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# Neutrophils Derived from Genetically Modified Human Induced Pluripotent Stem Cells Circulate and Phagocytose Bacteria In Vivo

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**Key Words.** Neutrophils • Human induced pluripotent stem cells • Circulation • Phagocytosis • AKT

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## ABSTRACT

**Bacterial and fungal infections are a major cause of morbidity and mortality in neutropenic patients. Donor-derived neutrophil transfusions have been used for prophylaxis or treatment for infection in neutropenic patients. However, the short half-life and the limited availability of large numbers of donor-derived neutrophils for transfusion remain a significant hurdle in the implementation of neutrophil transfusion therapy. Here, we investigate the in vitro and in vivo activity of neutrophils generated from human induced pluripotent stem cells (iPSC), a potentially unlimited resource to produce neutrophils for transfusion. Phenotypic analysis of iPSC-derived neutrophils reveal reactive oxygen species production at similar or slightly higher than normal peripheral blood neutrophils, but have an ~50%–70% reduced *Escherichia coli* phagocytosis and phorbol 12-myristate 13-acetate induced formation of neutrophil extracellular traps (NET). Signaling of granulocytic precursors identified impaired AKT activation, but not ERK or STAT3, in agonist-stimulated iPSC-derived neutrophils. Expression of a constitutively activated AKT in iPSC-derived neutrophils restores most phagocytic activity and NET formation. In a model of bacterial induced peritonitis in immunodeficient mice, iPSC-derived neutrophils, with or without corrected AKT activation, migrate similarly to the peritoneal fluid as peripheral blood neutrophils, whereas the expression of activated AKT significantly improves their phagocytic activity in vivo.** STEM CELLS TRANSLATIONAL MEDICINE *2019;8:557–567*

## SIGNIFICANCE STATEMENT

This study is a proof-of-concept of engineering methodology to generate efficient neutrophils to phagocytose bacteria in vitro and in vivo, for potential use in neutropenic patients with sepsis.

## INTRODUCTION

Functioning neutrophils are a vital component of the defense system against infection in humans. For patients undergoing stem cell transplantation or induction for acute leukemia, modern intensive chemotherapy often results in frequent and prolonged periods of neutropenia, a major risk factor for severe bacterial and fungal infections [1–4]. Despite the use of broad spectrum antibiotics and colony-stimulating factors (CSF), infection can account for over 20% of the mortality rate of these patients, and the severity of the infections depends on the degree and duration of neutropenia and the severity of immunosuppression [5–7]. Even if the numbers of neutrophils are normal, patients with a functional inability to adequately fight infections can suffer similar degrees of morbi-mortality [8]. Even with Clinical experience and data from animal studies suggest that control of infection in these patients requires the recovery of bone marrow neutrophil production [9].

Neutrophil transfusions are used to treat infection in patients with neutropenia. Data from neutropenic animal models indicate that neutrophil transfusion may resolve infections [10] [11]. However, it is unclear whether neutrophil transfusions are effective in humans. It has been suggested that the efficacy of neutrophil transfusions in neutropenic patients may be proportional to the dose of neutrophils transfused. Doses of at least $1 \times 10^{10}$ neutrophils per kilogram body weight (b.wt.) per transfusion once a day seem to be required to treat or prevent infection in infants [12, 13]. Although the prospective randomized Resolving Infection in Neutropenia with Granulocytes (RING) study, which evaluated the use of neutrophil

 

transfusion to treat infection in neutropenic patients, failed to reach enough statistical power to reach a conclusion on efficacy, a trend toward a beneficial effect for those patients who received the largest dose of neutrophils was seen [14].

Somatic cells can be reprogrammed to generate induced pluripotent stem cells (iPSC) and used as a renewable source of cells [15, 16]. iPSC are characterized by their ability to stably self-renew indefinitely, and produce differentiated progeny from each of the three embryonic germ layers. iPSC can also be directed to differentiate toward specific cells of therapeutic or scientific interest. For example, our collaborative team has successfully differentiated human iPSC into neutrophils from normal subjects and patients [17–19]. iPSC-derived neutrophils offer an advantage over donor-derived neutrophils and adult hematopoietic stem cell-derived neutrophils in that they represent a limitless source of large doses of neutrophils suitable for transfusion [17–21]. iPSC offer advantages over embryonic stem cells as they eliminate a number of ethical issues associated with the use of embryonic tissue. iPSC also offer the possibility of generating a repertoire of lines with different antigenic profiles which would potentially reduce the frequency of alloimmunization associated with granulocyte transfusion.

Here, we show proof-of-concept that viable, functional neutrophils can be generated ex vivo from human iPSC. These neutrophils, however, are defective in differentiation, survival and/or bactericidal potency. This dysfunction reflects a decreased AKT response to in vitro or in vivo sepsis-derived agonists, and can be ameliorated at levels similar to primary human neutrophils when hematopoietic progenitors developed using ex vivo differentiation of iPSC express activating forms of AKT.

## Materials and Methods

### Generation of iPSC from Peripheral Blood Mononuclear Cells from Human Donors

Peripheral blood from healthy donors and severe congenital neutropenia (SCN) patients was obtained at the Cincinnati Children's Hospital Medical Center using informed consent under an approved Institutional Review Board research protocol. iPSC were generated from peripheral blood mononuclear cells (PBMC) with lentiviral vectors or episomal plasmids as described [22, 23] from two male donors ages 15 and 25, and two female donors ages 29 and 34. Briefly, PBMC were purified from peripheral blood by density gradient separation (GE Health Care Chicago, IL) and plated at $5 \times 10^6$ cells per milliliter in X-Vivo 10 (Lonza, Walkserville, MD) supplemented with 10% fetal calf serum (FCS), recombinant human (rh-) stem cell factor, SCF (100 ng/ml), rh-thrombopoietin (TPO) (100 ng/ml), rh-interleukin (IL) IL-3 (100 ng/ml), rh-IL-6 (20 ng/ml), rh-FLT3 ligand (FLT3L) (100 ng/ml), and rh-granulomacrophage colony stimulating factor (GM-CSF) (10 ng/ml; all cytokines from Peprotech, San Jose, CA) on a low attachment plate (Corning, Corning, NY) and cultured at 37°C in 5% $CO_2$. After 2 days of culture, cells were transduced with a lentivirus containing OCT4, KLF4, MYC, and SOX2 [24] using Polybrene at a multiplicity of infection (MOI) of 20–40, or nucleofected with an Amaxa Nucleofector 4D (Lonza) with episomal plasmids containing OCT4, SOX2, KLF4, LIN28, and shp53 (Addgene Plasmid #27077, 27078, and 27080). Two days after lentiviral transduction or nucleofection, cells were plated onto irradiated mouse embryonic fibroblasts (MEF, GlobalStem Rockville, MD) in X-Vivo 10 supplemented with 10% FCS, SCF (100 ng/ml), TPO (100 ng/ml), IL-3 (10 ng/ml), IL-6 (20 ng/ml), and FLT3L (100 ng/ml). After 2 days of culture on MEF, the media was changed to hESC media (Dulbecco's modified Eagle's medium [DMEM] & Ham's F/12 (Life Technologies) 20% KnockOut Serum Replacement [KSR, Life Technologies, Carlsbad, CA], 1% L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol) supplemented with 10 ng/ml bFGF (Peprotech). Once iPSC like colonies were observed, they were plucked and passaged onto Matrigel (BD Biosciences, San Jose, CA) in mTeSR1 medium (Stem Cell Technologies, Vancouver BC, CA). All iPSC cell lines were characterized for pluripotency as described previously [25].

