Identification of a Novel Population of Bone Marrow-Derived Prominin-1/CD133^{+} Lung Progenitors with Regenerative Capacity

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Summary

Inflammation-induced lung fibrosis represents a common final pathway of various pulmonary disorders, such as the adult respiratory distress syndrome, or interstitial lung diseases, such as idiopathic pulmonary fibrosis. Endogenous stem/progenitor cells might represent a novel cell-based therapeutic option combining tissue repair and anti-inflammatory effects.

Here, we describe the identification of a novel population of prominin-1/CD133$^+$ progenitor cells from adult mouse lungs. Following digestion and culture of distal airways, we were able to expand high numbers of prominin-1$^+$ progenitor cells. Prominin-1$^+$ progenitors co-expressed stem and hematopietic cell markers, and were of bone marrow origin as suggested by the analysis of CD45.2 chimeric mice reconstituted with CD45.1 or GFP donor bone marrow. Immunohistochemistry revealed that bone marrow-derived prominin-1$^+$ cells resided in the alveolar epithelium. Prominin-1$^+$ progenitors showed multilineage differentiation capacities *in vitro*. Depending on culture conditions, they differentiated into alveolar type II surfactant protein-C positive epithelial cells, phagocyting macrophages, or fibroblast-like cells. After intratracheal administration into mice, prominin-1$^+$ progenitor cells engrafted in the alveolar epithelium and differentiated into type II pneumocytes. In order to evaluate the regenerative and anti-inflammatory capacity of prominin-1$^+$ progenitors, we used a mouse model of bleomycin (BLM)-induced lung injury. In this injury model, single intratracheal instillations of BLM into C57Bl/6 mice result in recruitment of inflammatory cells, infiltration of fibroblasts, and excessive collagen deposition and pulmonary fibrosis. In contrast to sham-treated control mice, following intratracheal transplantation of prominin-1$^+$ cells completely protected the animals

from fibrosis development. The extent of pulmonary inflammation and fibrosis was assessed by histology, immunohistochemistry, bronchoalveolar lavage fluid differentials, and real-time RT-PCR. Prominin-1^+ cells suppressed pro-inflammatory and pro-fibrotic gene expression and prevented the recruitment of inflammatory cells and fibrocytes. Mechanistically, the protective effect depended on the up-regulation of inducible nitric oxide synthase (iNOS) in prominin-1^+ progenitor cells and nitric oxide mediated suppression of alveolar macrophage proliferations. Accordingly, prominin-1^+ cells expanded from $iNOS^{-/-}$, but not $iNOS^{+/+}$ mice, failed to protect from BLM-induced lung injury.

In conclusion, the combined immunomodulatory and regenerative capacity of bone marrow-derived prominin-1^+ pulmonary progenitors makes them a promising option for novel cell-based treatment strategies against pulmonary fibrosis.

List of Abbreviations

AM – Alveolar Macrophages

AQP5 – Aquaporin-5

ARDS – Adult Respiratory Distress Syndrome

BALF – Bronchoalveolar Lavage Fluid

BASCs – Bronchioalveolar Stem Cells

BLM – Bleomycin

BM – Bone Marrow

BSA – Bovine Serum Albumin

CAB – Chromotrop Anilinblue

CC10 – Clara Cell 10-kd

CCL – Chemokine (C-C motif) Ligand

Col I – Collagen type I

cpm – Counts per Minute

CXCL1 – Chemokine (C-X-C motif) ligand 1

CXCR4 – Chemokine Receptor type 4

D-NAME – N^{ω}-Nitro-L-Arginine Methyl Ester

DAPI – 4',6-Diamidino-2-Phenylindole

DNAse I – Deoxyribonuclease I

dNTP - Deoxyribonucleotide Triphosphate

E. coli – Escherichia coli

ECM – Extracellular Matrix

EDTA – Ethylenediaminetetraacetic Acid

EMT – Epithelial-Mesenchymal Transition

eNOS – endothelial Nitric Oxide Synthase

EGF – Epidermal Growth Factor

ESC – Embryonic Stem Cells

FACS – Fluorescence-Activated Cell-Sorting

FCS – Fetal Calf Serum

GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase

GFP – Green Fluorescent Protein

H&E – Hematoxylin & Eosin

HSCs – Hematopoietic Stem Cells

HSPCs – Hematopoietic Stem and Progenitor Cells

IMDM – Iscove's Modified Dulbecco's Medium

IFN-γ – Interferon-gamma

IL – Interleukin

iNOS – inducible Nitric Oxide Synthase

IPF – Idiopathic Pulmonary Fibrosis

LPS – Lipopolysaccharide

L-NAME – N^{ω}-Nitro-L-Arginine Methyl Ester

MACS – Magnetic Activated Cell Sorting

MAPCs – Multipotent Adult Progenitor Cells

MCP-5 – Monocyte Chemoattractant Protein-5

M-CSF – Macrophage-Colony Stimulating Factor

MHC – Major Histocompatibility Complex

MIP-1α – Macrophage Inflammatory Protein-1 alpha

MMP – Matrix Metalloproteinase

MSCs – Mesenchymal Stem Cells

nNOS – neuronal Nitric Oxide Synthase

NO – Nitric Oxide

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

RT-PCR – Reverse Transcription - Polymerase Chain Reaction

SABM – Small Airway Basal Medium

SAGM – Small Airway Growth Medium

Sca-1 – Stem Cell Antigen-1

SCID – Severe Combined Immune Deficient

SD – Standard Deviation

SDF-1α – Stromal Cell-Derived Factor-1 alpha

SP-C – Surfactant Protein-C

TGF-β1 – Transforming Growth Factor-beta 1

TNF-α – Tumor Necrosis Factor-alpha

U – Unit

WT – Wild Type

Introduction

Idiopathic Pulmonary Fibrosis

Tissue fibrosis represents the common final pathway for a large variety of pulmonary diseases such as adult respiratory distress syndrome, cystic fibrosis, interstitial pneumonia, sarcoidosis or idiopathic pulmonary fibrosis. These disorders result in distortion of pulmonary architecture, which compromises pulmonary function. At the present, many end stage lung diseases are treatable with transplantation only. Due to its significant mortality and morbidity, this approach however remains palliative and the number of donor organs available is far outstripped by demand.

Idiopathic Pulmonary Fibrosis (IPF) is the most common form of interstitial lung diseases of unknown origin. It is associated with an extremely poor prognosis. Life expectancy after diagnosis varies, but is on average less than 5 years (1, 2). A recent study has estimated its prevalence ranges from 14 to 42.7 cases and incidence ranges from 6.8 to 16.3 cases per 100,000 persons (3). Although during the past decades significant advances have been made in the understanding of the pathogenesis of IPF, the exact mechanisms underlying the development of IPF still remain unknown (4).

More importantly, concepts of pathogenesis have shifted within the past years. Previously, the long held belief was that chronic inflammation plays an essential role in the pathogenesis of IPF (4-16). In this paradigm IPF is characterized by some degree of lung inflammation and abnormal tissue repair, resulting in the replacement of normal functional tissue with abnormal accumulation of fibroblasts and myofibroblasts and deposition of collagen and other extracellular matrix (ECM) in the

interstitial and alveolar spaces (6-16). This process is known to involve an intricate cytokine network that activates and mediates interactions between multiple cell types resulting in the elevation of collagen gene expression and abnormal collagen deposition in the lung (4-16) (Fig. 1).

Another hypothesis is founded on the fact that IPF results from epithelial cell injury and abnormal wound repair in the absence of preceding inflammation (4, 17). A more recent concept is based on the modification of these two hypotheses and postulates that inflammation is subsequent to injury and IPF occurs as a result of the body's immune response to lung injury (4). Thus, the pathogenesis of IPF may be much more complex than was previously thought, and IPF represents more likely a syndrome rather than a specific disease. The pathologic hallmarks of IPF are fibroblastic foci, representing areas rich in mesenchymal cells and ECM. These foci, consisting of fibroblast-like cells and myofibroblasts, are considered to be a key element in the diagnosis of IPF (11). Understanding the origin of these cells and the mechanism of their recruitment to the lungs should shed further insight into the basis for the development of progressive fibrosis, rather than successful healing and regeneration (11). Previous studies have suggested that fibroblasts and myofibroblasts in injured and fibrotic lung arise from fibroblast precursors residing in the adventitia of perivascular and peribronchial tissue. Recent evidence, however, rather supports the view that bone marrow-derived progenitor cells and circulating fibrocytes, which are known as progenitors for fibroblasts, may be recruited after lung injury and contribute to pathological tissue remodeling and fibrosis (11, 18-29).

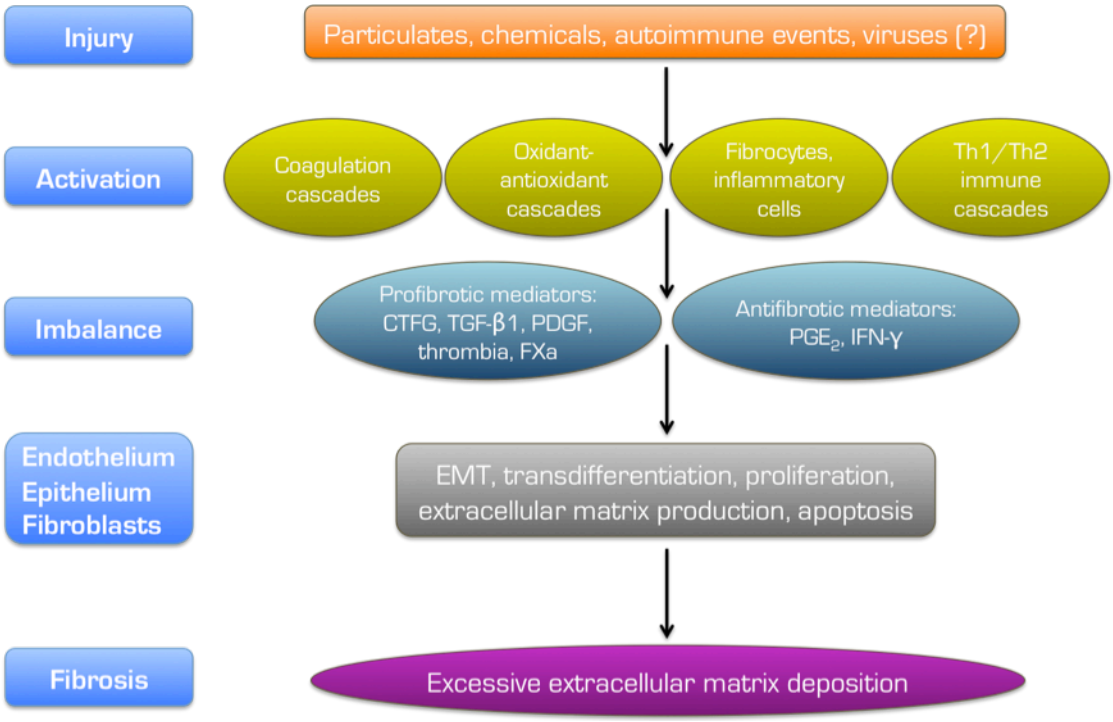

Figure 1. Pathogenesis of idiopathic pulmonary fibrosis: unknown stimuli promote injury activating multiple pro-inflammatory, reparative pathways. These cascades can result in an imbalance of pro- and antifibrotic mediators. In turn, these mediators activate multiple cell types, causing changes in cellular functioning and cell-to–cell interactions that ultimately result in progressive fibrosis. Th: T-helper cell; CTGF: connective tissue growth factor; TGF-ß1: transforming growth factor-ß; PDGF: platelet-derived growth factor; FXa: factor Xa; PG: prostaglandin; IFN-γ: interferon-gamma; EMT: epithelial–mesenchymal transition. (Adapted from Maher et al., *Eur Respir J,* 2007).

The development of idiopathic pulmonary fibrosis, as well as initiation of other interstitial lung diseases, might represent a well-orchestrated process between different cells, characterized by elevated levels of inflammatory cytokines-producing cells that may direct the recruitment of cells to the lung and enhance cytokine production (30-34). Various cells, such as eosinophils, alveolar macrophages, T cells, neutrophils, and natural killer cells, are believed to be implicated in the pathogenesis of IPF, with alveolar macrophages contributing to the disease via cytokine release (35). Nevertheless, while the role of T cells in the development of fibrosis in response

remains controversial (36-39), the development of fibrotic lesions is dependent on the release of chemokines, most notably CCL2 or CCL12 from the injured lung, and the recruitment of inflammatory cells such as monocytes, lymphocytes, and then fibrocytes (14, 40-44). The profibrotic cytokine transforming growth factor (TGF)-β1 is also critically involved in the development of pulmonary fibrosis (45, 46). Disordered coagulation cascades (47-49) and eicosanoid imbalances, which favor the overproduction of profibrotic leukotrienes and the underproduction of antifibrotic prostaglandins, are also noted (50).

Bleomycin injury as a mouse model for idiopathic pulmonary fibrosis

The bleomycin model of pulmonary fibrosis is the best-characterized mouse model in use today. Bleomycin was originally isolated from *Streptomyces verticillatus* (51) (Fig.2). It was subsequently found to be a potent anti-cancer drug, effective against squamous cell carcinomas and skin tumors (52); however, its usefulness as an anti-neoplastic agent was limited by dose-dependent pulmonary toxicity resulting in fibrosis (53).

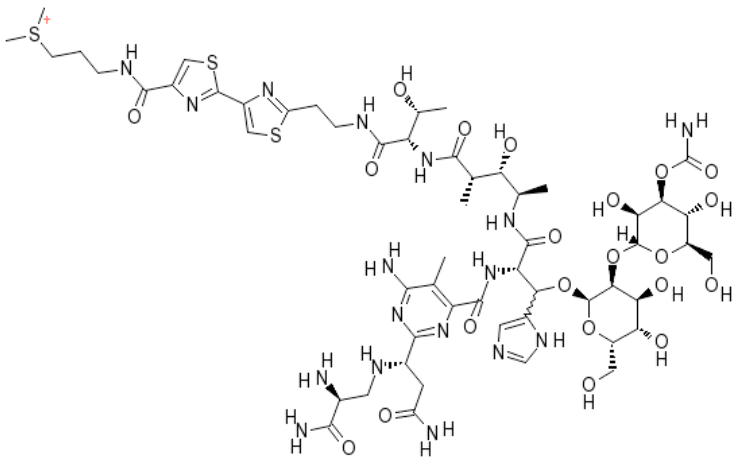

Figure 2. Chemical structure of bleomycin.

BLM has been shown to induce lung injury and fibrosis in a wide variety of laboratory animals including mice, rats, hamsters, rabbits, guinea pigs, dogs, and primates over a range of doses induced via intraperitoneal, intravenous, subcutaneous, or intratracheal delivery (53). One advantage of intravenous and intraperitoneal administration is that it more closely mimics the way humans are exposed to the drug regimen (54). Following intravenous administration of the drug, the first lesions affect the pulmonary endothelium. It is believed that initial damage of the pulmonary endothelium allows drug access to the lung interstitium where epithelial damage occurs subsequently (55). The pathological response to this injury has been well characterized (53) and includes leakage of fluid and plasma proteins into the alveolar space, alveolar consolidation, and formation of hyaline membranes. Later on, there is focal necrosis of type I epithelial cells and induction of type II epithelial cells metaplasia. Inflammatory infiltrates accumulate, and fibrosis develops in subpleural regions. A disadvantage of this model system, however, is that fibrosis does not develop in all animals, and the time frame for the development of fibrosis is relatively long (55).

The delivery of BLM via the intratracheal route has the advantage that a single administration of the drug produces lung injury and subsequently fibrosis in rodents (56-58) In addition, injury is restricted to pulmonary tissue. Intratracheal delivery of BLM results in direct damage of alveolar epithelial cells. This event is followed by the development of neutrophilic and lymphocytic pan-alveolitis within the first week (59). Subsequently, alveolar inflammation resolves, fibroblasts proliferate and synthesize extracellular matrix (60) The development of fibrosis in can be seen biochemically and histologically by day 14, with maximal responses around days 21–28 (59-62). Beyond 28 days, however, the response to BLM is more variable. Original

reports suggest that BLM delivered intratracheally may induce fibrosis that progresses or persists for up to 60–90 days (58, 63, 64). In contrast, others report a self-limiting response resolving after 30-40 days (62, 65, 66). In humans, pulmonary interstitial fibrosis is largely – but not always - an irreversible process. From this point of view, the BLM model might also offer the opportunity to study the plasticity of the fibrotic response in general.

In mice, the fibrotic response to BLM is strain-dependent. C57Bl/6 mice are much more susceptible to BLM-induced fibrosis than BALB/c mice (60, 67). This likely reflects strain-dependent differences in the expression of the inactivating enzyme, bleomycin hydrolase (68). Additionally, the fact that lungs are particularly sensitive to bleomycin toxicity is a reflection of the low levels of this enzyme in lung tissue compared with other body tissues (69).

Current treatments for idiopathic pulmonary fibrosis

In humans, IPF is a progressive and irreversible illness. So far, there are no drugs available with the capacity to modify the progressive natural disease course. Current treatments are based on the assumption that IPF is a chronic, unresolved inflammatory disease. Despite extensive research efforts over the past decades, no therapy has been shown to either reverse or even halt the progression of this disorder (5). The mainstay of therapy has been the use of corticosteroids with or without immunosuppressive drugs (70). However, it is now clear that therapies with anti-inflammatory drugs are associated with severe side effects and minimal objective benefit (70). Recent progress in understanding the mechanisms underlying the pathogenesis of fibrosis leads us to expect that inhibitors of pro-fibrogenic cytokines

and growth factors may be useful as novel therapeutic agents in controlling undesirable fibrosis. Therefore, potential therapeutic strategies could be developed for use at any of the steps that result in IPF. The major therapeutic agents in lung fibrosis include anti-inflammatory drugs, anti-fibrotic and anti-cytokine agents, receptor antagonists, signal transduction pathways inhibitors, anti-apoptosis agents, and angiogenic and MMPs inhibitors (Fig. 3).

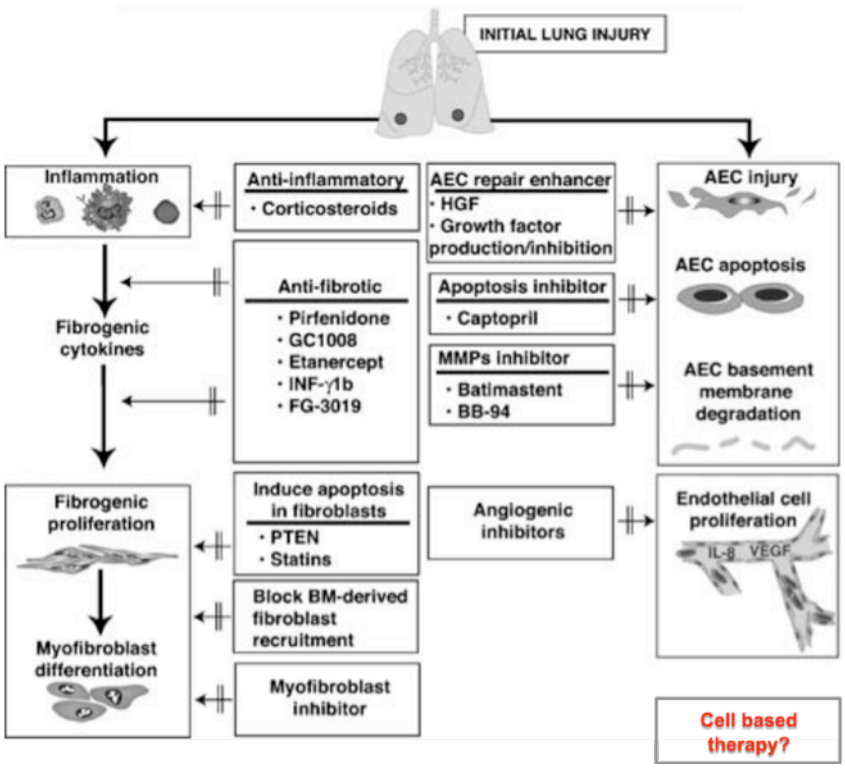

Figure 3. Major targets for therapeutic intervention in pulmonary fibrosis. Current treatment options include agents that interfere with the action of inflammatory mediators, agents that prevent epithelial cell damage, agents that prevent fibroblast proliferation and collagen synthesis, agents that down-regulate myofibroblast differentiation, agents that inhibit angiogenesis or stimulate angiostatic CXC chemokines, and agents that intervene with one or more key events in the pathogenesis or signal transduction pathways of IPF. Unfortunately, they cannot stop the condition from worsening, nor can they reverse the damage that has already occurred. Therefore, treatments for IPF are focused on minimizing further damage, along with relieving symptoms and improving a person's quality of life. Cell-based therapy may provide a new therapeutic option, contributing to regeneration of pulmonary epithelium. (Adapted from Gharaee-Kermani et al., *Pharmaceutical Research*, 2007).

Lung transplantation remains the only option for end-stage IPF (71). This procedure can prolong life and may improve the quality of the patient's life. However, many patients die because of shortage of donated organs, obliterative bronchiolitis, infections, and other complications, and the high cost associated with organ transplant. Thus, given the paucity of current therapeutic options, the research has been focusing efforts towards development of novel restorative approach, such as cellular therapies. In the field of cell-based therapy, significant advances have been reached in the biology of adult stem or progenitor cells in the last years. Stem cells may therefore offer a new hope for the patients with lung disorders such as IPF, emphysema, and other fibrotic lung diseases.

