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Research article

Optimized method for higher yield of alveolar macrophage isolation for *ex vivo* studies

Surya Prasad Devkota, Chinemerem Onah, Prabhu Raj Joshi, Sandeep Adhikari, Pankaj Baral [*,1]

Section of Microbiology and Immunology, Division of Biology, Kansas State University, Manhattan, KS, USA

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ABSTRACT

Alveolar macrophages (AMs) are a fully differentiated lung-resident immune cell population and are a critical component of lung immunity. AMs can be easily isolated from mice via bronchoalveolar lavage fluid (BALF) collection. The quality and quantity of AMs in BALF isolation are critical for generating reliable and high-quality data for *ex vivo* studies. Traditional techniques use ice-cold (4°C) buffer to collect AMs in BALF and result in low yield. Hence, a new method that consistently gives a higher yield of AMs is needed. We demonstrate here an optimized method that significantly increases the quantity of AM recovery in BALF (>2.8 times than the traditional method). Our method uses a warm-buffer (37°C) containing EDTA. We compared the viability and functional parameters (cytokine/chemokine expression, phagocytosis) of AMs isolated by our new and traditional methods. Our study revealed that AMs collected using our method have similar viability and functional characteristics to those collected using traditional method. Hence, our new method can be used for the collection of a higher number of AMs without altering their function. This protocol might also be useful for isolating tissue-resident immune cells from other anatomical sites for *ex vivo* and other downstream applications.

1. Motivation

Low AM yield in bronchoalveolar lavage fluid (BALF) collection poses a significant challenge for *ex vivo* studies of AMs. The inability to propagate and maintain these cells for a longer period also aids further challenges. Most protocols use ice-cold buffer (4°C) for AM collection in BALF which results in low cell yield. Here, we demonstrate an optimized protocol that gives significantly higher AM yield in BALF without affecting their function.

2. Introduction

Two populations of macrophages exist in the lungs: alveolar macrophages (AMs) and interstitial macrophages (IMs). The AMs are located near the alveolar type I and II epithelial cells [1] whereas IMs are present between the alveolar epithelium and the vascular endothelial layer [2]. AMs represent a tissue-resident macrophage (TRM) population in the lungs and are the major component of

* Corresponding author.
E-mail address: baral@ksu.edu (P. Baral).
[1] Lead contact.

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innate immunity [3]. TRMs are located at different anatomical sites (tissues and body cavities) throughout the body and are derived either from circulating monocytes or embryonic precursors [4]. AMs are prenatally derived, long-lived TRMs, capable of self-renewal and long-term maintenance [3,4]. They are essential for mediating host protection against pathogens and lung injury [5]. The inhaled pulmonary pathogens first encounter AMs before invading the lung tissues and disseminating to extra-pulmonary sites. Furthermore, activated AMs secrete multiple signaling molecules [6] (such as IL-1, TNF-α, IL-6, antimicrobial peptides, etc.) that communicate with other immune cells and play a critical role in mediating tissue homeostasis and inflammation [7,8]. AMs, under normal homeostatic conditions, are highly phagocytic and are the dominant cell type in pathogen recognition and elimination, and in the clearance of the cellular debris [2,9].

Bone marrow-derived macrophages (BMDMs) have been extensively used for the *in vitro* studies of macrophages because they can be propagated and cultured for a longer period. In contrast, the *in vitro/ex vivo* study of the primary AMs is challenging as they need to be freshly isolated every time and cannot be cultured for longer period [10]. Furthermore, BMDMs cannot accurately represent the AM immune responses that occur during primary lung infection/injury. In particular, BMDMs differ significantly from AMs in their ontogeny (monocyte-derived vs. embryonic-derived), immune response, metabolism, proliferation, and tissue repair abilities [11,12].

AMs are one of the most appropriate resident immune cell populations to study the innate immune responses in lung diseases as they constitute >90 % of the cells in the alveoli in the steady state and are critical in lung function and immunity [5]. Additionally, AMs highly express pattern recognition receptors to detect and respond rapidly to invading pathogens or any inhaled threats [6]. AMs represent the key immune cell types of lung immunity by mounting an appropriate immune response to destroy a wide variety of insults that reach to alveoli [2,9]. AMs can be isolated either via bronchoalveolar lavage fluid (BALF) collection with buffers or by digesting the lung tissue to make single cells, followed by antibody staining of the cells and fluorescence-activated cell sorting of $CD45^{+}CD11b^{-}Siglec\text{-}F^{+}CD11c^{+}F4/80^{+}$cells [13]. BALF collection using ice-cold buffer (4°C) is one of the most commonly used and reliable methods for the isolation of intact AMs [14–17]. However, this technique cannot fully isolate AMs because some AMs are firmly attached to the alveolar space [16]. Another significant problem with this method is the low AM yield and high variability in AM isolation. This causes a significant technical challenge and requires a labor-intensive effort to pool AMs from multiple mice for setting up *ex vivo* culture or for other downstream analyses.

Multiple studies using conventional ice-cold buffers have reported AM recovery in lavage collection in the range of 30,000–40,000 per mouse [18,19]. A few other studies also used chelating agents (e.g., Ethylenediaminetetraacetic acid, EDTA) for the AM isolation in lavage cells. Even after using the chelating agent, the AM recovery was reported in the range of 20,000–50,000 per mouse [20,21]. These along with other studies have used various concentrations (0.5–5 mM) of EDTA to collect lavage cells [20,22–24]. Similarly, these studies also used different volumes (0.4–4 mL) of buffer to lavage the lungs [18–22,24,25]. However, all these different approaches did not improve the yield and variability issue of AM isolation. Hence, there is a need to optimize the protocol that includes the optimal buffer composition, lavage volume, buffer temperature, etc. In this study, we altered the buffer composition and temperature that significantly increased the total AM yield in lavage without altering their functional characteristics. In our protocol, we incorporated 2 mM EDTA to the buffer and elevated the buffer temperature that facilitated the detachment of the AMs that are firmly attached to the alveolar wall. Since AMs are surrounded by fibroblasts, endothelial cells, and alveolar epithelial cells in a tight space, it