### Myeloid Differentiation of iPSC

Neutrophils were generated from iPSC using embryoid body (EB) based differentiation protocols modified from Lachman et al. [18]. In brief, 6 wells of confluent iPSC cultured on Matrigel were cultured in EB medium (DMEM/F12, 20% FBS, 1% NEAA) for 3 days. After 3 days, cells were washed and incubated with dispase for 5 minutes, then scored with an 18 gauge needle and scraped from the culture dish and placed in 3 wells of an ultra-low attachment plate (Corning?) in EB medium. After 4 days of culture, 15–20 EBs (starting from 1 to $2 \times 10^6$ undifferentiated iPSC) were transferred to each well of a 6-well culture dish and cultured in StemDiff APEL 2 culture media (Stem Cell Technologies) supplemented with 25 ng/ml IL-3 (Peprotech) and 50 ng/ml G-CSF (Peprotech). The EBs attached to the culture plate and formed mononuclear cell forming complexes which shed hematopoietic progenitors into the culture media. Cells including myeloid progenitors and precursors were harvested every 3 or 4 days from the culture supernatant and further differentiated to neutrophils in a culture medium consisting of RPMI medium, 10% FBS, and 100 ng/ml G-CSF for additional 5 days (hereafter called iPSC-derived neutrophils).

### Retroviral Transduction of iPSC-Derived Hematopoietic Progenitor Cells

Hematopoietic progenitors ($CD45^+$ $CD34^+$) were collected from the iPSC differentiation protocol and cultured in myeloid expansion medium containing 100 ng/ml recombinant SCF, 10 ng/ml IL-3, and 10 ng/ml GM-CSF for 12 hours, on retronectin-coated (CH-296, Takara Bio, Inc.) nontissue culture dishes. Cells were transduced with a recombinant retroviral vector expressing constitutively active human AKT mutant containing N-terminal Src myristoylation sequences (myr-AKT) and internal ribosome entry site-mouse Thy1.1 (cloned into MiT vector, kindly supplied by Dr. David Hildeman, Cincinnati Children's Research Foundation, Cincinnati, OH) for 8 hours. In parallel, cells were transduced with empty vector (mock) expressing mouse Thy1.1. Forty-eight hours post-transduction, the transduced iPSC-derived leukocytes were isolated by fluorescence assisted cell sorting (FACS) for cells double positive for human $CD45^+$ and the mouse $CD90.1^+$ marker transgene (FACSAria Cell Sorter, BD Biosciences). After sorting, the $CD45^+$ $CD90.1^+$ cells were cultured in neutrophil differentiation medium containing 50 ng/ml G-CSF for 5 days. Neutrophils transduced with empty vector or—myr-AKT were analyzed for in vitro reactive oxygen species (ROS) generation, and *Escherichia coli* phagocytosis both in vitro and in vivo. The effect of the expression of myr-AKT on the survival of iPSC-derived neutrophils was analyzed by measuring the cell surface binding of annexin V according to the manufacturer's

instructions (BD Pharmingen). Early apoptosis was determined as annexin V—positive events on $CD45^+$ hematopoietic cells after gating out large residual dead cells and debris with 7-AAD (Invitrogen).

### Morphological, Immunophenoypic, and Signaling Characterization of iPSC-Derived Neutrophils

At the end of granulopoietic differentiation, cells were cytospun onto a Superfrost Plus Microscope slide (Fisherbrand, Thermo-Fisher Scientific, Waltham, MA). The slides were Wright–Giemsa stained and scored for myeloid cell types (promyelocytes, myelocytes, metamyelocytes, bands, neutrophils, and monocytes) using an upright microscope (Motic BA310). For the immunophenotypic characterization of neutrophils, cells were stained for CD45-Pacific Blue (clone HI30, catalog #560367), CD34-PECy7 (clone 581, catalog #561440), CD33-APC (clone WM53, catalog #551378, CD11b-5APCCy7 (clone ICRF44, catalog #557754, clone ICRF44), and CD16-PE (clone W6D3, catalog #562370), and CD66b (clone G10F5, catalog #561927) from BD Biosciences. The neutrophil population (defined as $CD45^+/CD34^-/CD14^-/CD11b^+/CD16^+$) was gated and used for analysis of ROS generation, bacterial phagocytosis and phospho-FACS in a FACS-Canto flow cytometer (BD Biosciences).

### Analyses of ROS, Bacterial Phagocytosis, and Phospho-FACS of iPSC-Derived Neutrophils

Neutrophils derived from iPSC were analyzed for ROS generation and *E. coli* phagocytosis in vitro by FACS analyses. To quantify ROS generation, iPSC-derived neutrophils generated as described above and G-CSF stimulated human peripheral blood (PB) neutrophils were treated with 50 μM cell permeable 2′,7′-dichlorofluorescin diacetate (DCFDA, Invitrogen Molecular Probes) for 30 minutes in RPMI medium containing 10% FBS. Cells were washed with phosphate-buffered saline (PBS), and analyzed for ROS generation using flow cytometry by measuring the fluorescence emission of the 2′,7′-dichlorofluorescein generated by ROS induced oxidation of DCFDA. The ratio of mean fluorescence intensity (MFI) between DCFDA treated and control vehicle treated neutrophils was plotted.

For evaluation of *E. coli* phagocytosis, iPSC-derived and human PB neutrophils were stimulated by G-CSF and incubated with pH sensitive fluorophore tagged *E. coli* (pHrodo-*E. coli*, Invitrogen Molecular Probes) in Hanks' balanced saline solution containing 1% bovine serum albumin (BSA), 2 mM $CaCl_2$ and 1 mM $MgCl_2$, for 45 minutes. Cells were washed with PBS twice followed by flow cytometric and microscopic analyses. The internalization of *E. coli* particles was measured by analyzing the percentage of *E. coli*$^+$ neutrophils, and quantifying the ratio of MFI between *E. coli* treated and control neutrophils. To determine the effect of myr-AKT expression, iPSC-derived neutrophils generated from empty vector or myr-AKT transduced hematopoietic progenitors were analyzed for *E. coli* phagocytosis.

For phospho-FACS analyses of activated AKT (phopsho-AKT-S473), iPSC-derived and human PB neutrophils were cytokine starved in PBS for 3 hours. The cytokine starved neutrophils were treated with G-CSF (50 ng/ml), N-formylmethionine-leucyl-phenylalanine (fMLP, 10 μM) or interleukin-8 (IL-8, 25 ng/ml) for different time periods followed by fixation of with Cytofix solution (BD Biosciences, San Jose CA) for 30 minutes on ice. The fixed cells were permeabilized using Cytofix/Cytoperm permeabilization reagent (BD) and then washed twice with 1× Perm/Wash buffer containing 0.5% mouse serum. Cells were treated with Alexa-647 tagged isotype control IgG stained (Cell Signaling Technologies, catalog #3900) or phospho-AKT-S473 (Cell Signaling Technologies, catalog #4075) antibodies for 30 minutes on ice. Cells were washed, resuspended in PBS and FACS analyzed. The ratio of the mean fluorescence intensity of Alexa Fluor 647 between phospho-AKT and control IgG treated neutrophils was evaluated and presented. To evaluate the G-CSF induced ERK1/2 and STAT3 activation, the fixed and permeabilized cells were stained with Alexa-647 tagged isotype control IgG or phospho-ERK1/2 (Cell Signaling Technologies, catalog #612593) or phospho—Stat3 (Cell Signaling Technologies, Danvers, MA, catalog #557815) antibodies for 30 minutes on ice. Cells were washed, resuspended with PBS, and FACS analyzed. The MFI ratio of Alexa Fluor 647 between phospho-ERK1/2 or phospho-STAT3 and control IgG stained neutrophils was evaluated and presented.

