investigation showed a correlation between CD11b/CD66b and CD10 expression, with CD10 expression displaying a direct and significant association with CD11b and a slight association with CD66b (18). Our results regarding fMLP stimulation on neutrophils extracted across all different health statuses had a consistent increase/decrease pattern with these markers of neutrophil activation (18), which could be said that neutrophils from both healthy and people with cirrhosis could be successfully activated by fMLP stimulation.

Interestingly, our data revealed an increase in CD15 expression in response to fMLP stimulation, a unique and rarely reported phenomenon in previous investigations.

CD15 typically serves multiple essential roles in neutrophil functions (67, 68), including mediating cellular interactions, facilitating degranulation, and contributing to the respiratory burst. These functions are vital for enhancing pathogen clearance and overall immune defence. Since the fMLP challenge was employed to mimic an inflammatory condition in vitro, the observed elevation of CD15 expression (Figure. 9) on isolated neutrophils likely represents an activation and response of neutrophils to inflammatory stimuli. This consistent increase trend observed in both healthy individuals and patients suggests that neutrophils appeared to still play functional roles in liver cirrhosis. However, there has been limited exploration of CD15 expression levels in vitro, and further research on CD15 expression is necessary to gain a deeper understanding of its underlying processes in neutrophil activation in the cirrhosis background.

Our data also demonstrated that fMLP stimulation altered neutrophil forward scatter (FSC) in all groups, as shown in Figure. 7g and 8g. FSC is a measurement of the light scattered by cells in the forward direction when flowing through a flow cytometer, and it corresponds to cell size. Therefore, the decrease in FSC reflected a reduction in the size of neutrophils after incubation with fMLP. This phenomenon was reported in a very early study that focused on the light-scattering properties of human neutrophils in 1985. The study used flow cytometry to examine the light-scattering changes of human neutrophils before and after treatment with chemotactic peptides. They reported that after peptide addition, the forward scatter decreased by up to 5%, and the side scatter (SSC) increased by up to 15% between 3 to 15 minutes (82). They also suggested that the decreased neutrophil FSC after stimulation could indicate membrane ruffling or the polarisation of neutrophil morphology (82). Although our data did not demonstrate an increase in the SSC of neutrophils after stimulation, the shift in FSC appears to represent dynamic cellular responses during the innate immune response to neutrophil activation.

2. The expression pattern of age marker after fMLP stimulation

The essential chemokine receptors, CXCR2 and CXCR4, serve not only as activation markers but also as critical indicators of neutrophil ageing. Expressly, the pattern of CXCR4high and CXCR2low expression signifies aged neutrophils. We hypothesised that neutrophils extracted from advanced cirrhosis patients with acquired immunodeficiency may not be as capable of becoming activated as those from a hyperinflammatory state, such as those extracted from early-stage liver cirrhosis patients. From the flow cytometry data, it was shown that 30 minutes of fMLP incubation could induce neutrophil senescence in both healthy and early-stage individuals, as evidenced by the reduction in CXCR2 expression levels and an increase in CXCR4 levels in those isolated neutrophils. However, it is essential to note that the results for late-stage patients appeared to be different. Even though the CXCR2 expression on neutrophils was affected in advanced-stage patients, fMLP stimulation appeared to have no significant impact on their CXCR4 expression (Figure. 8d).

Nevertheless, it is worth noting that our analysis of unstimulated cells had already revealed elevated CXCR4 levels in advanced cirrhosis neutrophils. As a result, probably, these cells did not increase CXCR4 expression further in response to fMLP stimulation since they already had elevated baseline levels.

3. The expression pattern of purified neutrophils

3.1 CD66b

In the marker expression test of unstimulated neutrophils, we observed significantly higher expression of CD66b on neutrophils in patients with advanced cirrhosis compared to healthy individuals or those in the early stages of liver disease. Since neutrophils are typically recruited to infected sites to combat pathogens, the upregulation of CD66b in patients may be associated with this neutrophil recruitment process, as CD66b is one of the molecules that mediate neutrophil adhesion and migration (34). Early studies have demonstrated a significant increase in CD66b expression during the process of neutrophil transmigration through endothelial layers to the infection sites (34,87). Usually, patients with advanced liver cirrhosis often experience more severe chronic bacterial infections than those in the early stages (88). This much more severe infection environment likely leads to increased neutrophil recruitment, possibly resulting in the observed high CD66b expression pattern.

