Signaling and functional competency of neutrophils derived from bone-marrow cells expressing the ER-HOXB8 oncoprotein

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Abstract
Neutrophils play a central role in immunity and inflammation via their intrinsic ability to migrate into inflamed tissue, to phagocytose pathogens, and to kill bacterial and fungi by releasing large quantities of superoxide anions and lytic enzymes. The molecular pathways controlling neutrophil microbicidal functions are still unclear, because neutrophils have a short half-life and are resistant to genetic manipulation. Neutrophil-like cells (NLC) can be generated from myeloid progenitors conditionally immortalized with the ER-HoxB8 oncoprotein, but whether these cells can replace neutrophils in high-throughput functional assays is unclear. Here, we assess the ability of NLC derived from ER-HoxB8 progenitors to produce ROS and to perform chemotaxis and phagocytosis. We compare the Ca\(^{2+}\) responses and effector functions of NLC to primary murine neutrophils and document the molecular basis of their functional differences by mRNA profiling. Pro-inflammatory cytokines enhanced the expression by NLC of neutrophil surface markers and transcription factors. Ca\(^{2+}\) elevations evoked in NLC by agonists, adhesion receptors, and store depletion resembled the physiological responses recorded in primary neutrophils, but NLC expressed reduced amounts of Ca\(^{2+}\) signaling proteins and of chemotactic receptors. Unlike their myeloid progenitors, NLC produced H\(_2\)O\(_2\) when adhered to fibronectin, migrated toward chemotactic peptides, phagocytosed opsonized particles, and generated intracellular ROS. NLC phagocytosed as efficiently as primary neutrophils but produced 50 times less ROS and migrated less efficiently toward chemoattractant. Our data indicate that NLC can replace neutrophils to study Ca\(^{2+}\) signaling and phagocytosis, but that their incomplete granulocytic differentiation limits their use for chemotaxis and ROS production assays.

KEYWORDS
granulocytes, in vitro differentiation, myeloid cell lines, polymorphonuclear cells

1 | INTRODUCTION

Neutrophils are an essential component of the innate immune system, forming the first line of host defence against bacterial and fungal infections. These multifaceted cells release cytokines for autocrine and paracrine signalling, phagocytose pathogenic particles and kill them by releasing lytic enzymes from granules and by producing high levels of reactive oxygen species (ROS) and nitrogen species.\(^1,2\) Neutrophils derive from promyeloid progenitors and leave the bone marrow as fully differentiated cells to enter the blood circulation.\(^3,4\) Unlike other myeloid and lymphoid immune cells, neutrophils do not further differentiate outside the bone marrow but their bactericidal potency can be...
enhanced by cytokines, chemokines, or microbial products. During infection, the circulating cells are recruited from the blood stream to inflamed tissue following chemotactic gradients, a multistep process that is finely regulated.

Elevations in cytosolic $\text{Ca}^{2+}$ concentration regulate neutrophil adhesion, extravasation, migration, and phagocytosis, as well as several of their antimicrobial functions. Signal transduction from integrin, chemotactic, and phagocytic surface receptors involve $\text{Ca}^{2+}$ elevations mediated by phospholipase C that are sustained by receptor-operated or by store-operated $\text{Ca}^{2+}$ entry (SOCE) channels. TRPC, TRPM, and TRPV $\text{Ca}^{2+}$ channels have been implicated in the control of neutrophil functions and the role of the 3 ORAI1-3 SOCE channel isoforms and of their 2 regulatory proteins STIM1 and STIM2 has been clarified recently.

Novel functions of neutrophils in immunity and inflammation are continuously unravelled, but several fundamental questions regarding the biology of these versatile cells remain to be clarified. Progress is slow because neutrophils are terminally differentiated cells that cannot be expanded in vitro, have a short life-cycle preventing long-term assays, and are largely resistant to transfection or transduction methods of genetic manipulation. Consequently, neutrophil functions are usually explored in animal models or in cell lines using a combination of genetic and pharmacological approaches. The variability of approaches used to study these complex cells greatly complicates the interpretation and comparison of results emanating from different studies.

Current research practices favor the use of cell lines to comply with the 3R guidelines aiming to reduce, replace, and refine animal experimentation. The available cell lines HL-60 or PLB-985, derived from myeloma, accumulate further mutations in long-term cultures and require chemicals to induce their differentiation into neutrophil-like cells. In this study we explore the potential of murine myeloid progenitors conditionally immortalized with the homeobox oncoprotein HoxB8 driven by the estrogen receptor (ER-HoxB8), originally designed by Wang et al. These cells hold several advantages: (i) progenitor cells can be isolated from transgenic mouse strains and easily immortalized by the introduction of ER-HoxB8; (ii) the cells are from a non-cancerous background and recapitulate the physiological neutrophil differentiation program; (iii) the progenitors can be efficiently transduced using lentiviral vectors to provide a genetically tractable neutrophil model.

The HoxB8 system was recently used to establish the role of the non-muscle myosin Myh9 in neutrophil trafficking in vitro and to monitor neutrophil trafficking during inflammation in vivo. These studies validate the use of this cellular model to study neutrophil trafficking in vitro and in vivo and highlight its advantages for rescue experiments or adoptive transfer of cells bearing mutations resulting in lethal phenotypes in mice. However, whether sufficient numbers of neutrophil-like cells can be generated for high-throughput approaches and whether ER-HoxB8-derived neutrophils faithfully recapitulate the neutrophil $\text{Ca}^{2+}$ signaling toolkit is unknown. In this study, we compare neutrophil-like cells (NLC) immortalized from ER-HoxB8 progenitors to primary murine neutrophils. We evaluate the efficiency of chemotaxis, ROS production, and phagocytosis in these neutrophilic cells and their potential in high-throughput screens to evaluate regulators of store-operated $\text{Ca}^{2+}$ entry channels in neutrophils.

2 | MATERIALS AND METHODS

2.1 | Mice, cells, and cell lines

Primary murine neutrophils were isolated from bone marrow collected from 12- to 20-week-old male mice. Animals were control Stim1 or Stim2 floxed animals from a current mouse colony, established from B6.Cg- Tg(LysM-Cre)T29-1Stl/J × Stim1tm1Rao × Stim2tm1Rao animals (kindly provided by Prof. Marc Fivaz, DukeNUS, Singapore). Experiments were approved by the Geneva canton’s Direction Générale de la Santé (authorizations GE/87/15 and GE/142/16) and performed following the guidelines of the animal research committee of the University of Geneva. Mice were sacrificed, and femurs and tibias were collected. Bone-marrow was flushed from the bones, homogenized, and filtered to remove non-cellular debris. Neutrophils were isolated by negative isolation using the autoMACS® Pro Separator (Miltenyi Biotec, Germany). The ER-HoxB8 cell line was kindly provided by Prof. Thomas Kaufmann (University of Bern, Switzerland).

