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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Lung-derived SSEA-1⁺ stem/progenitor cells inhibit allergic airway inflammation in mice

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Abstract

Background: Asthma is characterized by chronic airway inflammation and airway hyperresponsiveness (AHR). Little is known about the role of pulmonary stem/progenitor cells (PSCs) in allergic airway inflammation.

Methods: To identify and investigate the role of PSCs in the bronchial epithelium of neonatal mice, we developed an enzyme-based digestion method to obtain single-cell suspension from lung tissues. Characterization of PSCs was performed using flow cytometry, real-time PCR, immunofluorescence staining, confocal microscopy, and scanning electron microscopy. The effects of SSEA-1⁺ (stage-specific embryonic antigen-1) PSCs was studied in an *in vivo* model of ovalbumin-induced allergic inflammation and an *in vitro* model of cell-based regulation using flow cytometry, real-time PCR, and immune-blotting.

Results: Single-cell suspensions derived from neonatal lung tissue included populations that expressed either SSEA-1⁺ or Sca-1⁺ (stem cell antigen-1). The SSEA-1⁺ PSCs were highly prevalent in neonatal mice, and they were rare in adult mice. Enriched neonatal SSEA-1⁺ PSCs had the ability of self-renewal and differentiated into pneumocytes and tracheal epithelial cells. SSEA-1⁺ PSCs reduced AHR and airway damage in asthmatic mice by decreasing eosinophil infiltration, inhibiting chemokines/cytokines production, and preserving the level of CCSP.

Conclusions: Here, we demonstrated that neonatal SSEA-1⁺ PSCs play an immunomodulatory role in the progression of asthma by reducing lung damage and inhibiting inflammatory responses. Further understanding the molecular mechanisms of neonatal SSEA-1⁺ PSCs might shed light on exploring the novel therapeutic approaches for allergic airway inflammation.

Asthma is a heterogeneous inflammatory disorder that is characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), and excessive airway mucous production. After exposure to allergens, various inflammatory cells infiltrate the airway (1). Eosinophils have been found to be the predominant effector cells for tissue damage and pulmonary dysfunction. The intensity of the pulmonary recruitment of eosinophils is strongly correlated with the severity of AHR (2).

In addition to function as a physical barrier, the airway epithelium plays a critical role in the process of T-helper type 2 (TH2) cell sensitization (3). Allergen-stimulated airway epithelial cells produce thymic stromal lymphopoietin (TSLP),

which attracts and activates the dendritic cells (DCs) to prime naive CD4⁺ T cells to differentiate into TH2 cells (3, 4). Eotaxin secreted by airway epithelial cells is an eosinophil-specific chemokine that plays a critical role in eosinophil recruitment in asthma (5, 6). Clara cell secretory protein (CCSP), which is produced by nonciliated bronchiolar Club cells (also known Clara cells), is critical in inhibiting the differentiation of naive T cells into TH2 cells via the DCs (7). In addition, CCSP not only inhibits the infiltration of inflammatory cells but also directly reduces allergic inflammation in the lung (8). A recent study showed that the LPS and house dust mite allergen-induced Toll-like receptor 4 triggering of lung structural cells was necessary and sufficient for the

development of TH2 immune response and allergic inflammation (9). These observations suggest that lung structural cells not only function as a barrier but also modulate the allergic immune response.

Asthma in most patients is only symptomatically controlled by the available medications (10). Therefore, the possibility that stem/progenitor cells could modulate the immune system has led to an increasing interest in using stem/progenitor cells as a potential therapeutic modality for severe refractory asthma (11). Airway stem cells were first identified in a lung injury model used to demonstrate that bronchiolar stem cells are pollutant-resistant, CCSP-expressing stem cells that contribute to the repair of injured airways (12). Ling et al. reported a serum-free culture system that can support the *in vitro* colony growth of primary neonatal pulmonary epithelial cells expressing octamer-binding transcription factor-4 (Oct-4). These cells express not only Oct-4 but also other markers, such as stage-specific embryonic antigen-1 (SSEA-1) and stem cell antigen-1 (Sca-1) (13). SSEA-1 and Sca-1 are cell markers for induced pluripotent stem cell and hematopoietic stem cell, respectively (14, 15). Previous study indicated that the progenitor epithelial colony cells from the lungs expressed stem cell markers such as Oct-4 and SSEA-1. The colony cells had extensive self-renewal and differentiation potential (16). These studies raised the possibility that pulmonary stem/progenitor cells (PSCs) could regulate immune responses and imply their therapeutic potential for treating asthma.

The aim of this study was to identify the PSCs in the bronchial epithelium of neonatal mice and to investigate their stem cell capacity *in vitro*. We also explored whether neonatal SSEA-1⁺ PSCs could exert a therapeutic benefits in the progression of allergic airway inflammation.

Results

Identification of potential stem/progenitor cells from neonatal mouse lungs

The development of the lungs occurs as a continuous process from embryogenesis to early adolescence in humans as well as mice (17). A stem cell pool present in the neonatal lung contributes to both the bronchiolar and alveolar lineages during lung development (18), whereas these stem cell populations are a rare and quiescent population in the adult lung (19, 20). Previous study showed that Oct-4⁺, SSEA-1⁺, Sca-1⁺, or CCSP-expressing pulmonary stem/progenitor cells undergo terminal differentiation to alveolar pneumocytes (13). Therefore, we speculated that mouse PSCs might reside among the Sca-1⁺, SSEA-1⁺, Oct-4⁺, and CCSP⁺ cells. To test this hypothesis, single-cell suspensions from lung tissues of neonatal and adult mice were prepared. CCSP and Oct-4 are expressed in cytosol and nucleus, respectively. Therefore, we used fluorescence-activated cell sorting (FACS) to analyze the expression pattern of cell surface Sca-1 and SSEA-1. Single cells were identified by forward scatter, and immune cells (CD45⁺) were excluded. FACS analysis showed that Sca-1⁺- and SSEA-1⁺-expressing cell populations were two distinct pulmonary cell populations in neonatal mice (Fig. 1A). To

evaluate the putative stem/progenitor cell population, we analyzed the total cell number of SSEA-1⁺ and Sca-1⁺ cells derived from the lungs of mice of different ages. We found that the number of SSEA-1⁺ cells significantly decreased in an age-dependent manner (Fig. 1B,C). The numbers of SSEA-1⁺ cells at postnatal day 1 and day 7 were $1.7 \pm 0.4 \times 10^5$ and $6.0 \pm 0.8 \times 10^5$, respectively. In contrast, the Sca-1⁺ cell population significantly increased with age. Compare with neonatal lung section, adult SSEA-1⁺ cells were difficult to detect in whole-mount view (Fig. S1). The frequency of lung SSEA-1⁺ cells presented in the adult mice was far fewer than the frequency seen in neonatal mice by whole-mount staining. These data were consistent with the results of Fig. 1. The enlarged immunofluorescence image showed that adult lung SSEA-1⁺ cells were localized in the bronchoalveolar duct junction (BADJ), which was almost the same as neonatal mice (Fig. S2).

Phenotypic characteristics of neonatal SSEA-1⁺ pulmonary cells

To characterize this potential stem/progenitor cell population, we performed an unbiased FACS-based screen of the SSEA-1⁺ pulmonary cells using a collection of monoclonal antibodies directed against cell surface markers. Fluorescence-activated cell sorting analysis showed that the neonatal SSEA-1⁺ pulmonary cells expressed epithelial lineages marker E-cadherin (CD324), while negative for CD31 (endothelial marker), CD34 (hematopoietic stem cell marker), CD90.2, CD73, and CD105 (mesenchymal stem cell; MSC markers) by FACS analysis. In addition, neonatal SSEA-1⁺ pulmonary cells expressed CD9, CD24, CD26, CD29, CD47, CD54, CD98, and CD147 (Fig. 2A). Lung is a complex organ that requires the specification of various epithelial cell types for proper homeostasis. To verify the cell lineage of SSEA-1⁺ cells, we checked the expression of p63 (a basal cell marker), T1 α (a type I pneumocyte marker), surfactant protein C (SPC, a type II pneumocyte marker), and CCSP (a Club cell marker) by FACS analysis, real-time quantitative PCR (RTQ-PCR), and immunoblotting. Interestingly, SSEA-1⁺ pulmonary cells were negative for p63 and T1 α , but positive for SPC (Fig. 2B) and CCSP (Fig. 2C,D). Immunofluorescence staining of whole airway tissue mounts revealed that SSEA-1⁺ cells resided in the bronchioles, terminal bronchioles, and the BADJ in the lungs of neonatal mice (Figs S1 and S2). The bronchoalveolar stem cells (BASCs) were defined as CCSP/SPC dual-positive population at the BADJ as described previously by Kim et al. (21). Therefore, these results raised the possibility that SSEA-1⁺ cells might be multifunctional and comprise the regenerative cell populations within the airway microenvironment.

