

## Original Article

# Umbilical cord blood-derived neutrophils possess higher viability than peripheral blood derived neutrophils

Qi Liu<sup>1,2\*</sup>, Yuliang Wu<sup>1,2\*</sup>, Genyin Chen<sup>3,4</sup>, Jiaqing Li<sup>1</sup>, Yingying Chen<sup>1</sup>, Yao Ge<sup>1,2</sup>, Chunyan Wang<sup>1</sup>, Bing Xiong<sup>1</sup>, Dandan Chen<sup>1</sup>, Xuguang Wang<sup>3,4#</sup>, Shupeng Liu<sup>1,2#</sup>, Zhongping Cheng<sup>1,2#</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China; <sup>2</sup>Institute of Gynecological Minimally Invasive Medicine, Tongji University School of Medicine, Shanghai 200072, China; <sup>3</sup>Department of Anesthesiology, Affiliated Hospital of Guangdong Medical University, Zhanjiang 524001, Guangdong, China; <sup>4</sup>Clinical Research Center, Guangdong Medical University, Zhanjiang 524001, Guangdong, China. \*Equal contributors and co-first authors. #Equal contributors.

Received September 15, 2023; Accepted February 22, 2024; Epub March 15, 2024; Published March 30, 2024

**Abstract:** Neutrophils, a primary type of immune cell, play critical roles in numerous biological processes. Both umbilical cord blood (UCB) and peripheral blood are rich in neutrophils. UCB is more abundant than peripheral blood, with cells generally at a more immature stage. However, comparative data between these two cell sources is lacking. This study aims to elucidate differences between UCB-derived neutrophils (UCBN) and peripheral blood-derived neutrophils (PBN). UCBN and PBN were isolated from fresh human umbilical cord blood and peripheral blood, respectively. Transcriptomic profiling was performed and compared against neutrophil RNA from three different donors. Bioinformatics analysis was employed to compare cell phenotypes. A cytokine cocktail (IFN- $\beta$ , IFN- $\gamma$ , and LPS) was used to activate UCBN and PBN in vitro. A united multi-omic approach, combining transcriptomic and proteomic analysis, was followed by experimental validation through flow cytometry, cell killing assays, and proteome profiler array to verify cell functions. Transcriptomic analysis revealed that the most upregulated genes in freshly isolated umbilical cord blood neutrophils (UCBN) compared to peripheral blood neutrophils (PBN) predominantly involve neutrophil activation and cell-killing functions. Validation through flow cytometry and cell-killing experiments demonstrated that highly viable UCBN exhibited significantly stronger ovarian tumor cell-killing activity in vitro compared to PBN. Both transcriptomic and proteomic analyses indicated that the primary upregulated genes in activated UCBN are chiefly involved in biological processes related to the regulation of cytokine secretion. Integrative multi-omic analysis, including a proteome profiler array, confirmed that UCBN indeed secrete elevated levels of cytokines. In conclusion: UCBN shows higher viability and cellular activity compared with PBN, particularly in tumor cell-killing and cytokine secretion.

**Keywords:** Neutrophils, umbilical cord blood, peripheral blood, viability

## Introduction

Neutrophils, a key component of the innate immune system, have been reported to play a role in tumor suppression. They induce tumor cell death in the presence of IgG1 antitumor antibodies through antibody-dependent cellular cytotoxicity (ADCC) [1]. Neutrophil cytotoxicity can be further enhanced by IgA antibodies or bispecific antibodies that combine the agonistic activity of Fc $\alpha$ RI targeting with IgG1 characteristics [2]. Additionally, neutrophils may promote long-term antitumor immunity in situ by releasing cytokines and chemokines and/or

by interacting with other immune cells such as dendritic cells (DCs) [3, 4]. Neutrophil infiltration has also been proposed as a prognostic factor in various tumors, including ovarian cancer [5, 6]. These findings suggest that neutrophils could play a significant role in cancer therapy.

However, the limited number and unstable status of autologous peripheral blood immune cells from tumor patients present challenges in developing off-the-shelf therapeutic products. To address these limitations, we have explored the use of immune cells sourced from umbilical

## High viability umbilical cord blood-derived neutrophils

cord blood (UCB) for off-the-shelf applications [7, 8]. Our previous work demonstrated that human umbilical cord blood-derived neutrophils (UCBN) exhibit anti-tumor properties in ovarian cancer following cytokine stimulation *in vitro* [9], indicating that allogeneic neutrophil transplantation might be an effective strategy for ovarian cancer therapy. Yet, there are no official reports comparing UCBN with peripheral blood-derived neutrophils (PBN).

In this study, we conducted transcriptomic profiling of freshly isolated UCBN and PBN from different donors using RNA-sequencing. The analysis revealed that the top upregulated genes in UCBN, compared to PBN, are mainly associated with neutrophil degranulation, activation, cell killing, nuclear division, nuclear chromosome segregation, and myeloid cell development. Further, UCBN exhibited high viability and functionality upon activation with a cocktail of cytokines. Subsequent transcriptomic and proteomic analyses, along with experimental verifications, confirmed that UCBN secrete higher levels of cytokines and demonstrate superior functionality compared to PBN.

### Materials and methods

#### *Isolation and activation of primary human neutrophils*

Umbilical cord blood (UCB) was collected from cesarean sections using lithium-heparin tubes (Greiner Bio-One 9mL LH, Austria) at Shanghai Tenth People's Hospital after obtaining informed consent from the mothers. Peripheral Blood (PB) samples collected in lithium-heparin tubes were obtained from adult volunteers. Studies involving human participants were reviewed and approved by the Ethical Committee of the Tenth People's Hospital. Characteristics of UCB and PB patients are listed in [Table S1](#). Neutrophils were isolated using density-gradient centrifugation with Histopaque®1077 (Sigma Aldrich, Steinheim, Germany) and Histopaque®1119 (Sigma Aldrich, Steinheim). Following erythrocyte lysis, granulocytes were washed once in 1× DPBS (Thermo Fisher, Germany) for 10 min at 800 g and resuspended in RPMI 1640 (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (Biochrom, Berlin, Germany). Isolated Neutrophils were seeded in 24-well plates ( $1 \times 10^6$  cells/well) and cultured at 37°C with 5% CO<sub>2</sub>. All procedures

were conducted at room temperature under sterile conditions. At 24 h post-isolation, neutrophils were activated with 1 ng/ml IFN-β (Cat. 300-03BC, PeproTech, USA), 50 ng/ml IFN-γ (Cat. 300-02, PeproTech) and 100 ng/ml LPS (Cat. 14011S, CST, USA) for 24 h.

#### *Neutrophil killing assay*

The human ovarian cancer cell line OVCAR3 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. OVCAR3 cells were authenticated by STR authentication (Beijing HuaKe Gene Technology Co., LTD.). Cells were cultured in the incubator at 37°C with 5% CO<sub>2</sub>. Medium was routinely replaced every 2-3 days. For the neutrophil killing assay, a total of  $5 \times 10^3$  ovarian cancer cells were first seeded in a 96-well plate. At 24 h after neutrophil activation, UCBN or PBN were co-cultured with OVCAR3 at ratio of 1:1, 5:1, 10:1, respectively. Additionally, OVCAR3 was treated with supernatant from  $1 \times 10^6$  neutrophils in 24-well culture plates. After 24 h of co-culture, ovarian cancer cell viability was assessed using functional assays.

#### *Cell counting Kit-8 assay*

Post 24 h treatment of OVCAR3 with neutrophils or supernatant in a 96-well plate ( $5 \times 10^3$ /well), Cell Counting Kit-8 solution (Cat. 40203ES60, Yeasen, China) was added to each well and cells were incubated at 37°C for 3 h. A microplate reader was then used to measure absorbance at 450 nm to assess OVCAR3 viability.

#### *EdU assay*

OVCAR3 proliferation was evaluated using the BeyoClick™ EdU Cell Proliferation Kit (Cat. C0075S, Beyotime, China) following the manufacturer's protocol. Briefly, OVCAR3 were incubated with 50 μM EdU-contained medium for 2 h, fixed in 4% paraformaldehyde, permeated in 0.5% TritonX-100/PBS, then stained with DAPI. A fluorescence microscope (Nikon ECLIPSE Ti Corporation, Japan) was used for imaging. Photoshop software was used for image processing.

#### *Apoptosis assay*

OVCAR3 were digested and harvested following incubation with neutrophil supernatant in

## High viability umbilical cord blood-derived neutrophils

24-well plates for 24 h at 37°C. Cells were suspended in 100  $\mu$ l Binding Buffer and stained with Annexin V-FITC/PI apoptosis detection kit (Cat No. 40302, YEASEN) following the manufacturer's protocol. OVCAR3 cell apoptosis was assessed using a Cyto FLEX LX flow cytometer (Beckman Coulter).

### *Flow cytometry*

Neutrophils were resuspended in PBS (Cat.02-023-1A, Biological Industries, USA) supplemented with 3% BSA (Cat. A8020, Solabio, China) for 20 min to block non-specific binding. Cells were stained with PE-conjugated mAb against human CD11b (Clone ICRF44, 562399, BD), PE-CF594 Mouse IgG1,  $\kappa$  Isotype (Clone X40, 562292, BD), Alexa Fluor® 647-conjugated mAb against human CD66b (Clone G10F5, 561645, BD), Alexa Fluor® 647 Mouse IgM,  $\kappa$  isotype control (Clone G155-228, 560806, BD), BV421 Mouse Anti-Human CD54 (Clone HA58, 564077, BD), BV421 Mouse IgG1,  $\kappa$  Isotype Control (562438, BD) for 30 min at 4°C protected from light. After staining for 30 min, samples were assessed using a Cyto FLEX LX flow cytometer. Data was analyzed by FlowJo.

