Video Article

Flow Cytometric Isolation of Primary Murine Type II Alveolar Epithelial Cells for Functional and Molecular Studies

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Abstract

Throughout the last years, the contribution of alveolar type II epithelial cells (AECII) to various aspects of immune regulation in the lung has been increasingly recognized. AECII have been shown to participate in cytokine production in inflamed airways and to even act as antigen-presenting cells in both infection and T-cell mediated autoimmunity [1-8]. Therefore, they are especially interesting also in clinical contexts such as airway hyper-reactivity to foreign and self-antigens as well as infections that directly or indirectly target AECII. However, our understanding of the detailed immunologic functions served by alveolar type II epithelial cells in the healthy lung as well as in inflammation remains fragmentary. Many studies regarding AECII function are performed using mouse or human alveolar epithelial cell lines [9-12]. Working with cell lines certainly offers a range of benefits, such as the availability of large numbers of cells for extensive analyses. However, we believe the use of primary murine AECII allows a better understanding of the role of this cell type in complex processes like infection or autoimmune inflammation. Primary murine AECII can be isolated directly from animals suffering from such respiratory conditions, meaning they have been subject to all additional extrinsic factors playing a role in the analyzed setting. As an example, viable AECII can be isolated from mice intranasally infected with influenza A virus, which primarily targets these cells for replication [13]. Importantly, through *ex vivo* infection of AECII isolated from healthy mice, studies of the cellular responses mounted upon infection can be further extended.

Our protocol for the isolation of primary murine AECII is based on enzymatic digestion of the mouse lung followed by labeling of the resulting cell suspension with antibodies specific for CD11c, CD11b, F4/80, CD19, CD45 and CD16/CD32. Granular AECII are then identified as the unlabeled and sideward scatter high (SSC^{high}) cell population and are separated by fluorescence activated cell sorting [3].

In comparison to alternative methods of isolating primary epithelial cells from mouse lungs, our protocol for flow cytometric isolation of AECII by negative selection yields untouched, highly viable and pure AECII in relatively short time. Additionally, and in contrast to conventional methods of isolation by panning and depletion of lymphocytes via binding of antibody-coupled magnetic beads [14, 15], flow cytometric cell-sorting allows discrimination by means of cell size and granularity. Given that instrumentation for flow cytometric cell sorting is available, the described procedure can be applied at relatively low costs. Next to standard antibodies and enzymes for lung disintegration, no additional reagents such as magnetic beads are required. The isolated cells are suitable for a wide range of functional and molecular studies, which include *in vitro* culture and T-cell stimulation assays as well as transcriptome, proteome or secretome analyses [3, 4].

Video Link

The video component of this article can be found at http://www.jove.com/video/4322/

Protocol

Details regarding required reagents and materials are listed in the table at the end of the protocol below. Before starting work, prepare 15 ml tubes (one per mouse) containing 4 ml of dispase and pre-warm them to 37 °C in a water bath. In a heating block, shortly heat small aliquots of 1 % low-melt agarose (in water) to 95 °C until liquefied and subsequently cool to 45 °C until use.

1. Preparation of the Mouse Lung

1. Sacrifice the mouse by CO_2 asphyxiation. **Note**: Do not perform cervical dislocation as this will injure the trachea so that following steps of the protocol, such as installation of the lung with liquid, cannot be performed successfully. Ensure loss of nociceptive reflexes.
2. Spray the mouse with ethanol and make a long cut along the ventral midline of the body. Pull the ventral fur and skin and subsequently also carefully cut and remove the peritoneum.

3. Exsanguinate the mouse by cutting the left and right jugular vein (*Vena jugularis*) as well as the renal artery (*Arteria renalis*). Remove flowing blood with tissue.
4. Carefully puncture and then remove the diaphragm to expose the heart and lung by cutting away the ribs. Take special care not to injure the lung.
5. With a 26G cannula and a 10 ml syringe filled with cold phosphate buffered saline (PBS), puncture the right ventricle of the heart and perfuse the lung with PBS until free of blood.
6. Cut and remove the salivary glands to expose the trachea. Also carefully cut the muscle surrounding the trachea.
7. Insert a 22G indwelling cannula into the trachea, remove the needle and push the plastic catheter towards the lung. Fix the catheter by tying a small piece of yarn around trachea and catheter.

