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INVITED REVIEW

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Current knowledge on the early stages of human neutropoiesis

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Summary

Polymorphonuclear neutrophils are no longer considered as a homogeneous population of terminally differentiated and short-lived cells that belong to the innate immune system only. In fact, data from the past decades have uncovered that neutrophils exhibit large phenotypic heterogeneity and functional versatility that render them more plastic than previously thought. Hence, their precise role as effector cells in inflammation, in immune response and in other pathophysiological processes, including tumors, needs to be better evaluated. In such a complex scenario, common knowledge of the differentiation of neutrophils in bone marrow refers to lineage precursors, starting from the still poorly defined myeloblasts, and proceeding sequentially to promyelocytes, myelocytes, metamyelocytes, band cells, segmented neutrophils, and mature neutrophils, with each progenitor stage being more mature and better characterized. Thanks to the development and utilization of cutting-edge technologies, novel information about neutrophil precursors at stages earlier than the promyelocytes, hence closer to the hematopoietic stem cells, is emerging. Accordingly, this review discusses the main findings related to the very early precursors of human neutrophils and provides our perspectives on human neutropoiesis.

KEYWORDS
immunophenotype of neutrophil progenitors, neutrophil progenitors, neutrophils, neutropoiesis

1 | INTRODUCTION

Polymorphonuclear neutrophils represent the most abundant circulating leukocyte type in human blood (roughly 60%, normally present at 2.5–7.5 × 10^9 cells/L) and typically act at the frontline of innate defense to potential threats.[1] Neutrophils contain heterogeneous cytosolic granules that are identified by staining with neutral dyes and that serve as reservoirs for enzymes, cationic proteins, preformed receptors, and other bioactive molecules that synergistically damage, and often kill, ingested microbes. Mature neutrophils are terminally differentiated, non-dividing cells that originate in bone marrow (BM) from $CD34^+$ hematopoietic stem cells (HSCs) under the regulatory effects of several colony-stimulating factors (CSFs), including granulocyte-CSF (G-CSF) and granulocyte-macrophage-CSF (GM-CSF).[1] Both mature and immature neutrophils can be morphologically identified by the peculiar shape of their nucleus, which evolves during maturation in the bone marrow from round to polymorphic, as well as by the spatial ratio of cytoplasm versus nucleus. However, the number of circulating neutrophils can dramatically increase as much as 10-fold under acute inflammatory conditions through accelerated cell production and release by the BM. In humans, circulating neutrophils are 10–20 nm in diameter and exist in

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dynamic equilibrium with a so-called "marginated pool" sequestered within the microvasculature of many organs.[1] Although neutrophils have long been viewed as effectors of acute inflammation and resistance to many pathogens, recent evidence has not only challenged but even subverted such a myopic and limited view.[2] A series of discoveries of their multifaceted functions have in fact made it clear that, in addition to innate immunity responses, neutrophils function as key effectors in chronic inflammation, angiogenesis, adaptive immunity responses, autoimmunity, and cancer.[3,4] In this context, the release of cytokines, extracellular traps, and/or humoral pattern recognition molecules represent some of the novel effector mechanisms whereby neutrophils exert their final effects.[3,4] An appreciation of neutrophils as very versatile cells is emerging as evidence demonstrates their capacity to acquire different, and even opposing, functions and activation status, depending on the physiological or pathological context.[5] Multicolor flow cytometry analysis has identified a variety of heterogeneous populations of mature and immature neutrophils, often displaying distinct functions, although a consensus on their identity as unique subsets has not been reached.[5] By virtue of accumulating evidence demonstrating neutrophil plasticity and their involvement in many human diseases, precise delineation of neutrophil ontogeny under steady state and pathological conditions is an important priority for investigators. Such an exercise is crucial, since precise knowledge of physiological and pathological neutrophil development in BM is prerequisite for a better understanding not only of myeloid leukemia pathogenesis but also of the mechanisms that promote the alterations of neutrophil output and generate novel pathological neutrophil populations.

In this review, we summarize the main findings concerning the identification of human neutrophil progenitors, with a focus on the early stages of neutropoiesis.

2 | NEUTROPOIESIS: "HISTORIA DE UN AMOR"

Granulopoiesis is a term conventionally referred to the development of granulocyte polymorphonuclear neutrophils, eosinophils, and basophils from BM precursors that, through a series of proliferation and differentiation steps, become fully mature.[6] The current knowledge of hematopoiesis mostly derives from observations obtained by performing a variety of in vitro and in vivo functional assays combined with flow cytometry analysis. These methodological approaches have progressively established the so-called *classical* model of hematopoiesis,[7] which relies on immunophenotypically defined cell populations and which has been considered by researchers in the field as the gold standard for the last three decades. According to such a hierarchical model, the myeloid lineage—including monocytes, dendritic cells and polymorphonuclear neutrophils, eosinophils, and basophils—is thought to originate from shared progenitors descending from HSCs[8] (Figure 1). The overall picture of human granulopoiesis presented in the literature assumes that the stepwise differentiation of the $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ HSCs dictates their commitment to the myeloid lineage and their differentiation into discrete multipotent progenitors (MPPs) that, in turn, generate mutually exclusive progenies. Along the progressive commitment of HSCs toward mature cells, the first bifurcation delineating the myeloid and lymphoid branches is proposed to occur at the level of the so-called $Lin^-CD34^+CD38^+CD10^-CD135^+CD123^{dim}CD45RA^-$ common myeloid progenitors (CMPs) and $Lin^-CD34^+CD38^-CD45RA^+CD10^-$ lymphoid-primed multipotential progenitors (LMPPs), the latter in turn generating the $Lin^-CD34^+CD38^+CD10^+CD45RA^-$ common lymphoid progenitors (CLPs) (Figure 1). In this scheme, CMPs would originate bipotent progenitors, namely Lin^-CD34^+CD

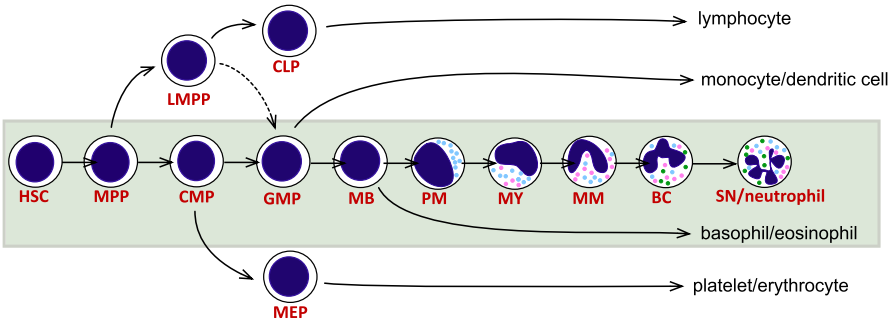

FIGURE 1 Human granulopoiesis. As evidenced by the green background, the illustration shows granulopoiesis according to the "classical" model of hematopoiesis (adapted from current literature). The "classical" model of hematopoiesis involves a hierarchical, stepwise, differentiation of the HSCs into multipotent (ie, MPPs), oligopotent (ie, LMPPs, CLPs, and CMPs), and bipotent (ie, GMPs and MEPs) progenitors. Commitment to eosinophil, basophil, or neutrophil lineages by granulocyte progenitors conventionally occurs at the MB stage. Development of neutrophils from MBs includes the sequential generation of PMs, MYs, MMs, BCs, SNs, and mature neutrophils, according to their content of distinct granule types, specific antigen expression, and peculiar nuclear segmentations. The light blue, pink, and green dots within the cytoplasm of neutrophils/neutrophil precursors represent primary/azurophil granules, secondary/specific granules and tertiary/gelatinase granules, respectively. BC, band cells; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte/macrophage progenitors; HSC, hematopoietic stem cells; LMPP, lymphoid-primed multipotential progenitors; MB, myeloblasts; MEP, megakaryocyte/erythroid progenitors; MM, metamyelocytes; MMP, multipotent progenitors; MY, myelocytes; PM, promyelocytes; SN, segmented neutrophils.

$38^+CD10^-CD135^-CD123^-CD45RA^-$ megakaryocyte-erythrocyte progenitors (MEPs) and $Lin^-CD34^+CD38^+CD10^-CD135^+CD123^{di}{}^mCD45RA^+$ granulocyte-macrophage progenitors (GMPs), the latter originated also from LMPPs (Figure 1). GMPs then differentiate into either granulocyte or monocyte precursors[9,10] (Figure 1). The development of neutrophils from GMPs would then proceed via the generation of myeloblasts (MBs), cells considered to be the most primitive granulocyte precursors and distinguishable by morphology, followed by the sequential generation of promyelocytes (PMs), myelocytes (MYs), metamyelocytes (MMs), band cells (BCs), segmented neutrophils (SNs), and fully mature neutrophils[11,12] (Figure 1). However, even though each of these neutrophil-specific precursors from MBs onwards is conventionally defined based on their nuclear morphology, cell size, and kinetics of granules appearance during development,[13] their characterization in the last decades has been progressively improved by the advent of multi-parametric flow cytometry. Consequently, PMs and the descending neutrophil precursors have been immunophenotypically better defined by the identification of discrete antigens, as well as their expression modulation during maturation. Nonetheless, several concerns persist. One unresolved issue is that the term *granulopoiesis* creates some confusion because it is used interchangeably to describe either the development of all three granulocyte types from MBs (Figure 1) or the production of neutrophils only. Since we focus on the generation of neutrophils only, in this article we will use the term *neutropoiesis*, as recently done by Overbeeke et al.[14] Another issue is that the *classical* model of hematopoiesis does not fully encompass the complexity of progenitor development, including that of human neutrophils. In fact, recent studies using single-cell transcriptional analysis coupled with multicolor flow cytometry have proposed that human hematopoiesis occurs as a gradual and continuous differentiation process, thus implying that HSCs, CMPs, GMPs, and other phenotypically defined cell compartments are in fact very heterogeneous.[10,15] According to this *continuum* model of hematopoiesis, cells already displaying a selective lineage commitment would directly emerge from HSCs instead of originating from intermediate multipotent progenitors such as CMPs or GMPs,[16] which consist of multiple clusters of lineage-specific cells that share a common immunophenotype.[16] The overall picture emerging from current studies of granulopoiesis reveals that neutrophils, eosinophils, and basophils, from very early progenitor stages, traverse independent pathways toward full maturation.[17–20] Furthermore, current characterization of neutrophil precursors is limited to stages that follow PMs, with little detail on characteristics of cells that precede PMs.

