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分離與鑑定 SSEA-1^{+}肺部前驅幹細胞治療過敏性氣喘

Isolation and Characterization of SSEA-1^{+} Pulmonary Stem/Progenitor Cells for Treatment of Allergic Asthma

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本論文係邱巧絨君（學號 D96449004）在國立臺灣大學醫學院免疫學研究所完成之博士學位論文，於民國 104 年 3 月 6 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

還記得才剛進來江伯倫老師實驗室的時候，我負責的助理工作是構築 H3N2 質體＆製備合成蛋白，到後來就讀免疫所，從充滿人氣的東址兒研三到有無敵夜景的兒醫 17F，晃眼間在台大醫院的時光已經邁入第九年了。最要感謝的是我的指導老師–江伯倫老師，謝謝您總是支持我嘗試對實驗各種天馬行空的想法，雖然老師身兼要職十分繁忙，但總是第一時間回應學生，即時與我討論研究進度與方向；在我投稿撞壁為期兩年的時間裡，謝謝您總是鼓勵我正向思考（很抱歉讓您一直替我煩惱畢業問題了><）。此外，我也要感謝中研院分生所賴明宗老師、成大免疫所楊倍昌老師、所上許秉寧老師及李建國老師，謝謝您們每年特地排開時間，前來聆聽我的進度報告，並給予學生許多的指正與建議。謝謝藥理所林泰元老師，在學生一開始接觸肺部幹細胞領域的時候，給予我許多實驗技術上的幫忙與指導。謝謝台大小兒過敏免疫科楊耀旭醫師、新生兒科曹伯年醫師、腫瘤所張純榮老師及醫技系莊雅惠老師，在每次 Stem cell meeting 中給予我許多建議與不同的想法。謝謝輔大營養科學系吳文勉老師，謝謝您待我如親姐妹般疼愛，總是在一旁默默為我打氣與鼓勵。謝謝成大生化所張明熙老師，每次您給我的大大擁抱，都是充滿愛與感動的力量。特別謝謝一路陪著我的陳瑪麻冠驊，我想我這八年研究生活只有妳最懂了，不管是生活雜事還是實驗上的疑難雜症，謝謝妳總是第一個伸出援手，還要謝謝陳瑪麻分享的畢姐妹生活記錄，讓我可以隨時開懷大笑! 也要謝謝我的好鄰居小茉，就像 7-11 一樣無不供應（還多了陪聊的娛樂功能 XD），謝謝妳的一路相伴! 謝謝江老師實驗室的每位夥伴們(特別是理性分析＆鼓勵我的演忠學長、剛進來實驗室就分一半位置給我的欣妙、常伴我挑燈夜戰的昆輝、陪我嚐盡酸甜苦辣的徽徽，以及所有兒醫實驗室夥伴們)，不管是問我午餐吃沒/還要留多晚/實驗需不需要幫忙/還是要不要幫妳買吃的回來？都代表著一句句貼心的問候，衷

心謝謝你/妳們平日所有的關心與幫忙。回想求學過程，除了能延續自己對研究的興趣與熱情外，最幸運的是我有始終無條件支持我的爸媽與我的先生許育祥，當我偶爾忙碌到忘記即時關心，回到家您們總還是張開雙手給我擁抱與鼓勵，您們是支持我繼續努力的原動力，感謝您們的付出與辛勞，讓我可以無後顧之憂地完成學業。另外，在此特別感謝在我的求學研究生涯中，每一隻付出生命的小鼠們，因為你們，才能讓我探索求證對科學的疑問與假設，謝謝你們的無比貢獻。

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ABSTRACT

Asthma is characterized by chronic airway inflammation and hyperresponsiveness (AHR). Little is known about the role of pulmonary stem cells (PSCs) in allergic airway inflammation. To identify the role of PSCs population in the bronchial epithelium of neonatal mice, we developed an enzyme-based digestion method to isolate PSCs from lung tissues. Characterization of PSCs was done using flow cytometry, real-time PCR, immunofluorescence staining, confocal microscopy, and scanning electron microscopy. The effects of SSEA-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, enzyme-linked immunosorbent assay, real-time PCR, and immune-blotting. Cell suspensions derived from neonatal lung tissue contained cells expressing either SSEA-1^+ (stage-specific embryonic antigen-1) or Sca-1^+ (stem cell antigen-1) that represent PSCs phenotype. The SSEA-1^+ PSCs were prevalent in neonatal mice, but rare in adult mice. Enriched SSEA-1^+ PSCs had the ability to differentiate into pneumocytes and tracheal epithelial cells. The expression of CCSP (Clara cell secretory protein) were higher in SSEA-1^+ PSCs as compared with that of SSEA-1^- pulmonary cells. Transplantation of SSEA-1^+ PSCs in asthmatic mice reduced AHR and airway damage by decreasing eosinophil infiltration, inhibiting chemokines/cytokines production, increasing regulatory T cells, and preserving the level of CCSP. Collectively, our results indicated that neonatal SSEA-1^+ PSCs contribute to ameliorate the progression of asthma by reducing lung damage and inhibiting inflammatory responses. Study about the molecular mechanisms of neonatal SSEA-1^+ PSCs might shed light on etiology of airway inflammation.

Keywords: Pulmonary stem/progenitor cell; asthma; airway inflammation.

中文摘要

氣喘主要的症狀為慢性呼吸道發炎與呼吸道收縮阻力（airway hyperresponsiveness, AHR）增加。目前尚無探討肺部幹細胞/前驅幹細胞（pulmonary stem/progenitor cells, PSCs）與調控過敏性呼吸道發炎的相關研究。為了尋找並進一步研究 PSCs，我們利用特殊的酵素分解法，由新生小鼠肺組織中分離出單一細胞懸浮液。利用流式細胞儀、即時聚合酶連鎖反應、免疫螢光染色、共軛焦顯微鏡及掃描式電子顯微鏡鑑定 PSCs 的特性。在體外細胞培養模式中，以流式細胞儀、酵素連結免疫吸附法、即時聚合酶連鎖反應及免疫轉漬法，分析 PSCs 的細胞調控機制；同時也探討 PSCs 對卵白蛋白引發過敏性呼吸道發炎動物模式的影響。結果發現，由新生鼠肺部組織所分離的單一細胞懸浮液，分別表現階段特異性胚胎抗原-1（stage-specific embryonic antigen-1, SSEA-1）或幹細胞抗原-1（stem cell antigen-1, Sca-1）。SSEA-1$^+$ PSCs 大量表現於新生鼠，但成鼠中則非常稀少。進一步純化後的 SSEA-1$^+$ PSCs 可分化為肺細胞（penumocytes）與氣管上皮細胞（tracheal epithelial cells）。此外，與 SSEA-1$^-$ 肺部細胞相較，SSEA-1$^+$ PSCs 表現高量的克拉拉細胞分泌蛋白（Clara cell secretory protein, CCSP）。移植 SSEA-1$^+$ PSCs 至過敏性呼吸道發炎小鼠體內，可降低嗜酸性白血球（eosinophils）浸潤、抑制細胞趨化素/細胞激素、增加調節型 T 細胞（regulatory T cells）及維持肺部 CCSP 表現，進而達到減少呼吸道收縮阻力與肺部受損情形。本研究發現新生鼠之 SSEA-1$^+$ PSCs 可降低肺部傷害與抑制發炎反應，進而對於氣喘進程具有免疫調節能力。深入了解 SSEA-1$^+$ PSCs 免疫調控能力的分子機制將有助於開發未來治療呼吸道發炎的新策略。

關鍵詞：肺部幹細胞/前驅幹細胞、氣喘、呼吸道發炎。

ABBREVIATIONS

AHR	Airway hyperresponsiveness
ALI	Air-liquid interface
AQP5	Aquaporin 5
BADJ	Bronchioalveolar duct junction
CCSP	Clara cell secretory protein
DCs	Dendritic cells
DAPI	4',6-diamidino-2-phenylindole
iTreg	Inducible Treg
LN	Lymph nodes
MSC	Mesenchymal stem cell
nTreg	Naturally occurring Treg
Oct-4	Octamer-binding transcription factor-4
proSPC	Prosurfactant protein C
PSCs	Pulmonary stem/progenitor cells
Treg	Regulatory T cells
RT-QPCR	Real-time quantitative-polymerase chain reaction
Sca-1	Stem cell antigen-1
SEM	Scanning electron microscopy
SSEA-1	Stage-specific embryonic antigen-1
TEC	Tracheal epithelial cell
TSLP	Thymic stromal lymphopoietin
TTF-1	Thyroid transcription factor-1

CONTENTS

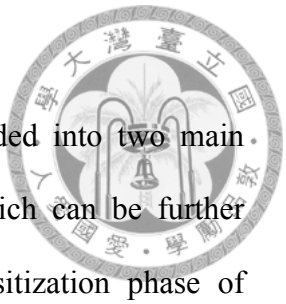
CONTENTS OF FIGURES

I. INTRODUCTION

Asthma is a heterogeneous inflammatory disorder that is characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), and excessive airway mucous production[1]. Prevalence of asthma in the developed world is approximately 7% in adult and approaching 30% in some pediatric studies[2]. The incidence of asthma and allergic airway diseases has increased over the past 50 years. In 2011, an estimated 300 million people worldwide have been diagnosed with asthma[3]. Current treatment of acute symptoms is primarily directed towards suppressing airway inflammation with inhaled corticosteroids and relieving bronchoconstriction with bronchodilators. Long-term use of high-dose corticosteroids therapy has potential to cause systemic side effects-impaired growth in children, decreased bone mineral density, skin thinning and bruising, and cataracts[4]. 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[5].

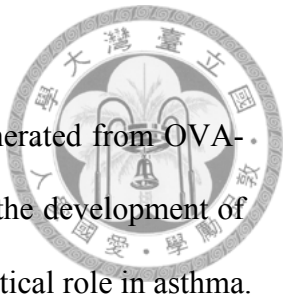
1.1 Pathogenesis of asthma

The immune system is constantly exposed to a large variety of antigens and has to distinguish pathogens from harmless antigens. The pathogenesis of asthma is associated with environmental factors, many cell types, and several molecular and cellular pathways[6]. Asthma is commonly divided into two types: allergic (extrinsic) asthma and non-allergic (intrinsic) asthma. Allergic asthma is triggered by exposure to allergens and associated with eosinophils in the airways and with allergic sensitization and adaptive immunity. Nonallergic asthma is associated with exposure to environmental factors, exercise, viral infection, stress, and obesity[6]; and often associated with neutrophils in the airways and innate immunity independent of T helper type 2 (TH2) cells[7-10].



The immune mechanisms underlying allergic asthma can be divided into two main phases: (i) sensitization and memory, and (ii) effector phase, which can be further subdivided into immediate and late responses[11]. During the sensitization phase of allergic diseases, the differentiation and clonal expansion of allergen-specific TH2 cells producing IL-4 and IL-13 is essential for the induction of B-cell class-switch to the ε-immunoglobulin heavy chain and the production of allergen-specific IgE antibody. Allergen-specific IgE binds to the high-affinity FcεRI on the surface of mast cells and basophils, thus leading to the patient's sensitization. During this step, a memory pool of allergen-specific T and B cells is also generated. The effector phase is initiated when a new encounter with the allergen causes cross-linking of the IgE-FcRI complexes on sensitized basophils and mast cells, thus triggering their activation and subsequent release of anaphylactogenic mediators responsible for the classical symptoms of the immediate phase.

Late-phase reactions are triggered in the continuous presence of allergen, leading to T-cell activation[12]. Activated allergen-specific TH2 cells produce IL-4, IL-5, and IL-13 play key roles in the induction and pathogenesis of asthma[13]. IL-4 induces the production of IgE by B cells and the expression of vascular cell adhesion molecule-1 on endothelial cells[14, 15]. IL-5 is crucial for the activation of eosinophils and their migration into the lung[16]. Activated eosinophils secrete a series of inflammatory cytokines and chemokines, and are a potent source of the chemical mediator leukotriene C4 and platelet-activating factor, which induce mucus secretion and smooth muscle contraction[17]. IL-13 is associated with various important events during the effector phase of asthma including AHR, mucus hyperproduction, and airway remodeling[18, 19].



In animal study, adoptive transfer of allergen-specific TH2 cells generated from OVA-specific T cell antigen receptor–transgenic DO11.10 mice results in the development of AHR and airway inflammation[20], suggesting that TH2 cells play a critical role in asthma.

1.2 Regulatory T cells in asthma

Regulatory T (Treg) have the ability to control and modify the development of allergic diseases altering the ongoing sensitization and effector phases, such as promoting the development of tolerogenic DC phenotypes[21], inhibition of allergen-specific TH2 cells[22], mast cells, basophils and eosinophils[23], and blocking the influx of effector T cells into inflamed tissues through a cytokine-dependent manner[24]. Moreover, reduced or altered function of Treg cell populations provides a possible explanation for the inappropriate immune response to allergens observed in patients with asthma[13]. In the study of asthmatic animal model, transfer of $CD4^+CD25^+$ Treg cells ameliorates the development of airway inflammation and AHR and prevents the allergen-induced activation of DCs in the airways[25-28]. In clinic, the percentage of $CD4^+CD25^+$ T cells in the airways of children with asthma is lower than in healthy children, which might be explained by the reduced response to chemokines of peripheral blood $CD4^+CD25^+$ T cells noted in patients with asthma[29]. The presence of $CD4^+CD25^+$ T cells in the airways is positively correlated with forced expiratory volume in 1 second (FEV1)[30]. Based on these clinic data and experimental evidences, Treg play a central role in controlling allergic disease.

1.3 Eosinophils in allergic inflammation

Eosinophils are bone marrow-derived leukocytes that are normally less than 5% of leukocytes in the blood, but can be found in higher numbers in tissues such as the bone marrow and gastrointestinal. Once recruited into the tissues, activated eosinophils have the capability to cause tissue damage and dysfunction[31]. Eosinophil-derived major basic protein and eosinophil peroxidase are toxic to a number of different cell types, including airway epithelial cells[32, 33] and may contribute to tissue damage and organ dysfunction in patients with asthma. CCR3 is selectively and abundantly expressed on eosinophils and promotes the accumulation and activation of eosinophils through binding high-affinity agonists secreted by airway epithelial cells including eotaxin-1 (also known as CCL11), eotaxin-2 (also known as CCL24), and eotaxin-3 (also known as CCL26)[34, 35]. Clinical study showed that increased expression of CCR3 and its ligands correlates with severity of disease in patients with asthma[36]. Eotaxin-1/2 double-deficient or CCR3-deficient mice were completely protected from allergen-induced AHR[37-41]. Therefore, the CCR3-eotaxin axis is crucial for the recruitment and accumulation of eosinophils in experimental models of asthma.

1.4 Airway epithelium is involved in the pathogenesis of asthma

The airway epithelium forms the first continuous line of defense against inhaled environmental insults, which include pathogens, pollutants and aeroallergens. In addition to functioning as a physical barrier, recent advances have also implicated a prominent role for the airway epithelium in the process of allergic sensitization[42, 43]. A recent study showed that the LPS and house dust mite (HDM) 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[44]. These observations

suggest that lung structural cells not only function as a barrier but also modulate the allergic immune response.

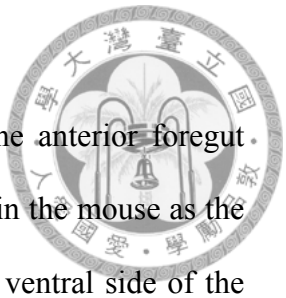
1.5 Cytokines secreted at the barrier that control TH2 immunity to allergens

Triggering of pattern recognition receptors on epithelial cells initiates NF-κB activation and leads to the release of pro-TH2 cytokines such as thymic stromal lymphopoietin (TSLP), GM-CSF, IL-1α, IL-25, and IL-33[44, 45].

TSLP is expressed at steady state in the lungs, intestines and tonsils, and this expression seems to be an important inhibitory signal that prevents TH1 responses at these sites[46]. The cell types that respond to TSLP include DCs, monocytes, B cells, mast cells and T cells[47]. Human bronchial epithelial cells also express TSLP receptor (TSLPR), and TSLP stimulates the proliferation of bronchial epithelial cells and IL-13 production by these cells[48]. Allergen-stimulated airway epithelial cells produce TSLP, which attracts and activates the DCs to prime naive $CD4^+$ T cells to differentiate into TH2 cells[43, 49]. Mouse models using either lung-specific expression of the TSLP transgene or intranasal administration of TSLP develops a spontaneous, progressive inflammatory disease with all the characteristics of human asthma[50]. Accordingly, TSLPR-deficient mice have impaired TH2 responses[51]. Blockade of TSLP during challenge alone partially reduces the response[52], and transfer of activated, OVA-specific wild-type CD4 T cells into TSLPR-deficient hosts rescue aspects of the inflammatory response[53]. Together, these data suggest that TSLP is required at both priming and challenge to generate a complete airway inflammatory response.