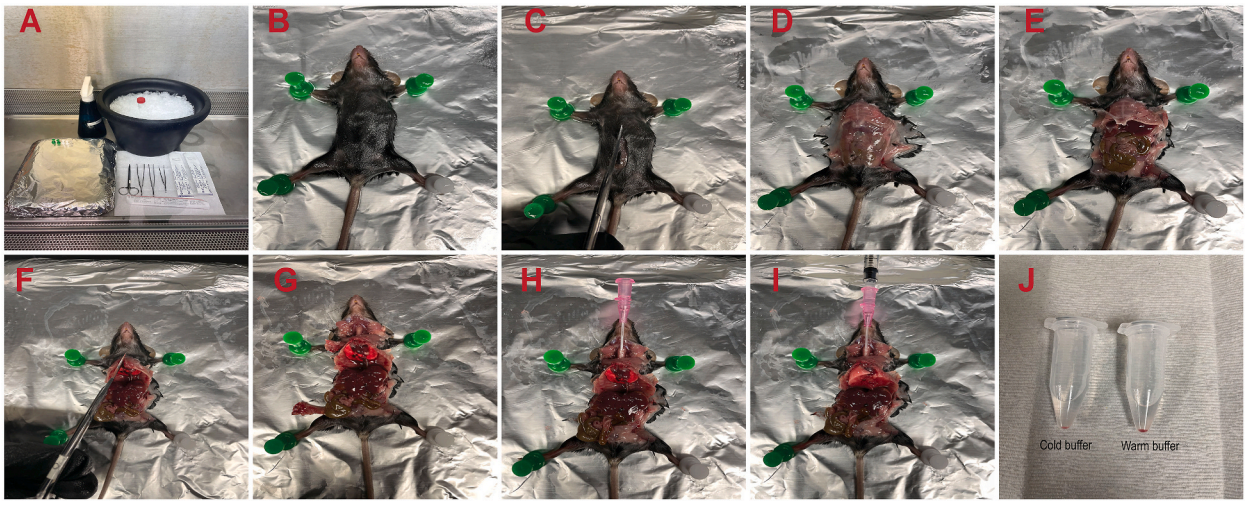


Fig. 1. Overview of dissection setup and BALF collection (A) Workbench set up for the mouse dissection and necessary tools. (B–I) Steps involved in mouse dissection and BALF isolation. (B) Fix the mouse on the dissection platform facing upside using thumb pin and spray 70 % ethanol throughout the exposed area. (C) Cut open the outer stomach layer. (D–E) Expose the abdomen and chest all the way to the trachea. (F) Cut the peritoneum, diaphragm, and chest cavity all the way to the neck without damaging the lungs and trachea. (G) Expose lungs and trachea clearly by cutting the muscles and tissue surrounding them. (H) Insert the 20 G cannula (BD Insyte Autoguard) into the trachea. (I) Lavage the lung with BALF buffer using 1 mL syringe containing 0.8 mL buffer four times, (J) Collect BALF in a 5 mL microcentrifuge tube, place it on ice, centrifuge the lavage, and follow the BALF analysis procedure.

is difficult to harvest them by the lavage using only phosphate-buffered saline (PBS) or culture media. Using a warm buffer (37°C) containing PBS, 1 % fetal bovine serum and EDTA for the lavage of the lungs may open the tight space in the alveoli, facilitating the release of AMs into the open space. The use of 1 % bovine serum helps to maintain the viability of detached AMs during lavage collection. EDTA chelates the calcium ions that are essential for maintaining cellular adhesion. It is possible that the combination of warm buffer and EDTA synergistically facilitates the release of AMs by weakening cell adhesion and widening the narrow cavities in the alveolar space.

After collection, we compared the viability of AMs isolated using our protocol vs. the traditional method (ice-cold buffer). In addition to this, we also analyzed the AMs of their ability to secrete cytokine and chemokine following lipopolysaccharide (LPS)

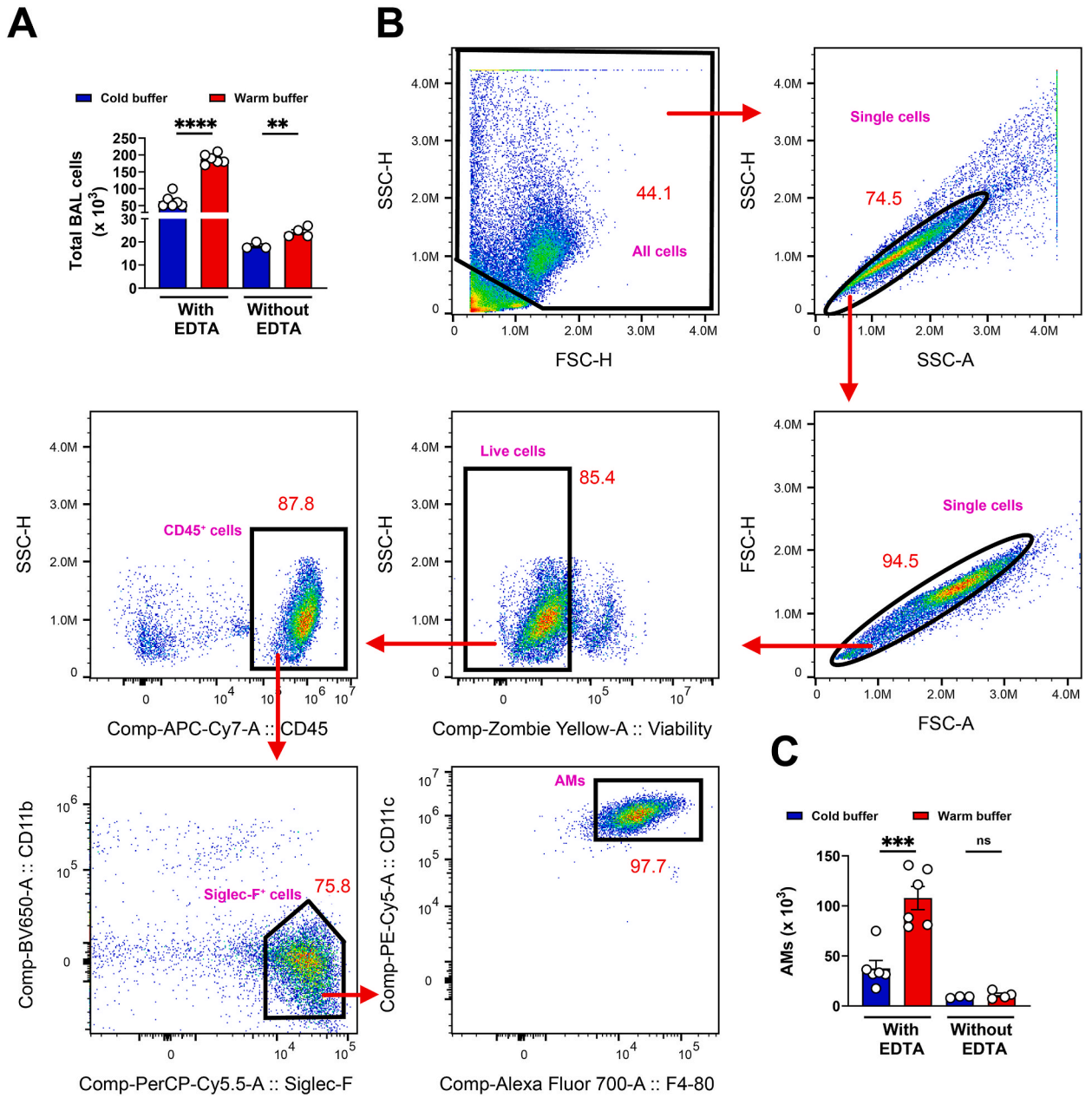


Fig. 2. Total BAL cells, and gating strategy to characterize and quantify alveolar macrophages. (A) Quantification of total BALF cells using a hemocytometer. BALF cells were isolated using cold buffer and warm buffer. (B) Characterization of alveolar macrophages (AMs) in BALF using flow cytometry. BALF single cells were gated for Live/Dead^{-} CD45^{+} leukocytes, which were further gated as CD11b^{-}Siglec-F^{+} (SF) cells. The CD11b^{-}SF^{+} cells were further separated as CD11c^{+}F4/80^{+} cells (AMs). (C) Total AM quantification in BALF isolated from ice-cold and warm buffers by flow cytometric analyses. Data in (A) & (C) are Means ± SEM & were analyzed by two-tailed unpaired *t*-test with the following significance level (*p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001). Each dot represents a single mouse.

stimulation, their steady-stage polarization pattern, and their abilities to phagocytose fungal conidia. Our new method may also produce a higher yield of other resident immune cell types (e.g., peritoneal macrophages) during their isolation.

3. Results

3.1. BALF collection using warm buffer containing EDTA yields significantly higher number of AMs than the AM number recovered in conventional method

First, we compared the total lavage cell and AM yield resulting from our technique with conventional technique. As in published literature, we performed post-mortem BALF collection, right after euthanizing mice with CO_2 overdose. The overall setup steps and necessary tools for BALF collection are shown in Fig. 1. We observed that the use of warm buffer (37°C) containing EDTA led to about three-fold increase in the total lavage cells (Fig. 2A) compared to conventional method. To confirm and compare the absolute AM count, we performed flow cytometry using AM-specific antibody panel. AMs were characterized as $CD45^+CD11b^-Siglec\text{-}F^+CD11c^+F4/80^+$ cells (Fig. 2B). Our results demonstrate a significantly higher yield of viable AMs using warm buffer containing EDTA (>2.8 times on average) (Fig. 2C). We believe that the increase in the number of AMs with warm buffer is more likely due to the increased number of total BAL cell isolation from the airspaces and lungs. We also determined the numbers of total BAL cells and AMs isolated from both cold and warm buffers without EDTA. Our results revealed that the numbers of both BAL cells and AMs isolated from each buffer without EDTA were very low compared to the numbers isolated from buffers with EDTA (Fig. 2A–C). These results thus suggest that the addition of EDTA in the BALF buffer is critical for the maximal yield of AMs.