### *RNA-sequencing*

Total RNA was isolated from  $3 \times 10^6$  cells using the TRIzol Reagent (Cat. 93289, Sigma-Aldrich) following the manufacturer's instructions. To obtain transcriptional information, RNA-sequencing (RNA-seq) of cells was conducted at the HaploX Genomics Center for sequencing and analysis. RNA sequencing data was collected using an Illumina NovaSeq 6000 System.

### *ROS detection assay*

ROS production in activated or inactivated UCBN or PBN was measured using a luminol-amplified chemiluminescence assay (Cat. D6883, Sigma). Cells were incubated with 60 mM luminol (Invitrogen, Germany). Cells were then stimulated by the addition of 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich). The resulting chemiluminescence was immediately analyzed using a Cyto FLEX LX flow cytometer.

### *Proteome profiler array*

Neutrophils were stimulated for 24 h and the supernatant was collected to quantify released

cytokines by the Human Cytokine Antibody Arrays V Kit Human XL Cytokine Array Kit (Cat. ARY022B, R&D Systems). The array enabled simultaneous detection of 106 human cytokine protein expression levels secreted by activated and inactivated neutrophils from UCBN and PBN in cell culture supernatants simultaneously. Spot color density was evaluated using ImageJ software.

### *Statistical analysis*

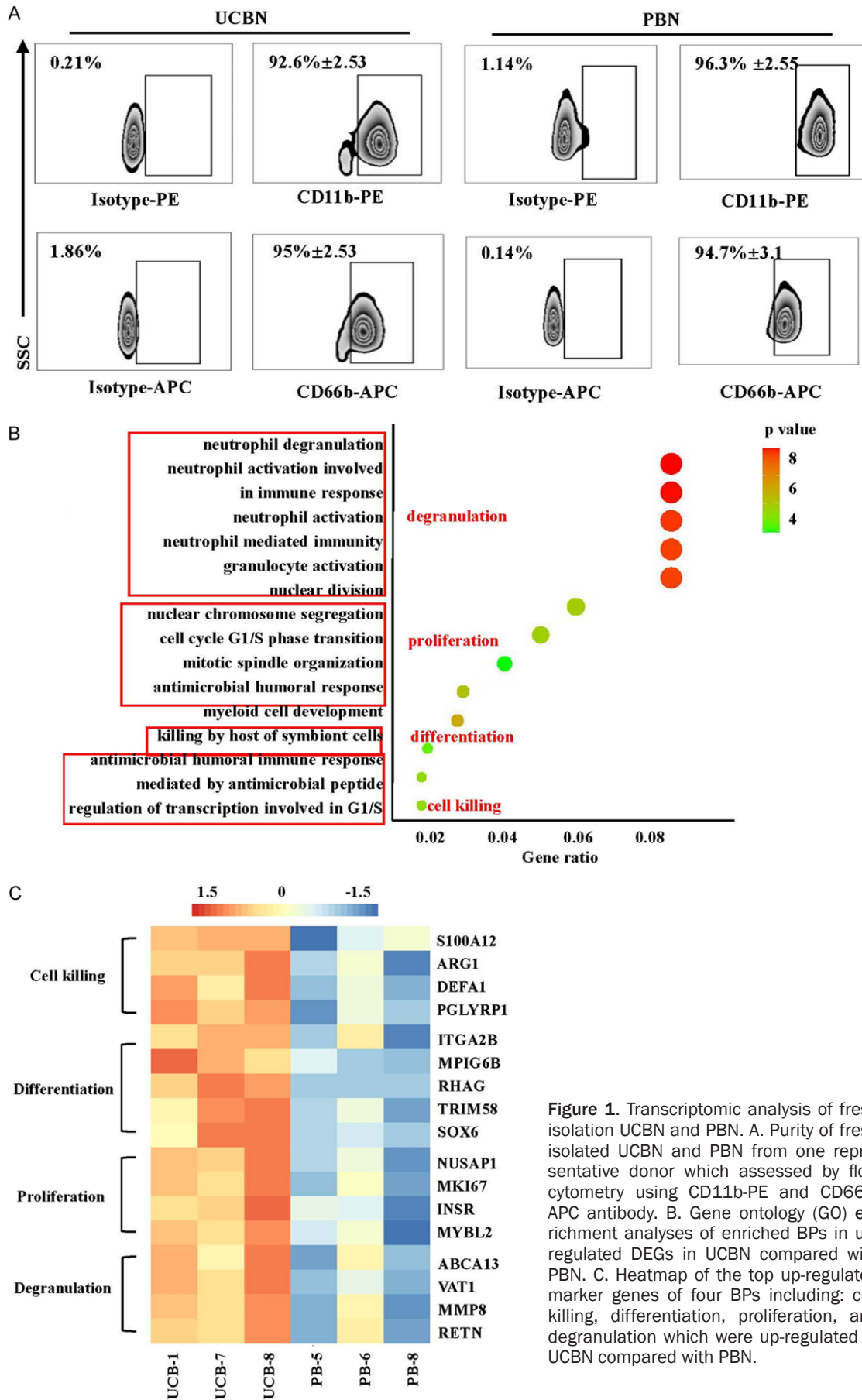
All cell experiments were performed in triplicate. Unless specified otherwise, data were generated from neutrophils isolated from different blood donors. Data was analyzed using GraphPad Prism Version 5.0 and are presented as mean  $\pm$  standard deviation. Comparisons between two groups were conducted using the Student's t-test. A  $P < 0.05$  was considered statistically significant (\* $P < 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ).

## Results

### *Transcriptomic analysis of UCBN and PBN*

The identity and purity of neutrophils freshly isolated from human umbilical cord blood and peripheral blood were confirmed by flow cytometry analysis of CD11b and CD66b expression. As shown in **Figure 1A**,  $92.6\% \pm 2.53$  CD11b<sup>+</sup> cells and  $95\% \pm 2.53$  CD66b<sup>+</sup> cells were observed in freshly isolated neutrophils from UCB, whereas  $96.3 \pm 2.55\%$  CD11b<sup>+</sup> and  $94.7 \pm 3.1\%$  CD66b<sup>+</sup> cells were observed in isolates from PB (**Figure 1A**). RNA-seq analysis was then performed and revealed 1217 differentially expressed genes (DEGs) between UCBN and PBN (FDR < 0.05) (**Figure S1**). Among them, 672 genes were significantly up-regulated and 545 were down-regulated in UCBN compared with PBN. Gene Ontology (GO) enrichment analyses indicated that biological processes (BPs) such as neutrophil degranulation, neutrophil activation, nuclear division, myeloid cell development, killing by a host of symbiont cells, and killing of cells of other organisms were enriched in up-regulated DEGs (**Figure 1B**). These BPs were closely associated with four biological characters of neutrophils: degranulation, proliferation, cell killing, and differentiation. Detailed analysis confirmed that most marker genes for these BPs were more highly expressed in UCBN than in PBN (**Figure 1C**). Collectively, these find-

# High viability umbilical cord blood-derived neutrophils



**Figure 1.** Transcriptomic analysis of fresh isolation UCBN and PBN. A. Purity of fresh isolated UCBN and PBN from one representative donor which assessed by flow cytometry using CD11b-PE and CD66b-APC antibody. B. Gene ontology (GO) enrichment analyses of enriched BPs in up-regulated DEGs in UCBN compared with PBN. C. Heatmap of the top up-regulated marker genes of four BPs including: cell killing, differentiation, proliferation, and degranulation which were up-regulated in UCBN compared with PBN.

## High viability umbilical cord blood-derived neutrophils

ings suggest that UCBN exhibit enhanced capabilities in degranulation, proliferation, and cell killing, along with a lower naïve status compared to PBN.

### *UCBN with higher viability in vitro than PBN*

To evaluate the viability of UCBN and PBN, we examined the expression of activation markers CD11b (integrin alpha M) [10] and CD66b (CEACAM8) [11] by flow cytometry. A significant reduction in the percentage of CD11b<sup>+</sup> and CD66b<sup>+</sup> cells was observed following 24- and 48-hour incubations, indicating a rapid decline in UCBN and PBN viability *in vitro* (**Figure 2A, 2C**). Interestingly, after 24 and 48 hours of incubation, UCBN displayed higher expression levels of both indicators than PBN, suggesting superior UCBN activity (**Figure 2A, 2C**).

Given the sequencing data indicating a propensity for activation in UCBN, we stimulated both UCBN and PBN *in vitro* to compare their states and functions post-activation. Neutrophil viability was maintained upon activation with cytokines such as IFN- $\beta$  and IFN- $\gamma$ , or stimulating agents such as LPS (lipopolysaccharide) [12]. Consequently, we treated neutrophils with a cytokine cocktail comprising IFN- $\beta$ , IFN- $\gamma$ , and LPS. Consistent with our earlier observation, higher percentages of UCBN expressing CD11b and CD66b were observed compared to PBN at both time points (**Figure 2B, 2C**). The degree of elevation of the two activated indicators was greater in UCBN than in PBN, reinforcing the notion of higher UCBN viability (**Figure 2C**).