GM-CSF overexpressed in the lungs of mice via adenovirus induces DC activation and spontaneous TH2 sensitization to the inhaled innocuous protein OVA[54, 55]. Neutralization of GM-CSF in mice abolishes sensitization to HDM and attenuates the adjuvant effects of diesel particles on allergic sensitization[45, 56-58]. Initially it was thought that IL-1 played only a minor role in asthma, as symptoms in the classical OVA-alum model of asthma were not reduced in IL-1 receptor deficient mice[59]. Recently study showed that autocrine release of IL-1α by HDM-exposed bronchial epithelial cells leads to TSLP, GM-CSF, and IL-33 production by epithelial cells, and suggested that IL-1α is required for the development of TH2 immunity to HDM[45]. Numerous cells of the innate immune system, such as DCs, macrophages, basophils, mast cells, and eosinophils express T1/ST2 (the receptor for IL-33) and stimulation of these cells by IL-33 leads to prolonged survival and/or activation, often leading to increased TH2 immunity in mouse models of allergy and asthma[60-63]. Neutralization of IL-33 blocks development of lung TH2 immunity to a number of allergens, such as HDM and peanuts, as well as to lung-dwelling parasites such as hookworms[45, 62, 64]. IL-25 expands a population of granulocytic myeloid cells that produce IL-5 and IL-13 and contribute to asthmatic lung pathology in mice and humans[65]. Epithelial IL-25 acts directly on fibroblasts and endothelial cells to promote airway remodeling and angiogenesis and boosts production of TSLP and IL-33, thereby amplifying TH2 immunity in the lung[66].

Altogether, these studies highlight a previously unrecognized role of innate pathways induced at the epithelial level, able to trigger TH2-type polarization and play a key interface between innate and adaptive immunity.



1.6 Lung development

The respiratory system, esophagus, thyroid and liver arise from the anterior foregut endoderm. Lung specification begins around embryonic day (E) 9.0 in the mouse as the transcription factor Nkx2.1 is expressed in endodermal cells on the ventral side of the anterior foregut. By E9.5, these epithelial cells evaginate and format the trachea and two lung buds and begin the embryonic stage of lung development (E9.5-E12.5). During this stage, the trachea completes its separation from the esophagus. During the embryonic and pseudoglandular stages (E12.5-E16.5), the two lung buds undergo a highly regulated branching process called branching morphogenesis to generate a tree-like network of airways with thousands of terminal branches. This is followed by the canalicular (E16.5-E17.5) and saccular [E18.5-postnatal day (P) 5] stages, during which these terminal branches narrow and form clusters of epithelial sacs that will later develop into alveoli in preparation for respiration at birth. Finally, full maturation of the alveolus occurs during the alveolarization stage (P0-P14)[67]. Therefore, development of the alveoli does not stop at birth; their number and surface area increases dramatically postnatally and continues for weeks (mice) and months (humans)[68]. Based on these observation, suggest that a stem cell pool present in the neonatal lung contributes to both the bronchiolar and alveolar lineages during lung development[69], whereas these stem cell populations are a rare and quiescent population in the adult lung[70, 71].

1.7 Lung stem cells

Depending on the composition and organization of respiratory epithelium, distinct regions of the lung contain different populations of epithelial cells that function as adult stem cells. These adult stem cells are defined by their ability to undergo long term self-renewal and give rise to different cell types during homeostatic turnover or cell

replacement after injury. Until now, several lung stem cell populations are identified including basal cells in the pseudostratified mucociliary epithelium, Club cells in the mouse bronchioles, and type II pneumocytes in the alveolar [68, 72].

1.7.1 Basal cells

The airways of human and mice (about 1-1.5 mm in diameter) are line by a pseudostratified mucociliary epithelium composed of multiciliated, secretary, neuroendocrine cells, as well as a population of basal cells. The basal cells of the mouse proximal airways characteristically express basal cell-restricted transcription factor Trp63 (transformation related protein 63), Ngfr (nerve growth factor receptor), Pdpn (podoplanin), GS1 lectin B4, and Krt5 (cytokeratin 5)[73-75]. The number and proportion of $Krt5^+$ basal cells decline with age in the mouse trachea[76]. From *in vivo* lineage tracing experiments, basal cells are suggested as stem cells that self-renew over the long term and give rise to ciliated and secretory (club) luminal cells during postnatal growth, homeostasis, and epithelial repair after loss of luminal cells[75, 77, 78].

1.7.2 Type II pneumocytes

Using an inducible Cre recombinase targeted to the endogenous Sftpc locus has been used to detect proliferation and clonal expansion of type II pneumocytes after adult lung injury as well as lineage-tagged type I pneumocytes deriving from type II pneumocytes *in vivo*[79, 80]. Moreover, a recent study has identified several type II pneumocytes self-renewal signals, including epidermal growth factor receptor (EGFR) and the GTPase KRAS[81]. These pathways are important for increases in type II pneumocytes and type II pneumocytes-derived type I pneumocytes during aging or after hyperoxic injury. Therefore, this population is suggested to function as a lung stem cell to proliferate in

response to lung injury, and display remarkable potential for multipotent airway and alveolar differentiation in mice study.

1.7.3 Club cells

The bronchiolar epithelium is quiescent until injured. Using naphthalene-induced secretory epithelial cell depletion, a subset of secretory cells expressing CCSP but not cytochrome p450 is spared from naphthalene toxicity. These $CCSP^{+}$ cells function as facultative progenitors, that expand rapidly to repopulate the damaged airway by forming both secretory and ciliated cell progeny[82-84]. Cell lineage-tracing experiments have shown that $CCSP^{+}$ cytochrome $p450^{-}$ cells can self-renew and differentiate into ciliated epithelium during normal homeostatic turnover as well as after naphthalene-induced injury[85]. Therefore, these cell population are named variant Club cells in some studies. CCSP is produced from club cells and is critical in inhibiting the differentiation of naive T cells into TH2 cells via the DCs[86]. In addition, CCSP not only inhibits the infiltration of inflammatory cells but also directly reduces allergic inflammation in the lung[87]. Therefore, it suggests that lung structural cells not only function as a barrier, but also have the potential to regulate the immune response.

1.7.4 Bronchioalveolar stem cells (BASCs)

The transition from bronchioles to alveolar sacs in the mouse lung is known as the bronchioalveolar duct junction (BADJ). It contains a few cells (<1 per BADJ) that coexpress CCSP and surfactant protein C (SPC) proteins and are proposed as putative BASCs[88]. The original description of BASCs was founded on an observation that BASCs at the BADJ could expand *in vivo* after bleomycin-induced lung injury, and cells proposed to be the same population could be purified from lung digests using a

defined flow-sorting algorithm (including $CD45^{-}CD31^{-}Sca1^{+}CD34^{+}$ gating)[88]. These sorted cells could be clonally expanded *in vitro*, where they show multipotent differentiation into bronchiolar and alveolar lineages[88, 89]. During development or after hyperoxic alveolar injury, these studies found little evidence in support of an CCSP lineage–tagged population contributing to alveolar reconstitution[85]. However, CCSP lineage tracing does not entirely distinguish airway secretory cells (including putative BASCs and club cells) from rare type II pneumocytes that also lineage trace with Scgb1a1 (CCSP)-driven Cre tagging[85]. Therefore, dual lineage-tagging approaches unique to the proposed BASCs will need to be used to rigorously define their potential *in vivo*[72].

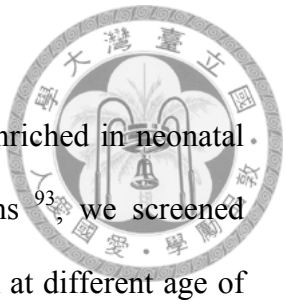
1.8 Lung stem cells and airway diseases

Asthma in most patients is only symptomatically controlled by the available medications[90]. 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[5]. Adult stem cells are thought to maintain and repair the tissue in which they are found[91]. Evidence suggests that stem cells reside in a specific area of each tissue and remain quiescent until they are activated for tissue maintenance or by disease or tissue injury[92]. Airway stem cells were first identified in a lung injury model that was used to demonstrate that bronchiolar stem cells are pollutant-resistant, CCSP-expressing stem cells that contribute to the repair of injured airways[83]. 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 not only express Oct-4 but also express other stem cell markers, such as stage-specific embryonic antigen-1

(SSEA-1) and stem cell antigen-1 (Sca-1)[93]. These studies raised the possibility that pulmonary stem/progenitor cells regulate immune responses and suggested their therapeutic potential for treating asthma.

The aim of the present study was to identify the PSC population in the bronchial epithelium of neonatal mice and to investigate their stem cell capacity *in vitro*. We also explore whether SSEA-1^+ PSCs could exert a therapeutic benefits in the progression of asthma.

II. MATERIALS AND METHODS



2.1 Study design

Lung development is ongoing after birth suggested that PSCs are enriched in neonatal and rare in adult mice. Based on previously reported observations [93], we screened surface SSEA-1 and Sca-1 expression on pulmonary cell suspension at different age of mice. The characteristics of PSC candidate were evaluated by a series of experiments including surface marker screening by FACS analysis, and the expression of stem cell-related factors by RT-QPCR, FACS, and confocal microscopy analysis. To verify the stem cell behavior of PSC candidate, self-renew and clonogenicity was evaluated by repeated spheres-forming assay, whereas the capacity to differentiate into pneumocytes or tracheal epithelial cells (TECs) was assessed by immunofluorescence techniques. To clarify the biological function of PSC, we performed *in vitro* and *in vivo* analyses to get insight into the immunomodulatory effect of PSC in cytokines/chemokines production and airway inflammation.

2.2 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.

2.3 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 Ca^{2+}- and Mg^{2+}-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 [94] 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. For the *in vivo* bromodeoxyuridine (BrdU)-labeling experiment, mice were given i.p. injections of BrdU (1.5 mg/mouse; Sigma) 24 hours before sacrifice to identify proliferating cells in lungs.

2.4 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.

2.5 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.

2.6 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 [95]. 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). 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 air-liquid interface (ALI) by changing the medium in the basal chamber every other day, and leaving the apical chamber empty for another 10-15 days as previously described [96, 97].

2.7 Real-time quantitative-polymerase chain reaction (RT-QPCR)

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.

2.8 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: acetylated α-tubulin (1:5000; clone 6-11B-1; Sigma-Aldrich), 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), BrdU (1:250; clone BU-33; Abcam), GFP (1:1000; Abcam), T1α (1:100; clone 8.1.1; Biolegend), SSEA-1 (1:50; clone MC480; BD Biosciences), Sox2 (1:50; clone O30-678; BD Biosciences), and Nanog (1:50; clone M55-312; 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.

2.9 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.

2.10 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 μl of permeabilization buffer containing rabbit anti-mouse CCSP polyclonal antibodies (Millipore). The cells were washed twice with PBS and then incubated with Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen). The cells were resuspended in PBS containing 0.5% FBS and analyzed using a FACSCanto II and a FACSCalibur (BD Biosciences).

2.11 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 μg/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.

2.12 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 μg/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 CCSP neutralization experiment, 5 μg/ml of sheep anti-CCSP polyclonal antibody or control sheep IgG (R&D Systems) was used. 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.

2.13 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.

III. RESULTS

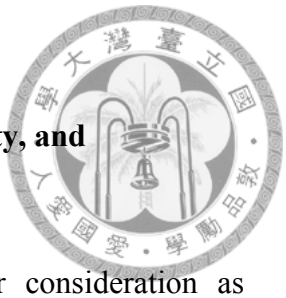
3.1 Identification of potential stem/progenitor cells from neonatal mouse lungs

The development of the lungs occurs as a continuous process from embryogenesis to early adolescence in humans as well as mice[98]. A stem cell pool present in the neonatal lung contributes to both the bronchiolar and alveolar lineages during lung development[69], whereas these stem cell populations are a rare and quiescent population in the adult lung[70, 71]. Previous study showed that Oct-4-, SSEA-1-, Sca-1-, or CCSP-expressing pulmonary stem/progenitor cells undergo terminal differentiation to alveolar pneumocytes [93]. Therefore, we speculated that mouse pulmonary stem/progenitor cells 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 cell suspensions 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 (**Figure 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 (**Figure 1B, C**). The numbers of SSEA-1^+ cells at postnatal day 1 and day 7 were $1.7 \pm 0.4\times10^5$ and $6.0 \pm 0.8\times10^5$, 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 (**Figure 2 and Figure 3**). 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 figure 1. The enlarged

immunofluorescence image showed that adult lung SSEA-1$^+$ cells were localized in the BADJ, which was almost the same as neonatal mice (**Figure 3**).

3.2 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. FACS 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 (**Figure 4A**). In addition, neonatal SSEA-1$^+$ pulmonary cells expressed CD9, CD24, CD26, CD29, CD47, CD54, CD98, CD133, and CD147 (**Figure 4A**). 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), T1α (a type I pneumocyte marker), SPC (a type II pneumocyte marker), and CCSP (a Club cell marker) by FACS analysis, RT-QPCR and immunoblotting. Interestingly, SSEA-1$^+$ pulmonary cells were negative for p63 and T1α, but positive for SPC (**Figure 4B**) and CCSP (**Figure 4B, C**). 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 (**Figure 3**). Since bronchoalveolar stem cells (BASCs) were defined as CCSP/SPC dual-positive population at the BADJ described previously by Kim and colleagues[88]. Therefore, these results raised the possibility that SSEA-1$^+$ cells might be multi-functional and comprise the regenerative cell populations within the airway microenvironment.



3.3 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 cells were re-suspended in Matrigel-based three-dimensional culture. We found that primary sphere colonies were observed 10-15 days after cell culture (**Figure 6A and B left**). To further clarify the self-renewal capacity of pulmonary SSEA-1$^+$cells, primary spheres were subsequently dissociated to single cell and then re-suspended to Matrigel-based three-dimensional culture. The formation of secondary spheres was observed after culture for 7-10 days **(Figure 6B 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/2,500–5,000 total cells) as determined by limiting dilution assay (**Figure 6C**). 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 (**Figure 4B and Figure 5**). However, after culture 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 (**Figure 7A**). In addition, we investigate that whether SSEA-1$^+$ pulmonary cells has the capacity to differentiate into TECs because SSEA-1$^+$ cells were located at the BADJ in the lungs of neonatal mice (**Figure 3**). 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 ALI cultures (**Figure 7B**). Scanning electron microscopy (SEM) also showed the similar results (**Figure 7C**).

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, suggested that neonatal SSEA-1$^+$ pulmonary cells are stem/progenitor cells with self-renewing, clonogenic, and multipotent properties.

3.4 SSEA-1$^+$ PSCs reduce TSLP and eotaxin production

Previous study[99] showed that human lung stem cells repair damaged mouse lung *in vivo* indicating PSCs might play a protecting role in lung damage. However whether PSCs play a critical role in the process of inflammation still 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 co-culture system in the presence of stimulators. Since 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[44]. 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[100]. 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 (**Figure 8**). To clarify whether the neonatal SSEA-1$^+$ PSCs-mediated inhibitory effect was dependent on soluble or cell-cell contact-dependent factors, co-culture of neonatal SSEA-1$^+$ PSCs and adult lung epithelial cells were physically separated by a Transwell insert, and found that neonatal SSEA-1$^+$ PSCs suppressed LPS- and IL-4-induced TSLP and eotaxin production (**Figure 9**). These results

indicated that the SSEA-1$^+$ PSCs-mediated inhibitory 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 o f CCSP neutralization antibody. We found that anti-CCSP antibody restored SSEA-1$^+$ PSCs-induced TSLP but not eotaxin, suggested that CCSP might not to be the predominant pathway for eotaxin inhibition (**Figure 10**).

3.5 Transplantation of SSEA-1$^+$ PSCs alleviates 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 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 OVA aerosol exposure (**Figure 13A**). 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 (**Figure 11A and B**). Moreover, almost GFP-labeled SSEA-1$^+$ PSCs maintained their SSEA-1 expression after repeated allergen challenge *in vivo* (**Figure 11C**). The SSEA-1$^+$ PSCs transplant did not change the anti-OVA IgE titer (**Figure 11D**). Notably, administration of SSEA-1$^+$ PSCs significantly suppressed the invasive AHR to methacholine (**Figure 13B**) and decreased the infiltration of inflammatory cells into peribronchovascular areas in the OVA-induced asthmatic mice (**Figure 15 and Figure 16**). The total cell counts and the proportion of eosinophils in

BAL fluid were significantly decreased in the SSEA-1$^+$ PSCs-treated group compared to untreated group (**Figure 13C and D**). 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 (**Figure 14**). To confirm the beneficial effects of SSEA-1$^+$ PSCs in asthmatic mice, we examined the effect of transplantation of SSEA-1$^+$ PSCs into mice before they received the OVA inhalation challenge. We found that both the cytokine profile of the BAL fluid and the inflammatory cell infiltration were decreased in the SSEA-1$^+$ PSCs-treated group (**Figure 12**).

