Review Article

Single cell sequencing of neutrophils demonstrates phenotypic heterogeneity and functional plasticity in health, disease, and cancer

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Abstract: A vital constituent of innate immunity, neutrophils had previously been considered functionally rigid with a fixed, defined role in host pathogen response, in part due to their fleeting lifespan. However, that consensus opinion has changed with evidence of functional neutrophil plasticity in a range of diseases including cancer. Typically difficult to sequence due to their low level of transcriptomic activity, advances in single cell RNA sequencing has allowed for closer examination of the neutrophil transcriptome in humans and mouse models and their interaction with other immune system constituents, both in health and disease, allowing for description of neutrophil phenotypes beyond previous descriptions reliant upon microscopic appearance, surface marker expression, and function. Transcriptomic analysis shows that neutrophils develop and mature along a fixed trajectory, but their transcriptome varies based on maturity, the insult that has provoked release from the bone marrow, and the tissue to which they are recruited. Thus neutrophil heterogeneity increases with maturity, with immature neutrophils being more transcriptomically rigid. Here, we review work done in neutrophil single cell RNA sequencing in mice and humans in health and a range of disease states including coronavirus disease 2019 (COVID-19) infection, and solid cancers to provide a template for understanding neutrophil biology in context.

Keywords: Neutrophil; single cell RNA sequencing; cancer

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Introduction

Background

Neutrophils, polymorphonuclear leukocytes, are the most abundant circulating blood cell and are produced in large numbers (~10^{11} daily) from the bone marrow (1). Forming a vital part of the innate immune system, neutrophils are activated from their quiescent state in the circulation (2) and respond when they encounter pathogens via phagocytosis, generation of reactive oxygen species (ROS), degranulation, and generation of neutrophil extracellular traps (NETs), a chromatin mesh dotted with granule products, released extracellularly. In addition to this direct killing response to extracellular pathogens, neutrophils also play an immunomodulatory role through crosstalk with other components of the innate and adaptive immune system, including T cells, B cells, NK cells, dendritic cells, and macrophages (3). Under inflammatory conditions, neutrophils gain the capacity to present antigen, and can acquire surface expression of MHC class II and T cell

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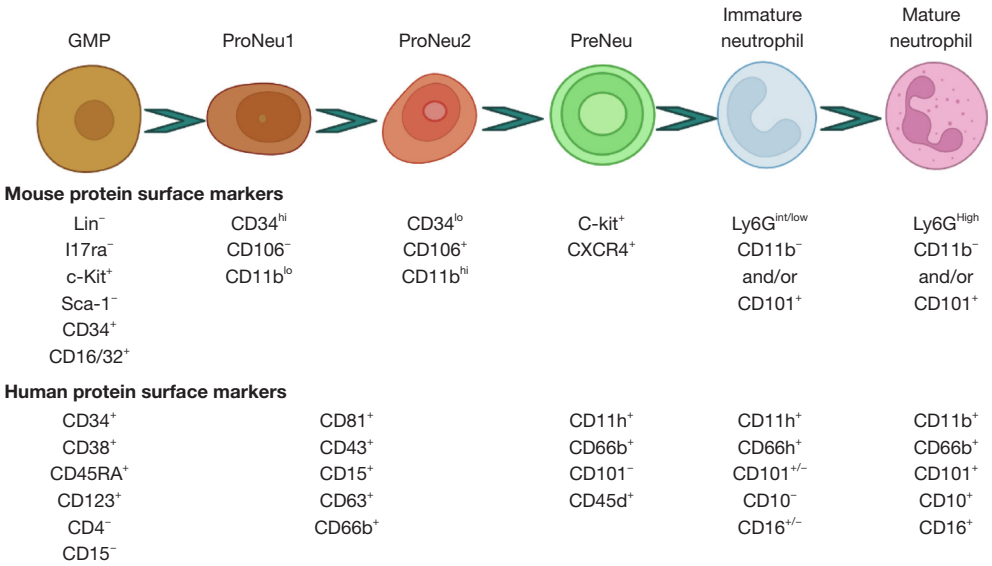

Figure 1 Neutrophil development from GMPs through mature neutrophils, and their surface marker expression in mice and humans. The figure was created with BioRendr.com. GMP, granulocyte-monocyte progenitor; proNeu, early committed neutrophil progenitors; preNeu, committed proliferative neutrophil precursors.

costimulatory molecules (4).

Classically, neutrophils were thought to consist of a terminally differentiated homogenous population, with similar appearances microscopically once mature (1). Produced in the bone marrow from haematopoietic stem cells, neutrophil maturation largely takes place in stepwise fashion from multipotent progenitors (MPPs) to lymphoid-primed multipotent progenitors (LMPPs) and then granulocyte-monocyte progenitors (GMPs). GMPs then commit to neutrophil cell lines by turning into myeloblasts, and subsequently through stages as promyelocytes, myelocytes, metamyelocytes, band cells and finally mature neutrophils (1,5,6). Primary (azurophil) granules are generated at the myeloblast to promyelocyte stage, secondary (specific) granules at the myelocyte and metamyelocyte stages, and tertiary (gelatinase) granules at the band cell stage (1). Under normal homeostatic conditions, mature neutrophils are mostly contained in the bone marrow, and only 1–2% are found in the circulation (7). Under conditions provoking an immune response, neutrophils are mobilised into the circulation via downregulation of the C-X-C chemokine receptor (CXCR)4/stromal cell-derived factor-1α (SDF-1α) pathway responsible for retention of mature neutrophils within the bone marrow, and upregulation of CXCR1 and CXCR2 signalling (8-11) and recruited through the leukocyte adhesion cascade (12) to sites of infection or inflammation. If this process consumes many neutrophils in response to a potent or chronic stimulus, emergency granulopoiesis (13) will occur, with increased production of neutrophils, and release of immature neutrophils from bone marrow into the circulation (14). Once in tissue, neutrophils were typically thought to have a short life span of 7 hours in humans (5,15) and 8–10 hours in mice (16), before undergoing apoptosis and phagocytosis by resident macrophages and dendritic cells (1).

