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Original Article

An optimized method for purification and functional evaluation of neutrophils from living mouse

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Abstract: Neutrophil is a group of innate immune cell that represents the first lines of defense during infection and injury. Recent studies indicate that neutrophils in the microenvironment of tumors can modulate tumor growth, even antitumor or protumor by various manners. However, neutrophils have a very short half-life, undergoing rapid death by apoptosis, thus there isn't a neutrophilic cell line used in laboratory so far. Most of isolating methods gained whatever peritoneal or circulating neutrophils via executing los of mice. Researchers encounter more problems not only in material waste, but also in technical terms including purification, viability, and recovery etc. In this study, we systematically established a method to isolate and identify highly enriched and purified neutrophils (the quantity can reach to 5×10^7 in 5 ml peritoneal lavages; the purity is more than 95%) from living mice. The results indicated that mouse strain greatly affected the inducing ability of neutrophils by peritoneal irritation. Neutrophils from four mouse strains: NOD/SCID, SCID, KM, and Balb/c strains, were compared, the highest purity of neutrophils were gained from NOD/SCID strain (more than 98%). These induced cells expressing more cytokine transcrips including IL-6, CCL3, TNF-α, Fas and Mcl-1 than circulating neutrophils. When co-cultured with tumor cells, induced neutrophils showed cytotoxic activities (the rate of inhibition was about 30%). The optimized purification of neutrophils from living mouse and cell functional characteristics provide a technical method for correlative investigations.

Keywords: Murine neutrophils, peritoneal lavage, mouse strains, purity, induced neutrophils, circulating neutrophils

Introduction

Neutrophil is the largest number type of leucocytes that play a critical role in host defense against invasion from microorganisms [1, 2]. Recent studies augmented its function with participating immuno-modulation, inducing antibody generation [3], involving in stress reaction [2, 4] and extruding strands of DNA with attached proteins that act as extracellular traps [5, 6]. Even in tumor associated immunity, neutrophils also play an intriguing role in antitumor and protumor reactions by altering its polarization direction, named as Tumor-Associated Neutrophil (TAN) [7-11]. Neutrophil's wide biological effects are more important than generally reflected in the literature and recognized more by scientists.

The mouse is an ideal model organism for studies of human disease, widely applied in the fields of screening potential drugs, radiology, bio-security and toxicity, pharmacodynamics evaluation, oncology, genetics and immunology. How to easily acquire highly enriched and purified mouse neutrophils becomes the primary question to study neutrophils function.

Presently, there are several main methods available to obtain neutrophils, including collecting peripheral blood to separate cells depending on the cell density [12, 13], immunomagnetic separation [14, 15] and inducing neutrophils by stimulating peritoneum. The first one needs a large volume of blood (generally, more than 5 ml meet the test requirements), and erythrocyte lysis is an essential step for most separating liquids. Only about 1 ml whole blood can be obtained by sacrificing one mouse that not only poses to resource waste but also might give rise to a change of cell biological characteristics and an effect on subsequent tests, such

Table 1. Stimulation methods

Reagent	Sodium thiosulfate	Glycogen
Concentration%	3-4	0.3-0.4
Injection site	Peritoneal cavity	Peritoneal cavity
Volume (ml)	1	2 (equal to 10% mouse body weight)
Injection frequency	1	4 (1 time/4 h)
Isolation of cells time	2 h or 4 h later	The next day (less than 12 h to last injection time)

as cell adhesion, chemotaxis, superoxide release, production of reactive nitrogen intermediates and granule exocytosis. Negative immunomagnetic separation yields relatively pure populations of murine neutrophils, but gain neutrophils by sacrificing mice, too. Kenneth T Kotz and Wenzong Xiao [16] designed and developed a microfluidic neutrophil-capture cassette based on immunomagnetic separation which can separate 4.5 ± 1.6×10^5 neutrophils from 150 uL whole blood. The purity and activity are more than 90%. Although this microfluidic neutrophil-capture cassette may be reliable, the tool is not yet universal so most of the laboratories could not carry out and the applications are limited. Inducing neutrophils by stimulating mouse peritoneum is convenient and many researchers selected this way to harvest neutrophils by the peritoneal lavage after the intraperitoneal instillation of some stimulants for several hours, such as casein protein acid salt [17], and Bacto NIH thioglycolate broth [18], thioglycolate [19] or thioglycollate [20], corynebacterium parvum [21] and glycogen [22] etc. We have tested parts of the stimulants and found that neutrophil's quantity and purity greatly depend on different stimulants. Some stimulants are even too tight for mice to withstand. Although there is an extensive literature on this isolation, different mouse strain, operation methods and the experimental conditions yield different results so that it is difficult for researchers' reference. Complete detailed method and identification protocol for isolation of neutrophils from mice and these cells' distinction from circulating neutrophils have not been addressed so far. We systemetically optimized a simply, economical and convenient method to acquire highly enriched and purified neutrophils from living mice. The ability to isolate 5×10^7 cells from 5 ml peritoneal lavages permitted us to use biochemical techniques to demonstrate that these induced cells by glycogen have an increased cytotoxic activity than the circulating neutrophils do. And we also highlighted the contrast of the effects on the neutrophils quantity and purity in different mouse strain to facilitate the researchers' reference.

