Published in final edited form as:

Histochem Cell Biol. 2008 November ; 130(5): 979–992. doi:10.1007/s00418-008-0466-z.

TLR9 is expressed in idiopathic interstitial pneumonia and its activation promotes in vitro myofibroblast differentiation

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Abstract

Infectious diseases can be cofactors in idiopathic interstitial pneumonias (IIP) pathogenesis; recent data suggests that toll-like receptors 9 (TLR9) ligands contribute to experimental chronic tissue remodeling. Real-time TAQMAN and immunohistochemical analysis of IIP normal surgical lung biopsies (SLBs), primary fibroblast lines grown from both IIP and normal SLBs indicate that TLR9 is prominently and differentially expressed in a disease-specific manner. TLR9 expression

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Electronic supplementary material The online version of this article (doi:10.1007/s00418-008-0466-z) contains supplementary material, which is available to authorized users.

was increased in biopsies from patients with IIP compared with normal lung biopsies and its expression localized to areas of marked interstitial fibrosis. TLR9 in fibroblasts appeared to be increased by profibrotic Th2 cytokines (IL-4 and IL-13) and this was true in fibroblasts cultured from the most severe form of IIP, idiopathic pulmonary fibrosis (IPF) SLBs, in non-specific interstitial pneumonia fibroblast lines, and in normal fibroblasts. Finally, confocal microscopy studies have shown that TLR9 activation by its synthetic agonist CpG-ODN significantly increased the expression of alpha smooth muscle actin, the main marker of myofibroblast differentiation. These data indicate that TLR9 expression may drive the abnormal tissue healing response in severe forms of IIP and its activation can have a key role in myofibroblast differentiation promoting the progression of disease during the terminal phase of IPF.

Keywords

Idiopathic interstitial pneumonia; TLR9; Th-2 cytokines; Confocal microscopy; α -SMA; Myofibroblast differentiation

Introduction

Idiopathic interstitial pneumonias (IIP) are a heterogeneous group of diffuse lung diseases involving the pulmonary parenchyma. Idiopathic pulmonary fibrosis (IPF) is the most common IIP and is associated with the classic pathologic pattern called usual interstitial pneumonia (UIP) (ATS/ERS 2002). IPF/UIP is a fatal and progressive lung disease of unknown etiology and is characterized by some degree of chronic inflammation, alveolar epithelial cell injury/activation, fibroblast proliferation, and exuberant accumulation of extracellular matrix (ECM) in the alveolar spaces (Selman and Pardo 2003). Idiopathic non-specific interstitial pneumonia (NSIP) is a milder form of IIP which represents a distinct clinical entity mostly occurring in non-smoking middle aged women who often have positive serologies for collagen vascular diseases (Flaherty et al. 2001a).

Although the pathogenesis of pulmonary fibrosis is very complex and still poorly defined, it is well-established that myofibroblasts have a pivotal role as their presence and persistence within the fibroblastic foci in IPF biopsies correlates with a more severe progression of the disease (Mitchell et al. 1989; Zhang et al. 1996).

Myofibroblasts express alpha smooth muscle actin (α -SMA) within cytoplasmic fibers, and is currently the most widely used myofibroblast marker (Darby et al. 1990; Skalli et al. 1989). These cells have a phenotype between fibroblasts and smooth muscle cells and are the major source of fibrogenic molecules, growth factors and ECM constituents (Darby et al. 1990; Fine and Goldstein 1987; Hinz 2007; Hinz et al. 2007). Tumor growth factor beta (TGF- β) directly promotes myofibroblast differentiation by inducing the expression of α -SMA (Desmouliere et al. 1993; Khalil et al. 1989). Recent evidence suggests that other molecules [like nerve growth factor, interleukin-6, Found in inflammatory zone-1 (Fizz1)] contribute to myofibroblast differentiation and further, these can optimally participate to the fibroproliferative response (Hinz 2007).

One recent hypothesis underlying the pathogenesis of IPF is that a combination of repeated microscopic injuries to alveolar epithelial cells results in an aberrant wound healing response mediated by fibroblasts and myofibroblasts (Noble and Homer 2005; Selman and Pardo 2003). Additionally, infections can directly contribute to of tissue damage, fibrosis and chronic inflammation as a result of the exaggerated and uncontrolled reparative immune response to invading pathogens or their byproducts (Meneghin and Hogaboam 2007).