Neonatal lung SSEA-1⁺ cells possess self-renewal, clonogenicity, and multipotency ability

To test whether pulmonary SSEA-1⁺ cells fulfill the criteria for consideration as stem/progenitor cells, we applied repeated sphere formation assay. Pulmonary SSEA-1⁺ single

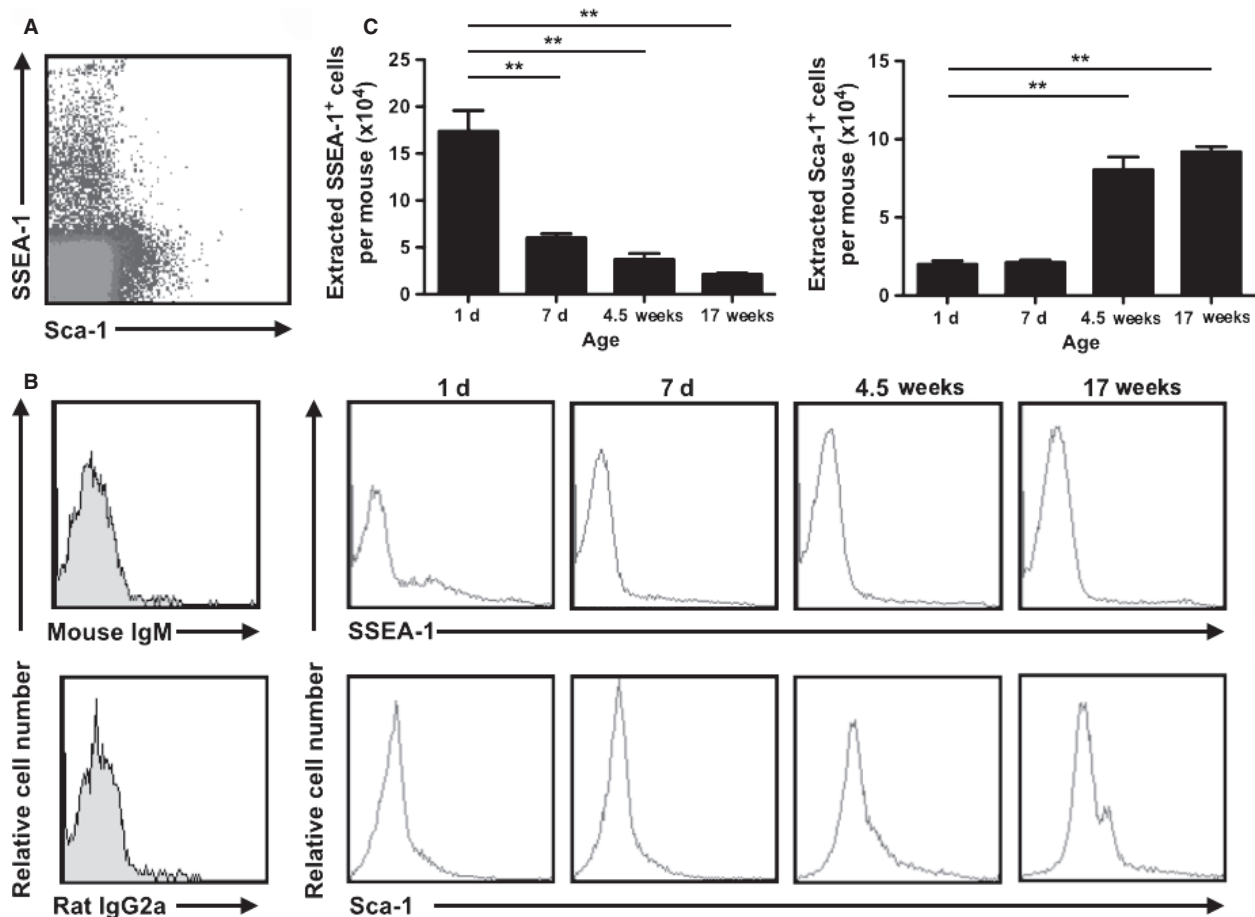


Figure 1 Identification of neonatal pulmonary stem/progenitor cells. (A) Neonatal pulmonary cells were labeled with Sca-1 and SSEA-1 antibodies and analyzed using flow cytometry. The data are representative of five independent experiments. (B) Representative flow cytometry histograms of SSEA-1 and Sca-1-stained cell popu-

lation in the lung single-cell suspension. (C) Cell number of SSEA-1⁺ and Sca-1⁺-enriched pulmonary cell extracted from whole lung/mouse ($n = 3-5$ mice per group). Data are means $\pm$ SD and representative of three independent experiments. $^{**}P < 0.01$.

cells were resuspended in Matrigel-based three-dimensional culture. We found that primary sphere colonies were observed 10–15 days after cell culture (Fig. 3A left). To further clarify the self-renewal capacity of pulmonary SSEA-1⁺ cells, primary spheres were subsequently dissociated to single cell and then resuspended to Matrigel-based three-dimensional culture. The formation of secondary spheres was observed after culturing for 7–10 days (Fig. 3A right). Sphere colony assay showed that SSEA-1⁺ cells exert higher sphere-forming ability than SSEA-1⁻ cells. The sphere formation efficiency of SSEA-1⁺ cells was 1–2 spheres/2500–5000 total cells, as determined by limiting dilution assay (Fig. 3B). These results indicated that SSEA-1⁺ pulmonary cells might expand through self-renewal. Although SSEA-1⁺ pulmonary cells only expressed SPC (type II pneumocyte marker) when initially isolated from neonatal mice (Fig. 2B), after culturing on Matrigel-coated plate for 15–20 days, the SSEA-1⁺ pulmonary cells differentiated into pro-surfactant protein C⁺ type II pneumocytes and AQP5⁺ type I pneumocytes (Fig. 3C). In addition, we investigate that whether SSEA-1⁺

pulmonary cells have the capacity to differentiate into tracheal epithelial cell (TEC) because SSEA-1⁺ cells were located at the BADJ in the lungs of neonatal mice (Figs S1 and S2). Immunofluorescence staining of tight junction marker ZO-1 and centrosome marker γ -tubulin showed that SSEA-1⁺ pulmonary cells differentiated into both ciliated and nonciliated cells 15 days after grown in air–liquid interface (ALI) cultures (Fig. 3D). Scanning electron microscopy (SEM) also showed the similar results (Fig. 3E). These observations suggested that neonatal SSEA-1⁺ pulmonary cells had the capability to differentiate into both pneumocytic and TEC lineages. Based on these *in vitro* studies, we suggested that neonatal SSEA-1⁺ pulmonary cells are stem/progenitor cells with self-renewing, clonogenic, and multipotent properties.

SSEA-1⁺ PSCs reduced TSLP and eotaxin production

Previous study (22) showed that human lung stem cells repair damaged mouse lung *in vivo*, indicating PSCs might play a

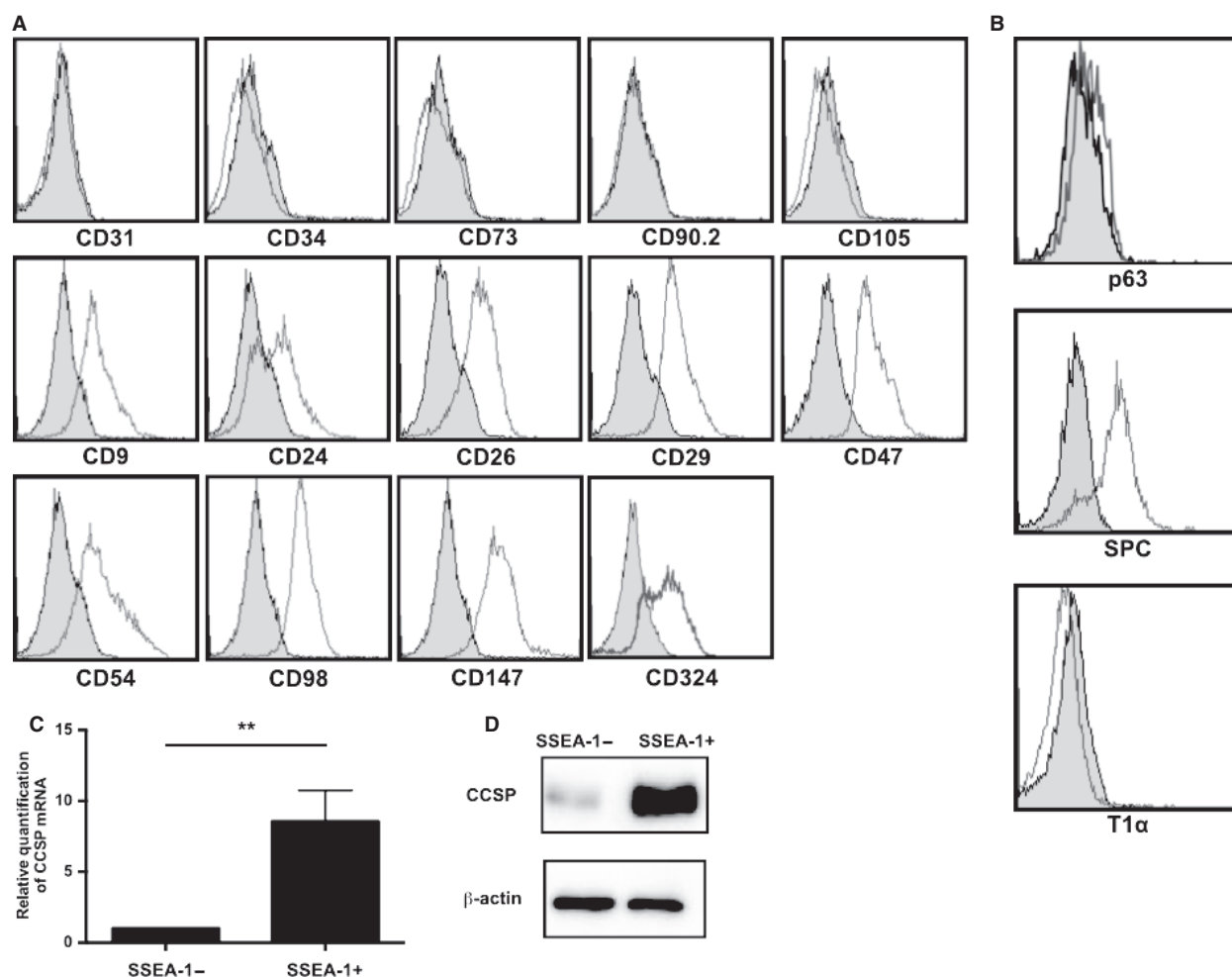


Figure 2 Characterization of neonatal SSEA-1⁺ pulmonary cells. (A) Flow cytometric analysis of surface marker expression in neonatal SSEA-1⁺ pulmonary cells. The gray areas represent matched isotype controls. Data are representative of three independent experiments. (B) Fluorescence-activated cell sorting analysis of intracellular surfactant protein C (SPC) and surface T1α expression in SSEA-1⁺ pulmonary cells. SSEA-1⁺ pulmonary cells expressed SPC (a type II pneumocyte marker) but were negative for T1α (a type I pneumocyte marker). Data are representative of two independent experiments. (C) Enriched SSEA-1⁺

pulmonary cells were purified from neonatal mice, and the Clara cell secretory protein (CCSP) expression levels were determined using RTQ-PCR with specific primers. The mRNA levels were normalized using the housekeeping gene GAPDH. Bars indicate mean $\pm$ SD of three independent experiments. $**P < 0.01$. (D) Lysates of enriched neonatal SSEA-1⁺ pulmonary cells were prepared, and the CCSP protein levels were determined from immunoblots stained with a specific antibody against CCSP. β -actin was used as the internal control. Data are representative of two independent experiments.

