Activation of Alveolar Macrophages via the Alternative Pathway in Herpesvirus-Induced Lung Fibrosis

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The etiology of idiopathic pulmonary fibrosis (IPF) is unknown. Because viral pathogenesis of IPF has been suggested, we have established a murine model of progressive pulmonary fibrosis by infecting IFN- γ R-deficient mice (IFN- γ R^{-/-}) with the murine γ -herpesvirus 68. Because alveolar macrophages in humans with IPF have been implicated in driving the profibrotic response, we studied their role in our model. Chronic herpesvirus infection of the lung was associated with recruitment of alveolar macrophages to areas with epithelial hyperplasia and fibrosis in infected lungs. Using immunohistochemistry, Western blot, and RT-PCR techniques, we demonstrated that recruited alveolar macrophages showed high levels of expression of the proteins Ym1/2, FIZZ1 (found in inflammatory zone 1), insulin-like growth factor-1, and arginase I, and also active transcription of fibronectin, indicative of activation of macrophages by an alternative pathway. Arginase I expression was also evident in interstitial fibroblasts, and increased arginase activity was found in lungs of infected animals. Lung tissue from patients with IPF showed increased expression of arginase I in epithelial cells, fibroblast foci, and alveolar macrophages compared with normal lung. These results suggest that virus-induced upregulation of arginase I could be a mechanism driving lung fibrogenesis.

Keywords: alternative pathway; fibrosis; γ -herpesvirus; lung; macrophages

Idiopathic pulmonary fibrosis (IPF) is a progressive, fibrotic, interstitial lung disease of unknown etiology (1). Although the pathogenesis of IPF is incompletely understood, it is suggested that IPF is the result of abnormal wound repair and remodeling after lung epithelia injury (2). Several studies have focused on chronic viral infection as a potential cause of lung injury in IPF, particularly herpesvirus infection (3–5). We have shown a link between IPF and virus infection by detecting one or more of three herpesvirus—cytomegalovirus, Epstein-Barr virus, or human herpesvirus 8—in the lungs of greater than 95% of patients with IPF (5). In addition, we found that, when mice defective in IFN- γ R signaling (IFN- γ R^{-/-}) are chronically infected with the murine γ -herpesvirus 68 (MHV68), a virus that is closely related to Epstein-Barr virus and human herpesvirus 8, there is progressive interstitial lung fibrosis with many histologic features reminiscent of IPF. IFN- γ R^{-/-} mice have a bias to develop T helper cell (Th) 2 responses, and, in concordance with this

phenotype, infected mice were found to have increased production of Th2 cytokines, high expression of transforming growth factor (TGF)- β and matrix metalloproteinase-7, myofibroblast transformation, and the accumulation of alveolar macrophages, particularly in fibrotic areas of the lung (6).

Macrophages are essential mediators of chronic inflammation as well as wound healing, where they function to phagocytose debris and to modulate inflammatory responses and fibroproliferation. In addition, macrophages produce extracellular matrix components (ECM) and can affect the degradation of ECM by producing matrix metalloproteinases and their inhibitors. During their recruitment, macrophages encounter various signals that direct their differentiation toward distinct phenotypes and functions. In parallel with the Th1/Th2 dichotomy, two major types of macrophage activation have been described (7, 8). Classically activated effector macrophages develop in response to proinflammatory stimuli, such as Th1 cytokines (IFN- γ). Classical activation of macrophages is characterized by the secretion of proinflammatory cytokines, such as TNF- α , IL-6, and IL-12. These macrophages are involved in promoting inflammation, extracellular matrix destruction, and apoptosis. Treatment of macrophages with Th2 cytokines has been shown to induce alternatively activated type 2 macrophages (9). Macrophages activated through the alternative pathway express a repertoire of proteins involved in repair and healing, cell proliferation, and angiogenesis, including the Ym1 and Ym2 chitinase-like secretory lectins, the resistin-like secreted protein FIZZ1 (found in inflammatory zone 1), and the growth factor IGF (insulin-like growth factor)-1. The activated macrophages secrete antiinflammatory molecules, such as IL-10 and TGF- β , inducing downregulation of the inflammatory processes initiated by Th1 cytokines (7, 10). TGF- β also functions indirectly to promote ECM formation by inducing fibroblasts to produce ECM components. Alternative activation of macrophages contributes further to this process by secreting the ECM component fibronectin (11). The presence of Th2 cytokines also promotes upregulation of arginase activity in macrophages and/or fibroblast (12). Arginase metabolizes L-arginine to L-ornithine, L-proline, and polyamine, which promotes fibroblast proliferation, collagen production, and, ultimately, fibrosis. In summary, activation of macrophages via the alternative pathway is driven by Th2 cytokines, and would be expected to promote fibrosis.

Macrophages with profibrotic functions have been implicated as key effector cells in the pathogenesis of IPF (13–15). Using our experimental model of chronic γ -herpesvirus infection, we addressed the question of whether the development of lung fibrosis could be due to alternative activation of macrophages. Our findings provide strong evidence for alternative activation of macrophages and/or fibroblasts as an important signaling pathway in the pathogenesis of lung fibrosis. Furthermore, our data show upregulation of this pathway in lung tissue from patients with IPF but not in normal lung.