However, this paradigm has shifted recently, and it has been shown that neutrophils form a highly heterogenous population both within the bone marrow as immature cells (6) with the discovery of early committed neutrophil progenitors (proNeus) (17), and committed proliferative neutrophil precursors (preNeus) (18), and also within the circulation and tissue as both immature neutrophils released prematurely as part of an inflammatory response (5,6), and mature neutrophils (6). An overview of neutrophil development and protein markers to identify these populations in mice and humans is given in *Figure 1*.

Neutrophils also are highly plastic, with variable function and lifetime depending on the tissue the neutrophil is recruited to (19). The function of immature neutrophils as well as younger mature neutrophils differs from mature neutrophils, with immature neutrophils (encompassing

polymorphonuclear myeloid-derived suppressor cells) having a reduced capacity for NETosis, reduced capacity for phagocytosis, and reduced ability to migrate, and, in a stimulus-dependent basis being functionally more immunosuppressive (14). Disease states can also polarise neutrophils (5), with cytokine signalling pathways such as transforming growth factor-β (TGF-β) and interferon-β (IFN-β) as well as tissue specific factors such as hypoxia playing a role in alteration of neutrophil function.

The positive role neutrophils play in host defence can be seen in congenital or acquired disorder of neutrophil number or function (2), for example acquired neutrophil deficiency following myeloablative chemotherapy, where individuals are more susceptible to life threatening infections (20). However, neutrophils may also play a detrimental role in autoimmune conditions such as systemic lupus erythematosus (SLE) (21,22) and rheumatoid arthritis (21).

Their role in development of cancers is emerging, with neutrophils shown to have both pro-tumour and anti-tumour effects at almost every stage of oncogenesis (5,6). Pro-tumour effects include amplification of DNA mutation from free radical generation (23), priming of the metastatic niche (24), suppressing the immune response through both chemokine release and direct interaction with T cells via programmed death ligand-1 (PD-L1) (25), and supporting tumour angiogenesis (6). NETs may capture circulating tumour cells, and degrade the extracellular matrix through proteinases on their chromatin mesh, promoting micrometastases (26). NETs directly interact with and prime T cells, and can contribute to T cell exhaustion and lack of immune response (27). Anti-tumour effects can be direct or indirect. Direct methods involve production of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (28), nitric oxide release (29), release of ROS (30), and trogoptosis, the ingesting of cancer cell plasma membrane after binding of antibody to the cancer cell (31). Indirectly, neutrophils can contribute to an anti-tumour microenvironment through cytokine release (32) and antigen presentation to T cells (33).

Rationale and knowledge gap

Bulk RNA sequencing of tumour microenvironments has given great insight into dynamics and factors that play a role in survival and outcome. However, with tumour heterogeneity, nuance is lost with bulk sequencing. Single cell sequencing, pioneered as a technique in 2009 (34), allows for detailed study of the transcriptome on a cell-by-cell basis, and therefore allows for study of transcriptional differences within a population of the same cells.

This technique allows for heterogenous populations, such as neutrophils to be examined in more granular detail. In humans, study may be possible on 2–3 g of tissue that may be obtained through surplus tissue at the time of surgery or percutaneous biopsy (35-37). Single cell RNA sequencing may be used to further knowledge about the role that neutrophils play in disease, particularly in malignancy.

Objective

Given the multifaceted role neutrophils can play in health and disease, efforts have been made to use single cell RNA sequencing to unpick the heterogeneity seen at the transcriptomic level. This review compares and summarises single cell sequencing of neutrophils in human health and disease, as well as murine models of human disease, and whether cross-comparison between the mouse and human can give insight into the role neutrophils play in the disease process.

Neutrophil single cell sequencing in healthy mice

RNA transcription signatures define neutrophil subpopulations, which correlate with previously defined groups

Xie *et al.*, 2020 (38) profiled the bone marrow, spleen and peripheral blood of healthy mice, isolating neutrophils based upon Gr1 antigen expression using FACS sorting. Some $GR1^{low}$ and $Gr1^{-}$ cells were included, as well as a sample enriched for c-Kit^{+} bone marrow haematopoietic stem progenitor cells to examine early granulopoiesis. Eight clusters of neutrophils were identified based on single cell RNA sequencing, five mostly in the bone marrow (labelled by the authors as G0–G4) and three mostly in the peripheral blood (G5a–G5c). There were 24 signature genes that distinguished each subpopulation, and gene ontology analysis revealed differing function of each subpopulation, with the least mature neutrophils having upregulation of ribonucleoprotein complex biogenesis, cytoplasmic translation, positive regulation of the cell cycle and protein localisation to the nucleus, suggesting these are actively proliferating and differentiating cells. Immature neutrophils and mature neutrophils had upregulation of leukocyte chemotaxis, myeloid leukocyte migration, defence response