3.6 Transplantation of SSEA-1$^+$ PSCs increase Foxp3$^+$ Treg population

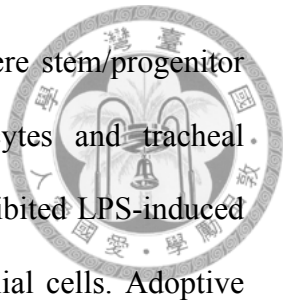
We further evaluate whether transplantation of SSEA-1$^+$ PSCs modulate airway inflammation through regulatory T cells (Treg). We developed allergic asthma model in Foxp3-GFP reporter mice to address this hypothesis. After 7-day consecutive challenge of OVA, thoracic and cervical draining lymph nodes (LN) were collected. Total cell number in thoracic LN but not in cervical LN was increased in asthmatic mice compared with that of healthy mice (**Figure 17A and B**). Analysis of cell composition by FACS showed that a significantly increased percentage and cell number of thoracic LN Foxp3-GFP$^+$ Treg in SSEA-1$^+$ PSCs-treated asthmatic mice (**Figure 17C**). In contrast, Treg population and cell number in cervical LN was not significant difference among these groups (**Figure 17D**). These data indicated that SSEA-1$^+$ PSCs increased Foxp3$^+$ Treg cells in inflamed lung draining LN, and may subsequently prevent the recruitment of inflammatory cells during the disease development of asthma.

3.7 Transplantation of SSEA-1$^+$ PSCs preserves the epithelium and inhibits unregulated lung structural cell proliferation in asthma

The levels of CCSP in both the BAL fluid and blood serum were lower in patients with asthma compared with healthy controls[101, 102]. Transplantation of SSEA-1$^+$ PSCs reduced the severity of asthma. Therefore, we hypothesized that SSEA-1$^+$ PSCs might reduce asthmatic injury through regulating the expression of CCSP. Immunofluorescence staining showed that CCSP was weakly expressed in asthmatic lung tissue compared with healthy lung tissue (**Figure 18A**). In the asthmatic model, CCSP was strongly expressed in the SSEA-1$^+$ PSCs recipients (**Figure 18A**). Furthermore, RT-QPCR confirmed the transcript of CCSP in these groups and obtained consistent results (**Figure 18B**). Therefore, Transplantation of neonatal SSEA-1$^+$ PSCs preserves CCSP secretion and thereby reduces the severity of asthma.

It has been suggested that the dysregulated proliferation of epithelial cells in asthma contributes to airway remodeling[103, 104]. To test whether SSEA-1$^+$ PSCs regulate the proliferation of lung structural cells, we adoptively transferred SSEA-1$^+$ PSCs into asthmatic mice and exposed to BrdU 18 hours before sacrifice (**Figure 19A**). BrdU immunostaining showed that the proliferation in the lung was increased in asthmatic mice (**Figure 19B**), which is consistent with observations in patients with asthma[103]. Interestingly, cell proliferation in the lung was dramatically inhibited in SSEA-1$^+$ PSCs-treated asthmatic mice (**Figure 19C**). Immunofluorescence staining confirmed that main BrdU-incorporated cells were expressed T1α (type I pneumocytes marker) (**Figure 20)**, but not expressed CD3 or thyroid transcription factor-1 (data not shown). These observations suggested that the proliferative rate of type I pneumocytes was increased under repeated allergen-challenge condition.

IV. Discussion



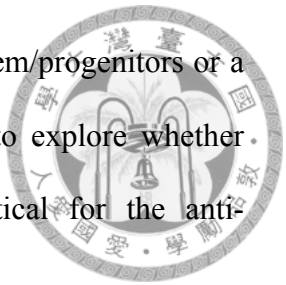
In this study, we found that neonatal SSEA-1$^+$ pulmonary cells were stem/progenitor cells capable of self-renewal and differentiating into pneumocytes and tracheal epithelial cells. The SSEA-1$^+$ PSCs highly expressed CCSP and inhibited LPS-induced TSLP and IL-4-induced eotaxin production in primary lung epithelial cells. Adoptive transfer of SSEA-1$^+$ PSCs reduced AHR and suppressed airway damage in acute OVA-induced asthmatic mice by preserving of the level of CCSP; decreasing the infiltration of eosinophils; inhibiting the production of IL-4, IL-5, IL-13, eotaxin, and TSLP; and increasing Foxp3$^+$ Treg cells. We have demonstrated that the SSEA-1$^+$ PSCs play an immunomodulatory role in the progression of asthma by inhibiting allergen-induced acute inflammatory responses and lung damage.

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[72]. We found that neonatal SSEA-1$^+$ PSCs were located in the bronchioles, terminal bronchioles, and BADJ in neonatal lung tissues. Moreover, neonatal SSEA-1$^+$ PSCs were able to self-renew, proliferate, and differentiate into pneumocytes, 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[88]. 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 in to 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[99], mouse Oct-4-expressing stem/progenitor cells comprise 0.0016-0.0022% of the neonatal lung cells[93], and a population termed bronchioalveolar stem cells represents 0.4% of the total lung cell preparations from adult mice[88]. Morphometric analysis of total lung sections showed that SSEA-1$^+$ PSCs constituted an average of 0.138 ± 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 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.

Phenotypic characterization of the SSEA-1$^+$ PSCs is pivotal to clarify their biological functions *in vivo*. We found that CD9, CD24, CD26, CD29, CD47, CD54, CD98, CD147, and CD324 are positive, but CD24 and CD54, and CD324 are distributed heterogeneously within the SSEA-1$^+$ cells. In our preliminary results, we found that enriched SSEA-1$^+$ CD133$^+$ cells simultaneously expressed CD24 and CD35, suggested that these SSEA-1$^+$ cells comprise two distinct cell populations that can be distinguished by the marker CD133. These results raised the possibility that SSEA-1$^+$ PSCs comprise



$CD133^+$ and $CD133^-$ populations and might with different lineage stem/progenitors or a different maturation status. Therefore, further studies are needed to explore whether distinct sub-groups of cells within the SSEA-1^+ PSCs are critical for the anti-inflammatory effects.

MSCs have been shown to improve inflammation in a variety of disease models including protection airway from allergen-induced pathology[105-107]. 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[108, 109]. 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[72]. 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[110]. TSLPR-deficient mice fail to develop an inflammatory lung response to inhaled antigen indicted that TSLP plays a critical role in the development of inflammatory and/or allergic responses in the asthma model[53]. Lung-specific overexpression of TSLP induces hyperreactivity and TH2-related inflammation[50]. TSLP induces the proliferation of bronchial epithelial cells and bronchial repair through regulating the production of IL-13[48]. 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.

Both naturally occurring Treg (nTreg) and inducible Treg (iTreg) cells are thought to effectively attenuate airway inflammation and improve airway function[111, 112]. Foxp3$^+$ Treg population in thoracic LN was increased in SSEA-1$^+$ PSCs-treated asthmatic mice which raises the possibility that engrafted SSEA-1$^+$ PSCs might increase the recruitment of Treg into inflamed site or promote the generation of iTreg in local tissue. Transgenic mice overexpressing IL-4 in the airway have decreased expression of CCSP[42, 113], suggested that IL-4 regulate CCSP expression. SSEA-1$^+$ PSCs inhibited IL-4 and increased airway CCSP production in our asthmatic mice, the possible molecular mechanism is that engrafted SSEA-1$^+$ PSCs ameliorate allergen-induced airway inflammation might associated with increase of Treg population or regulate original CCSP-positive epithelial cell through paracrine manner in the inflamed lung tissue, and subsequently inhibit TH2 cytokines and preserve airway CCSP expression and sustain its anti-inflammation effects.

Previous study indicated that CCSP suppressed allergen-induced inflammatory responses *in vivo*[87]. We found that anti-CCSP antibody restored SSEA-1$^+$ PSCs-induced TSLP but not eotaxin, suggested that CCSP might not to 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 the future.

V. CONCLUSION AND PROSPECTS

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 in primary lung epithelial cells. We also identified SSEA-1$^+$ PSCs play an immunomodulatory role in the pathogenesis of asthma. Transplantation of SSEA-1$^+$ PSCs ameliorated the allergen-induced airway inflammation and airway damage in the acute 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.

VI. FIGURES

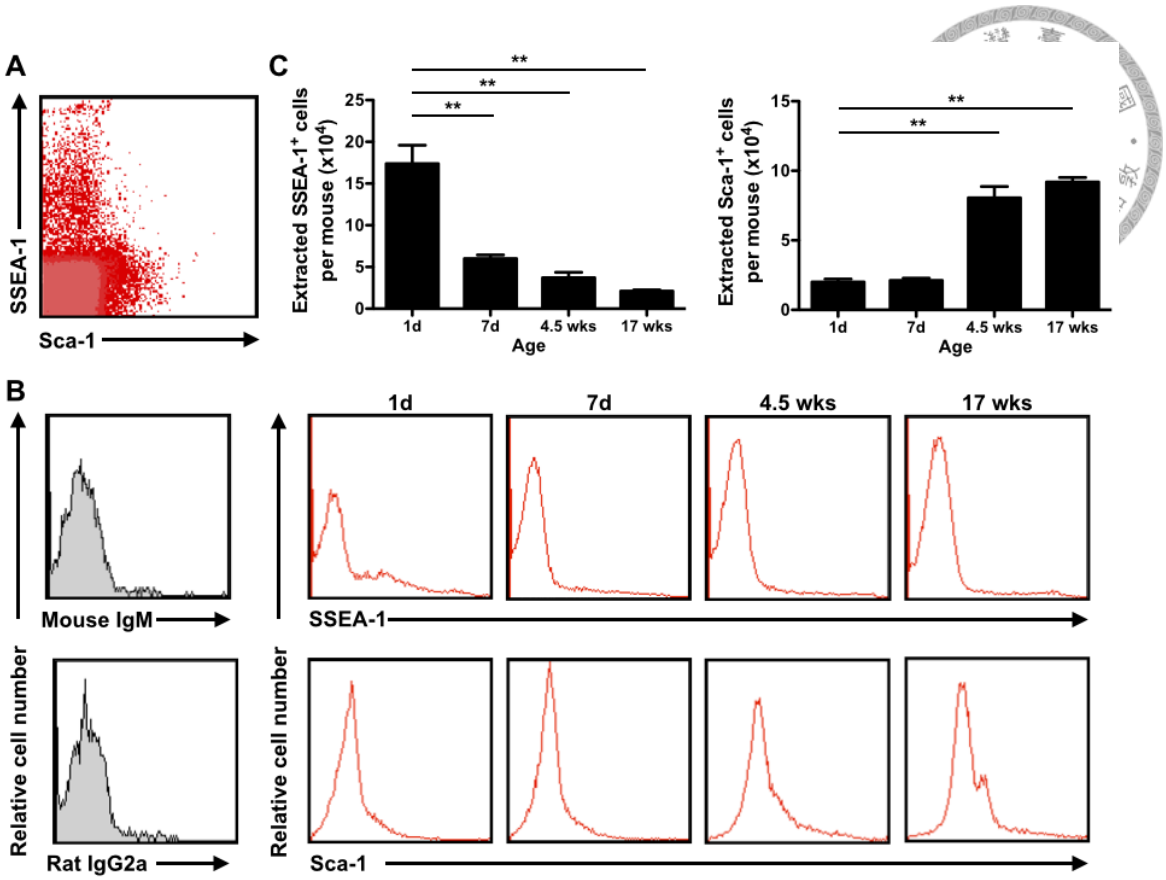


Figure 1. Identification of neonatal PSCs. (A) Neonatal pulmonary cells were labeled with Sca-1 and SSEA-1 antibodies and analyzed using flow cytometry. The data are representative of five independent experiments. **(B)** Representative flow cytometry histograms of SSEA-1 and Sca-1-stained cell population 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 ± SD and represent three independent experiments. ** P <0.01.

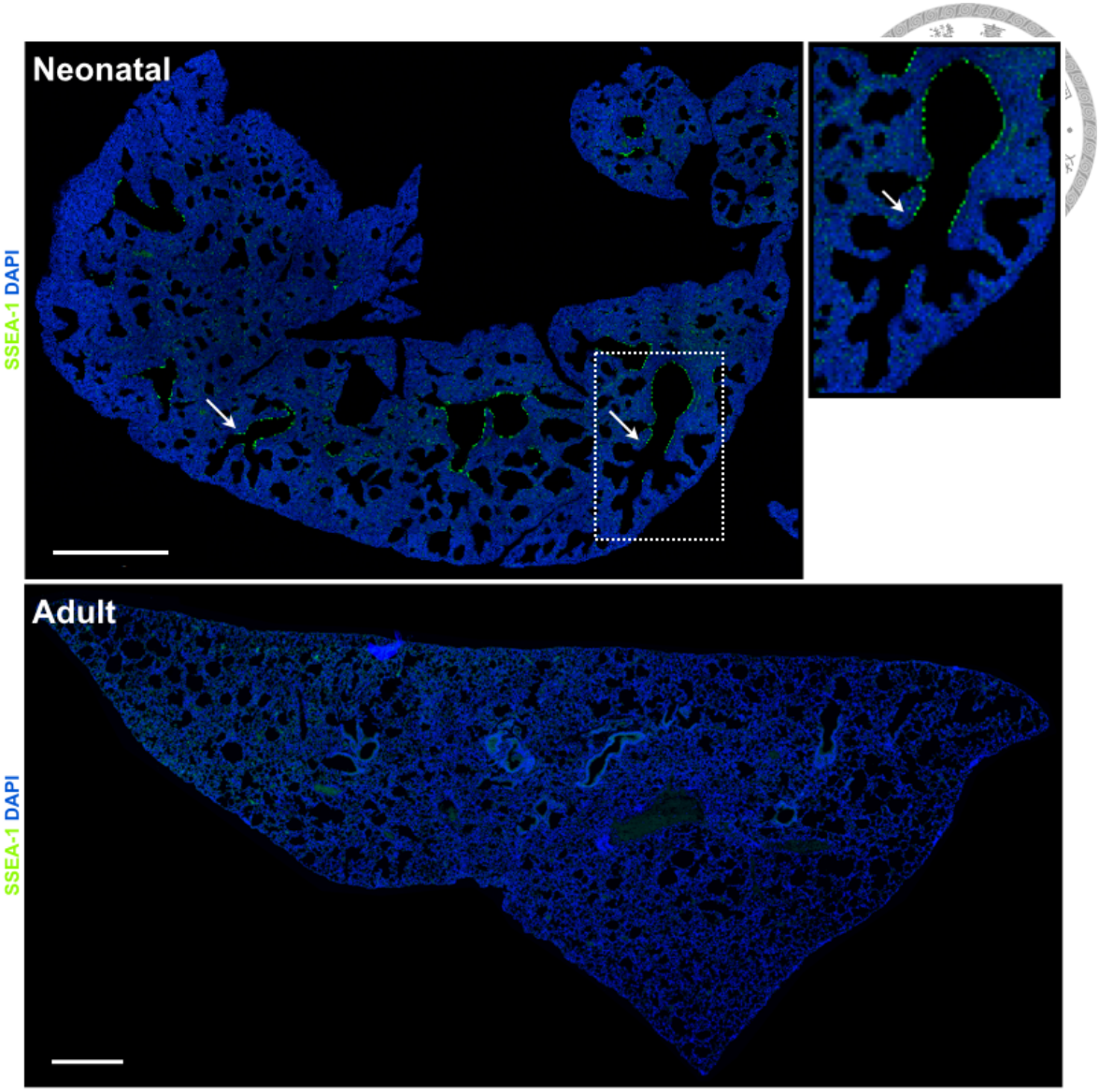


Figure 2. Whole-mount view of SSEA-1^{+} pulmonary cells in lung tissues. Neonatal and adult mice derived lung sections were stained with anti-SSEA-1 (green) and the nuclei were counterstained with DAPI (blue). Bar: 500 μm. Data are representative of at least five independent experiments.