Stem cell-based treatment as a novel therapeutic option

Stem cells are the self-renewing cells with the capacity to differentiate into specialized, or differentiated, cell types of a given tissue during a particular stage of life (72-79). Progenitor cells, in contrast, do not have self-renewal capability. Another defining characteristic of these cells is their niche (29). A niche is a subset of cells and extracellular components that can accommodate one or more stem cells indefinitely and control their self-renewal and progeny production *in vivo*. The stem cells undifferentiated character and capacity for unlimited self-renewal are a result of their interaction with the supportive cells and a unique microenvironment in their niche (26).

Stem cells may be classified into two categories based on their origin: embryonic stem cells (ESC), and adult stem cells. ESC are pluripotent cells and are derived from the inner cell mass of the blastocyst stage of embryos (80). They are

able to form any cell type and tissue in the animal body (Fig. 4A) and therefore have great therapeutic capacity to regenerate any damaged tissue (81). The therapeutic capacity of these cells that proliferate freely in culture has been shown in several animal models of diseases, such as Parkinson's disease (82, 83). Additionally, ESC can be generated in practically unlimited number. Although ESC have been suggested for use in tissue regeneration of pulmonary epithelium (84-87), their use is currently mired in substantial ethical controversy (88); moreover, they have to face a greater risk of teratoma formation and immune rejection (89). Use of autologous cells can be applicable, if new technology of reprogramming fibroblasts into ES-like cells becomes safe, reproducible and standardized for human cells (90). So far, all these barriers prevented experimental use of ESC in clinical trials.

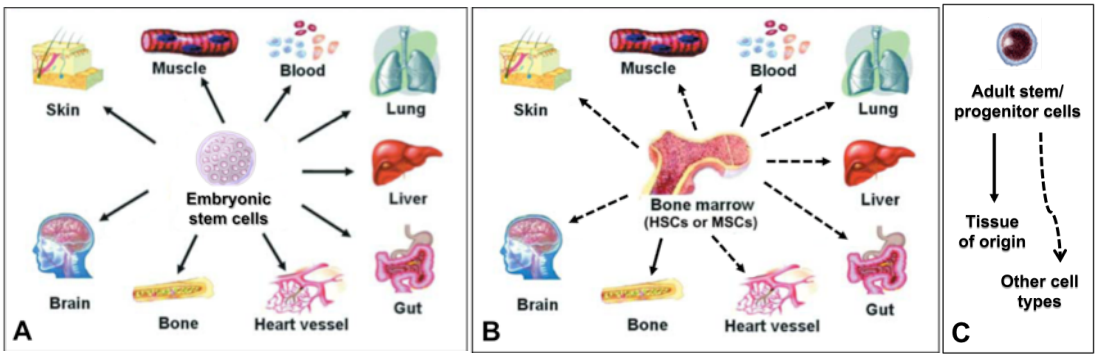

Figure 4. Stem cells for cellular therapy. (A) Embryonic stem cells are considered totipotent and therefore capable of giving rise to any cell type of an organism. (B) In contrast, bone marrow-derived cells can give rise to blood cells (HSCs), bone and adipose tissues (MSCs). Despite recent findings, the true contribution of bone marrow cells to other cell types is yet to be determined. (C) Adult stem/progenitor cells are believed to give rise mostly to cell type of their tissue origin.

Among the adult stem cells, the most characterized and possible candidates for cell-based therapies are bone marrow-derived stem cells (Fig. 4B). Bone marrow is

the source of several distinct stem cell populations: a primary category is hematopoietic stem cells (HSCs), which are traditionally thought to give rise solely to cells of the hematopoietic series, such as leukocytes and erythrocytes, therefore maintaining or restoring mature circulating blood cells. The bone marrow also contains marrow stromal or mesenchymal stem cells (MSCs), which are populations that give rise to a variety of connective tissues localized in different germ layers. Moreover, a growing body of evidence indicates that MSCs also possess immunomodulatory properties (91), both *in vitro* (92) and *in vivo* (93, 94). Several studies have illustrated that bone marrow-derived stem cells possess a broader plasticity and differentiation potential, can circulate in peripheral blood and migrate to distant tissues/organs, and thereby contribute to promote tissue repair at injured sites (95-102). The true potential for the adult, bone marrow-derived stem cell populations in lung tissue regeneration remains to be determined. There remains some uncertainty as well as to whether they play a harmful role in IPF, such as by promoting fibrogenesis, or a beneficial reparative and/or regenerative role.

Regarding tissue-specific not bone marrow-derived adult stem cells, the key to their identification resides in the ability to isolate them and show that they are capable of self-renewal and differentiation. Due to the intimate association between epithelial cells and their surrounding stroma, it was exceedingly difficult to identify solid tissue stem cells until recently (72, 103-105). Intense research on stem cells during the last decades has provided important information on developmental, morphological, and physiological processes that govern tissue and organ formation, maintenance, regeneration, and repair after injuries (95, 99, 106-110). In fact, in addition to stem cells from embryos, fetal tissues, amniotic membrane, and umbilical cord, adult stem cells with a self-renewal capacity have been identified within specific niches in most

human tissues/organs (21, 99, 103, 108, 111-120). Among them, there are heart, brain, adipose tissues, muscles, skin, eyes, kidneys, lungs, liver, gastrointestinal tract, pancreas, breast, ovaries, prostate, and testis (21, 99, 103, 111-115, 118, 119, 121-123). Non-hematopoietic adult stem cells can usually give rise to specialized cells of their tissue origin (Fig. 4C). Significant advancements in understanding of stem cell biology have provoked great interest and hold high therapeutic promise based on the possibility of stimulating their *ex vivo* and *in vivo* expansion and differentiation into functional progeny that could regenerate the injured tissues/organs in humans (97, 99, 100, 106, 114, 116, 118, 119, 123-130). Application of adult stem cells in cell-based therapy would overcome the ethical concerns that preclude the use of ESC in human trials. Nonetheless, in terms of transplantation, the issues of cell integration, survival still need to be addressed.

It is also possible that stem cells could be used to reconstitute more complex tissues and organs *in vitro*, and then transplanted to replace failed organs (Fig. 5). Tissue engineering is evolving rapidly and no tissue or organ has been excluded from active research. Only a few stem cell generated products, however, have entered clinical trials, namely cartilage and skin (131).

Stem cell treatment might also provide significant help for gene therapy. Gene therapy is the introduction of exogenous genetic material to correct or modify the function of cells (132, 133). However, high levels of gene expression cannot be reached without repeated gene transduction. The application of stem cells may resolve that problem. Gene therapy with stem cells is thought to be an ideal treatment strategy for many genetic diseases and lung injury (134). In principle, genetic modification of small numbers of stem cells produces a stable population of genetically altered cells and does not require repeated procedures. However, progress has been limited by the

low HSC gene transduction rates. Retroviruses are ideal vectors for integrating target genes into the DNA of host cells but it is difficult for them to infect relatively quiescent stem cells (132, 134, 135).

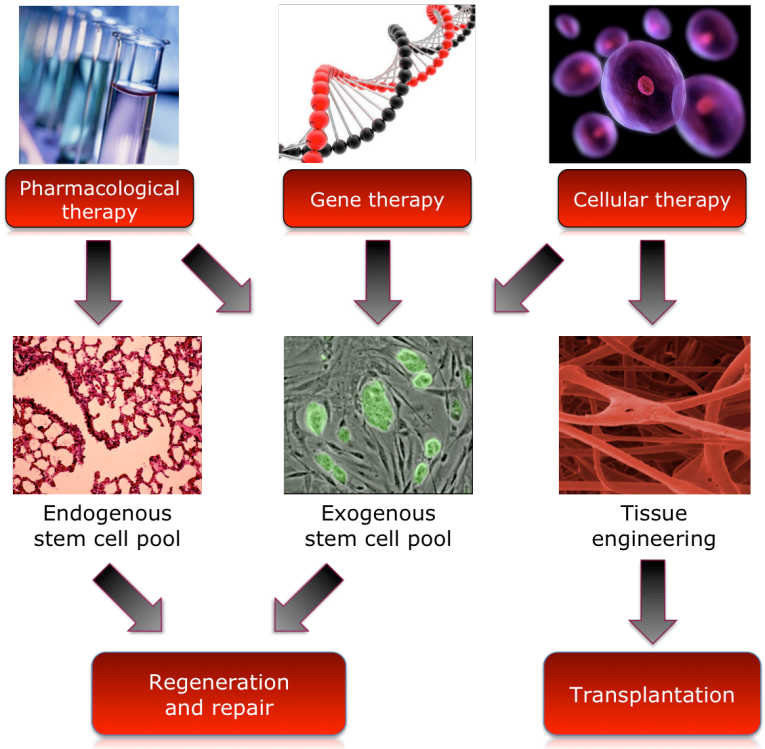

Figure 5. Prospects for cell-based therapies. Pharmacological therapy is the application of drugs or cytokines to stimulate endogenous stem cells or recruit exogenous stem cells for tissue regeneration and repair. Cellular therapy is the utilization of exogenous stem cells to help regeneration and repair and tissues or organs for transplantation after *in vitro* manipulation. Gene therapy with stem cells may be used to correct genetic defect. (Adapted from Yen et al., *Eur J Clin Invest,* 2006).

Lung stem cells

The architecturally complex mammalian lung contains cells from several distinct cell lineages, which interact during normal function, making it a difficult target for regenerative medicine. It has been reported and frequently cited that the

lung contains 40 different cell types (28). Current analyses, however, suggest that this number may be a gross underestimate, as new endogenous lung cell populations and circulating transient cells found in the lung have since been identified (24, 103, 136-138).

The lung is made up of a series of branching airways each smaller than the last, terminating in the gas-exchange units, the alveolar sacs. There are approximately 300 million alveoli in the human lung giving a surface area of about 80 m^2. The airways are lined with a continuous, confluent layer of epithelial tissue that forms a continuum from the main pulmonary artery to the capillary network of the distal lung, to the pulmonary vein and back to the heart. In between the epithelial and endothelial layers is the interstitium, made up of connective tissue cells, which form the supporting scaffold for the various lung cell layers.

The pulmonary tree can be divided into distinct anatomical regions according to its epithelial cell types (Fig. 5). The proximal, cartilaginous airways are lined by pseudo stratified epithelium containing ciliated and mucous secretory (or goblet) cells on the apical surface, with infrequent neuroendocrine cells and the less well differentiated basal epithelial cells lying underneath. Ciliated epithelial cells also line smaller airways but, rather than goblet cells, the bronchioles posses another cuboidal non-ciliated cell type known as the Clara cell, which is important for detoxifying inhaled pollutants and secrete Clara cell secretory protein (CCSP). Finally, the alveoli are lined by two epithelial phenotypes: flattened squamous (type I) and cuboidal (type II) pneumocytes. The type I pneumocyte is the cell type across which gas exchange occurs. Its long, thin cytoplasmic extensions present as little a barrier as possible to the diffusion of oxygen, while maintaining the integrity of the alveolar wall. The type II pneumocyte is far more numerous than type I but makes up only 5% of the alveolar

surface area. It is critical for maintaining alveolar homeostasis, clearing the alveolar airspace of edema and secreting pulmonary surfactant to lower surface tension and prevent airway collapse.

Cells have been described in the lung that seem to fulfill the criteria for stem cells (clonogenicity, the ability to self-renew an the capacity for multilineage differentiation), but their identification has been difficult. As well as the problem of the structural complexity and cellular heterogeneity of the lung, isolation of endogenous stem cells has been hampered by very slow cell turnover; in normal adult airways epithelial cell turnover is much lower than that in organs such as the gut and the skin.

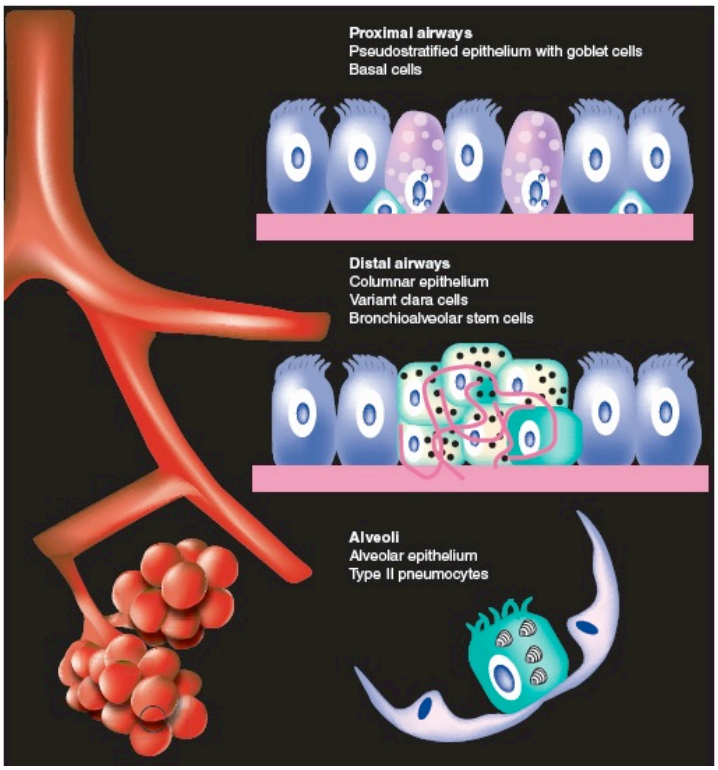

Figure 6. Lung stem cells. Pulmonary tree is divided into proximal (trachea) and distal airways (terminal bronchi, bronchioles and alveoli). Each of these compartments is believed to contain regional stem cell that in turn get activated following local injury. Classically, the basal cells of the trachea, the Clara cells of the bronchiole, bronchoalveolar stem cells (BASCs) at the bronchoalveolar duct junction and the type II pneumocyte of the alveolus are the potential cells which can repopulate the injured lung. (Lane et al., *Regen Med*, 2007).

The classical view of lung epithelial repair is that there are small populations of stem cells deposited throughout the pulmonary tree act to renew the local epithelial cell populations. Ciliated cells are widely agreed to be postmitotic and terminally differentiated in the adult lung. Therefore, in the proximal airways, the basal cell and goblet cell populations are thought to contain stem cells. Basal cells have been shown to have the capacity to produce all major cell types in the trachea, including basal cells, ciliated cells, goblet cells in the mouse (139-141) and a subset of basal cells in human proximal airways (142). Clara cells are the stem cells of distal airways epithelium. Variant Clara cells have been reported that express CC10, but are not typical Clara cells as they are resistant to airway pollutant, such as naphthalene. They reside in neuroepithelial bodies (143-145) and at the bronchoalveolar duct junction (146). Ablation of this population eliminates bronchiolar renewal entirely. A population expressing markers for both Clara cells and alveolar epithelium has recently been identified as a stem cell population for Clara cells and type II pneumocytes (103), known as bronchioalveolar stem cells (BASCs). The BASCs reside at the bronchoalveolar duct junction and are activated in response to bronchiolar and alveolar injury. In the alveoli, type II pneumocytes represent the stem cell population, proliferating and transdifferentiating to type I pneumocytes following injury of the alveolar wall (147). Unfortunately, type II cells are not sufficient in preventing pulmonary disorders (148), suggesting that exogenous cells are indeed necessary. Transplantation of alveolar type II epithelial cells into lungs of injured mice has been shown to ameliorate the effect of pulmonary fibrosis (149); nevertheless a similar approach would have anyway to face the risk of rejection in human patients.

Extrapulmonary adult stem cells for treatment of lung diseases

When local tissue-specific stem cells are not sufficient to repair serious injury, stem cells may be recruited from other sources to supplement repair. A population of multipotent adult progenitor cells (MAPCs) has been isolated from murine bone marrow that can differentiate *in vitro* at the single cell level in all three germ layers (150). The mechanism by which these bone marrow-derived stem cells are recruited to repair tissue is not known, although tissue injury clearly enhances engraftment (22, 151-156).

In 2001, Kotton and colleagues reported engraftment of bone marrow-derived cells into the alveolar walls of mice after bleomycin injury (153). Some of these engrafted cells had a type I pneumocytes phenotype. Theise and colleagues used a model of radiation pneumonitis and whole bone marrow transplantation to show that, 5 days after transplantation, a sub-population of type II pneumocytes in the irradiated lungs were of bone marrow origin (156). Bone marrow-derived cells engrafted as alveolar epithelium have been reported in irradiated lungs as long as 11 months after transplantation (134). MSCs isolated from bone marrow engraft in distal airways as epithelial-like cells to a greater degree after lung injury than in control mice (155). Circulating cells engraft in the lung as fibroblast-like interstitial cells and type I alveolar epithelium following radiation (151). Bone marrow-derived cells engraft as epithelium and endothelium in the alveolar walls of lipopolysaccharide-treated mice after whole bone marrow transplantation (157) (Fig. 7).

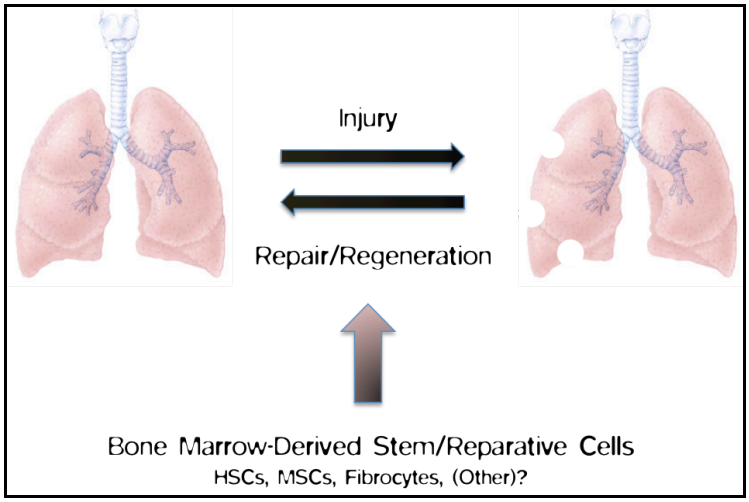

Figure 7. Extrapulmonary stem cells for treatment of lung disorders. Following injury, bone marrow-derived cells such as HSCs or MSCs can repopulate pulmonary epithelium; on the other hand, bone marrow-derived fibrocytes can migrate to lungs and contribute to the progression of fibrosis.

Recent studies have raised questions about the aforementioned results (158-160) and no clear consensus exists on the degree of lung chimerism following bone marrow transplantation. Engraftment of donor cells into epithelium and differentiation into epithelial cells has been shown to be very limited or to not occur at all (161, 162). The conflicting results could be the product of the differing injury models used. The differences in the data are likely due to differences in donor cell type, injury models, time after bone marrow transplantation, and detection techniques. In fact, Herzog and colleagues proved that a threshold of injury was necessary for bone marrow-derived cells to engraft in the lung (163). In addition, controversies arose about the functionality of bone marrow cells; some circulating bone marrow-derived cells seem to be disease-causing (151) and others appear to be protective (154). Until reproducible isolation, injury and transplantation procedures are established across laboratories, the controversy regarding the contribution of circulating cells to lung repair and their therapeutic potential will continue (164).

To date, investigation of human lung has either found no engraftment of bone marrow cells within the pulmonary epithelium, or has shown that it occurs only at a very low rate (165-171). These studies have been fraught with technical problems, not only because of the need to rely on the colocalization of histological markers, but also because of the scarcity and poor quality of appropriate human lung tissue samples. Nevertheless, if circulating cells can be recruited to the damaged pulmonary epithelium promoting tissue repair, this could be of major clinical interest.

Prominin-1/CD133 as a marker of stem and progenitor cells

Since its discovery 10 years ago, prominin-1 (alias CD133) has received considerable interest because of its expression by several somatic stem and progenitor cells originating from various sources, including the neural and hematopoietic systems, and in embryonic stem cell-derived progenitors (172-174). Murine prominin-1 was identified as a novel marker of neuroepithelial cells, primary progenitor cells of the mammalian central nervous system (172), whereas its human counterpart constituted a new hematopoietic stem and progenitor cell (HSPC) marker (initially referred to as AC133 antigen) (173, 175). As a cell surface marker, prominin-1 is now used for somatic stem cell isolation (173, 176-178). $CD133^{+}$ stem and progenitor cells might become clinically important, particularly with regard to brain injury/disease and bone marrow transplantation. It is important to note that, although various stem and progenitor cells express prominin-1, its expression is not limited to primitive cells. For instance, prominin-1 is detected in several epithelia in adult mice and humans where it appears to be restricted to the apical (luminal) side (172, 179-182). Additionally, prominin-1/ CD133 is often used for the identification and isolation of

putative cancer stem cell populations from malignant tumors of brain (183, 184), prostate (185), liver (186, 187), pancreas (188), lung (189), and colon (190-192). Its expression in cancer stem cells has broadened its clinical value, as it might be useful to outline new prospects for more effective cancer therapies by targeting tumor-initiating cells.

Cell biological studies of this molecule have demonstrated that it is specifically concentrated in various membrane structures that protrude from the planar areas of the plasmalemma. Prominin-1 binds to the plasma membrane cholesterol and is associated with a particular membrane microdomain in a cholesterol-dependent manner. Although its physiological function is not yet fully determined, a recent finding has shown that transgenic mice carrying human mutation for the prominin-1 gene (*PROM1*) undergo progressive photoreceptor degeneration in the retina consistent with that found in human patients (193), suggesting a functional role for prominin-1.