Furthermore, cirrhosis patients with bacterial infections may exhibit ongoing neutrophil activation, degranulation, and an attempt to increase ROS production (42), mediated by CD66b, to combat the chronic inflammatory environment within the body, potentially increasing CD66b expression. Additionally, it has been established that several bacterial infections, such as those induced by Staphylococcus aureus, may induce neutrophil dysfunction characterized by CD66b overexpression on neutrophils (34). Hence, the significant increase in CD66b level may also be associated with potential neutrophil dysfunction in advanced individuals.

3.2 CXCR4

Similarly, liver cirrhosis patients exhibited higher levels of CXCR4 expression than healthy individuals, with a particularly significant increase observed among late-stage patients. One reasonable explanation for this could be associated with dysfunction in neutrophil homeostasis. CXCR4 is typically highly expressed by immature or aged neutrophils. Therefore, the increase in CXCR4 levels in the circulation implies a potential increase in immature or aged neutrophils in the blood. Dysregulation of bone marrow neutrophil homeostasis can prematurely release immature neutrophils into the bloodstream and impair the homing of aged neutrophils. Beyond the homeostasis roles, CXCR4 plays a pivotal role in immune responses. First, neutrophils with high levels of CXCR4 expression display enhanced phagocytic activity, enabling them to combat pathogens (89) effectively. CXCR4 can also modulate the chemotactic activity of neutrophils, facilitating their transit across vascular endothelium to reach areas of inflammation (31,89). The highest levels of CXCR4 observed in advanced patients may

reflect that more severe and chronic infections usually challenge them. This upregulation of CXCR4 could respond to the heightened immune demands placed on these patients due to their advanced liver cirrhosis and increased susceptibility to infections.

3.3 HLA-DR

We also observed a progressive increase in the expression of HLA-DR on neutrophils with the advancement of cirrhosis (refer to Figure 10). Notably, neutrophils in cases with advanced disease exhibited the highest levels. While MHC-II expression on neutrophils is atypical, in vitro studies have demonstrated that neutrophils can express MHC-II and costimulatory molecules when exposed to specific inflammatory cytokines, such as IFN-γ. This phenotypic shift allows neutrophils to acquire the function of antigen-presenting cells, presenting antigens to CD4+ T cells in an HLA-DR-dependent manner (90). Since advanced individuals often experience severe infections, with chronic stimulation of immune cells and elevated levels of circulating inflammatory mediators (2), the neutrophils in the patient groups may upregulate HLA-DR expression to acquire antigen presentation abilities and enhance their immune response against pathogens. As mentioned earlier in Figure. 1, HLA-DR is predominantly expressed on the most immature neutrophils. Hence, the elevated HLA-DR levels observed in the patient group could also indicate the premature release of immature neutrophils from the bone marrow. This aspect aligns with our previous discussion concerning CXCR4.

3.4 CD15 & TLR-4

Again, our data revealed higher levels of CD15 expression in the patient groups compared to healthy donors (refer to Figure. 10d). It is essential to mention that CD15 is typically found at higher levels on the surface of immature neutrophils (17). Therefore, it is reasonable to infer that compared to healthy donors, the heightened CD15 expression in the patient groups is a consequence of an elevation in immature neutrophil levels in the circulation, potentially associating with the neutrophil homeostasis dysfunction, as discussed earlier in our study. Given that cirrhosis patients often encounter challenges related to bacterial translocation, contributing to systemic inflammation (2). In this context, constant exposure to bacterial products like lipopolysaccharides (LPS) could lead to the upregulation of TLR4 in response to the heightened microbial burden. In the early stages of cirrhosis, neutrophils are more reactive or sensitive (2), whereas advanced cirrhosis patients have reported immunosuppression and neutrophil dysfunction (2). These disease-stage-related features could explain our observation regarding the maintenance of TLR4 expression at low levels in advanced individuals but at high levels in early-stage patients.

