COMPOSITIONS AND METHODS FOR SUPPRESSING FIBROCYTES

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ABSTRACT
The present invention relates to the ability of anti-FcyR antibodies to suppress fibrocytes. Methods and compositions for suppressing fibrocytes are provided. These methods are useful in a variety of applications including treatment and prevention of conditions resulting from fibrosis in the liver, kidney, lung, heart and pericardium, eye, skin, mouth, pancreas, gastrointestinal tract, brain, breast, bone marrow, bone, gastrointestinal system, a tumor, or a wound.

10 Claims, 6 Drawing Sheets


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**FIGURE 1**

![Graph showing the number of fibrocytes per 2.5 x 10^5 cells for different conditions.](image)

- **Control**
- **FcγRII**
- **FcγRI**
- **FcγRII+FcγRI**

Legend:
- Open square: F(ab')_2 mAb
- Cross-linked F(ab')_2

**FIGURE 2**

![Graph showing the number of fibrocytes per 2.5 x 10^5 cells for different conditions.](image)

- **Control**
- **FcγRI**
- **FcγRII**
- **FcγRII+FcγRI**
- **X-linker only**

Legend:
- No inhibitor
- PP3
- PP2
- Syk inhibitor
**FIGURE 3**

![Graph showing fibrocyte count per 2.5 × 10⁵ cells with treatment groups: Control, PP2, PP3, and Syk. The graph compares two conditions: SFM and Agg. IgG. Statistically significant differences are indicated by asterisks.](image)

**FIGURE 4A**

![Graph showing fibrocyte count per 2.5 × 10⁵ cells in response to varying concentrations of IgG (µg/ml). The graph compares Monomeric IgG and Cross-linked IgG. Statistically significant differences are indicated by asterisks.](image)
FIGURE 5

![Graph showing number of fibrocytes vs. SAP (μg/ml) for different concentrations of IgG.]

FIGURE 6A

![Bar chart showing percentage of fibrocytes compared to control for different conditions: anti-OVA, OVA, OVA I.C. with asterisks indicating statistical significance.]

- 1 μg/ml IgG
- 10 μg/ml IgG
- 100 μg/ml IgG
- 1000 μg/ml IgG
FIGURE 6B

Number of fibrocytes compared to control

Ratio SRBC:monocytes

FIGURE 6C

Number of fibrocytes

Heat aggregated IgG  Heat aggregated F(ab)2  Control

100 µg/ml
FIGURE 7

Number of Fibrocytes (Percentage Control)

CD16  CD32  CD64  IgG Control
1 COMPOSITIONS AND METHODS FOR SUPPRESSING FIBROCYTES

PRIORITY CLAIM


FIELD OF THE INVENTION

The present invention relates to the ability of anti-FcγR antibodies, aggregated IgG, and/or cross-linked IgG to suppress fibrocytes. Accordingly, it may include compositions and methods for suppressing fibrocytes. These compositions and methods may be useful in a variety of applications, for example, those in which decreased fibrocyte formation is beneficial, such as treatment of fibrosing diseases and asthma.

BACKGROUND

Fibrocytes

Inflammation is the coordinated response to tissue injury or infection. The initiating events are mediated by local release of chemotactic factors, platelet activation, and initiations of the coagulation and complement pathways. These events stimulate the local endothelium, promoting the extravasation of neutrophils and monocytes. The second phase of inflammation is characterized by the influx into the tissue of cells of the adaptive immune system, including lymphocytes. The subsequent resolution phase, when apoptosis of the excess leukocytes and engulfment by tissue macrophages takes place, is also characterized by repair of tissue damage by stromal cells, such as fibroblasts.

In chronic inflammation, the resolution of inflammatory lesions is disordered, with the maintenance of inflammatory cells, fibroblast hyperplasia, and eventual tissue destruction. The mechanisms that lead to these events are complex, but include enhanced recruitment, survival and retention of cells and impaired emigration.

The source of fibroblasts responsible for repair of wound lesions or in other fibrotic responses is controversial. The conventional hypothesis suggests that local quiescent fibroblasts migrate into the affected area, produce extracellular matrix proteins, and promote wound contraction or fibrosis. An alternative hypothesis is that circulating fibroblast precursors (called fibrocytes) present within the blood migrate to the sites of injury or fibrosis, where they differentiate and mediate tissue repair and other fibrotic responses.