Materials and methods

Mice

Adult mice (including C57BL/6J, Balb/c, NOD/SCID, SCID and KM strain), 8-10 weeks, weighting about 18-22 g were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Animals were kept in collective cages (ten mice per cage) at a constant temperature of 23 (± 2)°C, relative humidity of 40-70% and a cycle lighting conditions (light/dark 12 h/12 h). All mice had free access to water and chow. All efforts were performed to minimize mice suffering according to the proposal of International Ethical Guideline for Biomedical Research.

Cell culture

The murine hepatoma cell line Hepa1-6 and the murine mammary cancer cell line MA782 were from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. They were cultured respectively in RPMI1640 (Gibco) and Dulbecco's Modified Eagle's Media (DMEM, Gibco) with 15% fetal bovine serum (FBS). Isolated neutrophils were resuspended in RPIM1640 medium supplemented with 10% FBS, 2 mmol/l L-glutamine and 0.1% (v/v) penicillin/streptomycin (Sigma-Aldrich) and counted by a Neubauer hemocytometer. Cells were cultured at 37°C in a humidified incubator with 5% CO_2.

Isolation of neutrophils from mouse peritoneal exudate

Mouse abdominal were exposed using a special mice shaving machine (Philips), then disinfected with iodine, 75% alcohol and the mouse

Table 2. Primers for qRT-PCR

Gene name	Primers
Mcl-1	Forward 5'-TGTAAGGACGAAACGGGACTG-3'
	Reverse 5'-GTAGTTTGGTGGCTGGAGCTT-3'
TNF-α	Forward 5'-TTCTGTCCCTTTCACTCACTGG-3'
	Reverse 5'-TTGGTGGTTTGCTACGACGTGG-3'
iNOS	Forward 5'-CTGTCGCAGCTCCCTATCTT-3'
	Reverse 5'-ACTGACACTTCGCACAAAGC-3'
Arginase-1	Forward 5'-CTGAGCTTTGATGTCGACGG-3'
	Reverse 5'-GAGTTCCGAAGCAAGCCAAG-3'
Fas	Forward 5'-TGCATGACAGCATCCAAGACA-3'
	Reverse 5'-GTTTCCATCATGGGTGGCAG-3'
CCL-3 (MIP-1α)	Forward 5'-TCACCTGCTCAACATCATG-3'
	Reverse 5'-TCAGGCATTCAGTTCCAGCT-3'
IL-6	Forward 5'-AGTTGCCTTCTTGGGACTGATG-3'
	Reverse 5'-GGGAGTGGTATCCTCTGTGAAGTCT-3'
β-actin (internal control genes)	Forward 5'-TCTACAATGAGCTGCGTGTG-3'
	Reverse 5'-GGTCAGGATCTTCATGAGGT-3'

was injected as above mentioned (**Table 1**, all drugs were purchased from Sigma-Aldrich and filtered by disposable filtrums). 2 h, 4 h or the next day, mice abdomens were disinfected with iodine and 75% alcohol again and injected with 37°C preheating RPMI1640 medium by 10 ml syringe equipped with 25 G needle (less than 10 ml/mouse). The state of mice was observed carefully. Injections were stopped when abdominal contents stirred up and the pinhole was immediately pressed for more than 5 minutes by the dehydration pressing lint ball to prevent the liquid from flowing along the pinhole. Then the mice were allowed to move freely for 3-5 minutes.

Afterwards, mice were held on an appropriate slope. After abdominal skins were disinfected with iodine and 75% alcohol, we thus could even see the liquid surface and extract the peritoneal lavage with 5 ml syringe at the bottom of the abdomen. Lest erythrocyte contamination, abdomen capillaries should be far away as possible when needle puncturing. The peritoneal lavages were centrifuged with 500 g for 10 minutes and the pellet was resuspended in complete RPIM1640 medium.

Isolation neutrophils from peripheral blood

Peripheral blood neutrophils were separated using Mouse Neutrophils Separation Liquid (TBD science, China) as described in the manufacturer's instructions.

Morphological analysis

Five isolated neutrophils smears were stained by Wright-Giemsa Stain (BASO Diagnostics Inc. Zhuhai, China) and observed under a light microscope (Olympus, Japan). Neutrophils were calculated following the percentage of 500 cells in different fields (more than 5 fields). Neutrophils viability was determined by exclusion of 0.1% trypan blue dye as previously described [23].

Percent of living cells = (1-A/500) ×100%, A represents stained cells.

Flow cytometric analysis

After centrifugation, the pellet was resuspended and 2×10^5 cells were studied by FACS analysis as previously described [24, 25]. FITC-conjugated anti-mouse CD11b and PE-conjugated anti-mouse Ly6G (BD Bioscience) were used to specific marker of neutrophils [8]. All flow cytometry was done by Cytomics™ FC500 Flow Cytometry (Beckman Coulter). Data analysis was performed by CXP software.

