Cultured alveolar macrophages from patients with idiopathic pulmonary fibrosis (IPF) show dysregulation of lipopolysaccharide-induced tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) inductions

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ABSTRACT. Regulation of the pulmonary host defence mechanism is crucial for protection of the lung without pathological consequences. This is exemplified in the normal lung by the induction of both the pro-inflammatory cytokine TNF-α, its receptors and the anti-inflammatory cytokine IL-10 by bacterial lipopolysaccharide (LPS). We have evaluated this mechanism in patients with idiopathic pulmonary fibrosis (IPF). Alveolar macrophages (AM) were obtained by bronchoalveolar lavage from 21 subjects with IPF and 12 healthy volunteers. Constitutive and LPS-stimulated AM production of TNF-α, TNF soluble receptors CD120a and CD120b, and IL-10 at the protein and mRNA level were measured by bioassay, ELISA and competitive PCR respectively. AM from IPF subjects were more susceptible to LPS induction of TNF-α protein ($P = 0.03$) and transcription of IL-10 mRNA ($P = 0.01$) and IL-10R1 ($P = 0.01$) expression in comparison to controls. In contrast, increased CD120b was present as protein and mRNA compared to controls ($P = 0.02$). AM from IPF subjects were at least as susceptible to down-regulation of LPS-induced TNF-α levels by exogenous IL-10 as normal controls (94% *versus* 63%). These data suggest that there is dysregulation of LPS-induced TNF-α and IL-10 in AM from IPF subjects. Further studies are required to elucidate these observations, which may, in turn, give additional insight into the pathogenesis of this disease.

Keywords: idiopathic pulmonary fibrosis, tumour necrosis factor alpha, interleukin-10

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disorder characterised by accumulation of inflammatory cells in the airspace and fibrosis of the lung parenchyma. The human alveolar macrophage (AM) plays a central role in this process due to its ability to recruit and regulate other cell types, including neutrophils, eosinophils and fibroblasts, principally *via* an extensive repertoire of cytokines and growth factors [1, 2]. Tumor necrosis factor-alpha (TNF-α), a pleiotropic pro-inflammatory cytokine, has, in several studies, been implicated in the pathogenesis of IPF. *In vitro*, TNF-α enhances adherence of inflammatory cells to the endothelium and their migration into alveolar inflammatory sites and activates the production of other inflammatory mediators by monocytes and neutrophils [3]. It has also been shown to stimulate the growth of fibroblasts and increase collagen deposition [4]. Further evidence for the role of TNF-α in IPF can be found in studies with transgenic animals. Expression of a TNF-α transgene in the murine lung causes spontaneous alveolitis with a progressive fibrotic reaction [5]. Similarly, there is a close association between pulmonary injury and increased pulmonary TNF-α levels in mutant, moth-eaten mice, who naturally develop progressive pulmonary inflammation and fibrosis [6].

The biological activity of TNF-α is mediated by binding to its specific cell-surface receptors, CD120a and CD120b, which can result in both the paracrine and autocrine effects of TNF-α [7]. Antigenic stimulation increases the shedding of these proteins into tissue fluids [8]. Such shed receptors act as physiological inhibitors of TNF-α, but in some circumstances may act as carrier proteins (especially CD120b) and may alter monocyte/T-cell interactions [9-11]. They can also inhibit the development of silica- or bleomycin-induced pulmonary fibrosis in animal models [12, 13]. They are also differentially expressed by fibroblasts from fibrotic lungs [14]. Cytokines considered to have an anti-inflammatory effect, such as IL-10, also have a regulatory role affecting TNF-α production and activity [15, 16]. Antigenic stimulation of monocytes induces IL-10 up-regulation, which reaches a maximal level at 24-48 hours. IL-10 also increases TNF-receptor expression [17]. Furthermore, TNF-α itself has been shown to stimulate IL-10 production [18]. Thus, TNF-α induces at least one molecule that provides negative feedback to its

own production. In transgenic mice that over-express TNF-α in lung, the mRNA level of IL-10 is constantly enhanced [19]. These observations provide insight into the complex homeostatic mechanisms designed to control the inflammatory response.