Table 1 Neutrophil clusters based on differentially expressed genes on single cell RNA sequencing, the locations in which they are found, and their correlations with previously defined neutrophil subpopulations

Paper	Neutrophil cluster	Location	Correlate	RNA expression significantly increased
Xie *et al.* (38)	G0	Bone marrow	Common myeloid progenitor (40)	*Cd34*, *Sox4*, *Rpl12*, *Elane*, *Prtn3*, *Mpo*, *Tuba1b*
	G1	Bone marrow	ProNeu (17,41)	*Rpl12*, *Elane*, *Prtn3*, *Mpo*, *Fcnb*, *Ltf*
	G2	Bone marrow	PreNeu (18)	*Chil3*, *Tuba1b*, *Fcnb*, *Ltf*, *Ngp*, *Camp*
	G3	Bone marrow → spleen, peripheral blood	ImmNeu (18)	*Chil3*, *Ltf*, *Ngp*, *Camp*, *Retnlg*, *Mmp8*
	G4	Bone marrow → spleen, peripheral blood	Mature neutrophils (18)	*Ltf*, *Ngp*, *Camp*, *Retnlg*, *Mmp8*, *Cxcl2*, *Ccl6*
	G5			
	G5a	Spleen, peripheral blood	–	*Ccl6*, *Gm5483*, *Stfa2l1*
	G5b	Spleen, peripheral blood → bone marrow	–	*Isg15*, *Rsad2*, *Ifit3*
	G5c	Spleen, peripheral blood	–	*Fgl2*, *Gm2a*, *Gngt2*
Grieshaber-Bouyer *et al.* (39)	P1	Bone marrow → spleen, peripheral blood	PreNeu (18)	*Ube2c*, *Stmn1*, *Tuba1b*, *Ptma*, *Ube2s*, *Tubb5*, *Chil3*, *Cebpe*, *Hmgb1*, *H2afz*, *Camp*, *Hmgn2*, *Hmgb2*, *Ngp*, *Ltf*, *Arhgdib*, *Calm2*, *mt-Co1*, *Lcn2*, *Lyz2*, *Wfdc21*, *Anxa1*, *Cybb*, *Dstn*, *Ly6c2*, *Ly6g*, *Cd177. Serpinb1a*, *Prdx5*, *Pglyrp1*
	P2	Bone marrow → spleen, peripheral blood	–	*Ngp*, *Ltf*, *Lcn2*, *Lyz2*, *Ifitm6*, *Wfdc21*, *Anxa1*, *Mmp8*, *Cybb*, *Dstn*, *Ly6c2*, *Ly6g*, *Cd177*, *Prdx5*, *Lgals3*, *AA467197*, *Mmp9*, *Pglyrp1*, *Mgst1*, *Mcemp1*
	P3	Bone marrow → spleen, peripheral blood	–	*Mmp8*, *Lgals3*, *Retnlg*, *S100a6*, *Prr13*, *Tmmc1*, *Mmp9*, *Mcemp1*, *Gm5483*, *Fth1*, *Ccl6*, *Msrb1*, *H2-D1*
	P4	Spleen, peripheral blood → bone marrow	–	*Wfdc17*, *Hbb-bs*, *Il1b*, *Ifitm1*, *Ifitm2*, *Btg1*, *Fxyd5*, *Hba-a1*, *Srgn*, *Malat1*, *Hba-a2*, *Dusp1*, *Rps27*, *Jund*, *Fth1*, *Ccl6*, *Msrb1*, *Csf3r*, *Junb*, *H2-D1*

ProNeu, early committed neutrophil progenitors; preNeu, committed proliferative neutrophil precursors; immNeu, immature neutrophils.

to bacterium, and positive regulation of defence response, highlighting the role of immature and mature neutrophils in host defence.

Grieshaber-Bouyer *et al.*, 2021 (39) sampled neutrophils from the bone marrow, spleen and peripheral blood from healthy mice, sorting neutrophils on positive expression of *Ly6g* and *CD11b*. Based on differentially expressed genes, 4 clusters of neutrophils were identified, labelled P1–P4. Here, gene ontology terms enhanced in the least mature neutrophils included metabolic processes and defence response, whereas in mature neutrophils there was upregulation of genes involved in response to the environment such as response to toxins, gas transport and immune response. A summary of upregulated differentially expressed genes in the neutrophil clusters described by Xie *et al.* (38) and Grieshaber-Bouyer *et al.* (39) is depicted in *Table 1*.

Work by both Xie *et al.* (38) and Grieshaber-Bouyer *et al.* (39) found correlates with previously identified immature neutrophil subpopulations from work by Olsson *et al.* (40) (common myeloid progenitors), Evrard *et al.* (18) (preNeus, immature neutrophils and mature neutrophils), and Muench *et al.* (41) and Kwok *et al.* (17) (proNeus). These are summarised in *Table 1*.

