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INTERSTITIAL LUNG DISEASE

Fcγ Receptor IIIb (CD16b) Polymorphisms are Associated with Susceptibility to Idiopathic Pulmonary Fibrosis

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Abstract An excess of neutrophils in the alveoli and lung interstitium has been described in idiopathic pulmonary fibrosis (IPF). Engagement of neutrophil Fcγ receptors with IgG complexes may contribute to the pathogenesis of IPF. The neutrophil FcγRIIIb receptor occurs in two codominantly expressed allelic variants, NA1 and NA2, which exhibit different binding affinities for IgG1 and IgG3 subclasses. The aim of this study was to investigate whether FcγRIIIb genotype is associated with IPF susceptibility or disease progression. In a case-control study we compared the distribution of FcγRIIIb NA1/2 polymorphisms in 142 patients with IPF and in 218 controls using allele-specific PCR amplification. Significant skewing in the distribution of FcγRIIIb genotypes was observed between patients with IPF and control subjects. In the IPF cohort, there was higher frequency of the NA1/NA1 genotype (0.19 vs. 0.07), and lower NA2/NA2 frequency (0.31 vs. 0.50; $\chi^2 = 17.71$, $df = 2, P < 0.001$). The overall frequency of the NA1 allele was increased in IPF patients compared to controls (0.44 vs. 0.29; $P < 0.0001$, odds ratio [OR] = 1.93, 95% confidence interval [CI] = 1.42–2.64). Heterozygotes and homozygotes of the NA1 allele were at higher risk of developing IPF (OR = 2.19, 95% CI = 1.40–3.41, $P = 0.0005$), whereas the NA2 allele was protective against IPF (OR = 0.34, 95% CI = 0.17–0.65, $P = 0.0014$). There was no association of FcγRIIIb genotype with disease progression as assessed by serial lung function measurements. FcγRIIIb NA1/2 polymorphisms are associated with IPF disease susceptibility. These results support a role for immunological mechanisms contributing to IPF pathogenesis.

Keywords Fc receptors · Idiopathic pulmonary fibrosis · Interstitial lung disease · Neutrophils

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease of unknown etiology that carries a poor prognosis and for which there is no effective treatment. Histologically, IPF is characterized by areas of fibrosis of various ages interspersed with normal lung [1]. This pattern of disease could be explained by repeated episodes of lung injury separated in time and place followed by an abnormal wound-healing response characterized by excessive fibrosis.

Although in IPF the nature of the initiating lung injury is unknown, several lines of evidence support a role for

immune complex-mediated lung injury: (1) lung fibrosis indistinguishable from IPF occurs in patients with autoimmune rheumatic diseases such as rheumatoid arthritis; (2) end-stage lung fibrosis may ensue in chronic hypersensitivity pneumonitis, the archetypal immune complex-mediated lung disease; (3) pulmonary fibrosis can be stimulated by immune complex-mediated lung injury in animal models [2, 3]; and (4) immune complexes have been detected in the serum and the lung of patients with IPF [4–12].

Neutrophil infiltration to the alveolar spaces is a characteristic feature of early IPF [13, 14] and there is an excess of neutrophils in the bronchoalveolar (BAL) fluid from IPF patients [15, 16]. Neutrophils bind immune complexes via the low-affinity IgG receptors FcγRIIa and FcγRIIIb [17], engagement of which triggers proinflammatory cascades, cell activation, and the production and release of histotoxic compounds [18, 19]. Since neutrophils and immune complexes have been linked to the development of IPF and FcγRIIIb is exclusively expressed by neutrophils [20], we hypothesized that genetic variation within the *FCGR3B* gene would be a genetic determinant for disease susceptibility. FcγRIIIb occurs in two codominantly expressed allelic variants with differential affinity for certain IgG subclasses [21]. In particular, FcγRIIIb is characterized by the presence of the NA1/NA2 polymorphic variants that comprise one synonymous and four nonsynonymous mutations, which affect N-linked glycosylation of the receptor and consequently the binding affinity for certain IgG subclasses (IgG1 and IgG3). The NA1 allele exhibits higher affinity for IgG1 and IgG3, and neutrophils from NA1 homozygous donors have been demonstrated to have increased capacity for phagocytosis of IgG1- and IgG3-opsonized particles [22, 23]. We have therefore investigated whether FcγRIIIb polymorphisms are associated with the pathogenesis and progression of IPF.