Prominin-1 is expressed on a subpopulation of $CD34^+$ HSPCs derived from various sources including fetal liver and bone marrow, adult bone marrow, cord blood and mobilized peripheral blood (173, 175, 194). Interestingly, an immunomagnetic selection of $CD133^+$ HSPCs allowed the enrichment of a sufficient amount of cells to perform hematopoietic stem cell transplantation (195), and pilot trials with leukemic children have proven the feasibility of $CD133^+$ selection for allogeneic transplantation (196, 197). Other studies have shown the successful transplantation of haploidentically mismatched peripheral blood stem cells using $CD133^+$ purified stem cells (198). Thus, the immunomagnetic isolation procedure of HSPCs based on prominin-1 appears to be an interesting alternative to CD34 (199). Moreover, accumulating studies illustrate that prominin-1/$CD133^+$ progenitor cells can exert

beneficial effects in treating of different pathological disorders, including cardiac and hepatic malignancies (96, 97, 125, 200-205).

Nitric oxide and lung injury

NO is a free radical produced in mammalian cells from the oxidation of L-arginine by three isoforms of an enzyme known as NO synthase (NOS), which occurs in three major isoforms: neuronal (nNOS or type I), inducible (iNOS or type II) and endothelial (eNOS or type III) (206-208). Neuronal and endothelial isoforms are constitutively expressed and are referred to as cNOS.

Nitric oxide is a pleiotropic mediator, which acts in a variety of physiological and pathophysiological processes (209-212). NO has a half-life of only a few seconds *in vivo*, but its solubility in both aqueous and lipid media is probably responsible for its pleiotropic effects in the cells. The constitutively expressed enzyme (cNOS) are calcium-dependent, release NO under physiological condition in various cells, including endothelial cells and neurons, and NO released by these isoforms are involved in the regulation of blood pressure in organ blood flow distribution, in the inhibition of the adhesion and activation of platelets and polymorphonuclear granulocytes and in neuronal transmission. Mice that overexpress eNOS in the lung are protected from ventilator-induced lung injury (213).

The inducible isoform of NOS (iNOS) is calcium-independent and can be induced by proinflammatory agents, such as endotoxins (bacterial lipopolysaccharide, LPS), interleukin-1β, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), in endothelial and smooth-muscle cells, in macrophages and in other cell types (208-212).

NO is well known to play critical roles in homeostasis, regulation of inflammatory processes, suppression of apoptosis and T-cell proliferation (206, 214-220). Moreover, current levels of evidence suggest a protective role for NO in various lung disease models (221-225).

Recent clinical trials using low-dose inhaled NO revealed benefits in prevention and alleviation of acute lung injury in adults (226), hypoxemic respiratory failure in infants and children (227) and chronic lung disease in neonatal infants (228), whose lungs are exposed to high oxygen and at risk of hyperoxic damage.

NO is a potent mediator of alveolarization and lung growth (229, 230). Whereas the normal response to lung injury is migration and proliferation of type II pneumocytes to reestablish an intact epithelial lining, the chronic and repetitive injury that culminates in fibrotic lung disease promotes hyperplasia and dysplasia of alveolar epithelial cells (AEC). Injured and activated AEC then participate in aberrant cytokine signaling that perpetuates the fibrotic response. Of particular recent interest is the possibility that AEC contribute directly to fibrosis through epithelial-mesenchymal transition (EMT) to a myofibroblast-like phenotype. Although myofibroblasts are integral to normal repair mechanisms, the persistence of the myofibroblasts beyond a period of normal repair has been associated with ECM deposition, structural remodeling, and destruction of alveocapillary units. Recent studies (231-233) have demonstrated alveolar EMT both *in vitro* and *in vivo* and have shown that the majority of myofibroblast-like cells after experimental injury are the result of alveolar EMT. There is mounting evidence that alveolar EMT is primarily mediated by local production and activation of transforming growth factor (TGF)-β1 (231, 232). Additionally, TGF-β1 down-regulates NOS expression in a variety of non-pulmonary cell types (234-236), and NO production is dramatically increased in TGF-

β1 null mice (237), indicating a reciprocal relationship between TGF-β1 and NO. In fact, recent evidence have illustrated that NO can play an important role in prevention of EMT in alveolar epithelial cells following bleomycin (238), suggesting a regulatory function in the context of lung injury.

Aim of the thesis

Idiopathic Pulmonary Fibrosis is a chronic, progressive and often fatal form of interstitial lung disease. IPF continues to pose a major clinical challenge since an effective therapeutic regimen has yet to be developed. My aim was to identify a population of stem/progenitor cells capable to suppress pulmonary inflammation and to mediate regeneration of injured lung tissue. I hypothesized that under certain conditions it must be possible to expand these cells from dissected adult lungs. Therefore, I planned to establish an efficient method for the isolation and generation of high numbers of progenitor cells and to address their regenerative potential *in vitro* and *in vivo*. As *in vivo* model I chose the mouse model of BLM-induced lung injury.

Materials and Methods

Mice: C57Bl/6 mice and C57Bl/6-GFP transgenic mice (GFP under the control of β-actin promoter) were purchased from Jackson Laboratory. iNOS$^{-/-}$ C57Bl/6 mice were kindly provided by Dr. Adrian J. Hobbs, Wolfson Institute for Biomedical Research, University College London. All mice were housed in optimized hygienic rooms (in accordance with the guidelines of the Department of Biomedicine at the University Hospital Basel) and received acidified (pH 2.5) water and sterile food. Newborn male and female mice were separated 3 weeks after birth in sterilized cages covered by a special protecting filter. No more than 5 mice were put together into each cage. All animal experiments were conducted in accordance with institutional guidelines and Swiss federal law and were approved by the local authorities.

Generation of bone marrow chimera: 5-7 week old C57Bl/6 CD45.2 mice were lethally irradiated with two doses of 6.5 Gy (4 hours between each round of irradiation) using a Gammatron (Co-60) system and reconstituted with $2x10^7$ donor bone marrow cells from C57Bl/6 GFP or C57Bl/6 CD45.1 mice. Cells were resuspended in 200 µl of sterile PBS and administrated intravenously into the tail vein. After reconstitution, all mice received prophylactic antibiotics (0.2% Bactrim, Roche, Basel, Switzerland) in the drinking water.

Bleomycin-induced lung fibrosis: 7-9 week old male C57Bl/6 mice were anesthetized using Ketalar (75 mg/kg; Parke-Davies, Zürich, Switzerland) and Xylasol (10 mg/kg; Gräub AG, Bern, Switzerland) resuspended in 0.9% NaCl by intraperitoneal injection. Individual mice were fixed on an oblique plane and their

throat was illuminated with a focused cold light lamp (KL 1500 LCD, Carl Zeiss, Germany) and the tongue of the animal was pulled out of its mouth (Fig. 8A, B). Instillation of 0.05 U/mouse bleomycin (Blenoxane, Axxora-Alexis, San Diego, CA, USA) dissolved in 50 µl of sterile PBS was done under view, directly into the tracheal lumen. Control animals received the same volume of PBS. Two hours after PBS/BLM instillation, the animals received intratracheally either 2 x 10^5 prominin-1^+ cells resuspended in 50 µl of PBS or PBS alone.

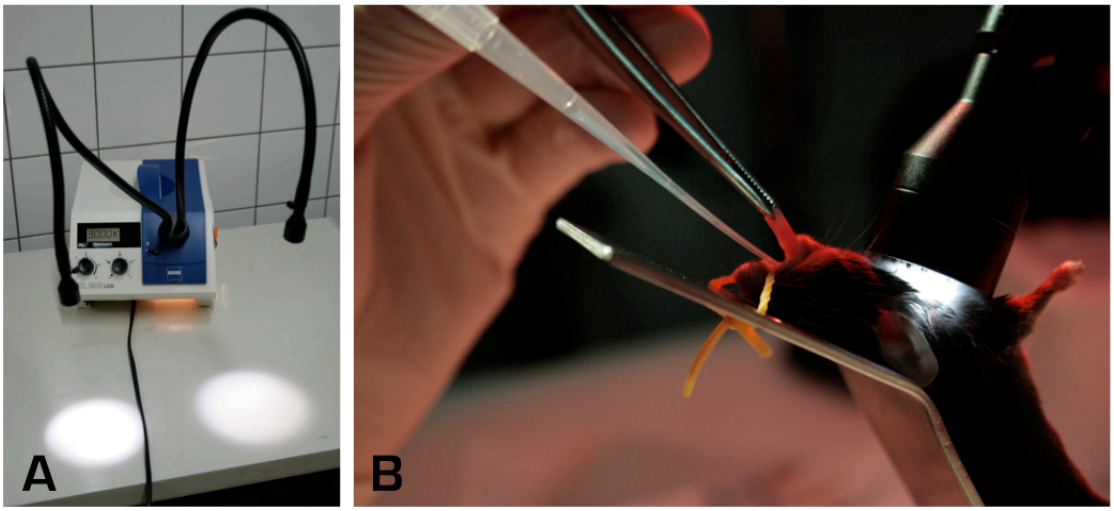

Figure 8. Intratracheal instillation is performed using a cold light lamp (A); the tongue of the mouse is pulled out and bleomycin, cells or PBS are injected into the tracheal lumen (B).

Isolation and expansion of prominin-1^+ cells: Lungs of 7-9 week old C57Bl/6 mice were perfused by injection of 5-10 ml of ice cold PBS through the right ventricle. Next, lungs were excised, separated from the trachea and the main bronchi, manually dissected into small pieces with carbon steel surgical blades (Swann-Morton), and digested for 90 min at 37°C in 15 ml of GKN (11 mM D-glucose (Sigma), 5.5 mM KCl (Fluka), 137 mM NaCl (Roth), 25 mM Na_2HPO_4 (Fluka), 5.5 $NaH_2PO_4 \cdot H_2O$ (Fluka)) containing 10% FCS, 1.8 mg/ml collagenase type 4 (Worthington Biochemical. Corp., Freehold, NJ, USA) and 0.1 mg/ml DNAse I (Sigma). The cell

suspension was filtered through 70 µm nylon mesh and washed with GKN containing 10% FCS. Cells were resuspended in IMDM (Iscove's Modified Dulbecco's Medium, Sigma) containing 2% FCS, 100 µM β-mercaptoethanol (Gibco), 100 U of penicillin and 100 µg of streptomycin/ml (Pen/Strep, Gibco), 2mM L-glutamine, 25 mM Hepes, and plated at 5 x 10^6 cells into 6 cm diameter tissue culture dishes. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed 2-3 times a week. Non-adherent cells were removed 48-72 hours after plating. Culture of lung homogenates gave rise to two main populations consisting in a round cell population and a fibroblast-like cell population that worked as a feeder layer.

Magnetic Activated Cell Sorting (MACS): After 3-4 weeks cells were removed with cell scrapers, washed, and filtered through 40-µm nylon mesh. Then, cells were stained for 30 min at 4°C with an anti-prominin-1 PE antibody 1:200 (eBiosciences San Diego, CA, USA), in MACS buffer (PBS supplemented with 2 mM EDTA and 1% FCS) and isolated using anti-PE antibodies coupled to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (purity > 95%).

***In vitro* differentiation:** For alveolar epithelial cell differentiation, MACS sorted prominin-1^+ cells were cultured two weeks onto sterile 0.2% gelatin-coated round cover slips (12 mm diameter, Menzel-Gläser, Germany) in 24 well plates in the presence of modified Small Airway Growth Medium (SAGM) (239) consisting of a basal medium (Small Airway Basal Medium, SABM, Cambrex, East Rutherford, NJ, USA) supplemented with 0.5 mg/ml BSA, 0.5% FCS, ITS supplement, 30 µg/ml

bovine pituitary extract, 0.5 µg/ml epinephrine, 6.5 ng/ml triiodothyronine, 0.1 ng/ml retinoic acid, 0.5 µg/ml hydrocortisone (all Sigma) and 1 ng/ml EGF (PeproTech, UK). For macrophage differentiation, prominin-1$^+$ cells were cultured two weeks in the presence of M-CSF (10 ng/ml) added to IMDM 2% FCS. For phagocytosis assay *Escherichia coli* BioParticles Alexa Fluor 488-conjugated (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were used according to the manufacture instruction. To induce fibroblast differentiation, prominin-1$^+$ cells were kept two weeks in the presence of TGF-β1 (10 ng/ml) (PeproTech, UK) added to IMDM 2% FCS.

Single cell-derived colony assay: prominin-1$^+$ cells were sorted from GFP culture and plated in presence of non-GFP cells in 24 well/plates. Up to 5 GFP$^+$/prominin-1$^+$ cells were co-cultured with 2 x 10^4 GFP negative cells. After 2-3 weeks single cell-derived colonies were selected, removed, washed and transferred into new wells to induce *in vitro* cell differentiation.

Flow cytometry: Cells were filtered through 40-µm nylon mesh, stained for 30 min on ice with the appropriate antibodies diluted in FACS buffer (PBS supplemented with 2 mM EDTA and 0.5 BSA%), and analyzed on a BD FACSCalibur™ (BD Biosciences, San Jose, CA, USA) and a CyAN (Dako-Cytomation, Carpinteria, CA, USA) using FlowJo 8.7.3 software (TreeStar, Ashland, OR, USA). The following antibodies and dilutions were used: Primary antibodies: anti-prominin-1-PE 1:200 (eBioscience), anti-CD11b-APC 1:400, anti-CD11c FITC 1:200, anti-CD45.1 FITC 1:200, anti-CD45.2 PerCP Cy5.5 1:200, anti-CXCR4-FITC 1:200, anti-MHC II 1:1000, anti-Gr1 FITC 1:500 (BD Bioscience, San Jose, CA, USA), goat anti-Sca-1 1:200 (R&D Systems, Abingdon, UK), biotin anti-c-kit 1:200, biotin anti-CD14

1:200, biotin anti-CD34 1:200, biotin anti-CD45 1:200, rat anti-CD31 1:400, (eBioscience, San Diego CA, USA), rabbit anti-collagen I (Rockland, Gilbertsville, PA, USA). Secondary antibodies: Alexa Fluor 488 donkey anti-goat 1:200, Alexa Fluor 488 chicken anti-rabbit 1:200, Alexa Fluor 488 donkey anti-rat 1:100 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and streptavidin-APC 1:200 (BD Bioscience, San Jose, CA, USA). Cells from adult mouse lungs were sorted with inFlux Cell Sorter (Cytopeia, Seattle, WA, USA).

Immunofluorescence: Cells were cultured on 0.2% gelatin-coated cover slips and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After blockade of non-specific binding with 10% FCS in PBS (30 min at room temperature), cells were stained for 1 hour at 37°C with the appropriate primary and secondary antibodies. Following nuclear staining with DAPI (10 min at room temperature, no-light exposed), cover slips were washed with distilled water and mounted with Fluorescent Mounting Medium (Dako Corporation, Carpinteria, CA, USA).

Paraffin sections: Paraffin-embedded lung sections were deparaffinized in two 5-min exposures to xylene; rehydrated in decreasing ethanol concentrations of 100%, 95%, 70%, and 50%; and subsequently washed in distilled water and PBS.

Frozen sections: Lung tissues were separated in single main lobes, placed in base mould disposable (Kaltek, Padova, Italy) and snap-frozen in O.C.T.™ Compound (Tissue-TeK®, Sakura, Netherlands) onto dry ice. Frozen samples were stored at -70°C until use. Serial 10-µm sections were cut with Microm HM 560 cryostat and thaw-mounted onto SuperFrost® microscope slides (76 x 26 mm, Menzel-Gläser, Germany). Frozen sections were initially stained with appropriate antibodies; next,

sections were fixed with 4% paraformaldehyde and then non-specific binding was neutralized with 10% FCS in PBS.

Primary antibodies: rat anti-prominin-1-PE 1:200 (eBioscience, San Diego CA, USA), CD45.2 FITC 1:200 (BD Bioscience, San Jose, CA, USA), rabbit polyclonal anti-surfactant protein-C 1:400 (SP-C, Chemicon, Temecula, CA, USA), rabbit anti-collagen I 1:200 (Rockland Immunochemicals, Gilbertsville, PA, USA), mouse anti-β-tubulin IV 1:400 (Sigma), rabbit anti-fibronectin 1:400 (Sigma), rabbit polyclonal anti-aquaporin-5 1:300 (Calbiochem, La Jolla, CA, USA), biotin anti-CD14 1:200 (eBioscience). Secondary antibodies: Green-fluorescent Alexa Fluor 488 rabbit anti-GFP 1:200, Alexa Fluor 488 chicken anti-rabbit 1:200, Alexa Fluor 546 goat anti-rabbit 1:400, Alexa Fluor 488 goat-anti mouse 1:200 (Molecular Probes, Invitrogen, Carlsbad, CA, USA), streptavidin-PE 1:200, streptavidin-PE Cy7 1:200 (BD Bioscience, San Jose, CA, USA). Cells and sections were stained 10 min with 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Samples were examined with Olympus BX61 Fluorescence Microscope ad with Olympus IX50 Inverted Microscope. Samples were stored at 4°C.

Histology: Animals were sacrificed on selected days after BLM instillation. Lungs were perfused, removed, fixed in 4% formaldehyde and cut in 5 µm sections. Samples were stained with Hematoxylin-Eosin (H&E); Masson's trichrome or Chromotrop Anilinblue (CAB) stain were used to visualize collagen depositions. Staining procedures were performed by the laboratories of Institute of Pathology, University of Basel.

Bronchoalveolar Lavage (BAL): Mice were sacrificed by intra-peritoneal injections

of Pentothal® (Abbott Laboratories, North Chicago, IL, USA). The trachea was exposed and cannulated with a size 20G sterile catheter (OPTIVA® 2, Medex, Rossendale, UK). Lavage was performed a total of 3-4 times by repeated flushing with 1 ml of ice-cold PBS. The BAL fluid (BALF) was stored on ice until centrifugation (4 min at 1500 rpm at 4°C). Collected cells were resuspended in 500 µl of PBS and counted. Next, cells were centrifuged again and re-suspended in 50-60 µl of PBS; then, were cyto-spun onto slides (7 min at 700 rpm), Diff-Quik stained (according to the manufacturer's protocol), and analyzed under a light microscope for cell differentials.

Alveolar macrophages isolation: BALFs from several mice were pooled and centrifuged at 300*g* for 10 min. The resulting pellet was re-suspended in RPMI-1640 medium (Gibco) supplemented with 10% FCS and incubated for 1 hour at room temperature on 10 cm diameter plastic dishes. Non-adherent cells were washed off with PBS and the adherent macrophages were collected. Cells were then removed with cell scraper, counted and transferred into 96 well plates for co-culture experiments.

Blood collection. Blood was collected via the inferior vena cava after opening the body cavity. A 1-mL syringe containing EDTA was used to bleed the mice. Cells were separated from erythrocytes using Lympholyte-M (Cedarlane Laboratories Ltd, Hornby, Canada) according to the manufacturer's instructions.

Co-culture experiments: MACS sorted prominin-1^{+} cells were irradiated (2000 rad) and cultured in RPMI with 10% FCS (Gibco) 100 µM β-mercaptoethanol (Gibco),

100 U of penicillin and 100 μg of streptomycin/ml (Pen/Strep, Gibco), in 96-well plates in the presence of alveolar macrophages (AM) at a ratio of 1:2. Cells were stimulated for 24 h with LPS (0.1 μg/ml) and IFN-γ (50 ng/ml). AM proliferation was measured by [^{3}H]Thymidine incorporation (0.5 μCi/well for the last 8 hours). Nitrite (NO_2^-) levels reflecting NO production in culture supernatants were assessed using the Griess Reagent System (Promega, Madison, WI, USA).

$CD4^+$ T cells were MACS sorted from BLM-treated animals and cultured for two days in RPMI with 10% FCS. 96 well plates were previously coated for two hours with anti-CD3/anti-CD28 antibody (1 μg/ml). 5 x 10^4 T cells were co-cultured with titrating amount of prominin-1^+ cells. Cell proliferation was measured by [^{3}H]Thymidine incorporation.