protecting role in lung damage. However, whether PSCs play a critical role in the process of inflammation is not well understood. To explore the biological functions of neonatal SSEA-1⁺ PSCs, we developed an adult lung epithelial cells and neonatal SSEA-1⁺ PSCs coculture system in the presence of stimulators. As TLR4 ligation on airway epithelial cells induces the release of innate cytokines including TSLP, which promote the development of pathogenic TH2 cells and asthmatic inflammation (9). In addition, IL-4 plays a critical role in the differentiation of TH2 cells and induces inflammation through stimulating the expression of eotaxin from lung epithelial cells (23). Therefore, we used TLR4 ligand-LPS and IL-4 to stimulate airway epithelial cells to produce TSLP

and eotaxin, respectively. ELISA measurements of cell culture supernatant indicated that the primary lung epithelial cells produced high levels of TSLP and eotaxin upon LPS and IL-4 stimulation, respectively. However, the neonatal SSEA-1⁺ PSCs inhibited LPS-induced TSLP and IL-4-induced eotaxin production (Fig. 4A). To clarify whether the neonatal SSEA-1⁺ PSC-mediated inhibitory effect was dependent on soluble or cell-cell contact-dependent factors, coculture of neonatal SSEA-1⁺ PSCs and adult lung epithelial cells was physically separated by a Transwell insert, and it found that neonatal SSEA-1⁺ PSCs suppressed LPS- and IL-4-induced TSLP and eotaxin production (Fig. 4B). These results indicated that the SSEA-1⁺ PSC-mediated inhibitory

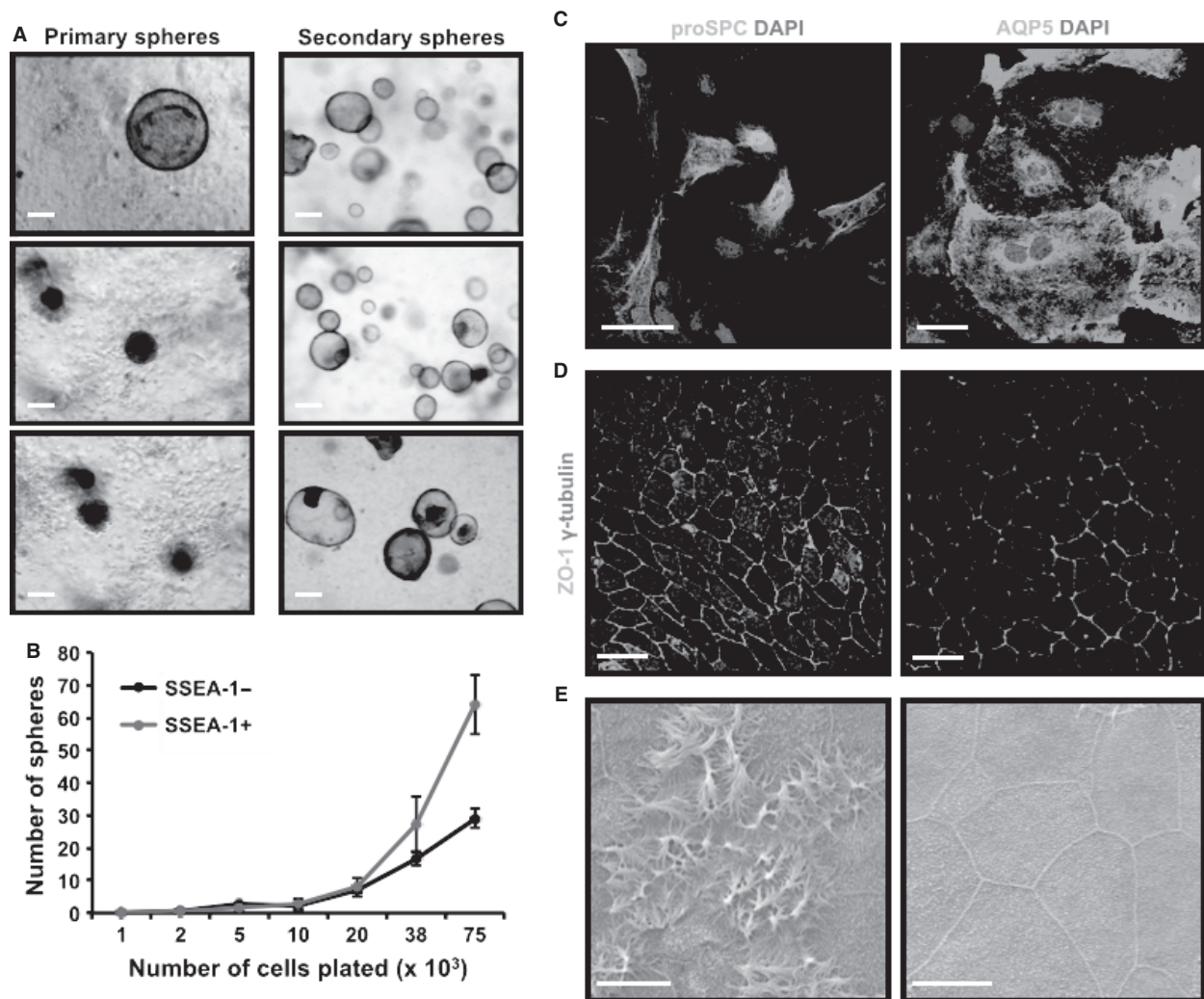


Figure 3 Neonatal SSEA-1⁺ pulmonary cells are self-renewal and multipotent. (A) Representative photographs of primary and secondary sphere colonies. Bars, 100 μ m. (B) Efficiency of secondary spheres formation. The results are expressed as means $\pm$ SD ($n = 3$). (C) SSEA-1⁺ pulmonary cells cultured on Matrigel-coated plate for 15–20 days were stained with pro-surfactant protein C (proSPC; green; left) and AQP5 (green; right)

antibody to identify the type I pneumocyte; the nuclei were counterstained with DAPI (blue). Bars, 20 μ m. (D) SSEA-1⁺ pulmonary cells seeded in an ALI culture for 15 days were labeled for ZO-1 (proSPC; green), γ -tubulin (red), and DAPI (blue). Bars, 20 μ m. (E) Scanning electron microscopic images of *in vitro*-cultured SSEA-1⁺ pulmonary cells at ALI for 15 days, showing ciliated and nonciliated cells. Bar, 10 μ m.

effect was mainly dependent on soluble factors. To clarify the mechanism of inhibition of inflammation and airway damage by SSEA-1⁺ PSCs, we tested whether SSEA-1⁺ PSCs could inhibit TSLP and eotaxin production in the presence of CCSP neutralization antibody. We found that anti-CCSP antibody restored SSEA-1⁺ PSC-induced TSLP but not eotaxin, suggesting that CCSP might not be the predominant pathway for eotaxin inhibition (Fig. S3).

Transplantation of SSEA-1⁺ PSCs preserved the epithelium and alleviated the severity of asthmatic features

Neonatal SSEA-1⁺ PSCs inhibited TSLP and eotaxin production; therefore, we hypothesized that transplantation of

neonatal SSEA-1⁺ PSCs into asthmatic mice might have therapeutic potential. Both SSEA-1-positive and SSEA-1-negative fractions isolated from neonatal mice were collected and used for further adoptive transfer studies. To provide a precise area of their niche that would permit the stem/progenitor cells to survive and to investigate the anti-inflammatory effects of this stem/progenitor cell population, SSEA-1⁺ PSCs were intravenously delivered into mice after the second ovalbumin (OVA) aerosol exposure (Fig. 5A). We used pulmonary cells isolated from enhanced GFP transgenic (EGFP-tg) mice to monitor the localization of the SSEA-1⁺ PSCs in the recipient animals. Six days after transfer, anti-GFP-labeled SSEA-1⁺ PSCs were detectable in the lung tissues (Fig. S4A,B). The SSEA-1⁺ PSCs transplant did not change