Laser scanning confocal microscope analysis

FITC-CD11b and PE-Ly6G labeled cells were drop in laser confocal small dish and detected immediately by Ultra VIEW VOX (Perkin Elmer). All Images were processed with Adobe Photoshop CS4.

mRNA analysis

After mouse neutrophils were prepared from the peritoneal lavages or circulating blood, cells lysis was immediately performed and total RNA was extracted using RNeasy kit (Invitrogen) as the manufacturers instructions. cDNA was synthesized using PrimeScript™ RT regent kit (TaKaRa). Quantitative Real-Time PCR analysis was carried out on the MX3000P System (Stratagene) using SYBR Green Premix ExTaq™ PCR kit following the manufacturer (TaKaRa). The condition of qRT-PCR amplified reaction was 95°C/10 minutes with a melt of 95°C/15

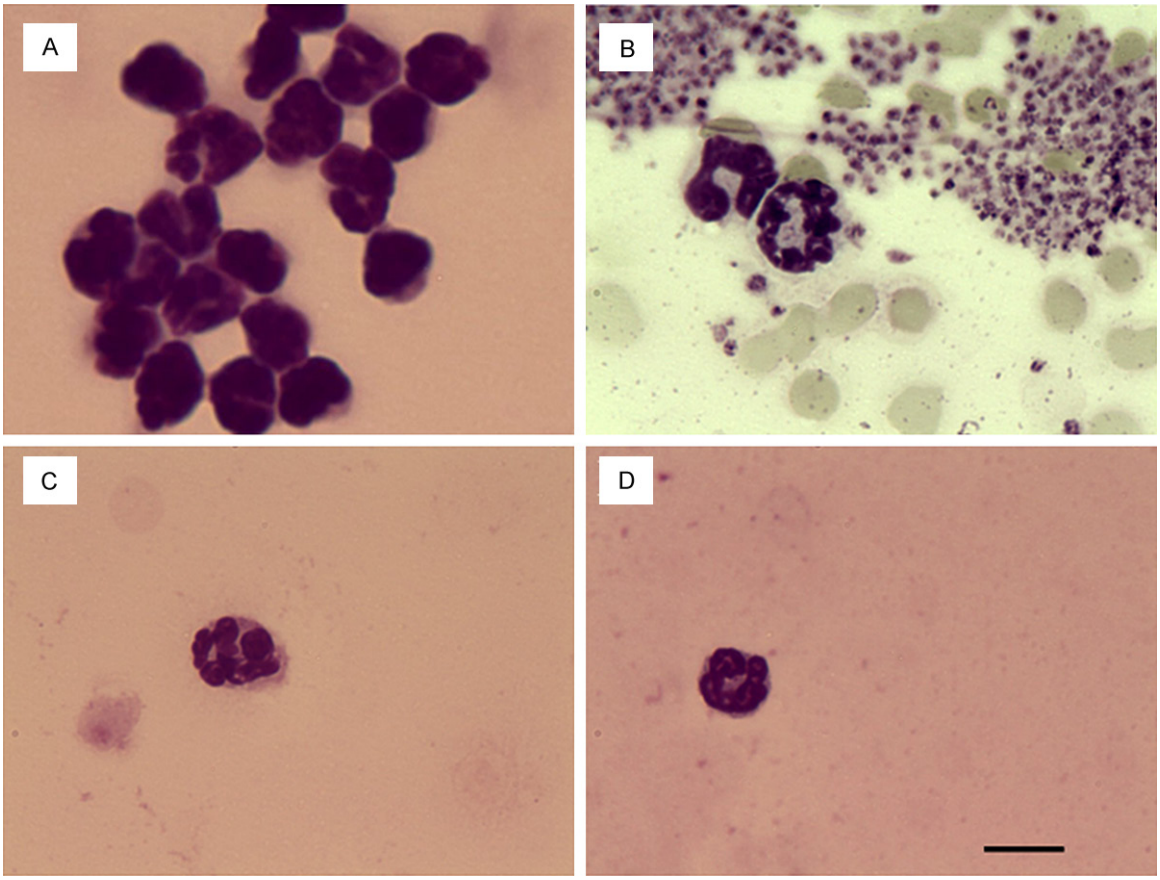

Figure 1. Neutrophil morphology contrast. Neutrophils (from the peritoneal lavage and peripheral blood) smears stained by Wright-Giemsa were observated under a light microscope. (A), (C) and (D) respectively represented neutrophils from peritoneal lavages: (C) for polymorphonuclear, and (D) for rod nuclear. (B) Represented neutrophils from peripheral blood of healthy mice, and typical polymorphonuclear and rod nuclear could be seen. Scale bars, 10 um.

seconds on first cycle and 45 cycles of 95°C/15 seconds, 60°C/20 seconds. The comparative cycle threshold (Ct) was used to measure the relative levels of gene expression. For analysis the mRNA expression of apoptosis and inflammatory chemokines, the following primer sequences were designed as **Table 2**.