Toll-like receptors (TLRs) are essential and evolutionary well-conserved receptors that recognize specific patterns of microbial components (Akira and Hemmi 2003; Janeway and Medzhitov 2002). Toll-like receptors 9 (TLR9) is localized in the endoplasmic reticulum and specifically recognizes bacterial and viral nucleic acids, which in contrast to endogenous DNA are largely hypomethylated (Latz et al. 2004; Leifer et al. 2004; Hemmi et al. 2000). The immunomodulatory effects of natural microbial DNA can be mimicked by short synthetic single-stranded oligodeoxyribonucleotides (ODNs) containing hypomethylated GC rich sequences namely CpG motifs (CpG-ODN) (Bauer et al. 2001; Ishii and Akira 2006; Krieg 2002; Takeshita et al. 2001). TLR9 expression was originally identified in B-lymphocytes, monocytes and plasmocitoid dendritic cells (Lund et al. 2003; Takeshita et al. 2001). More recent studies have shown that TLR9 is also expressed on normal and malignant structural cells and in many epithelial cell lines, implying that cells outside the immunological compartment also may be important sentinels of the innate system (Bowman et al. 2003; Droemann et al. 2005; Ewaschuk et al. 2007; Fischer et al. 2005; Merrell et al. 2006; Schmausser et al. 2005).

Because TLR9 had not been investigated in IIP, we evaluated the expression of TLR9 in lung biopsies derived from UIP and NSIP patients and in normal individuals using immunohistochemistry. Additionally, we investigated the influence of two profibrotic cytokines as IL-4 and IL-13 on TLR9 expression in primary fibroblasts derived from IIP and normal biopsies. Our results show enhanced TLR9 expression on pulmonary fibroblasts in IIP may provide a marker for measuring disease severity, and may also contribute to the understanding of how and why IPF progresses after infectious insults to the lung. Together, our results show how microbial byproducts can drive myofibroblast differentiation and add a novel field of investigation for unraveling the pathogenesis of pulmonary fibrosis.

Materials and methods

Isolation and culture of primary pulmonary fibroblast lines

UIP, NSIP and normal surgical lung biopsies (SLBs) were finely minced and the dispersed tissue pieces were placed into 150-cm² cell culture flasks (Corning Inc., Corning, NY) containing Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, Walkersville, MD) supplemented with 15% fetal bovine serum (DMEM-15, BioWhittaker), 1 mmol/L glutamine (BioWhittaker), 100 U/ml penicillin (BioWhittaker), 100 µg/ml streptomycin (BioWhittaker), and 0.25 µg amphotericin B (Fungizone; BioWhittaker). All primary lung cell lines were maintained in DMEM-15 at 37°C in a 5% CO₂ incubator and were serially passaged a total of five times to yield pure populations of lung fibroblasts as previously described in detail (Hogaboam et al. 2005). All three primary fibroblast cell lines from each patient group were used at passages 6 to 10 in the experiments outlined below and all of the

experiments were performed under comparable conditions. Regardless of the passage number, both the transcript and protein expression of the various target genes was stable during culture expansion and passage of primary human fibroblasts.

Each well in a six-well tissue culture plate was seeded with 2.5×10^5 fibroblasts and at the 75% confluence were stimulated for 24 h with 10 ng/ml of human recombinant IL-4 (Biosource, Worcester, MA), IL-13 (PeproTech, Rocky-Hill, NJ) or both IL-4 and IL-13, with or without 100 mM of CpG-ODNTCGTCGTTTTGTCGTTTTGTCGTT (Cell Sciences, CA) as synthetic agonist of TLR9. Twenty-four hours later, cell-free supernatants were removed from each well for immunocytochemistry and immunofluorescence analysis.

Preparation of RNA and cDNA from SLBs and primary pulmonary fibroblast lines

After treatments as describe above, Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) was added to each well and total RNA was then prepared according to the manufacturer's instructions. The same process was applied to 12 (upper and lower lobes) UIP, seven (upper and lower lobes) NSIP and seven normal SLBs after they were thawed on ice. Purified RNA from SLBs and the fibroblasts was subsequently reverse-transcribed into cDNA using a BRL reverse transcription kit and oligo (dT) 12–18 primers. The amplification buffer contained 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, and 2.5 mmol/L MgCl₂ (Invitrogen Life Technologies, Carlsbad, CA).

Real-time TAQMAN PCR analysis

Human TLR9 gene expression was analyzed by a real-time quantitative RT-PCR procedure using an ABI PRISM 7500 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The cDNAs from SLBs samples were analyzed for TLR9, and GAPDH (an internal control). Primers and probe used for TLR9 were purchased from Applied Biosystems. Gene expression was normalized to GAPDH, and the fold increases in targets gene expression was calculated by comparing of gene expression in all IIPs biopsies to untreated normal controls, and between Th2 cytokine treated UIP, NSIP and normal fibroblasts to the untreated cells within the respective disease group (as control). Gene expression in the control group of samples was given an arbitrary value of 1.