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MATERIALS AND METHODS

Animals and Animal Treatment

IFN- γ R^{-/-} mice on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were then bred, maintained, and used at Emory University, Atlanta, Georgia, in accordance with all university and federal guidelines after institutional approval. Mice were inoculated intranasally with three doses of 4×10^5 plaque-forming units of MHV68 mixed in Dulbecco's modified Eagle medium–10% FCS to a total volume of 40 μ l, at Days 0, 15, and 30 to simulate repetitive virus reactivation and were studied between 180 and 200 d after infection (6). Control mock-infected animals were inoculated with lysate from cells not infected with virus. All animal studies were performed according to National Institutes of Health (NIH) guidelines.

At 180–200 d postinoculation, mice were killed by cervical dislocation after isoflurane anesthesia, after which the following tests were performed in subsets of animals. Bronchoalveolar lavage (BAL) was performed through a tracheal canula by twice instilling then withdrawing 0.6 ml of serum-free complete medium (Cellgro, Herndon, VA). BAL fluid (BALF) was centrifuged and the supernatant collected and filtered through 0.22 μ m membranes. Samples were stored at -80°C for later determination of cytokine concentrations. White blood cells in the BAL pellet were counted on a hemocytometer, and cytologic examination was performed on cytopsin preparations fixed and stained using Diff Quick (American Scientific Products, Stone Mountain, GA). Differential counts were based on counts of 100 cells using standard morphologic criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes). Counts were performed by a single observer who was blinded to the study group. For isolation of mononuclear cells, BAL was performed by instilling five times with 600 μ l of serum free Dulbecco's modified Eagle medium media. BALF was incubated in 24-well plates for 2 h at 37°C in 5% CO_2 . Attached cells were analyzed by flow cytometry, and more than 80% of the cells were positive for CD11b (BD Biosciences, San Jose, CA), indicating mononuclear cell type. Isolated mononuclear cells were used for RT-PCR analysis, Arginase activity, or culture. For *in vitro* culture, cells were stimulated with recombinant mouse (rm)IL-4 (20 ng/ml) (BD Biosciences) and rmIL-13 (20 ng/ml) (Biosource, Camarillo, CA) for 1 h, washed, and cultured for an additional 20 h in the presence of macrophage colony stimulating factor (M-CSF) (20 ng/ml; Biosource). Supernatants were collected for fibronectin gene expression experiments.

After BAL, lungs were removed and processed for the following analyses: for histologic and immunohistologic examination, lungs were inflated with 4% paraformaldehyde; for immunofluorescence, lungs were inflated with OCT media (Tissue-tek; Sakura Finetek USA Inc., Torrance, CA) for the preparation of frozen sections. Additional lung tissue was used for RNA extraction for RT-PCR gene expression analysis of markers of alternative activation pathway or for preparation of whole-cell protein extracts and Western blot analysis.

Histology, Immunohistochemistry, and Immunofluorescence

An average of three to four mice was used per group at each experimental time point for histopathology analysis. After inflation and fixation with 4% paraformaldehyde for 24 h, lung tissue was paraffin embedded, sectioned, and stained with hematoxylin and eosin for routine histologic examination and Masson trichrome staining to delineate collagen. Immunohistochemistry was performed to identify macrophages and products of macrophages activated via the alternative pathway. Antibodies used were against antigens Mac-1 (BD Bioscience), Mac-3 (BD Biosciences), Ym1/2 (kindly provided by Dr. Toshihiko Iwanaga, Hokkaido University, Japan), FIZZ1 (kindly provided by Dr. Roger Johns, Johns Hopkins University, Baltimore, MD), IGF-1 (Abcam Inc., Cambridge, MA), and Arginase I (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Slides were deparaffinized and treated with 3% H_2O_2 in H_2O to quench endogenous peroxidase activity. Arginase I, Fizz1, and IGF-1 staining were performed at 4°C overnight, followed by exposure to anti-rabbit secondary antibody for 60 min. Anti-Mac3 and anti-YM1/2 staining was performed for 60 min at room temperature, followed by anti-rat secondary antibody treatment (Santa Cruz Biotechnology) for 30 min. Diaminobenzidine (DAB) (Vector, Burlingame, CA) was used as the chromogen. Indirect immunofluorescence was performed in sections

from frozen blocks. Slides were fixed with 4% paraformaldehyde for 20 min at room temperature. Anti-arginase I (Research Diagnostics Inc., Flanders, NJ) and anti-cytokeratin 5/8 (BD Biosciences) were used for immunostaining overnight at 4°C , followed by the respective secondary conjugated antibodies. Nuclei were detected by 4'-6-diamidino-2-phenylindole (DAPI) staining. Cytopsin slides of BALF cells were fixed with 4% paraformaldehyde and 0.2% Triton for 30 min at 37°C . Indirect immunofluorescence was performed using anti-arginase I antibody for 1 h, followed by the respective secondary antibody and nuclei staining with DAPI.