### *Cytokine activation enhances UCBN function in vitro*

To elucidate the mechanism of higher viability of UCBN juxtaposed with PBN, we conducted transcriptomic comparisons between activated UCBN and activated PBN from different donors. Consistent with phenotypic observations, GO term analysis revealed that the most significantly up-regulated DEGs in activated UCBN were concentrated in BPs related to neutrophil activation, including neutrophil degranulation, immune response involvement, and cytokine biosynthesis (**Figure 3A**). A heatmap highlights the top up-regulated genes in activated UCBN compared to PBN (**Figure 3B**). Neutrophils function in a multitude of ways to tissue damage, including release of oxidants as reactive oxy-

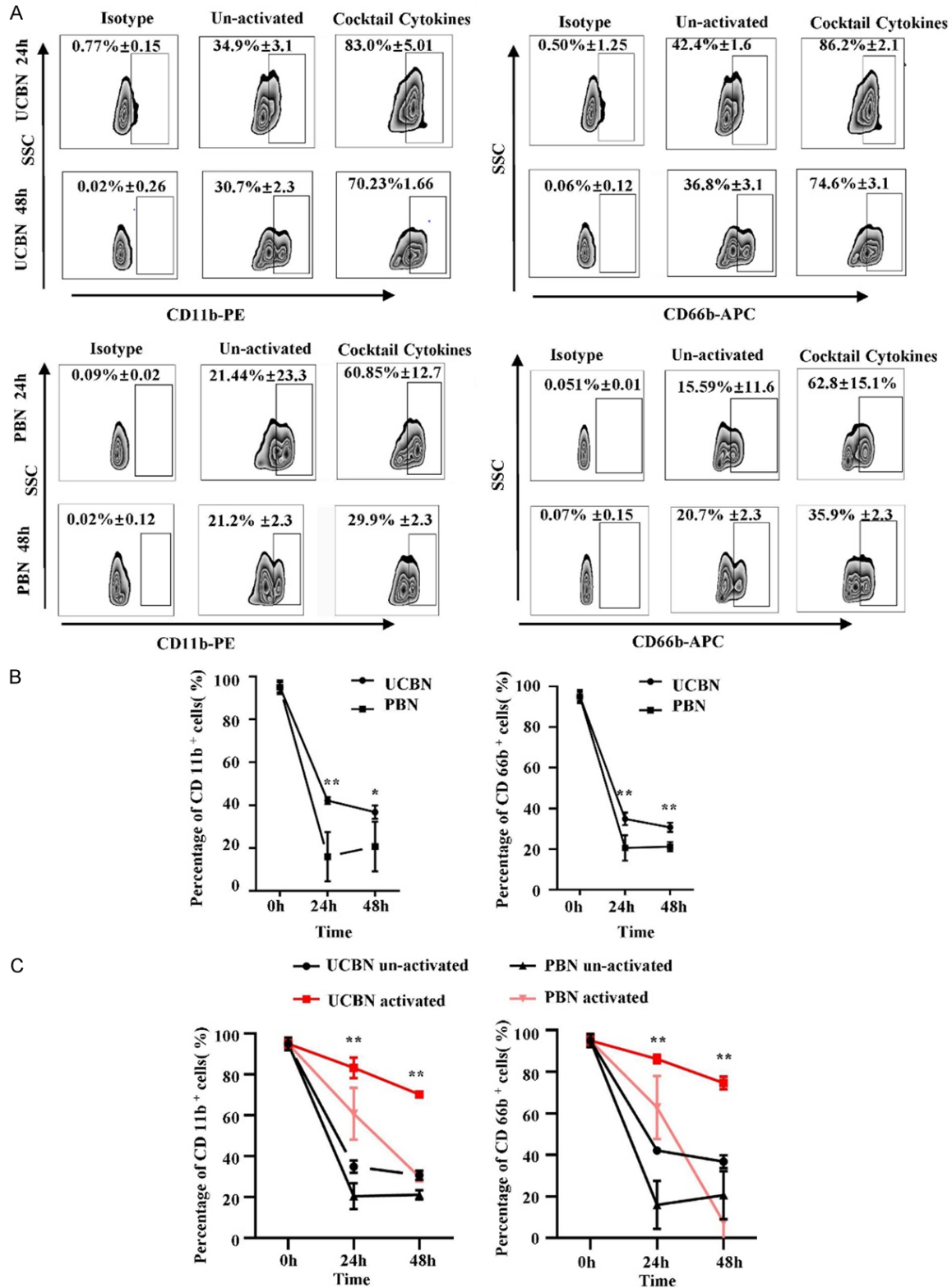
gen (ROS). Positive regulation of reactive oxygen species metabolic process means neutrophils possess high viability. ROS metabolic processes such as regulation of reactive oxygen species metabolic processes which represented neutrophils function were also significantly upregulated in activated UCBN (**Figure 3A**), suggesting higher ROS activity in activated UCBN than activated PBN. Positive regulation of reactive oxygen species metabolic process means activated UCBN possesses higher viability. Then, activated cells were further used for Functional verification: ROS function. Further luminol-based flow cytometry analysis confirmed a higher level of ROS in activated UCBN than that in activated PBN (**Figure 3C**).

We next sought to validate the function of activated UCBN through tumor cell killing experiments. We examined whether cytokine cocktail treatment may enhance the cytotoxicity of UCBN. After exposing the ovarian cancer cell line OVCAR3 to activate UCBN, PBN, or conditioned media (CM) derived from different donors, we assessed cancer cell viability using the CCK-8 assay. For UCBN, all three conditions induced cytotoxicity and one manifested increased cytotoxicity when compared with non-activated states. Only two conditions induced enhanced cytotoxicity when compared with cytokine cocktail treatment alone (**Figure 4A**). For PBN, activation led to a slight increase in cytotoxicity, but no condition showed enhanced killing compared to cytokine cocktail treatment alone (**Figure 4A**). These results suggest that cytokine-activated UCBN exerts superior cytotoxic effects than PBN.

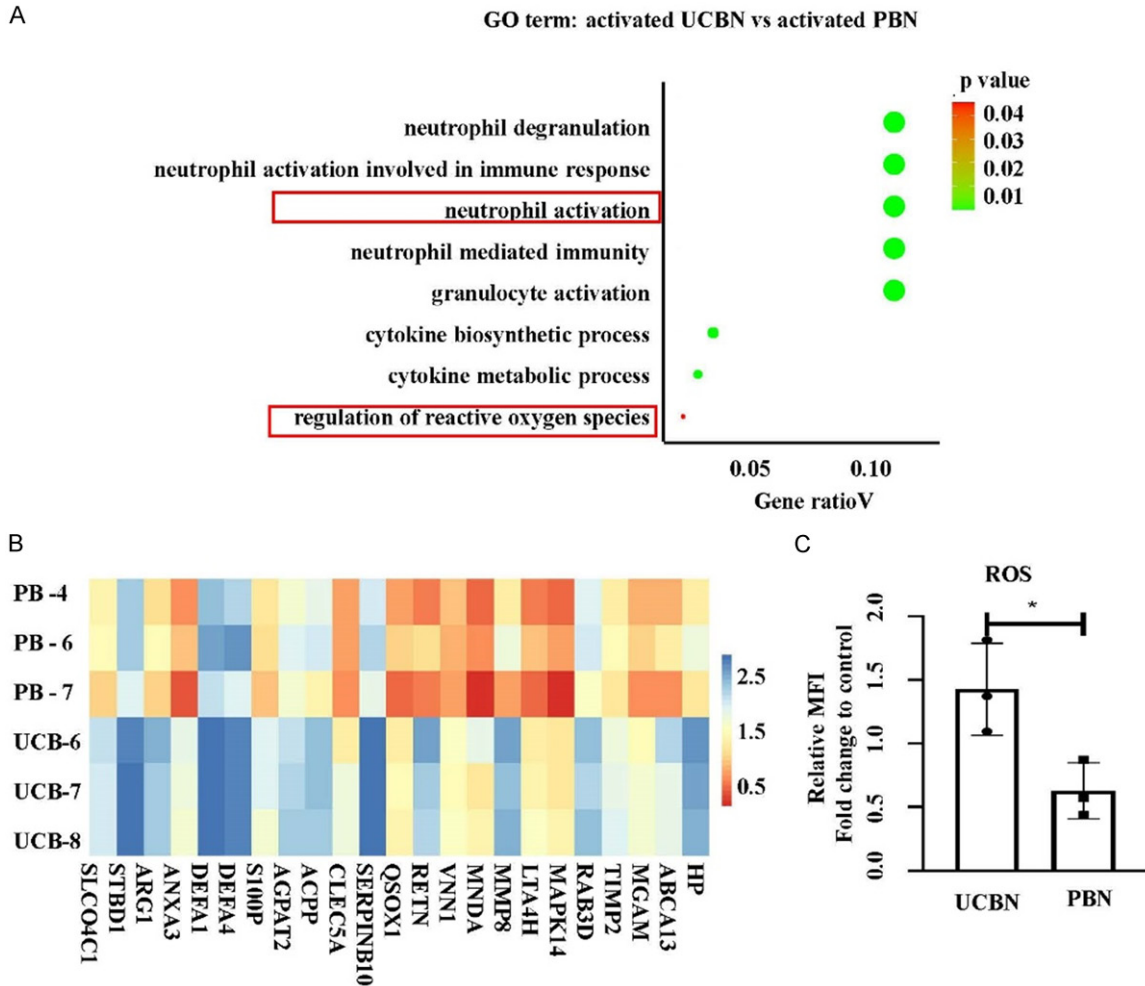
Notably, conditioned media (CM) from all five activated UCBN samples displayed increased cytotoxicity compared to non-activated samples, with four out of five showing enhanced killing effects over cytokine cocktail treatment alone (**Figure 4B**). This finding lends substantial support to the earlier results and suggests that cytokine cocktail activation augments the ovarian cancer cell killing activity of UCBN. In contrast, CM from activated PBN showed only marginal effects compared to non-activated samples, and only two out of five exhibited enhanced killing activity over cytokine cocktail treatment alone (**Figure 4B**).