In the normal human lung, we and others have shown that IL-10 is constitutively produced by AM thereby representing a normal, endogenous feedback factor for control of immune responses and inflammation [20, 21]. We have shown that in other forms of lung disease in which the inflammatory response may be part of the pathogenic process, the relative production of IL-10 or TNF receptors may be disproportionate [21, 22]. We hypothesised that in patients with IPF, the normal homeostatic mechanisms fail to control the TNF-driven inflammatory response. To test this hypothesis we have examined AM from patients with IPF and normal healthy controls for the protein and mRNA expression of TNF-α, IL-10 and their receptors in response to lipopolysaccharide (LPS) stimulation.

PATIENTS AND METHODS

Patients

This study was approved by the Ethics Committee of the North Bristol NHS Trust. Twenty-one patients were studied, all of whom had IPF, based on the international consensus statement [23]. None of the patients had any history of collagen vascular disease, exposure to asbestos or organic dusts. All the patients had been assessed both physiologically and by high resolution computed tomography (HRCT). All subjects had restrictive impairment FVC < 70% predicted and gas transfer (DLCO) less than 70% predicted. The HRCT on these patients fulfilled the radiological criteria. They had the usual interstitial pneumonia pattern of IPF [24, 25], and four had this diagnosis confirmed by open lung biopsy. Bronchoalveolar lavage and transbronchial biopsy findings were compatible with this diagnosis. None of the patients had received corticosteroid treatment. The IPF group consisted of 21 patients (15 male, 6 female) with a mean age of 71 years (range 53-84), 66% were smokers or ex-smokers *(table 1)*. Twelve, normal, healthy people (8 male/4 female) volunteers were used as control subjects. The normal controls had a mean age of 35 years (range 20-63), 58% of whom were smokers or ex-smokers.

Table 1
Subject details

	IPF	Normal Controls
Male / Female	15 M / 6 F	8 M / 4 F
Smoker / Non-smoker	14 Sm / 7 Non	7 Sm / 5 Non
Age (years)	68 (53 – 84)	35 (20 – 63)
Total white cells (x 10^6)	8.5 (1.5 – 20)	11.0 (1.5 – 20)
Macrophages	64.8% (21 – 83%)	92.9% (70 – 99%)
Neutrophils	25.9% (0 – 65%)	2.3% (0 – 7%)
Eosinophils	4.8% (0 – 15%)	1.3% (0 – 4%)
Lymphocytes	4.5% (0 – 25%)	3.5% (0 – 20%)
FVC% pred	56 (28-65)	99 (87-110)
DLCO	46 (32-63)	103 (89-115)

Subject details showing statistical median values with range of values shown in brackets.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed in the right middle lobe, and alveolar macrophage adherence was purified as previously described [21]. AM were cultured for 24 hours, 37 °C, 5% CO_2 in the presence or absence of LPS (*E. coli* 0111, Sigma). The supernatants were harvested and stored at -80 °C. For RNA extraction 1 x 10^6 AM were plated into sterile, 60 mm Petri dishes (Gibco Nunc) and isolated as above. AM from patients with IPF, and normal controls were incubated in 1 mL RPMI with or without 10 μg LPS for 1 hour. One x 10^6 AM were pre-incubated with 10 ng/mL IL-10 (R&D Systems, Abingdon, UK) for 30 minutes prior to stimulation with or without LPS for 1 hour. The time points for protein and mRNA had been previously determined in time course experiments as optimum, to allow us to perform relative measurements in samples from a single patient.

Measurement of TNF-α bioactivity

The WEHI 164 clone 13, mouse fibrosarcoma cell line displays dose-dependent cytotoxicity in response to TNF-α [26]. Confluent cultures were rinsed with sterile PBS, Ca^{++}- and Mg^{++}-free, before addition of trypsin: EDTA solution to detach the cells. After centrifugation at 80 g for 5 minutes, the cells were resuspended in RPMI 1640 medium (Sigma) containing 20% heat-inactivated foetal calf serum (Sigma). Five x 10^4 cells per well were left to adhere for 2 hours at 37 °C in 96-well, tissue culture micro-titre plates (Gibco-Nunc). Following cell attachment, 10 μL of 10 μg/mL actinomycin D (Sigma) was added to each well to arrest cell division. Forty μL of TNF-α standards (a gift from Bayer, UK, range 1 to 500 pg/mL) and samples were added in triplicate to bring the final volume in the well to 100 μL. WEHI cultures were then incubated for 20 hours at 37 °C, 5% CO_2 in a humidified incubator. Following this, 25 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Sigma) were added to each well and incubated for a further 2 hours. MTT dye is metabolised by viable cells to give a purple formazan product. The cells were then lysed with 100 μL of 20% sodium dodecyl sulfate/50% dimethyl formamide, acidified to pH 4.7 with acetic acid. The plates were incubated overnight to dissolve the formazan, before reading at 570 nm on a plate reader (Dynatech MR7000). Sample values were extrapolated from the standard curve using the Biolinx package (Dynatech, Billingshurst, UK). This bioassay has a detection limit of 3 pg/mL for exogenous TNF-α.