Neutrophils cytotoxicity analysis

Tumor cells (murine breast cancer cells MA782, murine hepatoma Hepa1-6) were inoculated in 96 well plate marked as group A (tumor cells control) and group B (co-cultured with neutrophils next day), with inoculation density of 3,000 cells and 50 ul per well. Afterwards cells were cultured at 37°C in a humidified incubator with 5% CO_2 for 24 hours. Neutrophils obtained by the peritoneal stimulation, were inoculated in group B of the 96 well plate and individual group C (neutrophils control). The cell density was adjusted to 1.2×10^6/ml, 50 ul/well. Each group was set 5~6 parallel wells. After continuing to be cultured for 24 h, each well was added with 10 ul CCK8 solution (Cell Counting Kit-8, Dojindo) and the 96 well plate was incubated for 2~4 h at 37°C. Then the 96 well plate was centrifuged with 500 g for 5 minutes and detected at 450 nm by enzyme-labeled instrument (OD value) (Multiskan Ascent, Thermo Labsysterms, Finland).

$I\% = (A-(B-C))/A\times100\%$

I% represents the inhibition rate to tumor cells.

Statistics analysis

Values were expressed as mean ± SD. Data were analyzed for statistical variance using a

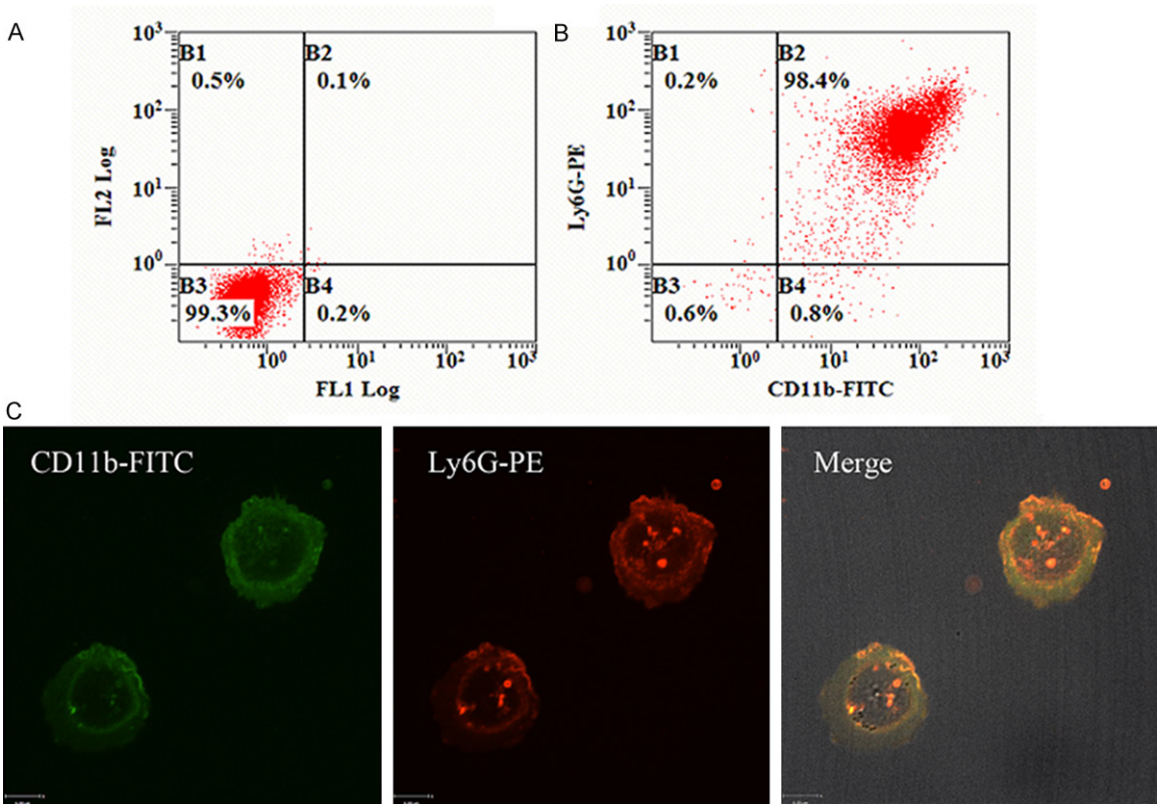

Figure 2. Flow cytometric analysis and the laser confocal appraisal of induced neutrophils. (A and B) Flow cytometry was performed to identify neutrophils from NOD/SCID strain by FITC- CD11b and PE-Ly6G labeling. (A) Shows the percentage of detected peritoneal lavage cells, and (B) shows representative FACS tracings of CD11b versus Ly6G expression in all these induced cells. (C) Shows the laser confocal results. The picture on the right side was merged with the first two pictures. Scale bars, 10 um. All results were repeated more than three times. **=P<0.001.

paired Student's *t*-test and P≤0.05 was considered significant.