Materials and Methods

Subjects

IPF ($n = 142$) was diagnosed in patients attending a clinic specializing in interstitial lung disease according to the ATS/ERS International Multidisciplinary Consensus Classification [16]. Surgical lung biopsy and/or BAL were performed in cases for which a confident diagnosis based on clinical, functional, and radiological grounds was not possible. A consensus diagnosis was made in each case following joint review by two respiratory clinicians and a radiologist (and a pathologist for cases in which biopsy was performed). The control group ($n = 218$) comprised healthy blood donors and age-matched patients with lung pathologies other than an interstitial lung disease. All the study subjects were ethnically matched (British) Caucasians and provided informed consent. Ethical approval was obtained from Lothian Research Ethics Committee (LREC/2002/4/65).

Pulmonary function tests at baseline and at 6 and 12 months (±1 month) following diagnosis were recorded to assess disease progression in 121 patients with IPF. The remaining 21 patients were lost to follow-up or were unfit to perform further testing.

Allele-specific PCR Amplification

Genomic DNA was extracted from peripheral venous blood using a QIAamp DNA Blood Midi Kit (Qiagen, Crawley, West Sussex, UK). FcγRIIIb NA1/2 polymorphisms were determined in 50-µl PCR reactions using allele-specific PCR primer pairs as previously described [24]. The efficiency and specificity of the allele-specific PCR genotyping was validated by direct sequencing using Applied Biosystems (Birchwood, Warrington, UK) Big-Dye 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer (College of Life Sciences, University of Dundee, UK).

Flow Cytometry

Neutrophil granulocytes were isolated by dextran sedimentation and discontinuous Percoll gradient centrifugation [17] from citrated peripheral venous blood drawn from subjects previously typed as NA1/NA1, NA1/NA2, and NA2/NA2. Neutrophils were immunolabelled, as previously described [25], using either allotype-specific mouse monoclonal antibodies (10 µg ml^{-1}) against human FcγRIIIb (NA1: CLB-gran/11, mouse IgG2a; NA2: GRM-1, mouse IgG2a) [21, 26] or isotype negative control monoclonal antibody (UPC-10, mouse IgG2a; Sigma, Poole, Dorset, UK), followed by Alexa Fluor 488-conjugated goat anti-mouse F(ab′)$_2$ (Invitrogen, Renfrew, Paisley, UK). Surface expression of FcγRIIIb (NA1 or NA2) was assessed by flow cytometry using a BD FACScan flow cytometer (BD Biosciences, Oxford, UK) and data were analyzed using BD CellQuest (BD Biosciences) or FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical Analysis

Hardy–Weinberg equilibrium was assessed by a χ^2 test with one degree of freedom. Differences in the genotype and allele frequencies between control and IPF patients were analyzed by the χ^2 test or Fisher's exact test. One-way analysis of variance (ANOVA) was used to test for differences in the mean values of quantitative variables. Unless otherwise stated, quantitative data are presented as

mean ± SD and $P < 0.05$ was considered to be statistically significant. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

IPF is Associated with Increased Frequency of the NA1 Allele of FcγRIIIb

A total of 142 IPF patients were diagnosed. The baseline characteristics of the IPF cohort are summarized in Table 1. Significant skewing in the distribution of the three FcγRIIIb genotypes (NA1/1, NA1/2, NA2/2) was observed between IPF patients and control subjects (Table 2). In the IPF cohort there was higher frequency of the NA1/NA1 genotype (0.19 vs. 0.07) and lower NA2/NA2 genotype frequency (0.31 vs. 0.50; $\chi^2 = 17.71$, df = 2, $P < 0.001$). Similarly, the overall frequency of the NA1 allele was increased in IPF patients compared to controls (0.44 vs. 0.29; odds ratio [OR] = 1.93; 95% confidence interval [CI] = 1.42–2.64, $P < 0.0001$). Heterozygotes and homozygotes of the NA1 allele (NA1/NA1 + NA1/NA2) were associated with higher risk of IPF (OR = 2.19; 95% CI = 1.40–3.41, $P = 0.0005$), while the presence of the NA2 allele had a protective effect against IPF (OR = 0.34; 95% CI = 0.17–0.65, $P = 0.0014$). Risk analysis of the NA1 allele revealed an additive association of this allele with disease susceptibility, with NA1 homozygotes being at higher risk than NA1/2 heterozygous ones (NA1/1 vs. NA2/2 OR = 4.14; 95% CI = 2.03–8.43, $P = 0.0001$; NA1/2 vs. NA2/2 OR = 2.23; 95% CI = 1.12–4.45, $P = 0.0257$).