Fibrocytes are fibroblast-like cells that appear to participate in wound healing and are present in pathological lesions associated with, inter alia, asthma, pulmonary fibrosis and scleroderma. Fibrocytes are known to differentiate from a CD14+ peripheral blood monocyte precursor population.
There are four distinct classes of FcγR. FcγRI (CD64) is expressed by peripheral blood monocytes and binds monomeric IgG with a high affinity. FcγRII (CD32) and FcγRIII (CD16) are low affinity receptors for IgG and only efficiently bind aggregated IgG. FcγRIII is expressed by NK cells and a subpopulation of monocytes. FcγRIV was recently identified in mice and is present on murine peripheral blood monocytes and neutrophils, macrophages and dendritic cells and efficiently binds murine IgG2a and IgG2b antibodies. There is a putative human FcγRIV gene, but the biological function of the protein, such as ligand specificity and cellular expression is, as yet unknown.

Peripheral blood monocytes express both FcγRI and FcγRII (a subpopulation of monocytes express FcγRIII), whereas tissue macrophages express all three classical FcγR. Clustering of FcγR on monocytes by IgG, either bound to pathogens or as part of an immune complex, initiates a wide variety of biochemical events.

FcγR activation and induction of intracellular signaling pathways may occur when multiple FcγR are cross-linked or aggregated. This FcγR activation leads to a cascade of signaling events initiated by two main kinases. The initial events following FcγR activation involve the phosphorylation of intracellular immunoreceptor tyrosine activation motifs (ITAMs) present on the cytoplasmic tail of FcγRIIs or the Fcγ-γ chain associated with FcγRI and FcγRIII, by Src-related tyrosine kinases (SRTK). In monocytes, the main Src-kinases associated with FcγRI and FcγRIII are hck and lyn. The phosphorylated ITAM then recruit cytoplasmic SH2-containing kinases, especially Syk, to the ITAMs and Syk then activates a series of downstream signaling molecules.

Anti-FcγR antibodies for FcγRI (anti-FcγRI) and for FcγRII (anti-FcγRII) are able to bind to either FcγRI or FcγRII, respectively. These FcγR may then be cross-linked by the binding of additional antibodies or other means. This process initiates intracellular signaling events consistent with FcγR activation.

Scleroderma

Scleroderma is a non-inherited, noninfectious disease that has a range of symptoms. It involves the formation of scar tissue containing fibroblasts in the skin and internal organs. The origin of the fibroblasts is unknown. In mild or early cases of scleroderma, there is a hardening of the skin, fatigue, aches and sensitivity to cold. Further evidence strongly suggests that disruption of the alveolar epithelium is an underlying pathogenic event. Given the role played by fibrocytes in wound healing and their known role in airway wall thickening, the source of these cells appears to be a specialized type of fibroblast called myofibroblasts.

In asthma patients, CD34+/collagen I+ fibrocytes accumulate near the basement membrane of the bronchial mucosa within 4 hours of allergen exposure. 24 hours after allergen exposure, labeled monocytes/fibrocytes have been observed to express α-smooth muscle actin, a marker for myofibroblasts. These observations suggest that in asthma patients allergen exposure causes fibrocytes from the blood to enter the bronchial mucosa, differentiate into myofibroblasts, and then cause airway wall thickening and obstruct the airways. Further, there is a correlation between having a mutation in the regulatory regions of the genes encoding monocyte chemoattractant protein 1 or TGFβ-1 and the severity of asthma. These observations suggest that recruitment of monocytes and appearance of myofibroblasts lead to complications of asthma.

Thickening of the lamina reticularis distinguishes asthma from chronic bronchitis or chronic obstructive pulmonary disease and is found even when asthma is controlled with conventional medications. An increased extent of airway wall thickening is associated with severe asthma. No medications or treatments have been found to reduce thickening of the lamina reticularis. However, it appears likely that reducing the number of myofibroblasts found in the airway walls may reduce thickening or help prevent further thickening.

Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a unique type of chronic fibrosing lung disease of unknown etiology. The sequence of the pathogenic mechanism is unknown, but the disease is characterized by epithelial injury and activation, the formation of distinctive subepithelial fibroblast/myofibroblast foci, and excessive extracellular matrix accumulation. These pathological processes usually lead to progressive and irreversible changes in the lung architecture, resulting in progressive respiratory insufficiency and an almost universally terminal outcome in a relatively short period of time. While research has largely focused on inflammatory mechanisms for initiating the fibrotic response, recent evidence strongly suggests that disruption of the alveolar epithelium is an underlying pathogenic event. Given the role played by fibrocytes in wound healing and their known role in airway wall thickening in asthma, it appears likely that overproduction of fibrocytes may be implicated in IPF.

SUMMARY

The present invention may include compositions and methods for suppressing fibrocytes. In the context of the present invention, the term "suppressing fibrocytes" refers to one or more of inhibiting the proliferation of fibrocytes, inhibiting the development of fibrocytes, including the development or differentiation of a cell into a fibrocyte, and promoting the differentiation of fibrocytes into non-fibrocytic cell types.