2. Enzymatic Digestion of the Lung Tissue

If desired, a bronchoalveolar lavage fluid sample can be prepared by flushing the lungs through the catheter inserted in the trachea with PBS or medium before the next step.

1. Using a 2 ml syringe, carefully instill a maximal volume of 2 ml of dispase (from the 4 ml aliquot) into the lung through the catheter so that all lobes are fully expanded. Exchange the syringe with a 1 ml syringe containing 0.5 ml of the liquefied agarose and also instill into the lung.
2. Leave the syringe on the catheter to prevent back-flow of the agarose and immediately cover the lung with laboratory tissue paper and ice. Let the agarose gel for a couple of minutes.
3. Remove the tissue paper, ice, syringe and catheter, cut the trachea and excise the lung, heart and thymus from the chest. Then rinse the lung in a dish with PBS and remove the heart, thymus and remaining trachea.
4. Put the lung into the remaining 2 ml of dispase and incubate at room temperature for 45 min.

3. Preparation of the Lung Cell Suspension

1. Remove the lung from the dispase and transfer it into a dish containing 7 ml of anti-CD16/32 antibody (1 µg/ml final concentration) in DMEM medium and 100 µl DNase.
2. Using forceps, completely disintegrate the lung tissue by pulling it apart and incubate for 10 min at room temperature while gently rocking on a rocker set to 200 rpm. If desired, the cell suspension can then be stored at 4 °C until the next step.
3. Filter the cell suspension through nylon meshes first with 100 µm and subsequently with 70 µm, 48 µm and 30 µm pores. Take care to rinse each mesh as well as the tubes and dishes thoroughly with DMEM to minimize loss of cells and maximize yield. If you are pooling samples, this can be achieved here by subsequently passing cells suspensions from mice of one group through the same filter. Renew the filter in case of clogging.
4. Depending on the volume, transfer the filtrate to one or more 50 ml tubes and centrifuge for 15 min at 160 x g and 4 °C. Remove the supernatant.
5. Resuspend the cell pellet in 2 ml erythrocyte lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA, pH 7.2) and quickly terminate lysis by addition of 13 ml of DMEM medium. Transfer the solution to a 15 ml tube and centrifuge for 12 min at 160 x g and 4 °C.

4. Antibody Staining for Flow Cytometric Cell-sorting

1. Resuspend the cells in 3 ml primary antibody cocktail, prepared in DMEM medium at dilutions suitable for flow cytometry. (Antibodies were previously titrated for optimal working dilutions by staining 1 x 10^6 splenocytes in a volume of 100 µl. Use 3 ml of the primary antibody cocktail containing the optimal concentrations for each of the used antibodies for cells pooled from up to five mice. If more mice are pooled to one sample, adjust the volume).
2. For staining, incubate the cells for 10 min in the dark at 4 °C.
3. Wash the cells by filling the 15 ml tube with DMEM medium and centrifugation for 12 min at 160 x g and 4 °C.
4. In case uncoupled or biotinylated antibodies were used in 4.1: Remove the supernatant and resuspend the cells in 2 - 3 ml secondary antibody cocktail. Stain for 10 min at 4 °C in the dark.
5. Wash the cells through filling the tube with DMEM and centrifugation for 12 min at 160 x g and 4 °C.
6. Resuspend the cells in 1 ml of DMEM and pre-filter through a 50 µm filter into a tube for cell-sorting. Optional: Count the cells to obtain the total cell number in the lung cell suspension.

5. Cell-sorting

1. Mix the cells by vortexing prior to sorting. Use a 100 µm nozzle for cell-sorting of AECII. Sheath fluid pressure as well as laser emission and detection wavelengths depend on the instrument used for flow cytometric cell-sorting as well as the fluorochromes used in the antibody staining procedure.
2. Gate on the SSC^{high} cells that are negative for the fluorochromes used for staining and sort into a tube containing DMEM. Use forward scatter height (FSC-H) vs. area (FSC-A) and sideward scatter height (SSC-H) vs. area (SSC-A) windows to exclude any doublets or cell aggregates (see details of the gating strategy in **Figure 1a**).
3. In order to collect the sorted cells centrifuge for 20 min at 280 x g and 4 °C.
4. Resuspend the cells in culture-medium or buffer as required for subsequent analysis.