There was agreement between genotypes observed and those predicted by the Hardy–Weinberg equilibrium in the control population ($\chi^2 = 0.53$, $P = 0.47$) and in the IPF group ($\chi^2 = 0.03$, $P = 0.86$). The specificity of PCR-based genotyping was validated by direct sequencing and by immunolabelling of isolated neutrophil granulocytes using monoclonal antibodies that specifically recognize the epitopes of the NA1 and NA2 alleles (Fig. 1) [21, 26].

Table 1 Characteristics and baseline pulmonary function of IPF patients

Patients (*n*)	142	
Age (years) (range)	70 ± 8.8 (50–87)	
Gender (F/M) (%)	47/95 (33.1/66.9)	
	Absolute value	% Predicted
FEV_1 (L)	2.16 ± 0.6	87.52 ± 20.0
FVC (L)	2.76 ± 0.8	87.65 ± 19.6
FEV_1/FVC (% predicted)		79.07 ± 9.9
TLC (L)	4.30 ± 1.0	74.20 ± 15.3
D_{LCO} (ml/min/mmHg)	4.11 ± 1.4	52.75 ± 15.9
K_{CO} (ml/min/mmHg/L)	1.10 ± 0.3	82.47 ± 22.8

Values are mean ± SD

IPF idiopathic pulmonary fibrosis, *F* female, *M* male, FEV_1 forced expiratory volume in 1 sec, *FVC* forced vital capacity, *TLC* total lung capacity, D_{LCO} diffusing capacity of the lung for carbon monoxide, K_{CO} D_{LCO} corrected for lung volume

Table 2 Genotype and allele frequencies in control and IPF patients

	Control ($n = 218$)	IPF ($n = 142$)
NA1/NA1	16 (7)	27 (19)
NA1/NA2	94 (43)	71 (50)
NA2/NA2	108 (50)	44 (31)
	$\chi^2 = 17.71$, df = 2, $P < 0.001$	
NA1	126 (29)	125 (44)
NA2	310 (71)	159 (56)
	$P < 0.0001$, OR = 1.93, 95% CI = 1.4–2.6	

Values are *n* (%)

IPF idiopathic pulmonary fibrosis

NA1/2 Polymorphisms have No Effect on IPF Disease Progression

We next determined whether FcγRIIIb NA1/2 polymorphisms are also associated with IPF progression. Serial pulmonary function measurements were collected during a follow-up period of 12 months in order to assess disease progression in 121 patients with IPF. In IPF, a drop of 10% or more in forced vital capacity (FVC) or 15% or more in diffusing capacity for carbon monoxide (D_{LCO}, marker for gas exchange capacity of the lung) from baseline in the first 12 months is generally associated with poor prognosis and a more progressive disease phenotype. Therefore, patients were categorized as either rapid progressors ($n = 49$) or slow progressors ($n = 72$) based on whether they displayed a fall from baseline of 10% or more in FVC or 15% or more in D_{LCO} in 12 months [15, 27–29]. Baseline measurements were similar between rapid and slow progressors (data not shown). Both rapid and slow progressor groups displayed similar genotype frequencies and no significant skewing in the genotype distribution was noted between the groups ($\chi^2 = 0.32$, df = 2, $P = 0.85$) (rapid progressors: NA1/1: 0.18, NA1/2: 0.49, NA2/2: 0.33; slow progressors: NA1/1: 0.17, NA1/2: 0.54, NA2/2: 0.29). Similarly, the percentage change in FVC and D_{LCO} in 12 months was similar for the three FcγRIIIb genotypes (Fig. 2), thereby excluding a role of FcγRIIIb NA1/2 polymorphisms in disease progression and aggressiveness.

