Original Article

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A novel method for the isolation of CD45-positive and CD45-negative cells from malignant pleural effusion

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Background: Patient-derived malignant pleural effusion (MPE) samples can be used to identify a patient-specific drug combination since MPE samples are readily available and cost-effective tumor cell sources. However, the isolation of target cancer cells from MPE has been inefficient because MPE samples contain a complex mixture of immune cells, non-cancerous cells, and cancer cells. Hence, new methods need to be developed to effectively isolate target cancer cells from MPE samples that can be used for 3-dimensional (3D) cell culture. Patient-derived *in vitro* 3D tumor models are expected to facilitate more precise drug treatment.
Methods: MPE samples were obtained from Seoul St. Mary's Hospital, The Catholic University of Korea with consent from patients previously diagnosed with lung adenocarcinoma. We isolated target cells from MPE samples using 2 different Percoll-gradient centrifugation methods.
Results: The use of 40% and 75% Percoll-gradient centrifugation led to a clearer separation of CD45-positive ($CD45^{pos}$) and CD45-negative ($CD45^{neg}$) cells than the traditional 44% and 67% Percoll-gradient centrifugation method.
Conclusion: Our findings strongly suggest that the 40% and 75% Percoll-gradient centrifugation method is more useful for the isolation of $CD45^{pos}$ or $CD45^{neg}$ cells than the previously described Percoll-gradient centrifugation method. Furthermore, our novel method was useful for the isolation of MPE-derived target cancer cells that can be used to construct *in vitro* patient-specific 3D tumor models.

Keywords: Adenocarcinoma of lung; Pleural effusion; Percoll-gradient; Three-dimensional culture; Patient derived organoids

Introduction

Patient-derived cells isolated from tumor biopsies can be used to track disease progression and screen for chemotherapy drug response, representing an ideal platform for precision medicine applications [1]. Patient-derived mouse xenografts (PDX) models have been shown to predict clinical outcomes more accurately than conventional cell line-derived xenograft models, particu-

larly when therapeutic compounds are evaluated with clinically relevant doses [2–4]. However, the time taken for engraftment and low tumor growth rates do not allow a timely assessment of a patient's chemosensitivity for therapeutic decisions. Furthermore, patient-derived PDX models are usually obtained from biopsies or resected primary tumors, but these samples often have limited availability or cellular vitality and are not adequately preserved, which causes low engraftment rates [5–7]. As an alternative to PDX models, *in vitro* 3-dimensional (3D) cell culture models mimicking *in vivo* environments have been applied to drug toxicity testing [8] and have offered better precision in drug discovery [9]. *In vitro* patient-derived 3D cell culture models have been developed by using extracellular matrix-based organoid culture protocols and 3D co-culture methods [10]. Both PDX and 3D cell culture models require the isolation of target cells from a limited number of patient-derived tumor biopsies. Hence, the efficient preparation of substantial amounts of target cells from tumor tissue biopsies is clearly needed to construct patient-derived tumor models allowing the identification of patient-specific combination regimens.

Malignant pleural effusion (MPE) is defined as a significant accumulation of exudate in the pleural cavity containing malignant cells or tumor tissue. MPE is a serious medical condition causing shortness of breath, pain, cachexia, and decreased physical activity [11,12]. MPE, which is a common occurrence in many end-stage epithelial cancers [13], contains cancer cells, as well as large numbers of immune cells including various lymphocytes and myeloid subsets [14]. Several mechanisms are involved in the generation of MPE, including pleural metastasis with increased permeability, pleural metastasis with obstruction of the pleural lymphatic vessels, mediastinal lymph node involvement, chest tube obstruction, bronchial obstruction, and pericardial involvement. In addition, pleural effusion can also occur as a result of hypoproteinemia, obstructive pneumonia, or pulmonary embolism in patients with malignant tumors [15].

Because MPE samples (liquid biopsies) are readily available and contain various cell types exhibiting the heterogeneity of lung adenocarcinoma, MPE samples can be used as cost-effective tumor cell sources to generate patient-specific lung cancer models for future precision medicine applications. In this study, we optimized the Percoll-gradient centrifugation protocol for isolating CD45-positive ($CD45^{pos}$) and CD45-negative ($CD45^{neg}$) cells from MPE aspirate biopsies in lung adenocarcinoma patients, followed by establishing patient-derived 3D organoid culture. In addition, we compared the 3D cell culture viability of $CD45^{neg}$ cells isolated from MPE samples with non-isolated cells ($CD45^{pos/neg}$).