Neutrophils display a typical maturation and aging trajectory

Grieshaber-Bouyer *et al.* (39) demonstrated a continuous

spectrum of neutrophil maturation they described as "neutrotime". Here, preNeus give rise to immature neutrophils and subsequently develop into mature neutrophils with no developmental branching off to form other neutrophil phenotypes. There were specific accumulation points found in early neutrophil development within the bone marrow, and the spleen had higher levels of immature but older neutrophils. Peripheral blood neutrophils were highly mature. Gene expression varied across neutrotime, with genes highly expressed early in neutrotime indicating host defence response, and those in late neutrotime involved in cellular response to toxins and oxygen transport. There was also peak expression of some genes such as *Retnlg*, *Mmp8*, and *Mmp9* at a midpoint through neutrotime.

Likewise, Xie *et al.* (38) found a continuous branch of neutrophil development within the bone marrow, with linear progression from G2 to G4. However, in addition to this, there was differentiation of neutrophils into different mature phenotypes. Some G3 neutrophils developed into G5a neutrophils without progression to G4, and G4 split into both G5a and G5b, but G5a did not develop into G5b neutrophils. G5c were terminally differentiated, the most mature with the highest apoptotic score, and both G5a and G5b neutrophils developed into G5c neutrophils (though there was still a significant proportion of apoptosis amongst G5a and G5b neutrophils).

Summary

Here, work done in single cell RNA sequencing corroborates previous efforts to identify neutrophil subpopulations in mice using alternative techniques, particularly immature neutrophils, with the finding of clusters that correlate with proNeus, preNeus and immature neutrophils, and granulocyte-monocyte precursors. Similarities in gene expression were found by the two groups, with genes such as *Camp*, *Retnlg*, and *Ccl6* expressed at similar points in time in neutrophil development. Grieshaber-Bouyer *et al.* (39) find only a single cluster of mature neutrophils, whereas Xie *et al.* (38) find multiple clusters, though similar gene expression from these, for example the interferon-responsive gene cluster, were found in this single mature neutrophil cluster. Differences seen in neutrophil clusters and their RNA expression between the two papers may be due to a difference in markers used to isolate neutrophil populations or may be due to plasticity in RNA expression between the same neutrophil subpopulation. Evidence from these papers supports a fixed neutrophil developmental trajectory in homeostasis within the bone marrow, culminating in mature neutrophils which are released into the peripheral blood, with identifiable RNA expression signatures that correlates with protein markers that may allow for isolation of these neutrophil subpopulations for further study.

Neutrophil single cell sequencing in murine disease models

Neutrophil differentiation maintains its trajectory after stimulation as in health

Grieshaber-Bouyer *et al.* (39) argue that neutrophils develop along a principle developmental pathway (termed "neutrotime"). Validating this model, Xie *et al.* (38) challenged mice intraperitoneally with *E. coli* at a single point in time, modelling acute inflammation. After challenge with *E. coli*, there is an expansion in neutrophil production, as expected (42). Neutrophil maturation trajectory was maintained as seen in health, and signature gene expression designating subtypes of neutrophils as defined in health were maintained. Proportions of each subtype were altered from in health, with expansion of the G1 population in the bone marrow, and G5b population of mature neutrophils, particularly in the subcapsular spleen. Whilst G3 and G4 neutrophils were not expanded proportionally, the time in which they developed and mobilised to peripheral blood was shorter, taking 2 days rather than three seen in healthy homeostasis.

Neutrophils are suggested to play both a detrimental and protective role at different times following ischaemic brain injury, and are a focus of research into improving outcomes (43). Early following ischaemia, neutrophils are recruited to the ischaemic tissue, and hyperactivation appears to be associated with secondary brain injury and worse outcomes clinically (43). Beyond the acute phase, it is suggested that neutrophils aid remodelling through promotion of angiogenesis and remodelling the extracellular matrix (43). Unpicking neutrophil heterogeneity in this setting through single cell analysis may provide valuable information as to the different roles of neutrophils in this setting. Zheng *et al.* (44). Identified 4 neutrophil clusters in their model of ischaemic stroke in the mouse, which correlated with G2–4 neutrophils, G5a, G5b, and G5c neutrophils described by Xie *et al.* (38). Twenty-four hours following middle cerebral artery

occlusion, altered proportions of these neutrophil clusters was identified, with a significantly higher proportion of G5b and G5c neutrophils with upregulated transcriptomic pathways suggesting activation on gene ontology analysis.

Type of challenge and tissue defines neutrophil phenotype

Upon *E. coli* challenge, there is an alteration in differentially expressed genes, suggesting alteration of function in each neutrophil subtype from health. Xie *et al.* (38) identified alteration in RNA expressed in subtypes as early as G0 and G1, with upregulation of genes involved in regulation of immunity and ROS production, suggesting a response to bacteria from early in neutrophil differentiation. Mature neutrophils showed upregulation of cytokine production and response to oxidative stress amongst others.

Grieshaber-Bouyer *et al.* (39) used both acute and subacute models of inflammation to study neutrophils under diseased conditions. Transcriptional concordance was found between neutrophils harvested from the peritoneum and the lung after acute inflammation, and between these neutrophils and those seen in the joint of the subacute inflammation model. However, as well as differing in maturity, with neutrophils from the joint being more mature than those seen from tissue in subacute inflammation, there were also transcriptional signatures that distinguished each neutrophil population from the other. Joint neutrophils, amongst other genes upregulated in inflammatory response had upregulated *Pgam1*, involved in glycolysis; *Rsad2* involved in innate immune signalling, and *Spp1*, which has been implicated in driving rheumatoid arthritis synovitis and production of PD-L1^{+} neutrophils, as well as severe coronavirus disease 2019 (COVID-19) (45). Whilst these changes could be due to the nature of the subacute stimulus, there were also differences between tissue neutrophils in acute interleukin-1β (IL-1β) pneumonitis and peritonitis in genes such as *Ccr1*, *Cd33*, and *Osm1* upregulated in pneumonitis, and *Marcks*, *Vasp*, and *Klf2* amongst others in peritonitis.