3.2. BAL cells collected using warm and ice-cold buffers showed no difference in cell viability

Next, we compared the viability of BAL cells that were collected using both buffers by apoptosis assay. Fig. 3A shows the FACS plots illustrating the gating strategy for characterizing lavage cells at various stages of viability/apoptosis. We observed no difference in the proportions of live (Annexin V^- Propidium $iodide^-$) cells isolated using both buffers. In both groups, around 73 % of cells were found to be alive (Fig. 3). In addition, the percentages of early apoptotic (Annexin V^+ Propidium $iodide^-$), late apoptotic (Annexin V^+ Propidium $iodide^+$), and dead cells (Annexin V^- Propidium $iodide^+$), were also comparable in BAL cells between the two groups (Fig. 3B). Altogether, our results demonstrate that BAL cells isolated using warm buffer exhibit similar viability as those isolated with the traditional method.

3.3. AMs collected using warm and ice-cold buffers showed similar cell viability

After the viability analysis of BAL cells, we further analyzed the viability of AMs by flow cytometry. For this, we first identified AMs in BAL cells by the surface staining of AM-specific markers and then determined their viability by Annexin V and Propidium iodide staining as described in Fig. 3. Our study showed that around 90 % of the AMs (Annexin V^- Propidium $iodide^-$ cells) isolated using cold (with EDTA) and warm (with EDTA) buffers were viable (Fig. 4). The percentage of early and late apoptotic cells was less than three percent in both cases (Fig. 4B). The proportions of dead cells were also similar for both groups (Fig. 4B). Hence, our new method of AM isolation using warm buffer doesn't impact AM viability.

3.4. AMs collected using both warm and ice-cold buffers were activated similarly following LPS treatment

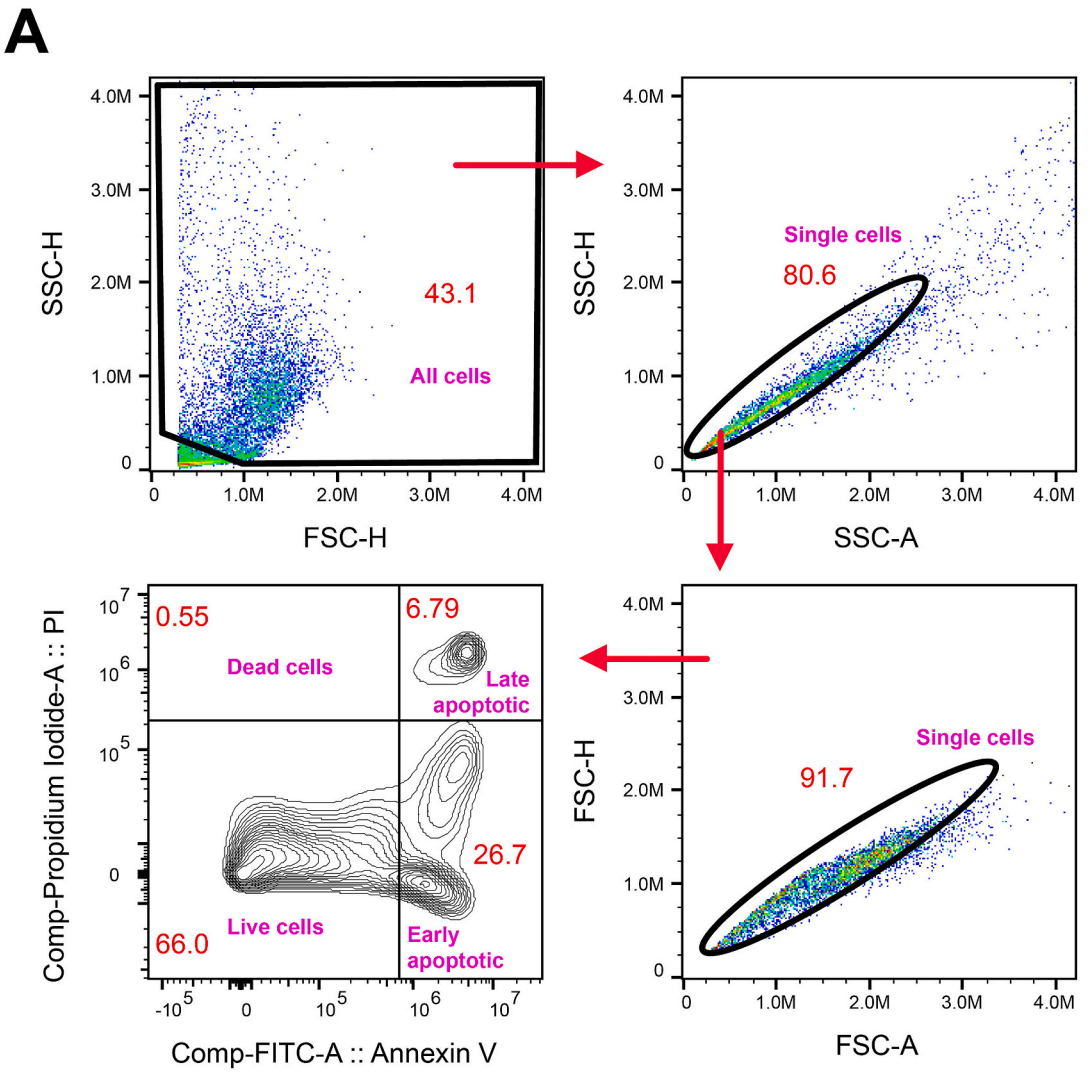
Here, we analyzed the immune activation of the AMs in response to LPS challenges. For this, we stimulated the AMs *ex vivo* with LPS for 4 h. The AM culture with media only was used as an unstimulated control. Culture supernatants from stimulated and unstimulated samples were analyzed for their cytokine production while the cell lysates were used for inflammatory gene expression analyses. TNF-α and MCP-1 production levels in the culture supernatants after LPS stimulation were comparable in both methods (Fig. 5A and B). In addition, we observed that the mRNA expression levels of *Il1b, Nos2*, and *Gpr18*, which are all M1-specific macrophage markers, were comparable in both groups (Fig. 5C–E). We also analyzed the gene expression of M2-specific macrophage markers (*Ym1,* Arg*1, Retnla*) and found no difference in the mRNA gene expression levels of *Ym1* and *Retnla* in both groups (Fig. 5F–H).

Our M1/M2 gene expression data is consistent with flow cytometry data. After AM isolation from BALF, we stained cells for M2-specific surface marker (CD206). As shown in Fig. 6A, CD206 Fluorescence Minus One (FMO) serves as a negative staining control. Our flow cytometry results showed a significant increase in the absolute number of M2 AMs when BALF is collected using EDTA-containing warm buffer as shown in Fig. 6B. The increase in the absolute number of M2 AMs with warm buffer isolation is because of the total number increase in AMs when using warm buffer. Although we found a minor decrease in the proportion of M2 AMs in warm buffer isolation, nearly 50 % of AMs collected using both techniques were M2 type (Fig. 6C).

In summary, our results confirm that the AMs collected using cold and warm buffer have similar baseline M2 polarization stages and inflammatory responses to LPS challenges.