3.5 FSC

Moreover, we observed a noteworthy reduction in the forward scatter (FSC) in neutrophils isolated from patients with advanced cirrhosis compared to the other two groups (refer to Figure. 10f). As a reduction in FSC could signal neutrophil activation,

the significantly lower FSC in advanced individuals reflected a higher activation (82) in neutrophils.

Furthermore, FSC is a critical parameter frequently utilized to discriminate between cells based on size or shape. The significant decrease in FSC among neutrophils from patients with advanced liver cirrhosis suggests the possibility of morphological alterations in these neutrophils. Neutrophils from late-stage liver cirrhosis patients may exhibit structural changes, such as irregular nuclear lobes or cytoplasmic vacuolization, which could substantially affect their light-scattering properties. Thus, the shifts in light scattering might serve as a distinct characteristic of neutrophils in individuals with advanced cirrhosis.

4. Morphology changes in cirrhosis context

4.1 Proportion of neutrophils in granulocytes

Our findings have revealed a significant decrease in the proportion of neutrophils within the granulocyte population among advanced-stage patients compared to healthy individuals or those in the early stages of cirrhosis (Figure 13a). This observation suggests a potential neutrophil deficiency in these individuals, consistent with expectations (91). It has been well-documented that cirrhosis patients commonly experience haematological abnormalities, including leukocytopenia, in which neutropenia, a specific type of leukocytopenia, is frequently observed in advanced chronic liver disease (91). Neutropenia in cirrhosis patients can be induced by hypersplenism (92). Additionally, a study conducted in 1988 investigated hematopoietic progenitor cell contents in the bone marrow of cirrhosis patients and suggested that these patients often exhibit serum humoral inhibitors of hematopoietic progenitors, which can lead to granulocytopenia, including neutropenia, in advanced liver cirrhosis (91).

4.2 Neutrophil proportion with normal lobe number

We then examined the proportion of normal mature segmented neutrophils across various groups representing different health conditions. Our observations indicated that more than 90% of neutrophils from healthy donors exhibited the typical mature lobulated morphology with the standard number of lobes (Figure 13b). However, the patient groups showed a significantly decreased proportion of neutrophils with this typical mature shape compared to healthy individuals. This finding aligns with our previous observations regarding neutrophil markers, where we reported elevated expression of neutrophil age markers such as CD15 and CXCR4. These markers imply an increase in immature or aged neutrophils in the patient groups, which often display distinct nuclear morphologies compared to normal mature cells.

4.3 Proportion of immature neutrophils

As expected, we observed a substantial increase in the proportion of immature neutrophils in both the early-stage and late-stage patient groups compared to healthy

individuals (Figure 14a). This finding was highly significant as it aligned with our earlier observations regarding changes in neutrophil surface markers. By using flow cytometry, we noted higher levels of CXCR4, CD15, and HLA-DR markers on neutrophils in the patient groups. It is worth noting that all these markers are reported to be highly expressed in immature neutrophils (17). The convergence of higher marker expression and the proportion of immature cells further supported our hypothesis that liver cirrhosis contributes to the premature release of immature neutrophils into circulation. Since this increase in immature cells is typically a result of urgent granulopoiesis. This urgent generation of neutrophils occurs due to the accelerated rate of neutrophil death and consumption during infections or injuries, such as liver injuries, leading to an increased demand for these immune cells (21). To counteract this substantial loss of neutrophil populations, the typical steady state of granulopoiesis in the bone marrow transitions into emergency granulopoiesis (20,21). This process induces the release of immature and mature neutrophils from the bone marrow into the peripheral circulation to replenish the supply.