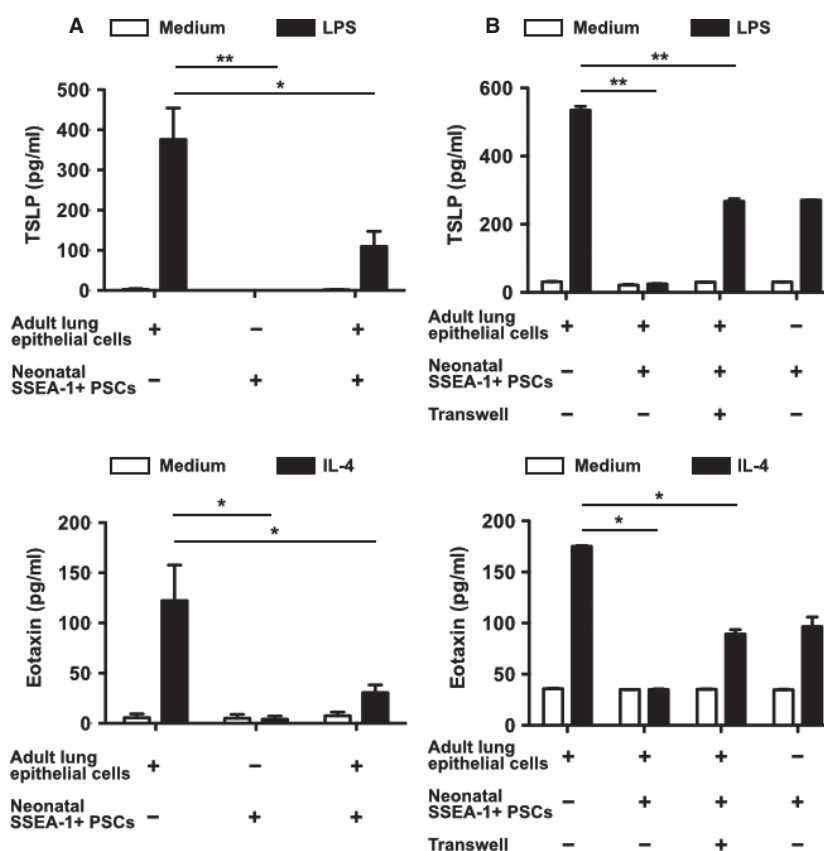


Figure 4 SSEA-1⁺ pulmonary stem/progenitor cells (PSCs) suppress thymic stromal lymphopoietin (TSLP) and eotaxin production. (A) Lung epithelial cells derived from adult mice were cocultured with neonatal SSEA-1⁺ PSCs in the presence of LPS or IL-4 for 24 h. TSLP and eotaxin contents in LPS- and IL-4-stimulated culture

supernatants were determined using ELISA, respectively. (B) Co-culture system of adult lung epithelial cells and neonatal SSEA-1⁺ pulmonary cells was separated with a Transwell. Data are means $\pm$ SD and representative of three independent experiments. * P < 0.05, ** P < 0.01.

the anti-OVA IgE titer (Fig. S4C). Notably, SSEA-1⁺ PSCs significantly suppressed the invasive AHR to methacholine (Fig. 5B) and decreased the infiltration of inflammatory cells into peribronchovascular areas in the OVA-induced asthmatic mice (Fig. 5C and Fig. S5). The total cell counts and the proportion of eosinophils in BAL fluid were significantly decreased in the SSEA-1⁺ PSC-treated group compared with untreated group (Fig. 6A,B). ELISA showed that neonatal SSEA-1⁺ PSCs treatment significantly inhibited the secretion of eotaxin, TSLP, IL-4, IL-5, and IL-13 in the BAL fluid of OVA-induced asthmatic mice (Fig. 6C). The levels of CCSP in both the BAL fluid and blood serum were lower in patients with asthma compared with healthy controls (24, 25). Reduction of CCSP expression in bronchiolar epithelium is associated with epithelial cell damage (26). Immunofluorescence staining showed that CCSP was weakly expressed in asthmatic lung tissue compared with healthy lung tissue (Fig. 6D). In the asthmatic model, CCSP was strongly expressed in the SSEA-1⁺ PSCs recipients (Fig. 6D). Therefore, transplantation of neonatal SSEA-1⁺ PSCs might preserve airway CCSP secretion and reduce bronchiolar epithelium damage.

Discussion

In this study, we found that mouse neonatal SSEA-1⁺ pulmonary cells were stem/progenitor cells capable of self-renewal and differentiating into pneumocytes and tracheal epithelial cells. Neonatal SSEA-1⁺ PSCs highly expressed CCSP and inhibited LPS-induced TSLP and IL-4-induced eotaxin production in primary lung epithelial cells. Transplantation of neonatal SSEA-1⁺ PSCs reduced AHR and suppressed airway damage in OVA-induced asthmatic mice, which might be associated by preserving the level of CCSP; decreasing the infiltration of eosinophils; and inhibiting the production of IL-4, IL-5, IL-13, eotaxin, and TSLP. We have demonstrated that neonatal SSEA-1⁺ PSCs play an immunomodulatory role in the progression of asthma by inhibiting allergen-induced inflammatory responses.

It is suggested that lung tissue comprises multiple spatially and temporally restricted stem or progenitor cell lineages that have varying abilities to respond to injury and disease (27). We found that neonatal SSEA-1⁺ PSCs were located in the bronchioles, terminal bronchioles, and BADJ in neonatal

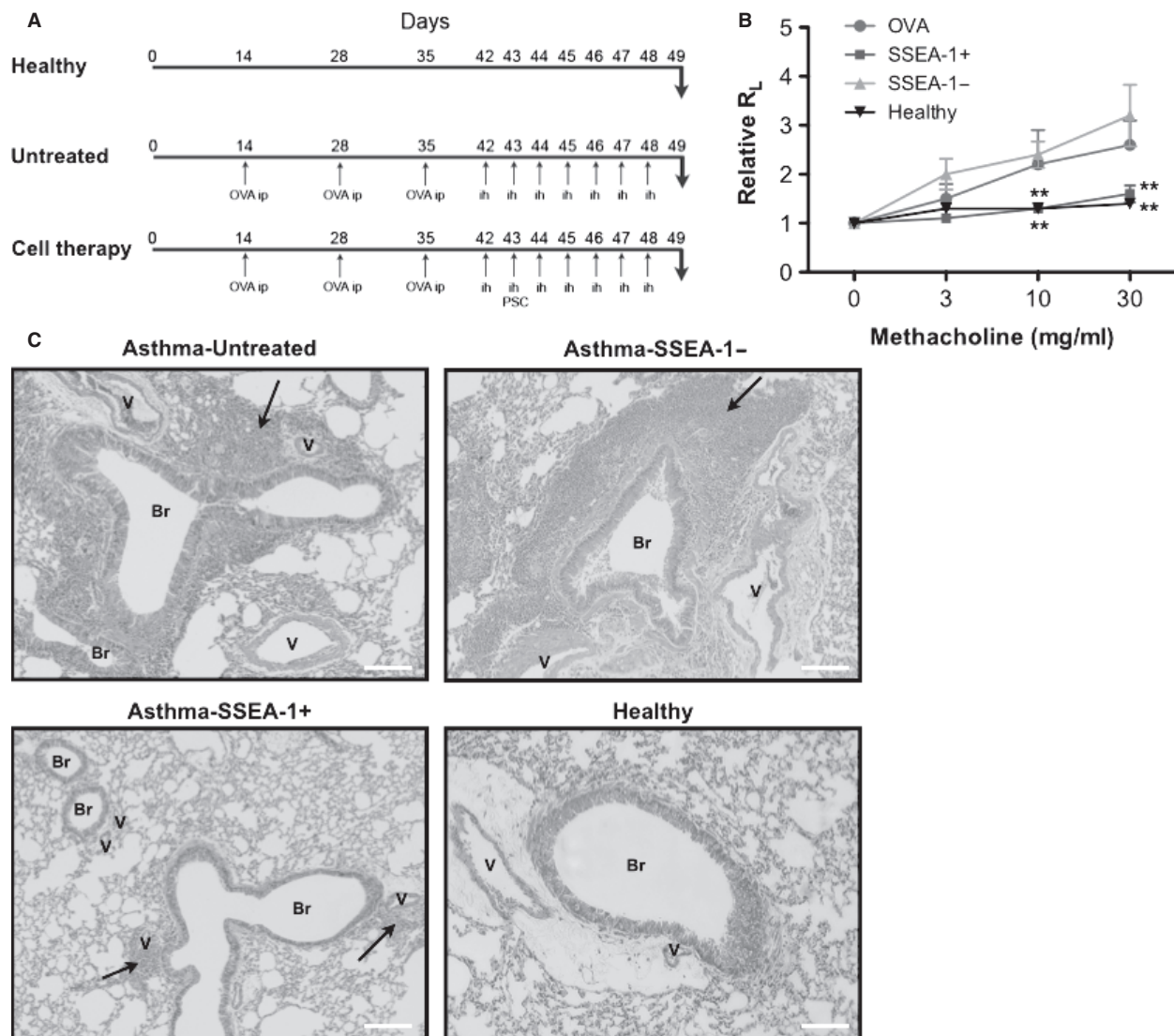


Figure 5 Transplantation of SSEA-1⁺ pulmonary stem/progenitor cells (PSCs) improves airway hyper-responsiveness in the asthmatic mice. (A) Flowchart of the method used to produce the ovalbumin-induced asthmatic murine model. (B) Airway function was measured by invasive body plethysmography. The results are expressed as means $\pm$ SEM of the lung resistance (RL) in the ratio

of RL after PBS nebulization. Data are representative of two independent experiments ($n = 4-7$ mice per group). ** $P < 0.01$. (C) H&E staining of lung sections in untreated, SSEA-1⁺ PSC-, SSEA-1⁻ pulmonary cells-treated, and healthy group. Bars, 200 μ m. Arrows denote infiltrated leukocytes; br, bronchus; v, vessel. Data are representative of two independent experiments.

lung tissues. Moreover, neonatal SSEA-1⁺ PSCs were able to self-renew, proliferate, and differentiate into pneumocytes, and ciliated and nonciliated TECs. Based on these findings, it is probable that SSEA-1⁺ cells might serve as progenitor/stem cells for all differentiated airway epithelial cells. In adult mice, BASCs were identified at the BADJ within the terminal bronchioles by expressing CCSP, SPC, and Sca-1(21). However, neonatal SSEA-1⁺ PSCs expressed CCSP and SPC, but not Sca-1; therefore, whether these two cell populations share similar functional therapeutic benefit remains further exploration. Moreover, SSEA-1⁺ PSCs were found in the bronchioles, terminal bronchioles, and BADJ in neonatal lung tissues,

but were located in the deep parenchyma after transplantation into asthmatic mice. In this model, we transplanted SSEA-1⁺ PSCs into asthmatic mice by intravenously injection, which suggested that SSEA-1⁺ PSCs should be localized inside lung parenchyma. However, whether SSEA-1⁺ PSCs migrate and localize in the parenchyma and execute their anti-inflammation or repair ability is still unknown.