Materials and Methods

1. Cell isolation

MPE samples were obtained from Seoul St. Mary's Hospital, The Catholic University of Korea with consent from patients previously diagnosed with lung adenocarcinoma. This study was approved by the Institutional Review Board (IRB) of The Catholic University of Korea (IRB no. KC18TNSI0033).

Each sample contained between 40 and 60 mL of fluid, yielding 1×10^7 cells for use in culture. Samples were transferred from the primary container to a 50-mL centrifuge tube, followed by centrifuging the tube at 440×*g* for 10 minutes at room temperature. The cell pellets were resuspended and washed 2 times with advanced dulbecco's modified eagle medium: nutrient mixture F-12 medium. A 90% Percoll solution was prepared by mixing the appropriate amount of Percoll (*d*=1.130 g/mL) with Hank's balanced salt solution (HBSS, with phenol red), and then 40% and 75% Percoll solutions were prepared by mixing an appropriate amount of 90% Percoll (*d*=1.130 g/mL) and buffer 1 (10% fetal bovine serum [FBS]+100 μM ethylenediamine tetraacetic acid [EDTA] in phosphate-buffered saline [PBS]). The MPE samples were strained with a 70-m cell strainer and centrifuged at 440×*g* for 10 minutes at room temperature, and the cell pellets were rinsed with rRoswell Park Memorial Institute medium. After spin-down, each cell pellet was resuspended in a single-cell suspension with 5 mL of 75% Percoll solution and transferred to a 15-mL centrifuge tube. The 40% Percoll solution was gently added to the 75% Percoll solution and centrifuged at 780×*g* for 20 minutes at room temperature without interruption.

2. Cell culture

Target cells were isolated from MPE of lung adenocarcinoma for 3D cell cultures. The isolated cells ($CD45^{neg}$ or $CD45^{pos/neg}$; 1×10^5 cells) were resuspended in 30 μL of 70% Matrigel (354230; Corning Life Sciences, Corning, NY, USA), and the Matrigel-cell mixture was spotted into each well of 4-well TCP plates (Thermo, Rochester, NY, USA), which were then incubated face down at 4°C for 15 minutes to allow cell aggregation at the bottom of the spots. The plates were then placed at 37°C in a humidity chamber to allow spot gelation. After gelation, fresh medium (CODRP_LOM; Medical & Bio Decision Co., Ltd., Suwon, Korea) was added to culture the cells isolated from MPE.

3. Flow cytometry analysis

Cells were incubated with an allophycocyanin (APC)-con-

jugated anti-CD3 antibody and fluorescein isothiocyanate (FITC)-conjugated anti-CD45 antibody in PBS containing 1% FBS for 30 minutes at 4°C. After washing, flow cytometry analysis of the fluorescent-labeled cells was performed to identify the population of $CD45^{pos}$ and $CD45^{neg}$ cells. Data acquisition and analysis were performed on a BD FACS Canto II (BD, Franklin Lakes, NJ, USA).

Results

1. The isolation of MPE-derived tumor cells from lung cancer patients

Tumor cells present in MPE samples from patients can be used for both *in vivo* PDX and *in vitro* 3D culture models to predict patients' chemosensitivity. However, the isolation of cancer cells from pleural fluid has been difficult because the fluid contains normal epithelial cells, fibroblasts, immune cells, and cancer cells. To isolate cancer cells from the pleural effusions of lung cancer patients, we used different Percoll-gradient centrifugation methods, which have been used for isolating lymphocytes and other cells from tumor tissues [16–18]. Rapp et al. [17] reported that 44% and 67% Percoll solutions have been used to isolate intratumoral leukocytes from tumor tissues. Using the previous method [17], we tried to isolate $CD45^{pos}$ cells, including leukocytes, and $CD45^{neg}$ cells, including tumor cells, from MPE. For Percoll-gradient centrifugation, appropriate amounts of Percoll (d=1.130 g/mL) and PBS were mixed to prepare 44% and 67% Percoll solutions. The next steps were to add 9 mL of 44% Percoll solution over 6 mL of the 67% Percoll solution in a 50-mL centrifuge tube. Finally, the resuspended single-cell suspension obtained from the MPE was added onto the top layer and centrifuged at 800×g for 30 minutes at 4°C without interruption (Fig. 1A). After centrifugation, a mixture of both $CD45^{neg}$ and $CD45^{pos}$ cells was found in the Percoll top layer and middle layer (Fig. 2A and 3B).