4.4 The percentage change in hyper segmented neutrophils

Our statistical analysis has revealed that late-stage patients exhibited a significantly higher percentage of hyper segmented neutrophils (Figure 14b). Building upon prior research, we assumed that the higher presence of hyper segmented neutrophils in advanced-stage patients may be associated with a specific type of anaemia, particularly megaloblastic anaemia, which falls under the category of macrocytic anaemia (93,94). As a common occurrence, anaemia is a frequent consequence of liver cirrhosis, occurring in approximately 75% of patient cases, and it has diverse aetiologies, often multifactorial in the context of liver disease (93). Significantly, the severity of anaemia usually progresses as liver cirrhosis progresses, making it a valuable indicator for identifying the severity of liver cirrhosis. It is crucial to note, however, that none of the individuals whose blood we obtained had a folate shortage, which is a common cause of this kind of anaemia. This implies that there must be additional underlying causes influencing the development of this disease.

Furthermore, it is worth noting that neutrophil hyper segmentation is frequently regarded as an age-related change. Those hyper segmented neutrophils usually have remained in circulation rather than migrating into tissues (95). The higher percentage of hyper segmented neutrophils in advanced patients suggests that there might be a more prominent number of senescent neutrophils in their blood. This observation aligns with our previous findings related to CXCR4. In the flow cytometry data, we noted a significant increase in the expression of CXCR4 in advanced-stage patients compared to the other two groups. CXCR4 is a marker of neutrophil ageing, with its levels typically rising as neutrophils age. Therefore, when considering the presence of increased hyper segmented neutrophils and elevated CXCR4 levels in advanced patients, it is reasonable to assume that aged neutrophils are more abundant in the blood of advanced patients.

4.5 The percentage change in vacuolated neutrophils

We also observed a significantly higher frequency of vacuolated neutrophils in late-stage patients than in healthy individuals and those in the early stages of the disease (Figure 14c). As early as 1984, researchers identified degenerative changes in neutrophils, including vacuolation (24), in blood smears from newborns with sepsis induced by bacterial infections. This discovery led to the hypothesis that vacuolation of neutrophils could serve as an excellent indicator of sepsis (96). In 1998, a study reported a significant correlation between the presence of vacuolated neutrophils and infection with microorganisms and associated it with the proliferation of many bacteria (125). Additionally, some studies have observed an association between the prominent vacuolation of neutrophils in patients with alcohol toxicity and liver failure (84). Since advanced-stage liver cirrhosis patients are more often afflicted with considerably severe bacterial infections and faster bacterial growth, even with severe liver failure, the enormous rise in vacuolated cells in late-stage patients most likely represents these conditions.

5. Mitochondrial content

After testing the level of mitochondria across individuals with different health conditions, we found that patients with early-stage cirrhosis appeared to exhibit higher mitochondrial levels. Several indications suggest that neutrophils in patients may contain higher levels of mitochondria. As previously discussed in our study on neutrophil morphology, we observed an increased presence of immature neutrophils in the circulation of individuals with liver cirrhosis, especially those in the early stages of the disease. The literature has reported abundant mitochondria in immature neutrophils (79), which was much higher than in mature neutrophils (97). Therefore, in individuals with a higher percentage of immature cells, the signal of mitochondria should also be higher.

Indeed, the rise in immature neutrophils might offer advantages to cirrhosis patients. For example, those immature cells exhibit an extended lifespan, apoptosis resistance, and the ability to develop ex vivo (80). Additionally, it is noteworthy that spontaneous neutrophil extracellular trap (NET) synthesis appears to be heightened in immature neutrophils from sepsis patients (98). All these advantages of immature cells appear to benefit liver cirrhosis patients with chronic infections. For example, they can have a higher NET production ability to kill bacteria and a longer lifespan to fight against apoptosis (80).

6. Non-cirrhosis related influence

6.1 PBC & NAFLD

Considering that both PBC and NAFLD hold the potential to progress to liver cirrhosis, the influence on neutrophils in affected individuals might be different due to their distinct underlying causes and risk factors. Our study systematically compared typical neutrophil markers in early-stage patients afflicted with either NAFLD or PBC as their primary etiological factors, and we found that there could be possible variances in the age and maturity of neutrophils in NAFLD and PBC-derived cirrhosis patients.