3.5. AMs collected using warm buffer showed similar phagocytic ability to the AMs collected using ice-cold buffer

To determine if AMs isolated using a warm buffer have an effect on the phagocytic ability, we examined the fungal conidia phagocytosis by these AMs. To assess phagocytosis, AMs isolated using both methods were treated with Alexa Fluor 488-labeled



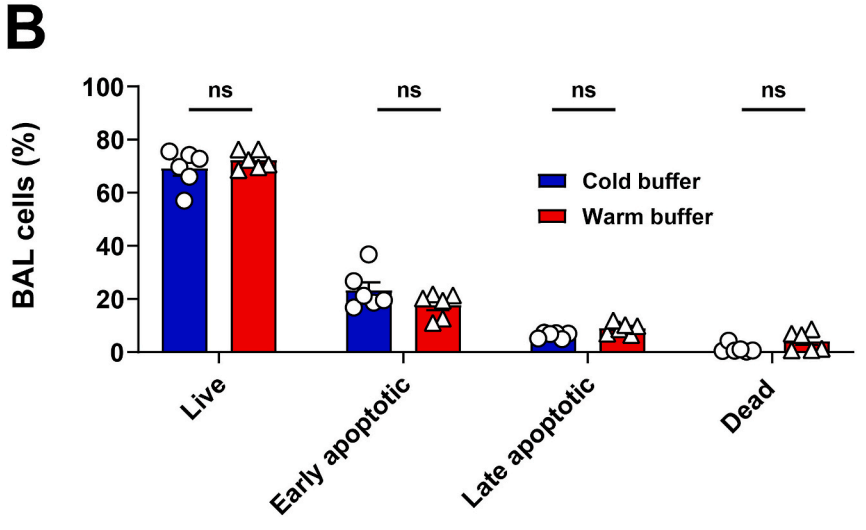


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Fig. 3. Gating strategy for the characterization and quantification of BAL cells at different live/apoptotic stages.
(A) BAL cells were analyzed for the viability and apoptosis using the apoptosis assay. Single cells were further gated for Annexin V and propidium iodide (PI) positivity. Cells which are both Annexin V and PI negative are live, only Annexin V positive are early apoptotic, both Annexin V and PI positive are late apoptotic, and PI only positive cells are dead as shown in the gating strategy. (B) Quantification of live, early apoptotic, late apoptotic, and dead cells by flow cytometry analyses. Each dot represents a single mouse. Total n = 6 mice was used for the cold buffer group and n = 6 mice was used for the warm buffer group. Data in (B) are Means ± SEM & were analyzed by two-tailed unpaired *t*-test with the following significance level (ns > 0.05).

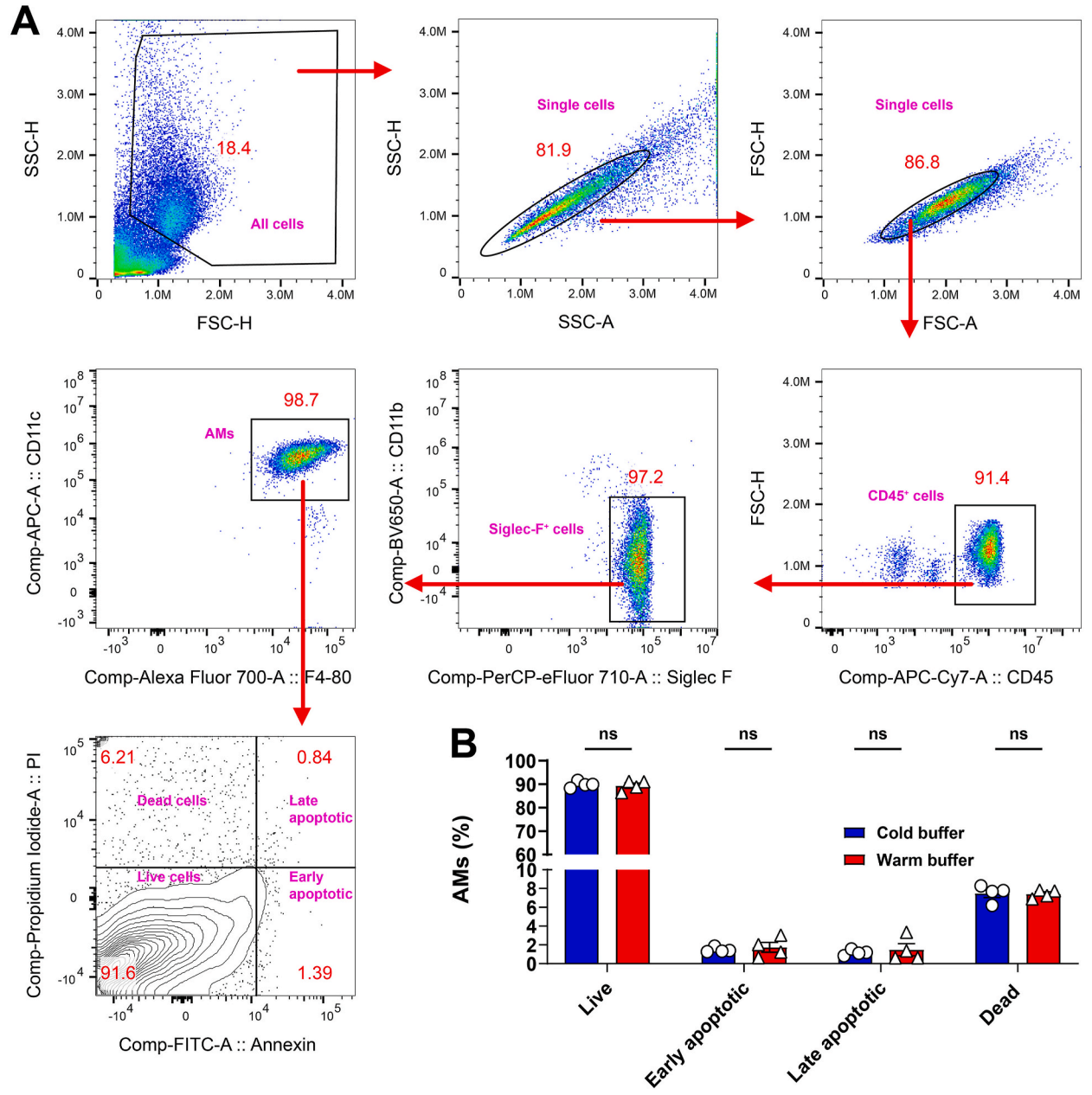

Fig. 4. Quantification of live, early apoptotic, late apoptotic, and dead AMs by flow cytometry analyses.
(**A**) Gating strategy showing characterization of live, early apoptotic, late apoptotic, and dead AMs in BALF isolated using cold and warm buffers. AMs were characterized as $CD45^+$ $Siglec\text{-}F^+$ $CD11b^-$ $CD11c^+$ $F4/80^+$ cells. Live cells were characterized as both PI and Annexin V negative. Annexin V only positive cells were early apoptotic and both PI and Annexin V positive cells were late apoptotic. Cells stained only for PI were dead. (**B**) Quantification of live, early apoptotic, late apoptotic and dead AMs. Each group has n = 4 mice. Data in (B) are means ± SEM and were analyzed by two-tailed unpaired *t*-test with the following significance level (ns > 0.05).