3 | PIONEER STUDIES FOCUSED ON MYELOID PRECURSORS PRECEDING PMS

Before the flow cytometric identification of CMPs and GMPs,[21–25] technical challenges in the isolation or assessment of cells of interest have determined a limited knowledge about the granulocyte progenitors preceding PMs. Standard colony forming unit (CFU) assays of samples containing putative myeloid progenitors have been long served as the analytical tool of choice for their characterization and classification.[26] CFU assays quantify the capacity of a given, single, progenitor cell, cultured in the presence of discrete cytokines and growth factors, to proliferate and differentiate into multiple types of cells, which altogether form a colony of erythroid, myeloid, or mixed cell populations. According to such CFU assays, myelopoiesis or erythropoiesis is represented as a hierarchy of progenitors starting from those forming granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) colonies[27] and continuing with those restricted to either megakaryocyte (CFU-MK) or granulocyte/macrophage (CFU-GM) colony generation. The CFU-GM differentiate into colonies of granulocytes (CFU-G) or macrophages (CFU-M) when incubated with, respectively, GM-CSF/G-CSF or GM-CSF/M-CSF.[28] CFU-GM-, CFU-G-, or CFU-M-generating cells were, however, associated with a very limited immunophenotype, whose main markers consisted of CD34, CD38, HLA-DR, CD13, and CD33.[29]

In this setting, Olweus et al[30] used the innovative approach of four-color flow cytometry to identify, and then purify for the first time, lineage-committed $CD34^+$ granulo-monocytic progenitors. By comparing the expression of several antigens by $CD34^+$ progenitor cells isolated from both fetal bone marrow (FBMs) or adult BMs in order to identify novel markers fully specific for both granulocyte and monocyte lineages, these investigators ultimately discovered CD64 (aka FcγRI) as the best candidate.[30] CD64 was found undetectable in $CD34^+CD38^-HLA\text{-}DR^+$ HSCs but expressed on a subset of more mature $CD34^+CD38^+HLA\text{-}DR^+$ cells.[30] The observation that the CFU of colonies arising from the $CD34^+CD38^+CD64^+$ cell population consisted of both granulocytes and monocytes confirmed that CD64 is specific for the granulocyte/monocyte lineages.[30] Flow cytometric analysis of the progeny generated by in vitro differentiation of the sorted $CD34^+CD38^+CD64^+$ population demonstrated the generation of granulo-monocytic cells.[30] A subsequent paper by the same group investigated the possibility that CD115/M-CSFR expression, and hence M-CSF responsiveness, could be useful to discriminate monocyte and granulocyte progenitors within FBM $CD34^+$ cells.[31] That proved correct, as the combined usage of CD64 and CD115 phenotypically distinguish monocytic precursors from those committed to the granulocytic lineage.[31] Accordingly, both CFU assay and flow cytometry analysis of cells derived from cultures of $CD34^{hi}CD64^+CD115^{hi}$ progenitors demonstrate that they are exclusively monocytic cells, whereas $CD34^{hi}CD64^+CD115^{lo}$ progenitors generate granulocytic cells.[31] However, the investigators did not pursue an exhaustive characterization of the granulocyte types derived from the cultures of $CD34^+CD64^+CD115^{lo}$ cells. It must be pointed out that Olweus et al[31] also described a subset of $CD34^{hi}CD64^-CD115^{hi}$ cells which precede the $CD34^{hi}CD64^+CD115^{hi}$ cell population, that was able to become almost exclusively monocytic cells. Therefore, based on the previous findings on $CD34^{hi}CD64^-CD115^{hi}$ and $CD34^{hi}CD64^+CD115^{hi}$ cells, Olweus et al[31] concluded that CD115 represents a marker that precedes CD64 along the monocyte developmental cascade. Moreover, the same group demonstrated that $CD34^+CD123^{hi}$

cells from FBM generate a CD1a$^+$ progeny that displayed a dendritic cell (DC) morphology and antigen-presenting function.[32] Thus, by using CD123/IL-3R in their flow cytometry panel, Olweus et al[32] were able to describe and isolate a CD34$^+$CD123^{hi} cell population resembling DC progenitors that, in turn, were generated by CD34$^+$CD123^{dim}CD115^{hi} cells, ultimately pointing to CD115 as a marker identifying both monocyte and DC progenitors.

Collectively, the studies by Olweus et al[30,31] demonstrated for the first time that granulocyte/monocyte-committed progenitors could be identified by their specific CD34, CD115 and CD64 expression, and thereby sorted by flow cytometry. Moreover, they also uncovered that DC progenitors express CD123.[32] Inexplicably, at least in our opinion, the seminal papers by Olweus and coworkers have been substantially neglected for a long time, maybe because a subsequent study by Manz et al[22] failed to reproduce the observation that BM Lin$^-$CD34$^+$CD38$^+$ cells express CD64.

4 | GMPS: THE MODERN ERA

More recently and in accordance with the *classical* model of hematopoiesis, both Lee et al[33] and Kawamura et al[34] have extensively characterized human GMPs by utilizing not only multiparametric flow cytometry and cell culture assays, but also transcriptional profiling. By using CD115 and CD123 as myeloid lineage markers,[30,32] Lee et al[33] fractionated GMPs into various cell compartments, namely (a) CD38$^+$CD34$^+$CD10$^-$CD45RA$^+$CD123$^{dim/-}$CD115$^-$ granulocyte-monocyte-DC progenitors (labeled GMDPs), which generate CD66b$^+$ cells (generally defined as granulocytes), CD14$^+$ monocytes, and three DC subsets (ie, cDC1s, cDC2s, and pDCs); (b) CD38$^+$CD34$^+$CD10$^-$CD45RA$^+$CD123$^{dim/-}$CD115$^+$ monocyte-DC progenitors (called MDPs), which generate both DCs and CD14$^+$ monocytes); and (c) CD38$^+$CD34$^+$CD10$^-$CD45RA$^+$CD123$^+$CD115$^-$ common dendritic progenitors (called CDPs), which are exclusively committed to the three DC subsets. Applying a similar approach but with a different panel of markers including CD64, Kawamura et al[34] identified a unilineage cluster of progenitors within GMPs, called common monocyte progenitors (cMoPs), which are characterized by a CD34$^+$CD38$^+$CD10$^-$CD123$^{dim/-}$CD45RA$^+$CD135$^+$CLEC12A$^+$CD64$^+$ phenotype. cMoPs was also described as CD115$^+$, and able to differentiate exclusively into CD34$^-$CD38$^+$CD10$^-$CD123$^{dim/-}$CD45RA$^+$CD135$^+$CLEC12A$^+$CD64^{++} pre-monocytes and ultimately mature into CD14$^+$ monocytes. As outlined in the previous section, Olweus et al[31] had previously demonstrated that co-expression of CD115 and CD64 specifically distinguishes human monocyte progenitors, thus already pointing to the existence of cMoPs. Kawamura et al[34] also postulated the existence of revised GMPs (rGMPs) as CD34$^+$CD38$^+$CD10$^-$CD123$^{dim/-}$CD45RA$^+$CD135$^+$CLEC12A$^+$CD64^{dim} myeloid progenitors, generating CD66b$^+$ granulocytes and CD14$^+$ monocytes. However, the phenotypic and morphological features of the CD66b$^+$ granulocytic cells originated by GMDPs as reported by Lee et al's study,[33] or by rGMPs as reported by Kawamura et al's study,[34] were not exhaustively investigated. Surprisingly, these investigators and other groups[33–37] exerted great effort to define the developmental cascades of human DC and monocyte differentiation from HSCs but never pursued a detailed characterization of human neutrophil ontogeny.