To solve this problem, we modified the Percoll-gradient centrifugation method. We prepare a 90% Percoll solution by mixing the appropriate amount of Percoll (d=1.130 g/mL) with HBSS (with phenol red). For Percoll-gradient centrifugation, 40% and 75% Percoll solutions were prepared by mixing an appropriate amount of 90% Percoll (d=1.130 g/mL) and buffer 1 (10% FBS+100 μM EDTA in PBS). The MPE samples were strained with a 70-m cell strainer and centrifuged at 440×g for 10 minutes at room temperature, and the cell pellets were rinsed with RPMI medium. After spin-down, each cell pellet was resuspended in a single-cell suspension with 5 mL of 75% Percoll

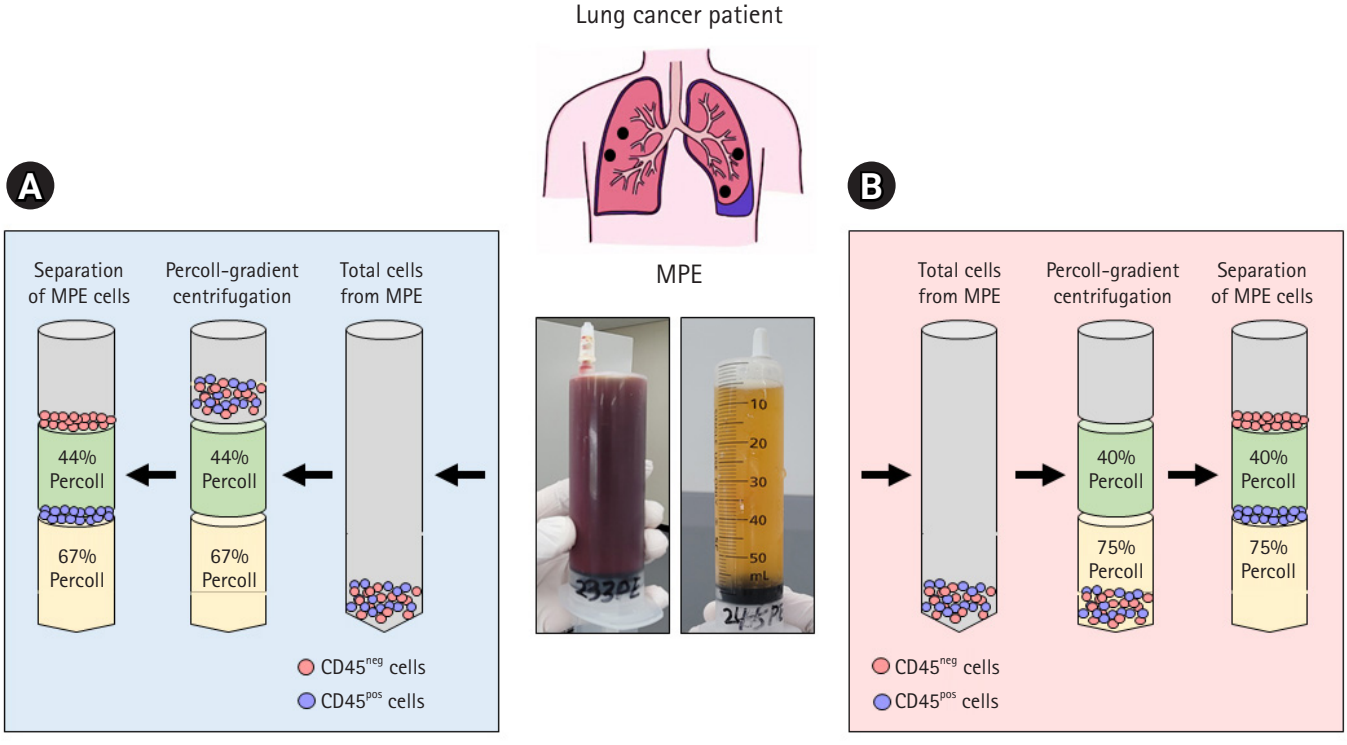

Fig. 1. Overview of the experimental design. (A) A previously proposed experimental method and (B) the new method for isolating CD45-negative ($CD45^{neg}$) or CD45-positive ($CD45^{pos}$) cells from patient malignant pleural effusion (MPE) samples.