To further confirm the results above, we performed EdU incorporation and Annexin V-PI

## High viability umbilical cord blood-derived neutrophils



**Figure 2.** Activation of UCBN and PBN by cytokine cocktail stimulation. A. CD11b and CD66b expression in UCBN and PBN after cytokines cocktail activation assessed by flow cytometry at 24 h and 48 h after stimulation respectively. B. The expression of the activation marker CD11b and CD66b in UCBN and PBN assessed by flow cytometry analysis at different time points. 0 h, freshly isolated cells; 24 h, cells cultured for 24 hours *in vitro*; 48 h, cells cultured 48 hours *in vitro*. C. Effect of cytokine cocktails on viability of UCBN or PBN assessed by flow cytometry using CD11b-PE and CD66b-APC staining at 24 h and 48 h (n = 3). \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 3.** Transcriptomic analysis of activated UCBN with activated PBN as control. **A.** The mainly enriched related BPs of top upregulated DEGs in activated UCBN compared with activated PBN from GO enrichment analysis. **B.** Expression of the most up-regulated marker genes of neutrophils degranulation BPs up-regulated in UCBN compared with PBN. **C.** Level of ROS production in activated UCBN and activated PBN assessed by luminol-based flow cytometry. N = 3 donors each group, \* $P < 0.05$ .

staining. As depicted in **Figure 4C**, CM from activated UCBN significantly inhibited ovarian cancer cell proliferation (**Figures 4C** and **S2**). The images in **Figure 4C** for the ovarian cancer and cytokine cocktail groups were identical to those in **Figure S2**, which were standardized to the same control. Conversely, CM from activated PBN showed minimal inhibitory effects on cancer cell proliferation (**Figures 4C, 4D** and **S2**). Additionally, CM from activated UCBN demonstrated strong apoptotic activity, whereas that from activated PBN showed minimal effects (**Figures 4E** and **S3A, S3B**). Collectively, these results strongly suggest that cytokine-activated UCBN exerts substantial proliferation-inhibitory and cytotoxic effects on ovarian

cancer cells, likely through a secretory mechanism.

#### Multi-omics analysis UCBN with PBN

To probe the underlying function of UCBN, we conducted transcriptomic analyses of UCBN with and without cytokine cocktail activation, using samples from different donors. GO enrichment analysis of DEGs revealed that the top upregulated genes in activated UCBN were mainly enriched in immunity-related BPs, such as response to interferon-gamma, positive regulation of cytokine production, response to lipopolysaccharide, cytokine secretion, peptide secretion, activation of the innate immune

## High viability umbilical cord blood-derived neutrophils

**A**

UCBN					PBN						
	-	+	+	-		-	+	+	-		
Cytokine cocktail	-	-	+	+	Cytokine cocktail	-	-	+	+		
UCB-7	1:1	1.00±0.05	0.76±0.09	0.73±0.03*	0.76±0.07*	PB-6	1:1	1.00±0.09	1.08±0.04	0.99±0.2	0.73±0.08
	5:1	1.00±0.05	0.60±0.12	0.51±0.03*	0.76±0.07**		5:1	1.00±0.09	0.86±0.06	0.74±0.1*	0.73±0.08
	10:1	1.00±0.05	0.63±0.06	0.42±0.02**	0.76±0.07**		10:1	1.00±0.09	1.11±0.17	0.89±0.08	0.73±0.08
UCB-8	1:1	1.00±0.03	1.04±0.06	0.92±0.07	0.80±0.06	PB-7	1:1	1.00±0.06	0.87±0.04	0.82±0.04	0.80±0.07
	5:1	1.00±0.03	1.02±0.09	0.87±0.06*	0.80±0.06		5:1	1.00±0.06	1.04±0.1	0.77±0.09**	0.80±0.07
	10:1	1.00±0.03	1.00±0.05	0.80±0.12**	0.80±0.06		10:1	1.00±0.06	1.11±0.13	0.74±0.08*	0.80±0.07
UCB-9	1:1	1.00±0.11	0.83±0.01	0.69±0.04*	0.73±0.06*	PB-8	1:1	1.00±0.09	1.06±0.07	1.02±0.07	0.82±0.07
	5:1	1.00±0.11	0.94±0.09	0.7±0.02*	0.73±0.06		5:1	1.00±0.09	1.12±0.09	0.81±0.04**	0.82±0.07
	10:1	1.00±0.11	0.96±0.14	0.61±0.06**	0.73±0.06*		10:1	1.00±0.09	1.07±0.02	0.82±0.1**	0.82±0.07

\*, \*\* Activated UCBN compared with Un-activated UCBN

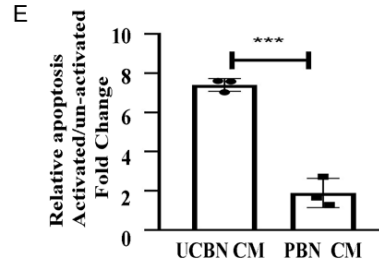
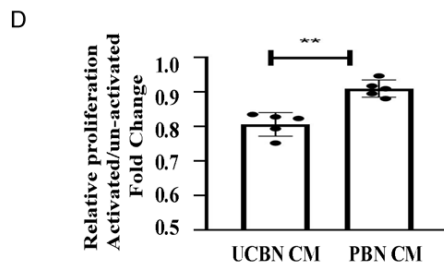
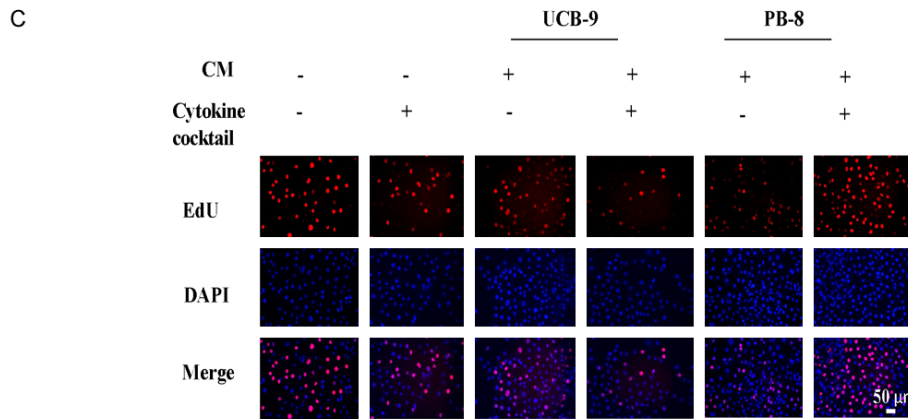
\*, \*\* Activated UCBN compared with Un-activated UCBN, except the effect of supplemented cytokine cocktail

**B**

UCBN CM					PBN CM				
	-	+	+	-		-	+	+	-
Cytokine cocktail	-	-	+	+	Cytokine cocktail	-	-	+	+
UCB-2	1.00±0.02	0.85±0.06	0.78±0.04*	0.91±0.04**	PB-1	1.00±0.02	1.24±0.06	0.94±0.02**	0.90±0.02
UCB-3	1.00±0.02	1.07±0.03	0.96±0.02*	0.90±0.02	PB-2	1.00±0.02	1.15±0.01	0.97±0.01	0.90±0.02
UCB-6	1.00±0.01	0.97±0.06	0.73±0.01**	0.78±0.03*	PB-3	1.00±0.02	0.70±0.05	0.76±0.01	0.91±0.04**
UCB-7	1.00±0.11	0.94±0.04	0.75±0.07**	0.82±0.08*	PB-6	1.00±0.11	0.77±0.08	0.74±0.07	0.82±0.08*
UCB-8	1.00±0.11	0.84±0.13	0.61±0.04**	0.82±0.08**	PB-7	1.00±0.09	1.08±0.10	0.80±0.08*	0.82±0.07

\*, \*\* Conditioned medium of Activated UCBN compared with Un-activated UCBN

\*, \*\* Conditioned medium of activated UCBN compared with un-activated UCBN, except the effect of supplemented cytokine cocktail