In a transient ischaemia model in aged mice developed by Li *et al.* (46), a proportional increase in all neutrophils was seen in brain tissue 3 days following ischaemic insult. These neutrophils appeared highly activated and mature in comparison with those found in sham mice.

Summary

Within mouse models of acute and subacute inflammation studied in these papers, neutrophil signatures are maintained from health that allow them to be phenotyped and grouped. However, Grieshaber-Bouyer *et al.* (39) have shown that there is also transcriptomic variation based upon type and nature of stimulus, as well as variation based upon tissue. Neutrophils deviate from their fixed developmental trajectory at different points in maturation due to environmental cues, with transcriptional differences governed by stimulus, tissue, and maturity at point of deviation from neutrotime, leading to different proportions of transcriptomically different neutrophils present in different tissues. This provides single cell RNA sequencing evidence corroborating Ballesteros *et al.* (19), showing that tissue environments influence neutrophil fate.

Human neutrophil single cell sequencing

Neutrophil single cell RNA sequencing has been performed in several disease states, including cancer (47-50), COVID-19 infection (51), and tissue burns (52), as well as in human healthy controls. Sequencing has also been performed in artificially stimulated neutrophil release by granulocyte colony-stimulating factor (G-CSF) or autologous stem cell transplant (49). Sites of neutrophil collection from which sequencing was performed, and inflammatory stimuli are summarised in *Table 2*.

Human neutrophil single cell sequencing in non-malignant disease

Immature neutrophils show little phenotypic diversity across disease states, but vary in transcriptome and proportion from health

Across all diseases sampled, there was transcriptomic evidence of immature neutrophils in the peripheral blood, with a higher proportion compared to the healthy state. These transcriptomic signatures were found by Montaldo *et al.* (49) in the bone marrow of healthy volunteers at a steady state but became evident in the peripheral blood after emergency myeloid stimulation for haematopoietic stem cell transplant (HSC-T), and after G-CSF challenge. Single cell sequencing and clustering of these immature neutrophils showed concordance with low density neutrophils as defined on flow cytometry and showed similar gene expression irrespective of disease state from which neutrophils were sampled. In comparison of neutrophils from peripheral blood of healthy controls, and burn patients, Huang

Table 2 Single cell sequencing of neutrophils in human health and disease

Site of neutrophil sample	Human health	Human disease	
		Paper	Condition
Bone marrow	Montaldo *et al.*, 2022 (49)	–	–
Peripheral blood	Huang *et al.*, 2022 (52)	Zilionis *et al.*, 2019 (47)	Lung cancer
	Montaldo *et al.*, 2022 (49)	Huang *et al.*, 2022 (52)	Tissue burn
		Xu *et al.*, 2021 (51)	COVID-19
		Wang *et al.*, 2023 (50)	PDAC
		Montaldo *et al.*, 2022 (49)	G-CSF treated; post HSC-T; PDAC
Tissue	–	Zilionis *et al.*, 2019 (47)	Lung cancer
		Alshetaiwi *et al.*, 2020 (48) [from data published by Azizi *et al.*, 2018 (53)]	Breast cancer
		Wang *et al.*, 2023 (50)	PDAC

COVID-19, coronavirus disease 2019; PDAC, pancreatic ductal adenocarcinoma; G-CSF, granulocyte colony-stimulating factor; HSC-T, haematopoietic stem cell transplant.

et al. (52) found conservation of immature neutrophil subtypes between health and disease based upon signature gene expression. Although similar across disease state, immature neutrophils showed transcriptomic differences from those seen in the healthy steady state, with upregulation of genes involved in neutrophil activation such as cluster of differentiation (CD)177, neutrophil granule products and degranulation such as lactoferrin (LTF), and phagocytosis such as cytochrome b-245, beta polypeptide (CYBB) in patients with burns (52). Chronic and acute inflammatory stimuli alike prompt immature neutrophil release across a range of stresses and disease states, with Huang *et al.* (52) demonstrating an increase in proportion of immature neutrophils as time from development of a burn increases, with expression of transcription factors CCAAT-enhancer binding protein (C/EBP)-B and C/EBP-D (seen in mature neutrophils) higher on day 1 than day 2 and 3 after development of a burn, corroborating emergency granulopoesis (13,14). A similar pattern was seen in patients by Xu *et al.* (51) with COVID-19, with upregulation of genes involved in NETosis, and cytokine signalling. Immature neutrophils have a role in host response, as proportion of immature neutrophils found in peripheral blood correlated with severity of disease across different disease types, with a higher proportion found in severe compared to mild COVID-19 infection (as defined by admission to intensive care or use of supplemental oxygen >50% or ≤50% respectively) by Xu *et al.*, 2021 (51), and in burn patients, a higher proportion of immature neutrophils was strongly associated with development of sepsis.