For example, the expression of CXCR4 and CXCR2, which are markers linked with neutrophil ageing, have been extensively explored in the preceding sections. We found that patients with NAFLD-related early liver cirrhosis had CXCR2low but CXCR4 high neutrophils, implying that neutrophils circulating in NAFLD patients were likely to be more senescent than in PBC-derived patients. One probable hypothesis was that because the liver is one of the places where aged neutrophils can be removed (99), the livers of NAFLD patients may have inflammation and liver damage caused by fatty acid accumulation (7), reducing their ability to clear the aged neutrophils, and thus inducing higher age-related signals. However, patients with PBC-derived early cirrhosis displayed significantly higher CD10 and CD15 signals on their neutrophils (Figure. 17 a-b). CD10 and CD15 are markers associated with neutrophil differentiation (17). As mentioned above, neutrophil activation can lead to an increase in CD10 and CD15 levels. The higher levels of these markers in PBC patients suggested increased activity of neutrophils (6). This observation is particularly understandable considering that PBC is an autoimmune disease characterized by an overactive immune system mistakenly targeting the bile ducts of patients.

6.2 Age

After separating the healthy donors into different age groups, we found that CD16, CD10, and CD15 expression was statistically significantly higher in the younger age group (≥20 and <30 years).

Firstly, CD16, one of the receptors involved in recognizing antibodies on the surface of bacteria, serves as a marker of phagocytic capability (17). Butcher et al. (100) demonstrated in 2001 that CD16 expression declines with age, reducing neutrophil phagocytic activity (100,101). This finding aligns with our observations. Next, a study has indicated that neutrophils with low CD10 and CD16 expression displayed characteristics often associated with aged neutrophils, such as hyper segmented nuclei and a decreased in vitro survival rate compared to typically mature neutrophils (102). Given that the neutrophils in our older donor group already exhibited lower levels of CD16 and CD10 than their younger counterparts (Figure. 17 a and c), it is reasonable to speculate that aged neutrophils were more prevalent in the circulation of the older group. Testing this possibility with an even older cohort of healthy donors

would be interesting.

Furthermore, our findings revealed a notable decline in CD15 expression with increasing age (Figure. 17b). However, the precise reasons for this shift still need to be discovered. While some current research suggests that the level of neutrophil CD15 modestly increases with age (103), this finding contradicts our observations in this investigation. Consequently, further comprehensive research is warranted to explore the relationship between CD15 and CD10 expression and age and to uncover the specific mechanisms of this phenomenon.

7 Neutrophils in blood

It is essential to highlight that the purified neutrophils in our study were isolated using density gradient centrifugation, precisely the Percoll Gradient method. After centrifugation, granulocytes settle at the interface just above the red blood cell pellets, effectively separating them from monocytes, which have greater buoyancy and remain in the upper part of the gradient tube (refer to Figure 5b). Importantly, our study shared blood samples with another investigation, and all monocytes obtained in our experiment were exclusively used for that separate study.

An important consideration is that early studies have identified neutrophil-like cells among the monocytes isolated using the density gradient centrifugation method mentioned above (17,104). These particular neutrophil subsets are found in the low-density fraction and, therefore, referred to as low-density neutrophils (LDNs) (17,104). Furthermore, LDNs are abundant in various clinical conditions, including immunosuppression, chronic inflammation, and malignancy (104). A possible absence of LDNs in the isolated neutrophils in our investigation raises a valid question about whether this loss could have introduced the possibility of data error in the marker expression level. To address this concern, we examined the expression levels of typical and atypical neutrophil markers on neutrophils in whole blood, as listed in Table 1. Unfortunately, the size of our whole blood sample was insufficient to yield statistically significant results.