#### *Confocal Immunofluorescence Microscopic Analyses for Neutrophil Extracellular Traps Formation*

G-CSF primed iPSC-derived and human PB neutrophils were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) in a poly-L-lysine coated glass chamber slide for 1 hour. PMA treated neutrophils were fixed using 4% paraformaldehyde for 30 minutes at 4°C followed by blocking with 5% protease free BSA in PBS for 30 minutes. The slides were stained with primary antibodies; anti-neutrophil elastase (Chicken IgY; kindly provided by Dr. Marshall Horwitz, University of Washington, Seattle, WA) or anti-myeloperoxidase (MPO) (Rabbit IgG) at 4°C overnight. The cells were washed and then treated with secondary antibodies (from Life Technologies) Goat anti-Chicken IgY Alexa Fluor 568 or goat anti-rabbit IgG Alexa Fluor 568 at 1:500 vol/vol concentration for 1 hour at room temperature. Cells were washed and mounted using Gold Antifade mounting media (Life Technologies) containing DAPI or treated with Sytox-green (Invitrogen) at the time of secondary antibody staining and then washed and mounted using Vectashield mounting media (Vector Laboratories, Burlingame, CA). The stained cells were analyzed for NET formation using a LSM 710 confocal microscope system (Carl Zeiss) equipped with an inverted microscope (Observer Z1, Zeiss) using a Plan Apochromat ×63 1.4 NA oil immersion lens. The chromatin spreading was analyzed by measuring the spreading of DAPI and SYTOX staining.

### Migration and Phagocytosis of Human or iPSC-Derived Neutrophils In Vivo in a Model of Bacterial Peritonitis in NSG Mice

To deplete mice neutrophils, anti-Gr1 antibody (clone 1A8, 500 μg, BioXCell) was intraperitoneally (i.p.) injected in NSG mice every 24 hours for 3 days [26]. Twenty-four hours after the last dose of anti-Gr1 antibody, $\sim 10^6$ human leukocytes isolated from healthy volunteers, or iPSC-derived neutrophils previously transduced with empty vector or myr-AKT, were injected intravenous (i.v.) in a final volume of 200 μl of PBS. After 15 minutes the mice received an i.p. injection of pHrodo Green *E. coli* conjugated BioParticles (200 μg, Invitrogen Molecular Probes) and fMet-Leu-Phe (fMLP) (15 μg) in 200 μl PBS [27]. After 1 hour, the animals were euthanized by $CO_2$ asphyxiation and peritoneal exudates were harvested in two successive washes with 10 ml cold PBS containing 0.2% BSA. Human iPSC-derived or PB-derived neutrophils were identified in the peritoneal exudates with hCD45,

hCD11b, and hCD16 staining, and phagocytosis was identified through uptake of pHrodo Green *E. coli* conjugated BioParticles.

### Statistical Analysis

All data are presented as mean ± SD. Statistical analyses were performed using Student's *t* test or analysis of variance test when more than two groups were compared. Statistical significance was defined as $p < .05$.

## RESULTS

### Neutrophil Differentiation from iPSC

In these studies, iPSC-derived neutrophil differentiation was initiated using embryoid body formation in suspension without the addition of exogenous cytokines to enable the spontaneous differentiation of the embryonic germ layers similar to other published studies [28, 29]. A key component of this differentiation method is that at 4–5 days after formation the embryoid bodies (EB) are switched into adherent culture conditions where they can generate their own stroma. Although a variety of different cytokine cocktails have been used in other studies, this study used IL-3 and G-CSF to support ongoing hematopoiesis, myelopoiesis, and granulopoiesis, over approximately 1 month of culture. Similar to other studies using comparable methodologies, abundant stroma developed surrounding the embryoid bodies as adherent cells migrated out, followed by the generation of cobblestone areas with adherent hematopoietic cells attached (Fig. 1A, 1B). At approximately 14–21 days after initiating differentiation, there was a peak release or "shedding" of cells into the media (Fig. 1C). The vast majority of these cells were hematopoietic (>95% $CD45^+$ cells). Specifically, most hematopoietic cells were a combination of myeloid progenitors ($CD34^+CD33^+$) and myeloid precursors ($CD34^-CD33^+$; Supporting Information Fig. S1A). The myeloid progenitors/precursors were further differentiated in culture media containing G-CSF (Neupogen, 50 ng/ml) without SCF, IL-3, or GM-CSF, for terminal neutrophil differentiation (Fig. 1A). There was continued production of hematopoietic cells ($CD45^+CD34^-$) for several more weeks, although at a lower pace (Fig. 1C). At the conclusion of the terminal neutrophil differentiation, there was a high frequency of neutrophils generated (∼30%–50%) as evaluated by flow cytometry analysis for CD11b, and CD16 cell surface expression (Fig. 1D). Furthermore, the cell surface expression of CD66b confirmed the generation of iPSC-derived neutrophils (Supporting Information Fig. S1E). To further validate the results obtained from FACS analyses, we performed Wright–Giemsa staining of the cells at the end of terminal differentiation. The Wright–Giemsa staining of hematopoietic cells obtained from different iPSC lines showed the presence of 40%–50% neutrophils in addition to the immature granulocytic cells and nearly 10% monocytes (Fig. 1F; Supporting Information Fig. S1B–S1D).

### Human Induced Pluripotent Stem Cell-Derived Neutrophils are Compromised in *E. coli* Phagocytosis with Reduced G-CSF Induced AKT Activation

Our group already identified a deficient neutrophil differentiation process in iPSC-derived granulocyte precursors as a possible source of dysfunctional neutrophils [19]. To investigate whether the terminal neutrophils generated from hematopoietic differentiation of iPSC are functional, we analyzed the bonafide characteristics of neutrophils, such as generation of ROS, phagocytosis of *E. coli* and NET formation, and compared these to G-CSF primed human PB neutrophils (isolated from seven different donors, Table 1). We analyzed the neutrophils derived from three independent normal donor-derived iPSC lines (iPSC 12E, iPSC 28L, and iPSC 35L) which were selected because of their ability to generate higher outputs of neutrophils upon differentiation. As shown in Figure 2A, and 2B, human iPSC-derived neutrophils produced similar or higher levels of ROS species in comparison to G-CSF primed healthy donor PB neutrophils. The increased production of ROS could be due to the continued in vitro culture in neutrophil differentiation medium containing human G-CSF, where PB neutrophils were primed with human G-CSF for 30 minutes. Next, we examined the phagocytic ability of iPSC-derived neutrophils by measuring the internalization of pH sensitive fluorochrome coupled attenuated *E. coli* (pHrodo-*E. coli*) in an in vitro phagocytosis assay. The PB- and iPSC-derived neutrophils were primed with G-CSF for 30 minutes followed by incubation with pHrodo-*E. coli* in a $Ca^{2+}$ and $Mg^{2+}$ containing medium. The iPSC-derived neutrophils are less efficient in internalizing the *E. coli* than healthy donor PB-derived neutrophils, as demonstrated by a significantly decreased number of *E. coli*$^+$ neutrophils as well as a reduced MFI (Fig. 2C, 2E). This effect was not cell line dependent, since neutrophils derived from different iPSC lines show similar levels of *E. coli* phagocytosis. The granulopoietic differentiation of iPSC-derived myeloid precursors generated nearly 10% monocytes (Supporting Information Fig. S1B–S1D). The phagocytic ability of PB monocytes and iPSC-derived monocytes were comparable (Supporting Information Fig. S1E), suggesting that monocytes derived from iPSC also contribute to bacterial phagocytosis with a potential therapeutic benefit. Furthermore, we analyzed the PMA induced NET-forming ability of iPSC-derived neutrophils and compared this with PB neutrophils. As shown in Figure 2F and Supporting Information Figure S2, PMA induced NET formation is highly compromised in iPSC-derived neutrophils in comparison to PB neutrophils.