5 | IDENTIFICATION OF CD34$^{+/-}$CD45RA$^{+/-}$CD64$^+$CD115$^-$ NEUTROPHIL-COMMITTED PROGENITORS (NCPS)

Taking advantage of the results concerning the identification of granulocyte[31] and mono/DC progenitors within GMPs[33,34] and recognizing the limited knowledge of CD34$^+$ neutrophil precursors, we recently assembled a flow cytometry antibody panel that detects all key markers conventionally used to identify myeloid and lymphoid progenitors, including CD34, CD38, CD10, CD123, CD45RA, CD64, and CD115.[38] We then focused our attention on the SSC^{lo}Lin$^-$CD45^{dim} cells of human BM, which include not only HSCs but also CD34$^+$ and CD34$^{dim/-}$ myeloid/lymphoid progenitors.[20,38,39] For our internal control, within CD34$^+$CD38$^+$CD45RA$^+$ GMPs we gated first on CD123$^+$CD64$^-$CD115$^-$ CDPs, CD123$^{dim/-}$CD64$^+$CD115$^+$ cMoPs, CD64$^-$CD115$^+$ MDPs, and CD64$^-$CD115$^-$ GMDPs. We hypothesized that the remaining CD64$^+$CD115$^-$ cell population within GMPs would correspond to neutrophil precursors based on (a) their phenotypic similarity to the granulocyte progenitors described by Olweus et al[31] (see above); (b) the already established myeloid developmental potential of the precursors within GMPs[33,34]; and (c) the previous discoveries of basophil and eosinophil precursors within CMPs/MEPs, but not GMPs.[17–20] In vitro differentiation assays confirmed our hypothesis, as CD34$^+$CD45RA$^+$CD64$^+$CD115$^-$ cells generated almost exclusively neutrophils without eosinophils or basophils. Hence, considering that CD115 expression within GMPs is a feature restricted to monocyte precursors,[31,33] but that CD64 expression is shared by both monocyte and granulocyte precursors,[31,34] we recognized that the CD64$^+$CD115$^-$ phenotype could define very early neutrophil progenitors, which we named neutrophil-committed progenitors (NCPs). Thus, by using the CD64$^+$CD115$^-$ expression as a phenotypic fingerprint, we could identify other NCPs within the SSC^{lo}Lin$^-$CD45^{dim} cells that variably expressed CD34, CD45RA, or both CD34 and CD45RA. Ultimately, we identified four populations, which we named CD34$^+$CD45RA$^-$CD64$^+$CD115$^-$ NCP1s, CD34$^+$CD45RA$^+$CD64$^+$CD115$^-$ NCP2s, CD34$^{dim/-}$CD45RA$^+$CD64$^+$CD115$^-$ NCP3s, and CD34$^{dim/-}$CD45RA$^-$CD64$^+$CD115$^-$ NCP4s (Table 1 and Figure 2).

To confirm convincingly the specific commitment of CD34$^{+/-}$CD45RA$^{+/-}$ NCPs to neutrophils, we sorted them for a series of experiments and defined their morphological features, functional properties, and bulk/single-cell transcriptomes. We observed that the morphology of CD34$^{+/-}$CD45RA$^{+/-}$ NCPs, as revealed by May Grunwald-Giemsa staining, displayed a high nucleus/cytoplasm ratio, a fine nuclear chromatin organization, a pronounced basophilia, and cytoplasmic basophilic granules. In other words, we

TABLE 1 Immunophenotypes of the human neutrophil progenitors discussed in the text

Progenitors	Immunophenotype	Methodology of investigation	References
Myeloid progenitors	$FSC^{int/hi}SSC^{lo/int}CD34^{+}CD16^{-}CD11b^{-}CD15^{-/dim}$	Flow cytometry, cell morphology, cytochemistry (MPO)	Terstappen et al[50] Lund-Johansen et al[51] Elghetany et al[54,59]
MBs	$FSC^{hi}SSC^{int}CD34^{-}CD16^{-}CD11b^{-}CD15^{dim/+}$		
PMs	$FSC^{int/hi}SSC^{int/hi}CD16^{-}CD11b^{-}CD15^{+/++}$		
MYs	$FSC^{int}SSC^{hi}CD16^{-}CD11b^{+/++}CD15^{++}$		
MMs	$FSC^{int/lo}SSC^{hi}CD16^{+}CD11b^{++}CD15^{++}$		
BCs	$FSC^{lo}SSC^{int/hi}CD16^{+/++}CD11b^{++}CD15^{++}$		
SNs	$FSC^{lo}SSC^{int/hi}CD16^{++}CD11b^{++}CD15^{++}$		
Granulocyte progenitors	$CD34^{hi}CD64^{+}CD115^{lo}$	Flow cytometry, in vitro differentiation assay, CFU assay	Olweus et al[31]
Myelo/monoblasts	$SSC^{lo/int}CD45^{dim}CD34^{+}CD117^{+}CD13^{+}CD33^{+}$	Flow cytometry	Van Lochem et al[39]
PMs	$SSC^{int}CD45^{dim}CD13^{+}CD11b^{-}CD16^{-}$		
MYs	$SSC^{hi}CD45^{dim}CD13^{dim}CD11b^{+}CD16^{-}$		
MM/BCs	$SSC^{int/hi}CD45^{dim}CD13^{dim}CD11b^{+/hi}CD16^{+/hi}$		
SNs	$SSC^{int/hi}CD45^{dim}CD13^{+}CD11b^{hi}CD16^{hi}$		
EPMs	$SSC^{lo}CD34^{-}CD15^{int}CD33^{+}CD49d^{+}CD11b^{-}CD16^{-}$	Flow cytometry, cell morphology, immunocytochemistry, microarray	Mora-Jensen et al[45] Rapin et al[60]
LPMs	$SSC^{hi}CD34^{-}CD15^{hi}CD33^{+}CD49d^{+}CD11b^{-}CD16^{-}$		
MYs	$SSC^{hi}CD34^{-}CD15^{hi}CD33^{hi}CD49d^{hi}CD11b^{int}CD16^{-}$		
MMs	$SSC^{hi}CD34^{-}CD15^{hi}CD33^{int}CD49d^{-}CD11b^{hi}CD16^{int}$		
BCs	$SSC^{hi}CD34^{-}CD15^{hi}CD33^{-}CD49d^{-}CD11b^{hi}CD16^{int}$		
SNs	$SSC^{hi}CD34^{-}CD15^{hi}CD33^{-}CD49d^{-}CD11b^{hi}CD16^{hi}$		
proNeu1s	$SSC^{lo}CD66b^{+}CD15^{+}CD11b^{-}CD49d^{hi}$	CyTOF, flow cytometry, Infinity Flow, in vitro differentiation assay (for proNeu1/2s only)	Evrard et al[46] Kwok et al[48] Cossarizza et al[71]
proNeu2s	$SSC^{hi}CD66b^{+}CD15^{+}CD11b^{-}CD49d^{int}$		
preNeus	$CD66b^{+}CD15^{+}CD11b^{+}CD49d^{+}CD101^{-}$		
Immature Neus	$CD66b^{+}CD15^{+}CD11b^{+}CD49d^{-}CD101^{+}CD16^{+}CD10^{-}$		
Mature Neus	$CD66b^{+}CD15^{+}CD11b^{+}CD49d^{-}CD101^{+}CD16^{+}CD10^{+}$		
eNePs	$Lin^{-}CD66b^{+}CD16^{-}CD10^{-}CD71^{+}CD117^{+}$	CyTOF, flow cytometry, cell morphology, CFU assay, in vivo adoptive transfer, bulk RNAseq	Dinh et al[49]
N1s	$Lin^{-}CD66b^{+}CD16^{-}CD10^{-}CD71^{+}$		
N2s	$Lin^{-}CD66b^{+}CD16^{-}CD10^{-}CD71^{-}$		
N3s	$Lin^{-}CD66b^{+}CD16^{+}CD10^{-}$		
N4s	$Lin^{-}CD66b^{+}CD16^{+}CD10^{int}$		
N5s	$Lin^{-}CD66b^{+}CD16^{+}CD10^{hi}$		
NCP1s	$SSC^{lo}Lin^{-}CD45^{dim}CD34^{+}CD45RA^{-}CD64^{dim}CD115^{-}CD15^{-}$	Flow cytometry, cell morphology, functional assays, in vitro differentiation assay, immunocytochemistry, in vivo adoptive transfer, bulk RNAseq, scRNAseq	Calzetti et al[38]
NCP2s	$SSC^{lo}Lin^{-}CD45^{dim}CD34^{+}CD45RA^{+}CD64^{dim}CD115^{-}CD15^{-}$		
NCP3s	$SSC^{lo}Lin^{-}CD45^{dim}CD34^{dim/-}CD45RA^{+}CD64^{dim}CD115^{-}CD15^{dim/+}$		
NCP4s	$SSC^{lo}Lin^{-}CD45^{dim}CD34^{dim/-}CD45RA^{-}CD64^{dim}CD115^{-}CD15^{dim/+}$		
PMs	$SSC^{hi}Lin^{-}CD66b^{+}CD10^{-}CD11b^{-}CD16^{-}$		
MYs	$SSC^{hi}Lin^{-}CD66b^{+}CD10^{-}CD11b^{dim/+}CD16^{-}$		
MMs	$SSC^{hi}Lin^{-}CD66b^{+}CD10^{-}CD11b^{+}CD16^{+}$		
BCs	$SSC^{hi}Lin^{-}CD66b^{+}CD10^{-}CD11b^{+}CD16^{++}CD10^{-}$		
SNs	$SSC^{hi}Lin^{-}CD66b^{+}CD11b^{+}CD16^{++}CD10^{+}$		

Abbreviations: BC, band cells; eNeP, early neutrophil progenitors; EPM, early promyelocytes; hNeP, human neutrophil progenitors; LPM, late promyelocytes; MB, myeloblasts; MM, metamyelocytes; MY, myelocytes; NCP, neutrophil-committed progenitors; PM, promyelocytes; preNeu, neutrophil precursors; proNeu, pro-neutrophils; SN, segmented neutrophils.