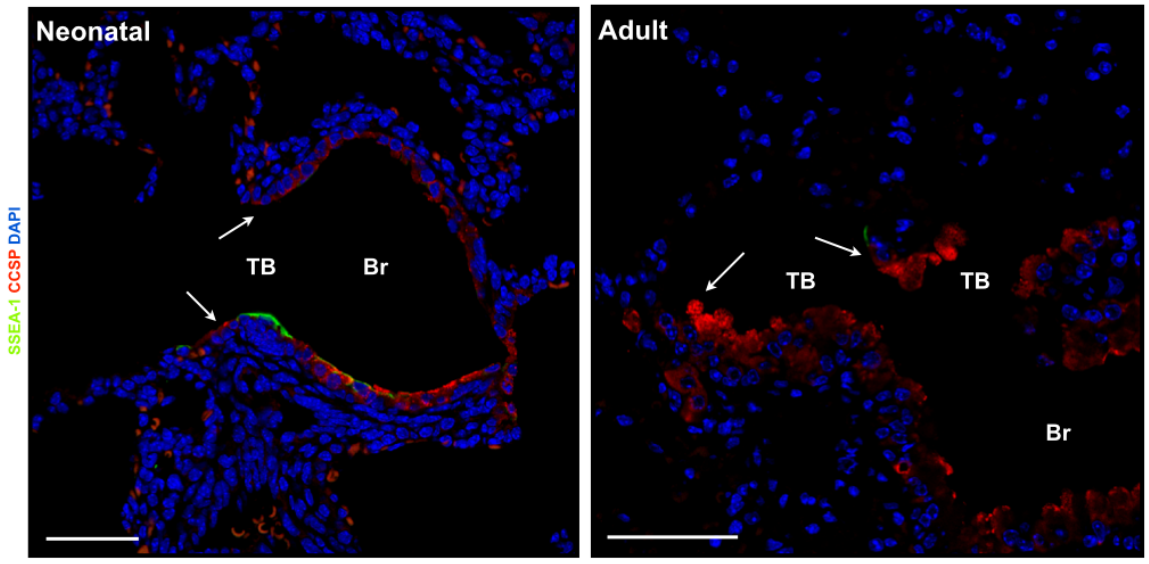


Figure 3. Expression of SSEA-1$^+$ pulmonary cells in lung tissues. Neonatal and adult mice derived lung sections were stained with anti-SSEA-1 (green) and CCSP (red); the nuclei were counterstained with DAPI (blue). The bronchiole (Br) and terminal bronchiolar (TB) airway were determined by CCSP (red) staining. Neonatal (left) and adult (right) SSEA-1$^+$ pulmonary cells were located within TB adjacent to the bronchioalveolar duct junction (BADJ). Bars: 50 μm. The arrows point to the BADJ. Data are representative of at least five independent experiments.

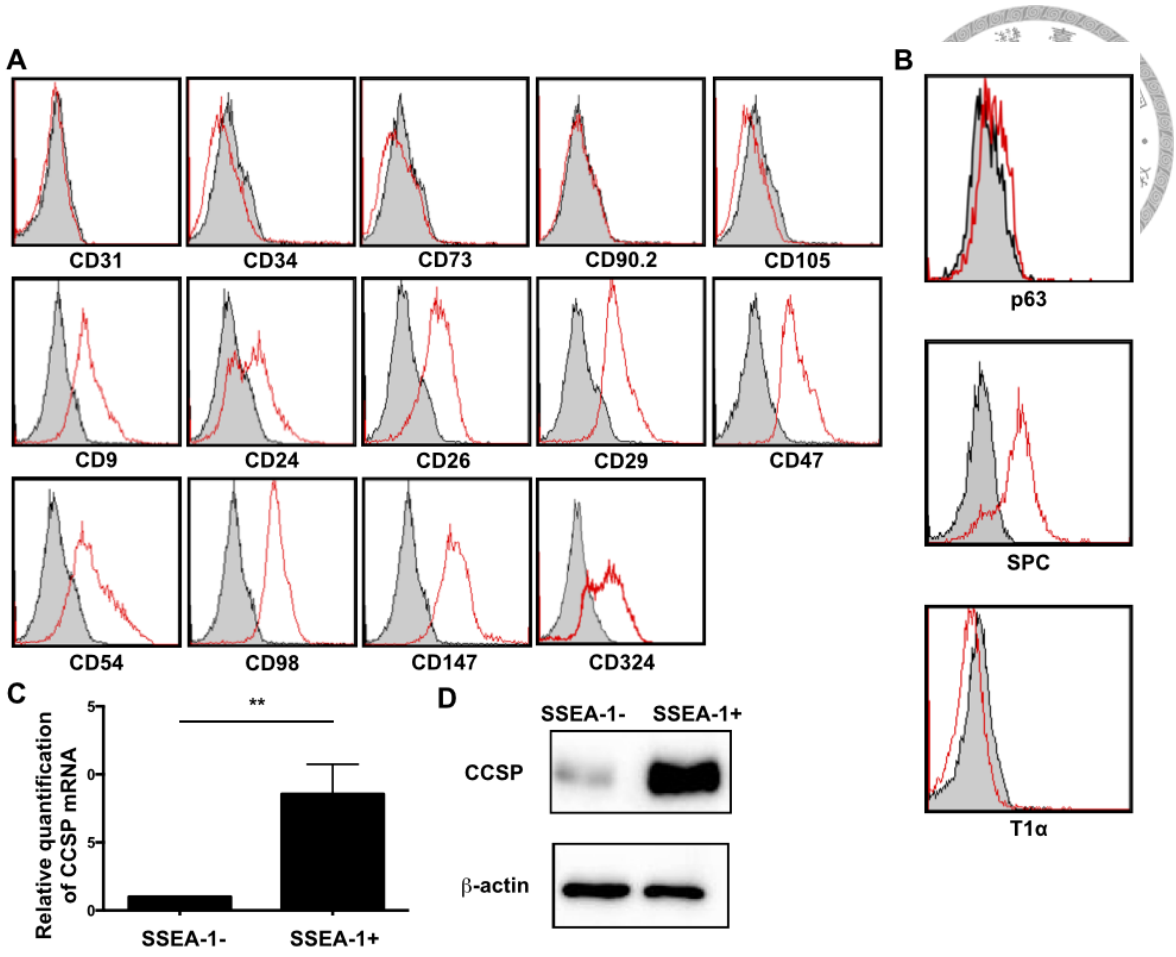


Figure 4. Characterization of neonatal SSEA-1$^+$ pulmonary cells. (A) Flow cytometric analysis of surface marker expression in neonatal SSEA-1$^+$ pulmonary cells. The gray areas represent matched isotype controls. Data are representative of three independent experiments. **(B)** FACS analysis of intracellular SPC and surface T1α expression in SSEA-1$^+$ PSCs. SSEA-1$^+$ PSCs 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$^+$ PSCs were purified from neonatal mice, and the CCSP expression levels were determined using RT-QPCR with specific primers. The mRNA levels were normalized using the housekeeping gene GAPDH. Bars indicate mean ± SD of three independent experiments. ** P <0.01. **(D)** Lysates of enriched neonatal SSEA-1$^+$ PSCs were

prepared, and the CCSP protein levels were determined by immunoblots stained with a specific antibody against CCSP. β -actin was used as an internal control. Data are representative of two independent experiments.

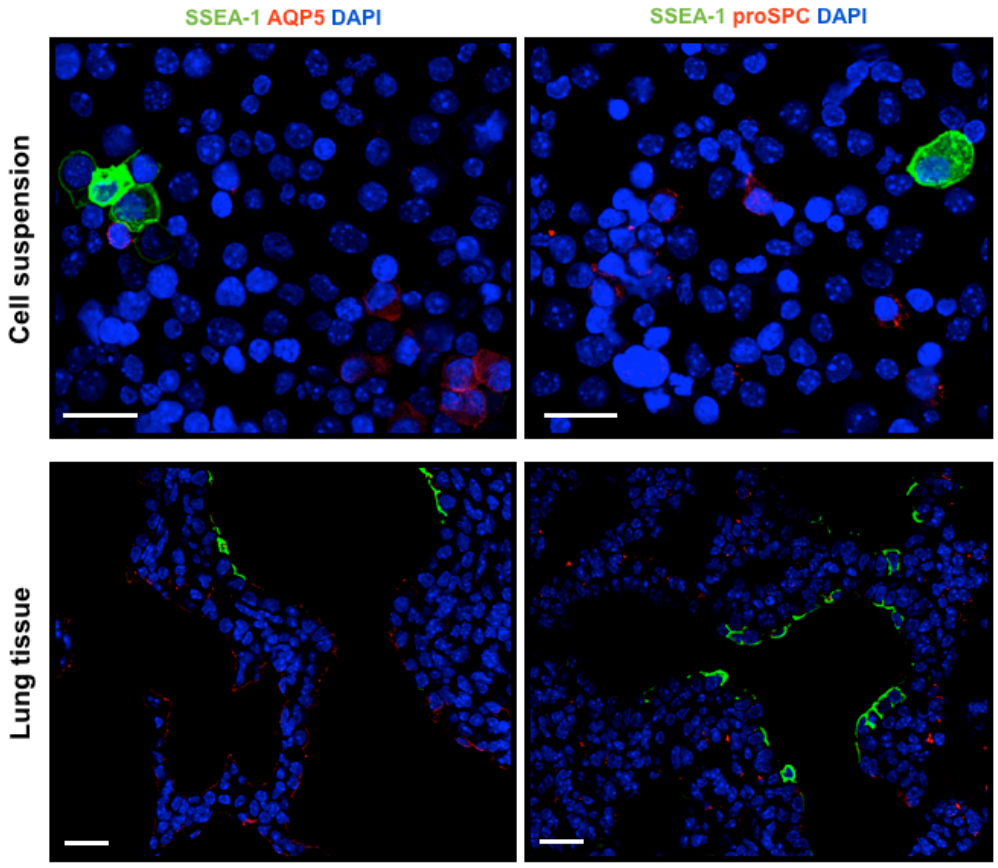


Figure 5. Neonatal SSEA-1^{+} pulmonary cells are negative for pneumocyte-related markers. Newborn mice-derived pulmonary single-cell suspensions and paraffin-fixed lung tissues were stained with SSEA-1 (green), alveolar cell markers (red) AQP5 (a type I pneumocyte marker), and proSPC (a type II pneumocyte marker). The nuclei were counterstained with DAPI (blue). Bars, 20 μm.

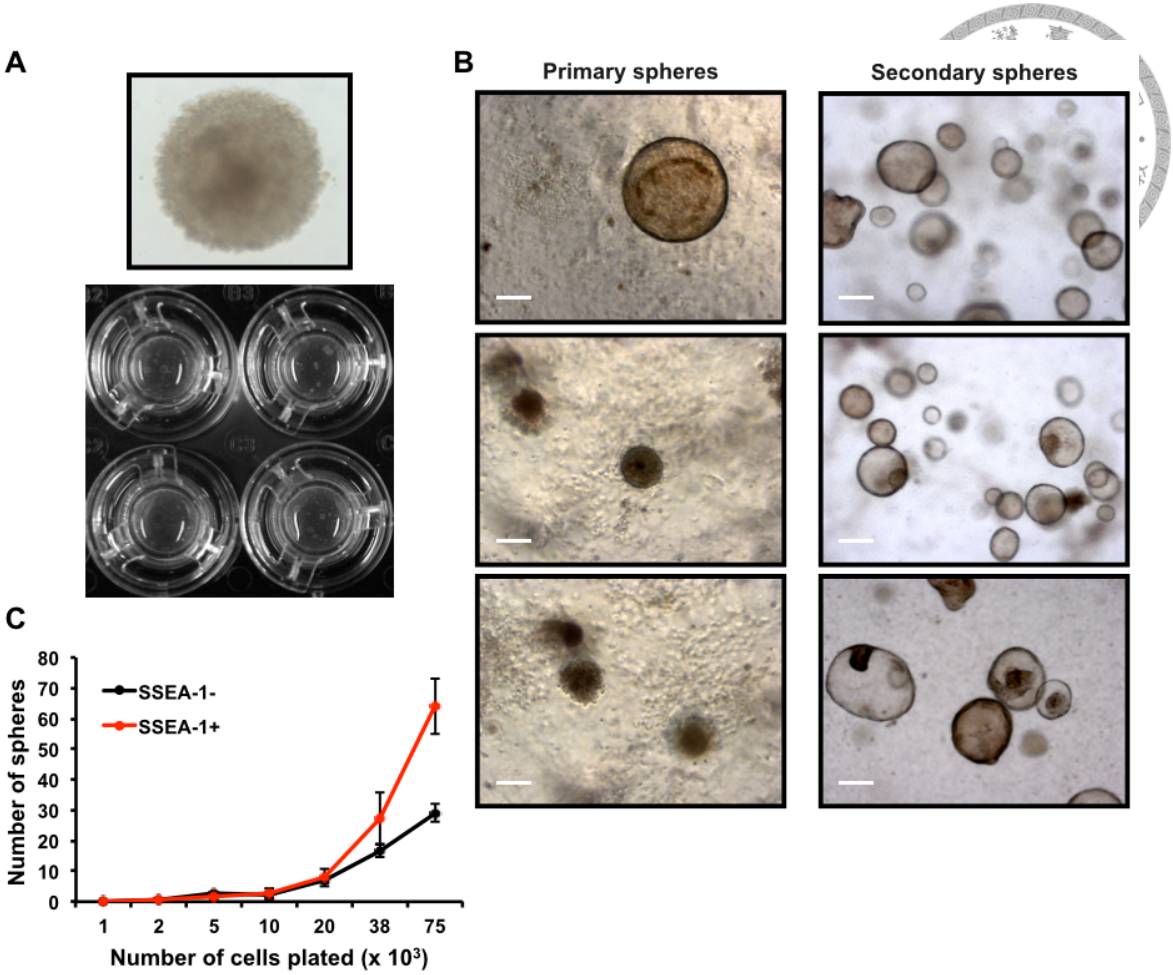


Figure 6. Neonatal SSEA-1$^+$ pulmonary cells are self-renewal and clonogenicity. (A) Representative photographs of sphere colonies cultured in Matrigel with Transwell plates. (B) Representative photographs of primary and secondary sphere colonies. Bars, 100 μm. (C) Efficiency of secondary spheres formation. The results are expressed as means ± SD (n = 3).

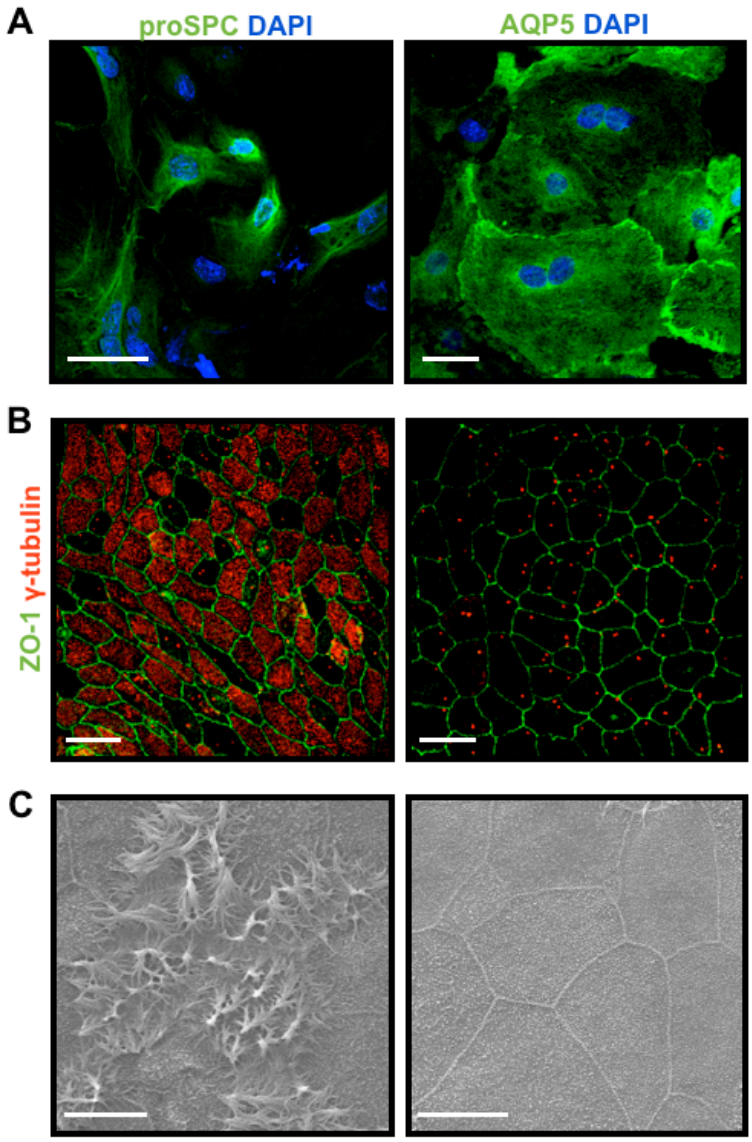


Figure 7. Neonatal SSEA-1$^+$ pulmonary cells are multipotent. (A) SSEA-1$^+$ PSCs cultured on Matrigel-coated plate for 15-20 days were stained with pro SPC (green; left) and AQP5 (green; right) antibody to identify the type I pneumocyte; the nuclei were counterstained with DAPI (blue). Bars, 20 μm. (B) SSEA-1$^+$ PSCs seeded in an ALI culture for 15 days were labeled for ZO-1 (green), γ-tubulin (red), and DAPI (blue). Bars, 20 μm. (C) Scanning electron microscopic images of *in vitro*-cultured SSEA-1$^+$ PSCs at ALI for 15 days, showing ciliated and nonciliated cells. Bar, 10 μm.

