Fraction of MHCII and EpCAM expression characterizes distal lung epithelial cells for alveolar type 2 cell isolation

Koichi Hasegawa 1, Atsuyasu Sato 1*, Kazuya Tanimura 2, Kiyoshi Uemasu 1, Yoko Hamakawa 1, Yoshinori Fuseya 3, Susumu Sato 1, Shigeo Muro 1 and Toyohiro Hirai 1

Abstract

Background: Alveolar type 2 (AT2) cells play important roles in maintaining adult lung homeostasis. AT2 cells isolated from the lung have revealed the cell-specific functions of AT2 cells. Comprehensive molecular and transcriptional profiling of purified AT2 cells would be helpful for elucidating the underlying mechanisms of their cell-specific functions. To enable the further purification of AT2 cells, we aimed to discriminate AT2 cells from non-AT2 lung epithelial cells based on surface antigen expression via fluorescence activated cell sorting (FACS).

Methods: Single-cell suspensions obtained from enzymatically digested murine lungs were labeled for surface antigens (CD45/CD31/epithelial cell adhesion molecule (EpCAM)/ major histocompatibility complex class II (MHCII)) and for pro-surfactant protein C (proSP-C), followed by FACS analysis for surface antigen expression on AT2 cells. AT2 cells were sorted, and purity was evaluated by immunofluorescence and FACS. This newly developed strategy for AT2 cell isolation was validated in different strains and ages of mice, as well as in a lung injury model.

Results: FACS analysis revealed that EpCAM+ epithelial cells existed in 3 subpopulations based on EpCAM and MHCII expression: EpCAMmedMHCII+ cells (Population 1: P1), EpCAMmedMHCII− cells (P2), and EpCAMlowMHCII− cells (P3). proSP-C+ cells were enriched in P1 cells, and the purity values of the sorted AT2 cells in P1 were 99.0% by immunofluorescence analysis and 98.0% by FACS analysis. P2 cells were mainly composed of ciliated cells and P3 cells were composed of AT1 cells, respectively, based on the gene expression analysis and immunofluorescence. EpCAM and MHCII expression levels were not significantly altered in different strains or ages of mice or following lipopolysaccharide (LPS)-induced lung injury.

Conclusions: We successfully classified murine distal lung epithelial cells based on EpCAM and MHCII expression. The discrimination of AT2 cells from non-AT2 epithelial cells resulted in the isolation of pure AT2 cells. Highly pure AT2 cells will provide accurate and deeper insights into the cell-specific mechanisms of alveolar homeostasis.

Keywords: Alveolar type 2 cell, EpCAM, MCHII, Cell isolation
conditions [6–8]. In turn, the loss of alveolar homeostasis could lead to lung disease development. In fact, damage to or dysregulation of AT2 cells has been implicated in various lung diseases, such as chronic obstructive pulmonary disease (COPD) [9], lung adenocarcinoma [10], and pulmonary fibrosis [11]. As evidence of the roles that AT2 cells play in alveolar homeostasis accumulates, elucidating mechanisms underlying their functions in normal lungs and their possible dysfunction in diseases is becoming more important.

To explore the functions of AT2 cells further, various studies have attempted to characterize AT2 cells isolated from digested whole lung cells [12–15]. Given the availability of novel, high-throughput technologies such as comprehensive transcriptional profiling, highly purified AT2 cells from the lung should offer deeper insights into cell-specific responses to various stimuli in the alveolar region. Negative selection for AT2 cell isolation has been reported previously [13, 14]. A certain level of contamination by non-AT2 cell populations is assumed from the reported purity for this selection; however, this contamination was not characterized in detail. More recently, positive selection using pan-epithelial cell markers such as epithelial cell adhesion molecule (EpCAM) or E-cadherin has increased the purity of this cell population [16, 17] without discriminating between AT2 cells and non-AT2 lung epithelial cells. In pursuit of further purification, identifying AT2 cell surface markers that can separate AT2 cells from non-AT2 lung epithelial cells is desirable.

We hypothesized that major histocompatibility complex class II (MHCII) is an AT2 cell surface marker. While MHCII is important in adaptive immunity in antigen presenting cells, it is also expressed on the surface of AT2 cells in both humans [18] and rodents [19, 20]. Although the role of MHCII in AT2 cells remains to be fully elucidated, recent studies have suggested that it modulates immunoresponses in the lung [20, 21].

In the present study, we fully characterized MHCII expression on murine AT2 cells. Then, we classified distal lung epithelial cells into subpopulations based on EpCAM and MHCII expression levels; one of these populations was found to be enriched by AT2 cells. Finally, we developed a new fluorescence activated cell sorting (FACS)-based strategy for AT2 cell isolation that is widely applicable in the study of AT2 cells.