Immunocytochemistry and immunohistochemistry

Purified fibroblast lines were added to 8-well Labtek (Nunc Inc., Naperville, IL) tissue culture plates at a cell density of 1×10^5 cells/well. We analyzed three different fibroblasts lines per each group of SLB and specifically for UIP and NSIP samples we investigated fibroblasts grown from the lower lobe biopsy. Twenty-four hours after treatments described above, fibroblasts were fixed with 3.7% paraformaldehyde and stained according to the procedures supplied with the Cell and Tissue Staining Kit from R&D Systems. Paraffin-embedded whole lung samples were analyzed for immunohistochemical localization of TLR9 as well. Five-micron histological sections were dewaxed with xylene, rehydrated in graded concentrations of ethanol, and blocked with normal mouse serum (R&D System). Immunohistological studies were performed in SLBs deriving from a minimum of seven different patients per each disease group. We also evaluated TLR9 expression in two SLBs obtained from two patients who met the criteria of acute exacerbation of IPF/UIP (Parambil

et al. 2005). After fixation with 3.7% paraformaldehyde, mouse anti-human TLR9 monoclonal antibody (Imgenex, San Diego, CA) and control normal mouse IgG1 (R&D System) were diluted in PBS to a final concentration of 0.5 µg/ml. Anti TLR9 or IgG were added to histological sections and to fibroblasts overnight at 4°C, after which both cells and tissue sections were washed thoroughly three times with PBS. A secondary rabbit anti-mouse biotinylated Ab (R&D System) was added to each sample for 1 h then washed and peroxidase (R&D Systems) conjugated to avidin was added to each sample. Receptor localization was revealed with 3-amino-9-ethylcarbazole. Coverslips were applied to each slide using an aqueous mounting solution and microscopic images (400× magnification) were captured using a digital camera.

Double immunofluorescence and confocal microscopy

UIP and normal fibroblasts were seeded onto 8-well glass Labtek tissue culture plates (Nunc Inc., Naperville, IL) at a cell density of 1×10^5 cells/well. Cells were treated as described above, and then were fixed with 3.7% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 2 min, and blocked with PBS/BSA2% for 2 h at room temperature. Wells were incubated sequentially with anti α -SMA FITC-conjugated antibodies (1:200) and Alexa Fluor 546 phalloidin (Invitrogen-Molecular Probes, Inc., Carlsbad, CA) or biotinylated mouse anti-human TLR9 (1:1,000) (Imgenex, San Diego, CA). Streptavidin Alexa Fluor 647-conjugated was used for 1 h incubation at room temperature for the detection of TLR9 (Invitrogen-Molecular Probes, Inc., Carlsbad, CA). Washes and all antibodies dilutions were performed in PBS/2% BSA. Well contents were mounted in Prolog Gold AntiFade with DAPI (to stain cell nuclei) (Invitrogen-Molecular Probes, Inc.) and analyzed with a Zeiss LSM510 confocal microscope with C-Apochromat 40× objective lens (Carl Zeiss, Inc., Germany). Images were recorded with the LSM5 software and processed with Adobe Photoshop CS2.

Statistical analysis

All the results are expressed as mean (SEM). The data were presumed to be non-parametric because of the inclusion of gene values (generated using TAQMAN) that were below the limit of detection and thus assigned a value of zero. Significance was set at $P < 0.05$.

Results

Expression of TLR9 transcript in idiopathic interstitial pneumonia biopsies

We have analyzed TLR9 expression in UIP ($n = 12$), NSIP ($n = 7$) and compared seven normal margins from tumor resection SLBs. Quantitative TAQMAN PCR analysis demonstrated that the expression of TLR9 mRNA was increased approximately sixfold and 2.5-fold in UIP and NSIP SLBs, respectively, compared to the normal SLB group (Fig. 1a). There was no significant difference in TLR9 gene expression between the two groups of IIP.

Next we looked at TLR9 levels in upper and lower biopsies. In the lower lobe, TLR9 transcript was detectable in 9 of the 12 UIP biopsies analyzed (75%) and in seven upper lobe UIP biopsies obtained from the same patients (58.3%) (Table 1). When TLR9 was absent in the lower lobe biopsy, it was also absent in the upper lobe biopsy ($n = 3$ patients (Fig. 1b).

Among the seven NSIP biopsies analyzed six were found positive for TLR9 transcript in the lower lobe (85.7%) and five exhibited TLR9 levels in the upper lobe (71.4%). Control biopsies weren't divided into upper and lower lobe as the section analyzed depended on the side from which the tumor was resected. All control samples showed low gene expression of TLR9.