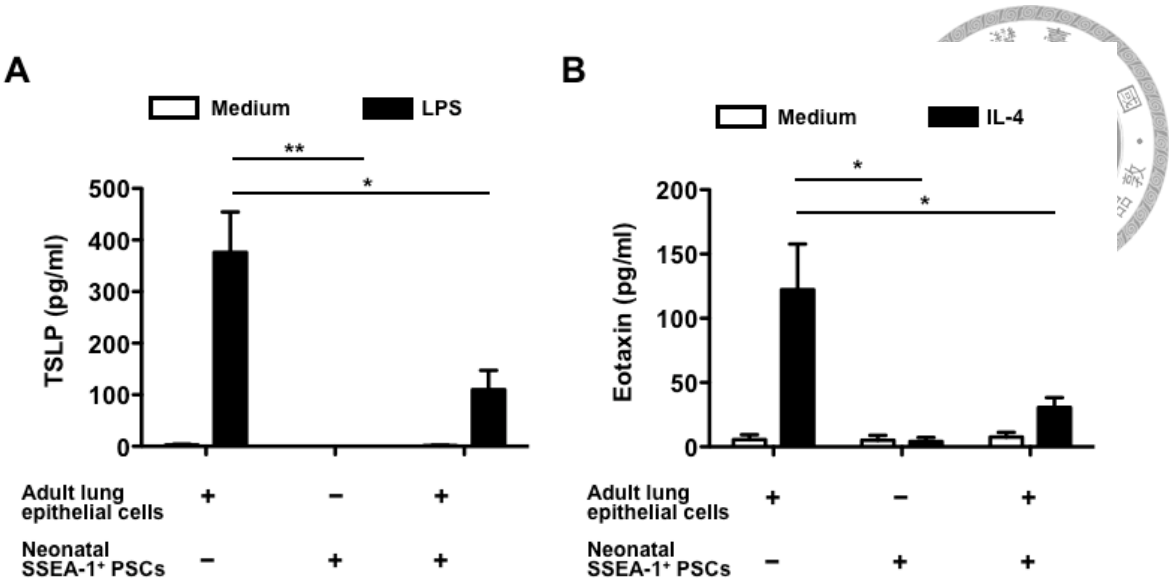


Figure 8. Neonatal SSEA-1$^+$ PSCs suppress TSLP and eotaxin production. Lung epithelial cells (2×10^4) derived from adult mice were co-cultured with neonatal SSEA-1$^+$ PSCs (1×10^4) in the presence of (A) LPS (2 μg/ml) or (B) IL-4 (100 ng/ml) for 24 hours. TSLP and eotaxin contents in culture supernatants were determined using ELISA. Values are the means ± SD. The data are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

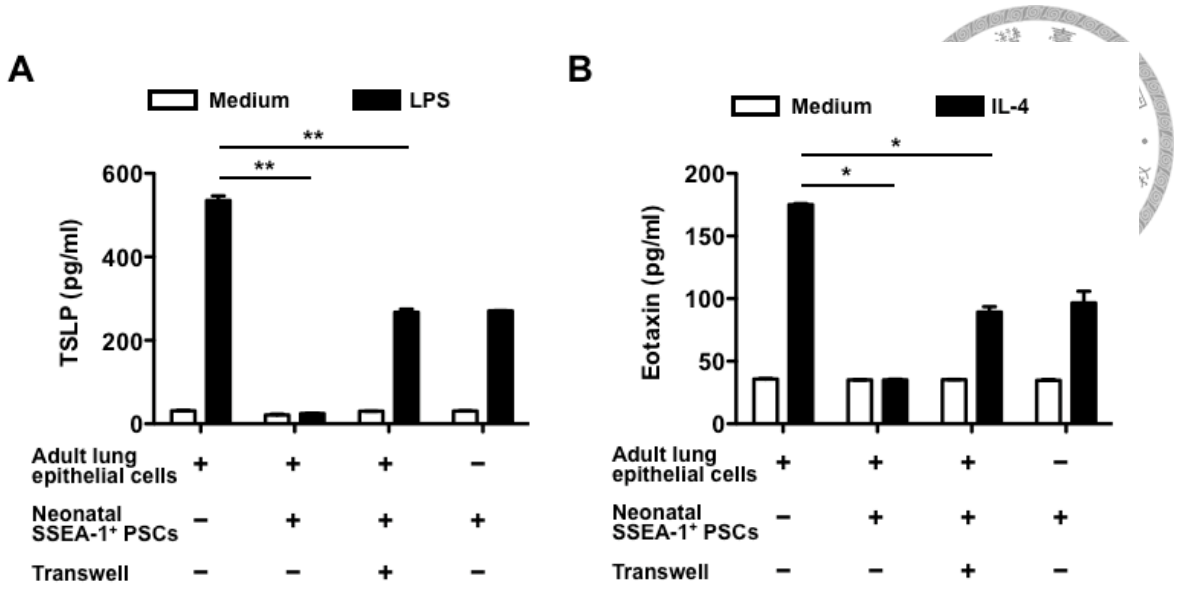


Figure 9. SSEA-1$^+$ PSCs suppress TSLP and eotaxin production through cell-cell contact independent manner, respectively. Co-culture system of adult lung epithelial cells (2×10^4; bottom) and neonatal SSEA-1$^+$ PSCs (1×10^4; insert) were separated with a Tranwell and stimulated with (A) LPS (2 μg/ml) or (B) IL-4 (100 ng/ml) for 24 hours. TSLP and eotaxin contents in culture supernatants were determined using ELISA. Values are the means ± SD. The data are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

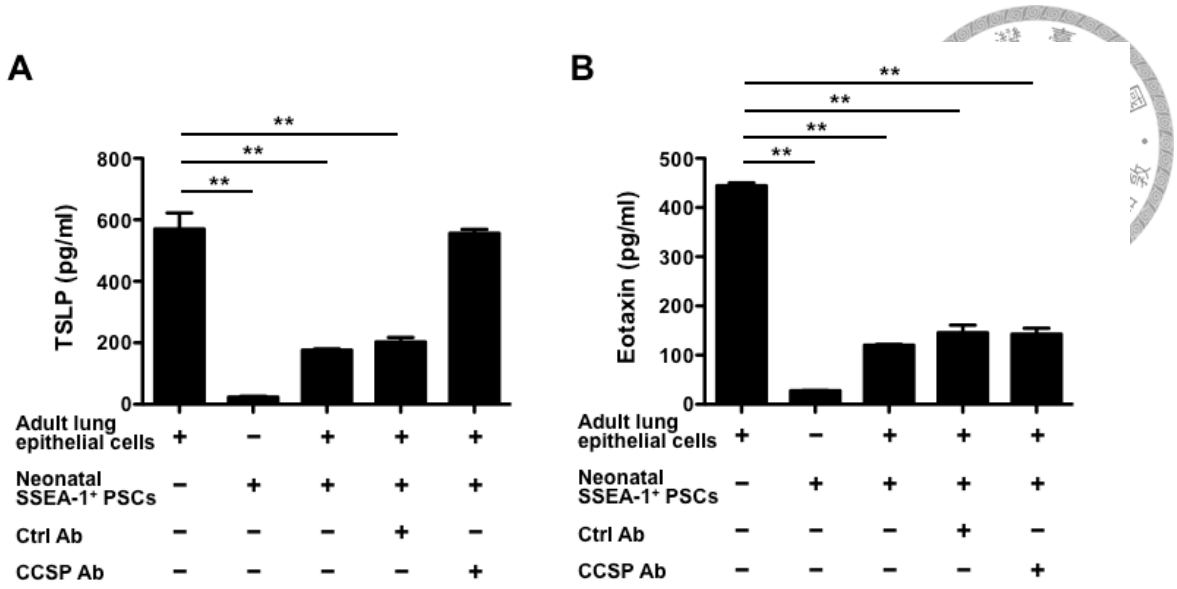


Figure 10. Neonatal lung SSEA-1$^+$ PSCs reduced TSLP and eotaxin production from adult lung epithelial cells through CCSP-dependent and -independent manner. Lung epithelial cells derived from adult mice were co-cultured with neonatal SSEA-1$^+$ PSCs and stimulated with (A) LPS or (B) IL-4 for 24 hours in the presence of control antibody or CCSP neutralizing antibody (5 μg/ml). TSLP and eotaxin contents in LPS- and IL-4-stimulated culture supernatants were determined using ELISA, respectively. Data are means ± SD and representative of three independent experiments. ** $P < 0.01$.

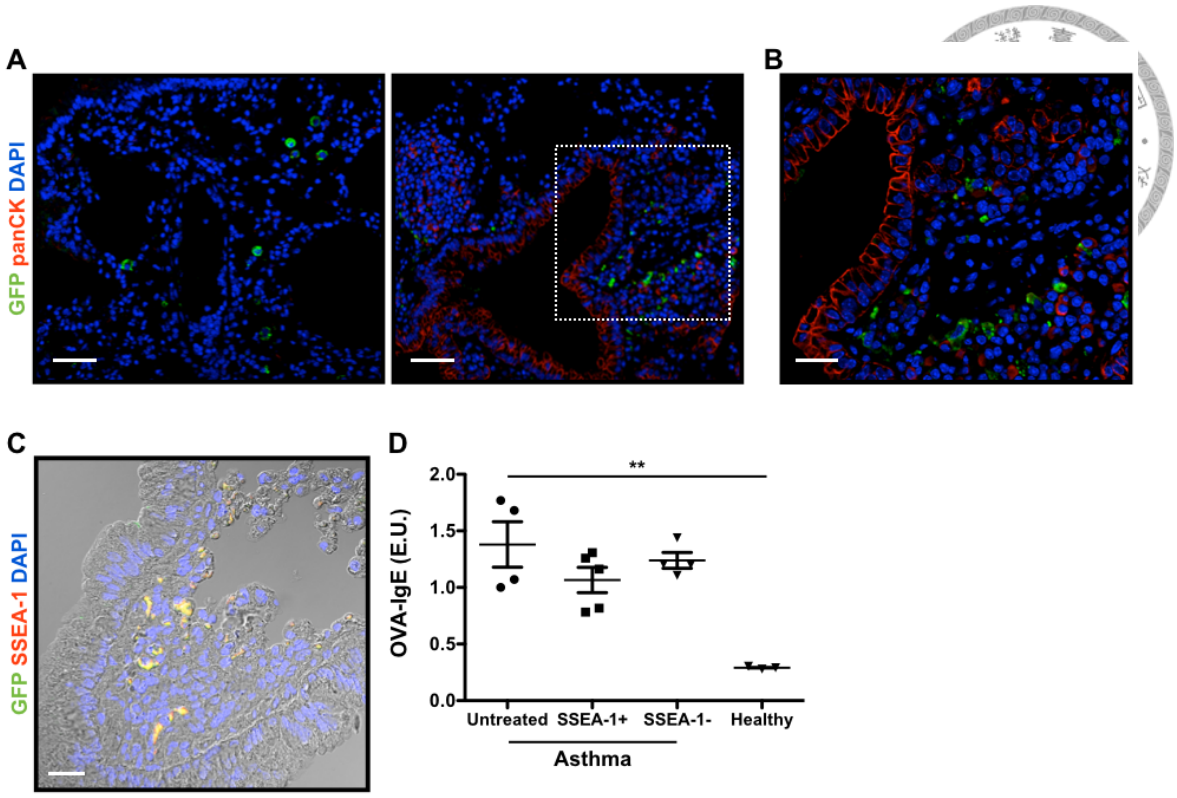


Figure 11. Transplantation of SSEA-1$^+$ PSCs did not change serum OVA-specific IgE in the asthmatic mice. (A) Distribution of SSEA-1$^+$ PSCs in adoptive transferred mice. The lung sections were stained with an anti-GFP- (green) and an anti-panCK (red) antibody, and they were counterstained with DAPI (blue). Bars, 50 μm. (B) Magnified view of the boxed area in A. Bar, 50 μm. (C) Transplanted PSCs maintained SSEA-1 expression after repeated OVA challenge *in vivo*. The lung sections were stained with an anti-GFP- (green) and an anti-SSEA-1 (red) antibody, and they were counterstained with DAPI (blue). Bar, 20 μm. (D) 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 ± SEM and representative of two independent experiments (n = 5 mice per group).

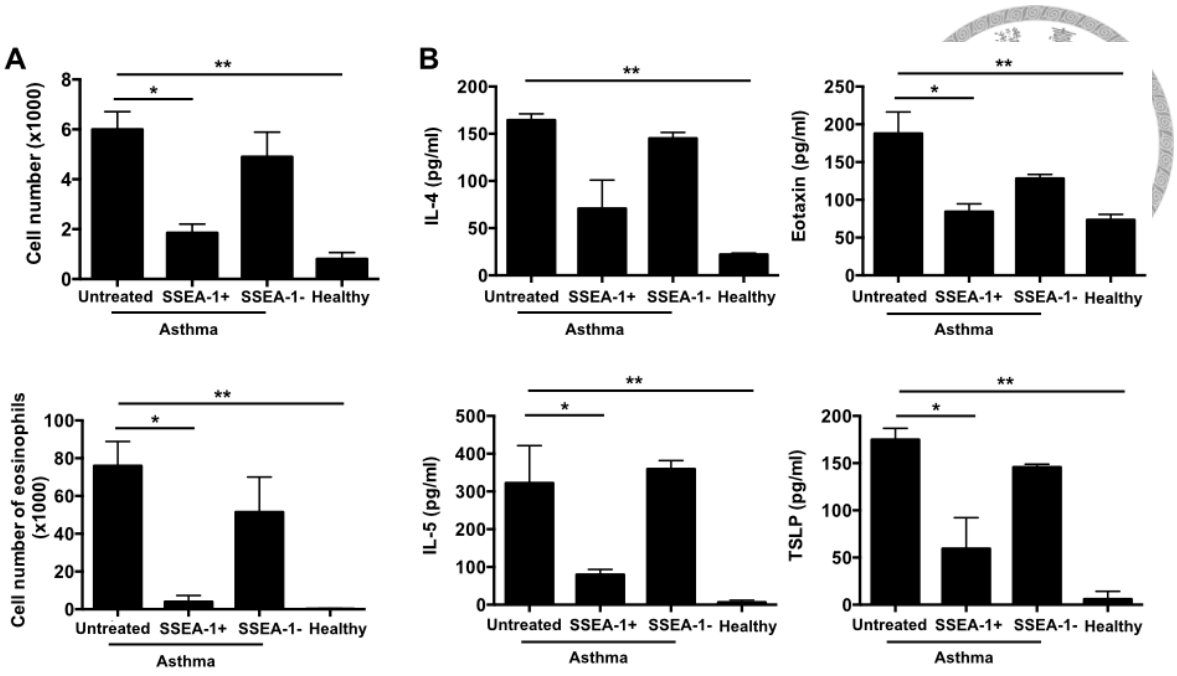


Figure 12. Transplantation of SSEA-1$^+$ PSCs before the OVA challenge reduced the level of inflammatory mediators and the cellular infiltration of the lungs. 1×10^5 SSEA-1$^+$ PSCs isolated from neonatal mice were i.v. injected into OVA-induced asthmatic mice prior to an OVA aerosol challenge. (A) The BAL fluid was taken 24 hours after the last aerosol OVA exposure. Differential counts of the cells in the BAL fluid were obtained by FACS analysis, and (B) The levels of IL-4, IL-5, TSLP, and eotaxin in the BAL fluid was determined by ELISA. n = 3-5 mice per group. Bars represent means ± SD. * $P < 0.05$, ** $P < 0.01$. The data from one representative experiment of two are shown.