TNF receptor ELISA

ELISA plates (Nunc Maxisorp) were coated with 5 μg/mL 5R13 monoclonal antibody for CD120a plates and 5 μg/mL 7R10 monoclonal antibody for CD120b plates (courtesy of Dr Sue Stephens, Celltech, UK) in coating buffer (carbonate, bicarbonate pH 6.5) 100 μL per well, and incubated for 12 hours at 4 °C. These antibodies showed no cross-reactivity with other known cytokines, and their activity was not affected in the presence of human plasma or serum. The plates were then blocked with PBS containing 0.5% BSA for 1 hour at 37 °C. The wells were then washed 3 times on a plate washer with PBS containing

0.1% Tween 20 (Sigma). Samples diluted 1:4 in sample buffer (PBS, 0.1% Tween and 2% FCS) were then added, together with the recombinant CD120a or CD120b, 100 µL per well and the plates incubated for 2 hours at 37 °C. The plates were washed 4 times in wash buffer, and 100 µL of biotinylated TNF-α, 50 ng/mL, were added to each well. The use of biotinylated TNF-α as a detector restricts this ELISA to the detection of free, rather than bound, receptors. The plates were incubated for 1 hour at 37 °C and then washed 4 times with wash buffer. The bound TNF-α was detected with streptavidin peroxidase 1: 400 dilution, incubated for 30 minutes at room temperature, and the plates washed 4 times. TMB substrate (200 µL per well) was added and the plates were left to develop in the dark for 15 minutes. The reaction was stopped with 50 µL of 1M H_2SO_4 and the plates read at 450 nm (reference filter 630 nm) on a platereader. Sample values were calculated from the resultant standard curve. The detection limit was 3 pg/mL for exogenous CD120a and 7 pg/mL for exogenous CD120b.

IL-10 ELISA

Microtitre plates (Nunc maxisorp) were coated overnight at 4 °C with coating antibody (Pharmingen, California, USA). The following day, diluted samples and standards were added after a 2-hour blocking stage. After 2 hours at 37 °C the plates were washed and biotinylated IL-10 antibody JES3-12G8 (Pharmingen) was added for a further 1-hour incubation. The plates were then washed and 1:400 avidin peroxidase was added for a further 30 minutes. One hundred µL of tetramethyl benzidine substrate (TMB) (Biostat diagnostics, UK) were then added to each well. The colour reaction was stopped by addition of 1 M sulphuric acid and the plates read at 450 nm on a plate reader (Dynatech). This assay had a detection limit of 1pg/mL.

RNA extraction

RNA was extracted from AM after culture with various stimuli. Cells were washed in sterile PBS, and cellular RNA extracted using RNABee (AMS Biotechnology, Oxon, UK). This method is based on the acid guanidium-phenol-chloroform (AGPC) method from Chomczynski *et al.,* [27]. Cellular RNA concentration was measured using a GeneQuant II (Pharmacia Biotech, Uppsala, Sweden).

Quantification of cytokine mRNA

To control for variation in both the reverse transcription (RT) and polymerase chain reaction (PCR), a synthetic RNA construct was designed and created. A nucleotide sequence composed of consecutive PCR primer sequences separated by a spacer region was ligated into a pSP64 Poly(A) vector (Promega, Madison, USA). The insert sequence contained primer sequences for the cytokines TNF-α, IL-10 plus their receptors, whilst also containing the primer sequences for 2 housekeeping genes, glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin *(table 2)*. The pSP64 Poly(A) plasmid has a stretch of 30 dA: dT residues inserted between Sac1 and EcoR1 restriction sites. Following linearisation of the vector with EcoR1, synthetic RNA was *in vitro* transcribed with the RiboMax® (Promega, Madison, USA) RNA production system using the vectors built in SP6 promoter. The result is synthetic RNA (sRNA) containing the primer sequences for the cytokines and housekeeping genes with a 3' poly A tail of 30 residues.