2.2 | Materials

Murine cytokines G-SCF (#250-05), INFγ (#315-05), TNF-α (#315-01A); Recombinant Murine MIP2 (CXCL2, #250-15) were purchased from Peprotech, USA. PMA (#79346), platelet activating factor (PAF, #P7568); fMLF (#F3506); Thapsigargin (Tg, #T9033); BSA (#B4287); Dulbecco’s PBS (10×) (DPBS, #D1408); (Z)-4-hydroxytamoxifen (4-OHT, #H7904), L-glutamine solution (#G7513), sodium pyruvate solution (#S8636), HEPES (#H3375), potassium chloride (KCl, #P5405), calcium chloride dihydrate (CaCl₂, #21098, Fluka™), D-glucose (#G8270), 2-APB (#D9754) were purchased from Sigma-Aldrich, USA. Cell culture water pyrogen free (#L0970-1000) for buffers was purchased from D. Dutscher, France. RPMI 1640-GlutaMAX (1x) (Gibco™, #61870) and penicillin-streptomycin (10,000 U/ml) (Gibco™, #15140122), HAT Supplement (50×) (HAT, #21060017), FCS (Gibco™), DRAQ™ Fluorescent Probe Solution (#62251) were purchased from ThermoFisher, USA. EGTA was purchased from ROTH (#3054.2; Germany), sodium chloride was purchased from Applichem (NaCl₂, #A1149; Germany), magnesium chloride hexahydrate was purchased from Merk-Millipore (MgCl₂, #105833; Germany), and GSK-7975A was purchased from Aobius (#AOB4124-1; USA).

2.3 | Media and buffers

ER-HoxB8-medium: RPMI 1640-GlutaMAX (1x) +10% FCS +1% PenStrep +5% SCF (from CHO cells); DMEM-medium: DMEM 22320 (1x) +1% PenStrep +10% FCS +1% HAT. All buffers and stock solutions were prepared using pyrogen-free cell culture water. All buffers used for experiments described below were prepared on the base of...
a standard ringer’s solution: cell culture water, NaCl (140 mM), KCl (5 mM), MgCl₂ (1 mM), HEPES (20 mM), glucose (10 mM), pH 7.4. This buffer was supplemented with CaCl₂ or EGTA to achieve specific Ca²⁺ concentrations or Ca²⁺-free conditions.

2.4 | Myeloid cell line culture and differentiation

ER-HoxB8 progenitor cells (ER-HoxB8) were originally isolated from healthy mice (C57/B6) and the line was kindly provided by Prof. Thomas Kaufmann (University of Bern, Switzerland) cultured in ER-HoxB8-medium + 0.1 μM 4-OHT in 75 cm² cell culture flasks. CHO cells (secreting murine SCF) were cultured in ER-HoxB8-medium until confluency. Supernatant was collected, filtered and stored at -20°C and added to every ER-HoxB8 culture and differentiation. Endothelial cells (Human umbilical vein endothelial cells, Eahy.926, kindly provided by Cora-Jean Edgell, University of North Carolina, NC, USA) were cultured in DMEM. To differentiate ER-HoxB8 progenitors to neutrophil-like cells, ca. 0.5 × 10⁶ cells/ml were washed twice (centrifugation, 800 rpm, 5 min) and resuspended in ER-HoxB8-medium (no 4-OHT), containing 10 ng/ml G-CSF and cultured for 4 days at 37°C (culture dishes, tissue culture treated, #734-0006, Falcon®, VWR, USA). On day 4, fresh medium containing INF-γ (10 ng/ml) and TNF-α (0.1 ng/ml) was added and cell density was adjusted if required. Cells were harvested at day 6 and used the same day.

2.5 | Flow cytometry analysis of surface marker expression

Expression of surface markers was determined by flow cytometry using the CyAn Flow Cytometer (Beckman Coulter, USA). A total of 0.5 × 10⁶ cells in FC-buffer (1× PBS +1% BSA) were used as per condition. Anti-Mouse CD16/CD32 antibody (Mouse BD Fc BlockTM) was added in a 1:200 dilution for 15 min (room temperature, RT) to avoid unspecific binding. CD11b/PE/Cy7 (#101215), Gr-1-FITC (#108405), and F4/80-APC (#123115) were added in a 1:200 dilution and incubated for another 15 min at room temperature (antibodies purchased from BioLegend, USA). Cells were washed and resuspended in FC-buffer. At least 10,000 cells were recorded per sample. Neutrophil population was gated in the forward-side scatter channel. Unstained controls served to define the background fluorescence levels. The percentage of positively stained cells and mean fluorescent intensity was measured for each fluorophore and used for further analysis. Single stained samples were used to establish settings and compensation using the CyAN software.

2.6 | May-Grünwald-Giesma staining

Cells for staining were isolated or cultured as described above, washed once, and seeded on polylysine-coated (0.1%) cover slips (CS, 12 mm) and allowed to adhere. Cells were air-dried and fixed with methanol (100%), washed once with PBS, and immediately stained. CS were stained in May-Grünwald solution (1:1 diluted in H₂O, #MG500, Sigma) for 5 min, followed by staining in Giemsa solution (1:9 diluted in H₂O, #G5500, Sigma) for 30 min, washed once in H₂O, allowed to dry and immediately analyzed by bright field microscopy (20x magnification).

2.7 | Calcium imaging, 96-well plate format

Ca²⁺ concentration changes were measured with Fura2 in 96-well microplates (black/clear bottom, Cell culture microplate, #655090, Greiner) using a FlexStation 3 Multi-Mode Microplate Reader (Molecular devices, US). Cells were seeded at 0.12 × 10⁶ cells/well on plates coated with fibronectin (5 μg/ml, 60 μl/well, 1 h at room temperature) or endothelial cells. Endothelial cells were seeded on plates 3 days before the experiment to ensure a monolayer. Cells adhered to fibronectin were loaded with Fura2-QBT (non-wash dye, # R8198, Molecular devices,US) following the manufacturer’s instructions. Cells adhered to endothelia were loaded with Fura2-AM (2μM) and washed before seeding on plates. Cells were loaded with dyes for 15 min in Ca²⁺-containing buffer with pluronic acid (Pluronic F-127, #P3000MP, Invitrogen) and probenecid (#P36400, ThermoFisher) to enhance loading and prevent dye extrusion. Measurements were performed at 37°C using bottom reading and 4 s time increment. Responses were evaluated as delta peak (ΔPeak = Max – Baseline) and area under the curve (AUC). Baselines for ΔPeak and AUC calculations are averaged from 5 data points, taken at least 1 min prior to the addition of active substances. Substances were dissolved in DMSO with equal final solvent concentrations of < 0.5%.