Real Time quantitative-PCR and Reverse Transcriptase-PCR Analysis: RNA was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) from total lung tissue according to the manufacturer's recommendations. First strand cDNA synthesis was performed as following: RNA (2 μg) was incubated with oligo$(dT)^{18}$ for 5 min at 70°C and chilled on ice. 5x reaction buffer, 10mM 4dNTP mix, RNase inhibitors and RevertAID™ M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) were added and the reaction mixture was incubated for 60 min at 42°C. For RT-PCR the following primers were used: AQP5 Fw 5'-GGC CAC ATC AAT CCA GCC ATT A-3', Rw 5'-GGC TGG GTT CAT GGA ACA GCC-3'; β-tubulin Fw 5'-GGA ACA TAG CCG TAA ACT GC-3', Rw 5'-TCT ACT GTG CCT GAA CTT ACC-3'; CC10 Fw 5'-CGC CAT CAC AAT CAC TGT GGT CA-3', Rw 5'-GAG GGT ATC CAC CAG TCT CTT CA-3'; E-cadherin Fw 5'-ACG TAT CAG GGT CAA GTG CC-3', Rw 5'-CCT GAC CCA CAC CAA AGT CT-3'; Islet-1 Fw

5’-GTT TGT ACG GGA TCA AAT GC-3’, Rw 5’-ATG CTG CGT TTC TTG TCC TT-3’; Keratin 5 Fw 5’-ACC CTT GTT CCA CGG AAT GCA A-3’, Rw 5’-AAA GCA CAG TTA AGA CCA GAA AC-3’; Nanog Fw 5’-AGG GTC TGC TAC TGA GAT GCT CTG CA-3’, Rw 5’-CAA CCA CTG GTT TTT CTG CCA CCG-3’; Oct4 Fw 5’-GTG GAT TCT CGA ACC TGG CT-3’, Rw 5’-GTC TCC AGA CTC CAC CTC AC-3’; SP-C Fw 5’-TAT GAC TAC CAG CGG CTC CT-3’, Rw 5’-GTT TCT ACC GAC CCT GTG GA-3’. The primers used for quantitative Real Time PCR are listed in Table 1. Real-time PCR was performed using a 7500 Fast Real time PCR System (Applied Biosystems, Foster City, CA, USA) in the presence of SYBR-green (Applied Biosystems); GAPDH was used as internal control. Amplification conditions were as following: 50°C (2 min); 95°C (10 min); 95°C (15 s), 60°C (1 min), 40 repetitions. Specificity of each reaction was ascertained by performing the Melt procedure (60–95°C; 1°C/15 sec) after completion of the amplification protocol, according to the manufacturer's instructions. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistics: Normally distributed data, such as proliferation responses and cytokine levels, were compared using the Student *t* test. Statistical analysis was conducted using Prism 4 software (GraphPad Software). $p < 0.05$ was considered to be statistically significant.

Table 1. Primers used in Real Time quantitative-PCR.

Gene	Forward Primer	Reverse primer
GAPDH	CCTGCACCACCAACTGCTTA	TCATGAGCCCTTCCACCATG
iNOS	CAGCTGGGCTGTACAAACCTT	TGAATGTGATGTTTGCTTCGG
IL-4	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA
IL-6	TGTATGAACAACGATGATGCACTT	GGTACTCCAGAAGACCAGAGGAAAT
IL-13	CGCAAGGCCCCCACTAC	AAAGTGGGCTACTTCGATTTTGG
IFN-γ	TGGAGGAACTGGCAAAAGGAT	GCCTGATTGTCTTTCAAGACTTCAA
TNF-α	CCCAGACCCTCACACTCAGATC	CCTCCACTTGGTTTGCT
CCL2	CATCACTGAAGCCAGCTCTCTCT	GCAGGCCCAGAAGCATGA
MIP-1α	TTTTGAAACCAGCAGCCTTTG	TCTTTGGAGTCAGCGCAGATC
MCP-5	AGAATCACAAGCAGCCAGTGTC	GTCAGCACAGATCTCCTTATCCAGT
TGF-β1	CAACGCCATCTATGAGAAAACC	AAGCCCTGTATTCCGTCTCC
Fibronectin	TACCAAGGTCAATCCACACCCC	CAGATGGCAAAAGAAAGCAGAGG
Col-I	GATGACGTGCAATGCAATGAA	CCCTCGACTCCTACATCTTCTGA
SDF-1α	CGTGAGGCCAGGGAAGAG	TGATGAGCATGGTGGGTTGA
CCL21/SLC	GGCAAAGAGGGAGCTAGAAAACA	TGGACGGAGGCCAGCAT
MMP-9	CCTGGAACTCACACGACATCTTC	TGGAAACTCACACGCCAGAA
KC/CXCL1	TGCACCCAAACCGAAGTCAT	GGAGCTTCAGGGTCAAGGC

Results

Expansion of prominin-1^{+} progenitor cells

Several lines of evidence suggest that activation of tissue resident progenitor cells represents an injury-triggered process (103, 240). Thus, we hypothesized that dissection of pulmonary tissue creates a specific "injured" microenvironment promoting the expansion of progenitor cells with specific differentiation capacity. We therefore separated distal airways from the trachea and main bronchi and dissected into small pieces (Fig. 9A). In fact, the culture of lung homogenates gave rise to a population of small, round, semi-attached cells, growing on a feeder layer (Fig. 9B, C). Immunohistochemistry revealed that round cells were all positive for prominin-1, whereas feeder layer cells were positive for Col I (Fig. 9D).

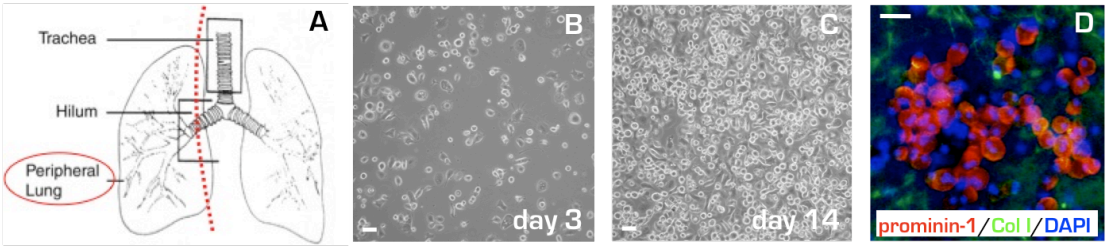

Figure 9. Characterization of lung explants. Distal part of adult mouse lungs (A) were cut, digested and plated in 6 cm dishes at a concentration of 5 x 10^6 cells/dish. Passage 0 cultures after 3 days exhibit a heterogeneous population (B); after 14 days a population of rounded, semi-adherent cells expanded growing on feeder layer cells (C). Immunohistochemistry showed that all round cells were positive for prominin-1 (red), but not Col I (green) (D). DAPI (blue) was used to visualize cell nuclei. Bars = 30 µm.

Characterization of prominin-1$^+$ progenitor cells

FACS analysis revealed that the vast majority of prominin-1$^+$ cells co-expressed markers characteristic of stem cells such as Stem Cell Antigen-1 (Sca-1) (Fig. 10A), c-kit (CD117) (Fig. 10B), Chemokine Receptor type 4 (CXCR4) (Fig. 10C), as well as the hematopoietic antigen CD45 and CD11b (Fig. 10D, E). In contrast, prominin-1$^+$ cells did not show co-expression with endothelial markers such as CD34 and CD31 (Fig. 10F, G), fibrocyte marker Col I (Fig. 10H), macrophage marker CD14 (Fig. 10I), dendritic cell marker CD11c (Fig. 10J), granulocyte marker Gr1 (Fig. 10K), and Major Histocompatibility Complex class II (MHC II) (Fig. 10L).

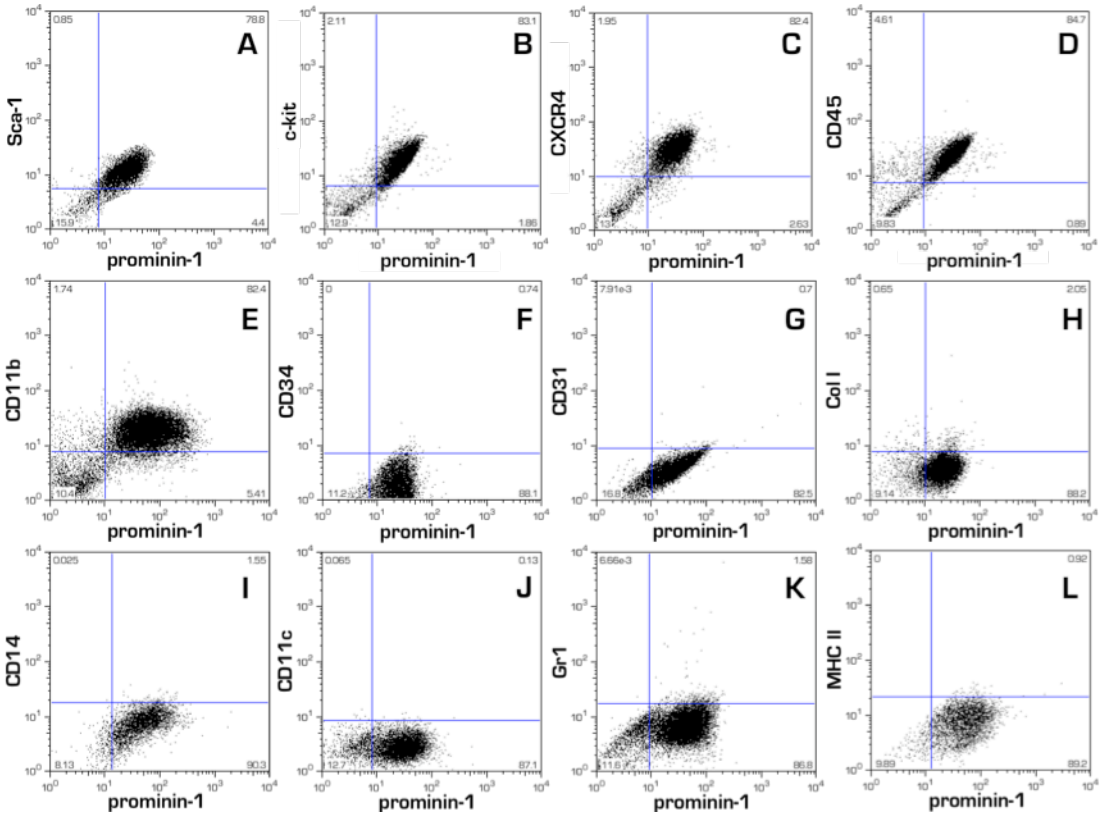

Figure 10. Characterization of prominin-1$^+$ progenitors by FACS. (A-L). Prominin-1$^+$ cells were positive for stem cell (A-C) and hematopoietic markers (D-E), but were negative for mature markers (F-L).

Prominin-1$^+$ progenitor cells were then purified by cell sorting and further analyzed. RT-PCR revealed that prominin-1$^+$ cells were negative for bronchial (Clara Cell 10-kd protein, *CC10*), alveolar type I (aquaporin-5, *AQP-5*), alveolar type II (surfactant protein-C, *SP-C*) and epithelial (E-cadherin and keratin 5, *K5*) genes (Fig. 11A), but expressed genes characteristic for stem and progenitor cells (Islet-1, Nanog, but not Oct4) (Fig. 11B).

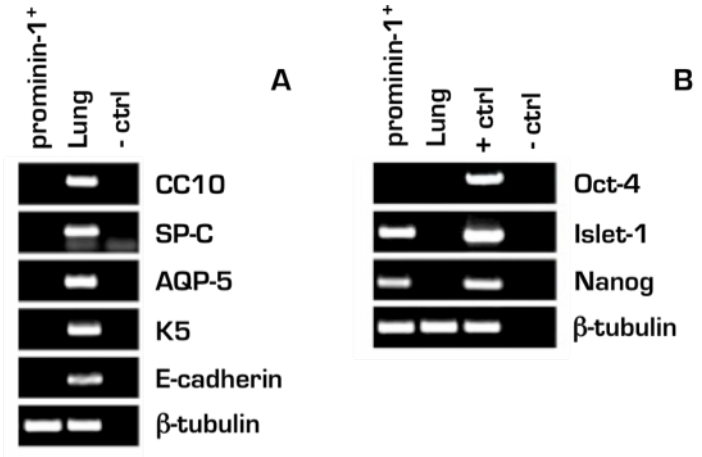

Figure 11. Characterization of prominin-1$^+$ cells by RT-PCR. Prominin-1$^+$ cells showed no expression of lung epithelial markers (CC10, SP-C, AQP-5, K5, E-cadherin) (A); whole lung tissue was used as positive control. In contrast, expression of genes characteristic for stem and progenitor cells (Islet-1, Nanog) was detected. cDNA from mouse embryonic stem cells was used as positive control (B). Amplification of reactions without cDNA was used as negative control.

Density is critical for maintenance of prominin-1$^+$ cell phenotype

Next we sorted prominin-1$^+$ cells and plated them at different densities in 6 well plates. Cells were cultured at densities between 0.25 x 10^4 (2.5K) and 6 x 10^4 (60K) cells/cm^2 (Fig. 12A-F). Morphological analysis by microscope showed that after 3 weeks prominin-1$^+$ cells plated at high density kept a round phenotype (Fig.

12J-L), whereas cells plated at low density became flat and only occasionally round cells were found (Fig. 12G-I). These data illustrated that density is definitely crucial for maintenance of prominin-1$^+$ round cell phenotype. 2 x 10^4 (20K) was identified as the minimal density necessary for prominin-1$^+$ cells to grow *in vitro*.

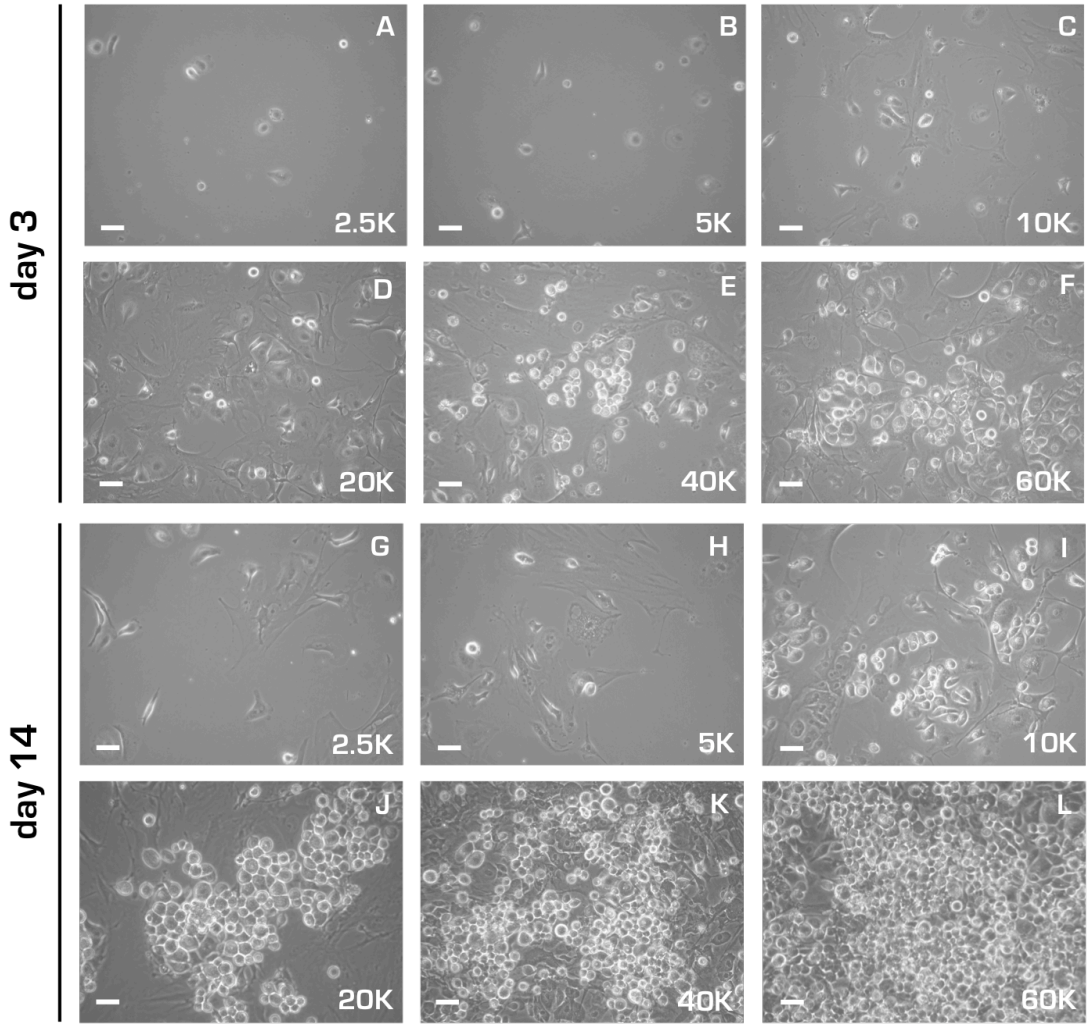

Figure 12. Density-dependent expansion of prominin-1$^+$ cells. MACS sorted prominin-1$^+$ cells plated at different densities (A-F) after 3 days. After two weeks cells plated at high density kept round cells phenotype (J-K), but not cells cultured at low density (G-I). (6 x 10^4 cells/cm^2 = 57.6 x 10^4 cells). Bars = 30 µm.

Prominin-1$^+$ cells show bone marrow origin

As shown in the Figure 10, nearly all prominin-1$^+$ cells co-expressed hematopoietic markers. To confirm the bone marrow origin of these cells, we generated chimeric mice. C57Bl/6 CD45.2 mice were lethally irradiated and reconstituted with bone marrow cells from or C57Bl/6 CD45.1 mice. FACS analysis of lungs of C57Bl/6 mice showed presence of two CD45.2$^+$ populations (Fig. 13A). Three weeks after bone marrow transplantation CD45.1$^+$ cells replaced nearly all CD45.2$^+$ cells (Fig. 13B).

Next, we dissected lung tissues from chimeric mice and expanded prominin-1$^+$ lung-derived progenitors as described. As illustrated in Fig. 13C, nearly all prominin-1$^+$ cells were CD45.1 positive, indicating clearly that prominin-1$^+$ cells are of bone marrow origin. These data were confirmed by culturing chimeric lungs from mice reconstituted with donor GFP cells. In fact, immunohistochemistry revealed that nearly all prominin-1$^+$ cells were also GFP positive (Fig. 13D).

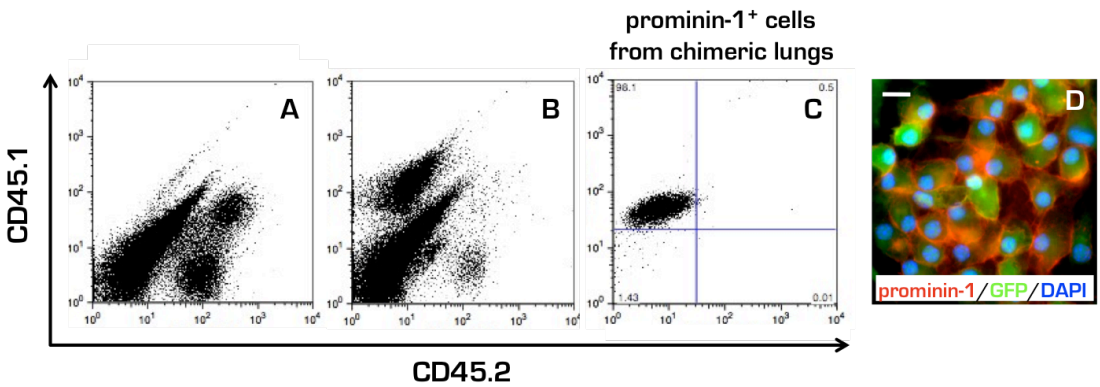

Figure 13. Bone marrow origin of prominin-1$^+$ cells. FACS analysis of lungs from C57Bl/6 CD45.2 mice lethally irradiated and reconstituted with CD45.2$^+$cells (A) or CD45.1$^+$cells (B) 3 weeks after bone marrow transplantation. FACS (C) and immunohistochemistry (D) demonstrated that prominin-1$^+$ cells expanded from chimeric lungs were of bone marrow origin. Prominin-1 (red), GFP (green), DAPI (blue). Bar = 30 μm.

In vitro multipotent capacity of prominin-1$^+$ cells

Bone marrow cells are well known to show high plasticity. We therefore, addressed the potential of prominin-1$^+$ progenitors to differentiate *in vitro* into various cell phenotypes.

To induce alveolar epithelial differentiation, sorted prominin-1$^+$ cells were cultured for two weeks in modified Small Airway Growth Medium (SAGM) (Fig. 14A). Immunofluorescence microscopy showed the expression of surfactant protein-C (SP-C) (Fig. 14B), which is specific for pulmonary type II cells. As illustrated in Fig. 14C, all cells differentiating into type II cells lost expression of prominin-1. RT-PCR confirmed the expression of SP-C, but not of CC10 and AQP5, which are characteristic for bronchial and alveolar type I epithelial cells, respectively (Fig. 14E).

To induce fibroblast differentiation prominin-1$^+$ cells were cultured two weeks in the presence of TGF-β1 (10 ng/ml). After 3 days cells changed phenotype, acquiring an elongated, bipolar phenotype (Fig. 14F); two weeks after differentiation induction, culture contained mostly cells with fibroblast-like phenotype (Fig. 14G). Prominin-1$^+$ cells were all positive for fibronectin (Fig. 14H) and Col I (Fig. 14I), fibroblast-specific marker. In addition, flow cytometry confirmed that, in presence of TGF-β1, prominin-1 expression was lost (Fig. 14L, M).

After two weeks in culture with M-CSF (10 ng/ml), prominin-1$^+$ cells acquired large and flat morphology; microscope analysis revealed that more than 80% of cells showed phagocyted bacteria within their cytoplasms (Fig. 14N, *green*). Moreover, immunohistochemistry (Fig. 14O) and flow cytometry (Fig. 14P) showed that more than 40% of cells were positive for the macrophage marker CD14.