Further research

While our work provided essential insights into neutrophil features in liver cirrhosis, certain limitations should be noted. First, our sample size was relatively limited in several instances, such as only having 4 late-stage liver cirrhosis data in the additional test of the new neutrophil surface maker, and our data on PBC related to liver cirrhosis was also insufficient. Future studies should investigate more diverse samples to ensure the validity of conclusions. Second, there may be some potential experimental or technical error; The method of neutrophil extraction used in this work, for example, could have resulted in the unexpected activation of purified neutrophils (105). Furthermore, the absence of LDNs in our separated neutrophils could add potential mistakes and impair the precision of our data. Alternative neutrophil isolation methods

could be explored in future research to solve these problems. For instance, immunomagnetic isolation may be used to prevent inadvertent neutrophil activation (105). In addition, antibody-mediated negative selection could be used to acquire neutrophils (with LDNs) from whole blood. These modifications to the isolation process may reduce potential sources of mistakes and improve the accuracy of our data.

Several further studies can be considered to improve our understanding of neutrophil dynamics in liver cirrhosis progression. For example, neutrophil functional alterations may be one of the opportunities to have more excellent knowledge of their properties. In this situation, the role of ROS production, NET formation, and phagocytic activity in the progression of liver cirrhosis can be examined. In addition, emerging imaging techniques such as confocal and electron microscopy can provide extensive information about neutrophil shape and intracellular changes. Also, looking into the clinical correlations between neutrophil features and outcomes, including infection rates, disease progression, and survival, will help us determine the clinical significance of our findings. Addressing these limitations and pursuing further research will expand our understanding of neutrophil behaviour in liver cirrhosis and may improve outcomes.