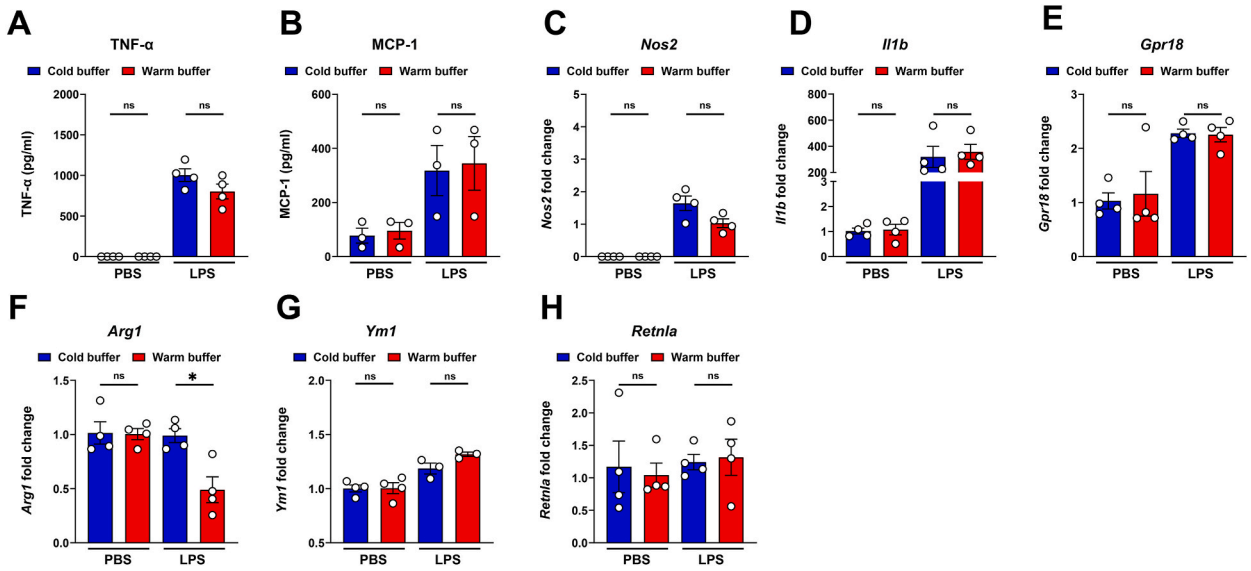

Fig. 5. mRNA level gene expression and cytokine secretion by alveolar macrophages following LPS stimulation.
(A to H) AMs were seeded in 96-well plates and stimulated with LPS after overnight culture. Culture medium was used for the unstimulated control samples. Following 4 h of stimulation, culture supernatants and cell pellets were collected. Quantification of TNF-α (A) and MCP-1 (B) levels in culture supernatants was determined by ELISA. The mRNA gene expression of *Nos2* (C) *Il1b* (D), *Gpr18* (E), Arg*1* (F), *Ym1* (G) and *Retnla* (H) were measured by real-time qPCR. Each dot represents a single mouse. Data in (A to H) are Means ± SEM and were analyzed using two-tailed unpaired *t*-test with the following significance level (ns > 0.05).

Aspergillus fumigatus conidia at multiplicity of infection (MOI) of 2 and co-cultured for 4 h. Confocal microscopy image analyses showed that the AMs isolated using warm buffer showed a similar level of conidia phagocytosis compared to the ones isolated with the conventional method (Fig. 7A and B). For the quantification of the phagocytic ability of AMs, we counted the total number of conidia engulfed by all AMs in one microscopic field of the confocal microscope and included at least three images per sample as shown in Fig. 7C. We also analyzed the average number of conidia engulfed by each alveolar macrophage as shown in Fig. 7D.

4. Discussion

Suboptimal AM yield in BALF collection is a major challenge encountered in high throughput *ex vivo* experiments for studying the role of AMs in various lung diseases. Here we show an optimized method that provides significantly higher AM recovery from BALF. Our study demonstrated that this technique could yield ~3-fold higher AMs compared to the conventional method. Obtaining an increased quantity of AMs suitable for protein and gene expression analyses will not only save time and reduce the cost, but also minimize variability issue in AM isolation. Therefore, our method is suitable for multi-parametric *ex vivo* analyses of AMs, facilitating lung research related to steady-state (or healthy) and disease conditions.

Given the importance of AM quality in analyses of subtle cellular changes and gene expression, we not only focused on quantitative but also compared the functional differences of AMs isolated by both methods. Therefore, we first conducted an analysis of the AM viability to examine whether there is an impact of using a warm buffer on the viability. Based on Annexin V and PI staining, our findings revealed that the viability of both total BAL cells (~73 %) and AMs (~90 %) collected using a warm buffer was comparable to those cells collected using a cold buffer. This outcome confirms that the warm buffer utilized for the BALF collection did not induce significant apoptosis (both early and late stage) in the AMs. The proportion of dead AMs was also similar in both groups, with approximately 8 % of cells in each group. This phenotype could be attributed due to the extended holding period and multiple centrifugation steps during processing and staining. Our findings indicate that warm buffer BALF collection provides higher quantity of AM recovery without altering the viability of isolated AMs.

Further, we investigated whether the use of warm buffer has any impact in the generation of M1/M2 immune responses by isolated AMs. To assess this, we stimulated AMs *ex vivo* with 100 ng/mL LPS and analyzed protein and mRNA levels of selected cytokines/chemokines as well as M1/M2 macrophage-specific markers. LPS is a TLR4 ligand that is extensively used for macrophage activation. Our analyses revealed no significant difference in the gene expression of inflammatory cytokines/M1 macrophage markers (*Il1b, Nos2, Gpr18*) and M2 macrophage markers (*Ym1, Retnla*) between the two groups. We also analyzed TNF-α and MCP-1 secretions by AM cultures following LPS stimulation and observed no notable difference in the cytokine secretion levels between the AMs collected by warm and cold buffers. Consistent with M1/M2 marker gene expression, most of the AMs isolated using both buffers were M2 type at the baseline level when examined by the surface staining of the CD206 marker.

Given that the pathogen clearance is a critical immune function of AMs, we investigated whether there are disparities in the phagocytic capacity of AMs isolated using our method compared to ice-cold buffer method. For this analysis, we treated both types of