2.8 | Three hundred eighty-four-well plate format

The dose-dependency of SOCE inhibition by 2-APB and GSK-7975A was performed with Calcium6 dye (#R8194, Molecular devices) on a Hamamatsu FDSSμCell Imager (Hamamatsu Photonics K.K., Japan). Cells were loaded as described above, washed, and re-suspended in 1.5 mM Ca²⁺-containing buffer and seeded on endothelial-coated microplates at 20,000 cells/well. Baseline Ca²⁺ levels were recorded for 2 min, inhibitors added for 9 min before Tg addition, and SOCE recorded for 9 min. To calculate IC₅₀ values, the fluorescence responses (F/F₀) obtained in the absence of inhibitors were set to 100% (maximal response) and the response obtained at a maximal dose of inhibitor were set to 0% (minimal response).

2.9 | H₂O₂ production

Cells were seeded on fibronectin-coated (0.1 to 10 μg/ml) microplates (96-well, black/clear bottom, Greiner bio-one, #655090). Extracellular ROS production was measured using AmplexUltraRed (#A36006, ThermoFisher) following the manufacturer’s instructions (#012001, ThermoFisher). All measurements were performed in buffer containing 1.5 mM Ca²⁺ using a SpectraMax Paradigm Multi-Mode Microplate reader (Molecular Devices, USA) at 37°C, excitation/emission 535/590, 30 s time increment. Relative fluorescence intensities were normalized to starting values (f = 0) and used to calculate ROS production kinetics as slope (from 2 to 20 min) or as total ROS produced after 45 min (average of 3 data points).
2.10 | Transwell-migration assay

Chemotaxis was measured in Millicell Hanging Cell Culture Insert (3 µm pore size, #PISP12R48, Merck-Millipore, USA). A total of 0.1 x 10^6 cells were seeded/insert in 75 µl buffer (1.5 mM Ca\(^{2+}\) and 10%FCS to improve migration). Inserts were placed in 24-well plates containing chemotactrant or solvent dissolved in 700 µl of Ca-buffer (1.5 mM Ca\(^{2+}\) and 10%FCS). The number of cells reaching the lower compartment after 5h was determined by flow cytometry using the live stain DRAQ5 and a BD AccuriTM C6 flow cytometer (BD Biosciences, Germany).

2.11 | Intracellular ROS production

Dihydrorhodamine 123 (DHR123, #D23806, ThermoFisher) was used to determine intracellular ROS production. Cells were seeded on 96-well V-bottom microplates (3 x 10^5 cells/well) in 100 µl Ca\(^{2+}\)-containing buffer (1.5 mM), loaded with DHR123 (5µM, 5 min at RT) and stimulated with PMA (1µM, positive control) or opsonized Zymosan particles (1:5, cells:particle ratio). Plates were centrifuged at 150,000 cells/condition were seeded on V-bottom 96-well plates and concentration ranges. Supplementing cells with G-CSF (10 ng/ml), and chemokine supplementation was tested, including IL-8, fMLF, and INF-α, for different time periods, combinations, and concentration ranges. Supplementing cells with G-CSF (10 ng/ml), INF-γ (10 ng/ml) and TNF-α (100 pg/ml) (Fig. 1B) eventually increased

2.12 | mRNA expression levels by quantitative RT-PCR

Total RNA was isolated from 1.5-3 x 10^6 cells using the QiaShredder (#79654, QIAGEN, Germany) and the RNeasy Mini Kit (#74104, QIA-GEN, Germany) following the manufacturer’s instructions and RNA was stored at -80°C. The quality of the extracted RNA was confirmed by loading 2 µl RNA on a 2% agarose gel and the concentration determined using a NanoDropTM 2000 Spectrophotometer (ThermoFisher, USA). Overall 0.5 µg RNA were used for cDNA transcription, using theTAKARA PrimerscriptRT reagent Kit (#RR037B, Takara Bio, USA) following the manufacturer’s instructions. cDNA was stored at -20°C or immediately used for quantitative PCR using SYBRGreen (PowerUpTM SYBR Green Master Mix, #A25743, Thermo Fisher, USA) and a 7900HT Real Time PCR (Applied Biosystems, USA) in 384-well plates with a reaction volume of 10 µl/well. Efficiency of all primers was determined in separate experiments and used with a final concentration of 0.3 µM. The expression of genes of interest relative to the expression of a reference gene (TBP) was determined using the ΔΔCT method (R = 2^(-ΔΔCT), with ΔΔCT = CT_{Sample} - CT_{Reference})

2.13 | Phagocytosis and preparation of Zymosan particles

Phagocytosis was measured by flow cytometry using opsonized Zymosan-Alexa488 coupled particles as phagocytic targets. A total of 150,000 cells/condition were seeded on V-bottom 96-well plates and incubated with phagocytic targets at 1:5 cell:target ratio. Measurements were performed in standard Ringer’s solution, supplemented with 1.5 mM Ca\(^{2+}\) or EGTA and 10% FCS. Phagocytosis was allowed for 30 min at 37°C (5% CO\(_2\)), after synchronization by plate centrifugation. One hundred microliters of FC-buffer was added to each well and cells were carefully resuspended. The BD AccuriTM C6 flow cytometer (BD Biosciences, Germany) was used for analysis, and at least 1000 cells were measured for each condition. Living cells were gated and Alexa488 intensity was analyzed in this population.

Living cells positive for Alexa488 were identified as phagocytosing. Preparation of phagocytic targets: 20 mg Zymosan A particles from Saccharomyces cerevisiae (# Z4250, Sigma-Aldrich) were dissolved in NHS Ester (Succinimidyl Ester). Four hundred microliter Zymosan suspension was incubated for 4 h (inverting/rotating, 4°C) with Alexa488 (final concentration of 1 mg/ml) in 0.1 M Na\(_2\)CO\(_3\) solution (pH 8.0).

Unbound probe was removed in 3 centrifugation steps, using DMSO (80%, 60%, 50% in 1x PBS). Zymosan particles were resuspended in 1x PBS and stored at –20°C. Prior to use, particles were incubated for 1 h at 37°C with ZymosanA BioParticles® Opsonizing Reagent (#Z2850, Thermo-fisher) and washed once in 1x PBS.