Electron Microscopy

Electron microscopy was performed on lung tissue after fixation in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. Samples were postfixed in 1% osmium tetroxide and embedded in eponate 12 resin (Ted Pella, Redding, CA). Ultrathin sections were cut, stained with lead citrate and uranyl acetate, and examined with a Zeiss EM 10 C electron microscope (Carl Zeiss Micro-Imaging, Inc., Thornwood, NY).

Determination of Cytokine Levels

Mouse IL-13, macrophage inflammatory protein (MIP)-1 α , and monocyte chemoattractant protein (MCP)-1 levels were measured in BALF and serum using a multiplex bead immunoassay (Linco Research Inc., St. Charles, MO) according to manufacturer's recommendations.

Western Blot

Whole-cell extracts from lung tissue samples were prepared using cell lysate buffer (0.15 Nonidet-P [NP]-40, 50 mM Hepes [pH 7.0], 250 mM NaCl, 5 mM EDTA [pH 8.0]). Aliquots of lung lysates (10 μ g) and BALF (12 μ l) were resolved in 4–20% SDS-PAGE, and transferred onto nitrocellulose membranes. Western blotting for anti-mouse arginase I (Santa Cruz Biotechnology) and anti-human Arginase I (Research Diagnostics Inc.) were performed according to manufacturer's recommendations. Antibodies against Ym1/2 and FIZZ1 were used at a 1:1,000 dilution. Filters were stripped and reprobed with an antiserum against β -actin or pro-surfactant B for lung extracts and BAL samples, respectively (Santa Cruz Biotechnology and Chemicon), as a loading control for lung homogenates. For arginase I immunoblot, lung samples from normal subjects and patients with IPF were run in nondenaturing conditions, and replica samples were run in denaturing conditions for β -actin control.

RNA Preparation and RT-PCR

Total RNA was extracted from lung tissue and lung mononuclear cells using an RNeasy mini kit (QIAGEN, Valencia, CA) according to manufacturer's recommendations. cDNA was generated from 0.5–5 μ g of total RNA using random hexamers and Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using SYBR Green and primers specific for the genes of interest in an iCycler iQ (Bio-Rad, Hercules, CA). Amplification was quantified and normalized using β -actin as a housekeeping gene. The primers used were: IL-13: 5' GGAGCTGAGCAACATCACACA 3' and 5' GGTCCTGTAGATGGCATTGCA 3'; Ym1/2: 5' TTATCCTGAGT GACCTTCTAAG 3' and 5' TCATTACCCAGATAGGCATAGG 3'; FIZZ1: 5' GAACTTCTTGCCAATCCAG 3' and 5' TCCAGT CAACGAGTAAGC 3'; arginase I: 5'TGGGAAGACAGCAGAG GAGGTG3' and 5' TGAGTTCGGAAACAAGCCAAGG 3'.

Fibronectin Gene Transcription

To evaluate fibronectin gene transcription, the pFN(1.2 kb)LUC promoter construct was introduced into murine NIH/3T3 fibroblasts from the American Type Culture Collection (Manassas, VA) via electroporation to create stable transfectants. The transfected NIH/3T3 fibroblasts were treated with BAL and macrophage culture supernatants and tested for luciferase activity as described previously (16).

Arginase Activity

Arginase activity was measured in lung lysates. Briefly, cells were lysed with 100 μ l of a buffer containing 0.1% Triton X-100, 25 mM Tris-HCl, and 5 mM MnCl_2 , pH 7.4. Arginine hydrolysis was performed by

incubating the lysate with 100 μ l of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 800 μ l of H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1/3/7, vol/vol/vol). The urea concentration was measured at 540 nm after addition of 50 μ l of α -isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95°C for 45 min (17).

Patient Population

With Emory University institutional review board approval, informed consent was obtained from four patients with IPF, and lung tissue was collected from the native lung at the time of lung transplantation. Normal control lung was obtained from archived lung samples. BAL was performed by standard technique. The BALF was immediately centrifuged at 600 $\times$ g for 10 min, followed by separation of cell-free BALF from the cellular pellet. Cyto centrifugation of BALF was performed using 50,000 cells/slide.

Statistical Analyses

Data were plotted and statistically analyzed using Instat 3 and GraphPad Prism 4 (GraphPad Software, San Diego, CA). Nonparametric analysis of number of macrophages, cytokine levels, and arginase activity were performed using a two-tailed Mann-Whitney test.

RESULTS

Lung Fibrosis in MHV68 Chronic Infected IFN- γ ^{-/-} Mice Is Associated with Macrophage Recruitment

We have shown previously that MHV68 infection in IFN- γ ^{-/-} mice causes severe pneumonia during the acute phase of the infection (< 15 d), followed by progressive pulmonary fibrosis during the chronic phase (> 15 d). To characterize the inflammatory response after infection, we performed differential counts of cell pellets harvested through BAL of infected animals. In the acute phase, the total cell counts increased four to seven times compared with uninfected animals, and there was a predominance of lymphocytes. In chronically infected mice, the number of cells in BAL was elevated at Days 15 and 180 after infection, with lymphocytes and macrophages being the predominant cell type (Figure 1A). The large number of macrophages in the lungs of infected animals was also evident by immunohistochemistry analysis using an anti-Mac3 antibody, an alveolar macrophage marker (Figure 1B). The alveolar macrophages were especially abundant in areas with hyperplasia of lung epithelial cells (Figure 1C) and alveolar remodeling with collagen deposition (Figure 1D). The recruitment of macrophages to the lung was associated with increased levels in the blood and BALF of MCP-1 (CCL2) and MIP-1 α (CCL3), both of which are chemotactic for mononuclear phagocytes (Figure 1E). The expression in epithelial cells of these two CC-chemokines can be regulated by the Th2 and profibrotic cytokine, IL-13. We found higher gene transcription in lung tissue and elevated protein levels in serum of IL-13 in MHV68 chronically infected IFN- γ ^{-/-} mice (Figures 1F and 1G).