Stress provokes different transcriptomic signatures in mature neutrophils depending on the disease, severity, and time from stress

Single cell neutrophil sequencing in mature human neutrophils across health and disease reveals a great degree of neutrophil plasticity. Proportion of neutrophil subtype expressed, as well as gene expression is affected by tissue that neutrophils are present in, the disease state, severity of the inflammatory insult, and time from the insult. There is a conflict in the literature regarding neutrophil phenotypes being preserved between health and disease. In peripheral blood from health controls and patients with burn injuries, Huang *et al.* (52). Found preserved transcriptomic signature genes shared between neutrophils in health and disease. Despite their similarities, transcriptomic changes were seen across all neutrophil subtypes, in upregulation of genes ontologically stated to be involved in neutrophil activation, degranulation and chemotaxis following a burn injury.

In contrast, other groups working in other diseases or immune stress found different clusters between health and disease in the peripheral blood with enhancement or suppression in different disease states. Montaldo *et al.* (49). found distinct clusters between healthy volunteers and patients challenged with G-CSF, and patients after

HSC-T. After G-CSF, neutrophil gene expression was ontologically increased in macrophage activation, leucocyte adhesion (and showed upregulation of CD44), and ATP metabolism. Neutrophils sampled early following HSC-T showed upregulation of cytokine-mediated signalling, cytokine response, and interferon responsive pathways, with upregulation of *OAS2*, *AIM2*, and *GBP5*. With time, this signature became less evident, and neutrophils returned to steady state as identified in health when sampled at later timepoints. In COVID-19 infection (51), 6 mature neutrophil clusters were identified, with different proportions seen not only in health and disease states, but also in the severity of COVID-19 infection. Clusters enhanced in ferroptosis, chemotaxis and positive regulation of apoptotic pathways were enhanced in healthy controls but attenuated in patients with COVID-19. In patients with COVID-19, oxidative stress response pathways and TNF signalling pathways amongst others were significantly activated in patients with severe infection compared to mild disease, where mild disease had broad activation of multiple pathways involved in antiviral response, including genes involved in response to other viral pathogens such as cytomegalovirus (CMV), hepatitis C and influenza that were downregulated in severe COVID-19 infection.

Summary

Single cell sequencing from neutrophils across human health and disease has shown great plasticity of neutrophils, with transcriptomic differences evident not only with different stressors, but also that this transcriptomic difference varies with time from stress. Neutrophil transcriptome also differs between more and less severe disease states, but whether this is reflective of the role neutrophils themselves play in the severity of disease, or is reflective of a dampened response that leads to severity is yet to be shown. What is not yet clear is whether neutrophil phenotypes are maintained between health and disease, or are sufficiently skewed to appear transcriptomically entirely different, with discordant findings seen in the literature.

Human neutrophil single cell sequencing in malignancy

Neutrophil single cell analysis has been carried out in human breast (48,53), lung (47) and pancreatic (49,50) primary cancer. Like other diseases, immature neutrophils appear less transcriptomically plastic, but mature neutrophils more so. In patients with pancreatic ductal adenocarcinoma (PDAC), Montaldo *et al.* (49) showed a higher proportion of immature neutrophils in peripheral blood than compared with healthy volunteers, but not transcriptomic differences from health, or other stimuli causing stress. Transcriptomic differences were seen in peripheral blood mature neutrophils in PDAC compared to health and other stimuli. Neutrophils from patients with PDAC also showed upregulated interferon response pathways, but with a different expression signature from that seen in patients who underwent H-SCT, involving *IRF1* and *GBP1*. Contrastingly, in Wang *et al.* (50).'s study of peripheral blood and tissue from PDAC, 6 subclusters of mature neutrophils from within peripheral blood of healthy controls and patients with PDAC had transcriptomic similarity, though there were key pathways that were upregulated in health and downregulated in disease, and vice versa. In health, positive regulation of neutrophil apoptosis was apparent, and in patients with PDAC, interferon response pathways were upregulated in peripheral blood neutrophils.

Studies support the role of tissue differentiation contributing to neutrophil plasticity, with neutrophil clusters identified exclusively within tumour tissue. Zilionis *et al.* (47), 2019 found a human neutrophil cluster that was exclusively within lung tumour, and these were also seen in PDAC (50). Pseudotime analysis of neutrophil clusters identified exclusively within the tumour supported differentiation within the tumour, with the distinct neutrophil clusters identified being transitional between peripheral blood neutrophils and the terminally differentiated neutrophil states they label as "TAN1" and "TAN2". These terminally differentiated neutrophils highly expressed genes associated with hypoxia response, endoplasmic reticulum stress, TNF, and angiogenesis. The presence of TAN1 neutrophils and their expression signature correlated with a worse prognosis.

Summary

Primary tumours influence neutrophil phenotype, both skewing those identified in peripheral blood, directly upon neutrophils trafficked into the tumour, with transcriptomically unique neutrophils found only within tumours. This change is tumour-dependent, as they differentiate within the tumour away from phenotypes also found within peripheral blood. This differentiation may be key in driving pro- and anti-tumour phenotypes.

Further work is required, not only in examining further types of cancer, but also in metastases, as there is evidence supporting the role of neutrophils in the pre-metastatic niche (54).