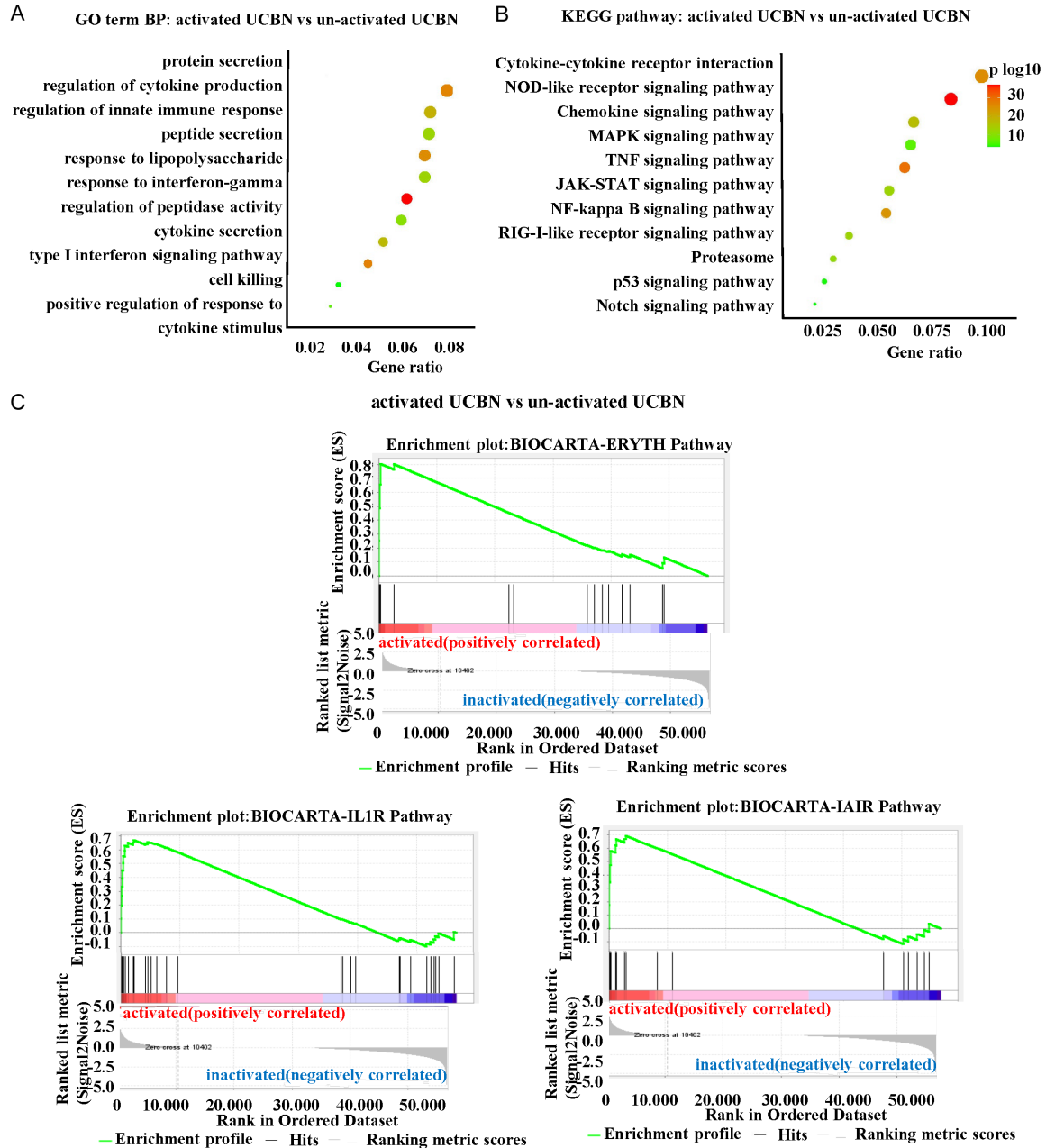


**Figure 4.** Cytokine cocktail activation enhanced the ovarian cancer cell killing activity of UCBN. **A.** The viability of ovarian cancer cells line OVCAR3 co-cultured with activated UCBN (donors of UCB-7, UCB-8, UCB-9) or PBN (PB-6, PB-7, PB-8) assessed by CCK-8 assay. \* $P < 0.05$ , \*\* $P < 0.01$ . **B.** The viability of ovarian cancer cells line OVCAR3 treated with conditioned medium (CM) of activated UCBN (donors of UCB-2, UCB-3, UCB-6, UCB-7, UCB-8) or PBN (donors of PB-1, PB-2, PB-3, PB-6, PB-7) assessed by CCK-8 assay. \* $P < 0.05$ , \*\* $P < 0.01$ . **C.** Represent images of



## High viability umbilical cord blood-derived neutrophils

immunofluorescence staining of EdU of ovarian cancer cells line OVCAR3 treated with conditioned medium (CM) of activated UCBN or PBN. Bar = 50  $\mu$ m. D. The proliferation capacity of ovarian cancer cells line OVCAR3 treated with conditioned medium (CM) of activated UCBN or PBN assessed by EdU analysis. E. The apoptosis of ovarian cancer cells line OVCAR3 treated with conditioned medium (CM) from activated UCBN or PBN by Annexin-V PI analysis. N = 3 donors each group, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

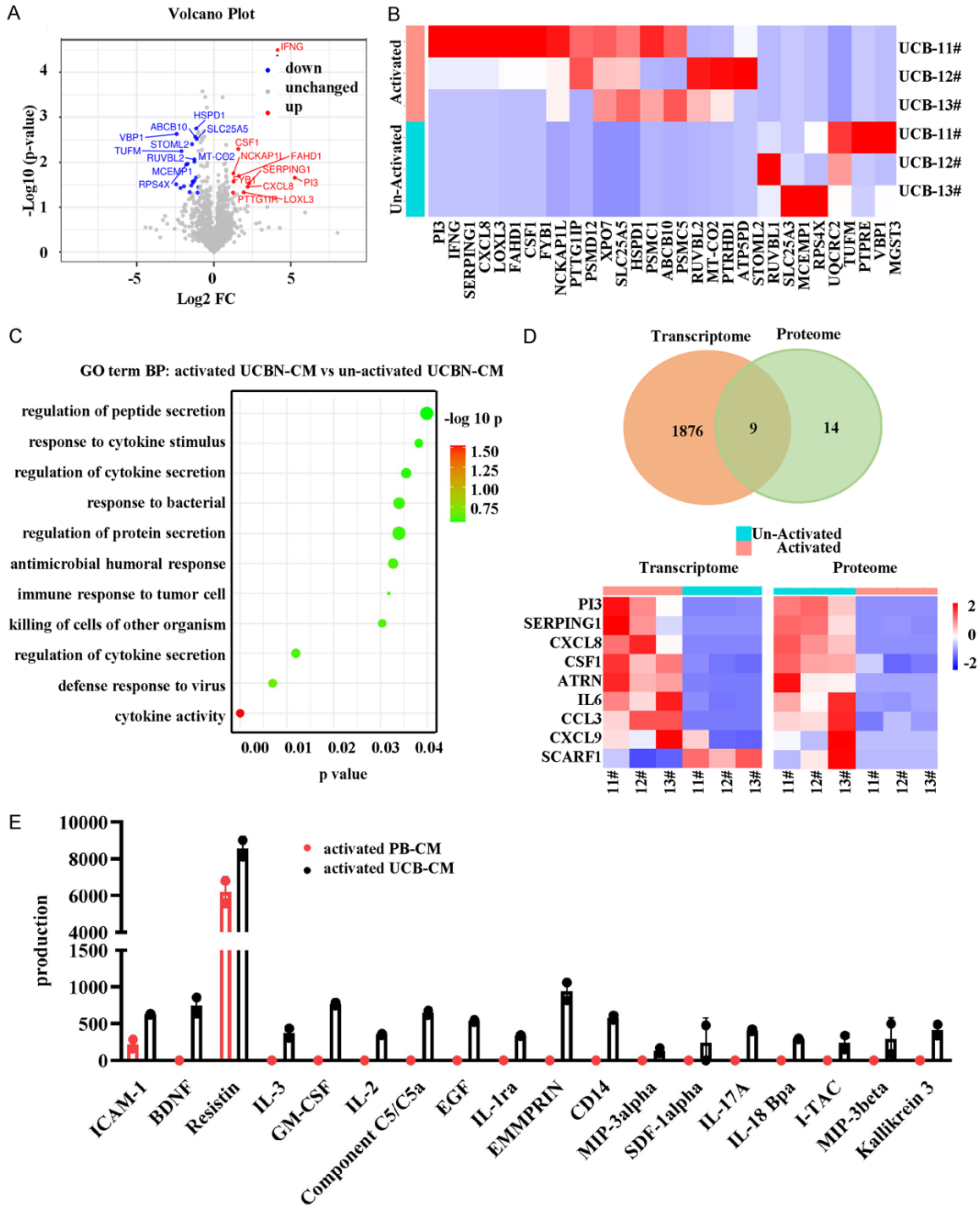


**Figure 5.** Transcriptomic analysis of activated UCBN with un-activated UCBN as control. A. The mainly enriched related BPs of top upregulated DEGs in activated UCBN compared with un-activated UCBN from GO enrichment analysis. B. KEGG pathway enrichment of top up-regulated genes in activated UCBN compared with un-activated UCBN. C. Represent pathways upregulated in activated UCBN compared to un-activated UCBN from GESA analysis.

response, and cell killing (**Figure 5A**). KEGG pathway analysis further confirmed that the top

upregulated genes in activated UCBN were mainly involved in immunity-related pathways,

# High viability umbilical cord blood-derived neutrophils



**Figure 6.** United Transcriptomic and proteomic analysis UCBN with PBN. A. Volcano plot of Expression of differentially expressed genes (DEGs) between activated UCBN-CM and un-activated UCBN-CM. n = 3 donors each group. B. Cluster Analysis of Expression of differentially expressed genes (DEGs) between activated UCBN-CM and un-activated UCBN-CM. n = 3 donors each group. C. Gene ontology (GO) enrichment analyses of enriched BPs in up-regulated DEGs in activated UCBN-CM and un-activated UCBN-CM. D. Multi-Omics analysis the molecule both up-regulated in transcriptomics and proteomics. E. Screened molecule headmap analysis in transcriptomics and proteomics.

including the NOD-like receptor signaling pathway, TNF signaling pathway, Cytokine-cytokine

receptor interaction, NF-kappa B signaling pathway, Chemokine signaling pathway, RIG-I-

like receptor signaling pathway, JAK-STAT signaling pathway, Proteasome MAPK signaling pathway, p53 signaling pathway, and Notch signaling pathway (**Figure 5B**). Gene Set Enrichment Analysis (GSEA) also indicated upregulation in ERYTH, IL1R, and LAIR pathways, which are associated with erythrocyte differentiation and inflammatory response, correlating with neutrophil functions, in activated UCBN (**Figure 5C**).