To further understand the underlying mechanisms that are responsible for impaired phagocytosis and NET formation, we analyzed the G-CSF induced signaling pathways such as activation of AKT, STAT5, and p42/44 MAPK (pERK) in iPSC-derived neutrophils and compared these with G-CSF induced activation in human PB neutrophils. As shown in Figure 2H, and 2I, G-CSF induced activation of AKT (phospho AKT-S473) is dramatically impaired in iPSC-derived neutrophils in comparison to human PB neutrophils. Pathologically relevant chemoattractants fMet-Leu-Phe (fMLP) or interleukin-8 (IL-8) activate AKT in respiratory burst and exocytosis [30] (Supporting Information Fig. S3A, S3B). Stimulation with fMLP or IL-8 did not significantly activate AKT in iPSC-derived neutrophils (Supporting Information Fig. S3A, S3B) demonstrating that iPSC-derived neutrophils are insensitive to AKT activation by multiple agonists. To define whether the defective AKT activation is unique or is part of a large f signaling deficiency in iPSC-derived neutrophils, we analyzed the levels of activation of two distinct downstream targets, p42/p44 MAPK and STAT3, in neutrophils derived from healthy donor PB and iPSC. Unlike AKT, similar levels of activation in the p42/44 MAPK and STAT3 signaling pathways were observed between neutrophils derived from iPSC and human PB (Supporting Information Fig. S3C, S3D). Furthermore, exposure to IL-8, tumor necrosis factor-α (TNF-α), IL-1, IL-2, interferon-c (IFNc), granulocyte–macrophage colony-

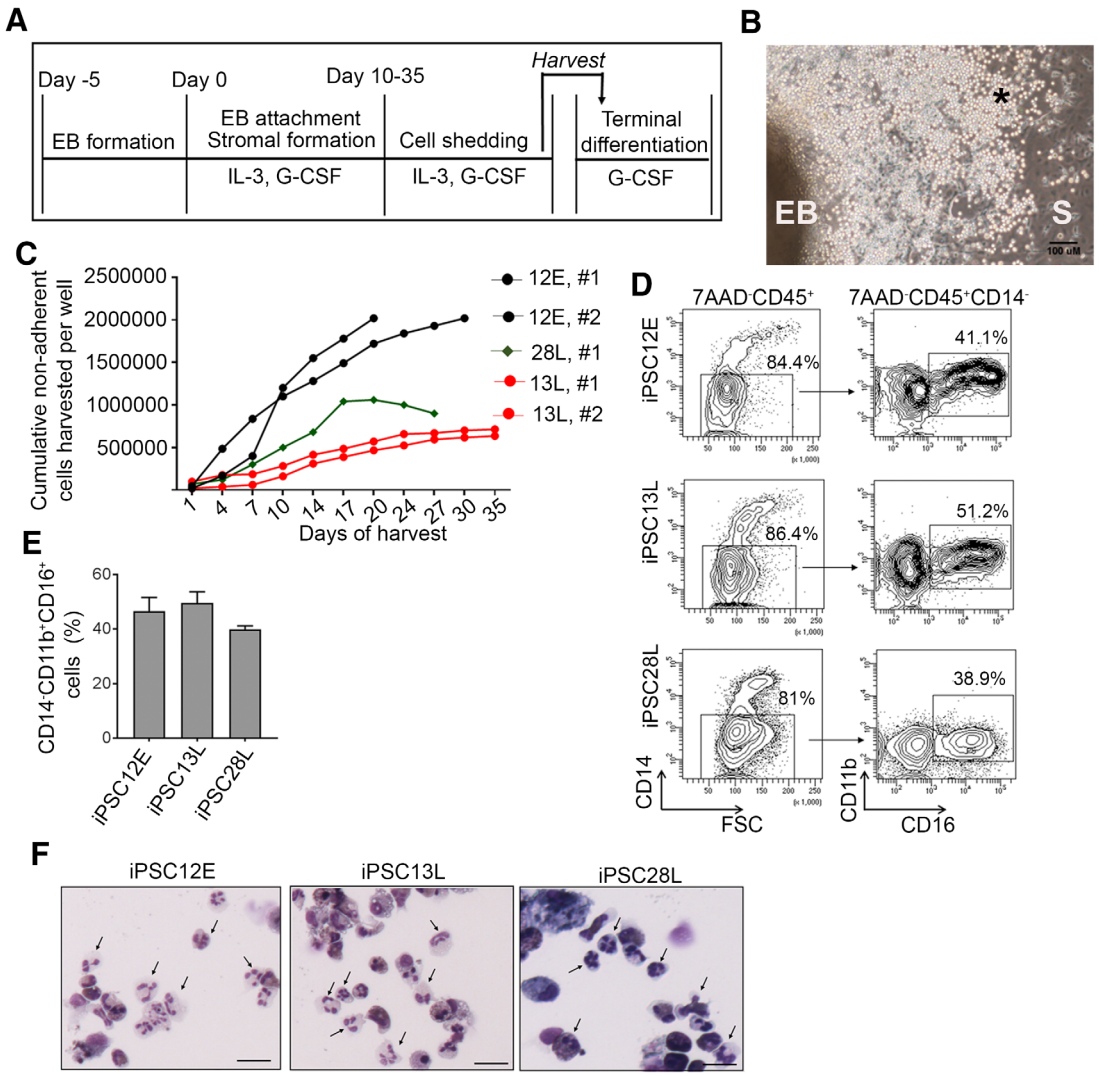


**Figure 1.** Myeloid differentiation from induced pluripotent stem cells (iPSC). **(A):** Overview of the differentiation process from iPSC to mature neutrophils. EB embryoid body **(B):** Microscopic image of the shedding of hematopoietic cells from attached embryoid bodies. Scale bar: 100 µm. EB-attached embryoid body. *, Nonadherent hematopoietic progenitors shed from stromal layer (S). **(C):** Cumulative equivalent number of cells harvested from each well of a 6-well plate throughout the 25–35 days differentiation process. **(D):** FACS contour plots showing neutrophil differentiation ($CD14^{-}CD11b^{+}CD16^{+}$) of iPSC12E, iPSC13L, and iPSC28L. **(E):** FACS quantification of neutrophil differentiation of iPSC12E, iPSC13L, and iPSC28L lines. **(F):** Wright–Giemsa stained images of neutrophil differentiation of iPSC lines. Arrows mark mature neutrophils. Bar = 20 µm. Data are presented as mean ± SD of a minimum of two independent experiments.

stimulating factor (GM-CSF), or granulocyte colony-stimulating factor (G-CSF) delays the apoptosis of neutrophils through p38-MAPK mediated AKT activation [31–33]. However, in a mouse genetic model it has been shown that Akt2, but not Akt1, is critical for chemoattractant-induced neutrophil $O_2^-$ production, degranulation, and cell migration [34]. AKT2 is also required for PMA-induced $O_2^-$ production independent of PI3K [34]. We speculate that the impaired activation of AKT might attenuate the phagocytic and NET forming abilities of iPSC-derived neutrophils.