Quantitatively, TLR9 gene expression was increased approximately fourfold in the lower lobe UIP SLBs compared to control SLBs, while TLR9 expression levels in the upper lobe UIP SLBs did not show any such increase (Fig. 1b). In patients with NSIP TLR9 transcript values were increased fourfold in the upper lobe SLBs compared to normal margin SLBs. Mean TLR9 gene expression values were significantly increased in upper lobes relative to lower lobes ($P > 0.05$) in NSIP biopsies (Fig. 1c). Overall, quantitative TAQM9 analysis showed that TLR9 transcript in all IIP SLBs is expressed in a disease specific manner where a higher fold increase was found in IPF/UIP patients than in NSIP patients compared to the normal patient group.

TLR9 is expressed in the interstitium of UIP and NSIP SLBs

Molecular analysis through real time PCR showed that TLR9 expression was higher in IIP SLBs than in normal margins SLBs; therefore to confirm these data and to localize cellular sources of pulmonary TLR9 mRNA expression, we performed immunohistochemistry of whole lung sections from IIP and normal margin SLBs. As shown in Fig. 2, TLR9 staining was detected in SLBs from both IPF/UIP and NSIP patients. Of interest, the staining was localized not only in the alveolar epithelium but also in the interstitium of both groups of IIP (Fig. 2b, d). These expression patterns were repeated in all specimens we examined ($n = 3$). Inflammatory cell infiltrates, comprising mainly small lymphocytes, macrophages, and occasional plasma cells (Flaherty et al. 2001b; Katzenstein et al. 2002), exhibited a variable staining intensity for TLR9. Specifically, a mild and heterogeneous inflammation was observed in UIP (Fig. 2b) samples while NSIP biopsies analyzed showed a more conspicuous and diffuse inflammatory infiltrates with a very intense staining for TLR9, particularly in morphologically-distinct macrophages (Fig. 2d). TLR9 expression was weak or undetectable in normal lung tissue, and appeared to be confined to small lymphocytes (Fig. 2f). We also analyzed biopsies obtained from two patients who died from acute exacerbation of pulmonary fibrosis. In both these specimens TLR9 was highly expressed within intra-alveolar spaces and in the scarring areas adjacent to diffuse alveolar damage that generally characterizes the histological pattern of this fatal clinical condition (Parambil et al. 2005) (Fig. 3).

TLR9 is expressed in primary fibroblast cell lines of idiopathic interstitial pneumonia and appears to be enhanced by Th2 type cytokines

Based on the observation that TLR9 expression is remarkably enhanced in the interstitium of the biopsies collected from patients affected by IIP we isolated and cultured pulmonary fibroblasts derived from UIP and NSIP SLBs for further investigation (Figs. 4, 5 and 6). Many in vivo and in vitro studies have suggested that the persistent imbalance in the expression of Th1 and Th2 type cytokines in the lung may be one of the important underlying factors for the progression of pulmonary fibrosis (Hashimoto et al. 2001;

Jakubzick et al. 2004; Sempowski et al. 1996). In particular, our group has shown that IIP primary fibroblast cell lines derived from IIP lungs display high levels of the receptors for IL-4 (IL-4R α) and IL-13 (IL-13R α 1 and IL-13R α 2) Th2 cytokines (Jakubzick et al. 2004). For this reason we evaluated whether these cytokines influenced TLR9 expression. We detected high levels of TLR9 in the cytoplasm of untreated (media alone condition) UIP fibroblasts (Fig. 4b); the pattern of expression appears to be quite heterogeneous throughout the cell population but mostly homogeneous within the intracellular compartment. TLR9 expression was enhanced when fibroblasts were exposed to IL-4 (Fig. 4d) and to IL-13 (Fig. 4f) treatment for 24 h. Incubation with IL-4 (Fig. 4d) clearly increased while when IL-13 did so, it was at a more subtle level (Fig. 4f). Additionally, the combination of IL-4 and IL-13 increased TLR9 expression as expected and a more homogenous distribution among all the cells in the culture (Fig. 4h). These data suggest that TLR9 expression appeared to be induced by Th2 type cytokine treatment and that IL-13 may act additively with IL-4 to effect this. In identical experiments with fibroblasts derived from NSIP SLBs, TLR9 was also detected in control untreated cells (Fig. 5b). Additionally, the expression of TLR9 appeared to be enhanced by the stimulation with IL-4 (Fig. 5d) or IL-13 (Fig. 5f) to the media. Again, enhanced staining was observed when IL-4 and IL-13 were added together (Fig. 5h).