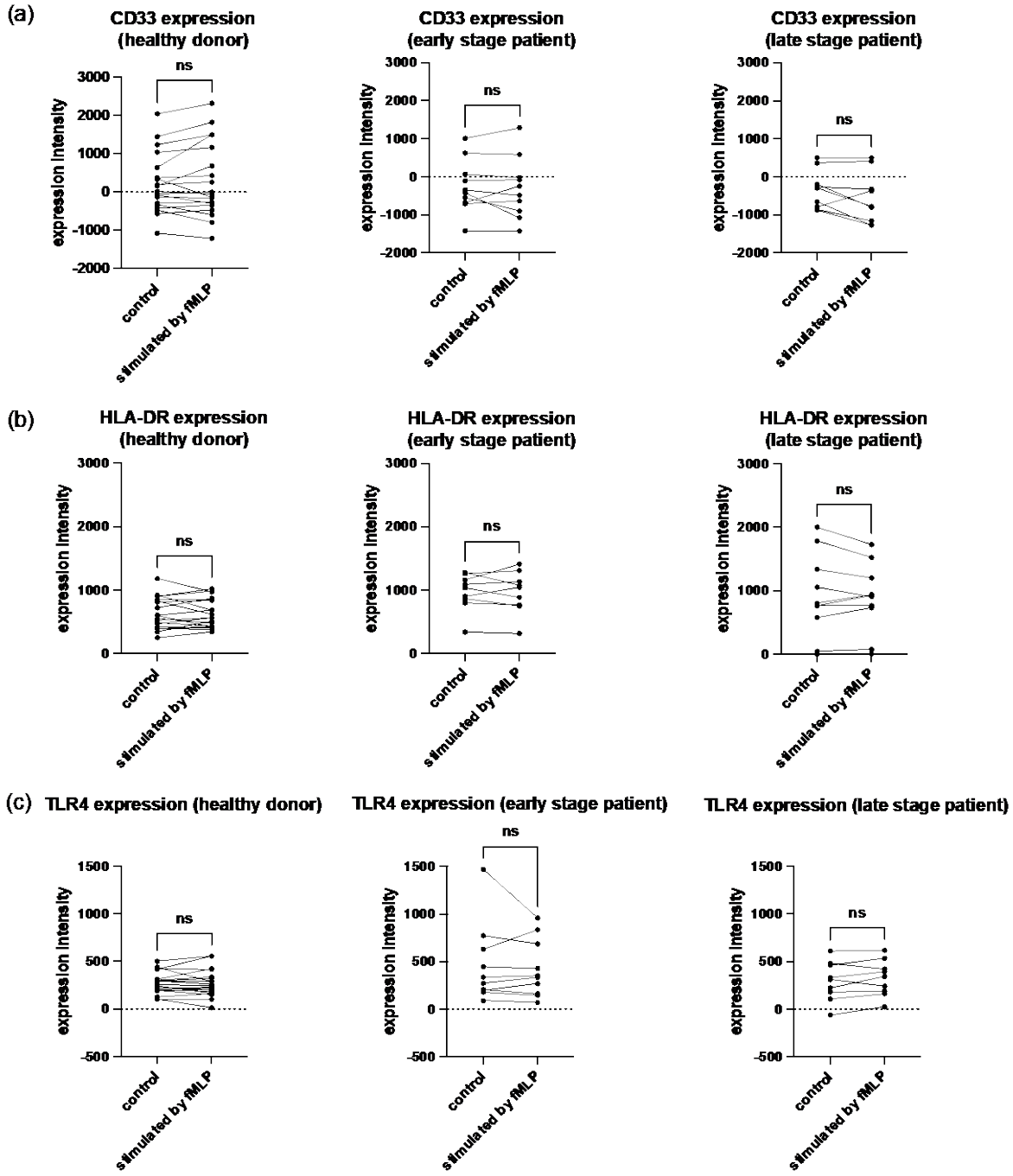
Conclusion

This comprehensive study delved into the intricate dynamics of neutrophil characteristics and functions in liver cirrhosis patients and healthy individuals. By examining surface expression, morphological changes, and mitochondrial levels, we gained valuable insights into the behaviour of neutrophils in varying stages of liver cirrhosis. While neutrophils from early and advanced cirrhosis patients displayed a regular activation pattern following stimulation with fMLP, isolated neutrophils from these patients exhibited a different trend than healthy donors. For instance, CD66b expression significantly increased in advanced cirrhosis patients, potentially indicating the severity of chronic bacterial infections and ongoing neutrophil activation. Concurrently, we noted elevated levels of HLA-DR, CD15, and TLR4, reflecting the intricate immune responses of advanced liver cirrhosis patients in the face of chronic infections. Additionally, neutrophils from cirrhosis patients displayed a more pronounced ageing pattern, with high CXCR4 but low CXCR2 expression.

Notably, the proportion of neutrophils and their morphological characteristics significantly changed across different liver cirrhosis stages. Advanced disease patients exhibited a higher presence of hyper segmented neutrophils, indicative of a prominent presence of senescent neutrophils in their blood, and a higher frequency of vacuolated neutrophils associated with severe infection. Therefore, neutrophil expression markers and morphological changes can serve as valuable indicators of disease progression.

In conclusion, our study unveils remarkable alterations in marker expression, ageing patterns, and neutrophil proportions within the context of liver cirrhosis. Utilising novel neutrophil-related parameters holds significant promise in understanding the liver cirrhosis process. However, it is essential to acknowledge the limitations of our study, including potential experimental errors and a relatively small sample size. Further research is imperative to explore the intricate relationship between neutrophil features and liver cirrhosis progression.