## Expression of Myristoylated, Constitutively Active AKT (myr-AKT) Enhances the *E. coli* Phagocytosis and NET Forming Ability of iPSC-Derived Neutrophils

The myristoylation of AKT fusion protein is constitutively active through its targeting to cholesterol rich plasma membrane microdomains [35]. To ectopically express the constitutively active AKT, we transduced iPSC derived hematopoietic progenitors with recombinant retroviruses carrying empty vector or constitutively active AKT mutant containing N-terminal Src myristoylation sequences (myr-AKT). The transduced cells, now expressing CD90.1 as a reporter, were cultured in granulopoietic differentiation medium for neutrophil generation. The iPSC-derived neutrophils expressing myr-AKT were analyzed for *E. coli* phagocytosis and PMA induced NET formation, and compared with G-CSF primed PB neutrophils. As expected, the expression of myr-AKT increased the survival of iPSC-derived neutrophils (Supporting Information Fig. S4A, S4B). However, the expression of myr-AKT did not affect the levels of neutrophil maturation (Supporting Information Fig. S4C). The expression of myr-AKT resulted in increased *E. coli* phagocytosis of iPSC-derived neutrophils as measured by the percentage of *E. coli*$^{+}$ neutrophils (Fig. 3A, 3B). Also, the number of *E. coli* per neutrophil is significantly increased upon expression of myr-AKT

**Table 1.** Migration of human neutrophils in mouse peritoneal cavity and phagocytosis of *Escherichia coli*

| Healthy donor | Age/ gender | WBC count ($10^3$/μl) | Neutrophil count ($10^3$/μl) | Human neutrophils in PF/$10^5$ infused neutrophils | % phagocytosis of *E. coli* by neutrophils (in vivo) | % phagocytosis of *E. coli* by neutrophils (in vitro) | ROS levels (MFI ratio) |
|---|---|---|---|---|---|---|---|
| Donor 1 | 32/F | 4.9 | 2.21 | 95 | 71.8 | **72.8** | **35.5** |
| Donor 2 | 35/F | 8.3 | 3.56 | 21 | 79.4 | **73.7** | **32.7** |
| Donor 3 | 35/M | 4 | 1.89 | 13 | 91.6 | 87.7 | 29.1 |
| | | 8 | 4.92 | 311 | 92.4 | 89.2 | 38.1 |
| | | 7.4 | 4.18 | 291 | 84.7 | **71.9** | **43.2** |
| Donor 4 | 38/M | 4.3 | 2.4 | 143 | 44.1 | 65.7 | 28.2 |
| | | 4.1 | 1.88 | 262 | 42.4 | 77.8 | 37.8 |
| | | 5.2 | 2.1 | 76 | 54.4 | 83.7 | 53.6 |
| Donor 5 | 33/M | 7.2 | 3.75 | 9 | 82.5 | **81.1** | **50.7** |
| Donor 6 | 30/F | 7.3 | 3.72 | 142 | 38.7 | **88.9** | **28.7** |
| Donor 7 | 33/F | 7.5 | 4.65 | 100 | 48.3 | N/A | N/A |

Leukocytes isolated from healthy volunteers were primed with G-CSF for 15 minutes and transfused (IV) in anti-Gr1 antibody treated NSG mice. After 15 minutes, mice received (IP) pHrodo Green *E. coli* and fMLP (15 μg). Peritoneal exudates were harvested after 1 hour, and neutrophil migration from circulation to peritoneal cavity and phagocytosis of *E. coli* by migrated neutrophils was assessed. For in vitro phagocytosis G-CSF primed neutrophils were incubated with pHrhodo Green *E. coli* for 45 minutes at RT.
Abbreviation: N/A, not available; MFI mean fluorescence intensity; ROS reactive oxygen species.

in iPSC-derived neutrophils (Fig. 3C). The increased phagocytosis of myr-AKT expressing neutrophils was further validated by FACS analyses of the internalization of *E. coli* (Supporting Information Fig. S5A–S5C), although the MFI of *E. coli* treated neutrophils was partially rescued. In addition to ROS generation and phagocytosis, neutrophils have been shown to form NETs to capture and kill pathogens [36]. We analyzed the PMA induced NET-forming ability of iPSC-derived neutrophils and compared this with PB neutrophils. As shown in Figure 3D, PMA treatment induced the formation of NET in PB neutrophils. However, the PMA induced NET-forming ability of iPSC-derived neutrophils was highly impaired. The myr-AKT expression in iPSC-derived neutrophils led to NET formation in response to PMA treatment as shown by confocal immunofluorescence microscopic images and the chromatin spreading (Fig. 3D, 3E; Supporting Information Fig. S5D, S5E). In the absence of PMA, expression of myr-AKT does not induce NET formation under granulopoietic differentiation culture conditions (data not shown).

### Expression of myr-AKT Enhances the Phagocytic Ability of iPSC-Derived Neutrophils In Vivo

To investigate whether iPSC-derived neutrophils can migrate from the circulation to the peritoneal fluid, and whether they efficiently phagocytose *E. coli* in vivo, $10^6$ healthy donor PB leukocytes or iPSC-derived neutrophils were injected intravenously into neutropenic NSG mice. pHrodo-*E. coli*, a nonviable, fluorescently labeled bacteria, and the stimulant fMLP were coinjected intraperitoneally to induced the migration of PB or iPSC-derived neutrophils from the circulation to the peritoneal fluid, and the phagocytosis of *E. coli* by the neutrophils pervaded into the peritoneum was assessed by flow cytometry (Fig. 4A; Supporting Information Fig. S6A). Our results from three independent experiments using seven different healthy donor PB and iPSC-derived neutrophils from three independent iPSC exhibited similar levels of migration of neutrophils from circulation into the peritoneum of NSG mice in presence of fMLP and *E. coli* (Fig. 4B, 4C; Supporting Information Fig. S6A–S6C; Table 1). To further examine whether AKT activation is critical for iPSC-derived neutrophil migration and *E. coli* phagocytosis, iPSC derived hematopoietic myeloid progenitors ($CD34^+$-$CD33^+$) were transduced with empty or myr-AKT retroviral vectors, and cultured in neutrophil differentiation medium. Overexpression of myr-AKT in iPSC-derived neutrophils had no effect on fMLP and *E. coli* induced neutrophil migration from the circulation into the peritoneal cavity (Fig. 4A, 4C), suggesting that AKT activation is not critically required for the migration of iPSC-derived neutrophils. We next evaluated the in vivo phagocytic activity of neutrophils infiltrated into the peritoneum of NSG mice. We found that iPSC-derived neutrophils from each of the three donor lines showed significantly reduced levels of *E. coli* phagocytosis in comparison to healthy donor PB neutrophils. However, overexpression of myr-AKT in these iPSC-derived neutrophils significantly increased the phagocytosis, compared with empty vector transduced iPSC neutrophils (Fig. 4D, 4E). In addition, overexpression of myr-AKT in iPSC-derived neutrophils increased the phagocytic ability as measured by MFI of *E. coli* in neutrophils (Fig. 4F). Taken together, these data demonstrate that iPSC-derived neutrophils are less efficient in *E. coli* phagocytosis, and expression of myr-AKT significantly enhances the phagocytic ability of iPSC-derived neutrophils.