**Methods**

The study protocols were approved by the Animal Research Committee of Kyoto University (ID: MedKyo 43,125).

**Animals**

Nine-week-old C57BL/6 J mice (Charles River, Japan), BALB/c mice (SLC, Japan), FVB/N mice (CLEA, Japan), and A/J mice (SLC, Japan) were purchased for use in this study. Double-transgenic Scgb1a1-rtTA (Line 1)/(tetO)7CMV-Cre mice (a gift from Dr. Machiko Ikegami and Jeffrey A. Whitsett) [22] were bred with ROSA mT/mG mice (the Jackson Laboratory) to generate triple-transgenic Scgb1a1-rtTA/(tetO)7CMV-Cre/ROSA mT/mG mice. Doxycycline (600 ppm) was added to the chow starting from 5 weeks old to 8 weeks old to activate cre-mediated recombination in the triple-transgenic mice.

**The preparation of single-cell suspensions of murine lung**

An overview flowchart of cell isolation protocol with estimated time and the yield of cells are depicted in Additional file 1: Figure S1. Single lung cells were obtained using Corti’s protocol [13] with some modifications. Briefly, mice were anesthetized via intraperitoneal injection of pentobarbital with 10 U of heparin. The abdominal cavity was opened, and mice were exsanguinated via transection of the abdominal aorta. The trachea was exposed and intubated with a 20-gauge catheter (Terumo, Tokyo, Japan). The thoracic cavity was opened, and the lungs were perfused with 10 mL of cold 0.9% saline from the right ventricle. In total, 1.5 mL of dispase solution (Corning, Corning, NY) was instilled into the lungs from the intubated catheter, followed by 0.2 mL of 1% low melting point (LMP) agarose (Wako Pure Chemical Industries, Osaka, Japan). The lungs were immediately covered with ice and incubated for 2 min.

After incubation, the lungs were isolated from the thoracic cavity and incubated for 45 min in 5 mL of Hank’s Balanced Salt Solution (HBSS) (Wako Pure Chemical Industries) at room temperature (RT). Then, the lungs were minced using scissors and a transfer pipette in Dulbecco’s Modified Eagle Medium (DMEM) (Nacalai Tesque, Kyoto, Japan) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Life Technologies, Gaithersburg, MD), antibiotics, antimycotic solution (Life Technologies). After mincing, 100 μL of 1 mg/mL DNase 1 (Sigma-Aldrich, St. Louis, MO) was added to the sample and incubated for 4 min at RT. The cells were sequentially filtered through 70-μm and 40-μm filters (BD Biosciences, San Jose, CA). Then, the cell suspension was centrifuged for 7 min at 340 g and washed once with Phosphate buffered saline (PBS) (Nacalai Tesque). Next, the cell pellet was suspended with 3 mL of lysis buffer (eBioscience, San Diego, CA), and incubated for 2 min at RT, followed by washing with PBS. The cell pellet was resuspended in PBS containing 3% fetal bovine serum (FBS) for cell isolation or in staining buffer (PBS/0.1% bovine serum albumin (BSA)/0.02% NaN₃/0.5 mM ethylenediaminetetraacetic acid (EDTA)) for cell analysis. The average number of cells in the single-cell lung suspensions was 9.0 ± 0.9 × 10⁶ /lung, and the viability was 83.0 ± 1.2% (n = 8). For the 3-week-old mice,
Flow cytometric analysis and sorting of distal lung epithelial cells

A list of antibodies used for flow cytometry is shown in Additional file 2: Table S1. For cell-surface antigen staining, the samples were incubated with antibodies for 20 min at 4 °C, washed and resuspended in PBS containing 3% FBS. To exclude dead cells, 7-amino actinomycin D (7-AAD, BD Biosciences) was added to the samples before sorting. We selected live, single cells using a FACSAria III Cell Sorter with FACS Diva ver. 8.0.1 (BD Biosciences). Sorted cells were collected in DMEM containing 10% FBS, antibiotics, anticytotoxic, and 25 mM HEPES for further analyses. For proSP-C staining, single cell suspension of the lung or the sorted cells were incubated with fixable viability dye eFluor780 (eBioscience) and surface antigens, fixed, and permeabilized with fixation and permeabilization buffer (eBioscience) according to the manufacturer's instructions. Permeabilized cells were incubated with anti-mouse proSP-C antibody or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and labeled with PE-conjugated F(ab')2 donkey anti-rabbit IgG (1:200; eBioscience). Fixed cells were analyzed using a BD LSR Fortessa (BD Biosciences). Appropriate isotype control samples were utilized for all FACS analyses. The data were analyzed using FlowJo software (ver. 7.6.5, Tree Star, San Carlos, CA).