2.14 | Analysis and statistics

Statistical analysis was performed using GraphPad Prism 7.02. Significance is indicated in the Figure legends with *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

3 | RESULTS

3.1 | Cytokines promote the differentiation of ER-HoxB8 myeloid progenitors into neutrophil-like cells

To assess the maturation state of neutrophil-like cells (NLC) derived from ER-HoxB8-immortalized murine hematopoietic cells, we measured the surface expression of granulocytic and Myeloid markers in NLC and progenitors by flow cytometry. Removal of tamoxifen from the culture medium to silence ER-HoxB8 expression did not induce full neutrophilic differentiation in our hands, as only 60% of cells stained positive for the neutrophil marker Gr-1. 6 days after estrogen deprivation (Fig. 1A). To improve the differentiation process, cytokine and chemokine supplementation was tested, including IL-8, fMLF, G-CSF, TNF-α, and INF-γ, for different time periods, combinations, and concentration ranges. Supplementing cells with G-CSF (10 ng/ml), INF-γ (10 ng/ml) and TNF-α (100 pg/ml) (Fig. 1B) eventually increased
the percentage of Gr-1 positive cells to ∼90%, compared to 5% in progenitors and 99% in primary murine neutrophils (Fig 1A and E). May-Grünwald-Giemsa staining confirmed the advantage of the adapted protocol as polymorphic nuclei were observed in NLC differentiated with cytokines and not in cells differentiated without cytokines (Fig. 1C). With this adapted protocol, Gr-1 expression levels remained significantly lower in NLC than in primary murine neutrophils (25 vs. 374, Fig. 1D), while expression of the myeloid integrin CD11b increased to reach the levels of primary murine neutrophils (Fig. 1D and E). The expression levels of the Mφ marker F4/80, detected in ∼80% of progenitors and neutrophils and ∼60% of NLC, remained constant (Fig. 1D and E). These results are consistent with the reported phenotype of ER-HoxB8-derived cells, which can be committed to both the granulocyte and Mφ lineages,22 and indicate that the addition of cytokines combined with repression of estrogen-driven ER-HoxB8 expression promotes neutrophil differentiation.

To identify the genetic pathways engaged by our modified protocol, we then monitored 8 transcription factors (TF) associated with

| Gene | Sequence (5′ → 3′) | Forward | Reverse |
|------|------------------|---------|---------|
| Stim1 short | GCTCTCAATGCCATGCTTCCAAT | TCTAGGCCATGGTCAAGGCTCAATA |
| Stim1 long | CCGGCTCACGAGGTGGGTGGT | AGGAGGCAACGACCACACTC |
| Stim2 | TTAGGCTGTAAGACCTTCCAGCTCATA | AGCACTGTAAGAGCTTCCTT |
| Stim2.1 | GTCTCAATGCCATGCTTCCAAT | TCTAGGCCATGGTCAAGGCTCAATA |
| Stim2.2 | CCGGCTCACGAGGTGGGTGGT | AGGAGGCAACGACCACACTC |
| Orai1 | GCTTGTTGGAAGGACCTCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Mpo | GCTCTCAATGCCATGCTTCCAAT | TCTAGGCCATGGTCAAGGCTCAATA |
| Lactotransferin (Itf) | GTCTCAATGCCATGCTTCCAAT | TCTAGGCCATGGTCAAGGCTCAATA |
| MMP9 | AGCGGACATGGTCATGCTCATT | AGGAGGCAACGACCACACTC |
| Itgam (CD11b) | TACCTCAATGCCATGCTTCCAAT | TCTAGGCCATGGTCAAGGCTCAATA |
| Itgal(CD11a) | CCGGCTCACGAGGTGGGTGGT | AGGAGGCAACGACCACACTC |
| Itgb2 (CD18) | CACCTGTAAGACCTTCCAGCTCATA | GCAGTACGTAAGAGCTTCCTT |
| Itga4 (CD49d) | CCGGCTCACGAGGTGGGTGGT | AGGAGGCAACGACCACACTC |
| Itgb1 (CD29) | CACCTGTAAGACCTTCCAGCTCATA | GCAGTACGTAAGAGCTTCCTT |
| Fgrl1 (CD64, FcyRI) | GCTCTCAATGCCATGCTTCCAAT | TCTAGGCCATGGTCAAGGCTCAATA |
| Fgrl2b (CD32, FcyRIIB) | CACCTGTAAGACCTTCCAGCTCATA | GCAGTACGTAAGAGCTTCCTT |
| Fgrl3 (CD16 FcyRIII) | CACCTGTAAGACCTTCCAGCTCATA | GCAGTACGTAAGAGCTTCCTT |
| Tlr2 (CD282) | CACCTGTAAGACCTTCCAGCTCATA | GCAGTACGTAAGAGCTTCCTT |
| Cxcl2 | TACCTCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Nfatc | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Cybb | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Ptaf | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Fprl1 | CACCTGTAAGACCTTCCAGCTCATA | GCAGTACGTAAGAGCTTCCTT |
| B2M | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Cebp-alpha | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Cebp-beta | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Cebp-gamma | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Cebp-delta | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Cebp-epsilon | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Gfi1 | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| Pu.1 (Sfpi-1) | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
| LEF-1 | GACGATCAGGAAATCCGCAACTC | GCAGTACGTAAGAGCTTCCTT |
Cytokines enhance the neutrophilic differentiation of ER-HoxB8 myeloid progenitors. The neutrophilic differentiation of ER-HoxB8 myeloid progenitors was initiated by tamoxifen removal using the protocol published by Wang et al.\textsuperscript{22} or modified to include G-CSF (10 ng/ml) and a mixture of INF-\(\gamma\) (10 ng/ml) and TNF-\(\alpha\) (0.1 ng/ml) added at day 0 and 4, respectively. (A) Effect of cytokines on Gr-1 surface expression in NLC (blue) derived from ER-HoxB8 progenitors (red). (B) Outline of the differentiation protocol published by Wang et al.\textsuperscript{22} and of our adapted protocol. (C) May-Grünwald-Giesma staining of progenitors, NLC obtained with or without cytokines, and murine neutrophils. (D) Median fluorescence intensity of progenitors, NLC, and neutrophils stained for the surface markers Gr-1 (FITC), CD11b (PE-Cy5), and F4/80 (APC). (E) Percentages of cells expressing the surface markers in (D). Significance of statistical differences is indicated with ****\(P < 0.0001\), unpaired t-Test with Welch’s correction for data from at least \(n = 3\) independent experiments.