## Discussion

Our data provides proof-of-concept for the development of a system capable of producing unlimited numbers of effective terminally differentiated neutrophils derived from iPSC. Effective iPSC-derived neutrophils were generated with enhancement of AKT activity in iPSC derived hematopoietic progenitors and their differentiated progeny. These iPSC derived myr-AKT expressing neutrophils had relatively conserved functional ability to phagocytose and induce NETs in vitro and circulate and phagocytose bacteria in vivo.

This approach is radically different from our current approach to the transfusion of neutrophils, where donor-derived neutrophils are collected through leukapheresis of healthy donor

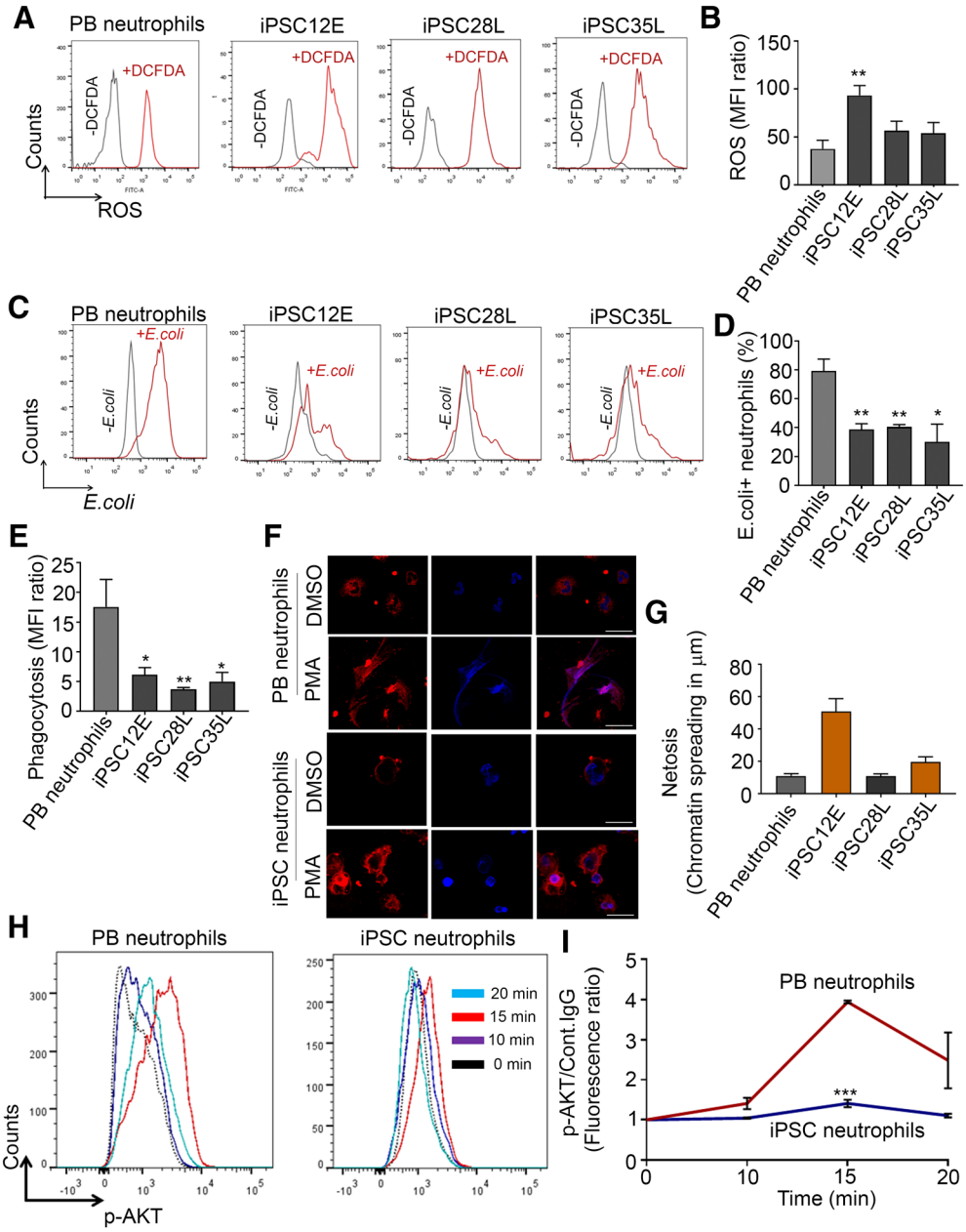


**Figure 2.** Analyses of reactive oxygen species (ROS), phagocytosis, and neutrophil extracellular traps (NET) formation of human PB and induced pluripotent stem cells (iPSC) derived neutrophils. **(A):** FACS histogram plots showing fluorescence emission resulting from the oxidation of cell permeant reagent 2′,7′-dichlorofluorescin diacetate (DCFDA) by intracellular ROS to 2′,7′-dichlorofluorescin (DCF) in healthy donor PB and iPSC-derived neutrophils. **(B):** Quantification of ROS levels in PB and iPSC-derived neutrophils as measured by mean fluorescence intensity (MFI) of DCF. **(C):** FACS histogram plots showing the phagocytosis of pH sensitive fluorescent *Escherichia coli* (pHrodo-*E. coli*) by PB and iPSC-derived neutrophils. **(D):** Quantification of *E. coli* phagocytosis as measured by the percentage of *E. coli*$^{+}$ neutrophils. **(E):** Quantification of *E. coli* phagocytosis as measured by ratio of the MFI of control (DMSO) and *E. coli* treated neutrophils. **(F):** Confocal microscopic images of PB and iPSC-derived neutrophils treated with phorbol 12-myristate 13-acetate (PMA) to induce NET formation. **(G):** Quantification of chromatin spreading (maximum length) during NET formation as measured by confocal microscopy. **(H):** FACS histogram plots showing G-CSF induced activation of AKT (phospho-AKT-S473) in PB and iPSC-derived neutrophils. **(I):** Quantification of AKT activation as measured by the ratio of the MFI of Alexa Fluor 647-anti-pAKT and Alexa Fluor 647-control IgG. Scale bars = 20 µm data are presented as mean ± SD of a minimum of two independent experiments. *, $p < .05$; **, $p < .01$; ***, $p < .001$.

volunteers. Normal donors do not have very high levels of circulating neutrophils in the peripheral blood, which results in doses of neutrophils that are only likely to be sufficient for small children [37, 38]. In addition, the exposure of a healthy volunteer donor to any form of medication with potential side effects presents ethical and safety issues [39–41]. Currently, two types of healthy-donor derived neutrophils are used in the clinic. In Europe, most neutrophil products are derived from pools of buffy coats of whole blood donations [42]. In the USA and Canada, healthy donors receive mobilizing agents (usually steroids and/or G-CSF) and are connected to an apheresis device that separates neutrophils from the peripheral blood [14, 43].