Results

Neutrophils morphological observation

The neutrophils isolated from the mice peritoneal cavity (NOD/SCID) had complete structure, clear envelope and nuclear structure, as shown in **Figure 1A**, characterized as the neutrophil-like typical polymorphonuclear and rod nuclear structure. Cell activity was appraised by typlan dye for more than 98%. Most cells were relatively concentrated (**Figure 1A**). A single cell was selected as shown in **Figure 1C** (polymorphonuclear) and **Figure 1D** (rod nucleariform). **Figure 1B** was a smear of peripheral blood and showed neutrophils morphology as a contrast. The peritoneal lavage and peripheral neutrophils showed no difference in morphology.

Flow cytometric analysis and the laser confocal microscopic appraisal of isolated neutrophils

To evaluate the purity more exactly, the obtained cells were subjected to FACS to detect $CD11b^+$ and $Ly6G^+$ cells. CD11b and Ly6G are specific antibodies of mouse neutrophils [8, 25], therefore depending on flow cytometric analysis we can confirm the cell purity from the peritoneal lavage. NOD/SCID mice were selected to isolate neutrophils by peritoneal stimulation. 10000 cells per sample were prepared for flow cytometric analysis. As **Figure 2A** showed, the double-positive ratio of neutrophils labeled by FITC or PE-conjugated anti-mouse CD11b and Ly6G is 98.4%. Therefore, we considered that the cell purity from the peritoneal lavage was more than 98%. Furthermore, laser confocal microscope results (**Figure 2B**) showed a more intuitive result.

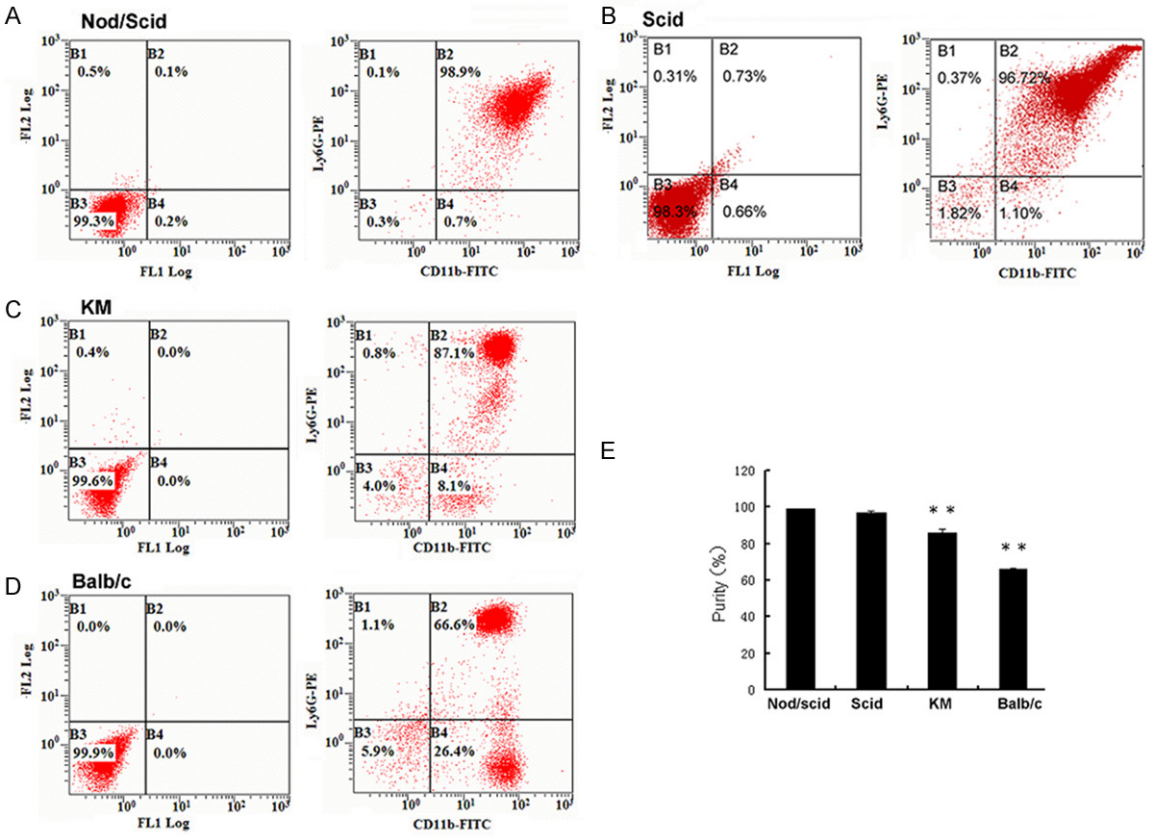

Figure 3. Purity contrast of neutrophils from different mice. Neutrophils were obtained from different strain mouse peritoneal cavity by glycogen stimulating. (A-D) represents flow cytometric results respectively for four kind of mice: NOD/SCID strain, SCID strain, KM strain, and Balb/c strain. The percentage of detected peritoneal lavage cells and the FACS tracings of CD11b versus Ly6G expression can be observed in each group of figures. (E) summarizes the percentage of $CD11b^+$ and $Ly6G^+$ cells from different strain mouse. **P<0.001.