Representative Results

When sorting lung cell suspensions isolated from healthy mice, the AECII gate will typically account for about 42 ± 10 % of all events. This percentage can be noticeably lower when mice with respiratory conditions such as a viral infection are used, as the initial cell suspension will contain a considerably higher proportion of lymphocytes and other immune cells recruited to the airways. For AECII isolated from IAV infected lungs on day 3 following infection we have observed a reduction of the frequency of cells in the AECII gate by roughly 50 %.

A typical sort as well as re-analysis of the sorted cells is depicted in **Figures 1b** and **1c**. As indicated, the purity of the isolated cells accounts roughly 92 ± 5 %. In **Figure 2a**,we show that successful separation of pure cell populations can be confirmed by staining for surfactant-proteins C, which is expressed exclusively by AECII [16, 17].

We have observed that the number of AECII isolated per single animal strongly depends on the mouse strain used. Whereas BALB/c mice typically yield 1 x 10^6 AECII/mouse, the C57Bl/6 strain only yields 1 - 3 x 10^5 AECII/mouse. The number of mice used per experiment therefore depends on the mouse strain as well as the type of subsequent analyses to be performed.

Regarding viability of the retrieved AECII, we typically find viability rates of around 90 **%** following flow cytometric cell-sorting. This high proportion of viable cells then allows direct functional analyses as well as short-term culture of the isolated primary murine AECII.

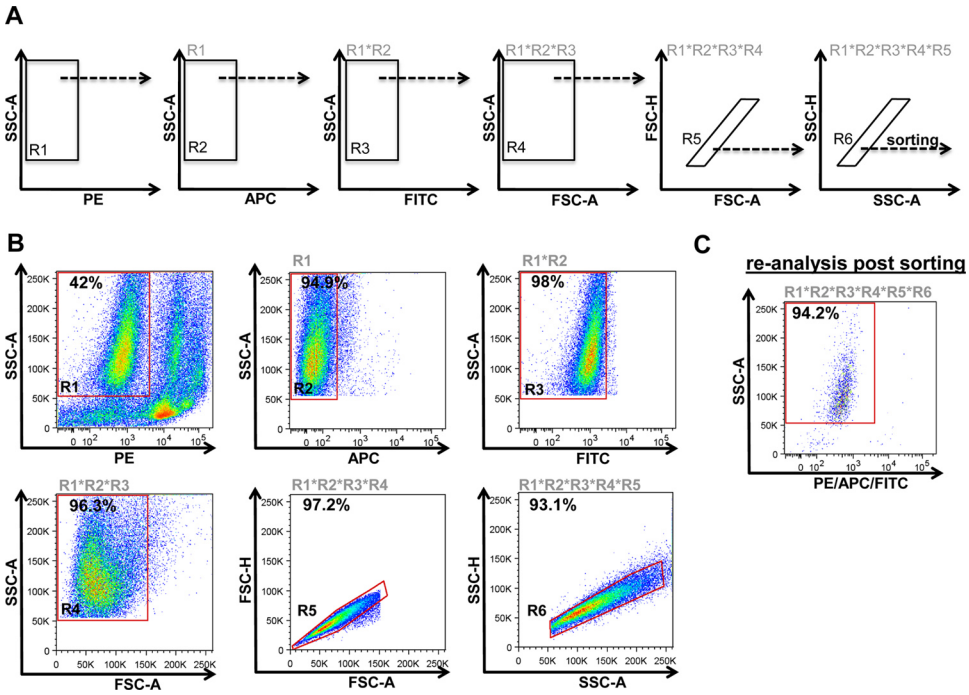


Figure 1. Overview over the gating strategy used for flow cytometric isolation of primary murine AECII by negative selection. (A) Schematic overview over the gating strategy. (B) Representative plots of a lung cell suspension sort. (C) Representative result of a re-analysis of the sorted cells. Click here to view larger figure.