Lipopolysaccharide (LPS)-induced lung injury

Mice were anesthetized with isoflurane and hung upright at a 45-degree angle. LPS from E. coli (Sigma-Aldrich, St. Louis, MO) (1 mg/kg body weight in 100 μL of PBS) or PBS (control) was aspirated intratracheally as reported previously [23]. The mice were sacrificed at 24 h after intratracheal instillation for further analyses. Methods for immunofluorescence and RT-PCR analyses are provided in the online Data Supplement.

Statistical analysis

The values are expressed as the means ± SEM. Statistical analyses were performed using JMP ver. 10 (SAS Institute, Cary, NC). Comparisons between two groups were performed using the Wilcoxon rank sum test.

Results

MHCIi expression in AT2 cells

To demonstrate the localization of MHCIi in adult murine lungs, we analyzed MHCIi expression by immunofluorescence. As shown in Fig. 1a, proSP-C + AT2 cells also expressed MHCIi, while AT1 cells were negative for MHCIi. In the alveoli, alveolar macrophages were also positive for MHCIi expression. All proSP-C⁺ cells were positive for MHCIi expression.

To investigate MHCIi expression in AT2 cells further, we performed FACS analysis of component cells of murine lungs. Single-cell suspensions obtained from enzymatically digested lungs were stained for surface antigens, fixed, permeabilized, and then stained for proSP-C. Using FACS analysis, CD45 CD31 EpCAM⁺ cells (henceforth, EpCAM⁺ cells) were analyzed for proSP-C expression (Fig. 1b). Among EpCAM⁺ cells, 90.4% ± 1.7% were positive for proSP-C expression, and almost all proSP-C⁺ cells expressed MHCIi (99.0 ± 0.2%) (Fig. 1c). In contrast, the majority of proSP-C⁻ EpCAM⁺ cells were negative for MHCIi expression (Fig. 1d). This observation suggests that EpCAM⁺ cells from enzymatically digested murine lungs primarily consist of AT2 cells but also contain a substantial amount of proSP-C⁻ epithelial cells. Thus, MHCIi could be a useful surface marker for classifying lung epithelial cells to identify AT2 cells.

In the two-dimensional plot of EpCAM and MHCIi, EpCAM⁺ cells were classified into 3 different subpopulations based on EpCAM and MHCIi expression: EpCAM^{high}MHCIi⁺ cells (Population 1; P1 cells), EpCAM^{low}MHCIi⁻ cells (P2 cells), and EpCAM^{low}MHCIi⁻ cells (P3 cells) (Fig. 1e). Most P1 cells were positive for proSP-C expression (97.8 ± 0.4%), while P2 and P3 cells were negative for proSP-C expression (Fig. 1f and g). To evaluate the efficiency of the gating strategy for AT2 cell identification, proSP-C⁺ cells were back-gated in the plot of EpCAM and MHCIi, demonstrating that 97.6 ± 0.3% of the cells were in the P1 gate (Fig. 1h).

Isolation of AT2 cells based on EpCAM and MHCIi expression

During AT2 cell isolation, we evaluated whether P1 cell isolation was superior to EpCAM⁺ cell isolation in terms of purity. Live single cells from enzymatically digested lungs were stained for surface antigens. EpCAM⁺ cells were classified into 3 subpopulations as shown in the fixed cell analysis (P1, P2, and P3) (Fig. 2a and Additional file 3: Figure S2). The average yield of P1 cells was 6.2 ± 0.7 × 10⁵ /lung (n = 8), and the viability was 89.0 ± 1.3% when assessed with fixable viability dye staining. EpCAM⁺ cells were also sorted (Fig. 2b), and the purity of both sorted cells was evaluated by immunofluorescence and FACS. The average yield of P2 and P3 cells were 1.8 ± 0.1 × 10⁴ and 2.3 ± 0.2 × 10⁵ /lung, respectively (n = 3).

Immunofluorescence analysis of cytopsin preparations of sorted cells demonstrated that the proportion of proSP-C⁺ cells in sorted P1 cells was 99.0 ± 0.3%, which was significantly higher than that in sorted EpCAM⁺ cells (94.0 ± 0.8%) (Fig. 2c and e).

22-gauge catheters were used for the tracheal intubation, and the instilled volume of dispase solution and 1% LMP agarose were reduced to 1 mL and 0.1 mL, respectively.
For FACS analysis of proSP-C expression, sorted cells were fixed, permeabilized and stained for proSP-C. The purity of sorted P1 cells was 98.0 ± 0.2%, whereas that of sorted EpCAM+ cells was 95.4 ± 1.5% (Fig. 2d and f), demonstrating again that the purity of P1 cells was significantly higher.