Alternative Activation of Alveolar Macrophages in MHV68 Chronically Infected IFN- γ ^{-/-} Mice

To characterize macrophages that were recruited to the injured lung by MHV68 infection, lung samples from chronically infected animals were analyzed by electron microscopy. These studies showed that the macrophages in infected animals contained numerous crystals similar to those described in murine models of chronic Th2-type inflammation (Figure 2A). The crystals in Th2 inflammation have been shown to be composed of the chitinase family proteins, Ym1 and Ym2 (18, 19). Th2 cytokines enhance Ym expression, and constitute one of the most abundant genes in alternative activated murine macrophages. Immunohistochemistry analysis using an antibody that recognizes both Ym1 and 2 showed constitutive expression of Ym in alveolar macro-

phages from mock-infected animals, and an intense increase in the staining for Ym in the numerous macrophages found in chronically infected animals (Figures 2B and 2C). The progressive increase in the expression of Ym proteins in infected animals was determined by Western blot analysis using lung lysates and BAL samples obtained at different time points after infection. Higher levels of Ym proteins were found in later stages of viral infection (Figures 2D and 2E). Because high levels of Ym in the lung can be the result of an increased number of alveolar macrophages in infected mice, we determined levels of expression by RT-PCR in isolated macrophages. Significant increases of these chitinase transcripts were found in equivalent numbers of macrophages isolated from virus infected animals compared with macrophages derived from the mock group (Figure 2F).

Recent studies have found FIZZ1 expression as another marker for alternative activation of macrophages. We analyzed FIZZ1 levels of expression by RT-PCR (Figure 3A) and Western blot technique (Figure 3B). We established early upregulation of FIZZ1 transcript and protein levels in BAL of infected animals. The immunohistochemistry of mouse lung sections showed that, in mock-infected animals, FIZZ1 had a minimal expression in epithelial cells and alveolar macrophages (Figure 3C). In contrast, virus infection increased FIZZ1 expression in airway epithelial cells, pneumocytes, and macrophages (Figure 3D). Human and mouse macrophages activated in the presence of Th2 cytokines also upregulate IGF-1 expression. IGF-1 produced by lung macrophages has long been implicated in the pathogenesis of pulmonary fibrosis (14). Immunostaining of lung sections of chronically infected mice with an anti-IGF-1 antibody showed high expression in alveolar and interstitial lung macrophages (Figure 3E).

Finally, discrimination between classically activated and alternatively activated macrophages is mainly demonstrated by the metabolism of L-arginine. L-arginine is the substrate for two enzymes: nitric oxide synthase (NOS) 2 and arginase I. Th2-activated macrophages use arginase I to promote the production of polyamines and L-proline, which are involved in cell proliferation and collagen production, respectively. To determine if chronically infected mice have upregulation in expression of arginase I, we performed immunohistochemistry analysis (Figures 4A–4C). Lung tissue from infected mice showed positive staining for arginase I in fibrotic areas (Figure 4B) and alveolar macrophages (Figure 4C). The levels of arginase I in lung tissue increased with the duration of infection, as demonstrated by Western blot analysis in lung lysates obtained at different time points (Figure 4D). In concordance, arginase I activity in lung lysates increased with virus infection (Figure 4E).

Increased Fibronectin Expression in Infected Mice

Alternatively activated macrophages can contribute to wound repair by multiple pathways, one of which is the induction of release of ECM components, such as fibronectin. We analyzed fibronectin transcription by incubating NIH/3T3 cells transfected with a luciferase reporter under the control of fibronectin promoter with BALF or supernatant of macrophages in culture. We found higher induction of fibronectin transcription with supernatants of cultured alveolar macrophages obtained from infected animals compared with macrophages from mock-treated animals (Figure 5). Fibronectin transcription was also stimulated to a similar degree by BAL samples from MHV68-infected mice compared with BAL from mock-treated animals (Figure 5).