Discussion

In this study we aimed to determine the association of FcγRIIIb NA1/2 polymorphisms with IPF disease

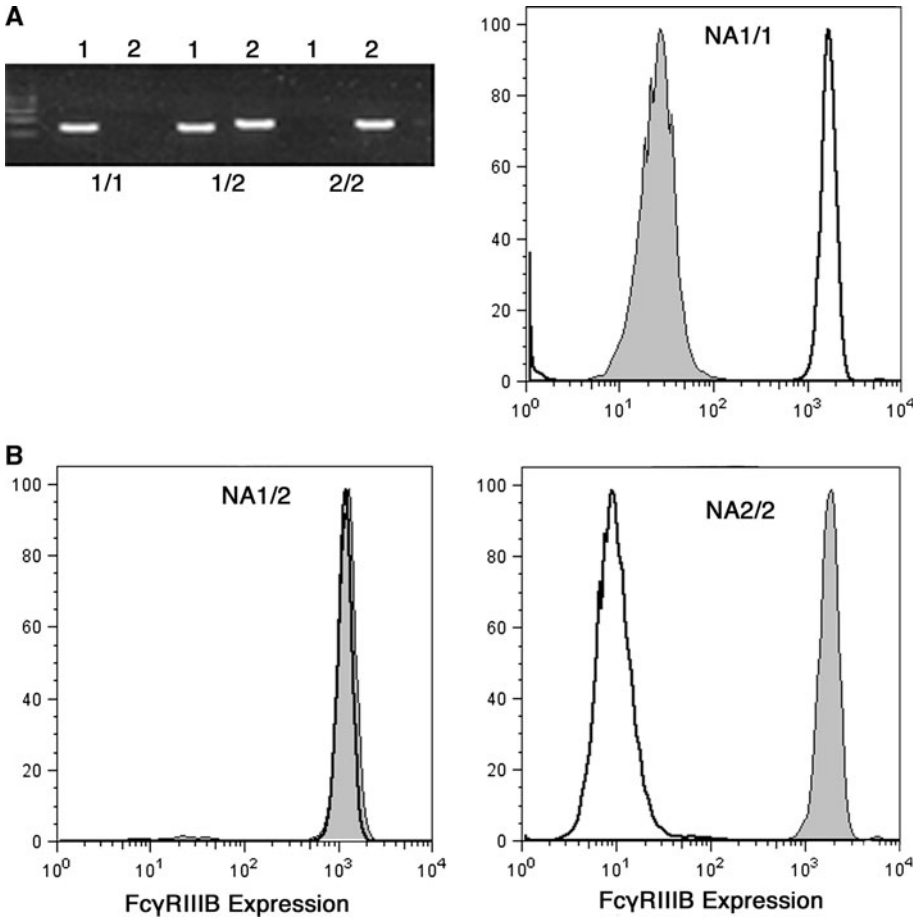

Fig. 1 Determination of FcγRIIIb NA1/2 genotypes. Genomic DNA was extracted from peripheral blood obtained from the participating subjects and the FcγRIIIb NA1/2 genotypes were determined by PCR amplification using allele-specific primer pairs, as previously described [24]. **a** Representative PCR amplification products from NA1/NA1, NA1/NA2, and NA2/NA2 donors, using NA1 [1]- or NA2 [2]-specific primers [24]. The efficiency and specificity of the PCR genotyping were validated using direct sequencing on an Applied Biosystems 3730 automated capillary DNA sequencer (College of Life Sciences, University of Dundee, UK). **b** As an additional control, neutrophil granulocytes were isolated by dextran sedimentation and discontinuous Percoll gradient centrifugation (described in [17]) from peripheral venous blood drawn from subjects previously typed as NA1/NA1, NA1/NA2, and NA2/NA2. Genotypes were confirmed using allotype-specific antibodies [21, 26]. Representative flow cytometry histogram overlays of neutrophils that were immunolabelled with allotype-specific monoclonal antibodies [for NA1: CLB-gran/11 (*thick line*); for NA2: GRM-1(*gray-filled*)]

susceptibility and progression. Given the substantial evidence that supports a clear role for immunological mechanisms, and more precisely for immune complexes, in IPF pathogenesis, we focused particularly on the low-affinity IgG receptor FcγRIIIb. Although a number of single-nucleotide polymorphisms (SNPs) have been described for Fcγ receptor genes, only a small number have a clear, well-established functional significance [20]. Among them, the FcγRIIIb NA1/2 polymorphisms exhibit differential affinity for human IgG subclasses and have been associated with a number of chronic inflammatory and autoimmune disorders (reviewed in [20]). In the present study, increased frequency of NA1 homozygotes along with overrepresentation of the NA1 allele was observed among patients with IPF.

In contrast, no significant association of the FcγRIIIb NA1/NA2 polymorphism with IPF disease progression was evident. Furthermore, since the baseline pulmonary function was similar irrespective of the NA1/NA2 genotype, disease severity at presentation might also not be linked with this polymorphism. Therefore, it could be suggested that FcγRIIIb-mediated interactions play a key role only during the initial stages of the disease pathogenesis, thereby altering disease susceptibility but not severity or progression.