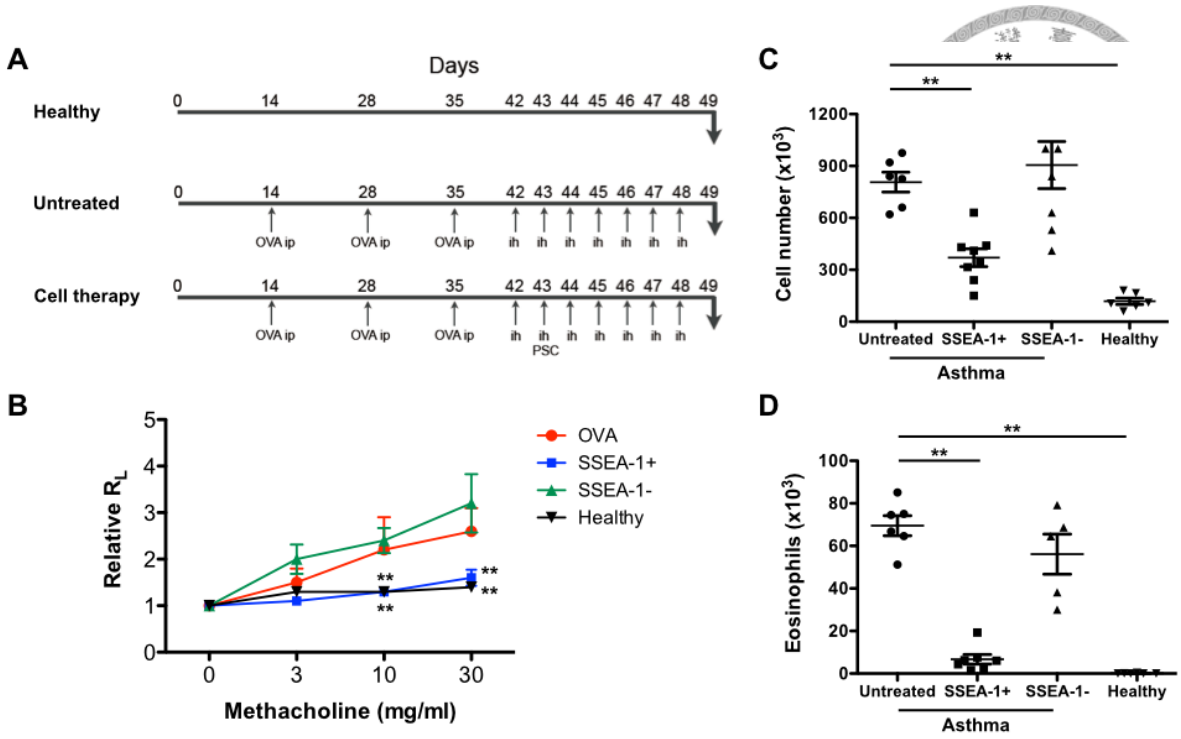


Figure 13. Transplantation of SSEA-1$^+$ PSCs improves AHR and airway eosinophilia in the asthmatic mice. (A) Flowchart of the method used to produce the OVA-induced asthmatic murine model. (B) Airway function was measured by invasive body plethysmography. The results are expressed as means ± SEM of the lung resistance (RL) in the ratio of RL after PBS nebulization. Data are represent two independent experiments (n = 4-7 mice per group). ** $P < 0.01$. (C) Cell number in the recovered BAL fluid was counted after staining by trypan blue. (D) Eosinophil counts in the BAL fluid were obtained by FACS analysis. Data are means ± SEM and representative of two independent experiments (n = 6-8 mice per group). ** $P < 0.01$.

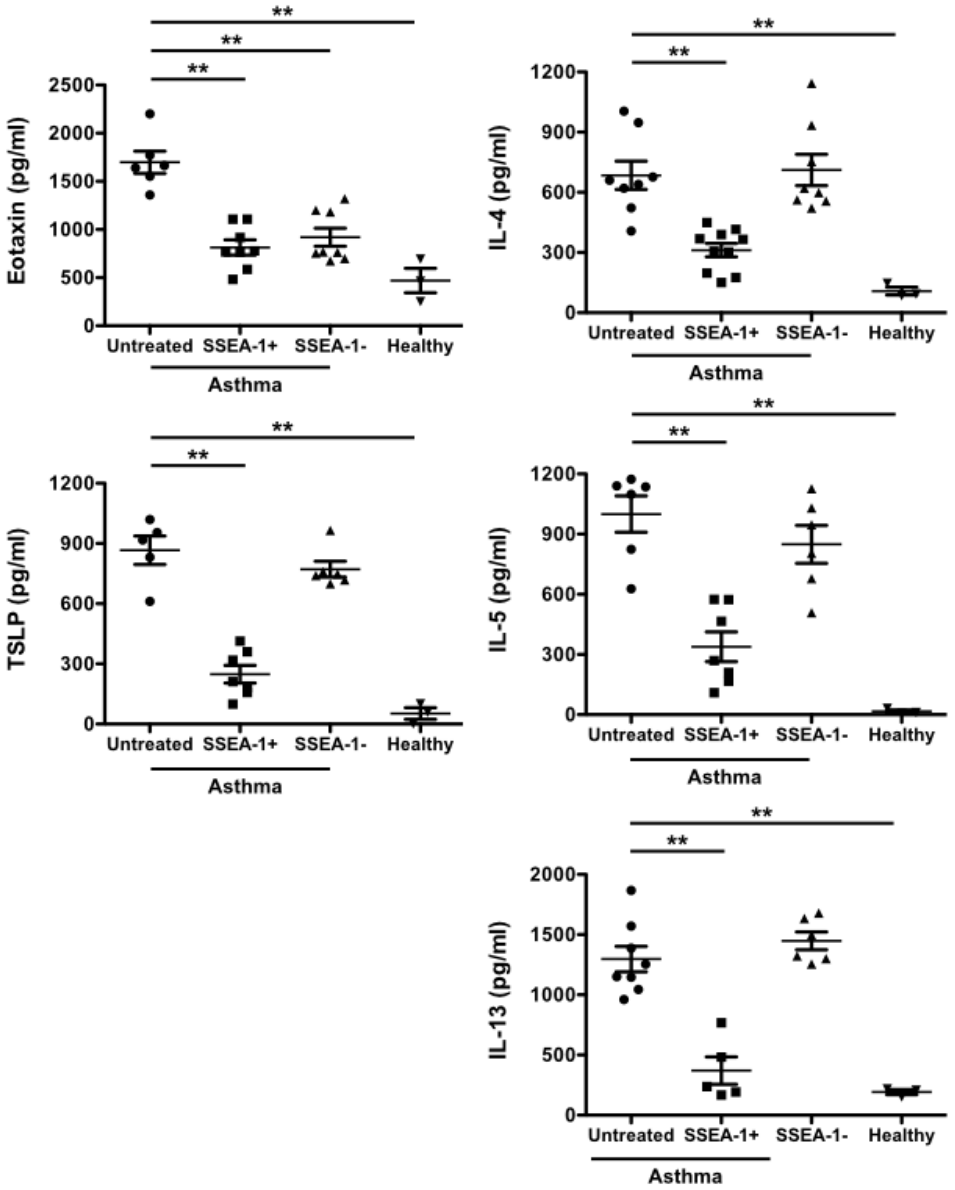


Figure 14. Transplantation of SSEA-1$^+$ PSCs reduces airway inflammatory cytokine/chemokine production in the asthmatic mice. BAL fluid was taken 24 hours after the last aerosol OVA exposure. The level of eotaxin, TSLP, IL-4, IL-5, and IL-13 in BAL fluid was determined using specific ELISA kits. Data are means ± SEM and representative of two independent experiments (n = 4-6 mice per group). ** $P < 0.01$.

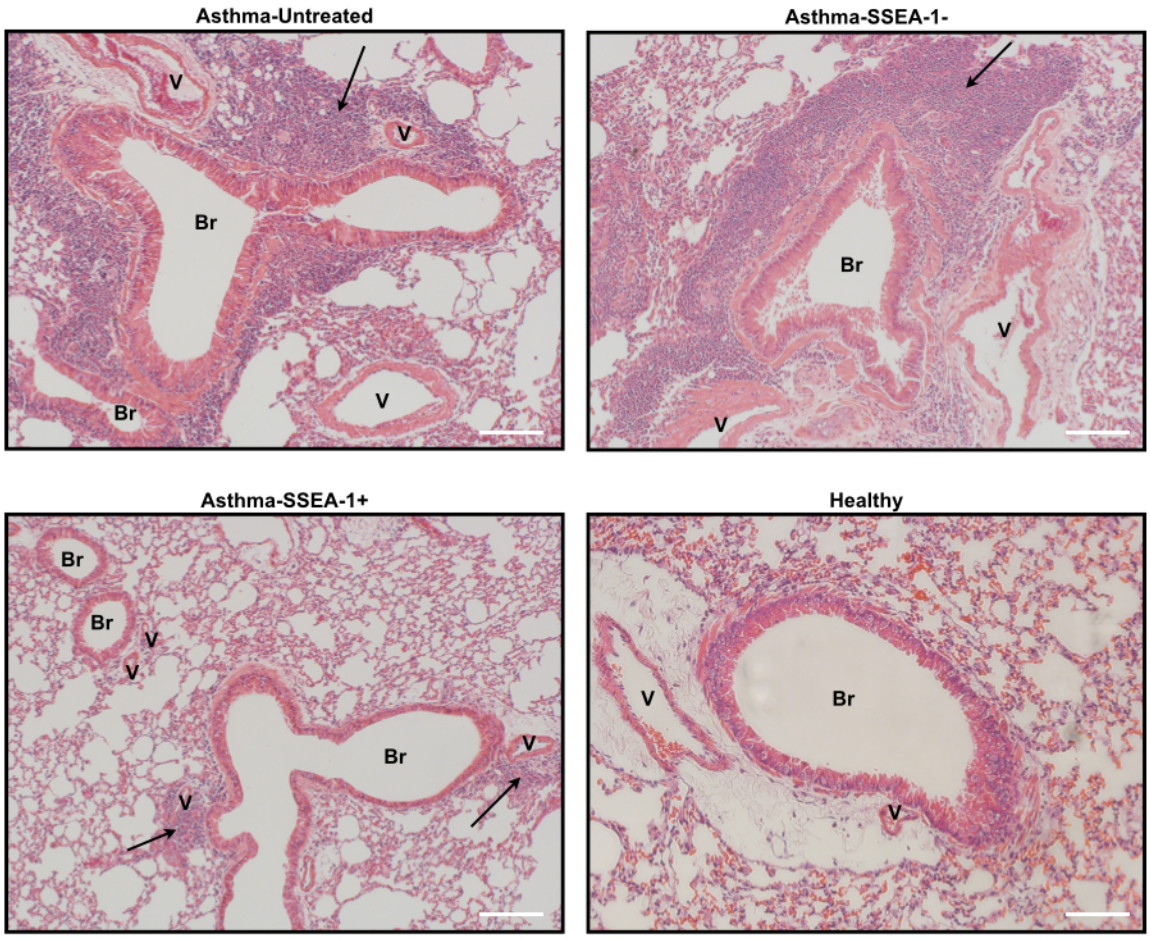


Figure. 15. Transplantation of SSEA-1^{+} PSCs reduces airway inflammation. H&E staining of lung sections in untreated, SSEA-1^{+} PSCs-, SSEA-1^{-} pulmonary cells-treated, and healthy group. Bars, 200 μm. Arrows denote infiltrated leukocytes; br, bronchus; v, vessel. Data are representative of two independent experiments.

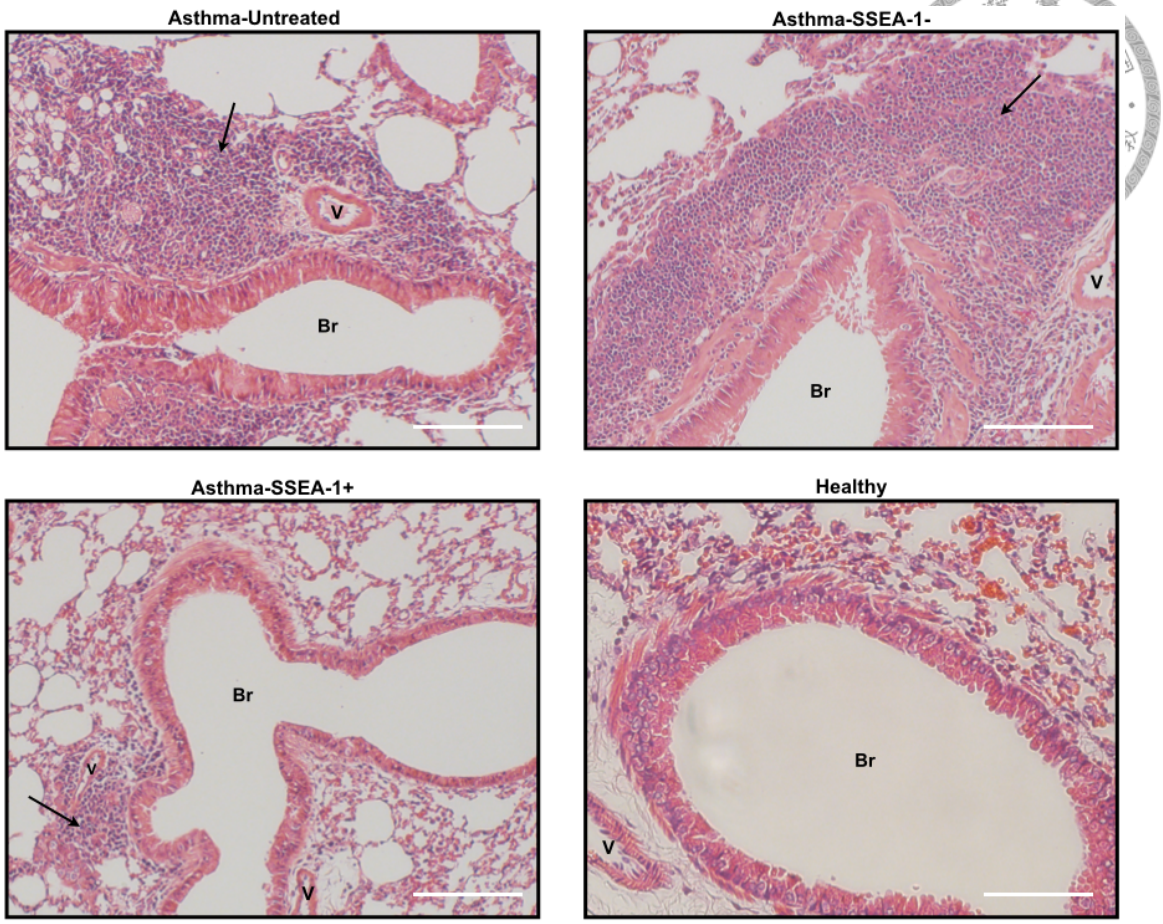


Figure 16. Transplantation of SSEA-1$^+$ PSCs decreased the infiltration of inflammatory cells into lung in the asthmatic mice. Enlarge view of Figure 15. Bars, 200 μm. Arrows denote infiltrated leukocytes; br, bronchus; v, vessel.

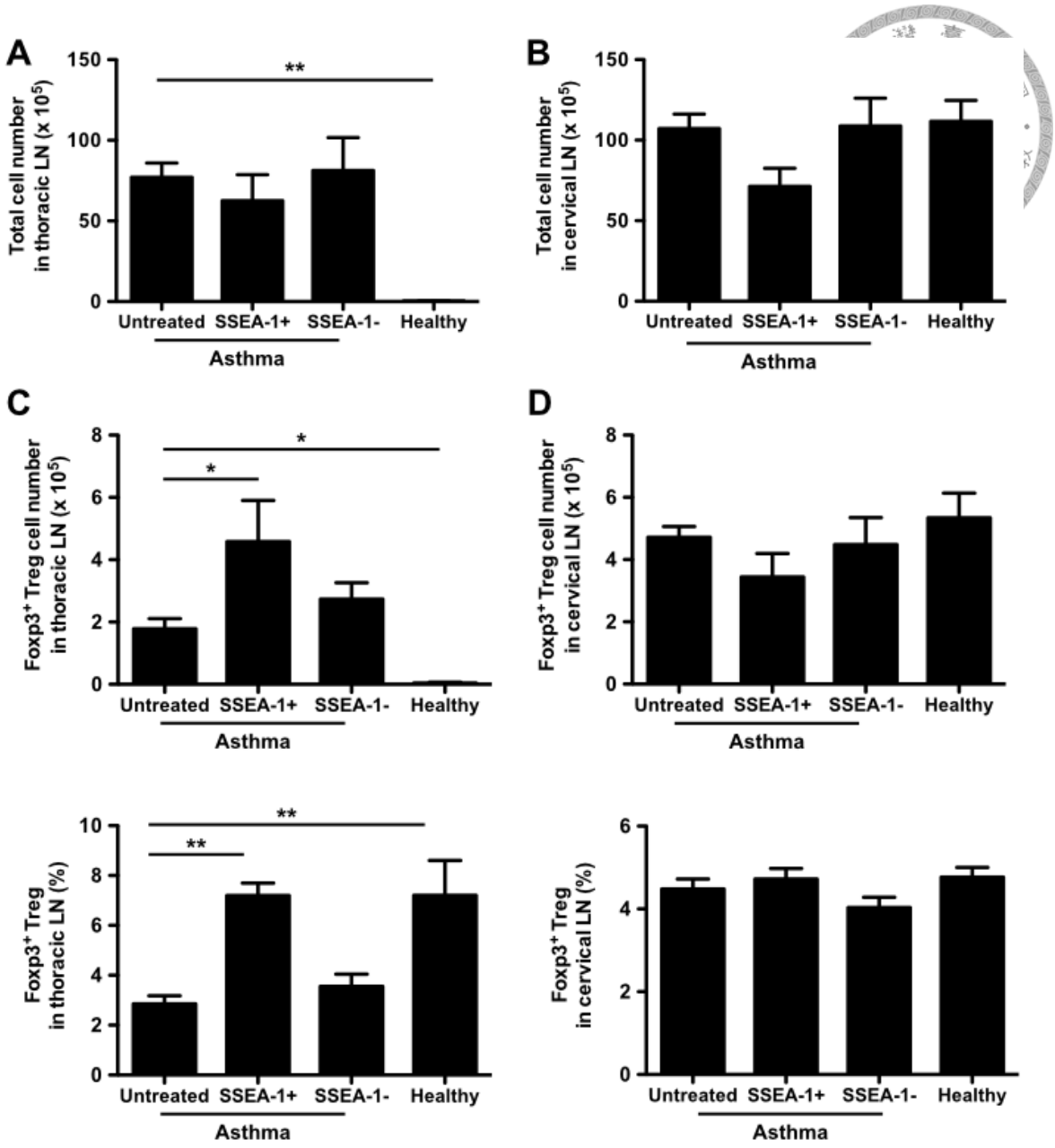


Figure 17. Transplantation of SSEA-1$^+$ PSCs increase Foxp3$^+$ Treg population.