Arginase I Is Expressed in IPF Lung

We performed immunofluorescence assays to investigate if, similar to our murine model of pulmonary fibrosis, there was upregulation of arginase I in lungs from patients with IPF. Using an

4–5 per group) are shown; statistical differences between the mock and infected groups were significant. (F) IL-13 levels were determined in serum samples of mock and infected IFN- γ R $^{-/-}$ mice at Day 180 after infection using an immunoassay ($n = 3$ per group). (G) Real-time PCR of mRNA IL-13 normalized against β -actin in lung of mock-treated or MHV68-infected IFN- γ R $^{-/-}$ mice at Day 180 after infection ($n = 2$ mock group; $n = 5$ infected group) (B and C: $\times 40$ and $\times 100$ magnification, respectively).

antibody against arginase I and a cytokeratin antibody to delineate lung architecture, we were able to determine minimal levels of arginase I in normal lung sections, which occurred around blood vessels (Figure 6A). Lung sections from patients with IPF with moderate fibrosis showed positive staining for arginase I in scattered fibroblast foci (Figures 6B and 6C). In contrast, IPF lungs with extensive fibrosis showed abundant arginase I staining in areas with pleura thickening, interstitial fibrosis, and some epithelial cells (Figure 6D). Alveolar macrophages were also positive for arginase I, visualized in lung frozen sections (Figure 6E) and cytospin preparations of BAL cells (Figures 6F and 6G). In concordance, we found higher levels of arginase I protein by Western blot using whole lung lysates from four different patients with IPF compared with lung lysate from normal lung (Figure 6H).

DISCUSSION

The pathogenesis of IPF is not fully delineated, but a critical event may be the ongoing injury of the lung epithelium. Chronic herpesvirus infection can be a potential cause of the injury that, in a susceptible host, persists and drives the fibrogenic response.

We found that herpesvirus lung infection in a mouse biased toward a Th2-type response resulted in progressive pulmonary fibrosis. The purpose of this study was to examine the host response to the virus, focusing on the alveolar macrophage, and how this response could influence the fibrotic process. We found that lung fibrosis mediated by γ -herpesvirus was associated with recruitment of macrophages to the injured lung. These macrophages were exposed to a Th2 environment, activated by the alternative pathway, and expressed a repertoire of genes, such as arginase I and IGF-1, characteristic of that pathway. Activation of alveolar macrophages via the alternative pathway may be an important molecular mechanism promoting ECM deposition and fibrosis. We found similar phenotypes in macrophages and fibroblasts in lungs of patients with IPF.

A generally held concept in IPF is that macrophages are recruited by chemokines derived from lung epithelial and participate in the pathogenesis of the disease by secreting growth factors. In our studies, we found enhanced levels of chemokines such as MIP-1 α and MCP-1 in infected animals during the acute and chronic phase of the infection. These two CC-chemokines, have been identified as chemotactic for mononuclear phagocytes

Figure 1. Accumulation of alveolar macrophages in fibrotic areas of MHV68-infected IFN- γ R $^{-/-}$ mice. (A) BAL was performed in mock- and virus-infected animals at different times after infection. Total and differential cell counts were obtained in cytospin preparations using Diff Quick staining. Cell counts show a significant elevation in the numbers of lymphocytes and macrophages ($n = 4$). Left Panel: open bars, macrophages; gray bars, lymphocytes; horizontally hatched bars, neutrophils; vertically hatched bars, basophils; diagonally hatched bars, eosinophils. (B) After viral infection in IFN- γ R $^{-/-}$ mice, infiltration with alveolar macrophages was increased as demonstrated by Mac-3 staining evaluated at Day 180 after infection (brown staining). Normal areas of lung showed less accumulation of macrophages compared with areas with alveolar remodeling. (C) Hematoxylin and eosin staining of the lung of IFN- γ R $^{-/-}$ mice at Day 180 after infection showed hyperplastic cuboidal lung epithelial cells associated with abundant macrophages (6). (D) Masson trichrome staining from IFN- γ R $^{-/-}$ mice at Day 180 after MHV68 infection. Large macrophages are stained red and are associated with abundant deposits of collagen (blue). (E) MIP-1 α and MCP-1 levels were measured in BALF and serum from IFN- γ R $^{-/-}$ mice at Day 180 after infection using a multiplex bead immunoassay and compared with mock-infected IFN- γ R $^{-/-}$ animals. Mean $\pm$ SEM ($n =$

Figure 2. Upregulation of Ym proteins in lung macrophages by γ -herpesvirus infection. (A) Ultrastructure analysis of lungs of infected mice at Day 180 after infection established the presence of numerous crystals and lamellar bodies inside alveolar macrophages; representative analysis of three animals. (B and C) Immunohistochemical staining for Ym1 and 2 showed positive staining for macrophages of mock- and virus-infected animals, respectively. Note that macrophages in infected lungs are large and had a foamy appearance. (D) Lung homogenate of a mock (M) and virally infected mice at the indicated time points were subjected to Western blot analysis for Ym proteins, and showed increase in Ym proteins at later stages of infection. Blots were stripped and reprobed with an anti- β -actin antibody as loading control. Samples with the same time point correspond to different animals. (E) BALF from mock-infected and infected mice collected at 15 and 180 d subjected to Western blot analysis for Ym proteins. Blots were stripped and reprobed with anti prosurfactant B antibody. (F) Quantitative RT-PCR was used to determine the levels of Ym transcripts in alveolar macrophages of mock- or MHV68-infected IFN- γ ^{-/-} mice at Day 180 after infection. Data were normalized against β -actin ($n = 2$ per group).