Purity contrast of neutrophils from different strain mouse

To determine whether the ability of neutrophils travelling to peritoneal cavity is similar to different strain mouse, we selected several laboratory commonly used model mice which were subjected to peritoneal cavity stimulation with glycogen following the above protocol. SCID mouse strain is known as severe combined immunodeficiency with impairing the differentiation of both T and B lymphocytes but has normal function NK cells and Macrophages [27]. NOD/SCID mouse strain is a hybrid strain of NOD/Lt and SCID with the characteristics that retains the SCID mouse strain feature and adds these features: low NK cell activity, non-circulating complement, and dysfunction macrophages and APC (antigen-presenting cell) [28]. KM and Balb/c strain are respectively outbred and inbred line with normal immunofunction. We found that the percentage of neutrophils was different in the different strains (**Figure 3A**). The double-positive ratio of neutrophils labeled by anti-mouse CD11b and Ly6G, i.e. the purity of neutrophils in NOD/SCID and SCID strain is > 95% (**Figure 3A**). The quantity of neutrophils can reach to 5×10^7 in 5 ml peritoneal lavages. The lowest purity belongs to Balb/c strain (**Figure 3A**). **Figure 3B** summarizes the purity contrast of neutrophils from the four different mice. Therefore, in subsequent experiments, NOD/SCID strain was selected as experimental subjects.

Induced neutrophils express multiple mRNA transcrips compared with circulating neutrophils

The previous experiments have established a complete detailed protocol of isolation of highly

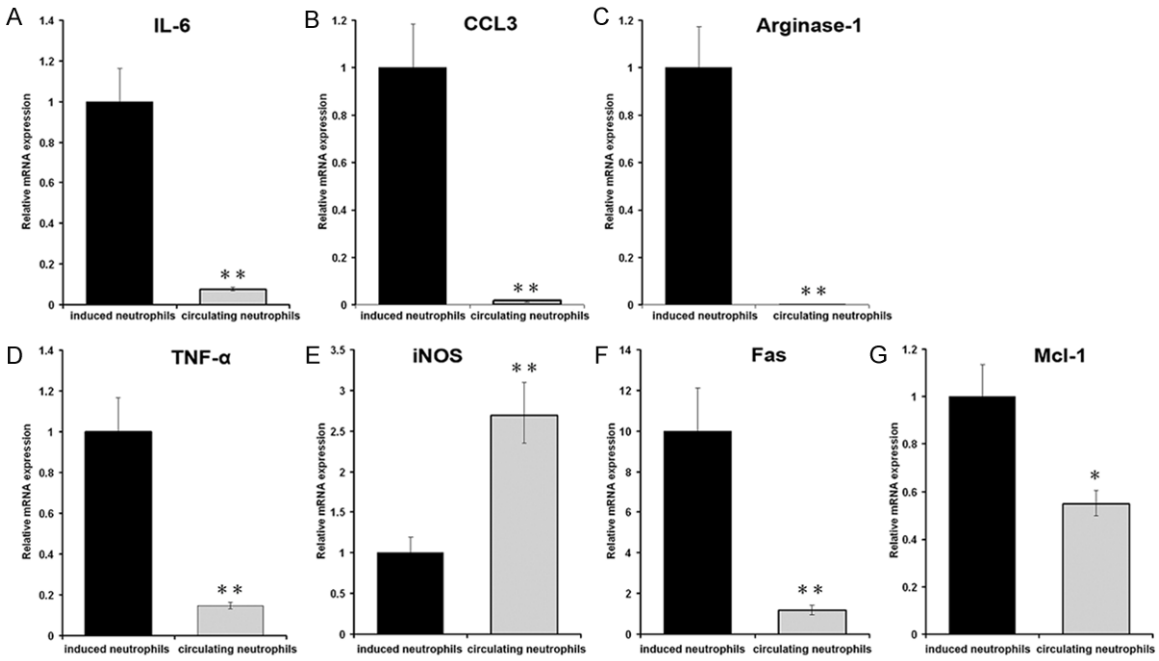

Figure 4. Correlative cytokine mRNA transcripts by the induced and circulating neutrophils. Relative mRNA expression of individual cytokine was compared. (A-E) Respectively represented pro-inflammatory cytokine mRNA transcripts: (A) IL-6, (B) CCL3, (C) Arginase, (D) TNF-α, (E) iNOS. While (F) and (G) represented apoptosis cytokine mRNA transcripts: (F) Fas, (G) Mcl-1. Unpaired Student's *t*-test was used to analyze, $^{**}P \leq 0.005$, $^{*}P < 0.05$, n=3.