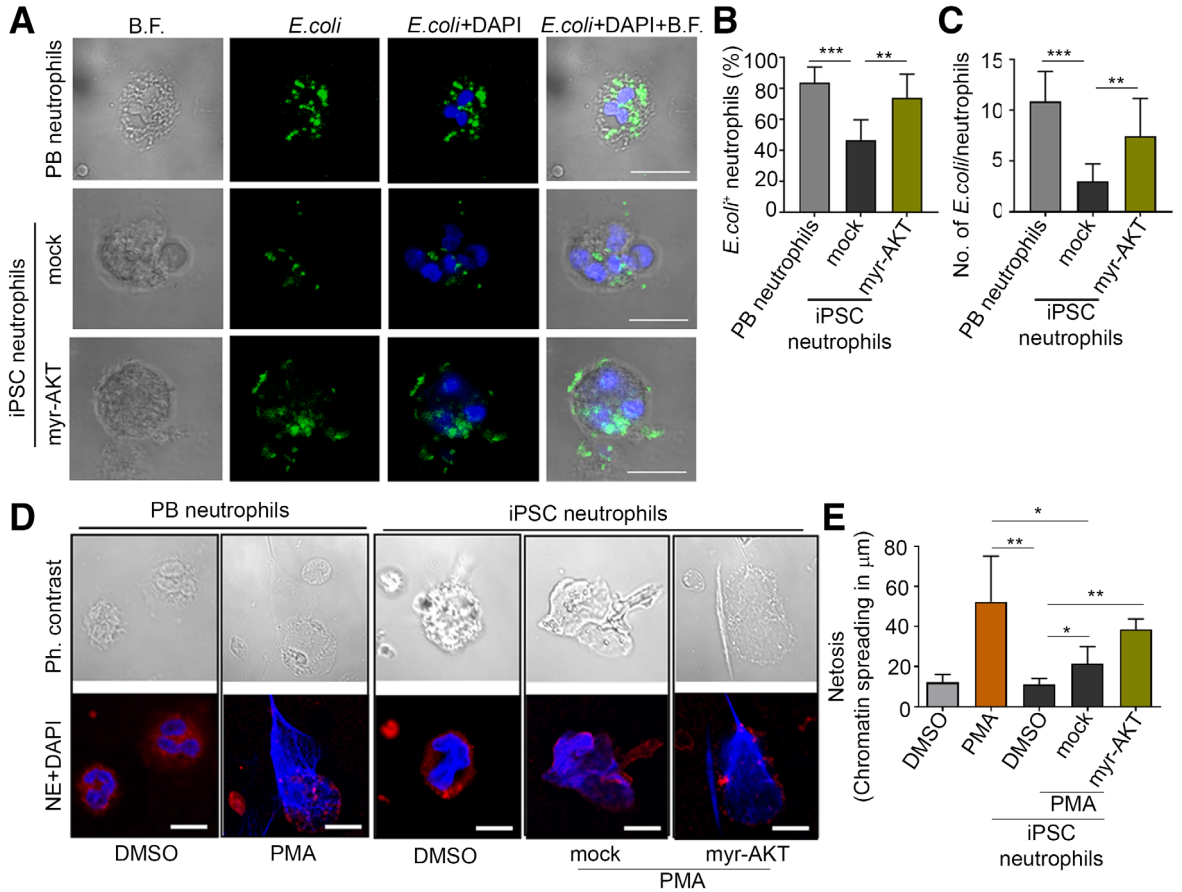


**Figure 3.** Forced expression of myristoylated AKT (myr-AKT) rescues impaired phagocytosis and NETosis of induced pluripotent stem cells (iPSC) derived neutrophils. Human PB neutrophils and mock/myr-AKT expressing iPSC myeloid differentiation cultures were treated with pH sensitive pHrodo-*Escherichia coli* in the presence of $CaCl_2$ and $MgCl_2$ for 45 minutes. Cells were surface stained for human neutrophil markers, and flow analyzed to evaluate the phagocytosis of *E. coli* by neutrophils. **(A):** Representative fluorescence microscopic images of the pHrodo-*E. coli* phagocytosis by healthy donor PB neutrophils and mock/myr-AKT expressing iPSC-derived neutrophils. **(B, C):** Quantification of the percentage of *E. coli*$^+$ neutrophils (B) and number of *E. coli* phagocytosed by each neutrophils (C). **(D):** Representative confocal microscopic images showing phorbol 12-myristate 13-acetate (PMA) induced NET formation of healthy donor PB neutrophils and mock/myr-AKT expressing iPSC-derived neutrophils. **(E):** Quantification of PMA induced NET formation as measured by chromatin spreading of healthy donor PB neutrophils and mock/myr-AKT expressing iPSC-derived neutrophils. Scale bars = 5 µm. Data are presented as mean ± SD of a minimum of two independent experiments. *, $p < .05$; **, $p < .01$; ***, $p < .001$.

Myeloid progenitors derived from hematopoietic stem cells and expanded ex vivo represent an alternative that is being tested in clinical trials (Romyelocel-L; Cellera Therapeutics, Inc.). These progenitors require in vivo expansion by G-CSF, have been shown to be efficacious in animal models of congenic transfusion [44, 45] and to be safe in Phase I clinical trials (NCT01297543 and NCT00891137) and efficacious in a Phase II clinical trial in acute myelogenous leukemia therapy (NCT02282215) [46, 47]. In this study, 76% of patients receiving myeloid progenitor-derived granulocytes showed a decrease in the incidence of serious infections compared with the control arm. The average total days in the hospital was 3.2 days less than in the control group, and there was a 29% reduction in the number of patients treated for a diagnosed infection, and a 32% decrease in microbiologically defined bacterial or fungal infections in the experimental arm versus the control arm, with no deaths due to infection in the experimental arm versus two in the control arm. Unfortunately, whether these myeloid progenitor products are efficacious in the context of allogeneic transfusion in other patient populations like, such as severe congenital neutropenia patients, is unclear since no significant circulation of allogeneic neutrophils derived from ex vivo expanded myeloid progenitors has been found in patients or in animal models [46, 47]. Our data indicate that the population generated from iPSC is heterogenous and includes myeloid precursors and monocytes which have phagocytic activity and a potential therapeutic benefit. Based on this data, we may speculate that the transfusion of iPSC-derived myeloid progenitors and precursors as well as monocytes may result in a better outcome for patients, in theory, and unlimited source of genetically manipulated cells readily available for harvest and transfusion.

Neutrophils are a critical cellular component of the innate immune response and work by releasing the superoxide anion free radical ($O_2^-$) and its toxic metabolites against invading microorganisms [48, 49]. The NADPH enzyme can release superoxide anions. In resting neutrophils, the NADPH oxidase complex consists of unassembled cytosolic and membrane components. After activation of neutrophils by chemoattractants, chemokines, complement components, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac-2 as the cytosolic components translocate to plasma, then