Semi-quantitative RT-PCR

Reverse transcription was performed using an oligo dT primer and M-MLV reverse transcriptase on 1 µg of cellular RNA with 10 ng of construct sRNA (Advanced Biotechnologies Ltd, Surrey, UK) in a 20 µl reaction. Using 2 µl of cDNA as a template, PCR was performed with 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds, in which the cDNA from both the patient mRNA and sRNA were co-amplified. Products were electrophoresed through a 1.5% agarose gel and visualised using ethidium bromide staining. mRNA and sRNA bands were distinguished by size, the sRNA band being approximately 100 bp shorter than the mRNA band for all primer sets *(figure 1)*. After being photographed, the Polaroid negatives of the gels were analysed on an imaging densitometer (Biorad, California, USA) and the density of the PCR bands measured. mRNA levels were quantified against the constant amount of the co-amplified synthetic RNA. GAPDH and β-actin were used to correct for mRNA content in the cellular RNA and cytokine mRNA levels compared to this. All RT and PCR reactions were performed in triplicate and values expressed as a median of these reactions. Standard curves for each primer set were prepared to ensure that amplification of both products was linear and parallel.

Table 2
PCR primers

	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	bp	bp
B'Actin	CACCTTCTACAATGAGCTGC	CACGTCACATTCATGATGG	598	400
GAPDH	GCCAAAAGGGTCATCATCTC	GTAGAGGCAGGGATGATGTT	287	200
TNF-α	CTCTGGCCCAGGCAGTCAGA	GGCGTTTGGGAAGGTTGGAT	519	220
IL-10	GACTTGCAAAAGAAGGCATGC	ATCATCTCAGACAAGGCTTGG	277	200
CD120a	CCTACTTGTACAATGACTGTC	TGCATGGCAGGTGCACACG	314	200
CD120b	CATCAGACGTGGTGTGCAAG	GGGTCATGATGACAGTTCA	389	220
IL-10 R1	GTACCACAGCAATGGCTACC	GCAAAGAAGATGATGACGTTGG	418	220
IL-10 R2	TTCTGTCCTGTGGATGACACC	TTCTTGTAAACGCACCACAGC	440	220