solution and transferred to a 15-mL centrifuge tube. Then, 40% Percoll solution was gently added to the 75% Percoll solution and centrifuged at 780×*g* for 20 minutes at room temperature

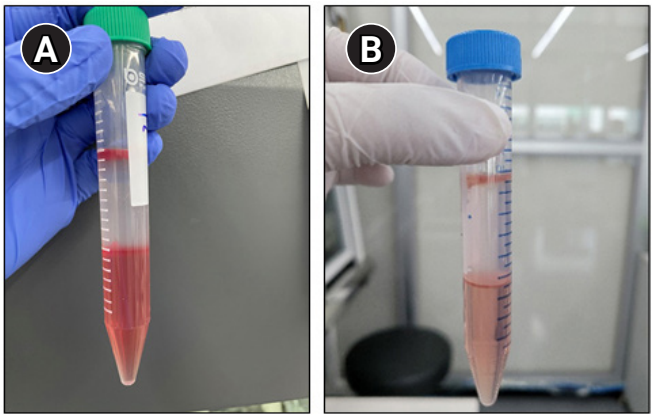

Fig. 2. Photos of the resulting Percoll-gradient layers after centrifugation. The top and middle layers containing CD45-positive and CD45-negative cell fractions were obtained from (A) 44% and 67% Percoll solutions and (B) 40% and 75% Percoll solutions.

without interruption (Fig. 1B). Surprisingly, we were able to observe a two clear layer at the interface of the 2 Percoll layers (Fig. 2B). In addition, >94% of the CD45^{pos} cells were found in the Percoll middle layer, whereas >98% of the CD45^{neg} cells were found on the top layer of 40% Percoll solution (Fig. 2B and 3C). No cells were found in the region between the top and middle layer of Percoll solution (data not shown).

2. Separation of both CD45^{pos} and CD45^{neg} cells from MPE

To measure the levels of CD45 expression in the cells in the top and middle layer of the Percoll solution, we performed fluorescence-activated cell sorting (FACS) analysis. The cells from each Percoll layer were stained with anti-CD45 antibody conjugated with FITC and anti-CD3 antibody conjugated with APC. The FACS data showed that the CD45^{pos} and CD45^{neg} cells were not separated using the 44% and 67% Percoll-gradient centrifugation method (Fig. 3B). However, the 40% and 75% Percoll-gradient centrifugation method led to a clear separation of CD45^{pos} and CD45^{neg} cells (Fig. 3C).