In selected embodiments, fibrocytes may be suppressed in a target location by providing anti-FcγR antibodies that are able to cross-link FcγR. The target location may be located in vitro or in vivo. Specifically, the target location may be located in a mammal, such as a human patient.
In vivo, the target location may include an entire organ or a portion thereof and the composition may be administered systemically or it may be confined to a particular area, such as an organ or tissue.

Suppressing fibrocytes may alleviate symptoms of numerous fibrosing diseases or other disorders caused by fibrosis. In a specific embodiment, administration of anti-FcyR antibodies may be used to treat the effects of unwanted fibrocytes. For example, it may be used to treat fibrosis in the liver, kidney, lung, heart and pericardium, eye, skin, mouth, pancreas, gastrointestinal tract, brain, breast, bone marrow, bone, genitourinary, a tumor, or a wound.

BRIEF DESCRIPTION OF THE DRAWINGS

The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention.

FIG. 1 shows the effects of cross-linked and non-cross-linked anti-FcyR antibodies on fibrocyte differentiation from Peripheral Blood Mononuclear Cells (PBMC). PBMC at 2.5 x 10^5 cells per ml were cultured in serum-free medium for 5 days in the presence or absence of 1 µg/ml of the indicated F(ab')2 anti-FcyR or control IgG1 antibodies, in the presence (black bars) or absence (white bars) of 500 ng/ml goat F(ab')2 anti-mouse IgG, which cross-links the F(ab')2. Cells were then air-dried, fixed, stained, and fibrocytes were enumerated by morphology.

FIG. 2 shows the effects of SRTK and Syk inhibitors on the ability of anti-FcyR antibodies on fibrocyte differentiation from PBMC. PBMC were incubated for 60 minutes at 45°C, with 10 nM PP2, PP3, or Syk inhibitor. PBMC at 2.5 x 10^5 cells per ml were then cultured in serum-free medium for 5 days in the presence or absence of 1 µg/ml of the indicated murine F(ab')2 anti-FcyR antibodies, in the presence or absence of 500 ng/ml goat F(ab')2 anti-mouse IgG. Results are expressed as the mean±SD of the number of fibrocytes per 2.5 x 10^5 cells (one of two separate donors)

FIG. 3 shows the effects of FcyR aggregation and the effects of SRTK and Syk on fibrocyte differentiation from monocytes. PBMC were at 2.5 x 10^5 cells per ml were incubated for 60 minutes at 37°C. Non-adherent cells were then removed by pipetting, resulting in a substantially monocyte cell sample. The adherent monocytes were incubated for 60 minutes at 4°C in the presence or absence of 10 nM PP2, PP3 or Syk inhibitor. Monocytes were then washed twice and cultured in the presence or absence of heat-aggregated human IgG at 60 minutes at 4°C. This IgG was not an anti-FcyR IgG, but instead was able to bind through its Fe region. The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention.

FIG. 4 shows the effects of cross-linking IgG and other antibody isotypes on and fibrocyte differentiation.

In FIG. 4A, PBMC were incubated with the indicated concentrations of monomeric human IgG for 60 minutes. PBMC were then washed and incubated in the presence (white boxes) or absence (black boxes) of 500 ng/ml goat F(ab')2 anti-human IgG. PBMC were then cultured at 2.5 x 10^5 cells per ml in serum-free medium for 5 days. PBMC were then air-dried, fixed, stained, and fibrocytes were enumerated by morphology. Results are expressed as the mean±SEM of number of fibrocytes per 2.5 x 10^5 cells (n=4 separate donors).

In FIG. 4B, PBMC were cultured as in FIG. 4A in the presence of the indicated concentrations of heat-aggregated human IgG or heat-aggregated F(ab')2. Results are expressed as the mean±SEM of number of fibrocytes per 2.5 x 10^5 cells (n=3 separate donors).

In FIG. 4C, PBMC were cultured as in FIG. 4A in the presence of 20 µg/ml of native or heat-aggregated human IgA, IgE, IgG or IgM.

FIG. 5 shows the effects of monomeric IgG on the ability of SAP to bind to monocytes and inhibit their differentiation. PBMC were cultured in serum-free medium in the presence of a range of concentrations of monomeric IgG for 60 minutes. SAP, at the concentrations indicated, was then added and the cells were cultured for 4 days.