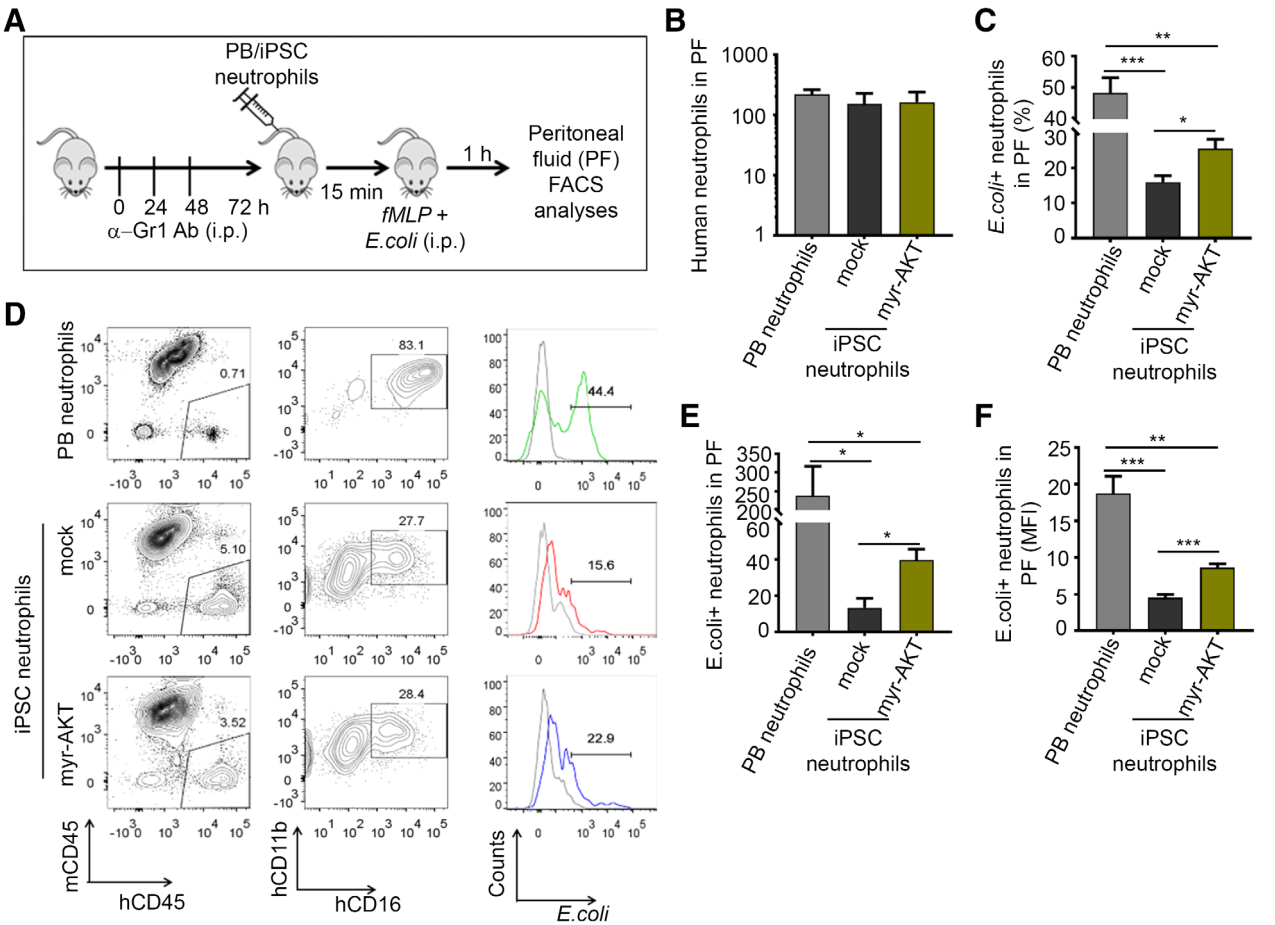


**Figure 4.** Expression of myr-AKT enhances the *Escherichia coli* phagocytosis of induced pluripotent stem cells (iPSC) derived neutrophils in vivo. **(A):** Schematic diagram of N-formylmethionine-leucyl-phenylalanine (fMLP) induced in vivo migration of healthy donor PB neutrophils and mock or myr-AKT expressing iPSC-derived neutrophils from PB to the peritoneal fluid (PF) and phagocytosis of pHrodo-*E. coli* particles. **(B):** FACS contour plots and histograms showing *E. coli*$^{+}$ neutrophils in the peritoneal fluid of NSG mice. **(C):** Quantification of the number of neutrophils (healthy donor PB and mock or myr-AKT expressing iPSC derived) in the peritoneal fluid (PF) of NSG mice. Expression of myr-AKT does not modify the in vivo migration of iPSC-derived neutrophils toward fMLP gradient. **(D, E):** FACS quantification of the percentage (D) and number (E) of *E. coli*$^{+}$ human neutrophils in the peritoneal fluid of NSG mice. **(F):** Quantification of the mean fluorescence intensity of *E. coli*$^{+}$ human neutrophils in the peritoneal fluid. Data are presented as mean ± SD of a minimum of three independent experiments. *, $p < .05$; **, $p < .01$; ***, $p < .001$.

interact with the flavocytochrome b558 for the activation of NADPH oxidase. Hence, activated neutrophils evoke a respiratory burst in which the oxygen consumption is increased and large amounts of ROS are generated [31]. Neutrophils derived from iPSC are quite similar in their immunophenotype and transcriptome, and in some crucial neutrophil functions (e.g., ROS production) but some signaling pathways and differentiation patterns may remain suboptimal in establishing the efficacy of these neutrophils [17–19]. Although the formation of NETs and neutrophil phagocytosis are controversial as an efficacy parameter, with potential undesirable consequences due to unspecific tissue damage, they are major antimicrobial mechanisms in vivo [50, 51] and thus a major area of research is to boost phagocytic activity of neutrophils generated from iPSC. AKT is a well-known inhibitor of apoptosis [52], prolongs cell survival and myeloid differentiation [53], and acts as a direct molecular switch to regulate the formation of the NET-apoptosis axis [54]. Although different forms of AKT have been shown to have different effects on neutrophil function, in general, AKT activation in neutrophils is required for the activation of NETs and phagocytosis [55]. AKT directly interacts with and phosphorylates Ser304 and Ser328 on $p47^{phox}$, then regulates the respiratory burst activity of neutrophils [49]. Additionally, the activation of AKT also participates in neutrophil chemotaxis [49, 56] and, as mentioned earlier, is responsible for superoxide production after stimulation [34]. Specifically, AKT, through its AKT2 form, is directly responsible for cell migration, granule enzyme release, and superoxide production, as well as phagocytosis by neutrophils [34]. The PI3K–AKT axis also plays a central role in Toll-like receptor 2 (TLR2)-induced activation of neutrophils [57, 58]. TLR2-mediated activation of AKT can phosphorylate the p65 subunit of NF-κB and promote NF-κB translocation to the nucleus, resulting in rapid release of pro-inflammatory cytokines and chemokines such as TNF-α and macrophage inflammatory protein 2 [59]. Therefore, AKT has a central role in the potentiation of TLR2-associated neutrophil responses. In this study, we used an active (myristoylated form) of AKT which results in activation of all downstream pathways dependent on AKT, and leads to increased survival, chemotaxis, ROS production, phagocytosis and formation of NETs as assessed in in vitro and in vivo.

## Conclusion

Our data indicate that genetically corrected neutrophils derived from iPSC were able to circulate and phagocytose bacteria in a model of bacterial peritonitis. These results along with the clinical experience using myeloid progenitor derived neutrophils for the prophylaxis of sepsis in neutropenic patients provide proof-of-concept that these unlimited source of granulocytes from myeloid progenitors, which have the ability to circulate and target infection sites, can be used for the prophylaxis and therapy of sepsis in neutropenic patients.

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## Disclosure of Potential Conflicts of Interest

J.A.C. discloses honoraria and consultant role with Hemanext, Inc.; Research Funding Cerus Co., Fresenius-Kabi, U.S. DoD/Westat, TerumoBCT, Hemanext, Inc. The other authors indicated no potential conflicts of interest.

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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