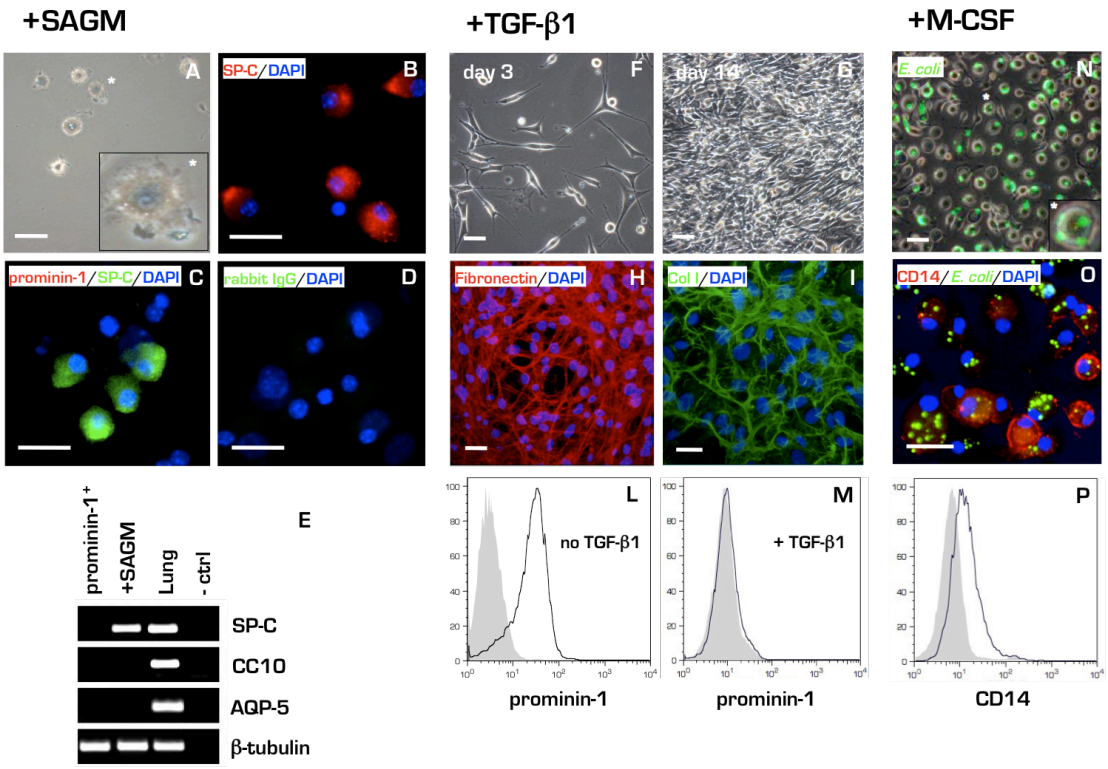

Figure 14. *In vitro* differentiation capacity of prominin-1$^+$ cells. Lung explants were cultured for 3-4 weeks and prominin-1$^+$ cells were MACS sorted. Phase contrast micrograph of prominin-1$^+$ cells incubated for 2 weeks in SAGM (A) and stained for surfactant protein-C (SP-C, red) (B). Expression of prominin-1 was lost after differentiation (C).; cells stained with rabbit IgG isotype control (D). RT-PCR confirmed expression of SP-C type II cell specific gene, but no other lung epithelial genes (E). In the presence of TGF-β1, prominin-1$^+$ cells acquired an elongated, flat phenotype (F, G). All cells were positive for fibronectin (H, red) and Col I (I, green) and lost prominin-1 expression (L, M). In the presence of M-CSF, prominin-1$^+$ cells differentiated into functional macrophages phagocyting *E. coli* bioparticles (N, O, green) and acquired CD14 expression, as illustrated by immunohistochemistry (O, red) and flow cytometry (P). Bars = 30 µm.

Given the bone marrow origin of prominin-1$^+$ cells, we wanted to confirm that cells expanded from chimeric mice had the same differentiation potential. Therefore, we expanded prominin-1$^+$ cells from GFP chimeric lungs. Cells were purified using magnetic beads and cultured in presence of appropriate media and factors.

Prominin-1^{+} cells cultured in presence of SAGM differentiated into alveolar type II cells and showed expression of SP-C (Fig. 15A-C). After being in culture two weeks with TGF-β1, prominin-1^{+} cells differentiated into fibroblast-like cells and confirmed expression of Col I (Fig. 15D-F). Finally, prominin-1^{+} cells cultured in presence of M-CSF showed phagocyting capacity, given by the presence of *E. Coli* bioparticles within the cytoplasm (Fig. 15G-I).

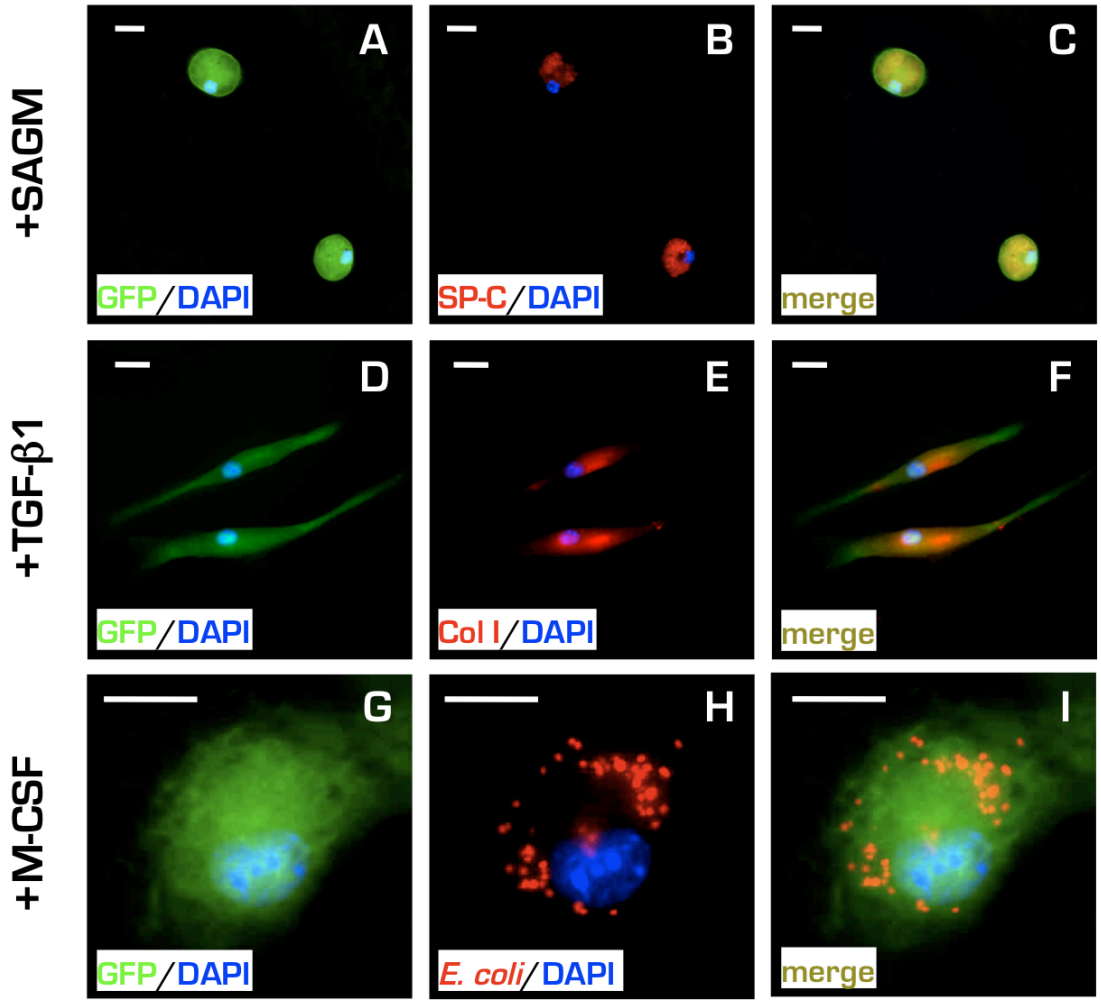

Figure 15. Differentiation capacity of prominin-1^{+} cells expanded from GFP chimeric mice. MACS purified prominin-1^{+}/GFP^{+} cells differentiated into SP-C^{+} alveolar type II cells when cultured in the presence of SAGM (A-C). In the presence of TGF-β1 and M-CSF, prominin-1^{+} cells differentiated into fibroblast- (D-F) and macrophage-like cells (G-I) respectively. SP-C (red, B), *E. coli* (red, E), Col I (red, H), 4',6-diamidino-2-phenylindole (DAPI) (blue). Bars = 20 μm.

Single cell-derived colonies of prominin-1$^+$ cells

One of the criteria that define stem cells relies on their clonogenic ability, which is the capacity to give rise to single cell-derived colonies. To address these issues, prominin-1$^+$ cells were plated as single cells. Cell cultured without feeder layer did not give rise to colonies and died after one week (data not shown). Therefore, we generated prominin-1$^+$ cells from GFP expressing transgenic mice and co-cultured them on a GFP negative feeder layer. Up to 5 GFP$^+$ cells were plated with 2 x 10^4 GFP$^-$ cells. As illustrated, a single GFP$^+$/prominin-1$^+$ cell (Fig. 16A) gave rise to a GFP$^+$ colony that expanded after 14 days (Fig. 16B).

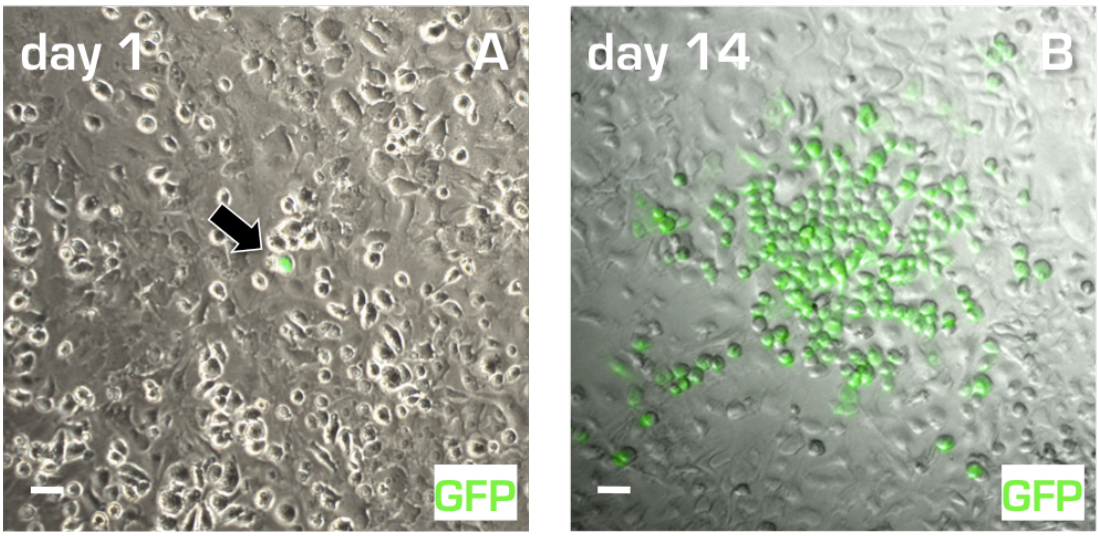

Figure 16. Prominin-1$^+$ cells gave rise to single cell-derived colonies. GFP$^+$/prominin-1$^+$ cells cultured as single cell in presence of 2 x 10^4 GFP$^-$ feeder layer cells (A) show ability to give rise to colonies after two weeks (B). Bars = 30 µm.

Next, we wanted to provide evidence that prominin-1$^+$ progenitors from single cell-derived colonies could also differentiate into specialized cells. Thus, after 2-3

weeks GFP$^+$ colonies were selected and transferred into new wells to induce *in vitro* differentiation into different lineages.

As shown in Fig. 17A-C, cells cultured two weeks in presence of SAGM differentiated into SP-C$^+$ alveolar type II cells. Single GFP$^+$ differentiated cells were detectable among GFP$^-$ cells. When plated in presence of M-CSF, cells differentiated into macrophage-like cells with phagocyting capacity (Fig. 17D-F). Finally, in presence of TGF-β1 cells differentiated into fibroblast-like which stained positive for Col I (Fig. 17G-I).

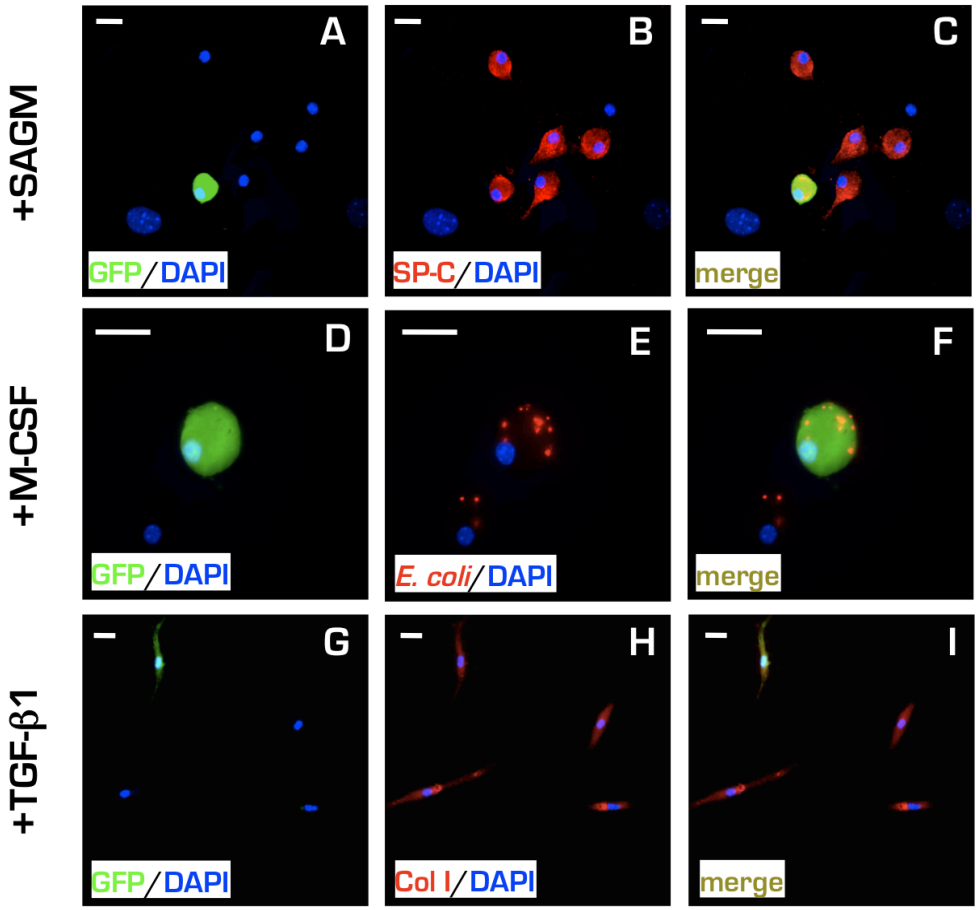

Figure 17. GFP$^+$/prominin-1$^+$ progenitors expanded as single cells give rise to specialized cells. Prominin-1$^+$ cells derived from single cell-derived colonies differentiated into type II pneumocytes (A-C), macrophages (D-F) and fibroblasts (G-I). SP-C (red, B), *E. coli* (red, E), Col I (red, H), GFP (green), 4',6-diamidino-2-phenylindole (DAPI) (blue). Bars = 20 µm.

Identification of two distinct prominin-1$^+$ cell populations in the adult mouse lung

We next wanted to address the presence of prominin-1$^+$ cells within the healthy mouse lungs. Distal airways were isolated from adult mice and digested as described. FACS analysis revealed that the percentage of prominin-1$^+$ cells correspond to 10.41 ± 0.98% (Fig. 18B); prominin-1$^+$ cells were then stained for CD45 antibody. Analysis of samples showed that among the prominin-1$^+$ subset, 6.82 ± 0.31% were co-expressing CD45 (Fig. 18D). Collectively, the percentage of prominin-1$^+$/CD45$^+$ cells in the adult mouse lungs corresponds to 0.71 ± 0.08%. In addition, staining of frozen sections confirmed the presence of two different cell phenotypes in the adult lung. In fact, rare, single round prominin-1$^+$ cells were detected in the alveolar epithelium (Fig. 18F, *asterisk*), differently located from prominin-1$^+$ cells sited along the bronchial epithelium (Fig. 18F, *arrows*).

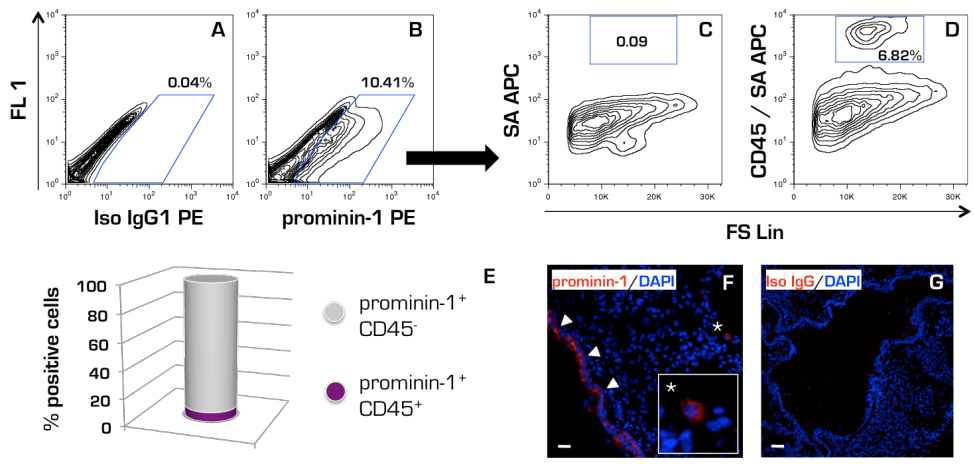

Figure 18. Characterization of prominin-1$^+$ cells in mouse lungs. FACS analysis of adult mouse lungs (A-D). Prominin-1$^+$ cells were gated (B) and stained for CD45 (D). The percentage of prominin-1$^+$/CD45$^+$ cells within lungs corresponds to 0.71±0.08. (*FL1 = unstained; Iso = Isotype control; SA = Streptavidin*). Graphical visualization of the two prominin1$^+$ subsets (E): a minority of prominin-1$^+$ cells (6.87 ± 0.59%, *violet*) co-expressed CD45, whereas the majority of prominin-1$^+$ cells (93.13 ± 0.59%, *grey*) was CD45$^-$. Immunohistochemistry on frozen sections confirmed the presence of two prominin-1$^+$ distinct cell phenotypes located in the bronchial epithelium (*white arrows*) and alveolar epithelium (*asterisk*) (F). Lung frozen section stained with isotype control PE-conjugated (G). Prominin-1 (red), DAPI (blue). Bars = 20 µm.

Next, we performed further stains on frozen sections in order to characterize the two prominin-1 expressing populations. As confirmed in Figure 19, round prominin-1^{+} cells were detectable only in the alveolar epithelium, but they were all negative for type II specific marker SP-C (Fig. 19A-C) and for type I marker AQP5 (data not shown). In addition they were all co-expressing CD45 (Fig. 19D-F).

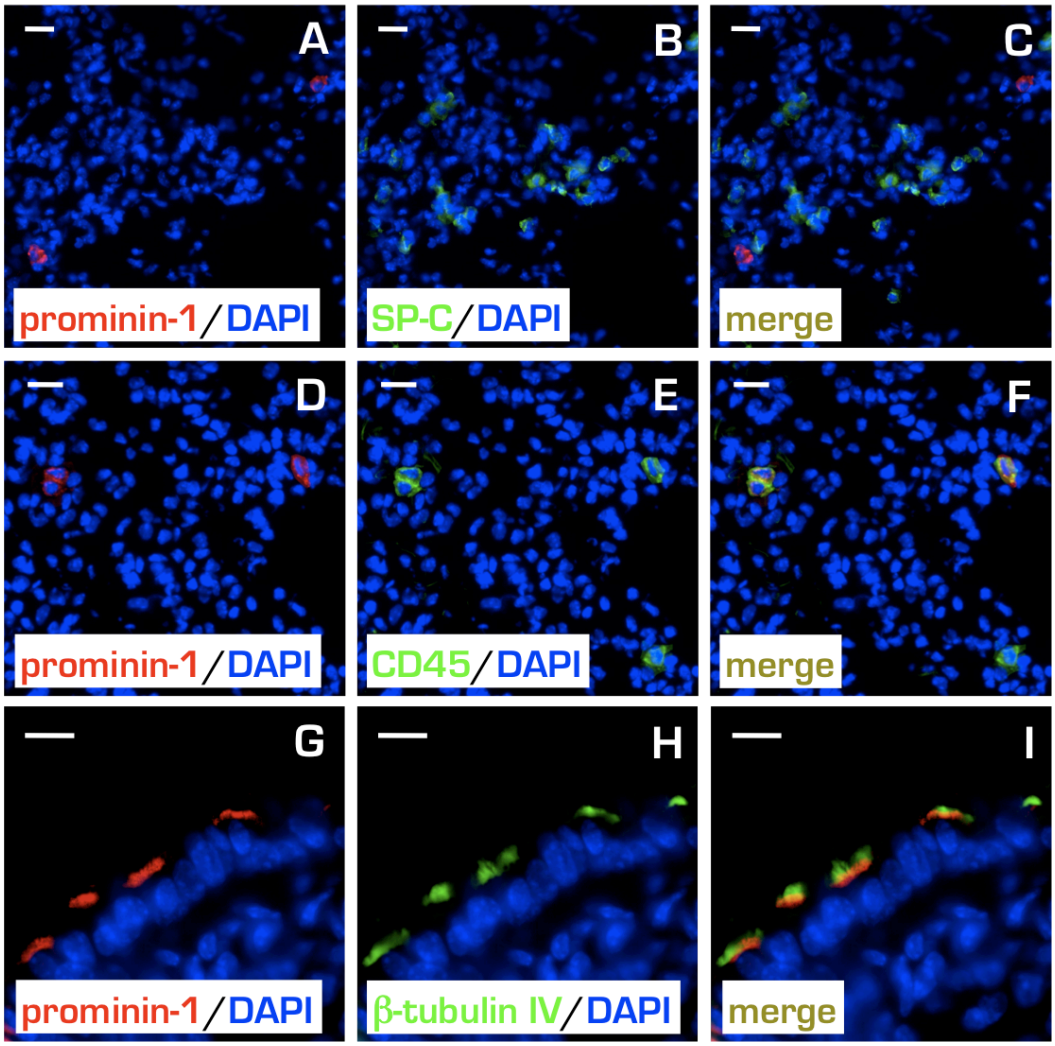

Figure 19. Characterization of pulmonary resident prominin-1^{+} cells. Stain of frozen sections from adult mouse lungs. Prominin-1^{+} cells located in the alveolar epithelium were negative for SP-C (A-C), but were all co-expressing CD45 (D-F). Prominin-1 was also expressed on the apical surface of bronchial epithelial (G) cells co-expressing β-tubulin IV (H-I). Prominin-1 (red), SP-C (green, B-C), CD45 (green, E-F), β-tubulin IV (green, H-I), 4',6-diamidino-2-phenylindole (DAPI) (blue).