The cytokine treatments were also included in fibroblasts grown from the normal margin of SLBs. These showed a weaker expression of TLR9 under media condition (Fig. 6b) compared to UIP and NSIP cells, and in contrast to results in IIP primary fibroblasts cell lines, TLR9 expression was not inducible via Th2 cytokine activation (Fig. 6d, f, h).

Using quantitative TAQMAN PCR analysis, we examined whether TLR9 gene expression was altered by Th2 cytokines stimulation (Fig. 7). After cytokine treatment, TLR9 transcripts in UIP fibroblast cell lines were significantly increased twofold by IL-4 or IL-13 stimulation and fivefold when treated with both together above the transcript levels detected in untreated UIP fibroblasts (Fig. 7a). TLR9 expression in NSIP fibroblast cultures was not affected by any of the treatments used and we did not observed any difference in gene expression between untreated NSIP fibroblasts and Th2 cytokines challenged NSIP fibroblasts (Fig. 7b). In normal fibroblasts cell cultures Th2 cytokines stimulation did not increase TLR9 gene expression compared with untreated normal fibroblasts (Fig. 7c).

CpG-ODN increases α -SMA expression in primary UIP fibroblast cell cultures

It is well-established that myofibroblasts have the potential to play an important role in the pathogenesis of pulmonary fibrosis. Its dual role as a key source of ECM and as inflammatory cell makes it the key cell in two processes that represent the hallmark of pulmonary fibrosis. Fully differentiated myofibroblasts express α -SMA (Darby et al. 1990). Since there are significant parallels in cell biology between inflammatory cells in inflammation and myofibroblasts in fibrosis we wanted to investigate the role of TLR9 expression in myofibroblast differentiation of UIP fibroblast primary cell lines. We have herein shown that UIP fibroblasts increase the neoexpression of α -SMA within cytoplasmic rich-actin stress fibers 24 h after treatment with synthetic agonist CpG-ODN (10 μ g/ml) (Fig. 8b) or IL-4 (10 ng/ml) (Fig. 8c). The combined stimulation with CpG-ODN and IL-4 did not significantly modify the level of expression of α -SMA any further compared the two

treatments alone (Fig. 8d). We also found that α -SMA positive fibroblasts expressed TLR9 (Supplementary Fig. 1). Under certain conditions, fibroblasts present morphological characteristics of myofibroblast but they do not express α -SMA, so the term “proto-myofibroblasts” has been coined to define these cells (Hinz et al. 2007). Usually, cultured fibroblastic cells in the presence of fetal calf serum exhibit a mixture of proto-myofibroblast α -SMA negative and differentiated myofibroblastic α -SMA positive (Skalli et al. 1989). In addition α -SMA incorporation within cytoplasmic stress fibers requires the generation of tension within a connective tissue matrix (Skalli et al. 1989). Our data are in line with what is reported in the literature (Hinz et al. 2007) namely in cells growing in media alone (Fig. 8a) a heterogeneous population was observed growing on glass and where cells positive for α -SMA (myofibroblasts) co-existed with other cells with similar morphology but these latter were negative for α -SMA and thus likely proto-myofibroblasts. Similar to IL-4, CpG-ODN incubation increases the number of α -SMA positive cells suggesting a novel role for this agent in driving myofibroblast differentiation. Thus our data support that in vitro CpG-ODN has in addition to fetal calf serum and mechanical tension, an important role in promoting the myofibroblast phenotype. These findings may have considerable pathophysiological significance in the understanding of the course pulmonary fibrosis.

α -SMA expression in primary normal fibroblasts is not inducible by CpG-ODN treatment

Despite the lower expression of TLR9 transcript in fibroblasts grown from normal SLBs compare to fibroblasts grown from IIP SLBs we investigated the effect of CpG-ODN stimulation on myofibroblast differentiation in this non-fibrotic context (Fig. 9). Immunofluorescence analysis showed that (Fig. 9a) normal fibroblasts express considerable levels of α -SMA when grown in media alone. Addition of IL-4 (Fig. 9b), CpG-ODN (Fig. 9c) or the combination of the two stimuli (Fig. 9d), did not increase α -SMA staining any further in contrast to what we observed in UIP fibroblasts. These findings support the notion that fibroblasts in the surrounding area of a tumoral mass become differentiated into myofibroblasts. Notably their differentiation is not driven by the profibrotic environment or by microbial challenges.

Discussion

It is well-established that pulmonary fibrosis is the outcome of excessive and relentless ECM deposition in the lung interstitial compartment and that fibroblasts and myofibroblasts play a pivotal role in this process (Selman and Pardo 2003). Recent data provide compelling new information regarding the natural history of IPF. In contrast to the chronic and progressive course of the disease, a growing body of evidence point to an apparently rapid fatal decline in lung function in patients who died from IPF related causes (Akira et al. 1997; Kim et al. 2006; Tajima et al. 2003). An explanation for the acute clinical deterioration in IPF patients is currently lacking but we have hypothesized that this rapid evolution of disease may be the consequence of the presence or persistence of infectious agents or their byproducts in the lung.