Recent studies demonstrated that c-kit⁺ human lung stem cells constitute an average of 0.0042% and 0.0244% of the lung cells of the adult and fetus, respectively (22), mouse Oct-4-expressing stem/progenitor cells comprise 0.0016–0.0022% of the neonatal lung cells (13), and BASCs

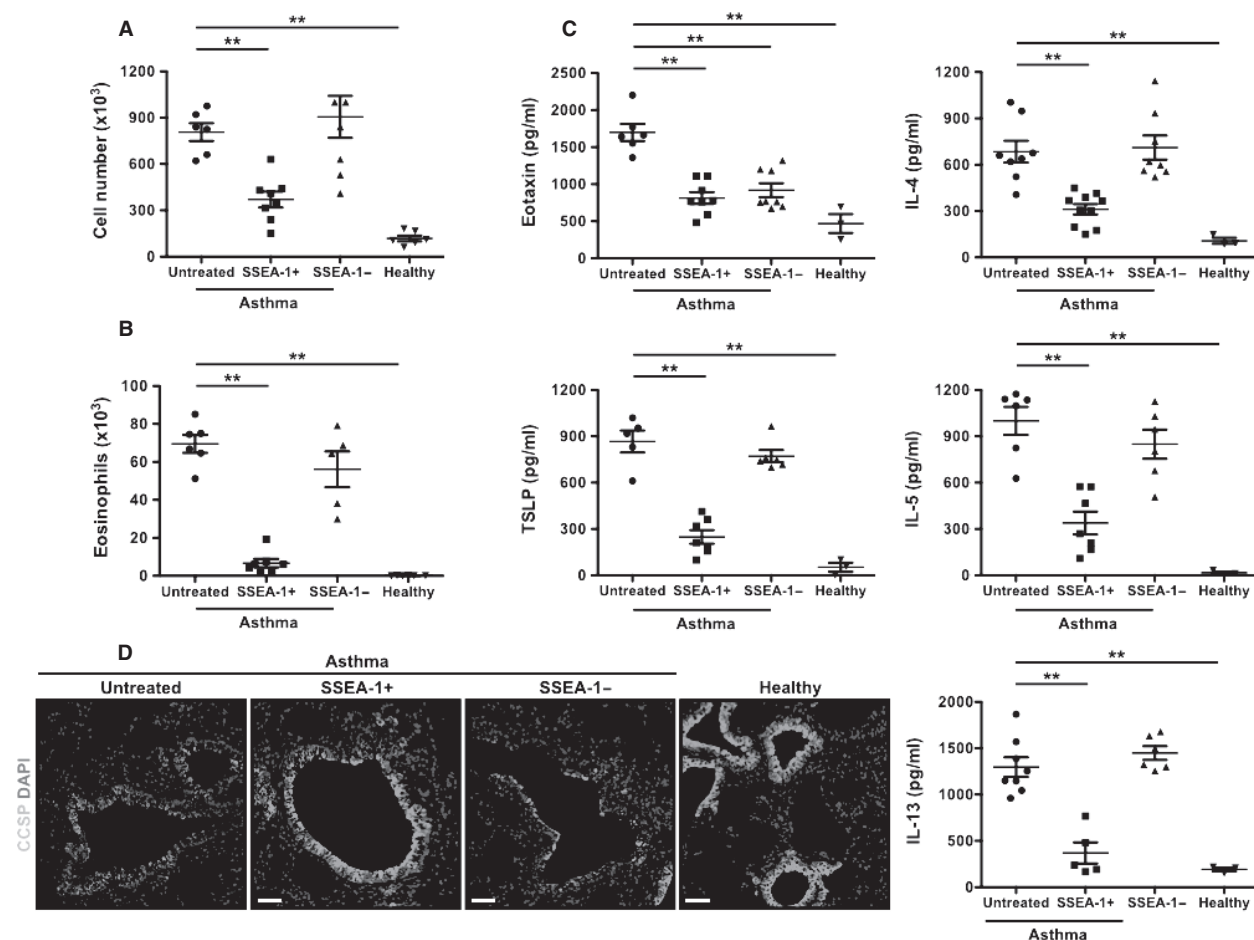


Figure 6 Transplantation of SSEA-1⁺ pulmonary stem/progenitor cells (PSCs) reduces airway inflammation and preserves epithelium stability. BAL fluid was taken 24 h after the last aerosol ovalbumin exposure. (A) Cell number in the recovered BAL fluid was counted after staining by trypan blue. (B) Eosinophil counts in the BAL fluid were obtained by fluorescence-activated cell sorting analysis. Data are means $\pm$ SEM and representative of two independent experiments ($n = 6-8$ mice per group). $**P < 0.01$. (C) The level of eotaxin, thymic stromal lymphopoietin (TSLP), IL-4, IL-5, and IL-13 in

BAL fluid was determined using specific ELISA kits. Data are means $\pm$ SEM and representative of two independent experiments ($n = 4-6$ mice per group). $**P < 0.01$. (D) The expression of Clara cell secretory protein (CCSP) in lung sections from untreated, SSEA-1⁺ PSC-, SSEA-1⁻ pulmonary cells-treated, and healthy mice was analyzed using immunostaining with an anti-CCSP antibody (green); the nuclei were counterstained with DAPI (blue). Data are representative of two independent experiments. Bars, 50 μ m.

represent 0.4% of the total lung cell preparations from adult mice (21). Morphometric analysis of total lung sections showed that SSEA-1⁺ PSCs constituted an average of $0.138 \pm 0.036\%$ of the lung cells in the neonatal mice that were used in our study. The reasons underlying the discrepancy of the size of stem/progenitor cell population between laboratories may be the differences in the species and age of the mice, the surface markers used, and the methodologies employed for sampling and analysis. In this study, the SSEA-1⁺ PSCs in the neonatal airways were found to represent approximately 2.5% of the pulmonary cell suspensions. These data indicated that the enzyme-based digestion method produced a high yield of stem/progenitor cells.

Mesenchymal stem cells (MSCs) have been shown to improve inflammation in a variety of disease models

including protection airway from allergen-induced pathology (28–30). Although MSCs could likely modulate an inflammatory microenvironment, various studies also indicated that MSCs promote fibrogenesis by directly differentiating to myofibroblasts and contribute to disease progression (31, 32). Most basic biological studies indicate that MSCs function as bone marrow stromal cells, modulating the marrow microenvironment and serving as precursors to differentiated skeletal lineages. Therefore, injection of MSCs into lung tissue is more likely an artificial therapy rather than an augmentation of naturally occurring mechanisms for lung repair (27). In this study, we identified that SSEA-1⁺ PSCs existed in lung and specifically differentiated into lung epithelial lineage; hence, it might have advantages over MSCs.

IL-5, eotaxin, and TSLP, secreted by airway epithelial cells, are critical for maintaining asthmatic inflammation and stimulating TH2 polarization (33). TSLP receptor-deficient mice fail to develop an inflammatory lung response to inhaled antigen indicated that TSLP plays a critical role in the development of inflammatory and/or allergic responses in the asthma model (34). Lung-specific overexpression of TSLP induces hyper-reactivity and TH2-related inflammation (35). TSLP induces the proliferation of bronchial epithelial cells and bronchial repair through regulating the production of IL-13(36). We found that the presence of SSEA-1⁺ PSCs was associated with reduced TSLP secretion *in vivo* and that SSEA-1⁺ PSCs inhibited TSLP and eotaxin production in activated airway epithelial cells *in vitro*. These results suggested that SSEA-1⁺ PSCs might decrease inflammation-induced epithelial damage and stabilize epithelium through interaction with lung structural cells to inhibit TSLP and eotaxin production, and suppress the infiltration of inflammatory cells. Moreover, SSEA-1⁺ PSCs inhibited the production of eotaxin and IL-5 in the airway epithelium and reduced the infiltration and activation of eosinophils. These data might explain the suppression of AHR in the SSEA-1⁺ PSCs recipient group.

Previous study indicated that CCSP suppressed allergen-induced inflammatory responses *in vivo* (8). We found that anti-CCSP antibody restored SSEA-1⁺ PSC-induced TSLP, but not eotaxin, suggesting that CCSP might not be the predominant pathway for eotaxin inhibition. Therefore, SSEA-1⁺ PSCs might inhibit the airway epithelial cells to produce inflammatory cytokines/chemokines through at least two different mechanisms. In this study, we reported that SSEA-1 was used as a marker for pulmonary stem cells and benefit for airway inflammation inhibition in asthmatic mice. Therefore, we suggested that lung SSEA-1⁺ cells might used to be a potentially useful airway inflammatory biomarker in diagnosis and management of asthma.

In summary, our findings provide evidence that neonatal SSEA-1⁺ PSCs can self-renew and differentiate into pneumocytes and TECs. SSEA-1⁺ PSCs inhibit TSLP and eotaxin production from primary lung epithelial cells. We also identified SSEA-1⁺ PSCs play an immunomodulatory role in the pathogenesis of asthma. Transplantation of neonatal SSEA-1⁺ PSCs ameliorated the allergen-induced airway inflammation and airway damage in the asthmatic mouse model. We therefore conclude that using SSEA-1⁺ PSCs to improve AHR, reduce lung damage, and inhibit inflammatory responses might shed light on exploring a potential therapeutic strategy for treating asthma or lung injury in the future.

Materials and methods

OVA-alum of asthma model

BALB/c mice were sensitized to OVA via intraperitoneal (i.p.) injection of OVA with aluminum hydroxide. See Materials and Methods SI for more details.

Preparation of pulmonary single-cell suspension and enrichment of SSEA-1⁺ PSCs

Pulmonary single-cell suspension was generated by enzyme-based digestion method. SSEA-1⁺ PSCs were enriched from pulmonary single-cell suspension using specific antibody conjugated with magnetic beads. See Materials and Methods SI for more details.