To further investigate the cytokine secretion by UCBN, we collected activated UCBN-CM and activated PBN-CM from different donors for proteomics analysis. We defined a protein as present if it appears two or more times in one group. Results showed that 14 proteins were highly expressed and 6 were lowly expressed in activated UCBN-CM (**Figure 6A**). Using another method of differential expression analysis, we found that 10 peptides were significantly up-regulated and 21 down-regulated in activated UCBN compared to activated PBN (**Figure 6B**). GO enrichment analysis of these up-regulated proteins revealed a focus on immunity-related BPs, including regulation of peptide secretion, regulation of response to cytokine stimulus, regulation of cytokine secretion, response to molecule of bacterial origin, regulation of protein secretion, positive regulation of immune response to tumor cells, killing of allogeneic cells, and positive regulation of cytokine secretion (**Figure 6C**), aligning with the transcriptomic analysis. KEGG pathway analysis again confirmed that the top upregulated genes in activated UCBN were primarily related to cytokine-cytokine receptor interaction, transcriptional misregulation in cancer, viral protein interaction with cytokine and cytokine receptor, and the JAK-STAT signaling pathway (**Figure S4A**). These results demonstrate that activated UCBN primarily functions in cytokine secretion and cell killing.

Combining gene sequencing data with proteome expression data for a multi-omics analysis, we defined that, for screening, a protein must be identified in the proteomics data and also have expression information at the transcriptome level. A total of 23 up-regulated proteins in the proteome were correlated with gene transcriptome data (**Figure 6D**). After analysis, 15 proteins were not expressed in the sequenced genome, and 9 proteins were co-

expressed at the gene level and protein level. We focused on these nine molecules, whose expression profiles are detailed in **Figure 6D**. Among these nine, eight proteins (PI3, SERPING1, CXCL8, CSF1, ATRN, IL6, CCL3, CXCL9) were highly expressed in both the transcriptomic and proteomic data in activated UCBN. However, SCARF showed opposite expression patterns. Proteome profiler array analysis further identified anti-tumor molecules (**Figure S4B**), revealing that 18 cytokines, including ICAM1 and BDNF, were altered in activated UCBN-CM (**Figure 6E**). This multi-omic analysis, corroborated by proteome profiler array verification, suggests that activated UCBN possess enhanced functions of cytokine secretion and cell killing.

### Discussion

In this study, we discovered that UCBN exhibits higher viability than PBN, with superior functional capabilities. Our transcriptomic analysis indicates that UCBN is better primed than PBN, being less susceptible to cell death and more readily activated. Co-culture with activated UCBN was found to inhibit ovarian cancer cell proliferation and promote apoptosis. Further, our combined transcriptomic and proteomic profiling suggests that a cytokine cocktail can stimulate UCBN in terms of degranulation, activation, proliferation, and survival, thereby enhancing and sustaining their viability.

Immune cells derived from PB are commonly utilized for cancer immunotherapy and have demonstrated clinical efficacy. However, limitations such as the need for prolonged amplification prior to transplanting, donor variability, and heterogeneity in cell vitality hinder their broader application [13]. In contrast, immune cells derived from UCB displayed a multitude of advantages across various donors. This suggests the potential for developing off-the-shelf therapies based on UCB-derived immune cells. Previous studies have reported that UCB-derived immune cells, including NK and T cells, exhibit notable cytotoxic activity against tumor cells [14, 15]. Additionally, it was also reported that UCB-derived immune cells may be more active than PB-derived cells. UCB-derived NK cells have been shown to be more responsive to cytokine activation than PB-derived NK cells [16]. In our study, we found that activated UCBN

showed higher cytotoxic activity towards ovarian tumor cells than activated PBN (**Figure 4**). Consistent with this phenotypic observation, we found an upregulation of the neutrophil activation-related signaling pathways in activated UCBN compared with activated PBN (**Figures 1 and 5**). These findings further suggested that UCB could be a superior source for cancer immune cell therapy.

Neutrophils can exhibit both tumor-promoting and tumor-killing activity [17, 18], depending on their tissue origin, contextual environment, and activation status [19, 20]. Previous reports showed that *in vitro* activation of PBN with cytokine cocktails exhibited anti-inflammatory functions, eliminating parasites [12]. However, no anti-tumor function had been reported with activated PBN. Consistent with these reports, our study found no significant increase in cytotoxicity with activated PBN. The only increase observed was with conditioned medium from un-activated PBN [21]. In contrast, both activated UCBN and their conditioned medium exhibited anti-tumor functions (**Figure 4**). Our transcriptomic analysis revealed UCBN is a better-primed neutrophil type compared with PBN in that they are less prone to cell death yet more readily activated. Treatment with a cytokine cocktail could further stimulate UCBN degranulation, activation, proliferation and survival, thus enhancing and sustaining their tumor-killing activity such as through up-regulation of Reactive Oxygen Species (ROS) metabolic processes [22], as verified experimentally.

In order to explain the mechanism of UCBN exhibits higher viability than PBN, we combining gene sequencing data with proteome expression data for a multi-omics analysis, we found eight proteins (PI3, SERPING1, CXCL8, CSF1, ATRN, IL6, CCL3, CXCL9) were highly expressed in both the transcriptomic and proteomic data in activated UCBN. PI3: peptidase inhibitor 3, this gene encodes an elastase-specific inhibitor that functions as an antimicrobial peptide against Gram-positive and Gram-negative bacteria, secreted by neutrophils to control mucosal immunity [23]. SERPING1: Serpin Family G Member 1 encodes a highly glycosylated plasma protein involved in the regulation of the complement cascade, play a potentially crucial role in regulating important

physiological pathways including complement activation, which have demonstrated act as part of activated neutrophils functions [24]. ATRN [25], CXCL8 [26], CSF1 [27], IL6 [28], CCL3 [29], CXCL9 [30], these gene are members of the chemokine family and is a major mediator of the inflammatory response, involved in the initial immune cell clustering during inflammatory response and may regulate chemotactic activity of chemokines. Among these cytokines, CCL3 [31], CXCL9 [32], CXCL8 [33], IL6 [34] and CSF1 [35] were affirmative disclosure secreted by neutrophils and behalf of cell functions on a certain extent. As ATRN, attractin, the gene encodes both membrane-bound and secreted protein isoforms, we need more research in the future to verify the relationship between the high expression of these genes and activated neutrophils. In conclusion, high expression of these genes may contributed to the higher viability of UCBN.

Nonetheless, our results raise several important questions in the neutrophil-based immunotherapy field. A primary challenge is the typically short lifespan of neutrophils, commonly described as “short-lived cells” [36], which impedes both basic and clinical research. Modifications to the genome or optimization of culture conditions may help elongate the lifespan of neutrophils. However, whether genetic modifications or culture conditions may significantly alter neutrophil properties remains a concern. Alternatively, although not ideal, conditioned medium could be a viable option, as demonstrated in our study. A second issue concerns individual differences among donors/patients, as we observed significant variations with PBN and, to a lesser extent, but still significant, variations with UCBN. Investigating these individual variations could provide critical insights into underlying mechanisms, greatly facilitating clinical applications. Given their accessibility and strong tumor cytotoxic activity, it is imperative to further investigate the molecular mechanisms underlying UCBN and their individual variations for more effective cancer immune cell therapy.

In summary, our study reveals that UCBN possesses higher viability and superior functions compared to PBN, positioning UCBN-based immunotherapy as a promising new strategy for cancer treatment.

## Acknowledgements

We would like to thank the support of all staff in the Department of Obstetrics and Gynecology, Shanghai Tenth People's Hospital, Tongji University School of Medicine. This work was supported by Shanghai Science and Technology Committee (Nos. 19QA1407700, 1841196-4100).

## Disclosure of conflict of interest

None.

**Address correspondence to:** Zhongping Cheng, Department of Obstetrics and Gynecology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China. E-mail: mdcheng18@tongji.edu.cn