found a series of features that supported that $CD34^{+/-}CD45RA^{+/-}$ NCPs are committed to become neutrophils. Interestingly, $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{dim/-}CD45RA^{+}$ NCP3s were found morphologically identical to $CD34^{+}CD45RA^{+}$ NCP2s and $CD34^{dim/-}CD45RA^{-}$ NCP4, respectively, with only NCP3s/NCP4s displaying granules, as expected given their higher level of maturation. The proliferative capacity and neutrophil differentiation of $CD34^{+/-}CD45RA^{+/-}$ NCPs in the presence of discrete growth factors (ie, SCF, FLT3, G-CSF, and GM-CSF) as well as their differentiation into neutrophils after adoptive transfer into humanized mice clearly demonstrated that they consist of very immature neutrophils precursors.[38] Consistent with their differential expression of CD34, $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{+}$ NCP2s generate PMs, MYs, and MMs in vitro, whereas $CD34^{dim/-}CD45RA^{+}$ NCP3s and $CD34^{dim/-}CD45RA^{-}$ NCP4s produce mostly MMs, BCs, and SNs, but very few PMs and MYs. Furthermore, $CD34^{+/-}CD45RA^{+/-}$ NCP-derived mature progeny are functionally active, as demonstrated by their ability to produce superoxide anion in response to agonists and to perform phagocytosis.[38] Finally, a detailed molecular analysis of $CD34^{+/-}CD45RA^{+/-}$ NCPs by bulk RNA-seq not only confirm specific features of, and commitment to, neutrophils, but also highlight remarkable differences among their gene expression profiles that correlate with their maturation status.[38] In fact, both principal component and hierarchical clustering analyses of RNA-seq data

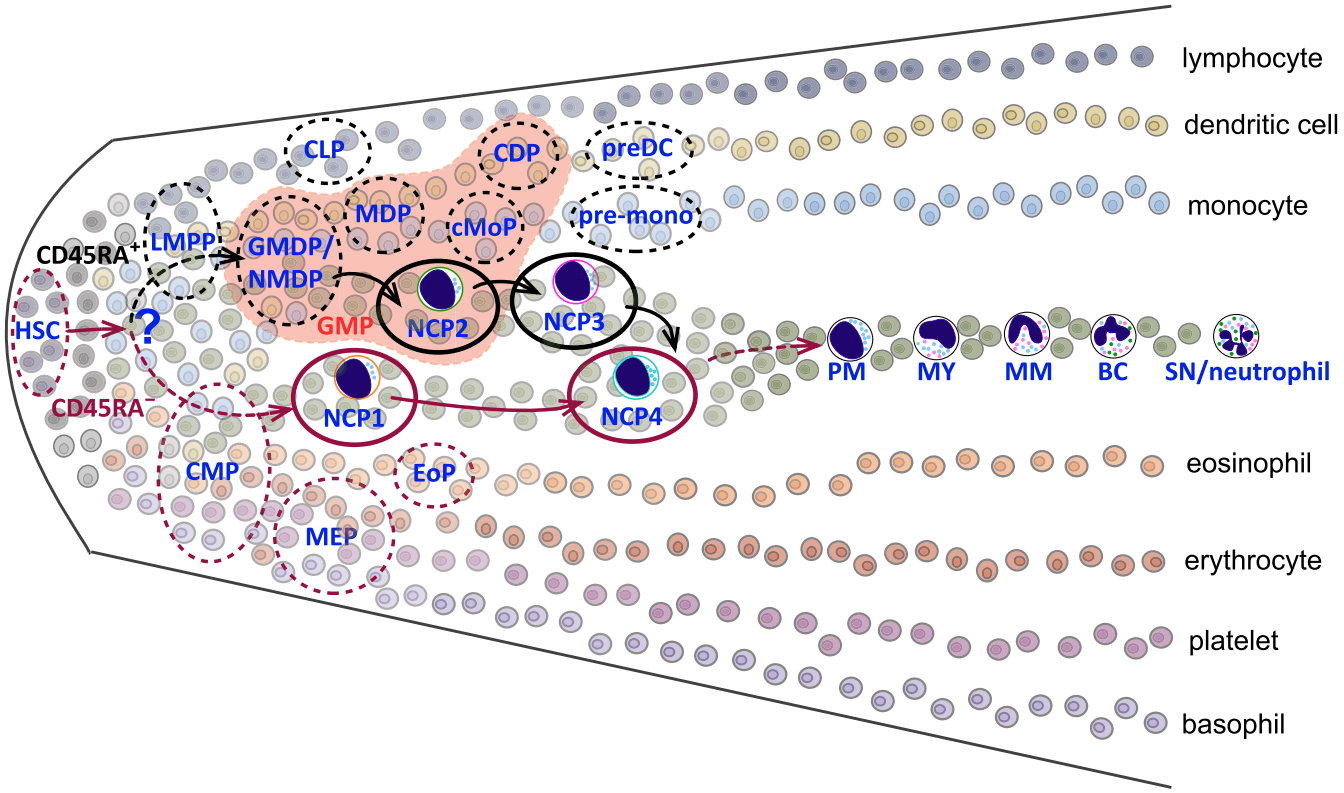

FIGURE 2 Continuum model of hematopoiesis revised by the inclusion $CD34^{+/-}CD45RA^{+/-}$ NCPs. According to this model, HSCs undergo a gradual commitment into unilineage progenitors, and then to terminally differentiated, mature, cells. In this graphical representation, a given developmental trajectory is represented by cells of the same color. Dashed circles identify intermediate progenitors as defined by the "classical" model (such as CMPs and GMPs), represented as compartments including pools of cells sharing a common phenotype, but already committed to a given lineage. According to our recent findings on $CD34^{+/-}CD45RA^{+/-}$ NCPs, neutropoiesis within this "continuum" model would follow two separate maturation trajectories: one including $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{dim/-}CD45RA^{-}$ NCP4s (represented by the purple ellipsoids), the other $CD34^{+}CD45RA^{+}$ NCP2 and $CD34^{dim/-}CD45RA^{+}$ NCP3s (represented by the black ellipsoids). NCP1s would directly differentiate into NCP4s, while NCP2s would develop into NCP3s and then NCP4s, the latter further maturing into PMs. The question mark indicates putative stage/s from which $CD45RA^{+}$- and $CD45RA^{-}$-committed precursors give origin to, respectively, $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{+}$ NCP2s, likely through the involvement of CMPs for $CD34^{+}CD45RA^{-}$ NCP1s (black dashed arrow) and LMPPs for $CD34^{+}CD45RA^{+}$ NCP2s (purple dashed arrows). CDP, common dendritic cell progenitors; CLP, common lymphoid progenitors; cMoP, common monocyte progenitors; CMP, common myeloid progenitors; EoP, eosinophil-committed progenitors; GMDPs/NMDP, granulocyte/neutrophil-monocyte-dendritic cell progenitors; GMP, granulocyte/macrophage progenitors; HSC, hematopoietic stem cells; LMPP, lymphoid-primed multipotential progenitors; MDP, monocyte-dendritic cell progenitors; MEP, megakaryocyte/erythroid progenitors; MMP, multipotent progenitors; NCP, neutrophil-committed progenitors; pre-DC, pre-dendritic cell progenitors; pre-mono, pre-monocytes.

place $CD34^{+/-}CD45RA^{+/-}$ NCPs before PMs along a sequential neutrophil maturation trajectory, with $CD34^{dim/-}CD45RA^{+}$ NCP3s and $CD34^{dim/-}CD45RA^{-}$ NCP4s positioned after $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{+}$ NCP2s.[38] Confirming the latter findings, immunocytochemistry (ICC) of discrete proteins specifically associated with primary and secondary granules of neutrophils demonstrated that all $CD34^{+/-}CD45RA^{+/-}$ NCPs express myeloperoxidase (MPO) but not lactoferrin, and that $CD34^{dim/-}CD45RA^{+}$ NCP3s and $CD34^{dim/-}CD45RA^{-}$ NCP4s, but not $CD34^{+}CD45RA^{-}$ NCP1s or $CD34^{+}CD45RA^{+}$ NCP2s, express neutrophil elastase and weakly defensin-alpha (Figure 3).

Because all observations gradually emerging from our experiments pointed to a strict, inter-related maturation cascade among $CD34^{+/-}CD45RA^{+/-}$ NCPs, we decided to further elucidate features of the differentiation process. To that end, we used in vitro culture assays to test the hypothesis that the NCP maturation cascade originates from $CD34^{+}CD45RA^{-}$ NCP1s to end into $CD34^{dim/-}CD45RA^{-}$ NCP4s, first via $CD34^{+}CD45RA^{+}$ NCP2s and then via $CD34^{dim/-}CD45RA^{+}$ NCP3s. The rationale for positing such a linear progression from $CD34^{+}CD45RA^{-}$ NCP1s to $CD34^{+}CD45RA^{+}$ NCP2s rested on the assumption that CD45RA acquisition by human cells represents a mandatory step for the progression of myeloid cell maturation, as illustrated by the origin of $CD34^{+}CD45RA^{+}$ GMPs from $CD34^{+}CD45RA^{-}$ CMPs.[9] Unexpectedly and in contrast to previous notions, our experiments demonstrated that $CD34^{+}CD45RA^{-}$ NCP1s mature directly into

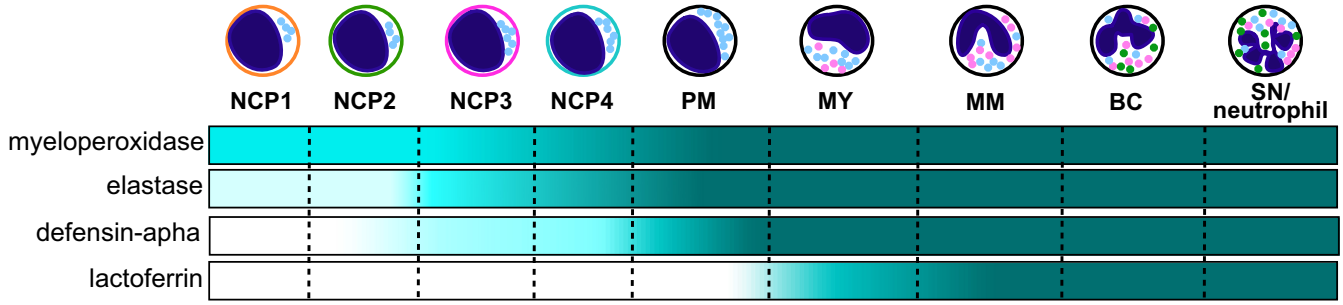

FIGURE 3 Kinetics of myeloperoxidase, elastase, defensin-alpha and lactoferrin appearance in either $CD34^{+/-}CD45RA^{+/-}$ NCPs or more mature neutrophil precursors. Myeloperoxidase, elastase, and defensin-alpha locate in primary granules, while lactoferrin locates in secondary granules. Light blue to green intensity directly correlates with the content of each given protein.