We selected “early” TF expressed in initial stages of granulopoiesis (Cebp\(\alpha\), Gfi-1, Lef-1, contributing to the suppression of proliferation and the repression of monocytic development), an “intermediary” TF, active before the entry into the banded/segmented neutrophil stage (Cebp\(\epsilon\), required for the development of secondary granules), and ”late” TF, active during the late stages of differentiation (Cebp\(\beta\), Cebp\(\gamma\), Cebp\(\delta\) and P.U.1, contributing to tertiary granule formation...
Expression of transcription factors associated with granulopoiesis. (A) Relative mRNA expression levels of granulopoiesis transcription factors in bone-marrow derived murine neutrophils and in NLC on day 6 (final day) of the differentiation. (B–E) mRNA levels of the transcription factors Cebp$\alpha$, Cebp$\varepsilon$, PU.1, and Cebp$\beta$ measured daily by qRT-PCR in progenitors and NLC obtained with the two protocols. Data show changes in expression levels ($2^{-\Delta CT}$) relative to day 1, from 2 to 3 independent experiments.

and the final maturation of the neutrophil). The expected change of TF expression is illustrated in Supplementary Fig. S1I (adapted from Fiedler et al.). Figure 2A shows the relative expression of these 8 TFs in NLC differentiated with the adapted protocol and in primary murine neutrophils. To compare the 2 differentiation protocols, cells differentiated with and without cytokines were sampled daily following tamoxifen removal together with undifferentiated progenitors, and the selected TFs determined by qRT-PCR. The expression patterns of the early and intermediate differentiation TFs Cebp$\alpha$ and Cebp$\varepsilon$ and of the late stage TFs Cebp$\beta$ and PU.1 significantly diverged between progenitors and NLC. The profiles of these 4 TFs are shown as $2^{-\Delta CT}$ values normalized to expression at day 1 (Fig. 2B–E, full dataset in Supplementary Fig. S1, $2^{-\Delta CT}$ values used for normalization in Table 2). A transient 2-fold increase of the early TF Cebp$\alpha$ expression was detected at days 2–4 regardless of cytokine supplementation (Fig. 2B). Expression of the intermediate TF Cebp$\varepsilon$ abruptly increased by 90-fold at day 4 and then slightly subsided in the presence of cytokines, while it continued to increase throughout the differentiation period in the absence of cytokines (Fig. 2C). Expression of the late stage TF PU.1 was observed with both protocols (Fig. 2D), while expression of Cebp$\beta$ was only detected with cytokine supplementation (Fig 2E). The failure of Cebp$\beta$ to increase and of Cebp$\varepsilon$ to decrease at day 6 suggests that in the absence of cytokines the cells may be locked in an intermediary differentiation phase. Four other TFs (Cebp$\gamma$, Cebp$\delta$, LEF1, and Gfi-1) showed similar expression patterns upon estrogen removal, compared to progenitors, and were not analyzed further (Supplementary Fig. S1E–H). Expression profiling, thus, indicates that cytokine supplementation promotes
We next assessed the functional activation of NLC by measuring the Stim/Orai-mediated store-operated Ca\(^{2+}\) entry (SOCE) pathway. SOCE is a predominant feature of proliferating cells while robust Ca\(^{2+}\) responses to bacterial peptides such as fMLF is a characteristic feature of primary neutrophils. We therefore compared the Ca\(^{2+}\) responses elicited in progenitors, NLC, and primary neutrophils by chemoattractants and by thasigargin (Tg), which induces SOCE by depleting intracellular Ca\(^{2+}\) stores. Tg, applied in Ca\(^{2+}\)-free medium, induced Ca\(^{2+}\) release in all cell types while subsequent Ca\(^{2+}\) readmission evoked massive SOCE in progenitors and a more modest response in NLC and neutrophils (Fig. 3A). In contrast, the prototypical bacterial peptide
**FIGURE 3**  
Ca²⁺ signaling competency of neutrophil-like cells. (A and B) Ca²⁺ responses evoked in progenitors, NLC, and neutrophils by thapsigargin (Tg, 1 µM) and by the chemotactic peptide fMLF (1 µM), measured with fura-2 on a plate reader in cells seeded on endothelial monolayers. Tg was applied in Ca²⁺-free medium and Ca²⁺ added back to evoke store-operated Ca²⁺ entry (SOCE). (C) Effect of increasing concentrations of fMLF on the peak amplitude of the Ca²⁺ responses evoked in NLC seeded on fibronectin (5 µg/ml) or endothelial cells (in Ca²⁺ containing conditions). (D and E) Statistical evaluation of the peak amplitude (D) and of the integrated Ca²⁺ responses (E, area under the curve, AUC) evoked by Tg, fMLF, and PAF. The integrated Ca²⁺ response evoked by Tg (E) reflects the mobilization of the content of intracellular Ca²⁺ stores (Tg applied in Ca²⁺ free condition, see Fig. 2A). (F) mRNA expression levels of SOCE channels (Orai1-3) and regulators (Stim1-2) and of receptors for fMLF (Fpr) and PAF (Ptafr) in NLC, progenitors, and neutrophils. (G and H) Effect of increasing concentrations of GSK-7975A (G) and 2-APB (H) on Tg-evoked SOCE in NLC, measured with Calcium 6 in 384 wells plates on a microplate imager. IC₅₀ values were determined by fitting a Hill equation to normalized ratio values. Data are from at least 2 independent experiments performed in duplicates/triplicates, * P < 0.05; ** P < 0.01; *** P < 0.001, unpaired t-test
fMLF evoked robust biphasic Ca\(^{2+}\) elevations in primary neutrophils and in NLC but failed to mobilize Ca\(^{2+}\) in progenitors (Fig. 3B). These data validate the proliferative state of progenitor cells and the Ca\(^{2+}\) signaling and bacterial-sensing capacity of NLC.

Neutrophils interact with the endothelial wall via adhesion receptors, primarily the P-selectin glycoprotein ligand 1 mediating neutrophil rolling and the \(\alpha_L\beta_2\) integrin LFA-1 whose switch to high-affinity conformational state induces neutrophil arrest, spreading, and crawling on the endothelium.\(^7\) Interactions between adhesion receptors and their endothelial ligands activate neutrophil tyrosine kinases and PLC\(\gamma_2\), enhancing the Ca\(^{2+}\) elevations triggered by GPCRand coupled chemokine receptors.\(^15,33\) To test whether this signaling cascade is recapitulated in NLC, we measured Ca\(^{2+}\) The AUC of the responses evoked by agonists, which integrates both content of stores decreases together with SOCE during differentiation (Fig. 3D). In contrast, the amplitude of the response evoked by f-MLF, which primarily reflects Ca\(^{2+}\) entry of fibronectin to recapitulate the effect of endothelial cells suggesting that L-selectin, rather than \(\beta_2\) integrins, may mediate this priming effect. Accordingly, plating cells on ICAM-1 did not potentiate the f-MLF Ca\(^{2+}\) responses (data not shown).