and to be essential pro-fibrotic mediators and are directly linked to the production of the Th2 cytokine IL-13 (20, 21). High expression of MIP-1 α has been reported in BAL immune cells from patients with progressive IPF. The release of MIP-1 α was absent in patients with IPF with stable disease (22, 23). More recently, CCL18, a marker of alternative activation by human alveolar macrophages, has also been shown to be upregulated in BAL-

derived cells from patients with IPF. Levels of expression of CCL18 correlated negatively with pulmonary function test parameters (24). Th2 cytokines contribute to the recruitment and activation of macrophages, and promote the metabolism of L-arginine by arginase to produce ornithine and other products that ultimately lead to collagen deposition and fibrosis. In contrast, Th1 cytokines activate NOS2 expression and metabolize

Figure 3. Expression of gene markers of alternative activation in lung macrophages by γ -herpesvirus infection. (A) Determination of FIZZ1 transcripts by quantitative RT-PCR in lungs of mock- or MHV68-infected IFN- γ ^{-/-} mice collected at the indicated time points (in days). Data were normalized against β -actin ($n = 2$ per group). (B) BALF from mock-infected and infected mice collected at different time points after infection were subjected to Western blot analysis for FIZZ1. Two different animals were analyzed for each time point. Notice two peaks in FIZZ1 expression, at the acute phase and at Day 180. Blots were stripped and reprobed with anti-prosurfactant B antibody. (C–E) Immunohistochemical staining for FIZZ1 in mock-infected and infected mice at Day 180, respectively. Positive staining was found in macro-

phages and epithelial cells from infected animals. (E) IGF-1 expression as determined by immunostaining of lungs of MHV68-infected IFN- γ ^{-/-} mice at Day 180 after infection. Intense positive staining was observed in macrophages (C and D: $\times 40$ magnification; E: $\times 100$ magnification).

Figure 4. Increased arginase I in lung of MHV68-infected IFN- γ R^{-/-} mice. (A) Arginase I expression as determined by immunostaining of lungs of mock-infected IFN- γ R^{-/-} mice at Day 180 after infection. Positive arginase I immunostaining in fibrotic areas (B) and macrophages (C) of the lung of chronically infected mice. (D) Western blot analysis for arginase I in lung lysates from mock- and virus-infected animals at the indicated time points. Arginase I protein levels increased with duration of infection. Two different animals were analyzed at Day 180. (E) Measurement of arginase I activity was determined in lung lysates from mock- and virus-infected animals at Day 200 ($n = 3$ per group). Virus infection was associated with significant increase of arginase activity (A: $\times 20$ magnification; B and C: $\times 40$ magnification).

L-arginine to generate L-hydroxyarginine, L-citrulline, and NO (25, 26). The divergent arginase I and NOS2 pathways have been investigated extensively in parasitic infections (27, 28). Infection with *Schistosoma mansoni* eggs induces Th2 immune responses and a preferential upregulation of arginase I expression. The *Schistosoma* granulomas are associated with extensive fibrosis, and studies using inhibitors of arginase I and NOS2 have shown that NOS2 slows the development of fibrosis, whereas arginase I activity accelerates this process (12, 29). The relevance of the arginase pathway is being studied in other lung pathologies, such as pulmonary hypertension (30) and asthma (31, 32). Increased arginase II metabolic pathway in endothelial cells from patients with pulmonary arterial hypertension affects NOS activity and vasodilation. In asthma, arginase I is involved in collagen deposition, smooth muscle cell proliferation, and airway obstruction

by reducing the production of bronchodilatory NO. Although alternative pathway can be activated by different factors that end in lung disrepair, there are models of pulmonary fibrosis that are not necessarily associated with macrophage differentiation via the alternative pathway. Such is the case in experimental silicosis, where macrophages express arginase I and NOS2 during the inflammatory phase, and only NOS2 during the chronic stages (33). In our model of MHV68-induced fibrosis, we observed different patterns of expression in the markers of activation via the alternative pathway. For instance, upregulation of FIZZ1 and arginase I begin at Day 7 after infection and remain high during the chronic phase. In parallel, secretion of Th2 cytokines peaks at Day 7 after infection and persists above control levels during later time points of infection. In contrast, upregulation of the marker Ym1/2 was only evident 180 d after infection, suggesting that it may require a second stimuli (19, 34).

Lung fibroblasts from patients with IPF present abnormal properties on survival and production of ECM components. They express high levels of Th2 cytokine receptors and elevated Th2-mediated proliferative responses (35). Fibroblasts, similar to macrophages, have the dual pattern of NOS2 and arginase I expression when stimulated with Th1 and Th2 cytokines, respectively (36). Th2 cytokines can upregulate arginase I directly in fibroblasts, increasing their collagen-producing potential. In concordance, wound healing fibroblasts have increased arginase activity compared with normal fibroblasts. In addition, alternative activation of macrophages in a paracrine fashion provides L-proline and stimulates collagen production in fibroblasts. High levels of the arginase I and II isoforms have been reported in the bleomycin-mediated lung fibrosis model (37). Arginase I was elevated in macrophages, whereas arginase II was found induced in several types of cells, including macrophages and myofibroblasts. Our current studies demonstrate the upregulation of arginase I in fibroblasts and macrophages from fibrotic

Figure 5. γ -Herpesvirus infection stimulates fibronectin gene transcription and arginase activity. Fibronectin reporter A549 stable transfected cells were cultured in the presence of supernatant of macrophage culture or BAL from mock- and virus-infected animals for 24 h. Afterwards, the cells were harvested and fibronectin gene transcription was measured by luminescence ($n = 3$ per group).