$CD34^{dim/-}CD45RA^{-}$ NCP4s, whereas $CD34^{+}CD45RA^{+}$ NCP2s mature separately into $CD34^{dim/-}CD45RA^{+}$ NCP3s, which ultimately generate $CD34^{dim/-}CD45RA^{-}$ NCP4s (Figure 2). Moreover, the separation between $CD45RA^{+}$ and $CD45RA^{-}$ NCPs occurs before their upregulation of CD64, as demonstrated by the observation that a pool of $CD34^{+}CD45RA^{-}CD64^{-}$ cells generate both $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{+}$ NCP2s, the latter via GMDPs (Figure 2). Subsequent cluster analysis of scRNAseq of sorted NCP1s, NCP2s, NCP3s, and NCP4s confirmed their restricted neutrophil-precursor status and provided interesting information.[38] In fact, we found that $CD34^{+/-}CD45RA^{+/-}$ NCPs consist of four clusters composed by either $CD45RA^{+}$ or $CD45RA^{-}$ cells distributed along two differentiation trajectories: one characterized by cells expressing elevated azurophilic granule mRNA levels, which we defined as "conventional trajectory," the other characterized by cells mainly expressing high mRNA levels for interferon-responsive genes (ISG, for instance ISG15, IFI6, IFIT3 and many others), which we defined as "ISG trajectory.".[38] Interestingly, the existence of neutrophil precursors accumulating ISG transcripts fits well with recently reported peripheral mature neutrophil subsets expressing ISGs under either healthy[40] or pathological[41–43] conditions.

In summary, by combining the detections of CD34, CD123, CD45RA, CD64, and CD115, our studies have proposed a relatively simple gating strategy to dissect the complexity of the BM $SSC^{lo}Lin^{-}CD45^{dim}$ cells. Such an immunophenotypical approach allowed us to define and identify novel human neutrophil precursors at their very early stages, namely $CD34^{+}$ NCP1s/NCP2s and $CD34^{dim/-}$ NCP3s/NCP4s preceding PMs. Moreover, the picture emerging from our work revises the step of human neutropoiesis that envisages the progression from CMPs to GMPs. In fact, our data point to the existence of maturation trajectories that separately include neutrophil progenitors as either $CD45RA^{+}$ or $CD45RA^{-}$ and that both generate mature cells. Furthermore, the identification of $CD34^{+/-}CD45RA^{+/-}$ NCPs together with previously described cMoPs, MDPs, CDPs, and GMDPs makes the characterization of myeloid progenitors included within GMPs as complete. Finally, our data also suggest that the peripheral mature neutrophils enriched for an enhanced type I IFN signature (named ISG^{+}-neutrophils)[40,44] might originate from already programmed precursors in BM, possibly from one of the scRNA clusters present in $CD34^{+/-}CD45RA^{+/-}$ NCPs.[38]

6 | ADDITIONAL NEUTROPHIL PROGENITORS AND THEIR INTERRELATIONSHIPS WITH NCPS

As already presented, our data point to $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{-}$ NCP2s as neutrophil precursors at the very early stages of neutropoiesis, preceding $CD34^{dim/-}CD45RA^{+}$ NCP3s and $CD34^{dim/-}CD45RA^{-}$ NCP4s, which would in turn precede PMs (Figure 2). However, the description of neutropoiesis is under continuous revision, mainly thanks to the adoption of novel, updated methodological approaches by researchers.[45–49] The first studies that coupled morphology with immunophenotype as analytical tools to better classify and align neutrophil progenitors along their maturation cascade were published in 1990–1993 by Terstappen et al[50] and Lund-Johansen et al,[51] whose findings were subsequently confirmed by other researchers.[52–58] The classification of BM neutrophil progenitors emerging from the experiments by Terstappen et al[50] and Lund-Johansen et al[50,51] is based on monitoring the gradual change of light scattering properties during cellular maturation combined with assessing the modulation of CD15, CD11b, CD16, and CD34 expression. The combination of flow cytometric phenotyping with the identification of morphological features, as assessed by both conventional optical light microscopy and cytochemistry, identified six discrete BM neutrophil precursors, namely $FSC^{hi}SSC^{int}CD34^{-}CD16^{-}CD11b^{-}CD15^{dim/+}$ MBs, $FSC^{int/hi}SSC^{int/hi}CD16^{-}CD11b^{-}CD15^{+/++}$ PMs, $FSC^{int}SSC^{hi}CD16^{-}CD11b^{+/++}CD15^{++}$ MYs, $FSC^{int/lo}SSC^{hi}CD16^{+}CD11b^{++}CD15^{++}$ MMs, $FSC^{lo}SSC^{int/hi}CD16^{+/++}CD11b^{++}CD15^{++}$ BCs, and $FSC^{lo}SSC^{int/hi}CD16^{++}CD11b^{++}CD15^{++}$ SNs. Notably, Lund-Johansen et al[51] specified that in their hands, MBs would derive from $FSC^{int/hi}SSC^{lo/int}CD34^{+}CD16^{-}CD11b^{-}$ cell populations, including $CD15^{-/dim}$ myeloid progenitors (Figure 4 and Table 1). However, all these early neutrophil progenitors were neither tested in either in vitro or in vivo assays to evaluate their effective differentiation potential, nor investigated for gene expression analysis, presumably

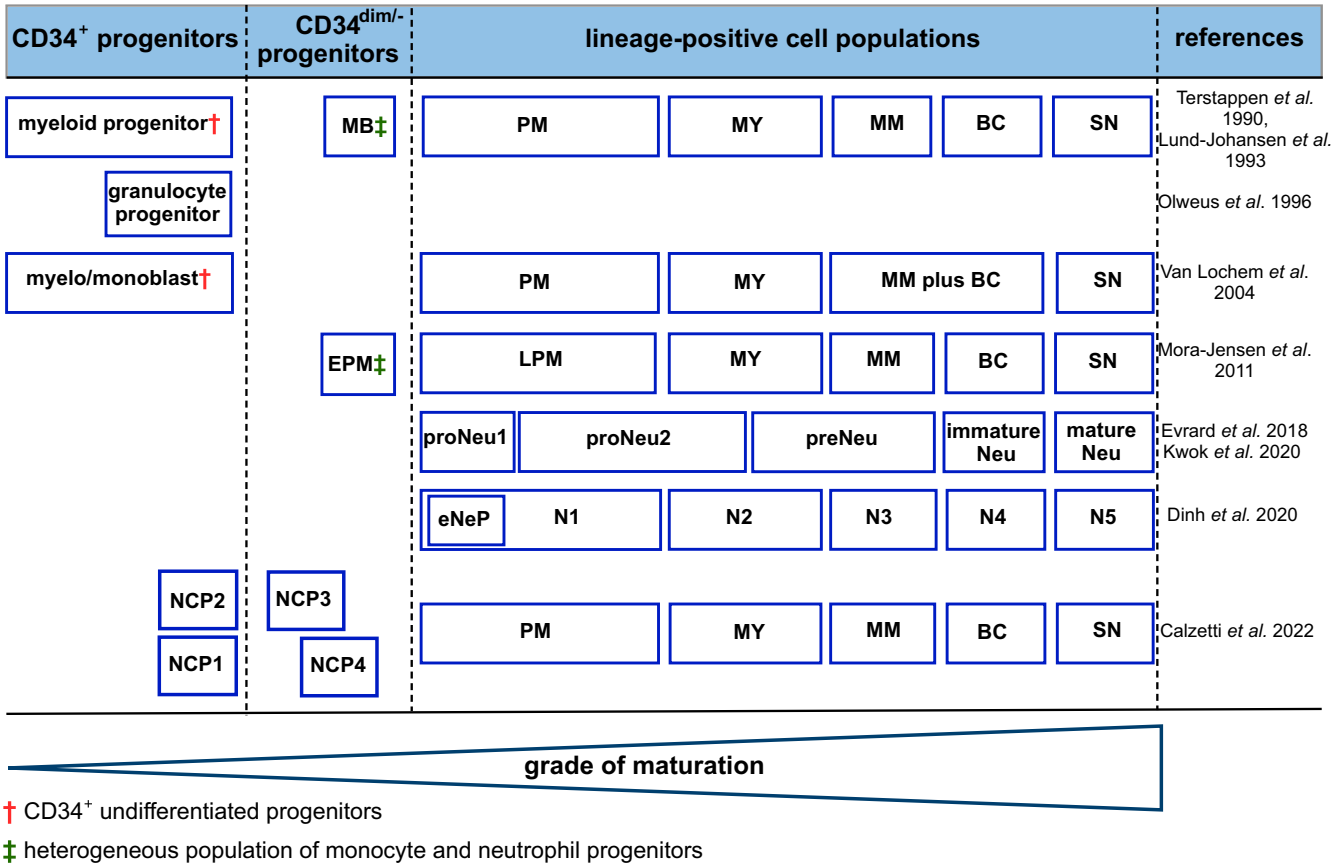

FIGURE 4 Comparison of the human neutrophil progenitors according to their grade of maturation. The figure includes the various cell populations discussed in the text. For the specific immunophenotypes of neutrophil progenitors, please refer to Table 1. BC, band cells; eNeP, early neutrophil progenitors; EPM, early promyelocytes; LPM, late promyelocytes; MB, myeloblasts; MM, metamyelocytes; MY, myelocytes; NCP, neutrophil-committed progenitors; PM, promyelocytes; preNeu, neutrophil precursors; proNeu, pro-neutrophils; SN, segmented neutrophils.