As already mentioned, the second subset of prominin-1$^+$ cells was found in the bronchial epithelium and the expression was restricted on the apical surface (Fig. 19G). As shown in Fig. 19H-I, these cells were all co-expressing β-tubulin IV, which stains for cilia and is specifically expressed on bronchial epithelial cells. These findings clearly show the presence of a hematopietic population of prominin-1$^+$ cells and a second population of non-hematopoietic prominin-1$^+$ epithelial cells. Analysis of lung suspensions from GFP chimeras revealed that only the CD45$^+$/prominin-1$^+$, but not the β-tubulin IV$^+$/prominin-1$^+$ fraction was of bone marrow origin (data not shown).

Furthermore, analysis of cells taken from BALF excluded the presence of circulating prominin-1$^+$ cells within pulmonary tissue. Accordingly, immunohistochemistry revealed that CD45$^+$ cells present in the BALF, composed prevalently by macrophages, leukocytes and neutrophils, were not co-expressing prominin-1 (Fig. 20A-D).

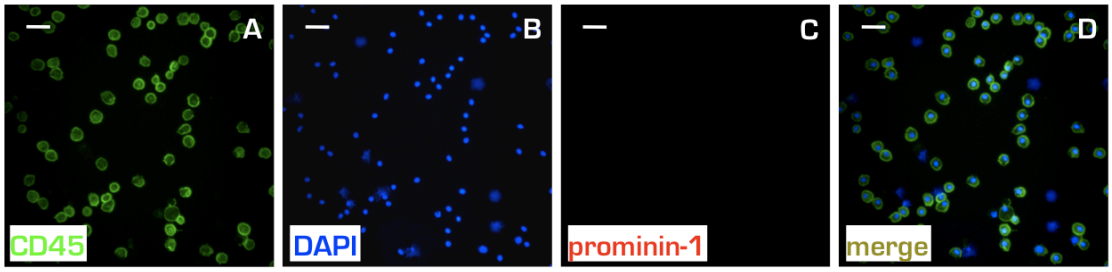

Figure 20. Prominin-1$^+$cells are not present in mouse BALF. BALF was collected from adult mice lungs; cells were cyto-spun and stained. The totality of CD45$^+$ cells was negative for prominin-1. CD45 (green), prominin-1 (red), DAPI (blue). Bars = 30 µm.

Prominin-1$^+$ cells sorted ex vivo expand in vitro and differentiate into alveolar type II epithelial cells

In order to confirm that progenitor cells specifically expanded from the hematopoietic prominin-1$^+$ population, we sorted *ex vivo* prominin-1$^+$/GFP$^+$ cells from chimeric mice and cultured them on a lung tissue-derived feeder layer. Up to 10 GFP$^+$ cells were cultured with 5 x 10^5 feeder layer cells (Fig. 21A-C). Cells were kept in culture for up to 30 days. Already after 1 week single prominin-1$^+$/GFP$^+$ cells started to expand (Fig. 21, G) and gave rise to colonies (Fig. 21E, F, H, I). Transfer of prominin-1$^+$/GFP$^+$ cells into SAGM resulted in their differentiation into type II alveolar epithelial-like cells expressing SP-C (Fig. 21J-L).

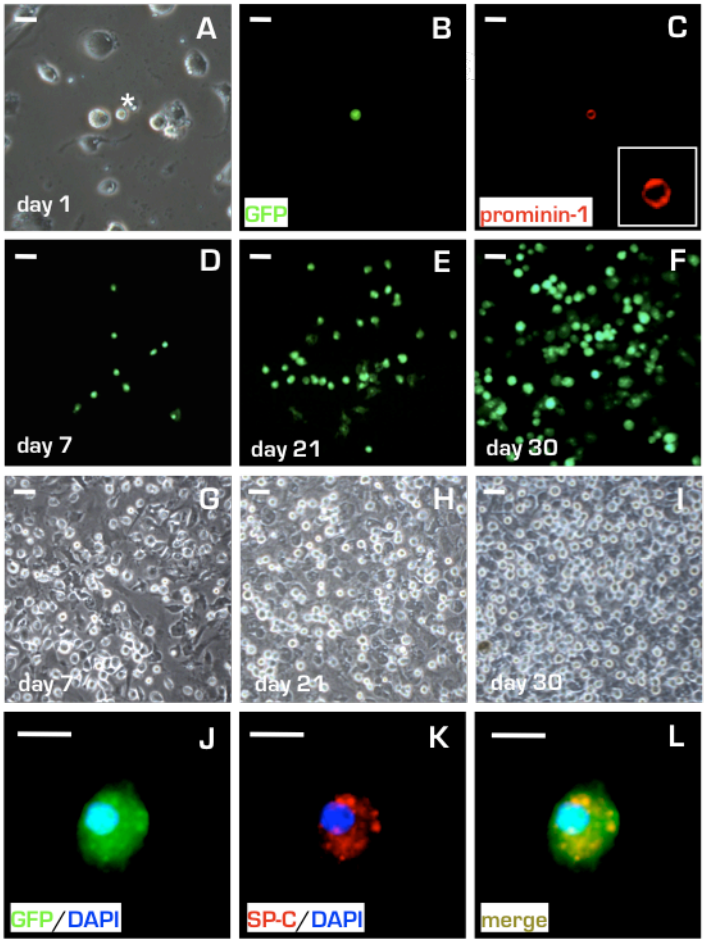

Figure 21. Prominin-1$^+$cells sorted from chimeric lungs give rise to colonies and differentiate into type II cells. Prominin-1$^+$/GFP$^+$ cells sorted from chimeric mice were FACS sorted, plated with 5 x 10^5 feeder layer cells (A-C) and expanded (D-I). After expansion, GFP$^+$ cells were incubated for 2 weeks in the presence of SAGM and differentiated into SP-C positive cells (J-L). SP-C (red), GFP (green), 4',6-diamidino-2-phenylindole (DAPI) (blue). Bars = 20 µm.

Blood-derived prominin-1$^+$ cells do not expand in vitro

Our findings have shown that prominin-1$^+$ cells originate from bone marrow. This indicates that prominin-1$^+$ cells circulate through the peripheral blood and then home to the lung, replacing the local niche impaired after injury. Based on these observations, we wanted to investigate whether it would be possible to expand a population of prominin-1$^+$ cells with similar feature from blood.

First, we isolated and purified cells from blood of adult mice. Flow cytometry revealed that the amount of prominin-1$^+$ cells in the blood corresponds 0.71±0.08% (Fig. 22A). Further analysis showed that nearly all prominin-1$^+$ cells co-expressed CD45, CD34, CXCR4, c-kit (Fig. 22B). These data showed that prominin-1$^+$ cells from blood share similar phenotype with human homologue AC133$^+$ cells (241).

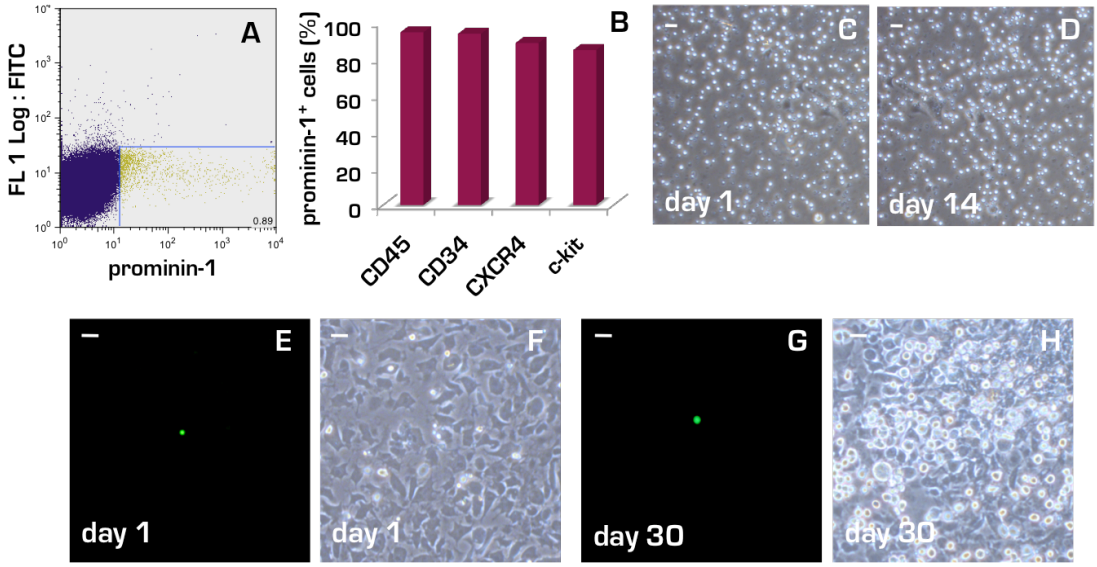

Figure 22. Characterization of prominin-1$^+$ from blood. Total amount of prominin-1$^+$ cells in the blood is 0.71±0.08%. FACS analysis showed that prominin-1$^+$ cells co-express with hematopoietic and stem cell markers (B). Prominin-1$^+$ cells MACS sorted from blood did not attach and did not expand. Purified single prominin-1$^+$/GFP$^+$ cells were plated with 5 x 10^5 feeder layer cells. Single cells attached (E, F), but failed to expand and did not give rise to colonies (G, H). Bars = 20 µm.

Next, prominin-1$^+$ cells were sorted from adult mice and plated in 6 well plates at a concentration of 5 x 10^5 cells/well. Microscope analysis revealed that prominin-1$^+$ cells did not attach to tissue culture plates (Fig. 22C) and did not expand (Fig. 22D). Therefore, we sorted prominin-1$^+$ cells from the blood of GFP$^+$ mice and plated as single cells with GFP negative lung-derived cultures. Prominin-1$^+$/GFP$^+$ cells attached to the plates (Fig. 22E, F), but did not expand and did not give rise to colonies (Fig. 22G, H).

Prominin-1$^+$ cells differentiate in vivo into alveolar type II cells

To address the differentiation capacity of prominin-1$^+$ cells *in vivo*, sorted prominin-1$^+$/GFP$^+$ cells were intratracheally delivered into C57Bl/6 mice after BLM instillation. At day 1 after delivery, GFP$^+$ cells were detected in the alveolar walls only and were negative for SP-C (Fig. 23A-C). In contrast, 7 days after delivery GFP$^+$ cells were still detected within the alveolar epithelium, but most of them also expressed alveolar type II specific marker SP-C (Fig. 23D-F). Importantly, GFP$^+$ cells did not engraft into the alveolar epithelium of unchallenged animals (data not shown).

Taken together, these findings clearly demonstrate that prominin-1$^+$ cells have the capacity to differentiate into type II pneumocytes *in vivo.*

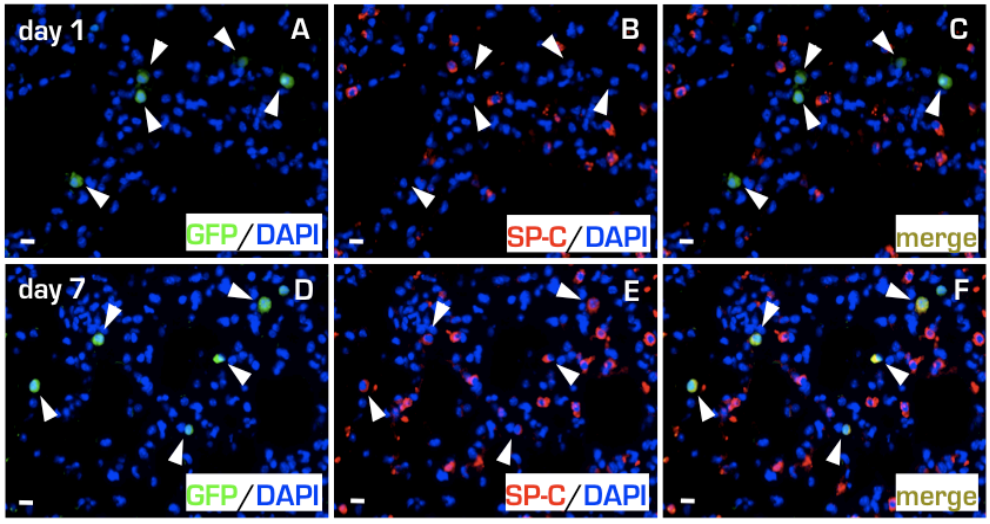

Figure 23. Prominin-1$^+$ cells differentiate into alveolar type II epithelial cells *in vivo*. Intratracheal injection of prominin-1$^+$/GFP$^+$ cells (E-J). GFP$^+$ cells were detected only in alveolar walls after 1 day (E-G) and differentiated into SP-C positive type II alveolar epithelial cells after 7 days (H-J). SP-C (red), GFP (green), DAPI (blue). Bars = 30 µm.

Prominin-1$^+$ cells protect from bleomycin-induced pulmonary fibrosis

At day 7 after intrapulmonary delivery of prominin-1$^+$/GFP$^+$ cells into BLM-challenged mice (Fig. 23D-F), alterations in the alveolar epithelial architecture, typically affected after BLM treatment, were not evident. Given this observation, we set out to specifically address the reparative and protective capacity of prominin-1$^+$ cells in the BLM model. Figures 24A, D and G illustrate the architecture of the alveolar epithelium in unchallenged, PBS-injected mice (control). In BLM-challenged mice intratracheally injected with PBS the alveolar epithelium was massively damaged; in fact, histological analysis revealed considerable infiltration of inflammatory cells (Fig. 24B, E), extensive collagen deposition and progressive fibrosis (Fig. 24H). In contrast, intratracheal delivery of prominin-1$^+$ cells to BLM-challenged mice resulted in the preservation of an almost completely normal architecture of the alveolar epithelium for up to at least 21 days (Fig. 24C, F, I).

Importantly, injection of prominin-1 negative fraction sorted from cultures was not able to prevent the progression of pulmonary fibrosis (data not shown).

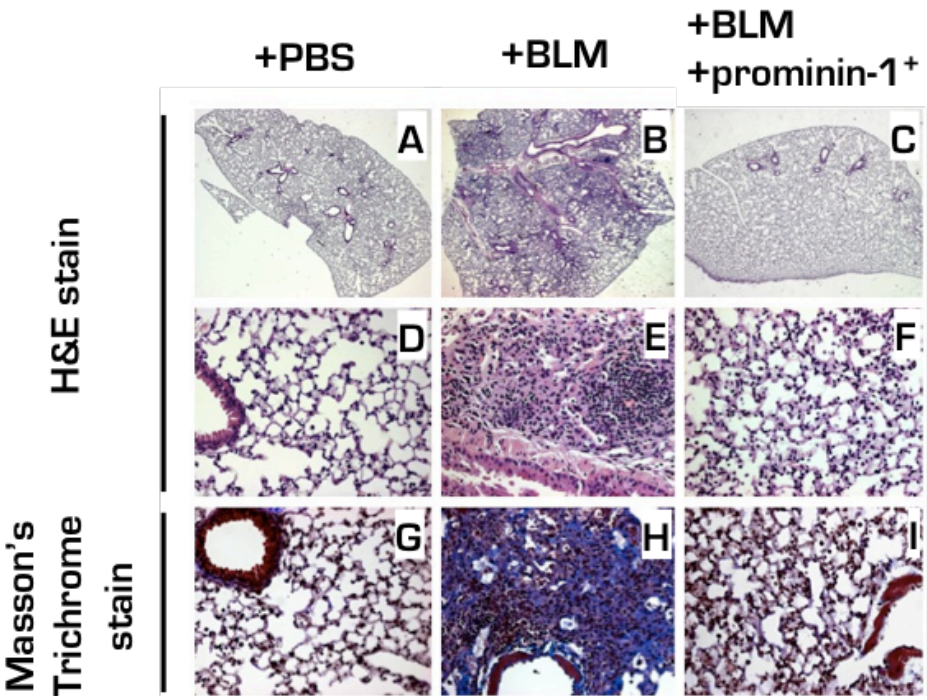

Figure 24. Prominin-1$^+$ cells administration prevents from BLM-induced lung fibrosis in mice. Hematoxylin and eosin (H&E)-stained histopathological sections from lungs of C57Bl/6 mice 21 days after saline exposure (A, D), BLM exposure (B, E) or BLM exposure and prominin-1$^+$ cells (C, F). Masson's Trichrome staining of lung sections from the same experimental groups (G, H, I). Original magnification: 25X (H&E) and 200X (H&E and Trichrome). Data are representative of at least five mice per condition.

In addition, mice that received BLM reported significant loss of weight (Fig. 25A, *black squares*); on the other hand, administration of prominin-1$^+$ cells into BLM-challenged mice also protected from progressive loss of body weight (Fig. 25A, *grey triangles*). Importantly, whereas BALF samples taken at days 3 and 7 from BLM-treated control mice injected with PBS only had increased numbers of total

cells, samples taken from BLM-challenged mice injected with prominin-1$^+$ cells did not (Fig. 25B). Injection of prominin-1$^+$ cells resulted in noteworthy reduction of neutrophils and macrophage recruitment to the injured lungs (Table 2).

Real Time PCR analysis of day 7 lung samples from prominin-1$^+$ cells-treated mice showed no up-regulation of IL-4, IL-6, IL-13 and TNF-α, nor of the chemokines CCL2, MIP-1α, and MCP-5 mediating inflammatory cell recruitment (41, 242) (Fig. 25C). Pro-fibrotic genes such as TGF-β1 and genes of cytokines mediating fibrocytes recruitment, such as SDF-1α or CCL21 (22, 24) were also not up-regulated in prominin-1$^+$ cells-treated animals at day 21 (Fig. 25D).

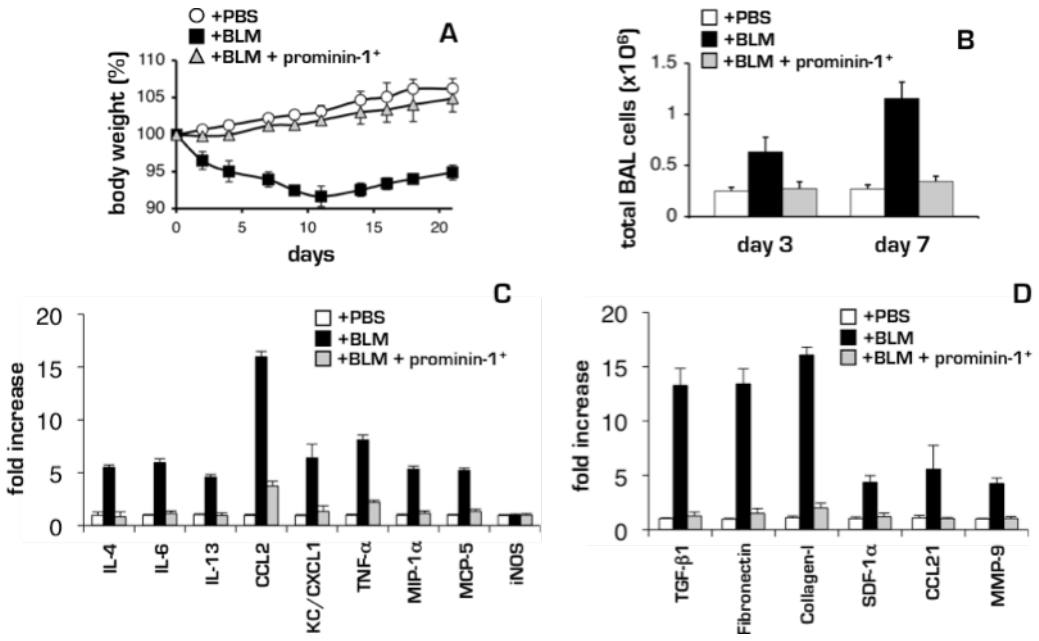

Figure 25. Effect of prominin-1$^+$ cells injection on BLM-induced lung fibrosis in mice. Body weight measurement from animals injected with saline (white), BLM (black) or BLM and prominin-1$^+$ cells (grey) (A). The number of total cells in BALF of the three groups at day 3 and day 7 (B). Quantitative Real Time PCR on lungs collected at the peak of inflammation (day 7) (C) and at the fibrotic stage (day 21) (D) after BLM instillation. The panels summarize the quantitative results after normalization to the GAPDH signal. Bars represent mean ± SD (*n=3*).