Quantification of the responses evoked by Ca\(^{2+}\) readmission to cells exposed to 1 \(\mu\)M Tg confirmed a 2-fold larger SOCE in progenitors compared to NLC and primary neutrophils (mean peak values 3.49 ± 0.25 vs. 1.57 ± 0.1 and 1.27 ± 0.09, respectively, \(P < 0.001\), Fig. 3D). In contrast, the amplitude of the response evoked by f-MLF, which primarily reflects Ca\(^{2+}\) release from stores, increased marginally in NLC, and significantly in neutrophils (mean peak values 0.4 ± 0.2 vs. 0.7 ± 0.2, \(P = 0.30\) and 1.2 ± 0.3, \(P < 0.02\), respectively, Fig. 3D). Unexpectedly, the response evoked by PAF, a potent phospholipid activator of neutrophil functions, was decreased in NLC compared to progenitors and neutrophils (mean peak values 0.8 ± 0.2 vs. 0. 5 ± 0.1, \(P = 0.07\) and 1.3±0.1, \(P = 0.13\), respectively, Fig. 3D). To test whether the reduced response of NLC reflected a reduced Ca\(^{2+}\) content of ER-stores, we quantified the extent of the Ca\(^{2+}\) that could be mobilized by Tg in Ca\(^{2+}\)-free medium. The AUC of the response evoked by Tg was significantly reduced in NLC compared to progenitors and marginally augmented in neutrophils (71.3 ± 4.3 vs. 34.5 ± 2.1, \(P < 0.001\) and 125.6±23.1, \(P = 0.14\), Fig. 3E). These data suggest that the Ca\(^{2+}\) content of stores decreases together with SOCE during differentiation, before increasing again in terminally differentiated neutrophils. The AUC of the responses evoked by agonists, which integrates both the Ca\(^{2+}\) release and influx components, were comparable in NLC and progenitors and significantly larger in neutrophils for both fMLF (31.52 ± 6.1 vs. 85.7 ± 23.0, \(P < 0.05\) and 174.7 ± 34.2, \(P < 0.05\), Fig. 3E) and PAF (65.2 ± 9.5 vs. 33.9 ± 8.8, \(P = 0.055\) and 111.2 ± 13.2, \(P < 0.05\), Fig. 3E).

To clarify the molecular roots of NLC Ca\(^{2+}\) responses, we measured the expression of genes coding for Ca\(^{2+}\) signaling proteins by qPCR. Consistent with the SOCE data, all the Stim/Orai isoforms were expressed at lower levels in NLC compared to progenitors (Fig. 3F; Supplementary Fig. S3). In contrast, neutrophils expressed higher levels of Oral isoforms and comparable levels of all Stim isoforms and splice variants, at odds with the reduced SOCE measured in these cells. Interestingly, the Stim1/Orai1 ratios were lower in both NLC (0.16 ± 0.03) and neutrophils (0.13 ± 0.01) compared to progenitors (0.26 ± 0.02), indicating a higher relative expression of the Orai1 channel than its activating ligand Stim1 in differentiated cells (Supplementary Fig. S3). The expression of the fMLF receptor was low in progenitors and increased in NLC and neutrophils (Fig. 3F; Supplementary Fig. S3), correlating with the ability of agonists to generate Ca\(^{2+}\) responses in NLC despite the reduced store content and entry of these cells.

Pharmacological inhibition of neutrophils is a therapeutic strategy to reduce the tissue damage associated with the recruitment of leukocytes during inflammation and infection.\(^34,35\) To test whether NLC can be used to screen for drugs targeting neutrophil activity, we tested different concentrations of two known SOCE inhibitors, GSK-7975A and 2-APB, using a high-throughput fluorescence assay. The responses evoked by Ca\(^{2+}\) readmission to cells treated with Tg were measured with Calcium 6 on a microplate reader allowing simultaneous dispensing of compounds and kinetic measurement in 384-well plates. A complete dose response (13 concentrations, ranging from 0.7 nM to 30 \(\mu\)M) was obtained and the IC\(_{50}\) values calculated from recordings performed in triplicates. GSK-7975A produced a partial inhibition (50% at 30 \(\mu\)M) with an IC\(_{50}\) of 3.09 \(\mu\)M (Fig. 3G) while 2-APB caused a more comprehensive inhibition of the SOCE response (70% at 30 \(\mu\)M) with an IC\(_{50}\) of 13.84 \(\mu\)M (Fig. 3H). These data validate the use of NLC as neutrophil surrogates for high-throughput drug screening assays and indicate that the SOCE channels expressed in NLC are inhibited by 2-APB and relatively resistant to GSK-7975A.

### 3.3 | ER-HoxB8-derived neutrophil-like cells recapitulate some, but not all, neutrophil effector functions

We next assessed whether NLC express a phagocytic NADPH oxidase (NOX2) able to produce the large amounts of ROS required for efficient neutrophils extravasation and pathogen clearance.\(^36,37\) Adhesion of neutrophils to the matrix glycoprotein fibronectin activates NOX2-dependent ROS production,\(^38,39\) an effect that is suppressed at high fibronectin concentrations due to the engagement of \(\alpha_\nu\beta_3\) integrins.\(^40,41\) When adhered to plates coated with low concentrations of fibronectin (0.3 \(\mu\)g/ml), NLC, but not progenitors, produced significant amounts of extracellular H\(_2\)O\(_2\) reported by AmplexUltra Red fluorescence (Fig 4A and B). Adhesion-mediated H\(_2\)O\(_2\) production was abrogated by the NADPH-oxidase blocker DPI and reduced by extracellular Ca\(^{2+}\) chelation and by the Ca\(^{2+}\) channel inhibitor 2-APB (Fig. 4A and B). Consistent with \(\alpha_\nu\beta_3\)-mediated suppression of ROS production, the rates of H\(_2\)O\(_2\) produced by NLC decreased as the fibronectin concentration increased from 0.1 to 10
FIGURE 4  Superoxide production, chemotaxis, and phagocytosis in neutrophil-like cells. (A) Kinetics of extracellular H$_2$O$_2$ production by NLC (open circles) and progenitors (closed circles) adhered to plates coated with fibronectin (0.3 µg/ml), measured in Ca$^{2+}$-containing and Ca$^{2+}$-free medium (EGTA, green) and with a NOX2 inhibitor (DPI, red). The AmplexUltra Red relative fluorescence intensity (RFI) recorded on a 96-well plate reader is shown, normalized to initial values. (B) Maximal rates of H$_2$O$_2$ produced by NLC in Ca$^{2+}$-containing and Ca$^{2+}$-free conditions and in the presence of DPI and 2-APB. (C) Effect of increasing fibronectin concentrations (0.1–10 µg/ml) on the rates of H$_2$O$_2$ produced by progenitors and NLC (black and white bars, left y-axis) and by primary murine neutrophils (grey bars, right y-axis). (D) mRNA expression levels of αMβ2 integrin (continued on the next page)
µg/ml (Fig. 4C, white bars). A similar dose-dependent H₂O₂ production was measured in neutrophils while ROS were undetectable in progenitors at all fibronectin concentrations (Fig. 4C, black and gray bars). Importantly, the rates of H₂O₂ production were 55-fold lower in NLC compared to neutrophils (2.8 ± 10^3 vs. 1.59 ± 10^5 at 1 µg/ml, Fig. 4C, white and gray bars, note different axis scales). Adhesion thereby activates NOX2-dependent ROS production in NLC, an effect partly dependent on the entry of Ca²⁺ across membrane channels, but the rates of ROS production are 1.5 order of magnitude lower than those of neutrophils.