Statistical analysis

All statistical analyses were performed with Prism 6.0 (GraphPad Software, San Diego, CA) software. Dual comparisons were made with Student's *t*-test. Groups of three or more were analyzed by ANOVA with Dunnett's post-tests for experiments comparing treatments to controls. *P* values < 0.05 were considered significant.

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Author Contributions

C.-J.C. performed all the experiments, analyzed data, and wrote the manuscript. T.-Y.L. provided the advice in the design of the *in vitro* experiments. B.-L.C. designed the experiments, wrote the manuscript, and coordinated and directed the project.

Conflicts of interest

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

Figure S1. Whole-mount view of SSEA-1⁺ pulmonary cells in lung tissues.

Figure S2. Expression of SSEA-1⁺ pulmonary cells in lung tissues.

Figure S3. Neonatal lung SSEA-1⁺ PSCs reduced TSLP and eotaxin production from adult lung epithelial cells through CCSP-dependent and -independent manner.

Figure S4. Transplantation of SSEA-1⁺ PSCs did not change serum OVA-specific IgE in the asthmatic mice.

Figure S5. Transplantation of SSEA-1⁺ PSCs decreased the infiltration of inflammatory cells into lung in the asthmatic mice

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Lung-derived SSEA-1⁺ stem/progenitor cells inhibit allergic airway inflammation in mice

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Supporting Information

SI Material s and Methods

Animals. BALB/c mice (6-8 weeks old) were purchased from the National Laboratory Animal Center (Taiwan) and maintained in the Animal Center of the College of Medicine, National Taiwan University. EGFP-tg mice with a BALB/c strain background were established at the National Laboratory Animal Center (Taiwan). Foxp3-GFP reporter mice on BALB/c strain background were obtained from The Jackson Laboratory (USA). Animal care and handling protocols were approved by the Animal Welfare Committee of National Taiwan University.

OVA-alum of asthma model. Mice were sensitized to ovalbumin (OVA; Sigma-Aldrich) via intraperitoneal (i.p.) injection of 100 µg of OVA with 4 mg of aluminum hydroxide (Pierce Chemical) on day 0. On days 14 and 28, the mice were boosted with 50 µg of OVA and 4 mg of aluminum hydroxide. On days 42-48, the mice were challenged for 30 min with an aerosol of 1% OVA in PBS delivered from a jet nebulizer. One day after the first OVA challenge, groups of mice received intravenous (i.v.) injection of SSEA-1⁺ PSCs (1×10^5 cells) and positive control mice were administered PBS in a volume of 100 µl. Healthy mice did not receive the OVA challenge. On day 49, lung resistance (RL) in response to increasing doses of aerosolized methacholine (Sigma) in anesthetized mice was measured by using a computer-controlled small animal ventilator (Harvard Rodent Ventilator, model 683; Harvard Bio-Science). Bronchiolar lavage (BAL) was performed with 1 ml washes of Ca²⁺- and Mg²⁺-free HBSS (Sigma-Aldrich), followed by lung resection. The lung tissue was immediately fixed with 4% paraformaldehyde in PBS. Paraffin-embedded tissue was cut into 5-µm sections for routine H&E staining. The cells in the BAL fluid were counted to determine their total number. Differential cell counts were performed by FACS analysis (1) or by examining at

least 400 cells of cytocentrifuged preparations (Shandon) stained with Liu's stain and categorizing them using standard morphological criteria. The results are expressed as the percentage of the total counted cells.

Preparation of pulmonary single-cell suspension and enrichment of SSEA-1⁺ PSCs. The lung tissues were washed with PBS and cut into small pieces and then digested in MEM (Sigma-Aldrich) in the presence of 1 mg/ml of protease-type XIV (Sigma-Aldrich) and 0.5 mg/ml of DNase I (Invitrogen) for 18 hours at 4°C. PBS containing 5% fetal bovine serum (Hyclone) was added to neutralize the protease activity, and pipetting was performed to obtain a pulmonary single-cell suspension. The cell extract was filtered through a 100-μm nylon mesh (BD Biosciences) to remove debris. The cell suspension was washed and re-suspended in PBS containing 0.5% BSA and EDTA. Thereafter, the single-cell suspensions were incubated with anti-SSEA-1 Microbeads (Miltenyi Biotec), and the SSEA-1⁺ cells were enriched using a magnetic separator. The purity of the SSEA-1⁺ PSCs was greater than 90%, as determined by FACS analysis. For the *in vivo* adoptive transfer experiments, 1×10^5 SSEA-1⁺ enriched cells were resuspended in 100 μl of PBS and i.v. injected into each mouse.

Spheres-forming assay. 1×10^5 SSEA-1⁺ PSCs derived from neonatal lungs were suspended in 50% growth factor-reduced Matrigel (BD Biosciences) with MCDB201 medium (Sigma-Aldrich) supplemented with 15% FBS, Insulin-Transferrin-Selenium (Gibco), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 25 ng/ml of epidermal growth factor (BD Biosciences). The medium was replaced daily, and the primary sphere colonies were generated obviously after 2 weeks. To test the self-renew and clonogenicity of SSEA-1⁺ PSCs, primary spheres were dissociated into single cell suspension by digestion with Dispase (BD Biosciences).

Briefly, each single sphere was collected into 10 units (0.2 ml) of dispase and incubated at room temperature for 30 minutes. Pipetted mixture up and down to disperse cell suspension and then re-suspended in 50% Matrigel for secondary spheres formation.

Induction of multipotent differentiation *in vitro*. A previous study reported that Matrigel, an extracellular matrix, enhanced the differentiation and maintenance of embryonic stem cells as SPC-expressing type II pneumocytes(2). To induce the differentiation of type I and type II pneumocytes, 3×10^5 SSEA-1⁺ PSCs derived from neonatal lungs were seeded on 10% growth factor-reduced Matrigel (BD Biosciences) in MCDB201 medium (Sigma-Aldrich) supplemented with 10% FBS, Insulin-Transferrin-Selenium (ITS; Gibco), 100 U/ml of penicillin, 100 µg/ml of streptomycin, 10 ng/ml of epidermal growth factor (EGF; BD Biosciences), 10 ng/ml FGF-10 (Biolegend), 100 ng/ml Wnt3a (R&D Systems), 10 ng/ml KGF (Biolegend), and 0.5 µM retinoic acid (RA; Sigma-Aldrich) for 15-20 days. The medium was replaced daily, and the cells were monitored by microscopy. To induce the differentiation of tracheal epithelial cells, SSEA-1⁺ PSCs were seeded on collagen I (BD Biosciences)-coated Transwell membranes (Corning) as previously described (3, 4). Briefly, 3×10^5 SSEA-1⁺ PSCs were maintained in in MCDB201 medium supplemented with 5% FBS, ITS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 25 ng/ml of EGF, 0.1 µg/ml of cholera toxin, 30 µg/ml of bovine pituitary extract (BD Biosciences), and freshly added 0.5 µM RA for 15-20 days, with daily medium changes. As cells reached 100% confluent, cells in the Transwell were then maintained at ALI by changing the medium in the basal chamber every other day, and leaving the apical chamber empty for another 10-15 days.

Real-time quantitative-polymerase chain reaction (RTQ-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized with random hexamers using MMLV reverse transcriptase (Clontech). TaqMan quantitative PCR with gene-specific probes (Applied Biosystems) was performed using the 7500 FAST Real-Time PCR System (Applied Biosystems) according to standard procedures. GAPDH was used as an endogenous control. The amount of each gene product was divided by the amount of GAPDH to obtain a normalized value.

Histology and immunohistochemistry (IHC). Lung tissues were dissected and fixed in 4% paraformaldehyde in PBS and processed into serial paraffin sections using standard procedures. In briefly, the sections were blocked with antibody diluent (Dako) for 1 hour at room temperature. The sections were then incubated with primary antibodies at 4°C overnight. Antibodies against the following proteins were used: γ -tubulin (1:5000; clone GTU-88; Sigma-Aldrich), panCK (1:500; clone PCK-26; Sigma-Aldrich), SPC (1:300; Sigma-Aldrich), AQP5 (1:100; Millipore), CCSP (1:2000; Millipore), proSPC (1:2000; Millipore), ZO-1 (1:100; Abcam), GFP (1:1000; Abcam), T1 α (1:100; clone 8.1.1; Biolegend) and SSEA-1 (1:50; clone MC480; BD Biosciences). Isotype-matched control antibodies were used as negative control. Alexa-Fluor-coupled secondary antibodies (Invitrogen) were used at a 1:500 dilution to detect the bound antibodies. Confocal microscopy (ZEISS, LSM 510 META and LSM780) was performed to visualize the stained cells.

Enzyme-linked immunosorbent assays (ELISA). Mouse IL-4, IL-5, IL-13, eotaxin, and TSLP DuoSet ELISA kits were used to analyze BAL fluid and culture supernatant according to the manufacturer's instructions (R&D Systems). Optical densities were measured with a

Molecular Devices VERSAmax microplate reader, and 540-nm values were subtracted from 450-nm values to calculate concentrations with a four-parameter logarithmic standard curve.