## References

- [1] Albanesi M, Mancardi DA, Jonsson F, Iannascoli B, Fiette L, Di Santo JP, Lowell CA and Bruhns P. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood* 2013; 122: 3160-3164.
- [2] Otten MA, Bakema JE, Tuk CW, Glennie MJ, Tutt AL, Beelen RH, van de Winkel JG and van Egmond M. Enhanced Fc $\alpha$ RI-mediated neutrophil migration towards tumour colonies in the presence of endothelial cells. *Eur J Immunol* 2012; 42: 1815-1821.
- [3] Carmi Y, Spitzer MH, Linde IL, Burt BM, Prestwood TR, Perlman N, Davidson MG, Kenkel JA, Segal E, Pusapati GV, Bhattacharya N and Engleman EG. Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity. *Nature* 2015; 521: 99-104.
- [4] van Egmond M and Bakema JE. Neutrophils as effector cells for antibody-based immunotherapy of cancer. *Semin Cancer Biol* 2013; 23: 190-199.
- [5] Lee W, Ko SY, Mohamed MS, Kenny HA, Lengyel E and Naora H. Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. *J Exp Med* 2019; 216: 176-194.
- [6] Carus A, Ladekarl M, Hager H, Nedergaard BS and Donskov F. Tumour-associated CD66b+ neutrophil count is an independent prognostic factor for recurrence in localised cervical cancer. *Br J Cancer* 2013; 108: 2116-2122.
- [7] Laughlin MJ, Eapen M, Rubinstein P, Wagner JE, Zhang MJ, Champlin RE, Stevens C, Barker JN, Gale RP, Lazarus HM, Marks DI, van Rood JJ, Scaradavou A and Horowitz MM. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med* 2004; 351: 2265-2275.
- [8] Rocha V, Cornish J, Sievers EL, Filipovich A, Locatelli F, Peters C, Remberger M, Michel G, Arcese W, Dallorso S, Tiedemann K, Busca A, Chan KW, Kato S, Ortega J, Vowels M, Zander A, Souillet G, Oakill A, Woolfrey A, Pay AL, Green A, Garnier F, Ionescu I, Wernet P, Sirchia G, Rubinstein P, Chevret S and Gluckman E. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. *Blood* 2001; 97: 2962-2971.
- [9] Liu Q, Yang W, Luo N, Liu J, Wu Y, Ding J, Li C and Cheng Z. LPS and IL-8 activated umbilical cord blood-derived neutrophils inhibit the progression of ovarian cancer. *J Cancer* 2020; 11: 4413-4420.
- [10] Fatemi A, Alipour R, Khanahmad H, Alsahebfofoul F, Andalib A and Pourazar A. The impact of neutrophil extracellular trap from patients with systemic lupus erythematosus on the viability, CD11b expression and oxidative burst of healthy neutrophils. *BMC Immunol* 2021; 22: 12.
- [11] Mauracher LM, Krall M, Roiss J, Hell L, Koder S, Hofbauer TM, Gebhart J, Hayden H, Brostjan C, Ay C and Pabinger I. Neutrophil subpopulations and their activation potential in patients with antiphospholipid syndrome and healthy individuals. *Rheumatology (Oxford)* 2021; 60: 1687-1699.
- [12] Ohms M, Moller S and Laskay T. An attempt to polarize human neutrophils toward N1 and N2 phenotypes in vitro. *Front Immunol* 2020; 11: 532.
- [13] Granot Z, Henke E, Comen EA, King TA, Norton L and Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 2011; 20: 300-314.
- [14] Shi L, Wang JX, Stevens L, Ness P and Shan H. Blood safety and availability: continuing challenges in China's blood banking system. *Transfusion* 2014; 54: 471-482.
- [15] Piccard H, Muschel RJ and Opendakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. *Crit Rev Oncol Hematol* 2012; 82: 296-309.
- [16] Alnabhan R, Madrigal A and Saudemont A. Differential activation of cord blood and peripheral blood natural killer cells by cytokines. *Cytotherapy* 2015; 17: 73-85.
- [17] Wang Y, Chen J, Yang L, Li J, Wu W, Huang M, Lin L and Su S. Tumor-contacted neutrophils promote metastasis by a CD90-TIMP-1 juxtacrine-paracrine loop. *Clin Cancer Res* 2019; 25: 1957-1969.

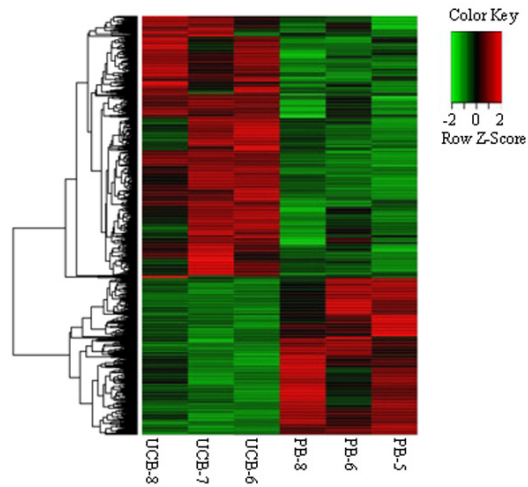
## High viability umbilical cord blood-derived neutrophils

- [18] Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, Damti P, Lumbroso D, Polyansky L, Sionov RV, Ariel A, Hovav AH, Henke E, Fridlender ZG and Granot Z. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep* 2015; 10: 562-573.
- [19] Eruslanov EB, Singhal S and Albelda SM. Mouse versus human neutrophils in cancer: a major knowledge gap. *Trends Cancer* 2017; 3: 149-160.
- [20] Coffelt SB, Wellenstein MD and de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer* 2016; 16: 431-446.
- [21] Cui C, Chakraborty K, Tang XA, Zhou G, Schoenfelt KQ, Becker KM, Hoffman A, Chang YF, Blank A, Reardon CA, Kenny HA, Vaisar T, Lengyel E, Greene G and Becker L. Neutrophil elastase selectively kills cancer cells and attenuates tumorigenesis. *Cell* 2021; 184: 3163-3177, e21.
- [22] Zivkovic M, Poljak-Blazi M, Zarkovic K, Mihaljevic D, Schaur RJ and Zarkovic N. Oxidative burst of neutrophils against melanoma B16-F10. *Cancer Lett* 2007; 246: 100-108.
- [23] Vandooren J, Goeminne P, Boon L, Ugarte-Berzal E, Rybakov V, Proost P, Abu EA and Opdenakker G. Neutrophils and activated macrophages control mucosal immunity by proteolytic cleavage of antileukoproteinase. *Front Immunol* 2018; 9: 1154.
- [24] Hair PS, Enos AI, Krishna NK and Cunnion KM. Inhibition of immune complex complement activation and neutrophil extracellular trap formation by peptide inhibitor of complement C1. *Front Immunol* 2018; 9: 558.
- [25] Li N, Gao S, Wang S, He S, Wang J, He L, Jiang D, Shi YS, Zhang J, Gu Y, Chen T, Kong M, Xu X and Zhao Q. Attractin participates in schizophrenia by affecting testosterone levels. *Front Cell Dev Biol* 2021; 9: 755165.
- [26] Ha H, Debnath B and Neamati N. Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases. *Theranostics* 2017; 7: 1543-1588.
- [27] Buechler MB, Fu W and Turley SJ. Fibroblast-macrophage reciprocal interactions in health, fibrosis, and cancer. *Immunity* 2021; 54: 903-915.
- [28] Rose-John S. Interleukin-6 family cytokines. *Cold Spring Harb Perspect Biol* 2018; 10: a028415.
- [29] Jia SN, Han YB, Yang R and Yang ZC. Chemokines in colon cancer progression. *Semin Cancer Biol* 2022; 86: 400-407.
- [30] Reschke R and Gajewski TF. CXCL9 and CXCL10 bring the heat to tumors. *Sci Immunol* 2022; 7: q6509.
- [31] Cheng D, Lian W, Wang T, Xi S, Jia X, Li Z, Xiong H, Wang Y, Sun W, Zhou S, Peng L, Han L, Liu Y and Ni C. The interplay of Cxcl10(+)/Mmp14(+) monocytes and Ccl3(+) neutrophils proactively mediates silica-induced pulmonary fibrosis. *J Hazard Mater* 2024; 467: 133713.
- [32] Korbecki J, Kojder K, Kapczuk P, Kupnicka P, Gawronska-Szklarz B, Gutowska I, Chlubek D and Baranowska-Bosiacka I. The effect of hypoxia on the expression of CXC chemokines and CXC chemokine receptors-a review of literature. *Int J Mol Sci* 2021; 22: 843.
- [33] Poto R, Cristinziano L, Modestino L, de Paulis A, Marone G, Loffredo S, Galdiero MR and Varricchi G. Neutrophil extracellular traps, angiogenesis and cancer. *Biomedicines* 2022; 10: 431.
- [34] Xiao Y, Cong M, Li J, He D, Wu Q, Tian P, Wang Y, Yang S, Liang C, Liang Y, Wen J, Liu Y, Luo W, Lv X, He Y, Cheng DD, Zhou T, Zhao W, Zhang P, Zhang X, Xiao Y, Qian Y, Wang H, Gao Q, Yang QC, Yang Q and Hu G. Cathepsin C promotes breast cancer lung metastasis by modulating neutrophil infiltration and neutrophil extracellular trap formation. *Cancer Cell* 2021; 39: 423-437.
- [35] McAlpine CS, Kiss MG, Rattik S, He S, Vassalli A, Valet C, Anzai A, Chan CT, Mindur JE, Kahles F, Poller WC, Frodermann V, Fenn AM, Gregory AF, Halle L, Iwamoto Y, Hoyer FF, Binder CJ, Libby P, Tafti M, Scammell TE, Nahrendorf M and Swirski FK. Sleep modulates haematopoiesis and protects against atherosclerosis. *Nature* 2019; 566: 383-387.
- [36] Kolaczowska E and Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013; 13: 159-175.