FcγRIIIb is expressed exclusively by neutrophils, so our findings support the pathogenic potential of this leukocyte subset in IPF. Uncontrolled recruitment or activation of neutrophils has been shown to be an important pathogenic mechanism in a variety of inflammatory diseases [30]. Interactions of immune complexes, which have been reported to be present in blood and lung tissue in IPF, with neutrophils through Fcγ-mediated binding can trigger a

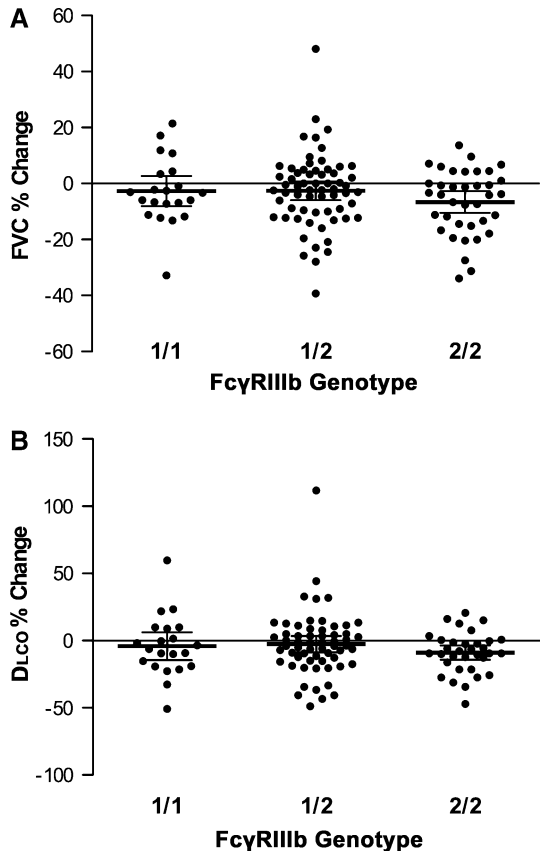

Fig. 2 NA1/2 polymorphisms are not associated with IPF progression. In IPF, a drop of 10% or more in FVC or 15% or more in D_{LCO} from baseline in the first 12 months is generally associated with poor prognosis [15]. Serial measurements of FVC and D_{LCO} were recorded at 12 months following baseline (date of first radiologic evidence for IPF) to determine whether FcγRIIIb NA1/2 polymorphisms are associated with deterioration in lung function, indicative of disease progression. No significant association between the FcγRIIIb genotypes and the percent change in FVC (**a**) and D_{LCO} (**b**) at 12 months following baseline was evident [for FVC: $P = 0.28$, nonsignificant (NS); for D_{LCO}: $P = 0.41$, NS). Results are presented as mean ± 95% confidence interval (CI)

range of effector and immunoregulatory functions, including degranulation, phagocytosis, and cell activation [31]. Such processes consequently lead to the production of histotoxic compounds, including proteolytic enzymes and reactive oxygen and nitrogen intermediates [19]. Ensuing damage to the alveolar walls and pulmonary interstitium could lead to fibroblast activation and aberrant deposition of fibrotic tissue, which characterize IPF.

In addition to the present study, association of the NA1 allele has also been reported for a number of diseases, including vasculitis and periodontitis, which are characterized by extensive tissue damage as a result of chronic and persistent inflammation [20]. The presence of the NA1 allotype on the neutrophil surface is likely to lower the threshold for neutrophil activation by immune complexes containing IgG1 and IgG3, the two most abundant IgG subclasses in serum. Increased neutrophil activation would enhance tissue damage and act as a key determinant for the establishment and progression of the disease.

Over the last decade, studies on the pathogenesis of IPF have suggested a strong genetic component for disease susceptibility and progression. Several reports describe associations of IPF with genes involved in proinflammatory pathways, including cytokines, chemokines, their corresponding receptors, as well as with genes having a role in tissue repair and fibrogenesis, such as TGF-β [32–43]. All these studies, along with the findings presented here, suggest that a number of different genes, mainly with a strong proinflammatory potential, are involved independently in disease pathogenesis. One of the main future challenges will be the identification of the precise mechanisms and pathways that confer disease susceptibility, as their therapeutic modulation could potentially influence disease pathogenesis and progression.

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