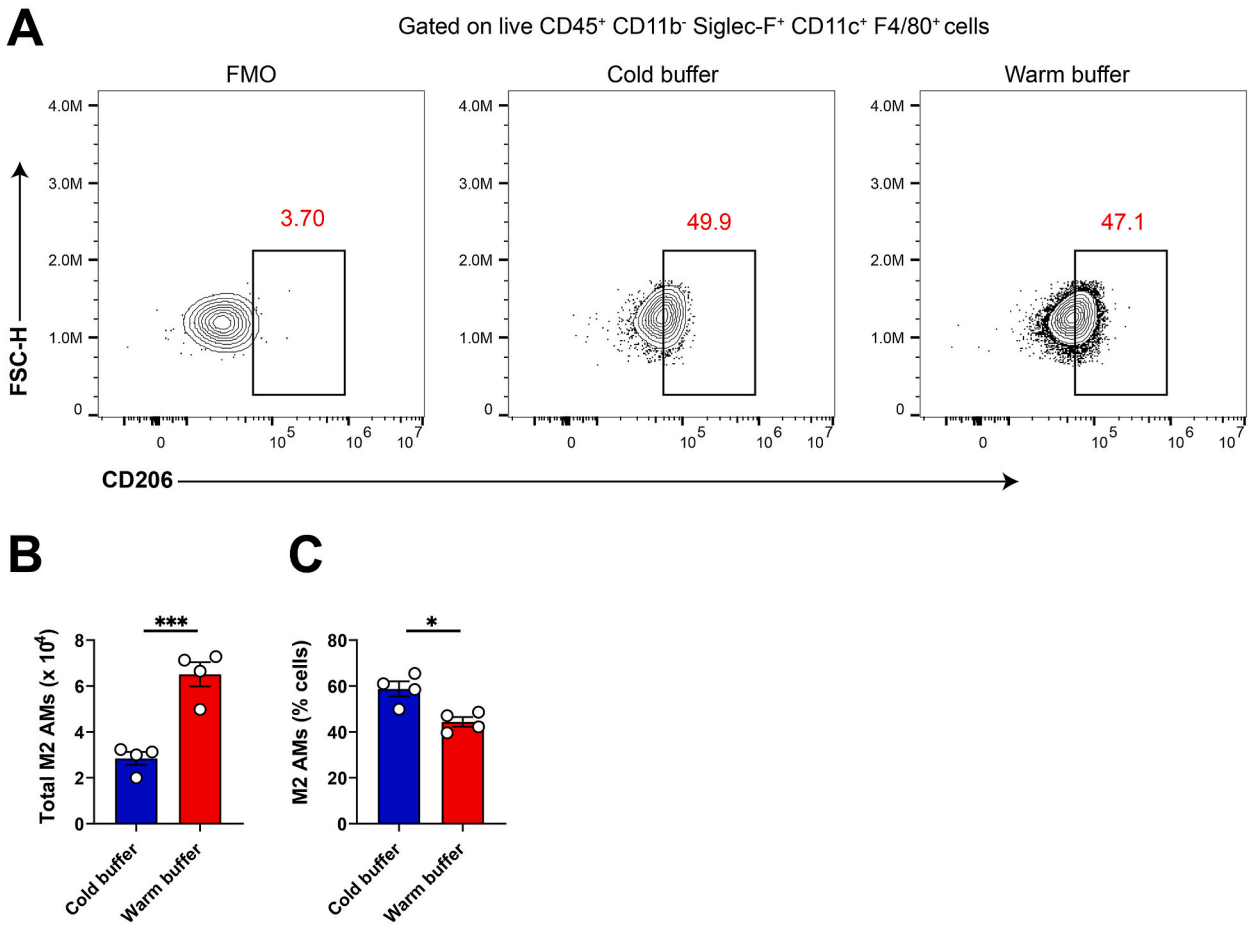

Fig. 6. Quantification of M2 AMs in BALF.
(A) Flow cytometry plots showing M2 alveolar macrophages ($CD45^+$ Siglec-$F^+CD11b^-CD11c^+$ $F4/80^+$ $CD206^+$ cells). We used fluorescence minus one (FMO) as a negative staining control to decide the appropriate gating for M2 AMs. (**B** and **C**) Total M2 AMs (B), and proportion of M2 AMs (C) quantified in BALF isolated from ice-cold (with EDTA) and warm buffers (with EDTA). Data in (B) & (C) are means ± SEM & were analyzed by two-tailed unpaired *t*-test with the following significance level (*$p < 0.05$, ***$p < 0.001$). Each dot represents a mouse.

AMs with Alexa Fluor 488-labeled *A. fumigatus* conidia. We selected fungal conidia for this experiment as they are inert, large enough to be engulfed by AMs, easy to label, and quantify through confocal microscopy. Our findings showed no difference in the phagocytic abilities between the two groups of AMs. Further, AMs collected by traditional method and AMs collected using our method exhibited a high phagocytic ability (Fig. 7). Particularly, the average engulfed conidia per cell was found to be 2–3 conidia/AM when using an MOI of 2 for the AMs isolated from both buffers (Fig. 7D), suggesting healthy and immunologically functional AMs in both groups.

In summary, BALF collection using warm buffer containing EDTA resulted significantly more AMs compared to routinely used cold buffer. Furthermore, the use of warm buffer in BALF collection has no effect on the viability of isolated AMs when compared to the viability of AMs isolated using acold-buffer. Likewise, AMs collected using our method and the conventional method exhibited nearly identical immune responses following LPS stimulation. In addition, AMs isolated using our technique demonstrate similar abilities in engulfing pathogens to those obtained by conventional method. With a significantly increased yield of AMs and uncompromised functional characteristics, our method is suitable for the high throughput and unbiased *ex vivo* studies of AMs in lung research.

5. Star★Methods

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Reagents		
EDTA	RPI research	Cat#E14000–250.0
FBS	Hyclone	Cat#SH30396.03IH25-40

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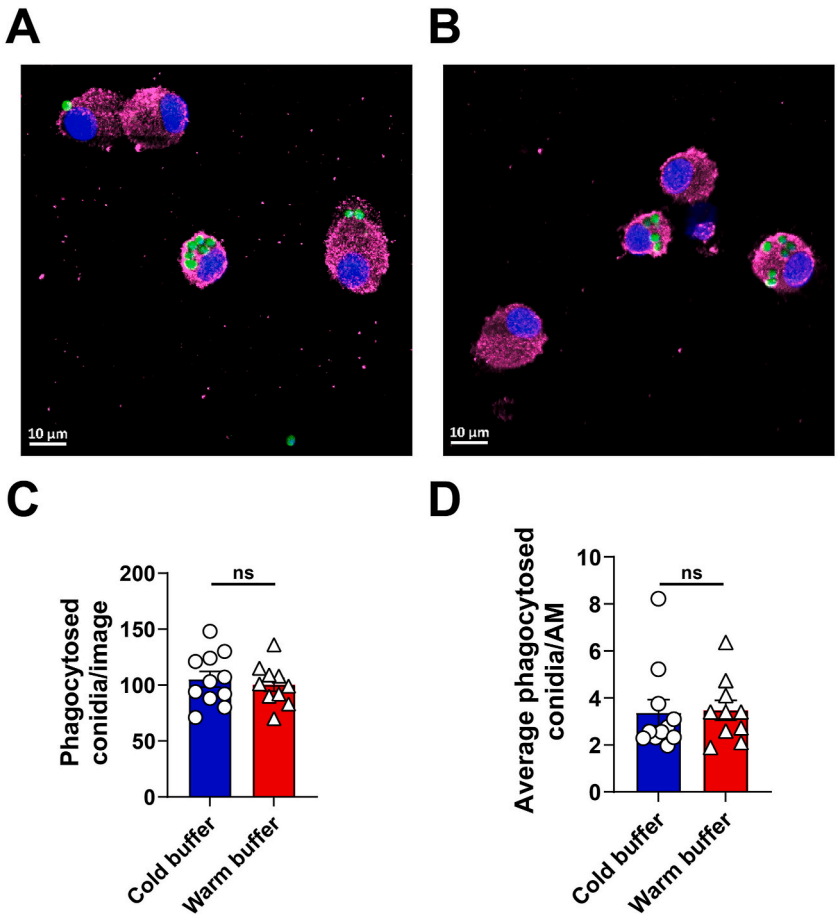

Fig. 7. Confocal microscopy images showing phagocytosis of fungal conidia by AMs.
Confocal images were taken using Zeiss LSM 880, EC Plan-Neofluar 40X/1.3 oil DIC objective (digital zoom 2). AMs isolated from cold-buffer (A) and warm buffer (B) were challenged with *A. fumigatus* conidia of MOI of 2 for 4 h, followed by immunofluorescence staining. Conidia were labeled with Alexa Fluor 488 (Green), nucleus with DAPI (Blue) and anti F4/80 antibody with Alexa Fluor 594 (Magenta) were used to stain AMs. Quantification of phagocytosis based on the number of conidia engulfed per image (C), and average number of engulfed conidia per AM (D). At least three images were taken per sample for quantification. For quantification purposes, images were taken using 40X objectives without zooming. Statistical analysis: two-tailed unpaired *t*-test with the following significance level (ns > 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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REAGENT OR RESOURCE	SOURCE	IDENTIFIER
PBS	Hyclone	Cat#SH30256.02
RBC lysis buffer	Invitrogen	Cat#00-4333-57
DMEM	Gibco	Cat#12800-017
LPS	Enzo life science	Cat#ALX-581-013-L001
Penicillin/Streptomycin	Gibco	Cat#15140-122
Trizol	Invitrogen	Cat#15596026
Trypan blue	Sigma	Cat#T6146-100G
Triton X-100	IBI Scientific	Cat#IBO7100
RNase free water	Ambion	Cat#100015637
Vectashield	Vector Laboratories, Inc.	Cat#H-1700
DAPI	Sigma	Cat#D9542
Tween-20	Sigma	Cat#P7949
Isoflurane	Akron Animal health	Cat#NDC 59399-106-01
Normal goat serum	Sigma	Cat#G9023
Dimethyl formamide	Sigma-Aldrich	Cat#227056
Poly d(T)	Integrated DNA technology	Cat#457476542
dNTP mix	Sigma	Cat#D7295
Alexa Fluor 488 NHS Ester (Succinimidyl Ester)	Thermofisher	Cat#A20100