Parameter	*PBS day 3 (n=3)*	*BLM day 3 (n=3)*	*BLM + prominin-1$^+$ day 3 (n=3)*	*PBS day 7 (n=3)*	*BLM day 7 (n=3)*	*BLM + prominin-1$^+$ day 7 (n=3)*
Total (x10^6)	0.251±0.036	0.630±0.144	0.261±0.067	0.270±0.040	1.145±0.163	0.343±0.045
Neutrophils	0.002±0.002	0.278±0.083	0.006±0.003	0.003±0.002	0.450±0.147	0.011±0.008
Lymphocytes	0.004±0.003	0.076±0.028	0.007±0.002	0.005±0.001	0.177±0.036	0.027±0.007
Macrophages	0.245±0.034	0.276±0.035	0.248±0.050	0.262±0.039	0.518±0.009	0.305±0.042

Table 2. Analysis of bronchoalveolar lavage (BAL) cell differentials. Data represent mean ± SD.

Accordingly, FACS analysis on blood samples revealed that the number of circulating fibrocytes (CD45$^+$/Col I$^+$/CXCR4$^+$) and did not increase in mice that had received prominin-1$^+$ cells (Fig. 26).

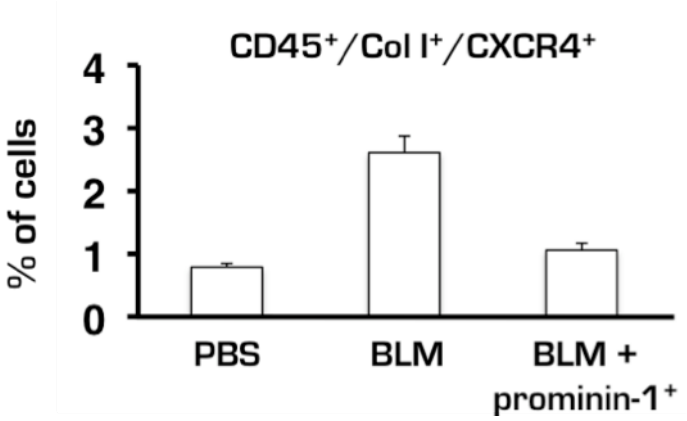

Figure 26. Injection of prominin-1$^+$ cells prevents from recruitment of circulating fibrocytes in mouse blood. FACS analysis on blood samples taken at day 14 from BLM or prominin-1$^+$ cells-treated mice. The percentage of fibrocytes (CD45$^+$/Col I$^+$/CXCR4$^+$) did not increase in blood of mice that received prominin-1$^+$ cells compared with blood taken from mice treated with BLM alone. Bars represent mean ± SD (*n=3*).

Taken together, these results indicate that intratracheal administration of prominin-1$^+$ cells specifically prevents the recruitment of inflammatory cells, abnormal extracellular matrix remodeling and pulmonary fibrosis in BLM-challenged mice.

Prominin-1$^+$ cells suppress alveolar macrophage proliferation

To specifically address the mechanism whereby prominin-1$^+$ cells mediate suppression of BLM-induced fibrosis, we focused on the early stages of disease development in this injury model. We compared the expression of various pro- and anti-inflammatory genes in day 1 lungs of control PBS-treated mice, and BLM-challenged mice treated or not with prominin-1$^+$ cells (Fig. 27). As illustrated, we did not observe up-regulation of Th2 cell specific cytokines such as IL-4 and IL-13 in mice that received prominin-1$^+$ cells.

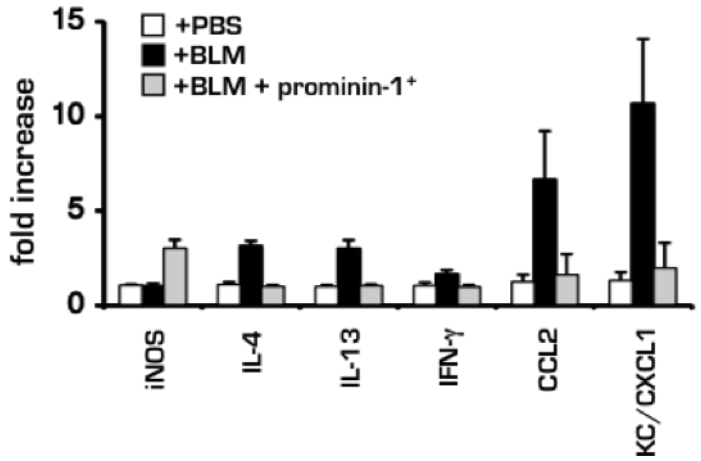

Figure 27. Injection of prominin-1$^+$ cells prevents from up-regulation of pro-inflammatory genes. Real Time PCR performed on lungs of mice challenged with buffer saline (PBS), bleomycin (BLM) alone or BLM + prominin-1$^+$ cells. Lungs were harvested at day 1 after treatment. The panel summarizes the quantitative results after normalization to the GAPDH signal. Bars represent mean ± SD (*n=3*).

Consequently, we hypothesized that T cells might be the targets of prominin-1^+ cells, and therefore involved in the pathogenesis of IPF, despite the fact that recent studies have been shown to be controversial regarding their role (36, 38). Advances in the study of immune reactions have shown that an imbalance between type 1 helper T cells (Th1) and type 2 helper T cells (Th2) plays a pivotal role in the inflammatory response in various diseases (243). Studies have revealed a predominance of the Th2-type cytokine pattern in inflammatory response of IPF. *In vivo* studies using a bleomycin (BLM)-induced pulmonary fibrosis model have also supported this concept by demonstrating that IL-4 mRNA expression is up-regulated in lung fibrosis lesions. The Th2-like pattern of immune response predominated in the infiltrating interstitial inflammatory cells and hyperplastic type II epithelial cells of patients with IPF (244, 245). We therefore isolated $CD4^+$ T cells from BLM-treated animals and co-cultured (5 x 10^4 cells) with irradiated prominin-1^+ cells at different cell ratio. As shown in Fig. 28, prominin-1^+ cells suppressed T-cells proliferation in a dose-dependent manner.

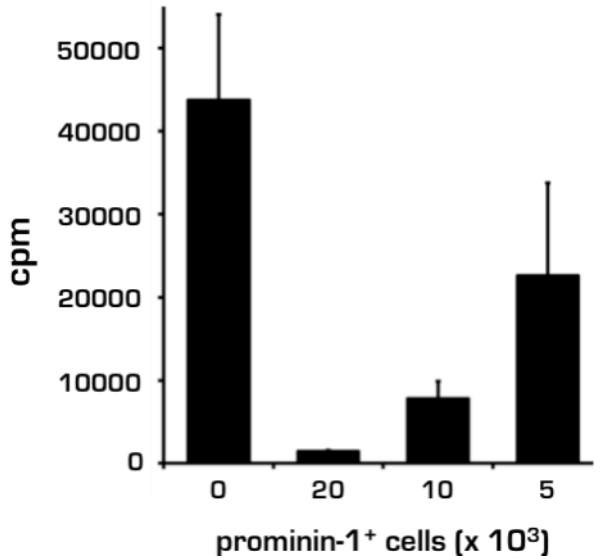

Figure 28. Proliferation of T cells is suppressed *in vitro* in the presence of prominin-1^+ cells in a dose-dependent fashion. 5 x 10^4 $CD4^+$ naïve T cells isolated from BLM-treated animals and previously stimulated with plate-bounds anti-CD3/anti-CD28 antibody were co-cultured with decreasing amount of irradiated prominin-1^+ cells. Cell proliferation was measured by [^{3}H]Thymidine incorporation *(cpm = counts per minute)*. Bars represent mean ± SD (*n=3*).

In order to prove the involvement of T cells in the pathogenesis of BLM-induced fibrosis, we next injected BLM into the lungs of RAG2$^{-/-}$ mice. RAG2$^{-/-}$ animals show total inability to initiate V(D)J rearrangement, leading to a severe combined immune deficient (SCID) phenotype; these mice lack the protein necessary to recombine the DNA at the loci of the T-cell receptor and the immunoglobulin genes, resulting in absent T cells and B cells (246).

As illustrated in Fig. 29, RAG2$^{-/-}$ mice injected with PBS showed normal pulmonary architecture, absence of infiltration of inflammatory cells (Fig. 29A) and collagen deposition (Fig. 29D). On the other hand, RAG2$^{-/-}$ mice that received BLM were not protected from development of pulmonary fibrosis (Fig. 29B, E) and showed a histological pattern comparable to RAG2$^{+/+}$ mice (Fig. 29C, F). Furthermore, an adoptive transfer of CD4^{+} T cells isolated from BLM-treated mice into lungs of non-treated animals did not result in initiation of pulmonary fibrosis (data not shown).

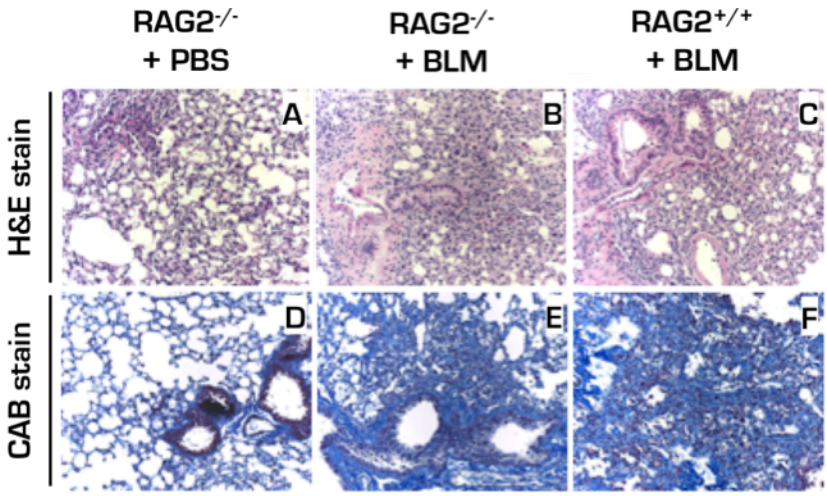

Figure 29. RAG2$^{-/-}$ mice are not protected from BLM-induced pulmonary fibrosis. Lungs of RAG2$^{-/-}$ and RAG2$^{+/+}$ animals were harvested at day 21 following BLM injection, sectioned, and stained with hematoxylin and eosin (H&E) (A-C) or CAB (D-F). Photomicrographs are representative of at least three mice per condition. Original magnification: 200X.

Taken together, these findings clearly explain that T cells are not essential players in the context of BLM-induced lung injury, and therefore they do not represent the main target of prominin-1^+ cells.

However, lungs of prominin-1^+ cells-treated mice also did not display up-regulation of KC/CXCL1 and CCL2 (Fig. 27), chemokines mainly produced by macrophages, which are considered as key players for the onset of pulmonary fibrosis in the BLM-induced injury model (41). Additionally, BLM-challenged mice injected with prominin-1^+ cells showed significant up-regulation of inducible nitric oxide synthase (iNOS) (Fig. 27). Current evidence suggest a regulatory effect of nitric oxide (NO) in many cell types (215, 217, 219, 220) and a protective role in various lung disease models (221, 222, 224). To clarify whether prominin-1^+ cells or alveolar macrophages (AM) were major NO producers, and to specifically assess effects of prominin-1^+ cells on the proliferation of AM, we performed co-culture experiments. As illustrated in Fig. 30A, the proliferation of AM was dramatically reduced when cultured in the presence of prominin-1^+ cells. Prominin-1^+ cells-induced suppression of AM proliferation required cell-to-cell contact between AM and prominin-1^+ cells (Fig. 30C). Compared with individual cultures of AM or prominin-1^+ cells, NO release was markedly increased in the supernatant of AM and prominin-1^+ cells co-cultures (Fig. 30B). To evaluate the role NO in growth arrest, we first assessed proliferation responses in the presence of the non-specific NO synthase inhibitor L-NAME. As shown in Fig. 30D, addition of L-NAME neutralized the effect of prominin-1^+ cells and restored AM proliferation capacity. We next expanded prominin-1^+ cells from iNOS deficient mice and co-cultured them with AM. iNOS$^{-/-}$ prominin-1^+ cells did not suppress the proliferation of AM *in vitro* (Fig, 30A). NO levels increased only in co-cultures of AM with iNOS$^{+/+}$, but not iNOS$^{-/-}$ prominin-1^+

cells (Fig. 30B), confirming that NO was produced specifically by prominin-1^{+} cells. It is noteworthy that NO production did not differ between irradiated and non-irradiated prominin-1^{+} cells and occurred only when cells were stimulated with LPS and IFN-γ (Fig. 30E). Taken together, our findings suggest that prominin-1^{+} cells directly inhibit the proliferation of AM, which are critical for disease development in the BLM-induced lung injury model (41). In addition, our *in vitro* data argue for a critical role of NO in regulation of AM proliferation.

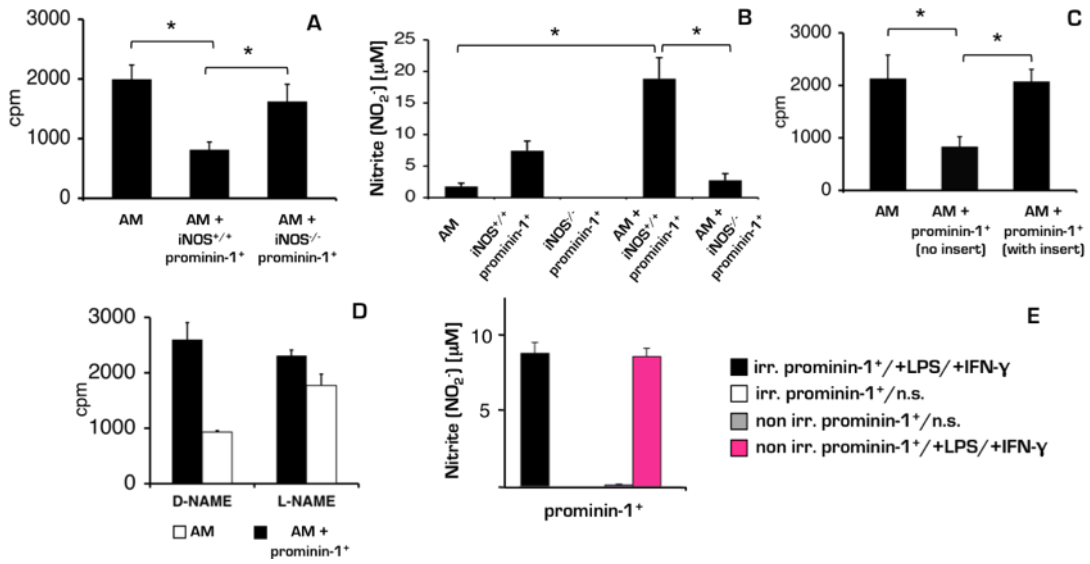

Figure 30. Proliferation of alveolar macrophages (AM) is suppressed in the presence of iNOS$^{+/+}$ prominin-1^{+} cells but not in the presence of iNOS$^{-/-}$ prominin-1^{+} cells (A). Nitrite levels reflecting NO production in culture supernatants increase when AM are cultured in the presence of iNOS$^{+/+}$ prominin-1^{+} cells but not in the presence of iNOS$^{-/-}$ prominin-1^{+} cells (B). The suppressive effects of prominin-1^{+} cells require close contact to AM (C). Inclusion of the NOS inhibitor L-NAME, but not its inactive enantiomer D-NAME, restores proliferation of AM in the presence of prominin-1^{+} cells (D) *(cpm = counts per minute)*. Prominin-1^{+} cells produced NO only when stimulated with LPS and IFN-γ. No difference was found between irradiated and non-irradiated prominin-1^{+} cells (E) (*irr. = irradiated*; *n.s. = not stimulated*). [^{3}H]-Thymidine incorporation was measured as the readout for proliferation. Bars represent mean ± SD (*n=3*). *$p < 0.01$.

Nitric oxide mediates the protective effects of prominin-1$^+$ cells

To address the role of iNOS and NO in the prominin-1$^+$ cells-mediated suppression of BLM-induced lung injury *in vivo*, we injected BLM-challenged mice with either iNOS$^{+/+}$ or iNOS$^{-/-}$ prominin-1$^+$ cells. BALF was collected at day 7. The number of total cells in BALF from mice injected with iNOS$^{+/+}$ prominin-1$^+$ cells was comparable to the BALF of mice challenged with PBS only (Fig. 31A). However, BALF from BLM-challenged mice injected with iNOS$^{-/-}$ prominin-1$^+$ cells contained a markedly increased number of total cells. H&E- and Masson's Trichrome-staining of lung sections from iNOS$^{-/-}$ prominin-1$^+$ cells-treated animals revealed the presence of inflammatory foci and collagen deposition (Fig. 31 E, I, M), comparable to that in lungs from BLM-challenged mice (Fig. 31 C, G, K).

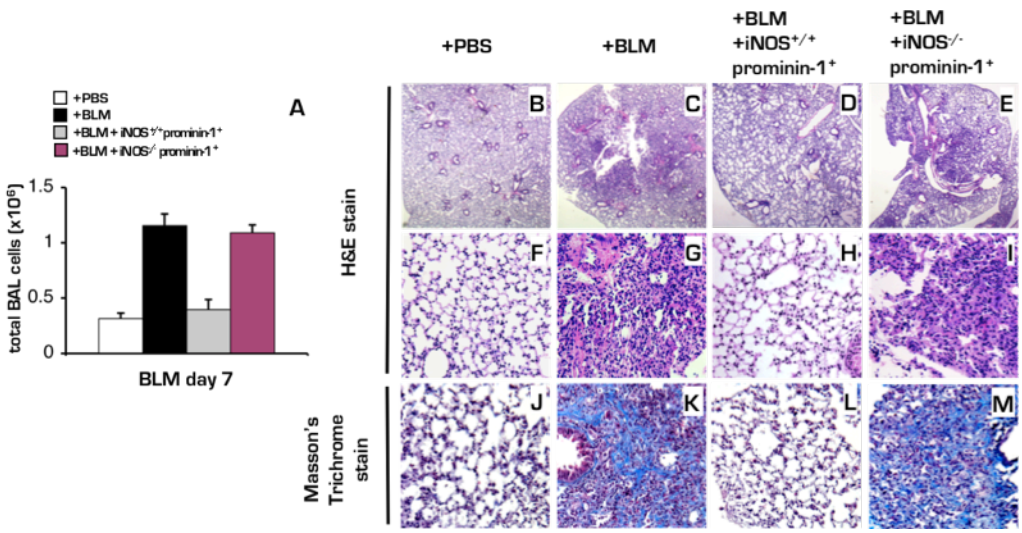

Figure 31. Intratracheal injection of iNOS$^{-/-}$ prominin-1$^+$ cells does not protect from BLM-induced pulmonary fibrosis. The total cell number in BALF is comparable in mice injected with BLM alone and BLM + iNOS$^{-/-}$ prominin-1$^+$ cells. The total number of cells is similar in BALF from mice exposed to saline buffer or BLM + iNOS$^{+/+}$ prominin-1$^+$ cells (A). *$p < 0.01$. Bars represent mean ± SD ($n=3$). Lungs were harvested, sectioned, and stained with hematoxylin and eosin (H&E) (B-I) or Masson's trichrome (J-M). Photomicrographs are representative of at least five mice per condition. Original magnification: 25X (H&E) and 200X (H&E and Trichrome).

In conclusion, bone marrow-derived prominin-1^{+} cells expanded from mouse lungs possess regenerative capacity and striking anti-inflammatory properties, and protect BLM-treated mice from pulmonary fibrosis in a NO-dependent manner.

Discussion

My thesis describes the discovery of a novel population of prominin-1/CD133$^+$ bone marrow-derived lung progenitors from adult mouse lungs with multilineage differentiation capacity *in vitro*, and anti-inflammatory and regenerative properties *in vivo*.

In support of the hypothesis that lung injury exceeding a certain threshold is necessary to activate stem/progenitor cell expansion, we found that dissection of pulmonary tissue resulted in the expansion of a small, round, undifferentiated population of prominin-1$^+$ cells growing on feeder layer cells with mesenchymal phenotype. Our data illustrated that density was critical in maintaining *in vitro* the round phenotype of prominin-1$^+$ cells.