Putative pathways altered in disease

Altered metabolism

Transcriptomic differences suggesting alterations in metabolism from steady state were found across diseases. In health, mitochondria are relatively sparse in neutrophils, and their main production of energy takes place through anaerobic glycolysis and production of pyruvate (55,56). In anaerobic conditions this is converted into lactate. In the presence of oxygen pyruvate is converted into acetyl coenzyme A, which then produces energy as a substrate in the Krebs cycle. Neutrophils also use the pentose-phosphate pathway to generate glucose-6-phosphate which is then broken down into pyruvate (57). In PDAC, the TAN1 subcluster identified by Wang *et al.* (50) strongly expressed genes involved in glycolysis such as *LDHA*, *HK2*, and the *GLUT1* glucose transporter, suggesting a switch to glycolysis. They concluded that this switch to glycolysis helped drive neutrophils to a pro-tumour phenotype. Changes in expression of genes involved in glucose metabolism, pyruvate metabolism and glycolysis were also seen by Huang *et al.* (52) in patients with burns, and were particularly pronounced in immature neutrophils.

IFN-stimulated population

Across multiple disease and stressors, as well as in murine models, a subset of neutrophils that are highly upregulating genes involved in response to interferons has been identified. Their role in disease, particularly cancer, is likely to be worthy of further study. Type I interferons have been shown to have anti-tumour function, and are said to be involved in driving neutrophils to an anti-tumour phenotype (58), which are able to suppress angiogenesis (59). In both studies that examined patients with PDAC, there was a higher proportion of neutrophils that highly expressed interferon responsive genes found in the peripheral blood. Interestingly, when recruited into the tumour, this cluster was seen early in pseudotime but disappeared as neutrophils differentiated. On histology, these neutrophils were found mostly in the stroma, rather than interacting with the tumour directly. This population were also identified by Zilionis *et al.* (47). in both mouse and human non-small cell lung cancers (NSCLCs), as well as peripheral blood.

In COVID-19, these interferon-responsive pathways were found to be upregulated in mild disease but suppressed in severe infection by Xu *et al.* (51). There are conflicting reports on the role of interferon response in pathogenesis of COVID-19, with patients with a severe COVID-19 infection shown to have a robust type I interferon response in comparison with mild disease (60), but also that peripheral blood immune cells had a significantly impaired response in patients with severe or critical COVID-19 (61). In patients with burns, this neutrophil subtype was particularly associated with development of post-burn infections, and their transcriptomic profile varied following a burn injury. Early after burn injury, they, like other neutrophil subtypes, had upregulated pathways involved in degranulation and neutrophil activation, but in subsequent days the interferon response became predominant.

Concordance between mouse and human neutrophils in cancer models

Mouse models of cancer and human disease have concordant and discordant populations of neutrophils

A number of studies utilised single cell RNA-sequencing to reconcile the different neutrophil populations in human and mouse, as summarised in *Table 3*. Grieshaber-Bouyer *et al.* (39). have shown that human neutrophils display a similar transcriptomic pattern to the continuous developmental spectrum they identified in mice. However, the full concordance between human and mouse neutrophil development is yet to be investigated in detail.

Zilionis *et al.* (47), 2019 analysed tumour-infiltrating immune populations in NSCLC from patient and mouse samples and identified conserved neutrophil subsets in both species. Neutrophils in both patient and mouse tumour samples exhibited a continuum of clusters (five and six subsets, respectively), which were further classified into three conserved modules based on gene expression: (I) neutrophils positive for canonical neutrophil markers, which continuously progressed to (II) pro-tumorigenic neutrophils and (III) a distinct neutrophils subset expressing a type I interferon response signature. *Table 2* summarises the different neutrophil subsets and representative markers.

Similarly, Alshetaiwi *et al.* (48), 2020 demonstrated that the neutrophil signature in breast cancer is largely conserved between human and mouse. Using a murine

Table 3 Conserved neutrophil clusters in h and m and their representative markers

Study	Neutrophil cluster	Markers identified
Zilionis *et al.*, 2019 (47)	hN1 and mN1	Canonical neutrophil markers: *MMP8*, *MMP9*, *S100A8*, *S100A9*, *ADAM8*
	hN5 and mN5	Cytokine markers: *CCL3*, *CSF1*, *CSTB*, *CTSB*, *IRAK2*
	mN6	*Hexb*, *Ptma*, *Fcnb*, *Ngp*
	mN4	*Hexb* and *Ptma*
	hN2 and mN2 (rare)	Type-I interferon response markers: *IFIT1*, *IRF7*, *RSAD2*
Alshetaiwi *et al.*, 2020 (48)	mC0	Mature neutrophils: *Camp*, *Ly6g*^{High}
	mC2	Immunosuppressive factors: *IL-1β* and *Arg2*
	mC4 and mC5	Overlapping markers: *Cebpe*, *Retnig*
	mC7 and mC8	Cell cycle markers: *Tuba1b*, *Cdc20*
	hN0	Immunosuppressive, tumor-infiltrating markers: *S100A9*, *CXCR2*
	hN1	*CXCL8*, *ENO1*
	hN2	*CD74*, *RPS18*
	hN3	*GATA2*, *CLC*

Cluster names as reported in the study. Prefixes h and m denote clusters in humans and mouse, respectively. h, humans; m, mouse.

breast cancer model, the authors identified six distinct clusters in the mouse spleen, characterised by high *Ly6g* and CXCR2 levels (*Table 2*). Moreover, they identified four distinct neutrophil clusters in published datasets from primary tumour samples of breast cancer patients (53), one of which largely recapitulated the immunosuppressive, tumour-infiltrating neutrophil signature observed in mice. Pseudotime analysis revealed that highly proliferative neutrophil progenitors bifurcate either into mature neutrophils or pro-tumorigenic neutrophils, with a transitional state at the bifurcation point. This suggests an alternative, yet aberrant, maturation process in the mouse spleen that gives rise to immunosuppressive neutrophils at the cost of normal neutrophil maturation. Whether this aberrant neutrophil trajectory translates to humans remains unexplored.