enriched and purified neutrophils from the peritoneal lavage. To better understand the biological effects of neutrophils recruited to the peritoneal cavity, we contrasted the relative mRNA expression of some cytokines between the peritoneal lavage and circulating neutrophils as **Figure 4** showed. Purified neutrophils RNA was analyzed by agarose gel electrophoresis and shown typical of intact 28S and 18S ribosomal RNA (data not shown). RNA purity was identified by optical density ratio read at 260:280 nm. The ratio range of 1.6~2.0 was accepted. Compared to circulating neutrophils, we found that some inflammatory cytokines had a significant increase of 5- to 10- fold at the mRNA levels in induced neutrophils than circulating neutrophils, including IL-6, CCL3, Arginase-1 and TNF-α, P<0.005 (**Figure 4A-D**). On the contrary, induced neutrophils expressed lower iNOs mRNA than circulating neutrophils, P<0.005 (**Figure 4E**).

As a short-life cell, whether induction affects the apoptosis processing of neutrophils is not known. Therefore, we analyzed two apoptosis associated genes: Fas and Mcl-1 as well. Fas gene mediates apoptosis and Mcl-1 is one member of Bcl-2 family acting to protect from apoptosis. Fas gene mRNA of peritoneal lavage neutrophils expressed considerably higher level (P<0.005) than circulating neutrophils (**Figure 4F**) and Mcl-1 gene mRNA was relatively higher (P<0.05) than peripheral blood neutrophils (**Figure 4G**).

Induced neutrophils can inhibit tumor cells growth

To evaluate a potential function of these cytokines, we assessed neutrophils cytotoxicity. Tumor cells and neutrophils were co-cultured as described in methods. Neutrophils from the peritoneal lavage liquid can inhibit tumor cells growth to some extent as **Figure 5** showed (P≤0.005). When efficient : target (neutrophil : tumor) was about 40:1, the inhibition rate to MA782 and Hepa1-6 was 32.7% and 26.0%, respectively.

Discussion

Neutrophils are mature terminal cells with short half-life. Circulating neutrophils can survive for 8- 20 h, but when entering the tissue, the lifespan may be extended [29]. Away from the body,

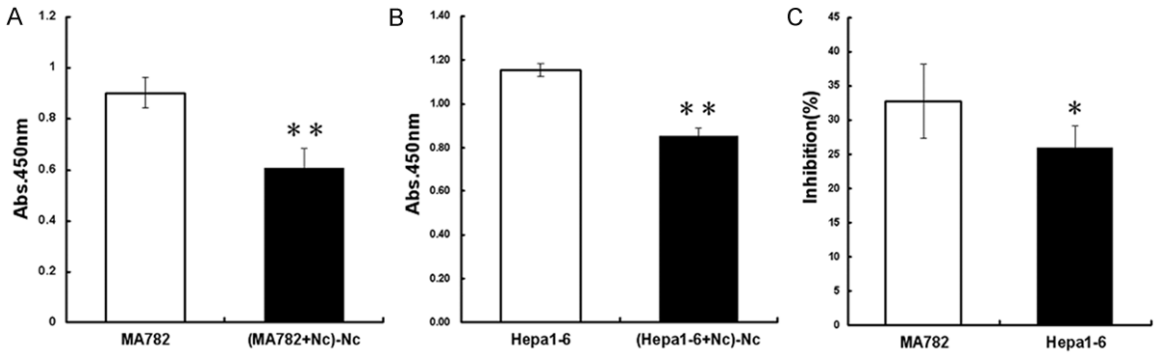

Figure 5. Neutrophils from the peritoneal lavages inhibiting tumor cell growth. Tumor cells (MA782 and Hepa1-6) and neutrophils were co-cultured in the 96 well plate and assayed for absorbance at 450 nm on enzyme-labeled instrument by CCK8 kit. Effector : target (neutrophils : tumor cell) was 40:1. (A and B) White column represents tumor cells control and black column represents tumor cells (treated with neutrophils) absorbance subtracting neutrophils absorbance itself. (C) Represents the inhibition of neutrophils to two types of tumor cells, *P≤0.005. Data are expressed as mean ± SEM and **P≤0.005 compared to respective control group, 3 mice per group, and two-tailed Student's *t*-test.

thus the neutrophils quickly undergo apoptosis which survival time is not more than 24 hours [30, 31]. Therefore, neutrophils could not continue to be cultured after separated and there is not a neutrophil cell line for experimental study as well. This makes it difficult to study neutrophil functions. This study was undertaken to establish a set of cheap-easy methods and evaluation systems to yield rich and purified neutrophils.

Peritoneal stimulant were used to yield neutrophils, especially Glycogen which was gentler and had little side effects than other stimulants (such as contrast stimulants: sodium thiosulfate, data not shown). Compared with previous reports [22], our experimental program increased inducing neutrophils by reducing concentration and increasing intraperitoneal frequency. And enough neutrophils could be yielded for subsequent tests, such as cell identification, cytotoxicity assay, PCR and Western blot, etc. Thioglycolate, many researchers selected it as peritoneum stimulant [18-20] was not recommended for poor immunity mice, such as NOD/SCID, SCID, nude mice etc. due to its severe stimulant.