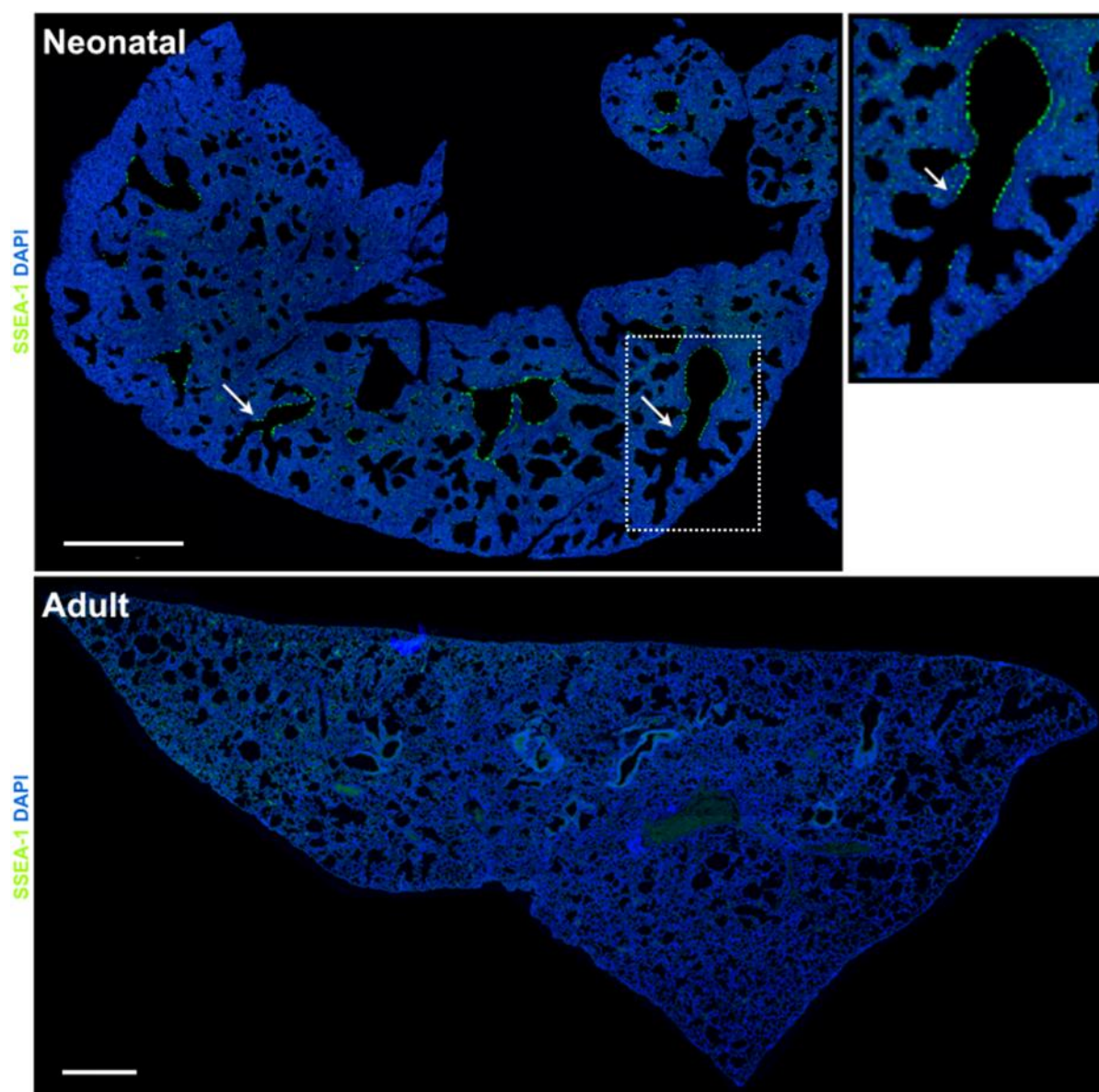
FACS analysis. For surface marker staining, 5×10^5 pulmonary cells were stained using the Mouse Cell Surface Marker Screening Panel kit (BD Biosciences). All procedures were performed according to the manufacturer's directions. For intracellular CCSP staining, 5×10^5 pulmonary cells were immersed in 0.5 ml of buffered formalin (BD Biosciences) at room temperature. After 30 minutes, 1 ml of permeabilization buffer (eBioscience) was added and the cells were collected by centrifugation. The cells were washed with permeabilization buffer and resuspended in 100 μ l of permeabilization buffer containing rabbit anti-mouse CCSP polyclonal antibodies (Millipore) at 4°C for 30 minutes. Then the cells were washed twice with permeabilization buffer and then incubated with Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) at 4°C for 30 minutes. The cells were resuspended in FACS buffer (PBS containing 5% FBS and 2 mM EDTA) and analyzed using a FACSCanto II and a FACSCalibur (BD Biosciences).

Adult lung epithelial cell culture. The lung tissue from adult mouse (≥ 8 weeks of age) was washed with PBS and cut into small pieces and then digested in MEM in the presence of 1 mg/ml of protease-type XIV and 0.5 mg/ml of DNase I for 18 hours at 4°C. PBS containing 5% FBS was added to neutralize the protease activity, and pipetting was performed to obtain a lung single-cell suspension. To avoid the contamination of leukocytes and endothelial cells, CD45⁺ and CD31⁺ cells were removed by using magnetic beads. The cell suspension was seeded in 100-mm dish with MCDB201 medium supplemented with 5% FBS, Insulin-Transferrin-Selenium, 100 U/ml of penicillin, 100 μ g/ml of streptomycin. Cells were sub-

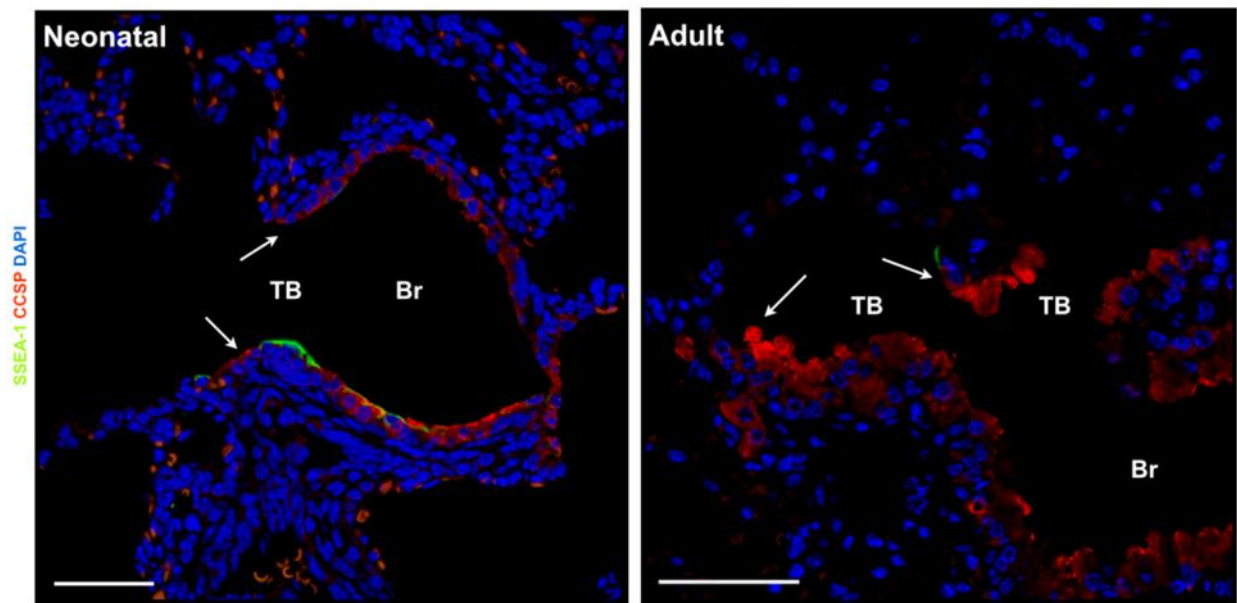
cultured with trypsin every 3-4 days. Primary adult lung epithelial cells were ready for use at passage 3-5.

Co-culture of SSEA-1⁺ PSC and adult lung epithelial cell. 2×10^4 adult lung epithelial cells and 1×10^4 SSEA-1⁺ PSCs /well were seeded and left to attach in a 24-well cell culture plate overnight. For stimulation of adult lung epithelial cells to produce TSLP and eotaxin, 2 μ g/ml LPS (Sigma-Aldrich) and 100 ng/ml IL-4 (BD Biosciences) were added, respectively. To measure the level of eotaxin or TSLP, supernatants were collected after incubation for 24 hours and analyzed using specific ELISA kit (R&D Systems). For Transwell culture, 1×10^4 SSEA-1⁺ PSCs and 2×10^4 adult lung epithelial cells were seeded in the upper and lower chamber, respectively.

SI Figures and Legends

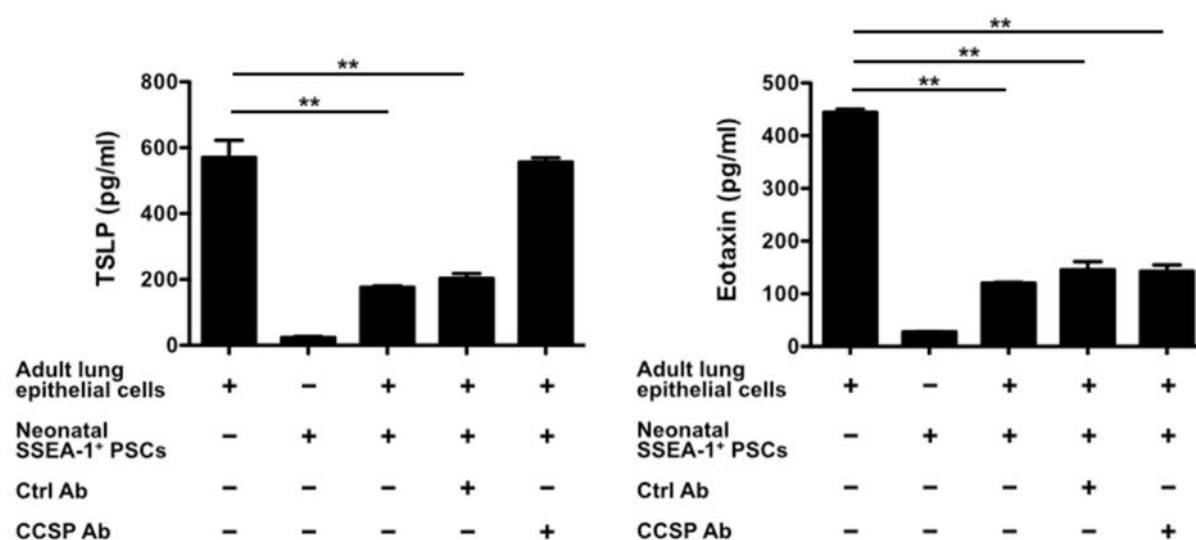


Supplementary Figure S1. Whole-mount view of SSEA-1⁺ pulmonary cells in lung tissues. Neonatal and adult mice derived lung sections were stained with anti-SSEA-1 (green) and the nuclei were counterstained with DAPI (blue). Bars: 500 μm. Data are representative of at least five independent experiments.