To clarify the molecular basis of the adhesion-mediated ROS responses, we measured the expression of genes coding for the adhesion receptor Mac1 (Itgam/Itgb2, α₅β₂, integrin CD11b/CD18) and for the cytosolic and membrane NOX2 subunits p47phox (Ncf1) and gp91phox (Cyb2). Cybb expression increased by 50-fold in NLC compared to progenitors while Itgam, Itgb2, and Ncf1 expression was conserved, all transcripts being 4–20 less abundant in NLC than in neutrophils (Fig. 4D; Supplementary Fig. S3). To establish the presence of a functional NOX2, we assessed gp91phox protein expression by western blot. A prominent ~60 kD band was detected by the anti-gp91phox antibody in WT neutrophils that was absent in neutrophils from gp91phox knock-out mice (Fig. S2D). This band was undetectable in ER-HOXX88-derived progenitors and barely detectable in NLC. Quantification of the gels indicated that NLC express ~20 times less gp91phox than primary neutrophils. The ability of NLC to produce ROS thus correlates with an increased expression of the rate-limiting membrane flavocytochrome gp91phox, but this expression remains at low levels, likely limiting the NOX2 activity of NLC.

We further examined the ability of NLC to migrate toward chemoattractants across a permeable membrane (3 µm pores) coated with fibronectin (5 µg/ml) or endothelial cells. On both substrates, fMLF and MIP2 increased the migration of NLC and of neutrophils, but not of progenitors (by 2- and 16-fold on fibronectin, P < 0.06 and P < 0.004 and by 4-fold on endothelial cells, P < 0.0001, and P > 0.001 for NLC and neutrophils, respectively, Fig 4E, ¹). Neutrophils migrated 3–4 times more efficiently than NLC in the presence of fMLF and MIP2 (P < 0.01 and P < 0.001 on fibronectin and endothelial cells, respectively, Fig. 4E, ²). Migration in absence of cytokines (chemokinesis) was enhanced in NLC and neutrophils compared to progenitors (P < 0.01 for NLC vs. progenitors, Fig. 4E, ³). NLC thus display enhanced chemokinesis and are able to migrate towards chemoattractants, but their chemotactic response to fMLF and MIP2 is poor, consistent with the moderate expression levels of the Fpr1 and Cxcr2 receptors in NLC (Figs. 3F and 4D; Supplementary Fig. S3).

We next assessed the capacity of NLC to phagocyte opsonized fluorescent yeast particles by flow cytometry. Zymosan particles, added at a 1:5 cell to target ratio for 30 min, were taken up 3–4 times more efficiently by NLC and neutrophils than by progenitors (14 ± 0.6 vs. 35 ± 2, P = 0.0001 and 46 ± 6%, P < 0.01 for progenitors, NLC, and neutrophils, respectively, Fig 4E, ⁴). Ca²⁺ removal decreased particle uptake (to 9.7 ± 0.4, 17 ± 1, and 23 ± 1%, Fig. 4F, ⁵) with NLC and neutrophils retaining a significantly higher phagocytic index (P < 0.001 and P < 0.0001 for NLC and neutrophils vs. progenitors, respectively, Fig. 4F). An EDTA-containing buffer was used to favor the detachment of non-internalized beads and particle internalization was verified microscopically. The increased phagocytic ability of NLC correlated with an increased production of intracellular ROS, measured by flow cytometry with DHR123 (1.1 ± 0.1 vs. 8.2 ± 2.2 and 1.2 ± 0.3 vs. 2.0 ± 0.3-fold for progenitors and NLC stimulated with PMA and zymosan, respectively, Fig. 4G). At the molecular level, phagocytic efficiency correlated with increased expression of the Tlr2 (0.16 ± 0.04 vs. 0.7 ± 0.1 in progenitors and NLC, respectively, Fig. 4D) and with a decreased expression of the immunoglobulin gamma FcγRIIb receptor (FcgR2b, CD32), but not with the expression of the FcγRII (FcgR3, CD16) and FcγRI (FcgR1, CD64) isomers (0.44 ± 0.02 vs. 0.13 ± 0.07 and 0.05 ± 0.01 for FcγRIIb, 1.12 ± 0.13 vs. 0.97 ± 0.32 and 1.7 ± 0.24 for FcγRII, 0.09 ± 0.03 vs. 0.14 ± 0.02 and 0.10 ± 0.12 for FcγRI (Fig. 4H; Supplementary Fig. S3). The ability of NLC to phagocyte thus likely reflects the relief of an inhibition conferred by the FcγRIIb receptor.

Finally, we checked the expression of components of 3 types of neutrophil granules. Unexpectedly, the expression of the primary (azurophilic) granule marker myeloperoxidase (MPO), although detectable, was low in both progenitors, NLC, and neutrophils (0.3 ± 0.1, 0.4 ± 0.3, and 0.4 ± 0.2 for MPO, respectively, Fig. 4H; Supplementary Fig. S3). In contrast, expression of the secondary (specific) granule marker lactoferrin (LF) and of the tertiary granule marker gelatinase (MMP9) was low in progenitors and NLC but 40–100-fold higher in neutrophils (0.03 ± 0.002, 0.2 ± 0.1, and 21.9 ± 2.8 for LF and 0.14 ± 0.06, 0.2 ± 0.1, and 7.5 ± 0.2 for MMP9, respectively, Fig. 4H; Supplementary Fig. S3). We further documented the presence of MPO, lactoferrin and MMP9 by immunohistochemistry. Based on the immunoreactivity, the levels of these granule proteins increased by 2-fold in NLC compared to progenitors but remained 2–3 times lower than that in primary neutrophils (Supplementary Fig. S2C). Together, these data indicate that NLC express low levels of protein components of primary, secondary, and tertiary granules and thus cannot replace neutrophils for degranulation experiments.
4 | DISCUSSION

In this study, we characterize the molecular and cellular basis of the neutrophilic effector functions acquired by cultured cells derived from progenitors immortalized with the ER-HoxB8 system. We show that these NLCs are suited for high-throughput screens and can partially replace primary murine neutrophils to study Ca$^{2+}$ signaling pathways, superoxide production, chemotaxis, and phagocytosis. A recent study used HoxB8 cells derived from liver progenitors of transgenic mice to establish the role of the non-muscle myosin Myh9 in neutrophil superoxide production, chemotaxis, and phagocytosis. Thus, neutrophils are an adequate model system to investigate signaling pathways, while NLC can be used to screen for modulators of the physiological Ca$^{2+}$ responses evoked in neutrophils by agonists, adhesion receptors, and store depletion.