FIG. 6 shows the effects of ligation and cross-linking of Fc receptors on monocyte to fibrocyte differentiation. Soluble immune complexes (ovalbumin-antibody), particulate immune complexes, including opsonised sheep red blood cells (SRBC) and heat-aggregated IgG were used. In FIG. 6A PBMC cultured for 4 days with ovalbumin or anti-ovalbumin mAb alone, or ovalbumin:anti-ovalbumin immune complexes. FIG. 6B shows the effects of SRBC alone and SRBC opsonised with rabbit anti-SRBC at 20.1 and 40.1 SRBC: monocyte ratios. Finally, FIG. 6C shows the effects on PBMC of heat-aggregated IgG and heat-aggregated F(ab')2. Stars in 6A and 6B indicate statistically significant differences.

FIG. 7 shows the effects of anti-FcyR antibodies on monocyte differentiation. Stars indicate a statistically significant difference from control.

DETAILED DESCRIPTION

The regulation of events leading to fibrosis involves the proliferation and differentiation of fibrocytes. Fibrocytes are a distinct population of fibroblast-like cells derived from peripheral blood monocytes that normally enter sites of tissue injury to promote angiogenesis and wound healing. Fibrocytes differentiate from CD14+ peripheral blood monocytes, and may differentiate from other PBMC cells. The presence of anti-FcyR antibodies, aggregated IgG, and/or cross-linked IgG may inhibit or at least partially delay this process.

Compositions containing anti-FcγRII antibodies and/or anti-FcγRII antibodies, and/or cross-linked or aggregated IgG, which may bind to FcγR through the Fc region, may be used to suppress fibrosis in inappropriate locations and in fibrosing disorders and chronic inflammatory conditions, inter alia.

In specific embodiments, compositions containing approximately 1 µg/ml anti-FcγR antibodies may be effective to inhibit fibrocyte proliferation or differentiation by approximately 50%. In other embodiments, compositions may contain an amount sufficient to deliver 1 µg/ml anti-FcγR antibodies to a target location (e.g., a tissue). In other specific embodiments, compositions may contain as little as 0.1 µg/ml cross-linked or aggregated IgG.

Anti-FcγR antibodies may be administered in a dose of approximately 10 µg/mL, in an amount sufficient to deliver 1 µg/ml anti-FcγR antibodies to the target tissue, or in another dose sufficient to inhibit fibrocyte proliferation or differentiation without causing an undesirable amount of cell death in the patient. Aggregated or cross-linked IgG may be administered in an amount sufficient to deliver at least 0.1 µg/ml IgG to the target location, or in another dose sufficient to suppress fibrocytes without causing an undesirable amount of cell death in the patient.

Anti-FcγR antibodies used in examples of the present disclosure include anti-FcγRII antibodies and anti-FcγRII anti-
bodies. Cross-linked or aggregated IgG may include any IgG able to bind the target FcγR through its Fe region, provided that at least two such IgG antibodies are physically connected to one another.

Antibodies of both types may include whole antibodies or a portion thereof, preferably the portion functional in suppressing fibrocytes. For example, they may include any antibody portion able to cross-link FcγR. This may include aggregated or cross-linked antibodies or fragments thereof, such as aggregated or cross-linked whole antibodies, F(ab')2 fragments, and possibly even Fe fragments.

Aggregation or cross-linking of antibodies may be accomplished by any known method, such as heat or chemical aggregation. Any level of aggregation or cross-linking may be sufficient, although increased aggregation may result in increased fibrocyte suppression. Antibodies may be polyclonal or monoclonal, such as antibodies produced from hybridoma cells. Compositions and methods may employ mixtures of antibodies, such as mixtures of multiple mono­clonal antibodies, which may be cross-linked or aggregated to either or different antibodies.

Anti-FcγR antibodies may include any isotype of antibody. Compositions may be applied locally or systemically. The compositions may also be supplied in combinations or with cofactors. Compositions may be administered in an amount sufficient to restore normal levels, if the composition is normally present in the target location, or they may be administered in an amount to raise levels above normal levels in the target location.

The compositions of the present invention may be supplied to a target location from an exogenous source, or they may be made in vivo by cells in the target location or cells in the same organism as the target location.

Compositions of the present invention may be in any physiologically appropriate formulation. They may be administered to an organism by injection, topically, by inhalation, orally or by any other effective means.

The same compositions and methodologies described above to suppress fibrocytes may also be used to treat or prevent conditions resulting from inappropriate fibrocyte proliferation or differentiation. For example, they may treat or prevent a condition occurring in the liver, kidney, lung, heart and pericardium, eye, skin, mouth, pancreas, gastrointesti­nal tract, brain, breast, bone marrow, bone, genito­urinary, a tumor, or a wound.