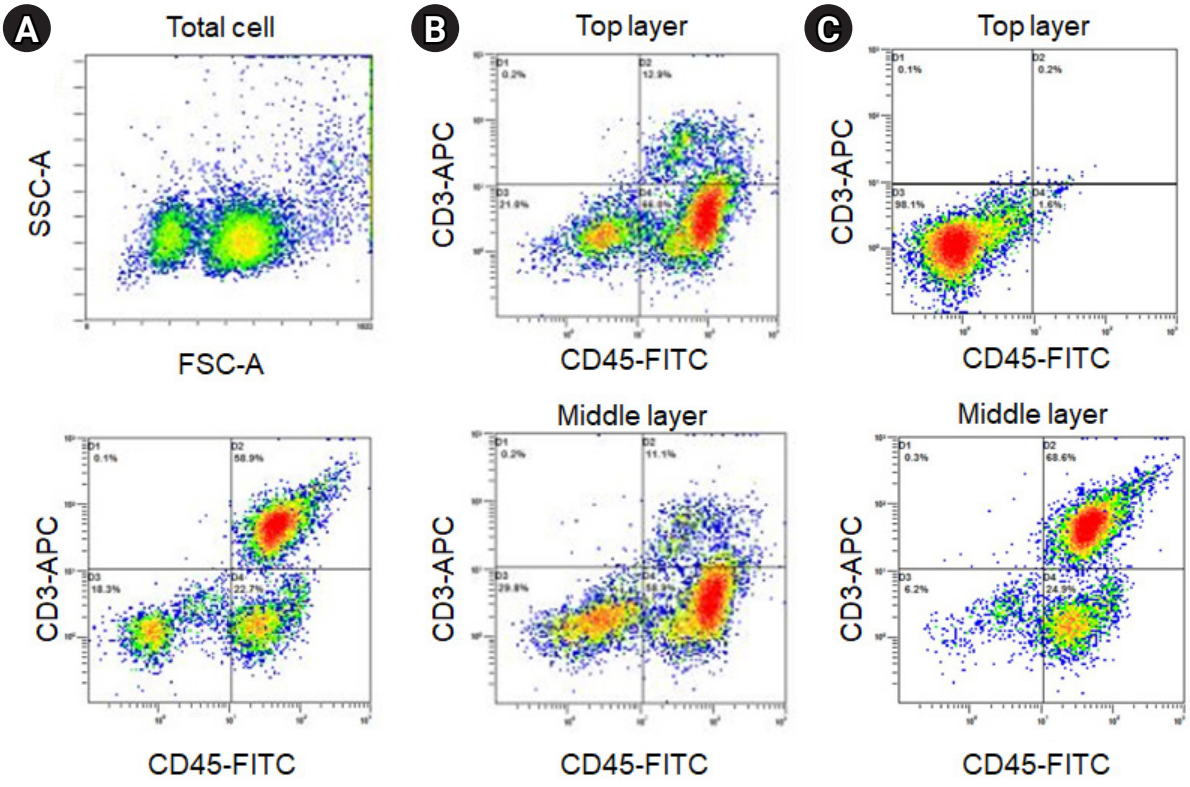

Fig. 3. Flow cytometry analysis of CD45-positive (CD45^{pos}) and CD45-negative (CD45^{neg}) cells. (A) The cell mixture from malignant pleural effusion samples were distributed as CD45^{pos} and CD45^{neg} cells. (B) Cell population in the top (upper panel) or middle layer (lower panel) of a 44% and 67% Percoll gradient. (C) Cell population in the top (upper panel) or middle layer (lower panel) of a 40% and 75% Percoll gradient. SSC-A, side scatter-A; APC, allophycocyanin; FITC, fluorescein isothiocyanate.

3. The effects of CD45^{pos} cells on the viability of CD45^{neg} cells in 3D cell culture

Next, we tested whether a cell mixture (CD45$^{pos/neg}$) could affect the viability of CD45^{neg} cells in a 3D cell culture condition. The cells collected from each top layer of 2 different Percoll concentration methods were embedded into Matrigel for 3D cell culture. After 5 days, the number of CD45^{neg} cells obtained from 44% and 67% Percoll solution was significantly reduced (Fig. 4A). In contrast, CD45^{neg} cells isolated from the top layer of the 40% and 75% Percoll gradients formed 3D aggregates (organoids) without cell death (Fig. 4B). These results indicate that the significant growth inhibition of the non-separated CD45$^{pos/neg}$ cell mixture could have resulted from immune cell cytotoxicity in CD45^{pos} cells, since there was no growth inhibition of the CD45^{neg} cell mixture in the absence of CD45^{pos} cells.

Discussion

MPE is associated with a poor prognosis and shortened survival of patients with non-small cell lung cancer. The development of MPE involves various mechanisms such as inflammation, angiogenesis promotion, and vascular leakage. We believe that aggressive cancers accompanied by MPE require novel therapeutic strategies of precision medicine. Therefore, it is necessary to expand patient-derived cancer cells to perform a customized chemosensitivity assay for patient-specific care.

Patient-derived organoids (PDOs) have recently been developed as a promising tool for personalized medical decisions [19–21]. When compared to cell lines and animal models, PDOs can offer more representative disease models, providing a genetically manipulable system that can be propagated, stored, and transferred into complex 3D *in vitro* or murine *in vivo* models [22]. These PDOs allow human *in vitro* immunotherapy modeling through co-culture with native syngeneic, autologous tumor-reactive tumor-infiltrating lymphocytes (TILs). However, the co-culture of primary tumor cells with endogenous, syngeneic TILs has been particularly troublesome. Recently, Neal et al. [23] developed an air-liquid interface method for propagating