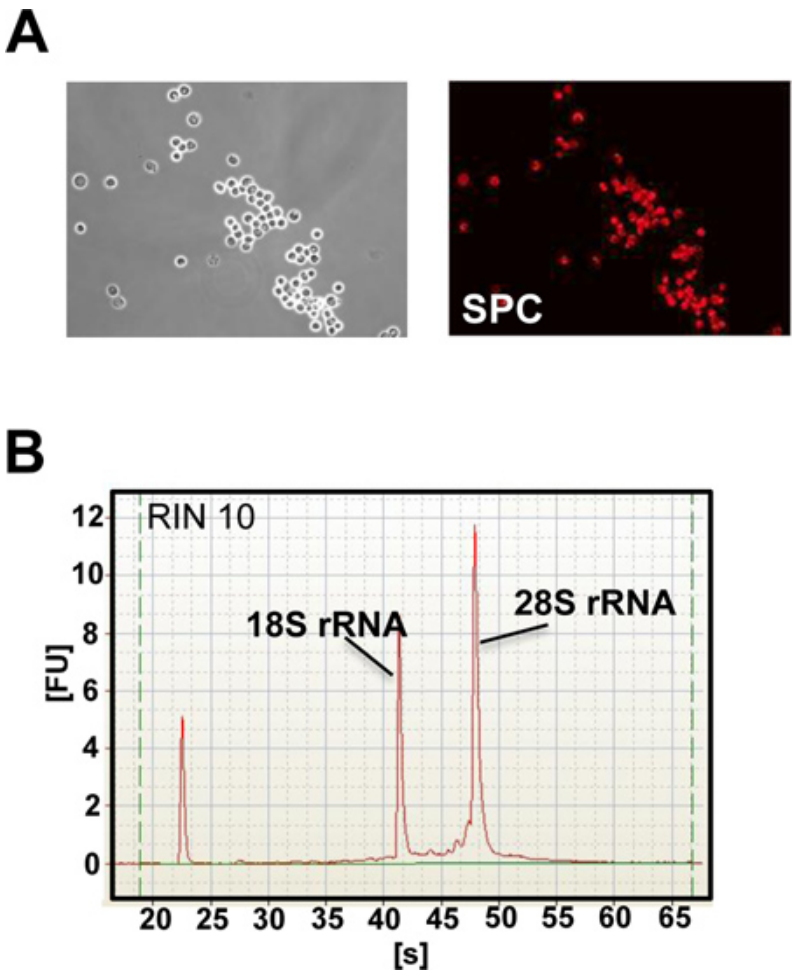
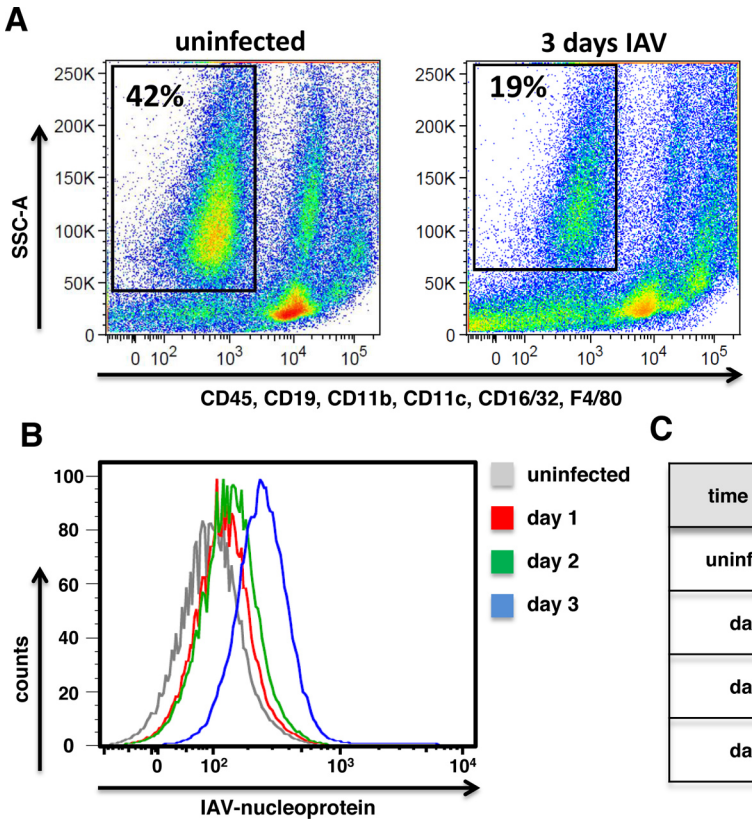