Generally, they may treat or prevent fibrosis resulting from conditions including but not limited to rheumatoid arthritis, lupus, pathogenic fibrosis, fibroblastic disease, fibrotic lesions such as those formed after Schistosoma japonicum infection, radiation damage, autoimmune diseases, Lyme disease, chemother­apy induced fibrosis, HIV or infection-induced focal sclerosis, failed back syndrome due to spinal surgery scarring, abdominal adhesion post surgery scarring, fibro­cytic formations, fibrosis after spinal injury, surgery-induced fibrosis, mucosal fibrosis, peritoneal fibrosis caused by dialysis, and Adalimumab-associated pulmonary fibrosis.

Specifically, in the liver, they may treat or prevent fibrosis resulting from conditions including but not limited to alcohol, drug, and/or chemically induced cirrhosis, ischemia-reperfusion injury after hepatic transplant, necrotizing hepatitis, hepatitis B, hepatitis C, primary biliary cirrhosis, and primary sclerosing cholangitis.

Relating to the kidney, they may treat or prevent fibrosis resulting from conditions including but not limited to proliferative and sclerosing glomerulonephritis, nephro­genic fibrosing dermatopathy, diabetic nephropathy, renal tubu­lointerstitial fibrosis, and focal segmental glomerulosclerosis.

Relating to the lung, they may treat or prevent fibrosis resulting from conditions including but not limited to pulmonary interstitial fibrosis, sarcoidosis, pulmonary fibrosis, idiopathic pulmonary fibrosis, asthma, chronic obstructive pulmonary disease, diffuse alveolar damage disease, pulmonary hypertension, neonatal bronchopulmonary dysplasia, chronic asthma, and emphysema. There are several sub­names or synonyms for pulmonary fibrosis including, but not limited to, cryptogenic fibrosing alveolitis, diffuse interstitial fibrosis, idiopathic interstitial pneumonitis, Hamman-Rich syndrome, silicosis, asbestosis, berylliosis, coal worker's pneumoconiosis, black lung disease, coal miner’s disease, miner’s asthma, an­thraxosis, and anthracosilicosis.

Relating to the heart and/or pericardium, they may treat or prevent fibrosis resulting from conditions including but not limited to myocardial fibrosis, atherosclerosis, coronary artery restenosis, congestive cardiomyopathy, heart failure, and other post-ischemic conditions.

Relating to the eye, they may treat or prevent fibrosis resulting from conditions including but not limited to exoph­thalmos of Grave’s disease, proliferative vitreoretinopathy, anterior capsule cataract, corneal fibrosis, corneal scarring due to surgery, trabeculectomy-induced fibrosis, progressive subretinal fibrosis, multifocal granulomatous cho­rioretinitis, and other eye fibrosis.

Relating to the skin, they may treat or prevent fibrosis resulting from conditions including but not limited to De­pytren’s contracture, scleroderma, keloid scarring, psoriasis, hypertrophic scarring due to burns, atherosclerosis, restenosis, and psuedoscleroderma caused by spinal cord injury.

Relating to the gastrointestinal tract, they may treat or prevent fibrosis resulting from conditions including but not limited to periodontal disease scarring, gingival hypertrophy secondary to drugs, and congenital esophageal stenosis.

Relating to the pancreas, they may treat or prevent fibrosis resulting from conditions including but not limited to pancreatic fibrosis, stromal remodeling pancreatitis, and stromal fibrosis.

Relating to the gastrointestinal tract, they may treat or prevent fibrosis resulting from conditions including but not limited to collagenous colitis, villous atrophy, cryo hyperplasia, polyf formation, fibrosis of Crohn’s disease, and healing gastric ulcer.

Relating to the brain, they may treat or prevent fibrosis resulting from conditions including but not limited to gli­al scar tissue.

Relating to the breast, they may treat or prevent fibrosis resulting from conditions including but not limited to fibrocystic disease and desmoplastic reaction to breast cancer.

Relating to the bone marrow, they may treat or prevent fibrosis resulting from conditions including but not limited to fibrosis in myelodysplasia and neoplastic diseases.

Relating to the bone, they may treat or prevent fibrosis resulting from conditions including but not limited to rheu­matoid pannus formation.

Relating to the genitourinary system, they may treat or prevent fibrosis resulting from conditions including but not limited to endometriosis, uterine fibroids, ovarian fibroids, and Peyronie’s disease.

Relating to radiation induced damage, they may treat or prevent fibrosis related to, but not limited to, treatment of head and neck cancer, ovarian cancer, prostate cancer, lung cancer, gastrointestinal cancer, colon cancer, and breast cancer.