Supplemental information



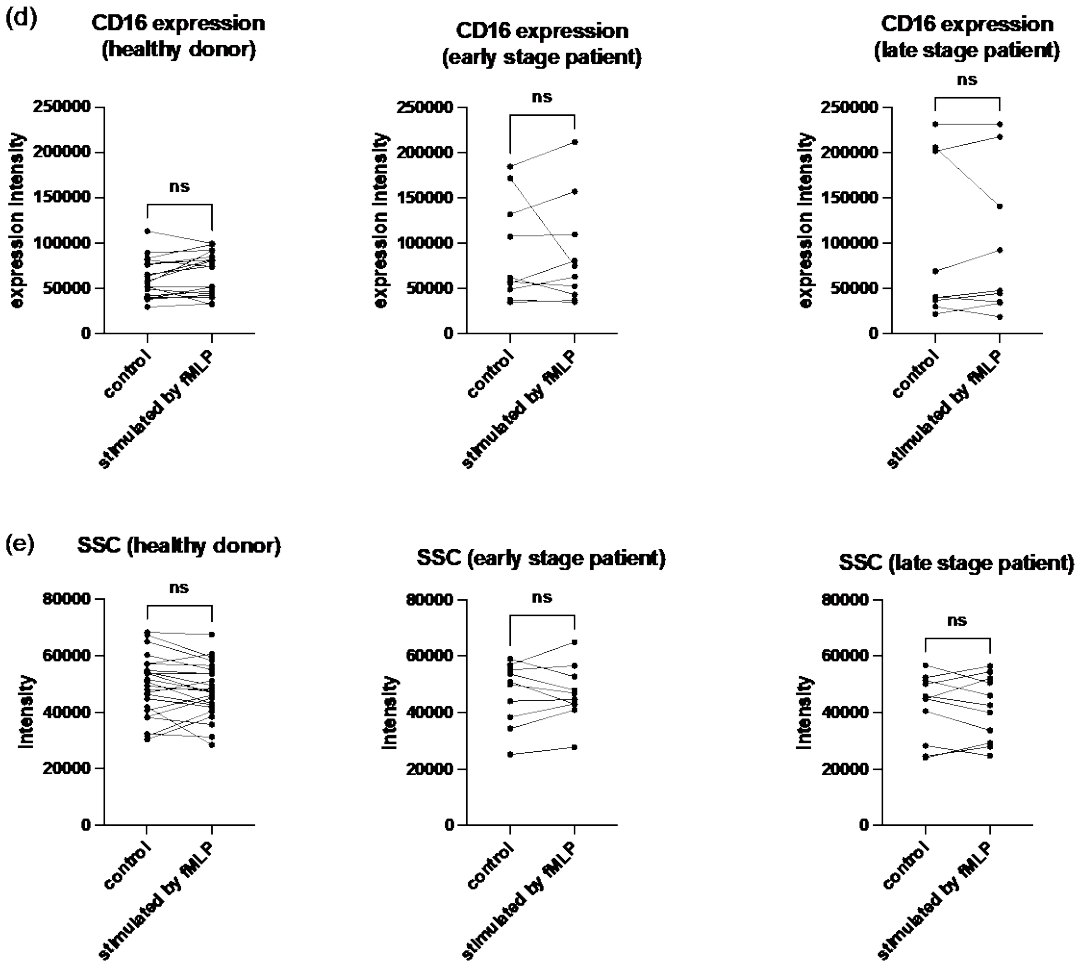


Figure S1. Effect of fMLP Stimulation on Side Scatter and Expression of CD33, HLA-DR, TLR4, and CD16 in individuals with different health status (Related to Figure. 7-9)

This figure shows the changes in the expression levels of CD33 (a), HLA-DR (b), TLR-4 (c), CD16 (d), and the alterations in the side scatter of neutrophils (e) in neutrophils from healthy donors and early to late-stage liver cirrhosis patients before and after fMLP stimulation.

The sample size: n=20 pairs for healthy donors, n=11 pairs for early-stage patients, and n=11 pairs for late-stage patients. Data validity and normality have been tested, and outliers have been removed. *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001, data representing two dependent experiments analysed with paired t-test.

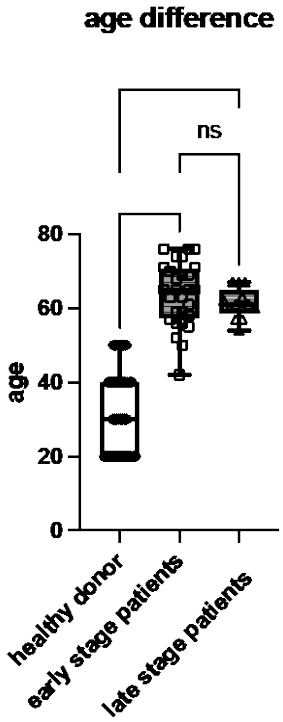


Figure S2 Age Differences in Each Group

The sample size comprises 45 healthy donors, 28 early-stage liver cirrhosis patients, and 13 late-stage patients. Data normality and logicality have been validated, with outliers removed. However, for the age of healthy donors, we only know their approximate age, as their ages were collected as age groups, such as age group 30 or 40.

*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001, data representing dependent experiments analyzed with one-way ANOVA.

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