However, whether the same results can be obtained from different mouse strains using this method hasn't been known. In our experiments, neutrophils quantity and purity of four common mouse strains in laboratory (NOD/SCID, SCID, KM and Balb/c) were identified by $CD11b^{+}Ly6G^{+}$ labeling. In NOD/SCID and SCID strain, the purity is more than in Balb/c strain. The reached cell quantity was enough to study. We can conclude that when exogenous activation occurs, immunodeficiency may lead to a large normal innate immune cells-neutrophils infiltrate into stimulant sites and perform the mainly activity. Regarding to KM and Balb/c mice with normal immune function, lower cell purity may be caused by all kind of immune cells infiltrating in peritoneal and the absolute proportion of neutrophils decreased (more than 60%, **Figure 3**). C57BL/6J strain was refused for its fierce temperament and black skin that enhanced the operation difficulty. After one time experiment, mice could be continued feeding and recover completely in about 7 days, and the purity of neutrophils had no difference with the first time (P<0.05, data not shown). Thus, these mice could be used repeatedly and plenty of experimental resources were saved. Operators can select corresponding mice according to their experimental purposes.

All these induced neutrophils in our methods had important biological function, especially as a receptor of some cytokines [34]. Cytokines could transfer information among cells and generate effects through immune regulation [32]. When cells were subjected to a certain stimuli, cytokines could be synthesized rapidly and secreted into the extracellular and even the pmol level can play a significant biological function. In addition, the different stages of the same type of cells could generate different

numbers and types of cytokines [33]. Therefore, the mRNA expression of some inflammatory factors and apoptosis gene in induced neutrophils were analyzed. As our results indicated, variety of mRNA (IL-6, CCL3, Arginase and TNF-α) were higher in induced neutrophils than in circulating neutrophils. Some cytokines were even multifunctional promote inflammatory or immune stimulation factor, such as TNF-α [35]. Induced neutrophils could highly express CCL3 (macrophage inflammatory protein 1α: MIP-1α), while CCL3 also affects the neutrophil chemotactic function [36]. IL-6 and arginase were also multi-function inflammatory cytokines, and played a key ingredient in inflammatory medium network, inflammatory reaction and inflammation related tumor [7, 35, 37]. It was not clear that whether the high expression of IL-6, CCL3 and TNF-α meant that neutrophils had cytotoxic functions. We then evaluated the cytotoxicity though co-culture the MA782/Hepa1-6 cell lines with neutrophils. Our research didn't agree with Alan Lichtenstein and Janet's research [21]. In our results, after being induced for 24 h by glycogen, the inhibition rate of neutrophils to tumor cells could reach to about 30% and the lysis capability of neutrophils to different tumor cells were different. And circulating neutrophils couldn't inhibit tumor-cell growth.

It was worth noting that iNOS mRNA expression of induced neutrophils was lower than circulating neutrophils in our data. All we know that NO could kill target cells through inhibiting the Krebs cycle, electron transfer and cellular DNA synthesis. NO is the substrate of iNOS. Therefore, iNOS can indirectly reflect the generation of NO [38, 39]. Zvi G.F ridlender *et al.* also stated where the tumor microenvironment polarized TANs (Tumor-associated neutrophils) toward a protumor (N2) versus an antitumor (N1) phenotype and the factors related N1 phenotype were Fas, CCL3, TNF-α and ROS (reactive oxygen species), except of iNOS [8]. Therefore, our data might indicate that the ability of inhibiting tumor growth derived from inflammatory factor IL-6, CCL3 and TNF alpha, but not iNOS. And the mRNA expression of induced neutrophils had similarities to N1 phenotype.

Fas is expressed on the surface of cells and forms FasL/Fas apoptosis pathway to induce apoptosis [40], but it won't cause cell death when Fas McAb co-cultured with leucocyte [41]. Therefore, at first, Fas biological function does not involve apoptosis, but early signs of cell activation [41]. Mcl-1 is one of antiapoptotic protein in Bcl-2 family, and characterized by a rapidly induced survival protein that is subject to rapid turnover [29]. The activating of apoptosis cytokines in induced neutrophils might affect nuetrophils lifespan.

In conclusion, we described an effective evaluation system of neutrophil isolation and provided a facility of mouse neutrophils study. Our studies emphasized the characteristics of these induced cells differing from the circulating neutrophils. This system might be convenient for researchers to isolate neutrophils and correctly choose appropriate animal recourse according to the experimental demands.

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Disclosure of conflict of interest

None.

Authors' contribution

Yinghong An and Shoujun Yuan conceived and designed the experiments. Yinghong An performed the experiments and Zixue xuan was the assistant. The data were analyzed mainly by Yinghong An, Chengwang Xu and Shanshan Wang. Dexuan Yang contributed reagents/materials/analysis tools. The whole paper was written by Yinghong An.

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