because the available technologies were not as advanced as are approaches currently. As highlighted by Van Lochem et al in 2004,[39] other authors reported the existence of $CD34^+CD15^{low}$ precursor cells that were committed instead to both monocytic and granulocytic development and consequently named as myelo/monoblasts[51] (Figure 4 and Table 1). Thus, at least for MBs, a specific commitment to the neutrophil lineage has remained speculative. In any case, Terstappen et al,[50] Lund-Johansen et al,[51] and Elghetany et al's[59] tested additional surface proteins, including CD10, CD24, CD33, CD35, CD43, CD67 (now CD66b), CD87, and various adhesion molecules, to further evaluate whether one or more of them could better mark neutrophil precursors. By doing so, it was discovered that the downregulation of CD49d/VLA-4 expression marks the transition from MYs to MMs (ie, from proliferative to non-proliferative stages of neutrophil precursors).[51] It was also found, using flow cytometry, that CD10 (expressed by segmented neutrophils),[54] CD24 (expressed from the MY to SN stages),[52] and CD87 (expressed only by BCs and SNs)[53] could function as novel markers that define discrete neutrophil progenitors.[59]

Taking advantage of the possibility of measuring up to eight fluorescences simultaneously, Mora-Jensen and colleagues subsequently proposed an antibody panel that would detect CD15, CD11b, CD16, CD34, CD33, and CD49d in a single sample.[45] By doing so, they identified and sorted six different putative BM neutrophil progenitors, namely $SSC^{lo}CD34^-CD15^{int}CD33^+CD49d^+CD11b^-CD16^-$ early promyelocytes (EPMs), $SSC^{hi}CD34^-CD15^{hi}CD33^+CD49d^+CD11b^-CD16^-$ late promyelocytes (LPMs), $SSC^{hi}CD34^-CD15^{hi}CD33^{hi}CD49d^{hi}CD11b^{hi}CD16^-$MYs, $SSC^{hi}CD34^-CD15^{hi}CD33^{int}CD49d^-CD11b^{hi}CD16^{int}$MMs, $SSC^{hi}CD34^-CD15^{hi}CD33^-CD49d^-CD11b^{hi}CD16^{int}$ BCs, and $SSC^{hi}CD34^-CD15^{hi}CD33^-CD49d^-CD11b^{hi}CD16^{hi}$ SNs (Figure 4 and Table 1), each associated with a distinct morphology[45] and subsequently profiled for mRNA expression by microarrays.[60] Therefore, Mora-Jensen and colleagues' experiments not only reproduced the progenitors proposed by Terstappen et al[50] and Lund-Johansen et al,[50,51] but also added EPMs to them. As a result, EPMs were described as the most immature neutrophil precursors ever identified at that time.[45] It is noteworthy that $CD34^{dim/-}CD45RA^+$ NCP3s and $CD34^{dim/-}CD45RA^-$ NCP4s heterogeneously express CD15,[38] although at lower levels than PMs, thus recalling, in part, the phenotypical characteristics of EPMs. However, as shown in Figure 5, for which the experiment dissects the gating strategies

used to define EPMs and analyze their CD64/CD115 expression to identify CD34$^{+/-}$CD45RA$^{+/-}$ NCPs, it is evident that EPMs include pre-monocytes and thereby indicates that CD15 expression alone is not sufficient to discriminate neutrophil from monocyte precursors. Figure 5 also shows that EPMs correspond to the fraction of CD34$^{dim/-}$CD45RA^{-} NCP3s and CD34$^{dim/-}$CD45RA^{-} NCP4s with higher CD15 and no CD34 expression. Moreover, even though EPMs resulted positive for MPO expression by ICC,[45] this does not ensure that that they are homogenous, since monocyte progenitors express MPO.[38,61] Furthermore, our unpublished analysis of the microarray datasets published by Rapin et al[60] revealed that EPMs express high levels of monocyte-specific transcripts, including *IRF8*, *CSF1R* e *VCAN*, a finding that once again demonstrates the mixed composition of this cell population. In addition, the fact that EPMs were not investigated for specific neutrophil differentiation potential by any in vitro or in vivo assays undermines any assertions that EPMs represent unipotent neutrophil progenitors. Taken together, Figure 5 and all these findings mentioned above doubt about CD15 as a suitable marker to exclusively identify early neutrophil progenitors, as previously hypothesized[39] and confirm that the combination of CD64 and CD115 may function instead much better.

To unequivocally mark neutrophil progenitors from PMs to SNs, CD66b represents an alternative to CD15.[51,62] CD66b (originally CD67),[63] one of the carcinoembryonic antigen family (CEA, comprising CD66a to CD66e), is expressed by neutrophils beginning in the PM stage[51] and in eosinophils,[64] but not by monocytes or by non-hematopoietic cells.[65] Preceding CD11b and CD16 expression, CD66b is absent from CD34^{+} cells but accumulates in secondary granules during neutropoiesis and persists at variable surface levels throughout neutrophil maturation.[39,51,66] Based on these observations, more recent papers that focused on neutropoiesis,[46–49,67] including our study on CD34$^{+/-}$CD45RA$^{+/-}$ NCPs,[38] have utilized CD66b as a specific neutrophil lineage marker detectable from PMs to SNs. In this context, Evrard et al[46] and Kwok et al[48] have classified the stages in the maturation cascade of human neutrophil precursors, from cord blood (CB) or FBM, according to the combinatory expression of CD66b, CD15, CD101, and CD49d. According to their classification,[46,48] the novel neutrophil progenitors include (a) SSC^{lo}CD66b^{+}CD15^{+}CD11b^{-}CD49d^{hi} proNeu1s and SSC^{hi}CD66b^{+}CD15^{+}CD11b^{-}CD49d^{int} proNeus2s, as collocated at the root of the proposed model of neutropoiesis; (b) CD66b^{+}CD15^{+}CD11b^{+}CD49d^{+}CD101^{-} preNeus; (c) then CD66b^{+}CD15^{+}CD11b^{+}CD49d^{-}CD101^{+}CD16^{+}CD10^{-}