To characterize P1 and EpCAM+ cells sorted by surface markers, we performed mRNA expression analysis to confirm cell specific gene expression. *SftpC* (Surfactant Protein C, AT2 cell specific surfactant protein) expression levels were 4.4-fold higher in P1 cells compared to whole lung cells, while the expression of other epithelial lineage markers including *Scgb1a1* (Secretoglobin Family 1A Member 1, club cell specific protein), *Pdpn* (Podoplanin, AT1 cell specific protein among lung epithelial cells), and *Foxj1* (Forkhead Box J1, ciliated cell specific nuclear protein) was minimal (Fig. 2g). In contrast, sorted EpCAM+ cells displayed *Pdpn* and *Foxj1* expression.
Fig. 2 (See legend on next page.)
To characterize P2 and P3 cells, we sorted these subpopulations and performed immunofluorescence and mRNA expression analyses. The majority of P2 cells were positive for acetylated tubulin (92.3 ± 2.0%, n = 3), with sparse SCGB1A1+ cells and unspecified cells as determined by immunofluorescence analysis (Fig. 2h). Foxj1 expression in sorted P2 cells was 116-fold higher compared to that in whole lung cells (Fig. 2i), suggesting that P2 cells are enriched with ciliated cells. Approximately half of P3 cells, which were negative for major epithelial cell markers including proSP-C, SCGB1A1, acetylated tubulin, and T1a as determined by immunofluorescence analysis, were positive for AQP5 (Fig. 2j). mRNA analysis revealed 3.9-fold higher Pdpn expression (Fig. 2k), suggesting that AT1 cells are the major cell type among P3 cells.

Validation of the gating strategy using AT2 cells with intrinsic GFP expression

FACS analysis with fluorescent antibody staining is a useful method to explore antigen expression. However, this approach is inevitably accompanied by possible non-specific antibody binding, which we verified using isotype controls. To evaluate the consistency of our gating strategy, we employed AT2 cells that have intrinsic GFP expression. We bred double-transgenic Scgb1a1-rtTA/(tetO)7CMV-Cre mice [22] with ROSA mT/mG mice to generate triple-transgenic Scgb1a1-rtTA/(tetO)7CMV-Cre/ROSAmT/mG mice (Fig. 3a). In these triple-transgenic mice, most AT2 cells and a subset of club cells display enhanced GFP expression following the addition of doxycycline to the chow (Fig. 3b), starting from 5 weeks old to 8 weeks old to minimize GFP labeling in AT1 cells and ciliated cells due to lung maturation after birth [24].

Using single lung cells obtained from these triple-transgenic mice, we analyzed GFP+ cells for EpCAM and MHCII expression and found that 97.4 ± 0.3% (n = 4) of GFP+ cells were gated with P1 cells (Fig. 3c). Then, we sorted GFP+ cells (5.8 ± 0.5 × 10⁵ cells /lung), analyzed proSP-C expression by FACS; the results indicated that most sorted GFP+ cells were positive for proSP-C (96.0 ± 0.6%) (Fig. 3d).

CD74 expression in AT2 cells

Because CD74 has been reported to be a potential surface marker of AT2 cells [25, 26], we evaluated whether CD74 can be utilized for the positive selection of AT2 cells. Live-cell analysis demonstrated that only a small fraction of EpCAM+ cells expressed CD74 (Fig. 4a). We further explored CD74 expression among EpCAM+ cells using fixed and permeabilized cells stained for proSP-C. When CD74 staining was performed before cell fixation and permeabilization (surface CD74 analysis), proSP-C+ cells were found to express CD74 weakly as a whole, with a small fraction of cells showing higher CD74 expression (Fig. 4b), while proSP-C+ cells did not express CD74. In contrast, when CD74 staining was performed after fixation and permeabilization (intracellular CD74 analysis), proSP-C+ cells displayed much higher CD74 expression compared with isotype controls (Fig. 4c). These observations suggest that epithelial CD74 expression is limited to AT2 cells, although CD74 is not suitable for the positive selection of AT2 cells due to its predominantly intracellular expression. Although why most of the epithelial cells were negative for CD74 in the live-cell analysis is unclear, surface CD74 may be unstable and may be transformed into a soluble form [27] without cell fixation.

The applicability of the AT2 isolation strategy to different strains and ages of mice

To investigate the applicability of our newly developed AT2 cell isolation strategy, we evaluated EpCAM, MHCII, and proSP-C expression levels in single lung cells from different strains and ages of mice (Table 1, Additional file 4: Figure S3).

Mouse MHCII is highly polymorphic [28]. We performed FACS analysis using BALB/c, FVB/N, and A/J...
mice. In BALB/c and FVB/N mice, EpCAM+ cells were similarly classified into 3 subpopulations, and proSP-C+ cells were enriched in P1 cells (Table 1) in these mice and in C57BL6/J mice. However, in A/J mice, MHCII expression among proSP-C+ cells was negative, resulting in the failed classification of EpCAM+/MHCII- cells, although non-EpCAM+ cells expressed MHCII, suggesting that AT2 cells in A/J mice lack MHCII expression.