Figure 2. Characterization and molecular analysis of sorted primary murine AECII. (A) Cytospins of sorted AECII were stained for the surfactant protein C (SPC). Compared to phase contrast microscopy, nearly 100 % of cells were found to be SPC positive. (B) Representative RNA profile of sorted primary murine AECII after RNA-isolation. Specific peaks for 18S and 28S ribosomal RNA together with the calculated RIN factor represent RNA integrity and quality for intact isolated RNA from AECII.



C

time point	NP MFI
uninfected	106
day 1	137
day 2	157
day 3	328

Figure 3. Analysis of the influenza A virus nucleoprotein (NP) expression in primary murine AECII isolated from infected mice. C57Bl/6 mice were intranasally administered phosphate buffered saline or influenza A virus PR8/A/34 on days 1, 2 or 3 prior to AECII isolation. (A) Representative plots of lung cell suspensions from an uninfected as well as a three-day infected C57Bl/6 mouse stained for sorting. (B) Influenza A virus NP expression in sorted AECII was analyzed through intracellular staining with an NP-specific antibody and subsequent flow-cytometric analysis. (C) The table shows the mean fluorescence intensity (MFI) of the NP staining.

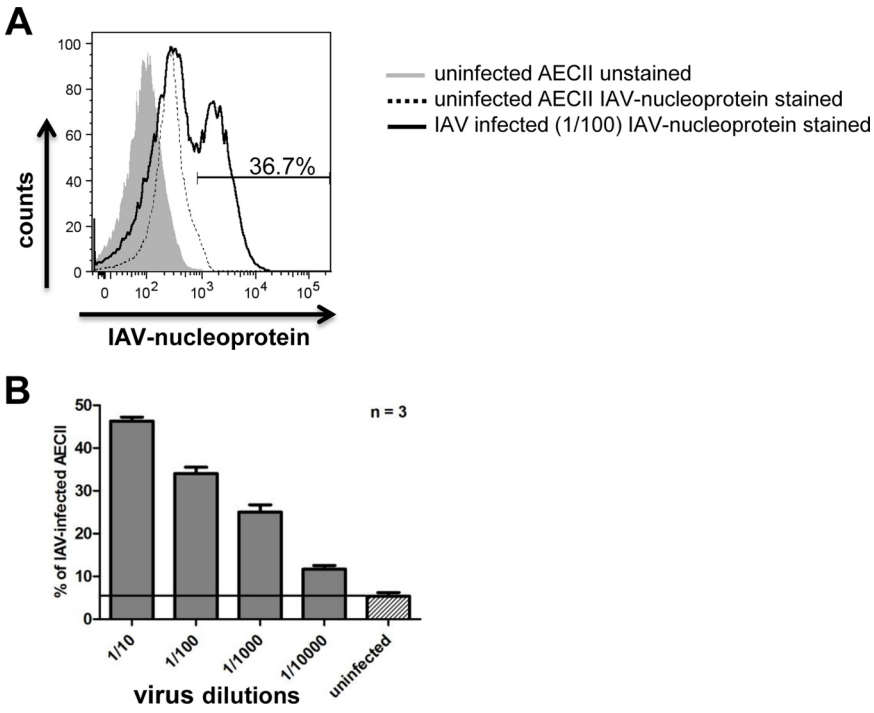


Figure 4. Analysis of the influenza A virus nucleoprotein (NP) expression in *ex vivo* infected primary murine AECII. (A) Representative flow-cytometric analysis of *ex vivo* influenza A virus PR8/A/34 infected AECII by intracellular staining for the influenza A virus NP 6 hr post infection. (B) Summary of representative results from NP-staining of *ex vivo* influenza A virus infected AECII 6 h post infection with different virus dilutions.