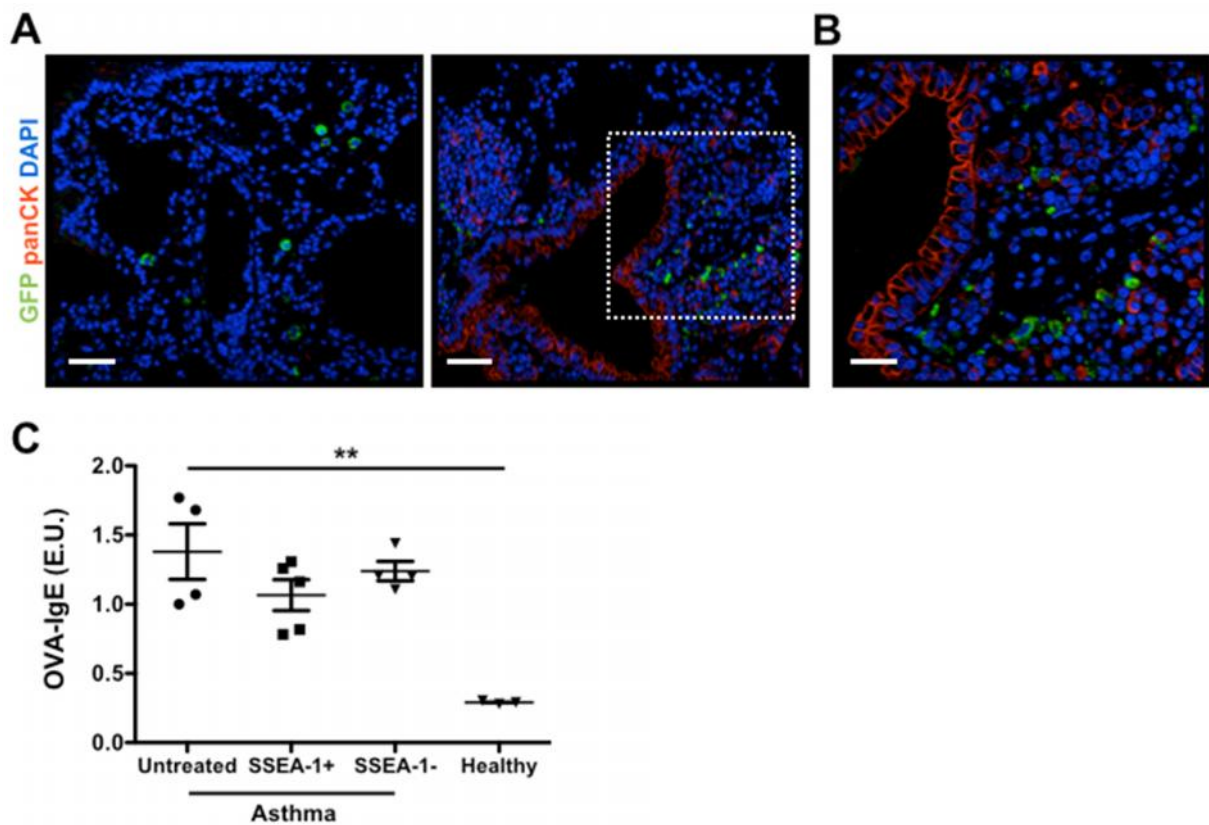


Supplementary Figure S2. Expression of SSEA-1⁺ pulmonary cells in lung tissues.

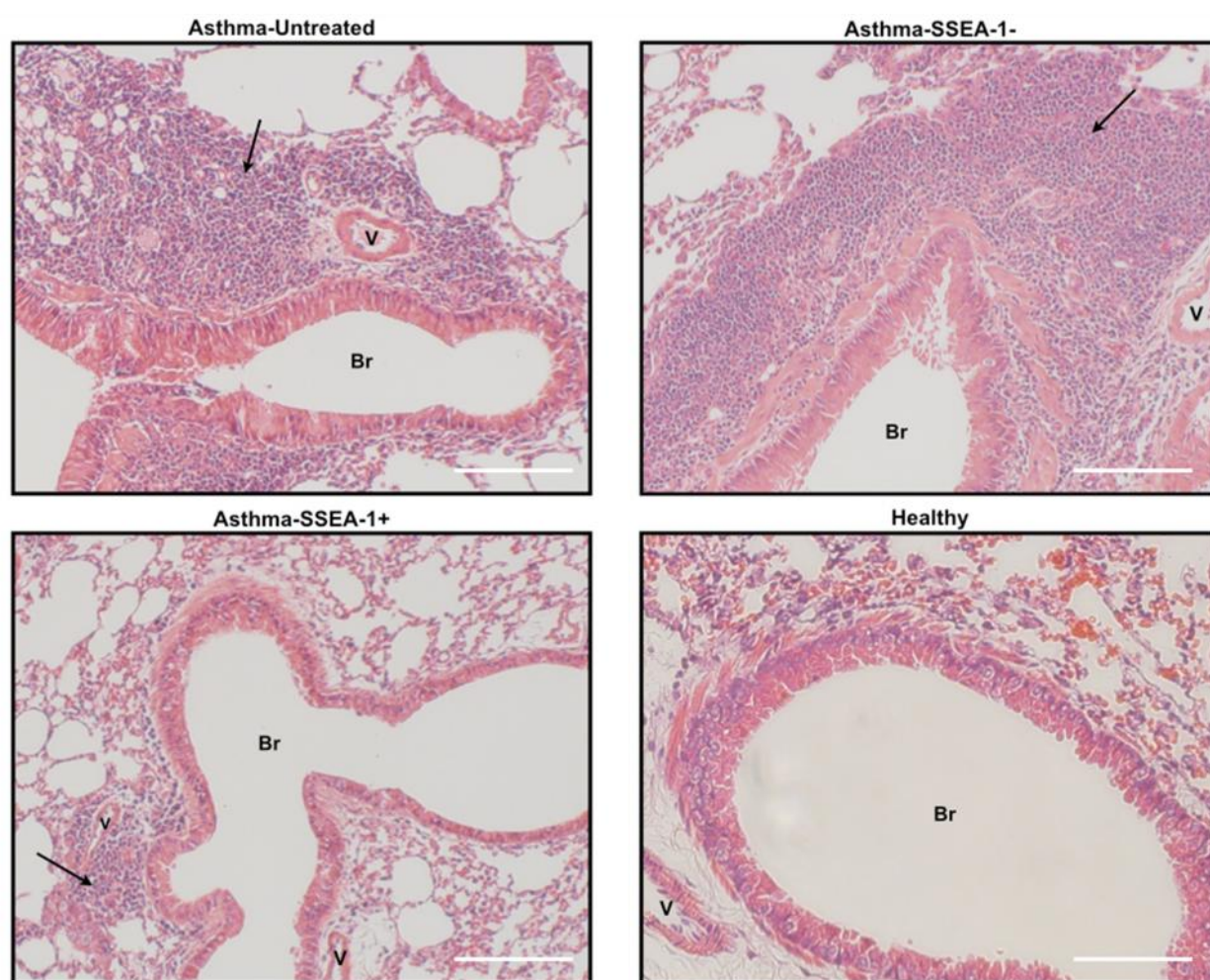
Neonatal and adult mice derived lung sections were stained with anti-SSEA-1 (green) and CCSP (red); the nuclei were counterstained with DAPI (blue). The bronchiole (Br) and terminal bronchiolar (TB) airway were determined by CCSP (red) staining. Neonatal (left) and adult (right) SSEA-1⁺ pulmonary cells were located within TB adjacent to the bronchioalveolar duct junction (BADJ). Bars: 50 μ m. The arrows point to the BADJ. Data are representative of at least five independent experiments.



Supplementary Figure S3. Neonatal lung SSEA-1⁺ PSCs reduced TSLP and eotaxin production from adult lung epithelial cells through CCSP-dependent and -independent manner. Lung epithelial cells derived from adult mice were co-cultured with neonatal SSEA-1⁺ PSCs and stimulated with LPS or IL-4 for 24 hours in the presence of control antibody or CCSP neutralizing antibody (5 μ g/ml). TSLP and eotaxin contents in LPS- and IL-4-stimulated culture supernatants were determined using ELISA, respectively. Data are means $\pm$ SD and representative of three independent experiments. ** $P < 0.01$.



Supplementary Figure S4. Transplantation of SSEA-1⁺ PSCs did not change serum OVA-specific IgE in the asthmatic mice. (A) Distribution of SSEA-1⁺ PSCs in adoptive transferred mice. The lung sections were stained with an anti-GFP- (green) and an anti-panCK (red) antibody, and they were counterstained with DAPI (blue). Data are representative of four independent experiments. Bars, 50 μ m. (B) Magnified view of the boxed area in A. Bar, 50 μ m. (C) The mice received an i.v. injection of 1×10^5 cells of SSEA-1⁺ PSCs or SSEA-1⁻ pulmonary cells isolated from neonatal GFP-mice after the second aerosol treatment. OVA-specific IgE was detected in serum 24 hours after the last inhalation exposure to OVA. Data are means $\pm$ SEM and representative of two independent experiments ($n = 5$ mice per group).



Supplementary Figure S5. Transplantation of SSEA-1⁺ PSCs decreased the infiltration of inflammatory cells into lung in the asthmatic mice. Enlarge view of Figure 5C. Bars, 200 μ m. Arrows denote infiltrated leukocytes; br, bronchus; v, vessel.

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