Despite the fact that the direct or indirect detection of microorganisms in the biologic samples of IIP patients has proven difficult (Akira et al. 1997; Kuwano et al. 1997; Tajima et

al. 2003), several workers have noted the presence of viral DNA and protein from hepatitis C virus (Ueda et al. 1992), adenovirus (Kuwano et al. 1997), Epstein Barr virus (Kelly et al. 2002; Stewart et al. 1999) and other herpesvirus (Tang et al. 2003). Thus, infections and microbial byproducts that accumulate near or in the lung parenchyma in IIP are common events that may account for the progressive nature and ultimately lead to the acute exacerbation of the disease. Considerable evidence has accumulated, showing that TLRs are not only expressed on cells of immune or bone-marrow origin but that these receptors can be expressed on structural cells in a variety of normal and diseased tissues (Bowman et al. 2003; Droemann et al. 2005; Ewaschuk et al. 2007; Fischer et al. 2005; Merrell et al. 2006; Schmausser et al. 2005).

Although TLRs are considered attractive therapeutic targets in other diseases, their expression pattern and role in IIP is presently unknown. Among the family of TLRs, it is TLR9 that recognizes bacterial and viral DNA (Hemmi et al. 2000) and for the first time in our study TLR9 has been detected in lung specimens from patients affected by IIP. We have investigated the pattern of TLR9 expression in lung biopsy samples from IIP and histologically normal biopsies. Quantitative mRNA transcript analysis and qualitative protein analysis revealed that TLR9 was dramatically increased in IIP relative to normal SLBs. UIP SLBs and to a lesser extent NSIP SLBs exhibited an intense TLR9 positive staining localized in inflammatory and non-inflammatory cells. Several inflammatory cell types were TLR9-positive in UIP and NSIP biopsies examined herein, namely small lymphocytes, macrophages (possibly of alveolar origin), and plasma cells. While the expression of TLR9 by these cells was consistent with the current literature (Lund et al. 2003; Takeshita et al. 2001), we were surprised by the high level of staining in interstitial areas that were devoid of immune cells within these biopsies.

To assess the positive immunostaining observed in the interstitial space of the biopsy sections, we subsequently grew primary fibroblast cell lines from IIP and normal SLBs and treated them with key Th2 cytokines such as IL-4 and IL-13 to test the influence on TLR9 expression. Little is known about the effect of cytokines on the TLR expression on structural cells. In a recent study, the effects of Th1 and Th2 cytokines on TLRs expression were investigated in intestinal epithelial cell lines showing that the latter are able to down regulate the expression of TLR3, TLR4 and MD-2 (Mueller et al. 2006). In the lung a similar relationship exists between IL-4 and TLR4 in patients affected by tuberculosis (Fenhalls et al. 2003). Thus, from these studies it appears that, at least for epithelial cells, during the course of infection the Th2 environment has an anti-inflammatory effect either reducing the Th1 response or reducing the recognition of microbial components through the impaired expression of different TLRs. In our study, we did not observe any down regulation of TLR9 on fibroblasts post IL-4 and/or IL-13 exposure and in fact it appeared that those cytokines induced TLR9 expression on these cells. Quantitative real-time PCR analysis has shown that TLR9 gene expression in UIP fibroblast cell lines is significantly increased by IL-4 or/and IL-13 stimulation compared the transcript levels detected in untreated UIP fibroblasts. So we speculate that the cytokine microenvironment in IPF, skewed towards a Th2 phenotype, leads to a proinflammatory and profibrotic environment, which is supported by the continued expression of TLR9. It is possible that observed absence of downregulation of TLR9 by Th2 cytokines can be related to traces of lipopolysaccharide (LPS) in the media as

this byproduct can increase TLR9 expression on immune cells (Bernasconi et al. 2003; Takeshita et al. 2001). The effect of LPS on TLR9 protein levels in non-immune cells is currently unknown, however all our experiments were carried out using highly purified CpG-DNA so this is an unlikely source of LPS.