From a functional standpoint, we show that NLC can mount a respiratory burst and can perform chemotaxis and phagocytosis, 3 critical functions of neutrophils. Specifically, we show that NLC produce substantial amounts of H$_2$O$_2$ when adhered to fibronectin, migrate toward chemotactic peptides across porous membranes coated with fibronectin or endothelial cells, phagocytose opsonized particles, and generate intracellular ROS. These signature neutrophil functions were undetectable in ER-HoxB8 progenitors, indicating that NLC had acquired a granulocytic phenotype. Although NLC qualitatively recapitulated these 3 effector functions, we observed important quantitative differences with neutrophils. NLC produced substantially less ROS than bone marrow-derived murine neutrophils and migrated less efficiently toward chemoattractants. In contrast, NLC took up zymosan particle as efficiently as neutrophils. NLC can therefore replace neutrophils to study phagocytosis but only provide qualitative information for the transcription of proteins involved in chemotactic sensing. ROS production, and phagocytosis. NLC chemotaxis correlated with an increased expression of the chemotactic receptor Fpr1, their H$_2$O$_2$ production with increased expression of the rate-limiting NOX2 subunit Cybb, and their ability to perform phagocytosis with a decreased expression of the inhibitory immunoglobulin receptor FcgR2b.

From a Ca$^{2+}$ signaling standpoint, we show that the responses evoked in neutrophils by chemotactic agents, adhesion receptors, and store depletion were recapitulated in NLC. In contrast, progenitors failed to respond to agonists and exhibited significantly larger SOCE rates than both NLC and neutrophils (Fig. 3). The high number of cells generated enabled us to test a whole range of concentrations of stimuli (Fig. 3C; Supplementary Fig. S2A and B) and of inhibitors (Fig. 3G and H) in 96- and 384-well microplate formats, thereby validating the use of NLC for high-throughput fluorescence screens. The IC$_{50}$ values for GSK79775A and 2-APB (3 and 14 µM, Fig. 3G and H) were in line with the values of ~4 µM for GSK79775A and >10µM for 2-APB reported in primary cells and in cells expressing the ORAI1 Ca$^{2+}$ channel. These agonists evoke physiological Ca$^{2+}$ response in NLC and their pharmacological profile matches that of primary cells. Interestingly, NLC mimicked the SOCE response of primary murine neutrophils, yet expressed reduced amounts of the Stim and Orai isoforms (Fig. 3; Supplementary Fig. S3). This suggests that Stim and Orai proteins might control specific neutrophil functions independently of global SOCE responses, consistent with our earlier report of Stim1-dependent local Ca$^{2+}$ signals promoting neutrophil phagocytosis. The reduced Stim/Orai levels of NLC might thus reflect the incomplete terminal differentiation of these cells, which do not recapitulate the whole range of neutrophil effector functions. On the other hand, the high Stim/Orai levels and large SOCE rates of undifferentiated progenitors are consistent with the role of the Stim/Orai machinery in sustaining high rates of cell proliferation. The ER-HoxB8-immortalized myeloid progenitor are thus an adequate model system to investigate the pharmacology of myeloid components of the Stim/Orai machinery, while NLC can be used to screen for modulators of the physiological Ca$^{2+}$ responses evoked in neutrophils by agonists, adhesion receptors, and store depletion.

In our initial validation experiments, we modified the original culture protocol by adding pro-inflammatory cytokines, a procedure that further promoted the granulocytic differentiation of NLC as verified by the expression of surface markers and of transcription factors related to granuloipoiesis (Figs. 1 and 2). Earlier studies obtained >70% of neutrophils with SCF, G-CSF, or both, and we could further enhance the enrichment by adding cytokines during the differentiation process. In future studies, additional protocol adaptations could further enhance the commitment of these cells toward the granulocytic lineage and the population of mature neutrophils could be selected by flow cytometry to exclude the fraction of Gr-1 negative NLC (~10%, Fig. 1E). The presence of residual myeloid precursors and the incomplete terminal differentiation of NLC reported by TF profiling could both account for the divergences in the functional responses observed between NLC and primary murine neutrophils.

Thus, agonists evoke physiological Ca$^{2+}$ response in NLC and their pharmacological profile matches that of primary cells. Interestingly, NLC mimicked the SOCE response of primary murine neutrophils, yet expressed reduced amounts of the Stim and Orai isoforms (Fig. 3; Supplementary Fig. S3). This suggests that Stim and Orai proteins might control specific neutrophil functions independently of global SOCE responses, consistent with our earlier report of Stim1-dependent local Ca$^{2+}$ signals promoting neutrophil phagocytosis. The reduced Stim/Orai levels of NLC might thus reflect the incomplete terminal differentiation of these cells, which do not recapitulate the whole range of neutrophil effector functions. On the other hand, the high Stim/Orai levels and large SOCE rates of undifferentiated progenitors are consistent with the role of the Stim/Orai machinery in sustaining high rates of cell proliferation. The ER-HoxB8-immortalized myeloid progenitor are thus an adequate model system to investigate the pharmacology of myeloid components of the Stim/Orai machinery, while NLC can be used to screen for modulators of the physiological Ca$^{2+}$ responses evoked in neutrophils by agonists, adhesion receptors, and store depletion.
functional differences observed between NLC and primary cells. On the other hand, our observation that NLC phagocytose zymosan particles as efficiently as neutrophils and that this ability correlates with a decreased expression of the low-affinity, inhibitory receptor rather than with an increased expression of the activating immunoglobulin receptors make this cell line a good model to study the impact of Fcy isoform expression on phagocytosis.

Complete reconstitution of neutrophil effector functions with the ER-HoxB8 system might require culture conditions mimicking processes occurring in the bone marrow niche. Indeed, our observation that adhesion to fibronectin or to endothelial cells modulate NLC Ca$^{2+}$ and ROS responses show that these cells quickly adapt to their microenvironment. Despite its current limitations, the ER-HoxB8 system has potential as an alternative model to animal experimentation, since cell lines could be derived from knockout mouse models or genetically engineered to provide sufficient material for large-scale experiments. Using CRISPR-Cas9 approaches, genetically modified HoxB8 cell lines were generated to visualize Ca$^{2+}$ signals during dendritic cells migration or to control their differentiation into osteoclasts.

In conclusion, we show that NLCs derived from murine bone marrow cells conditionally immortalized with the ER-HoxB8 oncoprotein recapitulate three major effector functions of neutrophils in vitro. We validate the use of NLC in high-throughput fluorescence screens and document the molecular basis of their signaling, chemotactic, phagocytic, and oxidative capacity. Our data indicate that NLC can replace neutrophils to study phagocytosis, chemotaxis, and ROS production, although their incomplete differentiation imparts important molecular and functional differences with primary neutrophils.

AUTHORSHIP

S.S. designed the study, performed experiments, analyzed data, and wrote the manuscript. C.C. and C.F. performed experiments, handled mice, and performed primary cell isolation. N.D. designed the study, supervised experiments, and wrote the manuscript.

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DISCLOSURE

The authors declare no financial conflict of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.