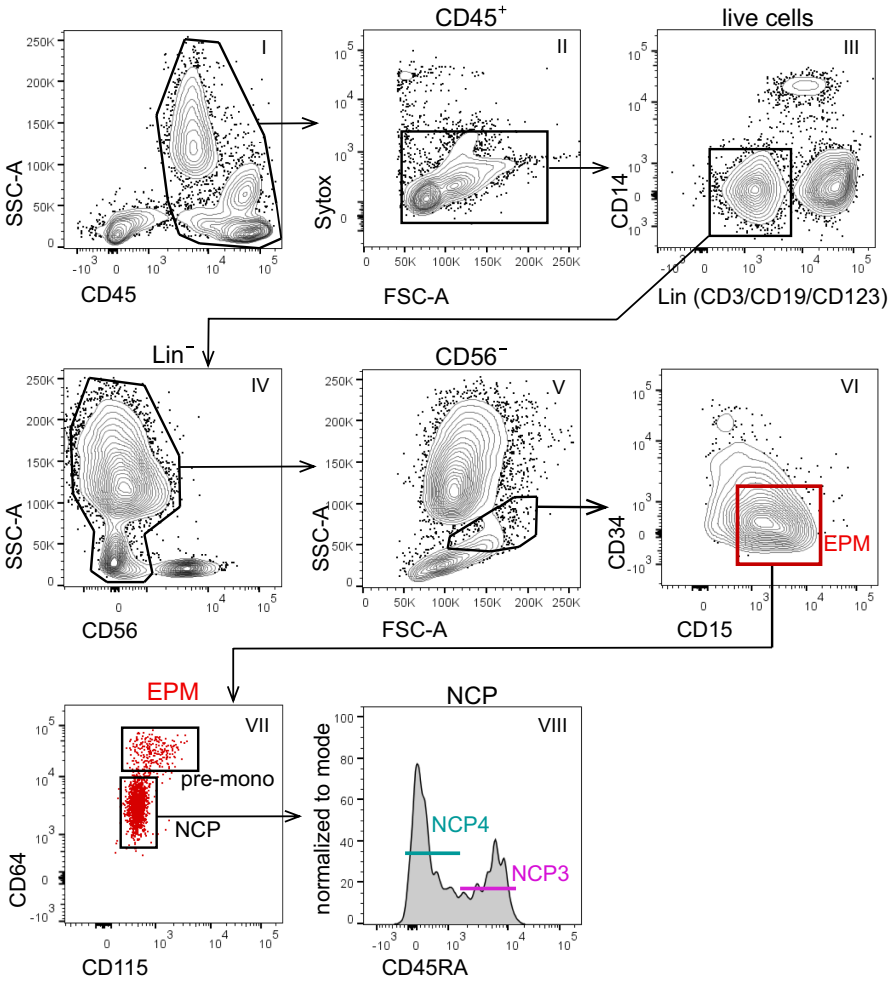

FIGURE 5 Evidence that EPMs include both neutrophil-committed progenitors and pre-monocyte populations. The figure illustrates the gating strategy used to identify EPMs. Precisely, BM cells were sequentially gated as CD45^{+}Lin^{-}CD56^{-} live cells (panel I-IV). Then, EPMs were gated as SSC^{lo}CD34^{-}CD15^{int} cells (red gate in panel VI) and hence displayed according to their expression of CD115 and CD64 (panel VII). This in turn highlights/demonstrates the presence of the CD34^{-} fraction of NCPs (gated as CD64^{+}CD115^{-} cells) and CD64^{++}CD115^{+} pre-monocyte populations within EPMs. The histogram of panel VIII shows CD45RA expression by NCPs detected within EPMs, in turn identifying both CD34^{-}CD45RA^{+} NCP3s (magenta gate) and CD34^{-}CD45RA^{-} NCP4s (turquoise gate). EPM, early promyelocytes; NCP, neutrophil-committed progenitors; pre-mono, pre-monocytes.

immature neutrophils (named immature Neus); and, lastly (d) $CD66b^+CD15^+CD11b^+CD49d^-CD101^+CD16^+CD10^+$ mature neutrophils (named mature Neus)[46,48] (Figure 4 and Table 1). Since proNeus1s and proNeus2s are $SSC^{lo}CD49d^{hi}$ and $SSC^{hi}CD49d^{dim}$, respectively, proNeus1s would precede proNeus2s as more immature. It is important to note that the description of human proNeu1s/proNeus2s/preNeus/immature Neus/mature Neus was extrapolated in large part from very extensive experiments focused on the investigation of murine neutropoiesis. In fact, while the five murine proNeu1s/proNeus2s/preNeus/immature Neus/mature Neus were clearly demonstrated to emerge from murine GMPs, as well as individually characterized in terms of phenotype, proliferative capacity, molecular signature and functions, a similar detailed investigation was not comprehensively performed for their human counterparts. Nonetheless, many recent reviews on neutrophil biology[68–70] often quote the proNeu1/proNeus2/preNeu/immature Neu/mature Neu sequence as an updated, general, model of neutropoiesis. Frequently when writing reviews, one indiscriminately discusses data from both human and mouse systems, consequently mixing them in illustrations as if precisely equivalent and perfectly interchangeable. In this *Immunological Reviews* issue, Bill Nauseef highlights how important it is to avoid extrapolating to human neutrophils, or to neutrophils in general, properties or features based on experiments that solely used murine cells.

A subsequent methodological article, presumably by the same group, in which the gating strategy used to identify proNeu1s/proNeus2s/preNeus/immature Neus/mature Neus was compared with the *classical* gating strategy used to detect PMs down to SNs,[51,55,57] found that: (a) human proNeu1s/proNeu2s localize within PMs/MYs; (b) preNeus consist of MMs/BCs; (c) immature Neus mainly consist of BCs but also include SNs; and (d) mature Neus mainly correspond to SNs (see figure 137 of reference[71]). Our phenotypic comparison between $CD34^{+/-}CD45RA^{+/-}$ NCPs and human proNeu1s/proNeu2s confirmed that all these progenitors share CD49d, whereas only proNeu1s/proNeu2s are, as expected, positive for CD66b.[46,48] Thus, even though human proNeu1s remain poorly characterized by functional studies or transcriptome analysis, their phenotype indicates that they reside within PMs, although displaying low SSC properties (Figure 4). Employing high dimensional mapping technologies (ie, CyTOF/mass cytometry), Evrard et al[46] discovered that the transition from non-dividing to dividing neutrophil progenitors occurs concomitantly with the CD49d downregulation that takes place at the level of immature Neus. Although using a much more advanced technology, these findings substantially confirm the data previously obtained by Lund-Johansen and colleagues[51] in 1993, who concluded that downregulation of CD49d expression marks the transition from MYs to MMs.

An additional revision of human neutropoiesis, different from that of Evrard et al[46] and Kwok et al,[48] comes from Hedrick's group.[47,49] In fact, by using CD66b and CD117 as main markers, Zhu et al[47] initially identified human $Lin^-CD66b^+CD117^+$ NePs (hNePs) within healthy BM cells. Similarly to the approach used by Kwok et al,[48] they discovered hNePs by extrapolation to human samples the phenotypical identification and extensive characterization of novel murine unipotent neutrophil progenitors, named NePs.[47] However, in a subsequent work by the same group,[49] hNePs were more extensively analyzed and, in turn, found to represent a highly heterogeneous population of neutrophil precursors. These findings led the authors to organize BM $CD66b^+$ neutrophils into five different populations differentially expressing CD16, CD10, and CD71, including $Lin^-CD66b^+CD16^-CD10^-CD71^+$ N1, $Lin^-CD66b^+CD16^-CD10^-CD71^-$ N2, $Lin^-CD66b^+CD16^+CD10^-$ N3, $Lin^-CD66b^+CD16^+CD10^{int}$ N4, and $Lin^-CD66b^+CD16^+CD10^{hi}$ N5.[49] Notably, a novel $Lin^-CD66b^+CD16^-CD10^-CD71^+CD117^+$ cell population within N1, named eNePs, was additionally described in the same paper by Dinh et al,[49] who also stated that the N1 to N5 substantially correspond to PMs/MYs/MMs/BCs/SNs (Figure 4 and Table 1). Subsequently, based on data obtained from proliferative capacity experiments, differentiation potential by CFU assay, adoptive transfer into animal models, and bulk RNAseq transcriptomic properties, Dinh et al[49] concluded that eNePs represent the most immature fraction of N1/PMs, thereby implying that PMs are also phenotypically heterogeneous. By performing a comparison at both phenotype and transcriptome levels, we confirmed that eNePs effectively overlap PMs, and that eNePs represent a more mature stage than do $CD34^{+/-}CD45RA^{+/-}$ NCPs along the neutrophil development pathway.[38] In fact, consistent with the approach by Dinh et al[49] to focus on BM $CD66b^+$ cells,[49] eNePs are $CD66b^+$, while $CD34^{+/-}CD45RA^{+/-}$ NCPs are $CD66b^-$.[38] Of note, eNePs were reported to include on average 8% $CD34^+$ cells,[49] whereas human proNeu1s were reported to be $CD34^+$ according to the Infinity Flow approach,[48] although a direct demonstration by multicolor flow cytometry for such CD34 positivity has never been demonstrated for human proNeu1s.[48,71] In any case, since $CD34^+$ cells notoriously neither express CD66b nor contain specific granules,[30,72,73] the fact that proNeu1s or a subpopulation of cells within eNePs would concomitantly express both CD34 and CD66b is not possible based on current understanding of the expression of those markers by cells in the neutrophil lineage. It is worth mentioning that the same Dinh et al[49] demonstrated that $CD117^+CD38^+CD34^+$ myeloid progenitors (including CMPs and GMPs) do not actually express CD66b. Nonetheless, future in-depth studies on concomitant membrane marker expression and scRNAseq analysis should be done to unequivocally clarify debatable issues.

To summarize, a careful analysis and comparison of the various, recently reported, novel populations of early neutrophil precursors[38,45,46,48,49,71] lead us to conclude that, even if human $CD66b^+$ proNeu1s/ proNeu2s and eNePs certainly stand at later stages of maturation than $CD34^{+/-}CD45RA^{+/-}$ NCPs (Figure 4), human proNeu1/2s require a much more detailed characterization to better define their status. Concerning EPMs, we have already remarked that the gating strategy to identify them does not clearly select pure neutrophil-committed progenitors.