## High viability umbilical cord blood-derived neutrophils

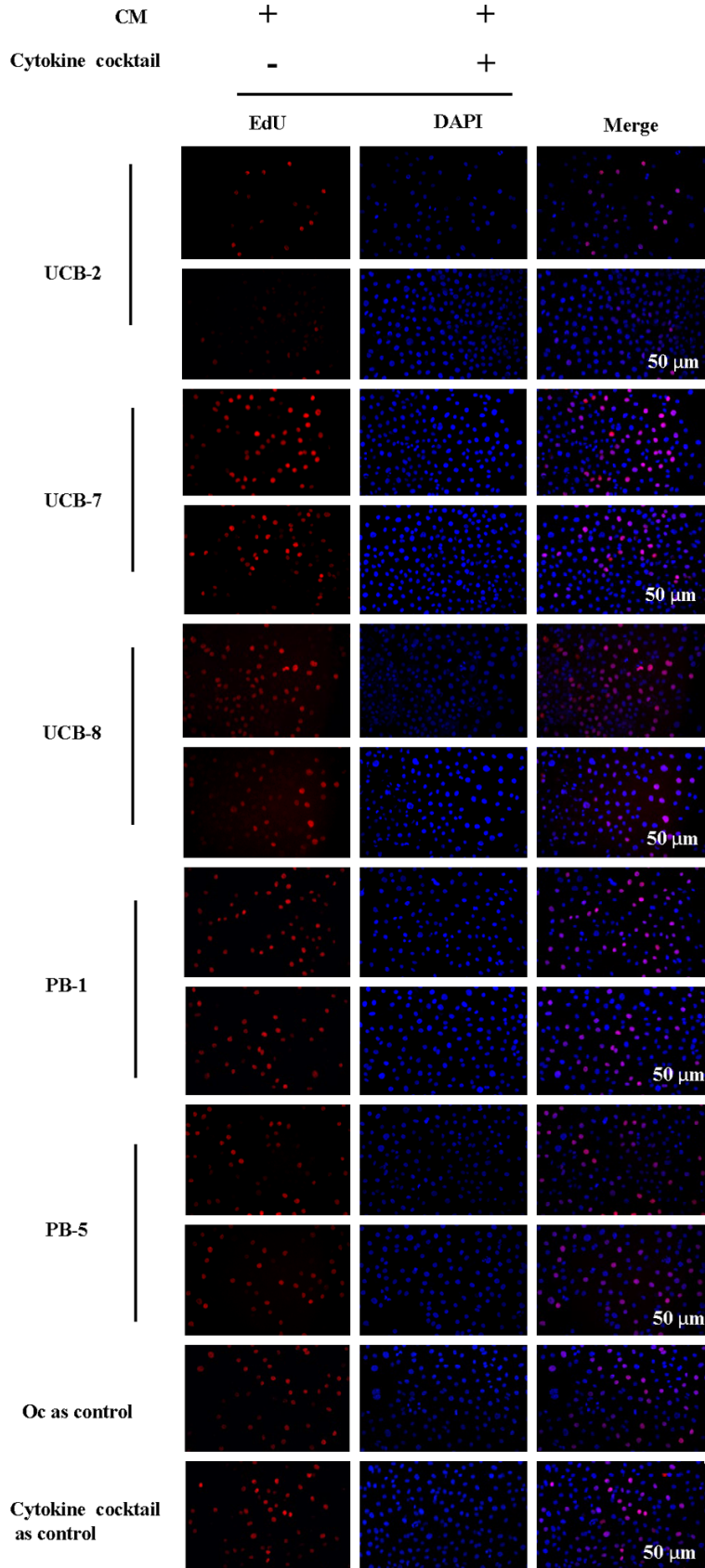
**Table S1.** Characteristics of UCB and PB samples

Sample	Sex	Age	Diagnosis
UCB-1	Female	38	Term labor
UCB-2	Female	29	Term labor
UCB-3	Female	31	Term labor
UCB-4	Female	32	Term labor
UCB-5	Female	26	Term labor
UCB-6	Female	30	Term labor
UCB-7	Female	31	Term labor
UCB-8	Female	30	Term labor
UCB-9	Female	32	Term labor
UCB-10	Female	26	Term labor
UCB-11	Female	28	Term labor
UCB-12	Female	32	Term labor
UCB-13	Female	24	Term labor
PB-1	Male	26	Healthy adult
PB-2	Male	27	Healthy adult
PB-3	Male	31	Healthy adult
PB-5	Female	39	Healthy adult
PB-6	Female	42	Healthy adult
PB-7	Female	38	Healthy adult
PB-8	Female	50	Healthy adult



**Figure S1.** Expression of differentially expressed genes (DEGs) between UCB and PBN. N = 3 donors in each group.

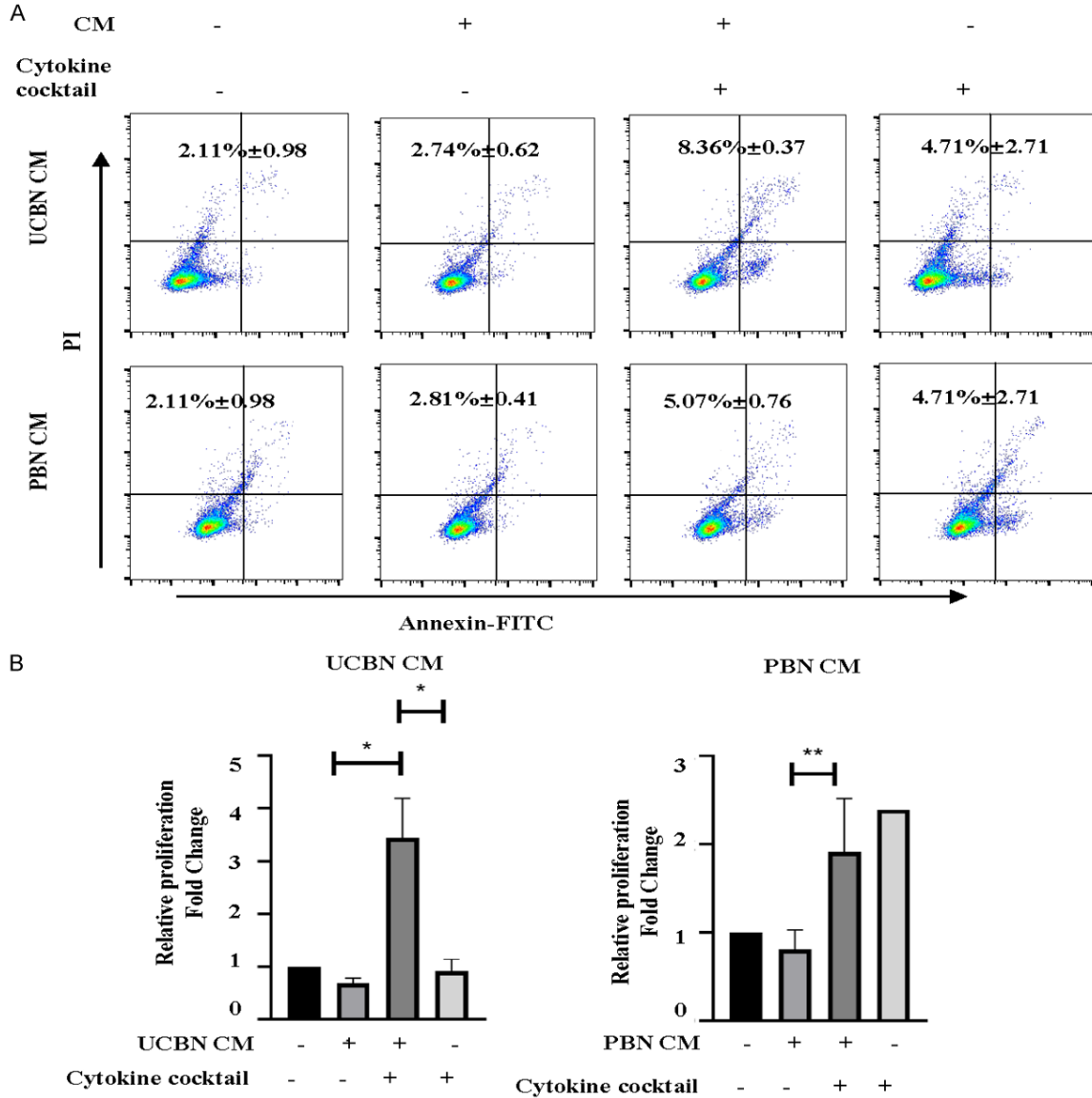
# High viability umbilical cord blood-derived neutrophils





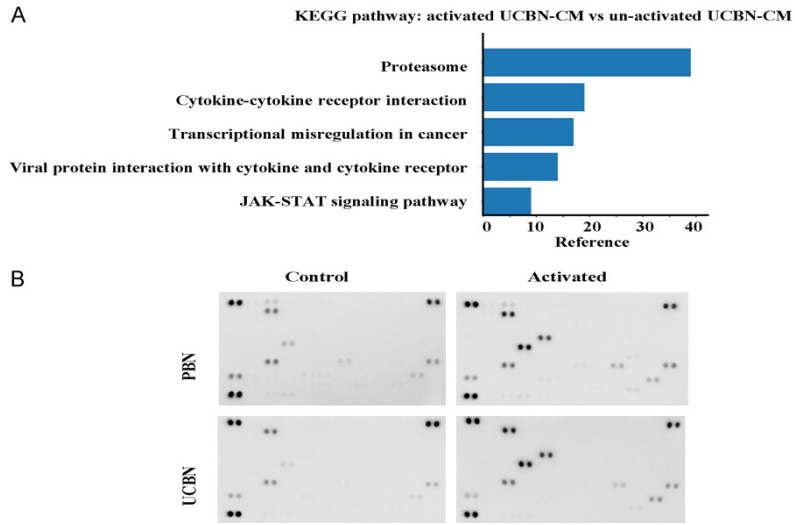
## High viability umbilical cord blood-derived neutrophils

**Figure S2.** Cytotoxicity of condition medium from activated UCBN and PBN determined by EdU analysis. A. Represent images of immunofluorescence staining of EdU of ovarian cancer cells line OVCAR3 treated with conditioned medium of activated UCBN from donors UCB-2, UCB-7, and UCB-8. Bar = 50  $\mu$ m. B. Represent images of immunofluorescence staining of EdU of ovarian cancer cells line OVCAR3 treated with conditioned medium of activated PBN from donors PB-1 and PB-5. Bar = 50  $\mu$ m.



**Figure S3.** Cytotoxicity of conditioned medium of activated UCBN and activated PBN determined by apoptosis analysis. A. Representative flow chart of OVCAR3 treated with conditioned medium of activated UCBN and activated PBN staining using Annexin V-FITC/PI apoptosis detection kit. B. Histogram of apoptosis OVCAR3 cells treated with conditioned medium of activated UCBN and activated PBN respectively (n = 5). \* $P < 0.05$ , \*\* $P < 0.01$ .

# High viability umbilical cord blood-derived neutrophils



**Figure S4.** Screened anti-tumor molecule CCL3 by multi-Omics analysis. A. KEGG pathway enrichment analyses in up-regulated DEGs in activated UCBN-CM and un-activated UCBN-CM. B. Lattice Diagram of Proteome Profiler TM Array.