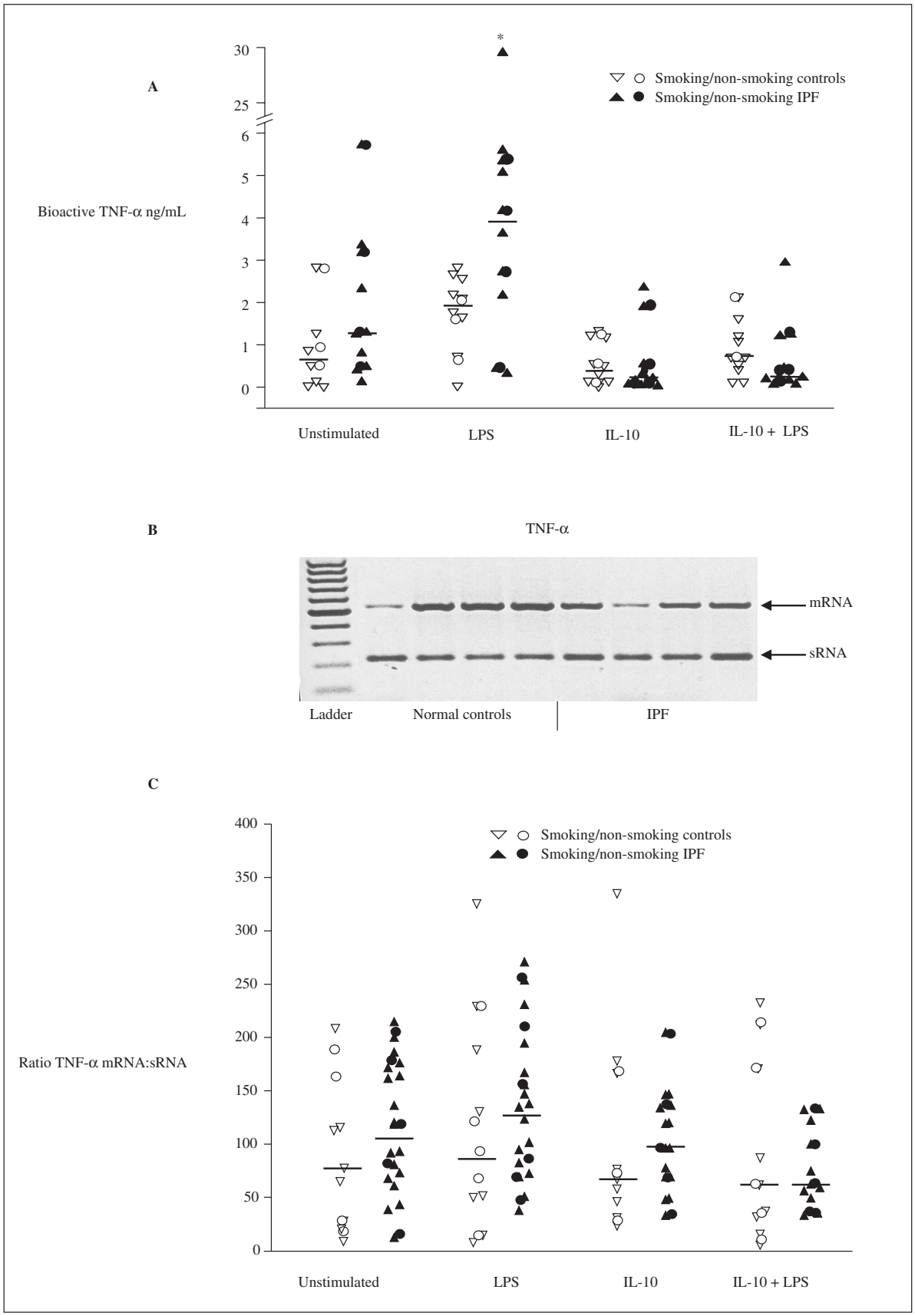

Figure 1

Expression of TNF-α in alveolar macrophages (AM). **A**) Comparison of bioactive TNF-α protein (ng/mL) in culture supernatant from AM in 12 normal controls (*open triangle*) and 21 patients with IPF (*closed triangle*). * $P = 0.03$ normal controls *versus* patients, in AM cultured with 10 μg/mL LPS. Median values are denoted by solid lines. **B**) Representative agarose gel of TNF-α RT-PCR products from normal controls and patients with IPF, showing co-amplified mRNA band and shorter control sRNA band. **C**) TNF-α mRNA expression from AM in 12 normal controls (*open triangle*) and 21 patients with IPF (*closed triangle*). AM were cultured in medium alone (unstimulated) or with addition of 10 μg/mL LPS, 10 ng/mL IL-10 or both IL-10 + LPS.

Statistical analysis

The data presented are shown as median values. The data were not normally distributed as determined by the Ryan Joiner normality test and were compared using the Mann-Whitney analysis on Minitab for Windows, with a p value of <0.05 regarded as significant.

RESULTS

Constitutive and LPS-induced TNF-α protein and mRNA

Measurement of culture supernatants by WEHI bioassay showed that AM derived from subjects with IPF spontaneously produced a median level of 1.265 ng/mL TNF-α, compared to 0.644 ng/mL TNF-α produced by healthy control AM *(figure 1A)*. When stimulated with 10 μg/mL LPS, these levels increased to 3.89 ng/mL and 1.95 ng/mL respectively. The LPS-stimulated values were significantly different (**p = 0.04*, 95% CI = 0.03, 3.59). Production of bioactive TNF-α by AM cultured with 10 ng/mL IL-10 + LPS was lower in the IPF group *versus* the control group (0.217 ng/mL *versus* 0.727 ng/mL respectively). These values represent an inhibition of LPS-induced TNF-α by IL-10 of 93.9% in IPF cultures and 62.8% in the control cultures. Analysis of TNF-α mRNA from cultured AM using semi-quantitative PCR showed no significant difference between the patients with IPF and normal controls *(figures 1B, 1C)*. Expression of TNF-α mRNA was seen in all samples measured with a slight, but non-significant, increase in mRNA level after culture for 1 hour with LPS in both groups.