Next, we evaluated young (3-week-old) and old (1-year-old) mice. In both groups, almost all proSP-C+ cells expressed MHCII, and P1 cells identified based on EpCAM and MHCII expression were enriched with proSP-C+ cells. These results suggested that MHCII expression does not alter as a function of age or mouse strain, except for A/J mice.

AT2 cell isolation in an LPS-induced lung injury model
To evaluate whether we can identify subpopulations of distal lung epithelial cells (P1, P2, and P3) during lung injury, we applied our isolation strategy to an LPS-induced lung injury model. LPS stimulation of the lung induced inflammatory cell infiltration in alveolar spaces (Fig. 5a). Single-cell suspensions of LPS model lungs contained higher numbers of cells (2.2 ± 0.2 × 10^7 per sample) with the same viability (91.6 ± 1.0% by trypan blue exclusion) as control samples, which were prepared in the same manner (8.3 ± 0.8 × 10^6 per sample and 89.7 ± 1.2%, respectively). FACS analysis indicated that EpCAM+ and MHCII+ cells were not significantly altered in distal lung epithelial cells following LPS instillation, which resulted in the successful identification of P1 cells in addition to P2 and P3 cells (Fig. 5b, c, and Additional file 5: Table S2). The yield of P1 cells in LPS model was 4.2 ± 0.3 × 10^5, which was slightly smaller than that of control samples (5.4 ± 0.3 × 10^5) due to the lower sorting efficiency in the LPS model, in which the cell suspension contained a greater number of non-AT2 cells. In the LPS model, sorted P1 cells showed...
15.6-fold higher Sftpc expression compared with whole-lung cells. In P1 cells, Sftpc expression was downregulated in response to LPS stimulation (Fig. 5d). Cxcl1 and Tnf were upregulated to higher levels in P1 cells following LPS stimulation (Fig. 5e and f) than those observed in whole-lung cell analyses. In P1 cells, Mmp9 was upregulated in response to LPS (Fig. 5h), while Mmp2 and Mmp12 expression was undetected in both control and LPS model cells (Fig. 5g and i). MMP12 is a metalloprotease that is expressed by macrophages, and the lack of MMP12 expression in sorted cells further supports the successful depletion of hematopoietic cells from the inflammatory lung injury model.
| Strain   | Age    | The yield of single cell suspension (×10^6/ lung) | proSP-C^+ cells/ Live single cells (%) | MHCII^+ cells/ proSP-C^+ cells (%) | proSP-C^+ cells/ P1 cells (%) |
|----------|--------|-------------------------------------------------|----------------------------------------|-----------------------------------|-------------------------------|
| C57BL/6 J| 3-wk.-old | 4.3 ± 0.6                                      | 25.2 ± 1.7                            | 99.2 ± 0.2                        | 97.2 ± 0.2                   |
|          | 9-wk.-old | 8.1 ± 0.4                                      | 34.5 ± 1.9                            | 99.0 ± 0.2                        | 97.9 ± 0.4                   |
|          | 1-yr.-old | 9.7 ± 0.8                                      | 31.1 ± 1.2                            | 99.2 ± 0.2                        | 97.4 ± 0.3                   |
| BALB/c   | 9-wk.-old | 8.2 ± 0.4                                      | 32.0 ± 3.5                            | 99.6 ± 0.0                        | 97.7 ± 0.5                   |
| FVB/N    | 9-wk.-old | 8.7 ± 0.7                                      | 33.3 ± 0.8                            | 99.1 ± 0.2                        | 97.6 ± 0.4                   |

The values are expressed as the means ± SE (n = 3/group). MHCII, major histocompatibility complex class II; proSP-C, pro-surfactant protein C

**Fig. 5** AT2 cell isolation in an LPS-induced lung injury model. a) Hematoxylin and eosin staining of the lung at 24 h after intratracheal instillation of LPS (1 μg/g body weight). Alveolar spaces were infiltrated with inflammatory cells. Scale bars, 50 μm. b) Representative FACS plot that identifies CD45^−CD31^− cells (left), which were analyzed for EpCAM and MHCII expression to sort P1 cells (right) in control samples. c) Representative FACS plots of the LPS-induced lung injury model. Compared to the control, the proportion of CD45^−CD31^− cells was lower due to increased numbers of inflammatory cells (left). P1 cells were successfully identified and sorted (right). In both the control and the LPS model, whole-lung samples and sorted P1 cells were analyzed for (d) Sftpc, (e) Cxcl1, (f) Tnf, (g) Mmp2, (h) Mmp9, and (i) Mmp12 mRNA expression (n = 3/group). In P1 cells, Cxcl1, Tnf, and Mmp9 were upregulated following LPS stimulation. Mmp2 and Mmp12 were undetected in P1 cells. EpCAM, epithelial cell adhesion molecule; MHCII, major histocompatibility complex class II; PE-Cy7, phycoerithrin-cyanin7; APC, allophycocyanin; LPS, lipopolysaccharide; SSC, side scatter.
Discussion