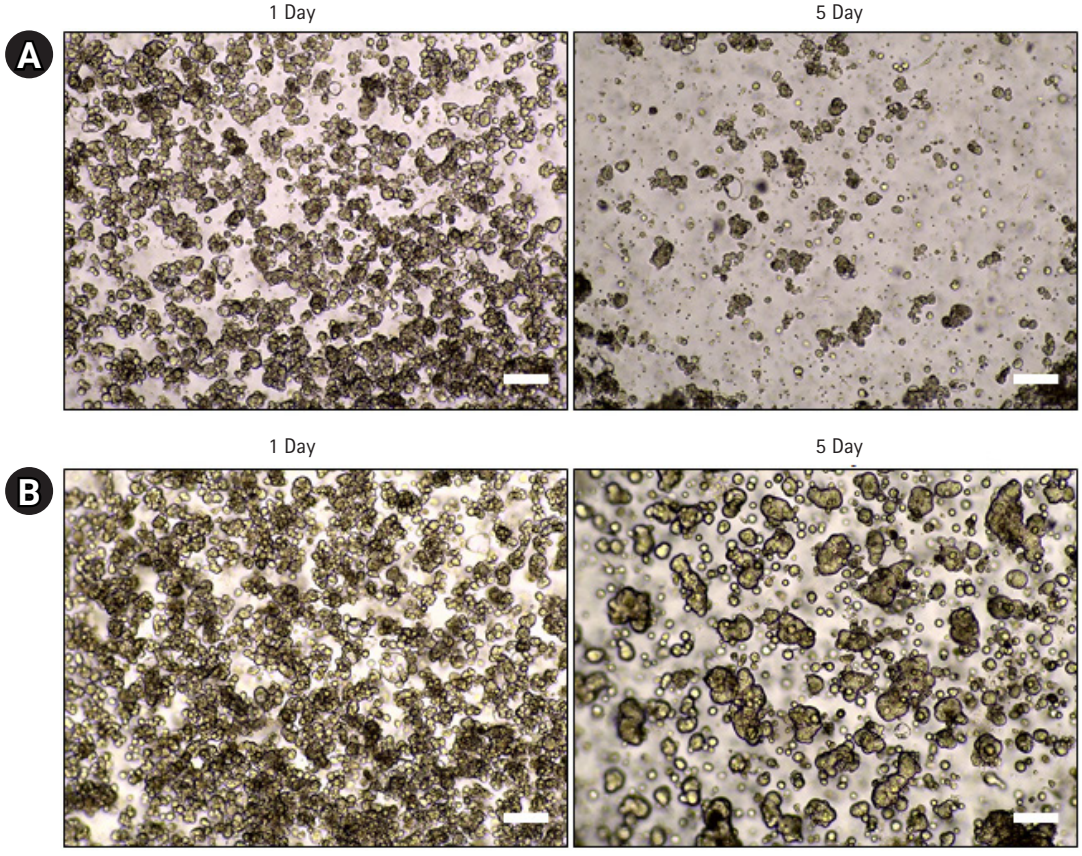

Fig. 4. Phase contrast images of 3-dimensional (3D) cell culture in Matrigel after Percoll-gradient centrifugation. (A) 3D cell culture of the cells (mixture of CD45$^{pos/neg}$ cells) obtained from the top layer with a 44% and 67% Percoll gradient. (B) 3D cell culture of the cells (mostly CD45^{neg} cells) obtained from the top layer of a 40% and 75% Percoll gradient. (A, B) Scale bars, 50 m. CD45^{pos}, CD45-positive; CD45^{neg}, CD45-negative.

PDOs containing native immune cells (macrophages, B cells, and natural killer cells) to facilitate personalized immunotherapy testing. Along with the previous study, we observed that growth inhibition of patient-derived cancer cells was observed in the presence of $CD45^{pos}$ cells. Therefore, to preserve and proliferate patient-derived tumor cells, it is critical to remove $CD45^{pos}$ cells from patient-derived tumor tissue samples.

The results from this study showed that $CD45^{neg}$ cells were successfully separated from a mixture of $CD45^{pos}$ and $CD45^{neg}$ cells in MPE samples using the 40% and 75% Percoll-gradient centrifugation method. Moreover, after separation, we were able to freshly store $CD45^{pos}$ cells, which can be used as patient-derived immune cells for application in the development of immunotherapies. In addition, 3D cell culture using isolated $CD45^{neg}$ cells can be used for chemosensitivity assays or immunotherapy using immune checkpoint modulators. In summary, 3D cell culture models using the $CD45^{neg}$ cells isolated from patient MPE samples might be a promising strategy for developing personalized medicine.

Notes

Conflict of interest
No potential conflict of interest relevant to this article was reported.

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Author contributions
Conceptualization: JC, SHL; Data curation: JC, JUL, WK; Formal analysis: Funding acquisition: SHL; Methodology: JC; Project administration: SHL; Investigation: DWL, SJK; Resources: JUL, SHL; Software: WK; Supervision: JUL, DWL; Validation: SJK; Writing-review & editing: SJK, SHL.

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Data availability
Please contact the corresponding author for data availability.

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