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REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45 APC/Cy7	BioLegend	Cat#103116, Clone: 30-F11 Dilution (1:200)
F4/80 AF 700	BioLegend	Cat#123130, Clone: BM8 Dilution (1:100)
CD11b BV650	BioLegend	Cat#10135, Clone: M1/70 Dilution (1:200)
Siglec-F Percp-Cy5.5	BD Pharma	Cat#56552, Clone: E50-2440 Dilution (1:100)
CD11c PE-Cy5	BioLegend	Cat#11731, Clone: N418 Dilution (1:150)
Siglec-F PerCp eFluor 710	Invitrogen	Ref# 46-1702-80 Clone:1RNM44N Dilution (1:100)
CD11c APC	BioLegend	Cat#117310, Clone: N418 Dilution (1:150)
Zombie yellow	BioLegend	Cat#B353069, Clone: N/A Dilution (1:200)
Rat mAb against F4/80	Abcam	Cat#90247, Clone: N/A Dilution (1:1000)
Goat anti-Rat AF 594	Abcam	Cat#150160, Clone: N/A Dilution (1:800)
Kits		
Apoptosis Assay	ABP Biosciences	Cat#A026
TNF-α ELISA	BioLegend	Cat#430916
MCP-1 ELISA	BioLegend	Cat#432704
PureLink™ RNA Mini Kit	Thermo Fisher Scientific	Cat#12183025
cDNA Synthesis Kit	Thermo Fisher Scientific	Cat#18064-014
Software and algorithms		
FlowJo	BD Biosciences	N/A
Prism 10	GraphPad	N/A

6. Lead contact

Any further requests or inquiries related to this article can be directed to Dr. Pankaj Baral (**baral@ksu.edu**).

7. Materials availability

This study did not generate new unique reagents.

8. Data and code availability

The manuscript text contains all the data generated in this study. Our study does not report any original code.

9. Experimental model and study participant details

Adult (8–12 weeks) C57Bl/6J male and female mice were used for the entire experiment. Initially, we purchased mice from the Jackson Laboratory, and colonies were maintained in the Animal facility at Kansas State University. All experimental procedures related to mice work were approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC)-approval numbers 4571 and 5021.

10. Method details

Buffers used for BALF collection.

Buffer composition	
Ingredients	**Composition**
EDTA	2 mM
FBS	1 %
PBS	1X
Cold buffer: 4°C	
Warm buffer: 37°C	

10.1. Bronchoalveolar lavage fluid (BALF) collection and cell count

Mice were euthanized by CO_2 overdosing and a cannula was inserted into the trachea after opening the chest cavity. Ice-cold and warm buffer (with or without 2 mM EDTA, 1 % FBS in PBS) were used to collect the BALF. Lavage buffer (0.8 mL) was used each time and repeated four times to collect around 3 mL lavage. During each BALF collection, the lung was flushed three times using the same buffer. Flushing the lungs at least 3 times using the same buffer maximizes the yield of cells. The collection of lavages four times for the same mouse facilitates efficient detachment of AMs and recovery of residual cells found in the airspaces. The BALF collection procedure we used in this study is very similar with other reported studies with a minor modification [26,27]. The BALF was collected in a 5 mL Eppendorf tube and placed on ice until further processing. RBCs were lysed by incubating BAL cells with 1X RBC lysis buffer for 8 min at room temperature. Next, 1 mL wash buffer (0.2 mM EDTA, 2 % FBS in PBS) was added to wash the cells and centrifuged (details are in Supplemental file). The pellets were resuspended in 200 μL wash buffer and cell count was performed by diluting the cells (20X) using trypan blue.

10.2. Apoptosis assay

ABP Biosciences apoptosis assay kit (A026) was used for analyzing the apoptosis of isolated BAL cells and AMs. After cell counting, BAL cells/AMs were washed twice with ice-cold 1X PBS and resuspended in 1X annexin binding buffer to 10^6 cells/mL. Then, 2.5 μL Annexin-V FITC was added to each tube, followed by addition of 1 μL Propidium iodide (PI) solution to the cells under dark condition. Tubes were vortexed gently to mix the content and incubated at room temperature for 15 min in the dark. After completion of the incubation, 200 μL 1X annexin binding buffer was added, mixed gently by vortexing, and kept on ice and covered with aluminum foil. Samples were analyzed immediately by flow cytometry using Cytek Northern Lights analyzer.

10.3. Flow cytometry analyses of AMs

BAL cells were transferred into the V-bottom 96-well plate and centrifuged at 1500 rpm for 5 min at 4 °C. The supernatant was removed, and then cells were resuspended with staining buffer (0.2 mM EDTA, 2 % FBS in PBS) containing antibody cocktail and FcR blocking solution (90 μL antibody cocktail + 10 μL FcR blocker). The antibodies cocktail used to characterize AMs include APC/Cy7 anti-mouse CD45, AF 700 anti-mouse F4/80, PE/Cy5 anti-mouse CD11c, PerCP/Cy5.5 anti-mouse Siglec-F and Brilliant Violet 650 anti-mouse CD11b. For the characterization of M2 AMs, we added Brilliant Violet 421 anti-mouse CD206 to the antibody cocktail. The plate was wrapped with aluminum foil and incubated on ice for 25 min. After incubation, 100 μL FACS wash buffer (0.2 mM EDTA, 2 % FBS in PBS) was added to each well and centrifuged at 1500 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellets were resuspended with 100 μL fixative buffer (0.5 mM EDTA, 2 % PFA in PBS) and kept on ice for 25–30 min. Following fixation, 100 μL wash buffer was added to wash the fixative buffer, and then centrifuged at 1500 rpm for 5 min at 4 °C. Then, pellets were resuspended in a 100 μL wash buffer and the stained cells were transferred to FACS tubes. Finally, 200 μL of wash buffer was added to each FACS tube through the wall making the final volume 300 μL as described previously [28]. FACS tubes were placed on ice, covered with aluminum foil and flow cytometry was performed on a Cytek Northern Lights analyzer. Data was analyzed using FlowJo (10.9).

10.4. LPS stimulation of alveolar macrophages

AMs (150,000/well) were seeded on flat-bottomed 96 well plates and incubated in 5 % CO_2 enriched environment at 37 °C overnight. The next day, culture supernatant was removed, and cells were washed with warm 200 μL 1X PBS with gentle shaking. PBS was replaced with 100 μL medium (10 % FBS, 1X DMEM, no antibiotic), and the plate was placed back into the incubator until ready to stimulate. LPS working dilution (200 ng/ml) was prepared using DMEM (10 % FBS, DMEM, no antibiotic). Once the working LPS dilution was ready, 100 μL LPS was added to the wells so that the final concentration of LPS became 100 ng/mL in each well. For unstimulated wells, 100 μL medium was added to make up the final volume. The plate was incubated at 37 °C in a 5 % CO_2 environment for 4 h to stimulate AMs with LPS. After stimulation, culture supernatant was collected, flash frozen and stored at −80 °C until use for the cytokine measurement. After supernatant collection, cell monolayer was washed with warm 1X PBS, lyzed with Trizol reagent, and the lysates were snap-frozen and kept at −80 °C for the RNA extraction.