The present study demonstrated that murine distal lung epithelial cells can be classified into 3 subpopulations (P1, P2, and P3) by FACS analysis of EpCAM and MHCII expression. AT2 cells were highly enriched in the P1 subpopulation (EpCAM$^{\text{high}}$MHCII$^{-}$) and were successfully sorted with high purity and viability. P2 (EpCAM$^{\text{low}}$MHCII$^{-}$) contained primarily ciliated cells, and approximately half of P3 (EpCAM$^{\text{low}}$MHCII$^{-}$) cells were identified as AT1 cells.

We revealed two important considerations for performing FACS analyses of distal lung epithelial cells. The first consideration is that EpCAM expression levels measured by fluorescence intensity vary widely among EpCAM$^{+}$ cells. The second consideration is that AT2 cells uniformly express MHCII and that the epithelial expression of MHCII is limited to AT2 cells. Together, these two factors enabled the clear classification of distal lung epithelial cells by FACS, leading to highly pure AT2 cell isolation.

EpCAM is known as a pan-epithelial cell marker, yet its relative expression among each type of lung epithelial cell has not been fully investigated. Because EpCAM is a principal marker for the positive selection of AT2 cells, we labeled EpCAM with an APC-conjugated antibody. APC has a high staining index and is superior to other common fluorescent proteins in discriminating positive and negative fractions by FACS analysis (https://www.bdbiosciences.com/documents/lsr_appnote02.pdf). This fluorescence property resulted in the demonstration of a wide range of EpCAM expression levels among distal lung epithelial cells by FACS analysis. In fact, a previous report implied that AT2 cells have lower EpCAM expression compared to bronchiolar cells using an anti-EpCAM antibody conjugated to PE [29], whose staining index is similar to that of APC.

Together with the wide distribution of EpCAM expression levels, the homogenous and specific expression of MHCII in AT2 cells was helpful for discriminating these cells from non-AT2 epithelial cells, particularly bronchiolar cells. In the FACS plot of EpCAM versus MHCII, lung epithelial subpopulations with different EpCAM expression levels were clearly separated based on MHCII expression. Although MHCII is primarily expressed in antigen-presenting cells, AT2 cells also constitutively express MHCII. Recently, the MHCII-dependent immunomodulatory functions of AT2 cells have received increasing attention. Debbabi et al. reported that AT2 cells can process mycobacterial antigens through the MHCII pathway but fail to prime naïve T cells, suggesting a regulatory role for AT2 cells in T cell inflammation [20]. In contrast, Gereke et al. reported that AT2 cells can prime naïve CD4$^{+}$ T cells for self-antigens or exogenous antigens and induce T-cell activation, while upon inflammation, AT2 cells induce regulatory T cells by producing antiproliferative factors such as TGF-β [21].

In the present study, we demonstrated that AT2 cells from A/J mice do not express MHCII; this lack of expression may be because A/J mice show less neutrophil infiltration in response to LPS compared to BL6 mice [30]. Furthermore, A/J mice are highly sensitive to the chemical induction of lung tumors [31]. Although its relevance is unknown, elucidating the relationship between MHCII expression in AT2 cells and lung disease development should be the subject of future research.

We developed a new strategy for isolating AT2 cells using EpCAM and MHCII as positive selection markers. Highly pure AT2 cells can provide accurate and cell-specific information for the study of AT2 cell functions. Even a small fraction of contaminating cells could complicate data interpretation, particularly in transcriptional profiling, as suggested by mRNA analyses of sorted EpCAM$^{+}$ cells in the present study. Although the purity of sorted EpCAM$^{+}$ cells exceeded 95%, Foxj1 expression derived from contaminating ciliated cells was clearly observed. Previous methods of AT2 cell isolation included negative selection that depletes other cell types, such as hematopoietic cells and endothelial cells from single lung cells [13–15, 26, 32, 33]. Recent strategies have added EpCAM as a positive selection marker, increasing the purity of isolated cells to approximately 95% [16, 17, 34] (Table 2). We further increased the purity of isolated AT2 cells by depleting non-AT2 lung epithelial cells, which primarily consisted of ciliated cells and AT1 cells. Although club cells were abundant in the distal lung tissue, they seemed to be depleted during the process of single lung cell preparation, as suggested by immunofluorescence and mRNA analyses of sorted EpCAM$^{+}$ cells. Analyses of triple-transgenic mice in which AT2 cells are labeled with GFP further support this conclusion. While GFP-labeled club cells were observed via immunofluorescence in the lung tissue, most sorted GFP$^{+}$ cells from the triple-transgenic mice were positive for proSP-C expression.