The following examples are included to demonstrate specific embodiments of the invention. It should be appreciated
by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

**Fibrocyte Differentiation Assay**

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Gulf Coast Regional Blood Center, Houston, Tex.) by ficoll-paque plus (Amersham Biosciences, Piscataway, N.J.). Cells were incubated in serum-free medium (SFM), which consists of RPMI (Invtrogen, Carlsbad, Calif.) supplemented with 10 mM HEPES (Invitrogen), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1×T1S-1 (500 μg/ml bovine serum albumin, 10 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 5 μg/ml linoleic acid, and 5 μg/ml oleic acid; Sigma-Aldrich, St. Louis, Mo.). Normal human serum (Sigma-Aldrich) was added at 1%. PBMC were cultured in flat-bottomed 96 well tissue culture plates (Type 350372, BD Biosciences, Discovery Labware, Bedford, Mass.) in 200 μl volumes at 2.5×10^5 cells per ml in a humidified incubator containing 5% CO₂ at 37°C for 5 days. Fibrocytes were identified by morphology in viable cultures as adherent cells with an elongated morphology in viable cultures as adherent cells with an elongated spindle-shape and the presence of an oval nucleus. All enumerations of fibrocytes were performed on cells cultured for 5 days. Cells were air dried, fixed in methanol and stained with eosin and methylene blue (Huma 3 Stain, Fisher Scientific, Hampton, N.H.). Fibrocytes from duplicate wells were counted in five different 900 μm diameter fields per well, using the above criteria of an elongated spindle-shape and the presence of an oval nucleus. All cultures were counted by at least two independent observers. The number of fibrocytes observed was 1.2±0.6x10^4 (mean±SD, n=12 healthy individuals) fibrocytes per ml of peripheral blood, with a range of 3.7x10^3 to 2.9x10^4 fibrocytes per ml. These results indicate that fibrocyte precursors account for approximately 1% of the total peripheral blood mononuclear cells.

**Example 2**

**Antibodies, Proteins, and Inhibitors**

Human IgA, IgG, IgM, and IgG F(ab')₂ fragments were from Jackson ImmunoResearch Laboratories, West Grove, Pa. Goat F(ab')₂ anti-human IgG, goat F(ab')₂ anti-murine IgG, goat F(ab')₂ anti-rabbit IgG, and whole mouse IgG1, whole mouse IgG2a and mouse F(ab')₂ IgG1 isotype control antibodies were from Southern Biotechnology Associates Inc., Birmingham, Ala. Sheep red blood cells (SRBC) and rabbit anti-SRBC were from ICN, Irvine, Calif. F(ab')₂ fragments of the blocking monoclonal antibodies to FcyRI (clone 10.1, IgG1 isotype) and FcyRII (clone 7.3, IgG1 isotype) were from Anceel, Bayport, Minn. The following primary monoclonal antibodies were used for immunohistochemistry: anti-CD14 (clone M3E2, IgG2a, BD-Biosciences, San Diego, Calif.), anti-CD45 (clone 30F11, IgG1, BD, antiprolyl 5-hydrolase (clone 5B5, IgG1, Dako, Carpinteria, Calif.) anti-alpha smooth muscle actin (clone 1A4, IgG2a, Sigma-Aldrich, St. Louis, Mo.). Collagen-I was detected using an affinity-purified rabbit polyclonal antibody from Rockland, Gilbertsville, Pa. P22 (AG 1879; 4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine), P39 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine and the Syk inhibitor (3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide) were from Calbiochem, EMD Biosciences, San Diego, Calif.

**Example 3**

**Inhibition of Fibrocyte Differentiation**

To determine if anti-FcyR antibodies activate FcyR to inhibit fibrocyte differentiation, PBMC at 2.5×10⁶ per ml were cultured in serum-free medium for 5 days in the presence or absence of 1 μg/ml of free or cross-linked F(ab')₂ antibodies to FcyRI or FcyRII. To crosslink individual FcyR, PBMC were incubated for 30 minutes at 4°C with 1 μg/ml F(ab')₂, anti-FcyRI or F(ab')₂ anti-FcyRII, and receptors were then cross-linked by the addition of 500 ng/ml F(ab')₂ goat anti-mouse IgG for 30 minutes at 4°C. PBMC were then warmed to 37°C and cultured for 5 days. After the cells were cultured in the presence or absence of F(ab')₂ antibodies to FcyRI or FcyRII, the cells were then air-dried, fixed, stained, and fibrocytes were enumerated by morphology. The results of this example are shown in FIG. 1.

**Example 4**

**Inhibition of Fibrocyte Differentiation is SYK- and SRC Kinase Dependent**

FcyR activation leads to a cascade of signaling events initiated by two main kinases. The initial events following FcyR
aggregation involve the phosphorylation of intracellular immunoreceptor tyrosine activation motifs (ITAM) present on the cytoplasmic tail of FcγRII or the Fcγ chain associated with FcγRI, by src-related tyrosine kinases (SRTK). In monocytes, the main src-kinases associated with FcγRI and FcγRII are hck and lyn. The phosphorylated ITAM then recruits ITAM-containing kinases, especially Syk, to the ITAMs and Syk then activates a series of downstream signaling molecules.