Total cell number in (A) thoracic and (B) cervical LN. Cell number and percentage of Foxp3-GFP$^+$ CD4$^+$ Treg in (C) thoracic and (D) cervical LN determined by FACS analysis (n = 5-9 mice/group). Values are presented as the means ± SD. The data are representative of two independent experiments. * $P < 0.05$, ** $P < 0.01$.

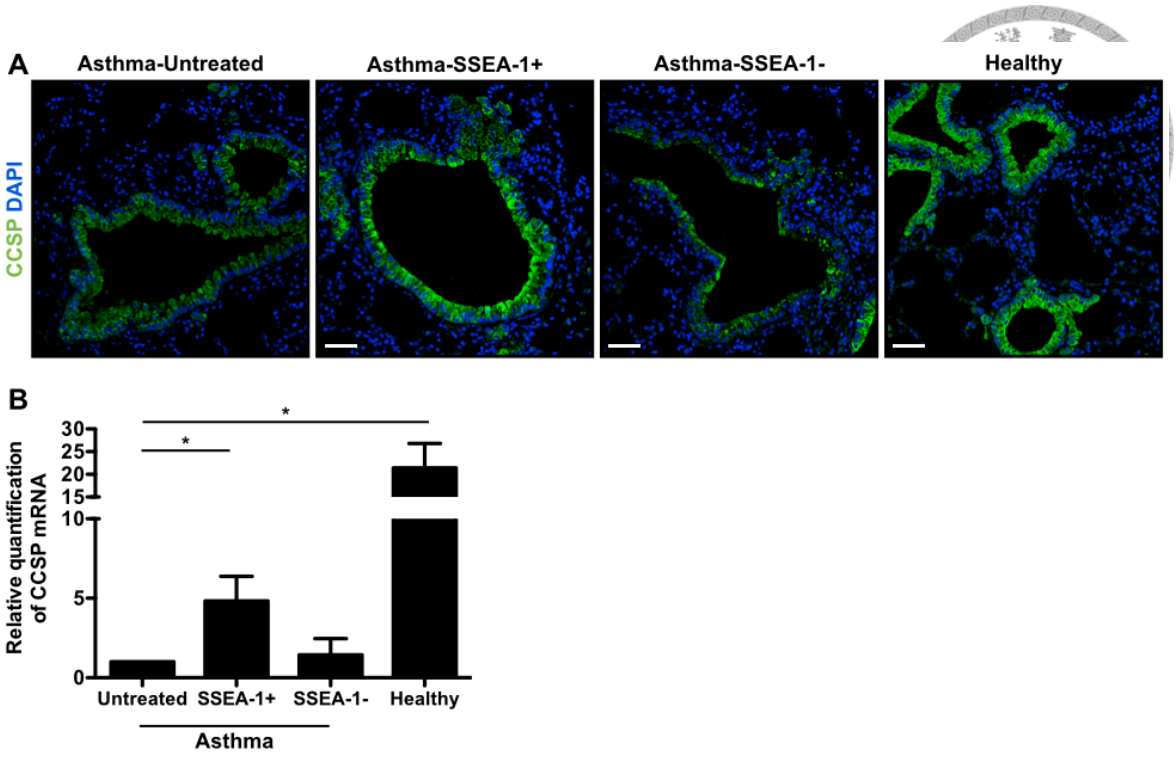


Figure 18. Transplantation of SSEA-1$^+$ PSCs preserve CCSP production. *(A)* The expression of CCSP in lung sections from untreated-, SSEA-1$^+$ PSCs-treated-, and healthy mice was analyzed using immunostaining with an anti-CCSP antibody (green); and the nuclei were counterstained with DAPI (blue). The data are representative of two independent experiments. Bars, 50 μm. *(B)* The transcript levels of CCSP in lung tissues were analyzed using RT-QPCR with specific primers. The mRNA levels were normalized with GAPDH as a housekeeping gene. * $P < 0.05$, ** $P < 0.01$.

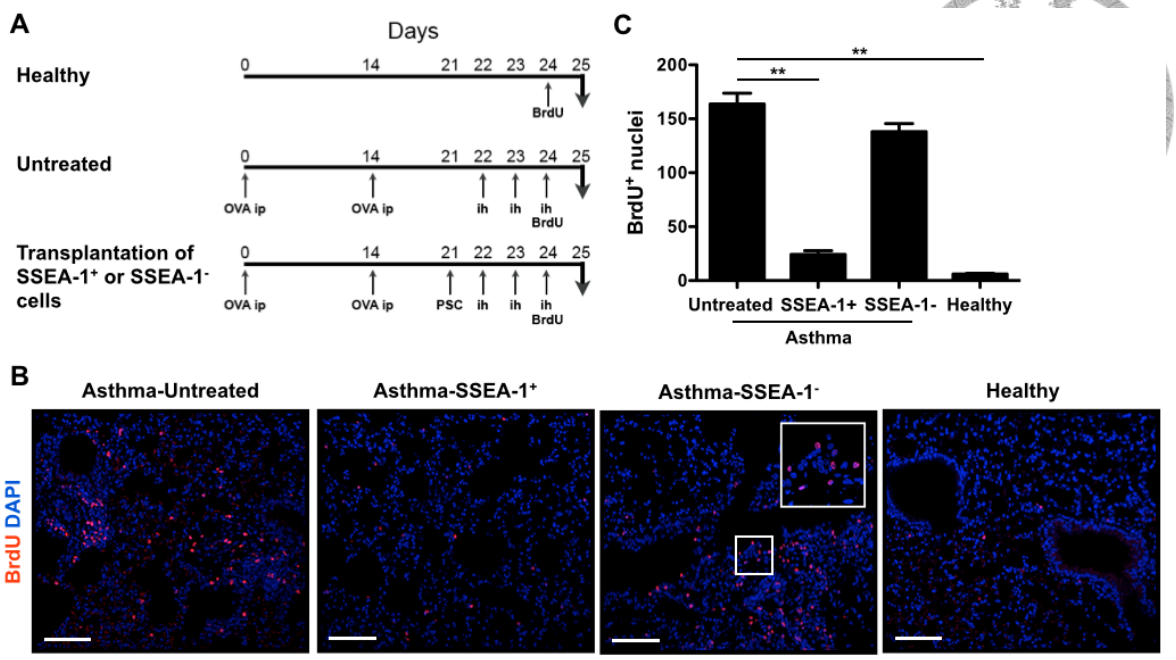


Figure 19. Transplantation of SSEA-1$^+$ PSCs inhibits unregulated cell proliferation in asthma. (A) Schematic representation of an OVA-induced asthma model with BrdU administration. (B) Representative BrdU immunostaining of proliferating cells (red); and the nuclei were counterstained with DAPI (blue). The data are representative of two independent experiments. Bars, 100 μm. (C) Graphic representation of decreased BrdU incorporation in the lungs of SSEA-1$^+$ PSCs-recipient mice. Two to three consecutive cryosections from each mouse lung were examined, and the average number of BrdU-positive nuclei per 1,000 nuclei was used as the value for the mouse. The data are expressed as the means ± SD, $n = 3$ mice/group. ** $P < 0.01$.

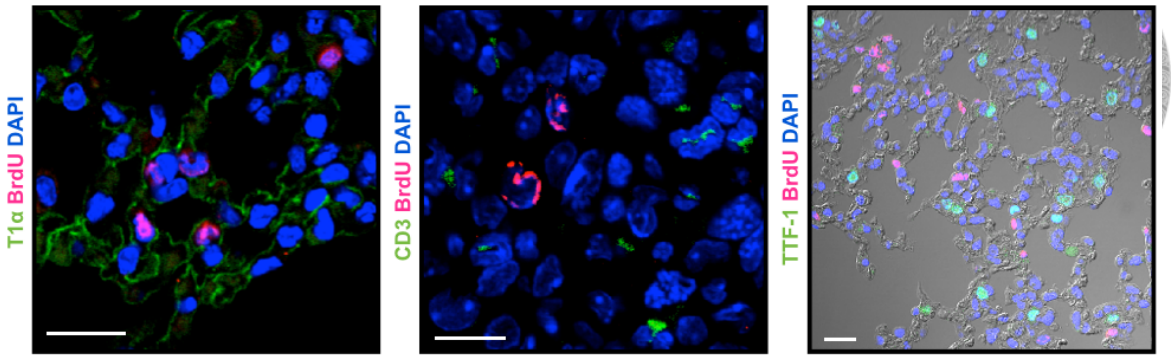


Figure 20. BrdU-incorporated cell population in asthma. Immunofluorescence staining of T1α, CD3 or TTF-1 (green), BrdU (red) and DAPI (blue) in asthmatic lung tissue. Bars, 20 μm.

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VIII. APPENDIX

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Allergy

ORIGINAL ARTICLE 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), T1α (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 T1α, 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

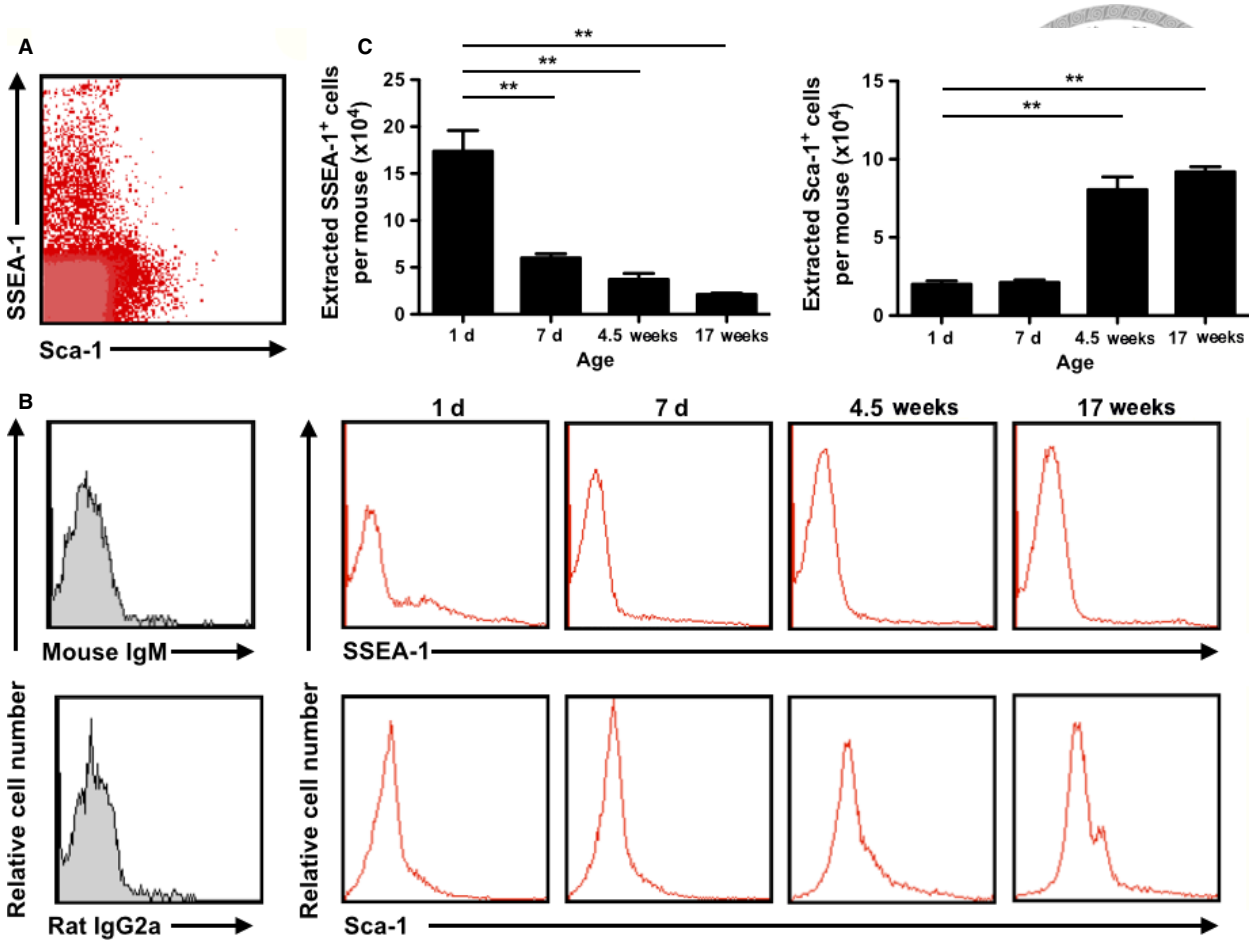

Figure 1 Identification of neonatal pulmonary stem/progenitor cells. (A) Neonatal pulmonary cells were labeled with Sca-1 and SSEA-1 antibodies and analyzed using flow cytometry. The data are representative of five independent experiments. (B) Representative flow cytometry histograms of SSEA-1 and Sca-1-stained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

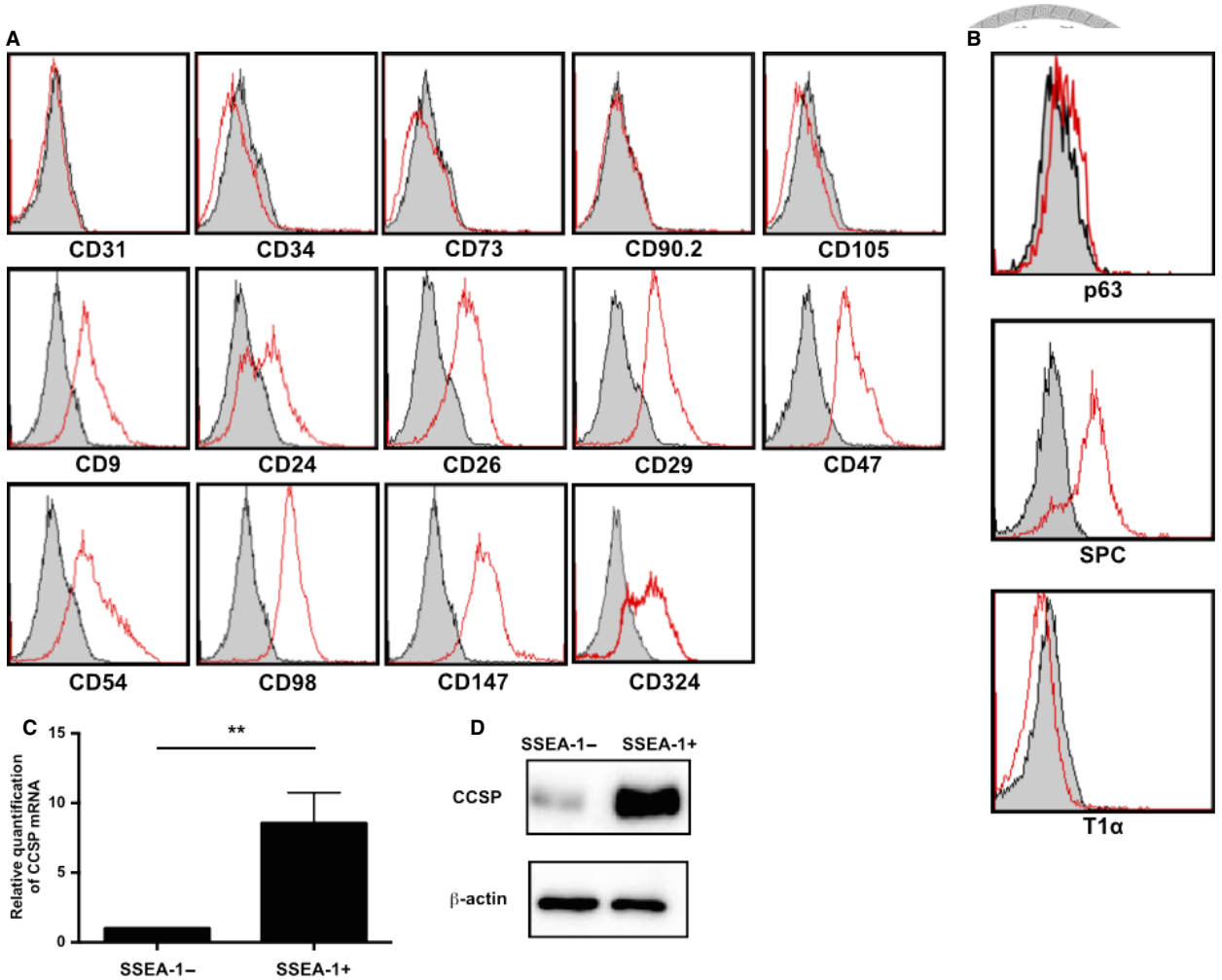

Figure 2 Characterization of neonatal SSEA-1$^+$ pulmonary cells. (A) Flow cytometric analysis of surface marker expression in neonatal SSEA-1$^+$ pulmonary cells. The gray areas represent matched isotype controls. Data are representative of three independent experiments. (B) Fluorescence-activated cell 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 ± 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