Discussion

Our protocol for the isolation of murine AECII by flow cytometry offers a rapid way of accessing primary cells from the mouse lung for a whole range of functional and molecular studies. The described procedure yields highly viable and pure populations of AECII that are sufficient in number for direct subsequent analyses, such as RNA isolation (see **Figure 2b**) and transcriptome studies. For functional applications, it is also possible to culture the isolated cells, allowing *e.g.* the generation of AECII conditioned medium or co-culture experiments. As a benefit especially for these functional studies, the isolation of primary cells by negative selection as described here yields untouched cells which have not been subject to antibody binding. However, despite the advantages of studies in primary AECII over those performed in cell-lines, there are practical limitations to the use of these primary cells. Next to the mere limitation in numbers, which might not meet the requirements of studies requiring extensive screening, primary AECII survive in culture only for a restricted time which we have observed to average around 48 hr. In these short-term culture experiments, we have based the choice of culture medium on the nature of the subsequent assays the AECII conditioned medium was used in and have achieved satisfactory results with IMDM (IMDM Glutamax, Gibco Cat. No. 31980) supplemented with 10 % FCS, standard antibiotics and 0.25 mM β-mercaptoethanol.

The protocol described here displays a relatively easy and rapid way of isolating viable and pure primary murine AECII in good numbers. The purity of the yielded cells after separation critically depends on the procedure of cell-sorting. A clear and strong staining of the cell populations which are to be excluded is equally important as stringent gating on the SSC^{high} events, so that contaminations with lymphoid cells as well type I epithelial cells are minimized. Regarding the yield of separated cells, we have observed that this is maximized by extensive flushing of tubes, filter meshes and plates during preparation of the crude cell suspension as well as by complete disintegration of the tissue following enzymatic digestion. One main benefit of working with primary AECII is that they can be isolated directly from hosts suffering from diseases of the respiratory tract. Such AECII will have been exposed to all factors present in their natural micro-environment and therefore well reflect the *in vivo* situation. Unlike for human AECII [18, 19], this can be extended to a wide range of experimental systems when working in murine models. We have been taking advantage of this in studies regarding autoimmune inflammation as well as viral infection of the lung. Thereby we found that AECII from mice suffering from $CD4^+$ T cell mediated respiratory inflammation express a wide range of inflammatory genes which demonstrates their potential for regulating lung immune responses [3]. Furthermore, we could show that AECII display self-antigens through major histocompatibility complex class-II molecules which results in the functional activation of auto reactive $CD4^+$ T cells. At the same time AECII maintain lung tolerance by secreting factors promoting the induction of Foxp3+ regulatory T cells [4].

Regarding viral infection of the lung, we have successfully isolated primary AECII from mice infected with influenza A virus. As mentioned above, here AECII account for a smaller proportion of the whole lung cell suspension, as considerable numbers of immune cells are recruited to the lungs in response to infection (see **Figure 3a**). Intracellular staining of the influenza A virus nucleoprotein (NP) within the isolated cells shows increasing expression of the viral protein over the course of time as shown in **Figures 3b and 3c**. These AECII, triggered by the virus *in vivo*, display a valuable tool for studies regarding the molecular and functional phenotype of the airway epithelium during respiratory viral infection as well as during recovery. However, as AECII are the primary target of influenza A virus and there is substantial destruction of the epithelial lining,

the rate of cells yielded from infected mice decreases over the course and with severity of the infection. Also, as the virus repeatedly replicates and infects new cells, the isolated AECII have not been subject to infection at a single defined point in time. Thus, these studies are ideally complemented by experiments using *ex vivo* infected primary murine AECII from healthy mice where infection is better synchronized and which, as assessed by intracellular viral NP staining shown in **Figures 4a and 4b**, can yield higher rates of infection depending on the viral dose used.

Taken together, flow cytometric negative selection as described here displays a rapid way of isolating pure and viable primary murine AECII. These cells are suitable for a wide range of analyses and are a valuable tool for extending our knowledge of the role of AECII in immune regulation in the respiratory tract *in vivo*.

Disclosures

No conflicts of interest declared.

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