10.5. ELISA for cytokine detection

AM culture supernatants was analyzed for cytokine measurements. The concentration of TNF-α and MCP-1 in the culture supernatant was determined using the ELISA kit (BioLegend) according to the manufacturer's instructions.

10.6. Real-time quantitative PCR

RNA extraction was carried out using PureLink™ RNA Mini Kit (Thermo Fisher Scientific). The cDNA was synthesized using the

Super Script II cDNA Synthesis Kit (Thermo Fisher Scientific). The PowerUp SYBR Green Master Mix (Applied Biosystems) was used for qPCR run in ABI 7000 System (Applied Biosystems). The mRNA level expression of targeted genes was determined using *Gapdh* as a normalizing gene. ΔΔCT method was employed for gene expression analysis. Primers used for gene expression analysis are listed in the Table 1.

10.7. Phagocytosis assay

AMs (200,000 cells/well) were seeded in an 8-chambered slide (containing 400 μL DMEM, 10 % FBS, 1 % antibiotics) and incubated at 37 °C with 5 % CO_2 enriched environment overnight. The next day cells were washed with warm 200 μL 1X PBS with gentle shaking. Alexa Fluor 488-labeled *A. fumigatus* conidia at MOI of 1:2 (macrophage/fungus conidia) was added to the wells and the slide was incubated for 4 h at 37 °C with 5 % CO_2. After completion of the incubation, cells were washed 3 times with 500 μL PBST-20 (0.05 % Tween-20 in PBS) and finally washed with PBS only. Cells were fixed for 15 min at 4 °C using 4 % paraformaldehyde solution in dark condition. Excess fixative buffer was removed by washing of the wells using 500 μL PBSTX-100 (0.05 % Triton-X100 in PBS). PBS was used for the last wash instead of PBSTX-100. Normal goat serum (500 μL) was added to the wells for blocking and incubated for 30 min at room temperature. F4/80 primary antibody (1:1000 dilution) was added to the wells and incubated for 2 h at room temperature to stain AMs. Unbound primary antibodies were removed by washing 3 times with 500 μL PBST-20. Then, Alexa Fluor 594 goat anti-Rat (1:800 dilution) secondary antibody was added and incubated for 1 h at room temperature (used PBSTX-100 as diluent). Excess secondary antibody was removed by three washes with 500 μL PBST-20 and the final wash was done using PBS. Then, mounting media containing DAPI (5 μg/ml) was added to the slides. Finally, cover slip was applied avoiding bubbles and the coverslip was shielded using colorless nail polish. The slide was wrapped with aluminum foil and placed at 4 °C before imaging.

Confocal images were acquired at Confocal Microscopy core facility located at the College of Veterinary Medicine, Kansas State University. Zeiss LSM 880 confocal microscope was used for imaging. Images were taken using EC Plan Neo-flour 40X oil immersion objective having 1.3 numerical aperture.

10.8. Statistical analysis

Statistical analyses used and sample size for each experiment are described in figure legends. GraphPad Prism 10 software was used for statistical analysis. All the experimental data are shown as mean ± s.e.m. Mean values of two experimental groups were compared using a two-tailed unpaired *t*-test. Differences between mean of two experimental groups were considered statistically significant as (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

10.9. Limitations

In this study, we couldn't identify whether the non-M2 AMs in steady state are the M1 and/or M0 types because of the lack of proper flow cytometry antibodies for the surface staining for M1/M0 characterization. More robust single-cell RNAseq analyses of AMs will help assess the non-M2 subtypes.

Our study only analyzed a few cytokine and chemokine responses by AMs. Multiplex-based cytokine and chemokine analyses are needed to assess the broad effects of buffers in AM activation.

Obtaining enough AMs for LPS stimulation and phagocytosis assay in a cold buffer group requires the pooling of several mice as the per-mouse AM yield is significantly low.

Table 1
List of primers used for gene expression analysis.

S.N.	Gene	Sequence
1.	*Il1b*	For: 5′-CAACCAACAAGTGATATTCTCCAT G -3′ Rev: 5′-GATCCACACTCTCCAGCTGCA -3′
2.	*Nos2*	For: 5′-GTTCTCAGCCCAACAATACAAGA-3′ Rev: 5′- GTGGACGGGTCGATGTCAC-3′
3.	*Gapdh*	For: 5′- TCGTGGATCTGACGTGCCGCCTG -3′ Rev: 5′- CACCACCCTGTTGCTGTAGCCGTA -3′
4.	*Gpr18*	For: 5′-CTGAAGCCCAAGGTCAAGGA-3′ Rev: 5′-TTGTAGCATCAGGACGGCAA-3′
5.	*Ym1*	For: 5′-AGACTTGCGTGACTATGAAGCATT-3′ Rev: 5′-GCAGGTCCAAACTTCCATCCTC-3′
6.	*Retnla*	For: 5′-GGGATGACTGCTACTGGGTG-3′ Rev: 5′-TCAACGAGTAAGCACAGGCA-3′
7.	Arg*1*	For: 5′-TTCTCAAAAGGACAGCCTCG-3′ Rev: 5′-TCTTCACAATTTGAAAGGAGCTG-3′

11. Troubleshooting

11.1. Problem: possible lung damage during chest cavity opening and lavage collection

If enough care is not taken while cutting the diaphragm, lung may be damaged by sharp edges of scissors. There is the possibility of cutting any of the lung lobes. Similarly, if excessive force is applied during lavage collection, there will be a leakage from the lung and hence we cannot collect the lavage.

11.2. Potential solution

While opening the chest cavity, make a very fine hole using scissors tip at the top right corner of the diaphragm which looks darker compared to other areas of diaphragm. After piercing diaphragm, the lung lobes will contract and move back further away from diaphragm. After this, pull the diaphragm muscle towards you and carefully cut the entire muscle. To prevent lung damage due to excessive force during lung flushing, apply moderate force to lavage the lungs. It is recommended to do couple of practice sessions of chest cavity opening and lung lavage so that the researcher knows how to open the chest cavity without damaging the lungs and optimize how much force is required to flush the lungs.

11.3. Problem: low BAL cell and AM yield in cold buffer group

BALF collection using cold buffer yields low lavage cells as well as AMs. In experiments that involve seeding cells for the monolayers for *ex vivo* analysis, this low cell yield creates a lot of technical problems and require pooling of several mice to set up the experiments.

11.4. Potential solution

Lavage can be done several times to collect as many cells as possible. For this reason, we suggest lavage the lungs four times per mouse and wash the lungs three times in each lavage collection step. To prevent cell death during lavage collection from multiple mice, euthanize one mouse at a time and place the lavage on ice immediately after collection. It is recommended to use a few extra mice (1–2 mice) so that you will have enough cells required for seeding and you don't need to discard the samples due to getting of insufficient cells for the seeding.

11.5. Problem: low RNA yield from cell lysate after PBS and LPS stimulation

The RNA yield might be low from cultured cell lysate in 96 well formats. This will affect the downstream procedures like cDNA synthesis and gene expression analysis**.**

11.6. Potential solution

Low RNA yield may be associated with incomplete cell lysis or partial cell/cell lysate collection at the initial stage. To avoid this, make sure you use a standard buffer that lyses all the cells present in the wells. Similarly collect cell lysates completely from the well. Any residual cells/cell lysate can be observed under microscope to make sure there are not any cells left. Since RNA extraction has multiple steps, perform all the steps very carefully especially pipetting the exact volume of reagents. One key point during RNA extraction steps is never use nuclease free water instead of buffers. Addition of water in the column in any steps except RNA elution steps will cause washing of the RNA causing very low RNA concentration or lack of RNA at all.

CRediT authorship contribution statement

Surya Prasad Devkota: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Chinemerem Onah:** Writing – review & editing, Investigation. **Prabhu Raj Joshi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Sandeep Adhikari:** Writing – review & editing, Investigation. **Pankaj Baral:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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