Strengths and weaknesses of this review

This review provides an oversight into neutrophil single cell sequencing in a variety of mouse models of health and disease. Having a comparison of mouse and human single cell sequencing is helpful, as understanding equivalence and variance in these is vital when making comparisons between mouse models and humans. It also highlights putative mechanisms by which altered neutrophils may have an effect, and picks out populations of note, such as the interferon-responsive neutrophil population seen expanded in all disease states that are worthy of further investigation. A weakness is that there are only a few tumour types that have been studied with single cell sequencing of neutrophils, so strong comparisons between neutrophils in different tumours cannot be made. Authors such as Grieshaber-Bouyer *et al.* (39) point out that single cell RNA sequencing of neutrophils is complicated by their lower RNA content, so conclusions are being drawn from small populations of neutrophils.

Conclusions

Single cell neutrophil sequencing in mice and humans has corroborated recent developments moving our knowledge of neutrophils from a rigid, homogenous population to one of a highly plastic, adaptable cell. Even in health, several subtypes of neutrophils can be identified, with their own transcriptomic signatures. When placed under stress or in response to disease, neutrophils can adapt, upregulating and downregulating pathways to vary gene expression, as well as altering the proportion of each subtype present, depending on the challenge. However, despite this plasticity, neutrophils maintain a fixed developmental trajectory from early differentiation within the bone marrow, to immature

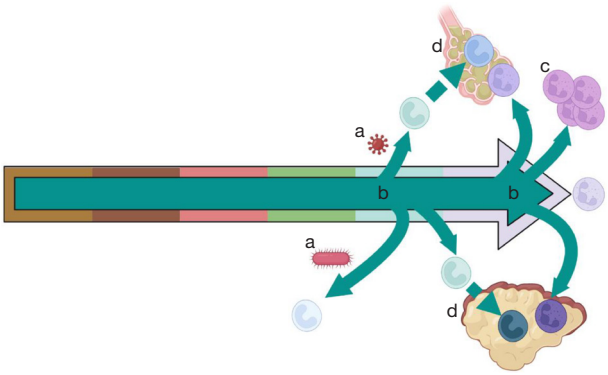

Figure 2 Neutrophil development maintains a fixed trajectory towards mature neutrophils. Different stimuli (a), at different times (b) may change the point at which neutrophils are mobilised and therefore maturity as well as the proportion of neutrophil phenotype present (c). Tissue, including tumours, into which the neutrophil is recruited also plays a role in the transcriptional phenotype of neutrophils (d). The figure was created with BioRendr.com.

and mature circulating neutrophils that appear to terminally differentiate within tissue (*Figure 2*). The different subtypes of neutrophils appear to play a role in response to disease, as neutrophil subtypes are associated with infection or sepsis following burn injuries, more or less severe COVID-19 disease, and worse prognosis pancreatic cancer.

More should and can be done with neutrophil single cell sequencing in cancer. Rare and common cancers such as colorectal cancer have yet to be sequenced in depth, and this work could provide insight into neutrophil-directed therapy that may alter immune response and allow for higher efficiency of existing therapies. Interferon-responsive neutrophils, identified in peripheral blood of patients with pancreatic cancer, and known to have an anti-tumour role, may be blunted in their efficacy through differentiation into other neutrophils that have a transcriptomically different appearance and therefore function. It could be possible to prevent this differentiation pharmacologically if the driving factor from within the tumour or microenvironment was identified. A key question remains: what signals to neutrophils in the tumour microenvironment, how are these recruited, and can they be modulated to function differently in the context of disease?

Studies comparing mouse and human neutrophils, albeit few, collectively highlight a conserved tumour-infiltrating neutrophil phenotype that is present in both species. However, further work is required to ascertain whether these signatures could be extended to other cancer types to permit translational studies in mice. In addition, individual neutrophil subsets have not been explored in detail and profiling more patient samples from paired primary and metastatic tumour sites might reveal additional populations. A thorough elucidation of the nature and function of these subsets could open a window of opportunity for identifying potential diagnostic tools and therapeutic targets.

In the future, single cell sequencing of neutrophils could be performed not only on tissue that has been dissociated, but in a spatial manner, using platforms such as the CosMx spatial molecular imager (NanoString, Seattle WA, USA). This will allow for deeper comprehension of cell-cell interactions, cellular neighbourhoods within areas of the tumour such as the tumour centre invasive edge and tertiary lymphoid structures, and potentially allow for understanding on positive or negative regulation of these interactions. This could then be exploited pharmacologically to promote tumour death, either with novel therapies, or augmenting current therapies such as checkpoint inhibitors. As single cell RNA sequencing becomes cheaper, it could move from the research laboratory into the clinical laboratory, with development of biomarkers based on single cell sequencing that predict efficacy of treatment. This could be used to stratify patients for further treatment, such as surgical resection of metastases, and adjuvant, neoadjuvant and palliative systemic anti-cancer therapy.

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Footnote

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