7 | NEUTROPHILS MATURE INDEPENDENTLY FROM OTHER GRANULOCYTES

As already mentioned, although long considered as arising from common progenitors (ie, MBs and/or even GMPs), neutrophils, eosinophils, and basophils instead have been demonstrated by both traditional flow cytometry-based assays and more innovative single-cell RNA sequencing to originate via separate pathways. Accordingly, human unilineage $CD34^+CD38^+CD123^+CD45RA^-IL5R\alpha^+$ eosinophil-committed progenitors (hEoPs) were described in 2009,[17] while $CD34^+CD38^+CD45RA^-CD133^{low}$ cells (EoBPs) giving rise to both basophils and eosinophils, and $CD34^+CD38^+CD45RA^+CD133^+$ cells giving rise to neutrophils and macrophages, were reported in 2013.[18] Moreover, Drissen et al[74] in 2019 reported that the $CD114^-CD131^+$ fraction of CMPs selectively generates basophils, eosinophils, and mast cells, and that $CD114^+CD131^-$ cells generate neutrophils and monocytes. In 2020, Grootens et al[75] defined a $CD34^+FC\varepsilon R1^+CD203c^+$ population able to generate mast cells and basophil-like cells, but not neutrophils or monocytes. In addition to the previous studies, by performing scRNAseq experiments integrated by the retrospective identification of each cell immunophenotype, Velten et al[19] suggested that BM granulocytes derive from distinct haematopoietic compartments. In fact, these researchers reported that—in contrast to neutrophil-primed progenitors—Eo/Baso/Mast cell progenitors display a $Lin^-CD34^+CD38^+CD10^-CD45RA^-CD135^{mid}$ immunophenotype, a property that implicitly excludes them from the GMPs.[19] Pellin et al[20] in 2018, by performing scRNAseq experiments and in vitro differentiation assays of BM samples, identified basophil progenitors within the $Lin^-CD34^+CD38^-CD135^-$ cell compartment, thereby demonstrating that basophil and neutrophil developments are clearly mutually independent. More recently, Hassani et al[64] reported a detailed immunophenotypic and morphologic characterization of eosinophil progenitors from PMs to mature cells. Accordingly, eosinophils and their precursors, identified within the BM $SSC^{hi}CD16^-CD64^{low}CD193^+$ cells, were sequentially categorized as $CD11b^-CD62L^-$ eosinophil PMs; $CD11b^{+/++}CD62L^-$ eosinophil MYs; $CD11b^{++}CD62L^+$ eosinophil MMs; and $CD11b^{++}CD62L^{++}$ mature eosinophils.[64] Notably, none of the eosinophil progenitors was found to display banded nuclei but exhibit several differences from neutrophil progenitors at the phenotypic level.[64] For instance, the expression of CD49d was found to reach peak levels at the eosinophil MY stage and persist throughout eosinophil maturation, in marked contrast to the behavior of neutrophil precursors, which lose CD49d after MY stage.[64] Moreover, CD64 expression was present on only eosinophil PMs, but at lower levels than that on neutrophil PMs.[64]

In summary, current evidence is clearly in favor of distinct origins and differentiation routes for each granulocyte type. This notion is in contrast with the *classical* model of granulopoiesis, since it excludes the existence of multipotent progenitors (such as MBs) specifically committed to each of the three granulocyte types. However, such progenitors might exist among clusters of very immature multipotent cells close to HSCs and thus at much earlier stages than that of PMs. Some authors, in fact, propose that, alongside their gradual lineage commitment demonstrated by the *continuum* model, some HSCs also generate intermediate, oligo/multipotent, cells that still retain some plasticity.[76]

8 | REVISING WITH THE NOTION THAT CD45RA ACQUISITION REPRESENTS A MANDATORY STEP FOR MYELOID DEVELOPMENT

CD45, also known as the leucocyte common antigen, is a transmembrane protein expressed only by cells of the hemopoietic system with the exception of platelets and mature erythrocytes.[77,78] Alternative splicing of exons 4, 5, and 6 produces different CD45 mRNA isoforms,[78,79] namely CD45RA, CD45RB, CD45RAB, CD45RBC, CD45RABC, and CD45RO.[80] CD45 triggers intracellular signaling pathways,[81–83] but the discrete functions of each CD45 isoforms have not been clearly defined. Specific antibodies have demonstrated that the expression of CD45RO and CD45RA isoforms is mutually exclusive and that $CD34^+CD45RB^+CD45RO^+$ progenitors expressing low or undetectable levels of CD45RA represent the most immature fraction of $CD34^+$ cells.[77,84] It has been then established that $CD34^+$ cells remain $CD45RO^+CD45RB^+$ upon erythroid differentiation, whereas they upregulate CD45RA once committed to the myeloid/lymphoid lineages.[84] Consequently, the upregulation of CD45RA expression by CMPs to become GMPs has always been considered a fundamental step for the development of the myeloid lineage. Such dogma has been recently challenged not only by the discovery that $CD45RA^-$ progenitors give origin to eosinophils/basophils (see the previous section) but also by the establishment of the maturation trajectories proposed by the *continuum* model of hematopoiesis.[16] Furthermore, our identification of $CD34^{+/-}CD45RA^{+/-}$ NCPs confirms that the dogma associating the upregulation of CD45RA expression with the generation of myeloid progenitors and neutrophil commitment requires revision, in that $CD34^+CD45RA^-$ NCP1s and $CD34^+CD45RA^+$ NCP2s were shown to be substantially identical by scRNAseq analysis, as well as to independently mature into neutrophils. These latter observations spawn questions about the origin of the maturation trajectories that include $CD34^+CD45RA^-$ NCP1s and $CD34^+CD45RA^+$ NCP2s. From our in vitro differentiation experiments, it was evident that $CD34^+CD45RA^+$ NCP2s directly arise from $CD34^+CD45RA^+CD64^-$ GMDPs, which we have proposed to rename as NMDPs (ie, neutrophil-monocyte-DC progenitors)[38] (Figure 2). By contrast, $CD34^+CD45RA^-$ NCP1s arise from $SSC^{lo}CD45^{dim}CD10^-CD123^{dim/-}CD34^+CD64^-CD45RA^-$ cells, which were renamed by us as $CD64^-CD45RA^-$ subset.[38] Since the latter cells restore $CD34^+CD45RA^-$ NCP1s, GMDPs/NMDPs, and $CD34^+CD45RA^+$ NCP2s in in vitro differentiation assays, we concluded that $CD34^+CD45RA^-$ NCP1s and $CD34^+CD45RA^+$ NCP2s originate from separate maturation routes. Consequently, it remains to be established when $CD34^+$ progenitors bifurcate to

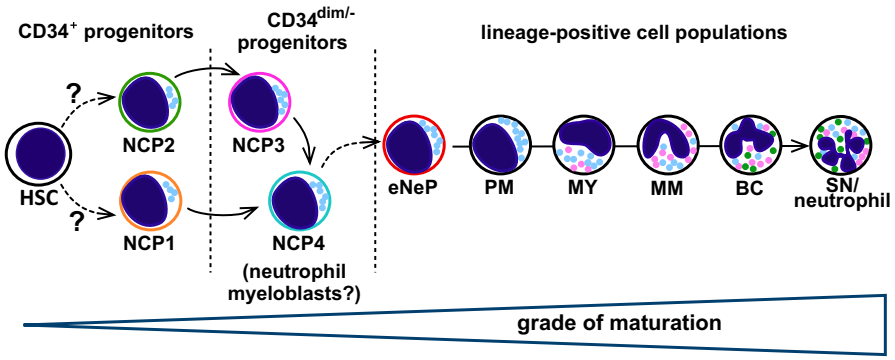

FIGURE 6 Our proposed model of human neutropoiesis. The scheme depicts our proposed model of neutropoiesis which includes $CD34^{+/-}CD45RA^{+/-}$ NCPs, and, prior PMs, eNePs (see the text).

give origin to the $CD45RA^{+}$ and $CD45RA^{-}$ maturation trajectories to which $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{+}$ NCP2s belong (see Extended Data figure 6 of reference[38]). In this context, various studies have reported that granulocytes arise from $CD34^{+}CD38^{-}CD45RA^{+}CD10^{-}$ lymphoid-primed multipotent progenitors (LMPPs),[18,36,76,85] which consist of very immature progenitors, directly differentiated from MPPs and already expressing CD45RA. Since GMDPs/NMDPs have been recently shown to derive from LMPPs according to the *classical* model of hematopoiesis,[16,36] (Figure 2), we speculate that the immunophenotype of neutrophil precursors more immature than $CD34^{+}CD45RA^{+}$ NCP2s could be detected within LMPPs.

9 | CONCLUDING REMARKS

At the end of our *excursus* on the knowledge of recently described early neutrophil progenitors in humans, we propose our own model of neutropoiesis. As shown in Figure 6, we include PMs, MYs, MMs, BCs, and SNs, as we do not deem it necessary to replace them. We believe it to be more advantageous to keep PMs, MYs, MMs, BCs, and SNs along the final stages of human neutropoiesis, since these neutrophil progenitors are morphologically well-defined, immediately recognizable, and still currently used by hematologists and other clinicians for the classification of myeloid diseases. Our neutropoiesis model also includes eNePs and positions them immediately before PMs (Figure 6), as justified by the evidence presented above and by their identification as the most immature fraction of PMs.[49]

For the immunophenotypic, transcriptomic, morphological and functional features, our proposed model of neutropoiesis includes the $CD34^{+/-}CD45RA^{+/-}$ NCPs (Figure 6), which to date represent the most immature, reasonably pure, sortable neutrophil precursors available for research and, hopefully, clinical applications. Accordingly, $CD34^{+}CD45RA^{-}$ NCP1s and $CD34^{+}CD45RA^{+}$ NCP2s represent the earliest myeloid progenitors and are logically placed downstream of HSCs, whereas, in accordance with the phenotype proposed by Terstappen et al[50] and Lund-Johansen et al,[51] $CD34^{dim/-}CD45RA^{+}$ NCP3s and $CD34^{dim/-}CD45RA^{-}$ NCP4s might putatively represent the neutrophilic myeloblasts (Figure 6). Of note, our neutropoiesis model does not include EPMs, even though by morphology and immunophenotype, they would precede PMs. However, as explained above, EPMs include substantial numbers of monocyte progenitors and thus do not represent a pure population of cells in the neutrophil lineage. For these reasons, EPMs are replaced by $CD34^{dim/-}CD45RA^{+}$ NCP3s and $CD34^{dim/-}CD45RA^{-}$ NCP4s in our neutropoiesis model (Figure 6).

Although we offer our model of neutropoiesis (Figure 6), we realize that it is mandatory to reach a general consensus to classify and accommodate all the various recently described early neutrophil progenitors (discussed in this review), in order to very accurately represent human neutropoiesis. Among the many benefits of reaching a consensus, a shared view of the origins and development of human neutrophils would promote more informed exchanges and fruitful scientific dialogues among immunologists, hematologists, and others interested in these important biological processes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

We agree on the data availability statement. All our data are available.

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