The majority of the studies looking at the effect of TLR9 activation on structural cells have been carried out with epithelial cell lines or on specimens from cancer biopsies from different organs like breast (Merrell et al. 2006), prostate (Ilvesaro et al. 2007), gastrointestinal tract (Ewaschuk et al. 2007; Fenhalls et al. 2003; Mueller et al. 2006; Schmausser et al. 2004) glia (Bowman et al. 2003), uterine cervix (Andersen et al. 2006; Lee et al. 2007; Schaefer et al. 2004) and respiratory system (Droemann et al. 2005; Parilla et al. 2006). These studies also support the notion that non-immune cells can be “alert sentinels” for the immune system and participate either to the host response to the tumor or in cancer progression. Cells grown from lung tumors exhibit an increase of proinflammatory, profibrotic and anti-apoptotic responses after TLR9 activation (Droemann et al. 2005). Notably, TLR9 is generally weakly expressed in lung tissue; TLR9 expression on airway epithelium is in fact only modest compared to other epithelium (Platz et al. 2004). We found a low TLR9 expression pattern on fibroblasts grown from normal margins SLBs but this data was predictable as TLR9 is present in non-malignant tissue only sporadically (Droemann et al. 2005).

Myofibroblast activation has been identified as a predictor of pulmonary fibrosis progression both in experimental animal models and in patients. In the present study we wanted to investigate the role of TLR9 activation through its synthetic ligand CpG-ODN. Here we report for the first time that CpG-ODN promotes the differentiation of primary fibroblasts grown from patients affected by IPF into myofibroblasts. It should be noted that only a few factors (included IL-6, NGF and Fizz1) have been identified that potentially promote myofibroblast differentiation in the absence of TGF- β the most potent inducer of myofibroblast transformation. (Desmouliere et al. 1993). With confocal microscopy we have shown that UIP/IPF fibroblasts challenged for 24 h with CpG-ODN increased the expression of α -SMA. Although there are several studies showing the ability of CpG-ODN to promote cell differentiation of immuno-competent cells, its effect on non-immune cells is still poorly understood. We do not think the increase of α -SMA expression observed in our study can be related to the stimulation of TLR4. However, our findings agree with those of Watanabe et al. who showed that hepatic stellate differentiated to myofibroblasts through TLR9 activation by CpG-ODN and also by apoptotic DNA, which increased TGF- β 1 and collagen type 1 mRNA transcripts in these cells (Watanabe et al. 2007). Just as hepatic stellate cells in liver fibrosis can differentiate into myofibroblasts, so we propose that resident fibroblasts can differentiate into myofibroblasts in pulmonary fibrosis (Hinz et al. 2007). As in all of these studies, our experiments have been carried out without the simultaneous inhibition of TGF- β signaling, but the possible cooperation with endogenous autocrine/paracrine released TGF- β or other profibrotic growth factors, cannot be ruled out.

Taken together our data show for the first time that TLR9 is expressed in lung samples and pulmonary fibroblasts from IIP patients and that its expression is enhanced by a Th2 lung microenvironment, which is known to be an important factor in maintenance and

progression of the fibrotic response. In addition, the activation of TLR9 by its synthetic agonist CpG-ODN plays a novel role in promoting myofibroblast differentiation of UIP fibroblasts in vitro enhancing the expression of α -SMA positive cells. Thus, our discovery of TLR9 expression on pulmonary fibroblasts can explain how infections might initiate, maintain and cause progression of this chronic fibrotic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the National Heart Lung and Blood Institute P50 HL56402 (CMH, SLK, KRF, FJM, and GBT), for financial support. We also thank Ms. Robin G. Kunkel for her artistic assistance in the preparation of the manuscript, as well as Dr. Judith Connett for her critical reading of the manuscript.

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Fig. 1.

Quantitative TAQMAN polymerase chain reaction analysis of TLR9 gene expression in IIP and normal SLBs. Each patient in this study had biopsies of both upper and lower lung lobes except for normal controls, which had one sample. **a** The data shown are the mean of all the combined upper and lower lobe mRNA values compared to the mean of normal SLBs mRNA values (standardized to GAPDH housekeeping gene). The *error bar* shows the SEM of all the data in the UIP and NSIP patient groups. Analysis of TLR9 gene expression in upper and lower lobe surgical lung biopsies from patients with UIP ($n = 12$) (**b**) and NSIP (n

= 7) (C). The *horizontal lines* represent the means and each positive biopsy is represented as single dot. Q test for rejection of outliers eliminated one value in each group in **b** and **c**. $P > 0.05$ compared with normal margin tumor patient group. *TLR9* toll-like receptor 9, *NSIP* non-specific interstitial pneumonia, *UIP* usual interstitial pneumonia

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Fig. 2. Representative immunohistochemical analysis of TLR9 in SLBs from (a, b) UIP, (c, d) NSIP, and (e, f) normal margin tumor patient groups. *Panels (a), (c), and (e)* show control staining. TLR9 immunoreactivity (*red staining*) was seen in the interstitial fibrotic areas in SLBs from (b) UIP, and (d) NSIP, but not (f) normal margins. Original magnification, $\times 200$. *TLR9* toll like receptor 9, *NSIP* non-specific interstitial pneumonia, *SLB* surgical lung biopsy, *UIP* usual interstitial pneumonia