We extensively validated EpCAM and MHCII expression in lung epithelial cells using different strains and ages of mice, as well as a lung injury model. The stability of EpCAM and MHCII surface expression is crucial to identifying AT2 cells based on surface antigen expression. Except for A/J mouse cells, distal lung epithelial cells were classified in the same manner based on EpCAM and MHCII expression.

In the LPS model, transcriptional analyses revealed the prominent upregulation of Cxcl1 and Tnf in AT2 cells following LPS instillation. Using in situ hybridization, Elizur et al. demonstrated that club cells and AT2 cells express Cxcl1 following LPS stimulation, while alveolar macrophages are the primary types of cells that express Tnf in the distal lung [35]. These authors also reported
that TNFα produced by macrophages is important for CXCL1 production by club cells [36], whereas Skerrett et al. reported that Tgα is expressed in bronchiolar epithelial cells following LPS inhalation through NFκB activation, leading to neutrophil recruitment [37]. In contrast with the known role of bronchiolar cells in the immune response, the role of AT2 cells in LPS-induced lung injury has remained largely unknown. Here, we isolated AT2 cells by FACS and investigated specific transcriptional changes in response to LPS stimulation. The upregulation of Cxcl1 and Tgα mRNA expression implies that AT2 cells also have a role in modulating immunological responses by recruiting neutrophils into alveolar spaces, and further investigations are warranted to elucidate the coordinated responses between epithelial cells and hematopoietic cells in the alveolar region.

Whether our isolation strategy is applicable to human AT2 cell isolation is also of interest. For human lung epithelial cell isolation, a previous report demonstrated that EpCAM+T1α− cells were enriched with AT2 cells at a purity of 94.0%, as assessed by proSP-C staining, while AT1 cells and club cells were found in the EpCAM+T1α−/lo fraction [38]. Interestingly, AT2 cells displayed higher EpCAM expression compared to AT1 cells, as shown in the murine lung. Because the constitutive expression of MHCII in human AT2 cells has been reported previously [39], human AT2 cell isolation using EpCAM and MHCII as positive selection markers is worth considering.

Our study has some limitations. First, the preparation of single cells by protease digestion might affect cell status or surface-antigen expression. Tissue digestion using dispase, which is relatively gentle, has been widely used for many bioassays using AT2 cells. Because our protocol is not appropriate for club cell isolation, optimal digestion methods including proteases would depend on targeted cells in the lung. Second, tissue digestion and cell sorting require a certain amount of time, which might affect the transcriptional status of AT2 cells. To further optimize our methods, we attempted tissue dissociation using a gentle MACS dissociator (Miltenyi Bio-Tech, Bergisch-Gladbach, Germany). This modification shortened the amount of digestion time by 20 min with 3 samples and resulted in approximately 3 times the number of cells in a single-cell suspension without compromising cell viability (>90% with trypan blue exclusion).

Third, the number and purity of the sorted cells were not high enough to analyze the P2 and P3 subpopulations themselves. Although we identified major types of cells that were enriched in these subpopulations, we could not characterize a few of P2 cells and approximately half of P3 cells by immunofluorescence. One possible explanation for this difficulty in characterization is that dispase negatively affected surface-antigen expression [40].

**Conclusion**

In conclusion, we demonstrated that distal lung epithelial cells can be classified into 3 subpopulations based on
EpCAM and MHCII expression. We successfully discriminated AT2 cells from non-AT2 epithelial cells and sorted AT2 cells with high purity. Highly pure AT2 cells will serve as a powerful tool for molecular and transcriptional analyses and will provide cell-specific information in both normal and diseased lungs.

Additional files

Additional file 1: Figure S1. An overview flowchart of cell isolation protocol. (PPTX 73 kb)
Additional file 2: Table S1. List of antibodies for flow cytometry. (DOCX 17 kb)
Additional file 3: Figure S2. Gating strategy for isolation of AT2 cells based on EpCAM and MHCII expression. (PPTX 301 kb)
Additional file 4: Figure S3. The applicability of the AT2 isolation strategy to different strains and ages of mice. (PPTX 572 kb)
Additional file 5: Table S2. The yield of single cell suspension and the proportion of each cell population in control and LPS-induced injury mice. (DOCX 14 kb)

Acknowledgments
The authors thank Dr. Machiko Ikegami, Dr. James Bridges and Dr. Jeffrey Whitsett for supporting this study. The authors also thank Dr. Mitsuhiro Yamada and Dr. Hiroshi Kubo for their technical support.