To determine the roles of SRTK and Syk in the regulation of fibrocyte differentiation, PBMC were pre-incubated with the specific SRTK inhibitor PP2, PP3 as a control for PP2, or the specific Syk inhibitor 5-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide, before the addition of anti-FcγR antibodies. This Syk inhibitor was used instead of the standard Syk inhibitor piceatannol, as piceatannol at concentrations used to inhibit Syk in whole cells (10 μM) also inhibits a variety of other enzymes and transcription factors. These proteins include the catalytic subunit of protein kinase A, protein kinase C, myosin light chain kinase, TNF-induced NF-κB activation, and interferon α-mediated signaling via STAT proteins.

Inhibition of fibrocyte differentiation by activating either FcγRI or FcγRII alone or both receptors together was dependent on SRTK and Syk, as the inhibition was lost when PBMC were pre-incubated with either PP2 or the Syk inhibitor (FIG. 2). Compared to control cultures or cultures incubated with 500 ng/ml goat F(ab')2 anti-mouse IgG (X-linker only), PBMC cultured with 500 ng/ml goat F(ab')2 in addition to anti-mouse IgG anti-FcγRI, anti-FcγRII or both antibodies significantly inhibited fibrocyte differentiation (p<0.05), as determined by ANOVA. The presence of PP2 or Syk inhibitor, but not the control compound PP3, inhibited this inhibition. These data suggest that anti-FcγR antibodies inhibit fibrocyte differentiation through a pathway involving both Syk and SRTK.

Similar results were found when monocyte samples, rather than PBMC, were used to perform tests. Specifically, in FIG. 3, compared to monocytes incubated with 10 μg/ml aggregated human IgG (able to bind to FcγRII through its Fc region), pre-incubation with PP2 (p<0.01) or Syk inhibitor (p<0.05) significantly inhibited the ability of IgG to inhibit fibrocyte differentiation as determined by ANOVA.

Example 5

IgG Immune Complexes Inhibit Fibrocyte Differentiation

In addition to FcγR, monocytes express IgA receptors, low numbers of IgE receptors, and the recently characterized IgM receptor. To determine if other immunoglobulins inhibit fibrocyte differentiation, native or heat-aggregated IgA, IgE, IgG or IgM were added to PBMC. The results of this example are shown in FIG. 4C. Only heat-aggregated IgG, but not monomeric IgG or monomeric or heat-aggregated IgA, IgE or IgM, could inhibit fibrocyte differentiation. This suggests that ligation and cross-linking of FcγR receptors is an inhibitory signal for fibrocyte differentiation, but that ligation of the other immunoglobulin receptors has no effect on fibrocyte differentiation.

Example 6

Cross-Linked IgG Inhibits Fibrocyte Differentiation

PBMC were incubated with the indicated various concentrations of monomeric human IgG for 60 minutes. PBMC were then washed and incubated in the presence or absence of 500 ng/ml goat F(ab')2 anti-human IgG. PBMC were then cultured at 2.5×10⁵ cells per ml in serum-free medium for 5 days. PBMC were then air-dried, fixed, stained, and fibrocytes were enumerated by morphology. Results are shown in FIG. 4A. Specifically, compared to monomeric IgG, cross-linked human IgG clearly inhibited fibrocyte differentiation, as compared to non-cross-linked IgG at 0.1 μg/ml. At 10 and 100 μg/ml inhibition of differentiation was significant (p<0.03 and p<0.003, respectively, as determined by Student’s t test. Additional experiments using sheep red blood cells (SRBC) either opsonized or not opsonized with rabbit anti-SRBC IgG indicated that the opsonized SRBC significantly inhibited fibrocyte differentiation (p<0.018) (data not shown).

PBMC were also cultured as above in the presence of the indicated concentrations of heat-aggregated human IgG or heat-aggregated human F(ab')2. Results of this example are shown in FIG. 4B. Compared to heat-aggregated human F(ab')2, heat-aggregated whole IgG significantly inhibited fibrocyte differentiation at concentrations of 25 μg/ml and higher, as determined by Student’s t test. Although only exemplary embodiments of the invention are specifically described above, it will be appreciated that modifications and variations of these examples are possible without departing from the spirit and intended scope of the invention.