Fig. 3. Intense interstitial immunostaining for TLR9 expression of SLBs from two different patients (**b** and **d**) who died from acute exacerbation of idiopathic pulmonary fibrosis. *Panels (a)* and (**c**) show control staining for IgG1 antibody. Original magnification, $\times 200$

Fig. 4. Representative immunocytochemical analysis of TLR9 expression in primary UIP fibroblasts. Cells were treated for 24 h with DMEM plus 15% FBS alone (**a, b**), and containing 10 ng/ml of IL-4 (**c, d**), IL-13 (**e, f**), or IL-4 and IL-13 (**g, h**). *Panels (a), (c), (e), and (g) show control staining for IgG1 antibody.* Original magnification, $\times 200$. *UIP* usual interstitial pneumonia, *IL-4* interleukin 4, *IL-13* interleukin 13

Fig. 5. Representative immunocytochemical analysis of TLR9 expression in primary NSIP fibroblasts. Cells were treated for 24 h with DMEM plus 15% FBS alone (**a, b**), and containing 10 ng/ml of IL-4 (**c, d**), IL-13 (**e, f**), or IL-4 and IL-13 (**g, h**). *Panels (a), (c), (e), and (g) show control staining for IgG1 antibody. Original magnification, $\times 200$. NSIP* non-specific interstitial pneumonia, *IL-4* interleukin 4, *IL-13* interleukin 13

Fig. 6. Representative immunocytochemical analysis of TLR9 expression in fibroblasts grown from normal margins of resected lung tumors. Cells were treated for 24 h with DMEM plus 15% FBS alone (**a**, **b**), or containing 10 ng/ml of IL-4 (**c**, **d**), IL-13 (**e**, **f**), or IL-4 and IL-13 (**g**, **h**). *Panels (a), (c), (e), and (g) show control staining for IgG1 antibody.* Original magnification, $\times 200$. *IL-4* interleukin 4, *IL-13* interleukin 13

Fig. 7.

Quantitative TAQMAN polymerase chain reaction analysis of TLR9 gene expression in UIP (a), NSIP (b) and normal (c) fibroblast cell lines treated for 24 h with 10 ng/ml of IL-4, IL-13 or IL-4 and IL-13. Fold increase is calculated within each group of disease compared with the respective untreated fibroblasts. Data are mean $\pm$ SEM. *TLR9* toll-like receptor 9, *NSIP* non-specific interstitial pneumonia, *UIP* usual interstitial pneumonia

Fig. 8.

Immunofluorescence staining demonstrating that TLR9 agonist CpG-ODN promotes the differentiation of primary UIP/IPF fibroblasts to myofibroblasts. Cultures of primary human UIP fibroblasts in glass chamber slides were treated for 24 h without (**a** and **c**) or with CpG-ODN (10 $\mu\text{g}/\text{ml}$) (**b** and **d**) in presence (**c** and **d**) or absence (**a** and **b**) of IL-4 (10 ng/ml) then stained for alpha smooth muscle actin (*green*) and actin-rich stress fibers (phalloidin) (*red*). Myofibroblast differentiation is characterized by α -SMA positive stress fibers and appears *yellow*. DAPI (*blue*) was used as a nuclear counter stain. Original magnifications, $\times 400$. The *panels* shown are representative of three independent experiments

Fig. 9.

Cultures of primary fibroblasts grown from normal margins of tumor SLBs in glass chamber slides were treated for 24 h without (**a** and **c**) or with CpG-ODN (10 μg/ml) (**b** and **d**) in presence (**c** and **d**) or absence (**a** and **b**) of IL-4 (10 ng/ml) then stained for alpha smooth muscle actin (*green*) and actin-rich stress fibers (phalloidin) (*red*). DAPI (*blue*) was used as a nuclear counter stain. Double immunofluorescence staining show unmodified level of α -SMA expression after CpG-ODN or IL-4 treatment. Original magnifications, $\times 400$. The *panels* shown are representative of three independent experiments

Table 1

Percentage of IIP biopsies found positive for TLR9 gene expression with real-time TAQMAN analysis

SLBs	Upper lobe	Lower lobe
UIP (<i>n</i> = 12)	7 (58.3%)	9 (75%)
NSIP (<i>n</i> = 7)	6 (85.7%)	5 (71.4%)

N = total number of patients biopsied in both upper and lower lung lobes. The number (and percentages) shown in the columns represent the samples that were positive for TLR9 gene expression