Funding
Supported by Grants-in-Aid for scientific research (C) No. 25461156 and No. 25893106.

Availability of data and materials
Not applicable.

Authors’ contributions
Conception and design: KH, AS, KT, SS and SM; Analysis and interpretation: KH, AS, Randell SH, Noble PW, Hogan BL. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. Proc Natl Acad Sci U S A. 2011;108:E1475–83.

Consent for publication
The study protocols were approved by the Animal Research Committee of content: KH. And AS. All authors read and approved the final manuscript.

Conception and design: KH, AS, KT, SS and SM; Analysis and interpretation: KH, AS, Randell SH, Noble PW, Hogan BL. Type 2 alveolar cells are stem cells in adult lung. J Clin Invest. 2013;123:3025–36.

Consent for publication
The study protocols were approved by the Animal Research Committee of content: KH. And AS. All authors read and approved the final manuscript.

Ethics approval
The study protocols were approved by the Animal Research Committee of Kyoto University (ID: Medkyo 43,125).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1 Department of Respiratory Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara Shogoin Sakyō, Kyoto 606-8507, Japan. 2 Pulmonary Medicine, Kishiwada City Hospital, 1001 Gakuhara Kishiwada, Osaka 596-8501, Japan. 3 Pulmonary Medicine, Otsu City Hospital, 2 Chome-9-9 Motomiya Otsu, Shiga 520-0804, Japan.
Received: 8 May 2017 Accepted: 2 August 2017 Published online: 07 August 2017

References
1. Herzog EL, Brody AR, Colby TV, Mason R, Williams MC. Knowns and unknowns of the alveolus. Proc Am Thorac Soc. 2008;5:778–82.
potential mechanism for macrophage migration inhibitory factor-induced epithelial repair. Am J Physiol Lung Cell Mol Physiol. 2009;296:L442–52.

27. Assis DN, Leng L, Du X, Zhang CK, Grieb G, Merk M, Garcia AB, McCran C, Chapro I, Meinhardt A, et al. The role of macrophage migration inhibitory factor in autoimmune liver disease. Hepatology. 2014;59:580–91.

28. Hood L, Steinmetz M, Malissen B. Genes of the major histocompatibility complex of the mouse. Annu Rev Immunol. 1983;1:529–68.

29. McQualter JL, Yuen K, Williams B, Bertoncelli I. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. Proc Natl Acad Sci U S A. 2010;107:1414–9.

30. O'Malley J, Matesic LE, Zink MC, Strick JD, Mooney ML, De Maio A, Reeves RH. Comparison of acute endotoxin-induced lesions in a/J and C57BL/6J mice. J Hered. 1998;89:525–30.

31. Meuwissen R, Bens A. Mouse models for human lung cancer. Genes Dev. 2005;19:643–64.

32. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell. 2005;121:823–35.

33. Herold S, von Wulffen W, Steinmueller M, Pleschka S, Kuzel WA, Mack M, Srivastava M, Seeger W, Maus UA, Lohmeyer J. Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. J Immunol. 2006;177:1817–24.

34. Messier EM, Mason RJ, Kosmider B. Efficient and rapid isolation and purification of mouse alveolar type II epithelial cells. Exp Lung Res. 2012;38:363–73.

35. Elizur A, Adair-Kirk TL, Kelley DG, Griffin GL, deMello DE, senior RM: Clara cells impact the pulmonary innate immune response to LPS. Am J Physiol Lung Cell Mol Physiol. 2007;293:L383–92.

36. Elizur A, Adair-Kirk TL, Kelley DG, Griffin GL, Dernello DE, Senior RM. Tumor necrosis factor-alpha from macrophages enhances LPS-induced Clara cell expression of keratinocyte-derived chemokine. Am J Respir Cell Mol Biol. 2008;38:8–15.

37. Reuttershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS, Ley K. Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. J Clin Invest. 2006;116:955–702.

38. Fujino N, Kubo H, Ota C, Suzuki T, Suzuki S, Yamada M, Takahashi T, He M, Suzuki T, Kondo T, Yamaya M. A novel method for isolating individual cellular components from the adult human distal lung. Am J Respir Cell Mol Biol. 2012;46:422–30.

39. Cunningham AC, Zhang JC, Muy JV, Ali S, Kirby JA. A comparison of the antigen-presenting capabilities of class II MHC-expressing human lung epithelial and endothelial cells. Immunology. 1997;91:458–63.

40. Auegner A, Recke M, Hansen G, Hennig C, Bruder D. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. Eur J Microbiol Immunol (Bp). 2012;2:112–20.