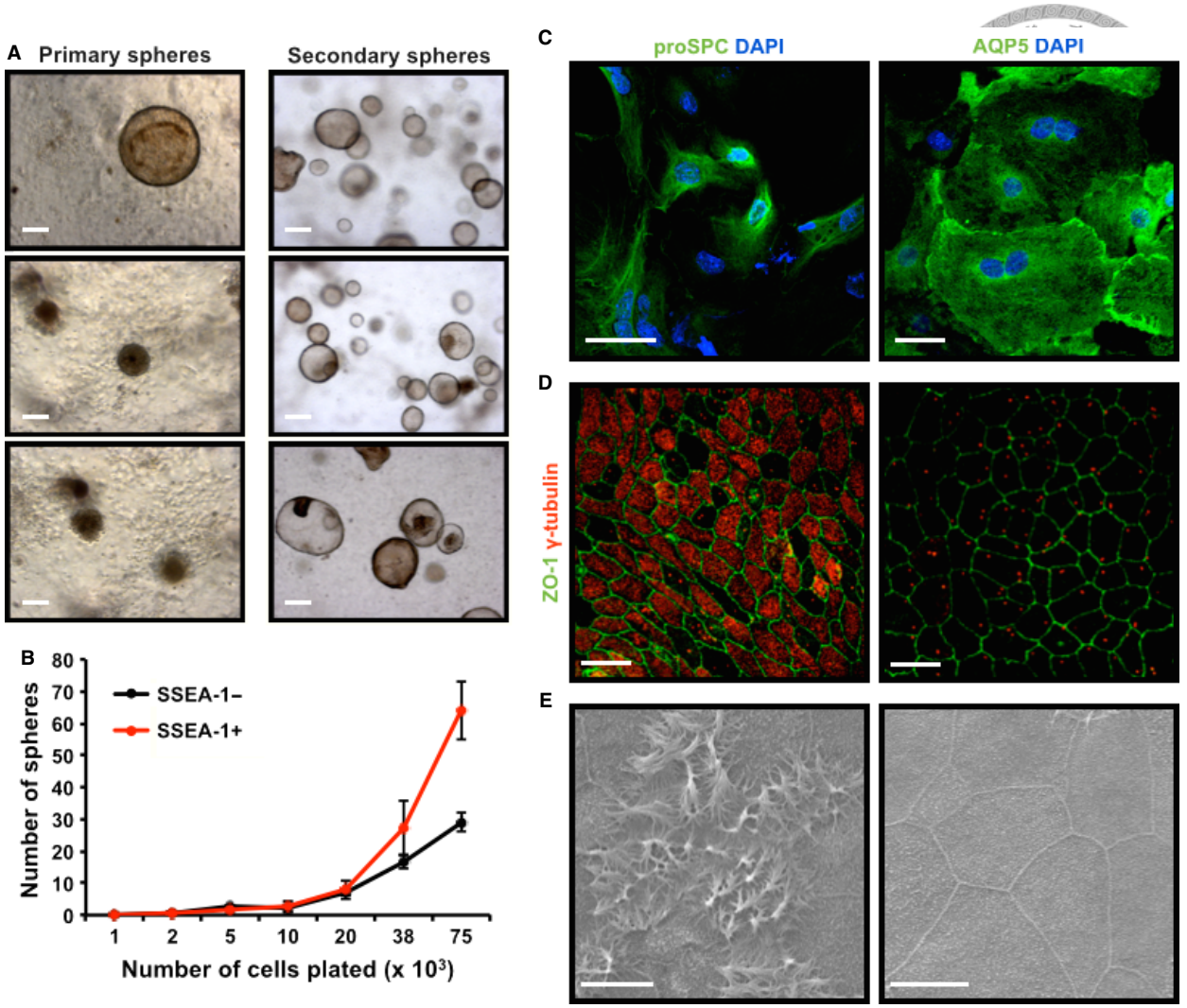

Figure 3 Neonatal SSEA-1$^+$ pulmonary cells are self-renewal and multipotent. (A) Representative photographs of primary and secondary sphere colonies. Bars, 100 μm. (B) Efficiency of secondary spheres formation. The results are expressed as means ± SD ($n = 3$). (C) SSEA-1$^+$ pulmonary cells cultured 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 μm. (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 μm. (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 μm.

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 to 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

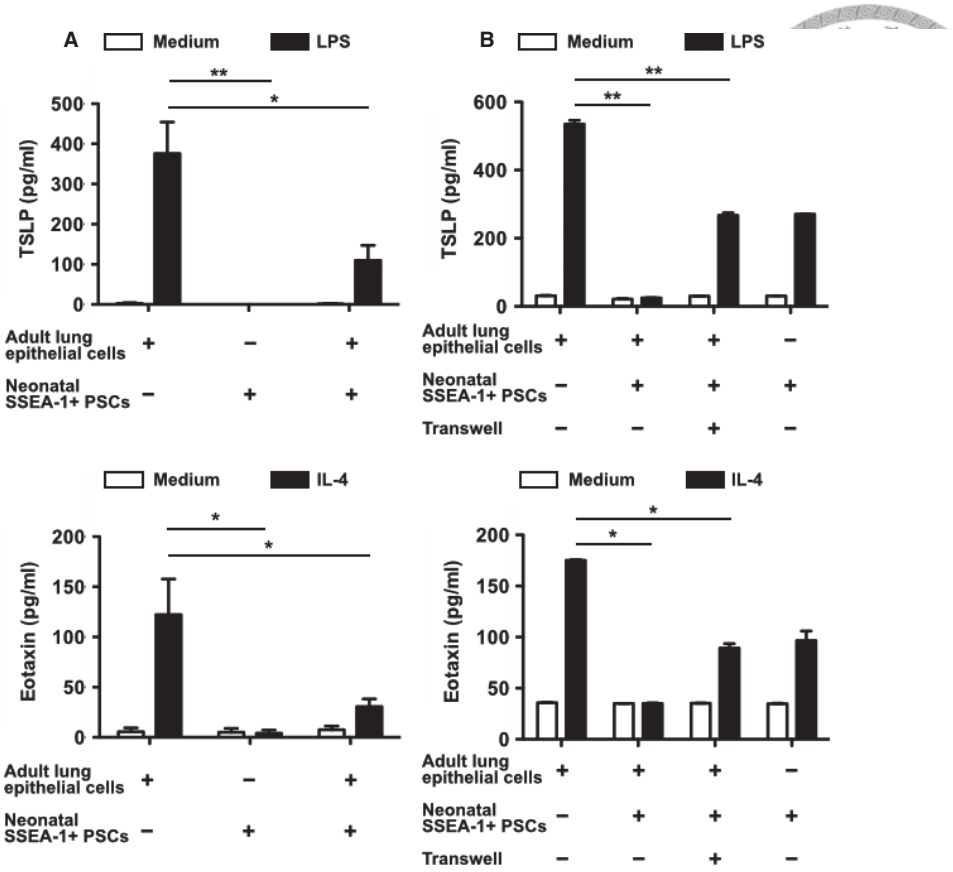

Figure 4 SSEA-1$^+$ pulmonary stem/progenitor cells (PSCs) suppress thymic stromal lymphopoietin (TSLP) and eotaxin production. (A) Lung epithelial cells derived from adult mice were cocultured with neonatal SSEA-1$^+$ PSCs in the presence of LPS or IL-4 for 24 h. TSLP and eotaxin contents in LPS- and IL-4-stimulated culture supernatants were determined using ELISA, respectively. (B) Coculture system of adult lung epithelial cells and neonatal SSEA-1$^+$ pulmonary cells was separated with a Transwell. Data are means ± 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 of 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

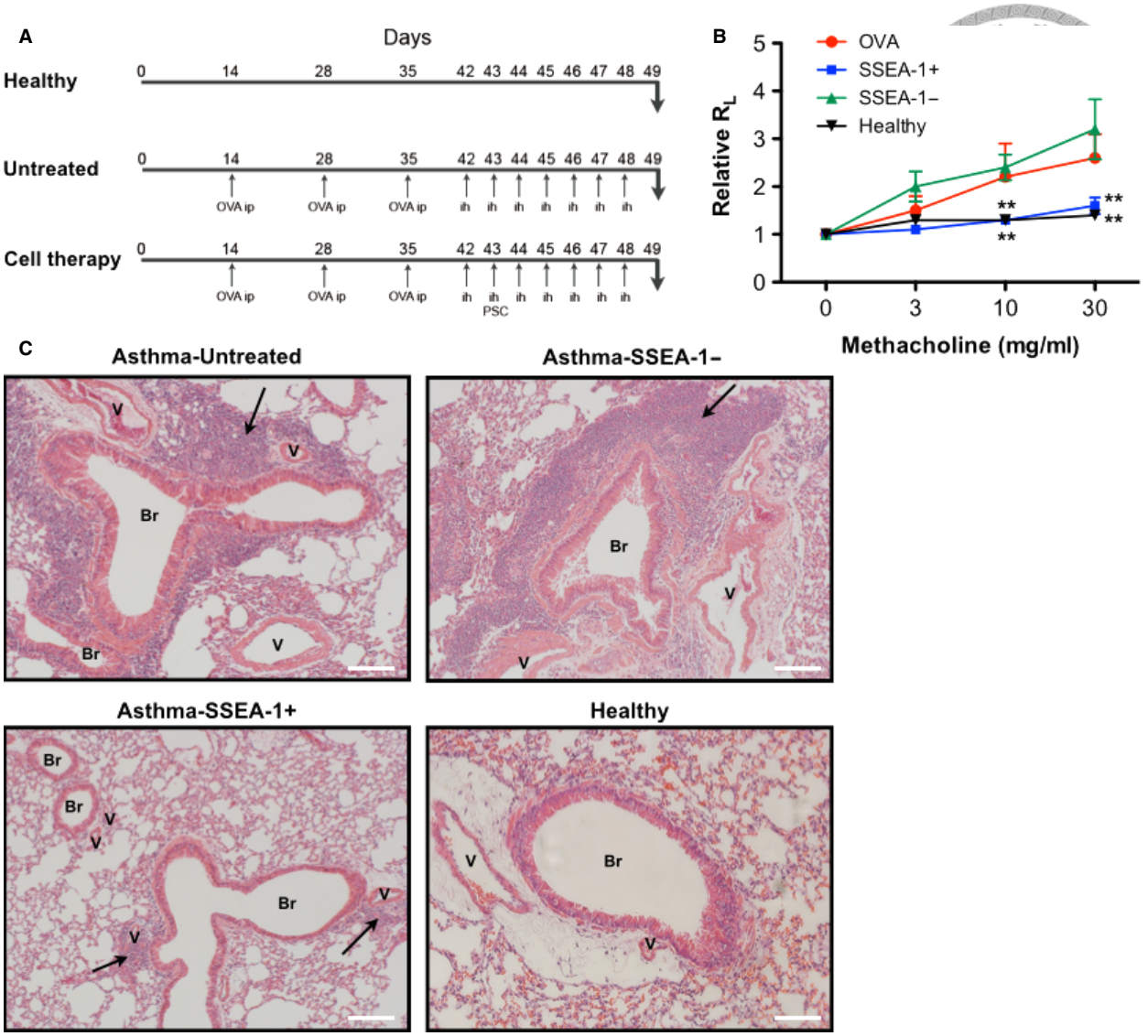

Figure 5 Transplantation of SSEA-1$^+$ pulmonary stem/progenitor cells (PSCs) improves airway hyper-responsiveness in the asthmatic mice. (A) Flowchart of the method used to produce the ovalbumin-induced asthmatic murine model. (B) Airway function was measured by invasive body plethysmography. The results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

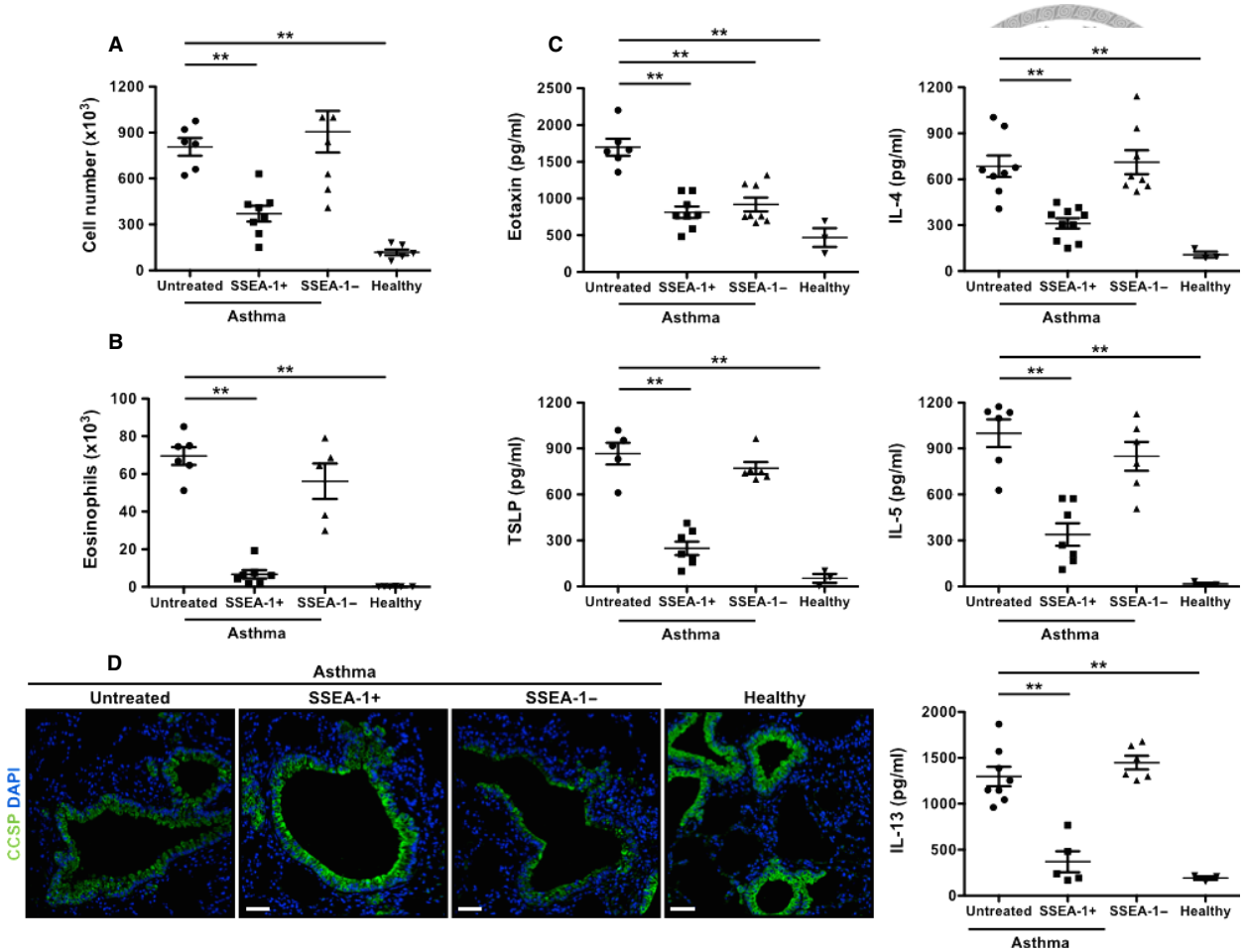

Figure 6 Transplantation of SSEA-1$^+$ pulmonary stem/progenitor cells (PSCs) reduces airway inflammation and preserves epithelium stability. BAL fluid was taken 24 h after the last aerosol ovalbumin exposure. (A) Cell number in the recovered BAL fluid was counted after staining by trypan blue. (B) Eosinophil counts in the BAL fluid were obtained by fluorescence-activated cell sorting analysis. Data are means ± 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 ± 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 μm.

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 ± 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 indicted 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 to 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 meads. 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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