Figure 6. Arginase I expression in human IPF. (A) Immunostaining of lung frozen sections from normal lung using anti-cytokeratin 5/8 (red) and anti-arginase I (green) antibodies. Modest endothelial and perivascular positive staining was found in subpleural areas. Pleura localization is shown with an arrow. (B) Immunostaining of lung frozen sections from patients with IPF with moderated alveolar remodeling using anti-cytokeratin (red) and anti-arginase I (green) antibodies. Notice arginase I-positive staining in fibroblast foci (asterisks). (C) Higher magnification of a positive arginase I fibroblast foci. (D) Immunostaining of lung frozen section from patient with IPF showing severe areas of fibrosis. Notice extensive arginase I staining in pleura (arrow) and areas with interstitial fibrosis. Merged image shows some yellow cells indicating epithelial cells expressing arginase I. (E) Immunostaining for cytokeratin 5/8 (green) and arginase I staining (red) of lung frozen section from a patient with IPF. Notice alveolar macrophage positive for arginase I. (F and G) Immunofluorescence analysis of BAL cell cytospin from a patient with IPF, incubated with rabbit IgG and anti-arginase I (red), respectively. Forty percent of the nucleated cells in the BAL were positive for arginase I. Slides were counterstained with DAPI, which stain nuclei blue. (H) Arginase I expression as determined by Western blot analysis in whole-lung lysates of a normal control (lane 1), and four different patients with IPF (lanes 2–5). The same set of samples were run in reducing conditions and probed for β -actin expression as loading control (A, B, and D: $\times 10$ magnification; C and F: $\times 100$ magnification).

lungs associated with chronic murine γ -herpesvirus infection and in lung samples of patients with IPF.

Inhibition of arginase activity can be a therapeutic approach to control fibrosis. Synthetic inhibitors of arginase I activity, like NOHA (L-hydroxy arginine) and the 40-times more potent nor-NOHA, have been used successfully to regulate the fibrosis and control replication in animal models of parasitic infections (38, 39). However, chronic treatment with these compounds would cause alteration of the liver urea cycle function and hepatotoxicity. Recent identification of regulatory elements for expression or function of extrahepatic arginase I could provide potential ways to manipulate arginase I activity in immune cells while sparing liver function (40).

In summary, we have demonstrated that chronic γ -herpesvirus infection in IFN- γ R $^{-/-}$ mice induces macrophage recruitment and alternative activation of macrophages in the lung. In parallel, lungs from patients with IPF show upregulation of several markers of this pathway. Our studies suggest that interruption of this pathway may represent an important therapeutic intervention strategy for the treatment of lung fibrosis.

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References

1. American Thoracic Society/European Respiratory Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. *Am J Respir Crit Care Med* 2000;161:646–664.
2. Selman M, Pardo A. The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2003;29(Suppl.):S93–S97.
3. Stewart JP, Egan JJ, Ross AJ, Kelly BG, Lok SS, Hasleton PS, Woodcock AA. The detection of Epstein-Barr virus DNA in lung tissue from

- patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1999;159:1336–1341.
4. Lok SS, Egan JJ. Viruses and idiopathic pulmonary fibrosis. *Monaldi Arch Chest Dis* 2000;55:146–150.
 5. Tang YW, Johnson JE, Browning PJ, Cruz-Gervis RA, Davis A, Graham BS, Brigham KL, Oates JA Jr, Loyd JE, Stecenko AA. Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. *J Clin Microbiol* 2003;41:2633–2640.
 6. Mora AL, Woods CR, García A, Xu J, Rojas M, Speck SH, Roman J, Brigham K, Stecenko AA. Lung infection with γ herpesvirus induces progressive pulmonary fibrosis in Th2 biased mice. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L711–L721. Epub 2005 Feb 25.
 7. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3:23–35.
 8. Mosser DM. The many faces of macrophage activation. *J Leukoc Biol* 2003;73:209–212.
 9. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992;176:287–292.
 10. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005;115:56–65.
 11. Gratchev A, Guillot P, Hakiy N, Politz O, Orfanos CE, Schledzewski K, Goerdt S. Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein β IG-H3. *Scand J Immunol* 2001;53:386–392.
 12. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, Pearce EJ, Wynn TA. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines *in vivo*: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* 2001;167:6533–6544.
 13. Wynes MW, Frankel SK, Riches DW. IL-4-induced macrophage-derived IGF-I protects myofibroblasts from apoptosis following growth factor withdrawal. *J Leukoc Biol* 2004;76:1019–1027.
 14. Wynes MW, Riches DW. Induction of macrophage insulin-like growth factor-I expression by the Th2 cytokines IL-4 and IL-13. *J Immunol* 2003;171:3550–3559.
 15. Uh ST, Inoue Y, King TE Jr, Chan ED, Newman LS, Riches DW. Morphometric analysis of insulin-like growth factor-I localization in lung tissues of patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1998;158:1626–1635.
 16. Roman J, Rivera HN, Roser-Page S, Sitaraman SV, Ritzenthaler JD. Adenosine induces fibronectin expression in lung epithelial cells: implications for airway remodeling. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L317–L325.
 17. Corraliza IM, Campo ML, Soler G, Modolell M. Determination of arginase activity in macrophages: a micromethod. *J Immunol Methods* 1994;174:231–235.
 18. Nair MG, Cochrane DW, Allen JE. Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and FIZZ1 that can be partly replicated *in vitro*. *Immunol Lett* 2003;85:173–180.
 19. Webb DC, McKenzie AN, Foster PS. Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction: identification of a novel allergy-associated protein. *J Biol Chem* 2001;276:41969–41976.
 20. Blease K, Mehrad B, Standiford TJ, Lukacs NW, Kunkel SL, Chensue SW, Lu B, Gerard CJ, Hogaboam CM. Airway remodeling is absent in CCR1^{-/-} mice during chronic fungal allergic airway disease. *J Immunol* 2000;165:1564–1572.
 21. Gao JL, Wynn TA, Chang Y, Lee EJ, Broxmeyer HE, Cooper S, Tiffany HL, Westphal H, Kwon-Chung J, Murphy PM. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1–type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* 1997;185:1959–1968.
 22. Ziegenhagen MW, Schrum S, Zissel G, Zipfel PF, Schlaak M, Muller-Quernheim J. Increased expression of proinflammatory chemokines in bronchoalveolar lavage cells of patients with progressing idiopathic pulmonary fibrosis and sarcoidosis. *J Invest Med* 1998;46:223–231.
 23. Standiford TJ, Rolfe MW, Kunkel SL, Lynch JP III, Burdick MD, Gilbert AR, Orringer MB, Whyte RI, Strieter RM. Macrophage inflammatory protein-1 α expression in interstitial lung disease. *J Immunol* 1993;151:2852–2863.
 24. Prasse A, Pechkovsky DV, Toews GB, Jungraithmayr W, Kollert F, Goldmann T, Vollmer E, Muller-Quernheim J, Zissel G. A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18. *Am J Respir Crit Care Med* 2006;173:781–792.
 25. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4⁺ T cells correlates with Th1/Th2 phenotype. *J Immunol* 1998;160:5347–5354.
 26. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* 2004;4:583–594.
 27. Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A. Alternatively activated macrophages during parasite infections. *Trends Parasitol* 2004;20:126–133.
 28. Pearce EJ, MacDonald AS. The immunobiology of schistosomiasis. *Nat Rev Immunol* 2002;2:499–511.
 29. Hesse M, Cheever AW, Jankovic D, Wynn TA. NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. *Am J Pathol* 2000;157:945–955.
 30. Xu W, Kaneko FT, Zheng S, Comhair SA, Janocha AJ, Goggans T, Thunnissen FB, Farver C, Hazen SL, Jennings C, et al. Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *FASEB J* 2004;18:1746–1748.
 31. King NE, Rothenberg ME, Zimmermann N. Arginine in asthma and lung inflammation. *J Nutr* 2004;134:2830S–2836S. (Discussion 2853S).
 32. Vercelli D. Arginase: marker, effector, or candidate gene for asthma? *J Clin Invest* 2003;111:1815–1817.
 33. Misson P, van den Brule S, Barbarin V, Lison D, Huaux F. Markers of macrophage differentiation in experimental silicosis. *J Leukoc Biol* 2004;76:926–932.
 34. Ward JM, Yoon M, Anver MR, Haines DC, Kudo G, Gonzalez FJ, Kimura S. Hyalinosis and Ym1/Ym2 gene expression in the stomach and respiratory tract of 129S4/SvJae and wild-type and CYP1A2-null B6, 129 mice. *Am J Pathol* 2001;158:323–332.
 35. Jakubzick C, Choi ES, Carpenter KJ, Kunkel SL, Evanoff H, Martinez FJ, Flaherty KR, Toews GB, Colby TV, Travis WD, et al. Human pulmonary fibroblasts exhibit altered interleukin-4 and interleukin-13 receptor subunit expression in idiopathic interstitial pneumonia. *Am J Pathol* 2004;164:1989–2001.
 36. Witte MB, Barbul A, Schick MA, Vogt N, Becker HD. Upregulation of arginase expression in wound-derived fibroblasts. *J Surg Res* 2002;105:35–42.
 37. Endo M, Oyadomari S, Terasaki Y, Takeya M, Suga M, Mori M, Gotoh T. Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L313–L321.
 38. Iniesta V, Gomez-Nieto LC, Corraliza I. The inhibition of arginase by N(ω)-hydroxy-L-arginine controls the growth of *Leishmania* inside macrophages. *J Exp Med* 2001;193:777–784.
 39. Tenu JP, Lepoivre M, Moali C, Brollo M, Mansuy D, Boucher JL. Effects of the new arginase inhibitor N(ω)-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. *Nitric Oxide* 1999;3:427–438.
 40. Erdely A, Kepka-Lenhart D, Clark M, Zeidler-Erdely P, Poljakovic M, Calhoun WJ, Morris SM Jr. Inhibition of phosphodiesterase 4 amplifies cytokine-dependent induction of arginase in macrophages. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L534–L539.