Example 7

Antibody Studies

SAP and CRP augment phagocytosis and bind to Fcγ receptors on a variety of cells. CRP binds with a high affinity to FcγRII (CD32), a lower affinity to FcγRI (CD64), but does not bind FcγRIII (CD16). SAP binds to all three classical Fey receptors, with a preference for FcγRI and FcγRII. Monocytes constitutively express FcγRI. Because this receptor binds monomeric IgG, it is saturated in vivo. In order to determine whether the presence of monomeric human IgG could prevent SAP from inhibiting fibrocyte differentiation, PBMC were cultured in serum-free medium in the presence of a range of concentrations of monomeric IgG for 60 minutes. SAP, at the concentrations indicated in FIG. 5, was then added and the cells were cultured for 4 days. As described in the above examples, 2.5 μg/ml SAP in the absence of IgG strongly inhibited fibrocyte differentiation. (See FIG. 5.) Monomeric IgG in a range from 0.1 to 1000 μg/ml, which corresponds to approximately 0.001 to 10% serum respectively, had little effect on the suppression of fibrocyte formation by SAP.

To determine whether ligation and cross-linking of Fc receptors could also influence monocyte to fibrocyte differentiation, three test samples were used: soluble immune complexes (ovalbumin-antibody), particulate immune complexes, including opsonised SRBC and heat-aggregated IgG. PBMC cultured for 4 days with ovalbumin or anti-ovalbumin mAb showed that the two proteins alone had a modest effect on the differentiation of monocytes compared to cultures where no reagent was added. (See FIG. 6A.) However, the addition of ovalbumin/anti-ovalbumin immune complexes led to a significant reduction in the number of differentiated fibrocytes (See FIG. 6A). A similar effect was observed when PBMC were cultured with opsonised SRBC. SRBC opsonised with rabbit anti-SRBC at 20:1 and 40:1 SRBC:monocyte ratios significantly suppressed fibrocyte differentiation as compared to cells cultured with SRBC alone (See FIG. 6B). Finally, PBMC cultured with heat-aggregated IgG, but...
not heat-aggregated F(ab')2, also showed potent inhibition of fibrocyte differentiation (See FIG. 6C.) Together these data suggest that ligation and cross-linking of Fe receptors is suppressor of monocyte to fibrocyte differentiation.

The observation that immune complexes inhibit fibrocyte differentiation suggests that one or more FcyR influences fibrocyte differentiation. To examine the role of FcyR in fibrocyte differentiation PBMC were cultured in the presence or absence of blocking antibodies to FcyRI (CD64), FcyRII (CD32) or FcyRIII (CD16) before the addition of SAP, or as a control CRP. When samples were pre-incubated with a blocking mAb for each of the three FcyR, SAP was later able to modestly suppress fibrocyte differentiation. However, in the absence of exogenously added SAP, the FcyRI (CD64) blocking mAb had a profound effect on fibrocyte differentiation. Incubation of PBMC with blocking mAb to FcyRI, but not FcyRII or FcyRIII, promoted fibrocyte differentiation as compared to cells cultured with isotype-matched control mAb or cells cultured with no mAb (P<0.01) (See FIG. 7). These data suggested that SAP or IgG, might have been produced by some cells in the culture system over 4 days, or that SAP or IgG was retained by cells from the blood. Western blotting failed to show the presence of SAP or IgG after cells had been cultured for 4 days in vitro. This suggests that the FcyRI blocking mAb has a direct effect on fibrocyte differentiation or that SAP or IgG were only present during the early time points of the cell culture.

The invention claimed is:

1. A method of suppressing fibrocyte formation in a subject in need thereof comprising administering to the subject having pulmonary fibrosis an anti-FcyR antibody in an amount sufficient to suppress fibrocyte formation in a lung.

2. The method of claim 1, wherein the anti-FcyR antibody is administered at a concentration of at least 1.0 µg/ml.

3. The method of claim 1, wherein the anti-FcyR antibody is administered at a concentration of at least 0.1 µg/ml.

4. The method of claim 1, wherein the antibody is an IgG.

5. The method of claim 1, wherein the antibody is an anti-FcyRI antibody.

6. The method of claim 1, wherein the antibody is an anti-FcyRII antibody.

7. The method of any one of claims 4-6, wherein the antibody comprises an F(ab')2 fragment.

8. The method of any one of claims 4-6, wherein the antibody comprises an Fe fragment.

9. The method according to claim 1, wherein the pulmonary fibrosis comprises a condition selected from the group consisting of: Adalimumab-associated pulmonary interstitial fibrosis, sarcoidosis, idiopathic pulmonary fibrosis, asthma, chronic obstructive pulmonary disease, diffuse alveolar damage disease, pulmonary hypertension, neonatal bronchopulmonary dysplasia, and emphysema.

10. The method of claim 1, wherein the antibody is an anti-FcyRIII antibody.