Recent studies have shown the presence of stem cell populations within lungs (103, 247). Our sorted prominin-1$^+$/CD45$^+$ cells were negative for SP-C and CC10. Accordingly, they represent a distinct subset from bronchioalveolar stem cells (BASCs) (103), which in contrast co-express both SP-C and CC10. BASCs were identified at the bronchioalveolar duct junction within the terminal bronchioles, whereas prominin-1$^+$/CD45$^+$ cells were located in the alveolar epithelium. Furthermore, our prominin-1$^+$ cells were of hematopoietic origin and clearly expressed a phenotype different from the BASCs and lung Side Population (SP) mesenchymal stem cells described by Summer and colleagues (247). Similarly to BASCs, prominin-1$^+$ progenitor cells exhibited multipotent properties. In fact, depending on culture conditions, prominin-1$^+$ cells showed multineage capacity and differentiated *in vitro* into three different cell types, acquired a different phenotype and changed their gene and protein profile. Since single cell-derived clones showed

capacity to differentiate into epithelial (alveolar type II cells), mesenchymal (fibroblasts) and hematopietic (macrophages) phenotypes, prominin-1$^+$ progenitors can be considered as multipotent progenitor cells. These data clearly highlight the plasticity of prominin-1$^+$ progenitors *in vitro*. Given their capacity to differentiate into inflammatory cell and fibroblast phenotype, it remains to be established whether prominin-1$^+$ cells could play a role in the context of inflammatory pulmonary disease. Since prominin-1$^+$ cells acquired the expression of collagen type I after fibroblast differentiation, they might also contribute to collagen deposition and progression of pulmonary fibrosis. Data from our laboratory illustrated that cardiac prominin-1$^+$ progenitors differentiated *in vivo* into macrophages and fibroblasts when injected during inflammatory stage within adult heart tissue in a mouse model of autoimmune myocarditis. Collectively, these data emphasize the plasticity of prominin-1$^+$ cells in different organs.

However, prominin-1$^+$ cells did not give rise to progenies when plated as single cells without feeder layer. The ultimate test for stem cell activity is a single-cell clonogenic transplantation assay, as has been done in the hematopoietic system (248, 249) However, such assays are particularly challenging when applied to stem cells of epithelial tissues. Epithelial cells are closely associated with endothelial cells, stromal fibroblasts, inflammatory cells, and accompanying extracellular matrix and cell-to-cell interactions. Some or all of these components may be required to allow survival, engraftment, and proper differentiation of putative stem and progenitor cells. Although there is an established protocol in order to allow stem cells to repopulate the mammary tissue without injection of additional exogenous supporting cells (104, 250-252), a comparable system remains to be created for other epithelial tissues, including the lung.

We have demonstrated that the healthy mouse lungs displayed two distinct prominin-1$^+$ phenotypes. Prominin-1/CD133 expression has already been described for different adult epithelial cells (253). We identified for the first time the presence of a population of prominin-1$^+$/CD45$^+$ cells within the adult mouse lung. On the other hand, the majority of prominin-1$^+$ cells belonged to the bronchial epithelial ciliated population. Accordingly, our findings are consistent with recent work of Shmelkov and collaborators (182) demonstrating the presence of prominin-1$^+$ cells along the bronchial epithelium. Moreover, our data have shown that the cuboidal prominin-1$^+$/β-tubulin IV$^+$ cells were not of bone marrow origin, whereas the prominin-1$^+$/CD45$^+$ cells were also GFP positive and clearly bone marrow-derived. Notably, prominin-1$^+$/GFP$^+$ cells sorted *ex vivo* from chimeric lungs expanded only in the presence of a feeder layer. This observation suggests that lung-derived cells might specifically trigger the expansion of bone marrow-derived resident prominin-1$^+$ pulmonary progenitors. On the other hand, in the same conditions it was not possible to expand *in vitro* prominin-1$^+$ cells isolated from mouse blood. These records clearly exclude that prominin-1$^+$ cells expanded from lungs could represent a residual of blood cells.

Immunohistochemistry showed that prominin-1$^+$/CD45$^+$ cells were associated with the alveolar epithelium *in vivo*. Prominin-1$^+$ cells expanded from adult tissue differentiated *in vitro* into type II epithelial cells. Based on their expression of type II alveolar specific gene SP-C, but not bronchial epithelial CC10, prominin-1$^+$ cells may represent a specific early committed alveolar progenitor population. In support of this view, intratracheal injection of prominin-1$^+$ cells in BLM-challenged mice resulted in straight differentiation towards a type II epithelial phenotype. These data underscore the striking regenerative potential of prominin-1$^+$ cells *in vivo*. It is important to

underline that prominin-1$^+$ cells engrafted into the alveolar walls of the lungs of BLM-challenged animals only. These findings support the view that tissue injury is necessary to create a specific microenvironment that promotes engraftment and subsequent differentiation of prominin-1$^+$ cells. Our data are consistent with other studies that have demonstrated the occurrence of tissue-specific differentiation of engrafted bone marrow cells only in challenged animals (155, 160, 254, 255).

Besides, it is of particular interest the finding that prominin-1$^+$/CD45$^+$ cells reside within the pulmonary alveolar epithelium. A recent work has shown that the expression of prominin-1 in human lung tissue was associated with rapid progression of IPF (256), and prominin-1 expression was predominantly expressed in the areas of the alveolar lesions. In addition, in a model of lung cancer, mice develop atypical adenomatous hyperplasias (hyperproliferations of alveolar type II cells) that progress to adenomas and then overt adenocarcinomas (257). Together with the observation that the adenocarcinomas were positive for the alveolar type II cell-specific marker SP-C, these data implicate that type II cells are the target cells in rodent and human lung adenocarcinomas. Recent findings have already shown that lung tumors can arise from expansion of stem cells (103); furthermore a novel population of prominin-1$^+$ expressing tumorigenic stem cells has been identified in human in small cell and non-small cell lung cancer (189). Given the location of prominin-1$^+$/CD45$^+$ as regional pulmonary stem cells and as precursors *in vivo* for type II cells, they might possibly represent a target cell population in tumorigenesis or fibrosis. In addition, unpublished observations have shown that the percentage of prominin-1$^+$/CD45$^+$ cells increase after BLM treatment; moreover, immunohistochemistry revealed that a significant increase of prominin-1$^+$ cells within the alveolar epithelium (data not shown). It remains to be established whether the increase of prominin-1$^+$/CD45$^+$ cells is due to

migration of bone marrow-derived cells from the circulation or due to activation of resident prominin-1^{+}/CD45^{+} progenitor cells. If so, it might be possible to target and activate these cells and redirect towards epithelial differentiation in order to restore the damaged tissue. Similarly, administration of both keratinocyte growth factor and hepatocyte growth factor to animals with lung injury has been shown to stimulate alveolar epithelial cell proliferation (258-260). Given these remarks, prominin-1 expressing progenitors might symbolize a double-edged sword: on one hand, prominin-1^{+} cells could represent a cellular source for tissue regeneration; on the other hand, they might contribute to fibrosis or even lung cancer. Nevertheless, contribution, involvement and activation of pulmonary prominin-1^{+} progenitors after alveolar injury will need to be carefully investigated. A major task will be required to figure out under which conditions resident cells will exert a protective effect *in vivo*.

Accumulating evidence supports a beneficial role of administered stem or precursor cells in several injury models (200, 261-265). Here we have demonstrated that intratracheal injection of prominin-1^{+} cells in the BLM-injury model resulted in complete protection from pulmonary fibrosis. It is important to underline that administration of prominin-1 negative fraction did not decrease the pro-inflammatory and pro-fibrotic response to BLM (data not shown). This finding clearly states the specific immunomodulatory effect of prominin-1^{+} cells. Other studies have shown that injection of bone marrow-derived stem cells can ameliorate BLM-induced lung fibrosis (25, 155). In line with these studies, we found that beneficial effects occur only when prominin-1^{+} cells are injected during the early phases after BLM instillation. Intrapulmonary delivery of mesenchymal stem cells has been shown to attenuate the effects of pulmonary injury in different animal models when administered at early stages (266, 267), indicating that hematopoietic and

mesenchymal stem cells share similar immunological properties. As illustrated, only a small amount of GFP^+/prominin-1^+ cells engrafted into the alveolar walls and differentiated into type II epithelial cells. Our data are in line with other studies showing relatively minimal contribution of donor cells to the alveolar epithelium. Given these findings, we believe that the primary effect of prominin-1^+ progenitors results in an immunomodulatory action, whereas the engraftment might represent only a secondary consequence. Besides, it remains to be established whether bone marrow-derived stem cells might exert a similar protective potential in reverting BLM-induced effects at the late stages of fibrosis. In the presence of pro-fibrotic cytokines and massive damage to the whole alveolar epithelium, a higher number of cells might be required to exert an adequate response.

Our study also demonstrates that prominin-1^+ cells exerted an anti-inflammatory effect *in vivo* and suppressed alveolar macrophages proliferation *in vitro*. Baran and colleagues (41) have shown that the presence of macrophages is crucial for development of BLM-induced fibrosis. The fundamental role of alveolar macrophages in the context of lung injury has been similarly noted in other studies (268-270). We found that injection of prominin-1^+ cells suppressed the production of cytokines secreted by macrophages, such as KC/CXCL1, which might explain the consequential lack of neutrophils recruitment to the lungs of prominin-1^+ cells-treated BLM-challenged mice. In addition, no up-regulation of SDF-1α was found in lungs of the prominin-1^+ cells-treated mice. Lack of SDF-1α up-regulation, as well as CCL21, cytokines mainly produced also by macrophages, might be responsible for the impaired recruitment of circulating fibrocytes (24) and bone marrow-derived fibroblasts to injured lung (22). Accordingly, flow cytometry analysis of blood samples taken from animals that received prominin-1^+ cells confirmed that no

recruitment of fibrocytes was found, suggesting that the anti-inflammatory effect resulted also in the abolishment of a more systemic reaction.

The suppressive effect of prominin-1^+ cells-treatment on AM proliferation was critically mediated by NO production. *In vitro*, AM growth suppression was restored in presence of NO inhibitor, L-NAME. In accordance with this finding, prominin-1^+ cells expanded from iNOS$^{-/-}$ mice could not suppress alveolar macrophages proliferation *in vitro* and failed to protect from BLM-induced injury *in vivo*. Interestingly, our data show that prominin-1^+ cells do not secrete NO without LPS and IFN-γ stimulation. This observation suggests that a specific environment (e.g. inflammation) is necessary to render prominin-1^+ cells capable to exert NO-mediated immunomodulatory effects. Of note, the administration of NO-producing cells such as bone marrow-derived macrophages and bone marrow-derived dendritic cells did not exert any protective effect, therefore emphasizing the specific immunomodulatory role of prominin-1^+ cells. Unlike prominin-1^+ cells, other NO-producing cells might secrete pro-inflammatory factors that in turn compensate the beneficial effect of NO. Likewise, a recent study from our lab has shown that bone marrow-derived prominin-1/CD133$^+$ cardiac progenitors protect from autoimmune myocarditis in mice through a NO-mediated mechanism (200). Our data are consistent with the reported benefits of NO inhalation in other lung injury models (223, 224), confirming that low concentration of NO exert a protective effect, despite the evidence that iNOS$^{-/-}$ mice are protected from BLM-induced fibrosis (271). Recent findings have shown that exogenous NO can attenuate the EMT that affects alveolar type II cells (238). In fact, EMT has been shown to occur both *in vitro* (231, 232) and *in vivo* (231-233) and involve significantly type II pneumocytes that, consequently, give rise to myofibroblast-like cells. Thus, we cannot exclude that prominin-1^+ cells targeted not

only macrophages, but also type II pneumocytes, preventing in this way also the transition to myofibroblasts. Collectively, these findings strongly support the view that NO-dependent mechanisms play a critical role in prominin-1^+ cells protection from BLM-induced pulmonary fibrosis.

Prominin-1^+ progenitors represent a population of bone marrow-derived cells. Prominin-1^+ cells are also found in blood and bone marrow of healthy animals, but do not share analogous regenerative and immunomodulatory properties with prominin-1^+ cells (data not shown). We believe that either the specific microenvironment created by disintegration of whole pulmonary tissue, or inflammatory processes are critical for the activation and expansion of immunomodulatory and regenerative prominin-1^+ cells. Appropriate adaptation and optimization of culture conditions for blood- or bone marrow-derived cells might enable isolation and expansion of vast numbers of immunomodulatory/regenerative cells from the peripheral circulation.

Autologous stem cell transplantation could be used to overcome the problem of immune rejection in regenerative medicine. Adult stem cells could be transplanted into a new niche where they would be able to repopulate the cells of defective tissue. Stem cells purified from bone marrow, blood, or umbilical cord could be also stored before a major operation or when it is predicted that a patient is at risk of acute lung injury. As an agent of combination therapy, stored stem cells would be injected back into patients with acute lung injury to enhance lung repair and to generate functional alveolar structures or enhanced surfactant. There is great excitement and potential with these therapies, but at present the field is littered with controversies and questions (272). Although we are some way off from clinical trials with bone marrow stem cells in parenchymal lung disease until some issues are clarified, such is the promise of this therapy that it has already been used in human cardiac trials.

Collectively, there is a considerable safety record with clinical use of stem cells, consisting in transplantation of bone marrow-derived cells. Initial exploratory trials, phase 1 or otherwise, can be safely designed and implemented with appropriately measurable outcome assessments. In fact, there are many patients with end-stage lung diseases who are already offering to participate in clinical trials and investigations. Although patients with end-stage lung diseases may not be the optimal population to study, initial investigations might provide important data about safety and will generate future hypotheses for study. Unfortunately, pulmonary and critical care medicine has traditionally lagged behind other fields, for example, cardiology and hematology, in translational studies of potential new therapies, including the use of reparative cells. In fact, clinical trials in cardiology (273) have demonstrated the feasibility and safety of administering progenitor cells derived from autologous bone marrow to the myocardium of patients with ischemic heart disease. The few controlled trials that have been completed so far show a tendency to improved heart function in transplanted patients after myocardial infarction, and in those with chronic ischemic heart failure (273).

Despite many safety records for patients who underwent bone marrow transplantation, there is not enough information about long-term effects of stem cell engraftment within adult tissues. Cogent examples are the publications demonstrating development of gastric cancer from transplanted stem cells in a mouse model of chronic *H. pylori* infection and the potential role of adult stem cells in development of cancer (274, 275).

Advances in stem cell biology and technology will eventually turn cell transplantation into a useful treatment for patients with a variety of diseases and injuries, including genetic diseases involving lung and acute lung injury. However,

much more research on how to purify stem cells and how to direct the differentiation of stem cells and to stimulate regeneration and functional recovery is needed. Although stem cell therapy in the lung has great promise, further basic science research is needed before this treatment can be confidently moved to the bedside.

Conclusion

We successfully expanded a population of bone marrow-derived progenitor cells with multilineage differentiation capacity from adult mouse lungs. The cells were characterized by prominin-1^{+} expression and showed *in vitro* and *in vivo* immunosuppressive and regenerative capacities. Our study therefore represents an important step toward the development of novel cell-based therapies for pulmonary fibrosis, the common final pathway of several interstitial lung diseases. Future projects must focus on the identification of related prominin-1 expressing progenitors in human lung biopsy samples (Fig. 32).

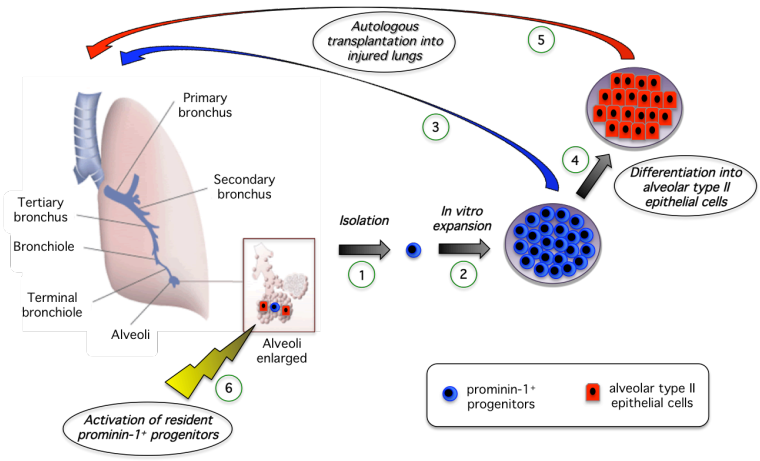

Figure 32. Potential therapeutic applications of prominin-1^{+} cells. Prominin-1^{+} progenitors might be isolated from human biopsies (1), expanded *in vitro* (2) and injected back into donor patients affected by pulmonary disorders (3); alternatively, prominin-1^{+} cells might differentiate into alveolar type II cells (4) and injected to replace damaged cells (5). In addition, following pharmacological activation (6), resident prominin-1^{+} progenitors might be redirected towards epithelial differentiation in order to restore the injured tissue.

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Education

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  Final grade: 102 out of 110
  Faculty of Pharmacy, University of Padua, Italy

  Final project done at ISREC (Institut Suisse de Recherce Expérimentale sur le Cancer), Epalinges s/Lausanne, Switzerland

- 09/1994 – 07/1999
  Diploma of humanistic secondary school
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Research and work experiences

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  PhD in Experimental Critical Care Medicine Laboratory, University Hospital Basel, Switzerland
  Head: Prof. Urs Eriksson
  Project: Identification of a Novel Population of Bone Marrow-Derived Prominin-1/CD133$^+$ Lung Progenitors with Regenerative Capacity
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- 03/2005 – 06/2005
  Training period at Centre Pluridisciplinaire d'Oncologie, Lausanne, Switzerland
  Head: Dr. Curzio Rüegg
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- 03/2004 – 02/2005
  Internship in Breast Cancer and Mammary Gland Development Group, ISREC (Institut Suisse de Recherce Expérimentale sur le Cancer), Epalinges s/Lausanne, Switzerland
  Head: Dr. Cathrin Brisken
  Project: Role of RANKL (Receptor Activator of NF-kappaB Ligand) in the Mammary Gland Development
  Supervisor: Dr. Cathrin Brisken

Laboratory skills

Cellular Biology: expansion and culture of stem cell-like colonies; *in vitro* differentiation (type II pneumocyte-, fibroblast-, macrophage-, dendritic cell-, hepatocytes-like phenotype); basic mammalian cell culture (3T3, 293T, melanoma cell lines, transformed primary mammary epithelial cells); Magnetic Activated Cell Sorting (MACS); proliferation assay; migration and invasion assays; cytokine measurements (ELISA); cell transfection and infection; production and titration of retrovirus.

Animal Experimentation: maintenance of transgenic colonies; genotyping; mammary transplants; mouse dissection and microdissection; mammary gland whole mount preparation; intratracheal, intraperitoneal, intravenous injection; bronchoalveolar lavage (BAL); generation of chimeric mice; blood and bone marrow cell isolation; bone marrow cell transplantation; hepatectomy.

Microscopy: basic light microscopy; fluorescence microscopy; whole mount analysis by stereoscope.

Immunohistochemistry: cut and stain of frozen and paraffin sections; stain of *in vitro* cultured cells grown on Matrigel- and gelatin-coated cover slips.

Flow cytometry: BD FACSCalibur™ and CyAn™ ADP Analyzer; data analysis using FlowJo software.

Molecular Biology: DNA and RNA extraction; cloning by enzymatic digestion; electrophoresis; Reverse Transcription and Real Time PCR; gene transduction by retroviral system.

Biochemistry: protein gel electrophoresis; Western Blot.

Languages

Italian: mother tongue

English: fluent written and oral

French: basic knowledge

German: basic knowledge

Informatic skills

Operating System: MacOS; Windows;
competent in all Microsoft Office package (including Word, Excel, Powerpoint) and other scientific package (Endnote);
good knowledge of Adobe Photoshop; Gimp; Aperture.

Bioinformatic: search for homology against nucleotide and protein sequence databases (BLAST); primers design.

Course certificates

"Radiological Safety Protection Course" organized by the "Department of Biomedicine" of the University Hospital Basel, September 2006, Basel, Switzerland.

LTK1 Module 1E: Introductory Course in Laboratory Animal Science (Swiss Ordinance on the Education and Training of Persons Conducting Animal Experiments), 2005, Basel, Switzerland.

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Available on request

Publication list

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Germano D, Blyszczuk P, Valaperti A, Kania G, Dirnhofer S, Landmesser U, Lüscher TF, Hunziker L, Zulewski H, Eriksson U. Prominin-1/CD133^{+} lung epithelial progenitors protect from bleomycin-induced pulmonary fibrosis (submitted).

Blyszczuk P, Berger CT, Kania G, Marty RR, Valaperti A, **Germano D**, Dirnhofer S, Lüscher TF, Bogdan C, Hunziker L, Eriksson U. Type I IFN signaling promotes heart-specific autoimmunity (submitted).

Mauermann N, Burian J, von Garnier C, Dirnhofer S, **Germano D**, Schuett C, Tamm M, Bingisser R, Eriksson U, Hunziker L. Interferon-gamma regulates idiopathic pneumonia syndrome, a Th17^{+}CD4^{+} T-cell-mediated graft-versus-host disease. *Am J Respir Crit Care Med.* 2008.

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Abstracts

Ecublens/Lausanne, EPFL, 15 December 2006, *3th Swiss Stem Cells Network (SSCN) annual meeting*. **Poster presentation**.

Basel, 23 October 2007, *BioValley Science Day*. **Poster presentation.**

Zürich, 16 December 2007, *4th Swiss Stem Cells Network (SSCN) annual meeting*. **Poster presentation.**

Philadelphia, 11-14 June 2008, *6th Annual Meeting of the International Society for Stem Cell Research (ISSCR).* **Poster presentation.**

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