Constitutive and LPS-induced CD 120a and 120b protein and mRNA

Soluble CD120a receptor was released at low levels by AM cultures from both groups. Spontaneous CD120a levels were 0.05 ng/mL for patients with IPF compared to 0.08 ng/mL for healthy controls, as measured by ELISA. These levels did not change significantly in response to LPS or IL-10 in either group *(figure 2A)*. Increased soluble CD120b was present in unstimulated AM cultures from patients with IPF, 0.167 ng/mL compared to 0.071 ng/mL from healthy controls (**p = 0.02*, 95% CI 0.02, 0.18) *(figure 3A)*. Although LPS stimulation of AM cultures increased CD120b shedding in both groups, there was no longer any significant difference (0.206 ng/mL and 0.106 ng/mL for IPF and controls respectively, *p = 0.2*). IL-10 had no measurable effect on CD120b shedding.

CD120a and CD120b, were both constitutively expressed at the mRNA level in AM from normal controls and patients with IPF *(figure 2B*, *figure 3B)*. However, there was no significant difference between the normal controls and patients, in the relative expression of CD120a mRNA after culture for 1 hour either unstimulated or with LPS *(figure 2C)*. The mRNA expression of CD120b was significantly increased in patients with IPF compared to normal controls in unstimulated AM (**p = 0.02*) *(figure 3C)*. A similar, significant increase in CD120b mRNA expression was seen in patients with IPF compared to normal controls after culture with LPS (†*p = 0.01*) and IL-10 (**p = 0.02*), although the increase compared to normal controls was no greater than that seen in unstimulated cells. An increased CD120b mRNA level was also observed in the AM from patients with IPF after culture with IL-10 + LPS, but the differences were not significant.

Constitutive and LPS-induced IL-10 protein and mRNA

Evaluation of IL-10 protein levels in unstimulated AM cultures showed 0.038 ng/mL and 0.016 ng/mL IL-10 in IPF and controls respectively *(figure 4A)*. These levels increased in response to 10 μg/mL LPS to 0.066 ng/mL and 0.058 ng/mL respectively. Although more IL-10 protein was seen in the AM culture supernatant from patients with IPF, the differences did not reach statistical significance.

Analysis of total RNA extracted from AM of patients with IPF, and normal controls showed that the expression of IL-10 mRNA was significantly altered between the two groups. Constitutive expression of IL-10 mRNA was detected in 67% of unstimulated samples from IPF patients while only 30% of normal control samples showed IL-10 mRNA in the unstimulated samples *(figure 4B)* (**p = 0.02*). Culture of the AM with LPS for 1 hour resulted in an increase in the expression of IL-10 mRNA compared to unstimulated AM in both groups *(figure 4C)*. However, the median expression of IL-10 mRNA after LPS stimulation was over 10 times greater in the patients with IPF compared to normal controls (†*p = 0.01*). Culture of AM from IPF patients with IL-10 also resulted in increased IL-10 mRNA expression compared to normal controls (‡*p = 0.03*), with the biggest differences seen after culture with IL-10 + LPS (†*p = 0.01*).

IL-10 receptor mRNA expression in AMs

The mRNA expression of both IL-10 receptors was also quantified. The ligand binding IL-10R1 (IL-10Rα) mRNA was present in all unstimulated samples analysed *(figure 5A)*. Although the patients with IPF had higher levels than the normal controls, there was no statistically significant difference between groups in either the unstimulated or LPS incubated AM *(figure 5B)*. However, culture of AM with IL-10 showed a significant increase in IL-10R1 mRNA in IPF patients compared to normal controls both with (†*p = 0.02*) and without subsequent LPS challenge (**p < 0.01*). Up-regulation of IL-10R1 expression by IPF AM compared to normal subjects was also shown by immunocytochemistry (data not shown). No significant differences were seen between IPF patients and normal controls in the expression of IL-10R2 (IL-10Rβ, CRF4B) mRNA, although again, constitutive expression of the receptor was seen in all subjects *(figure 6A, 6B)*. In general, higher levels of IL-10R2 mRNA were seen in the AM from IPF patients compared to the normal control, but the differences did not reach statistical significance.

DISCUSSION

The regulation and interaction of LPS-induced TNF-α and IL-10 are complex, and are crucial for the appropriate regulation of the inflammatory response [21, 22, 28]. In

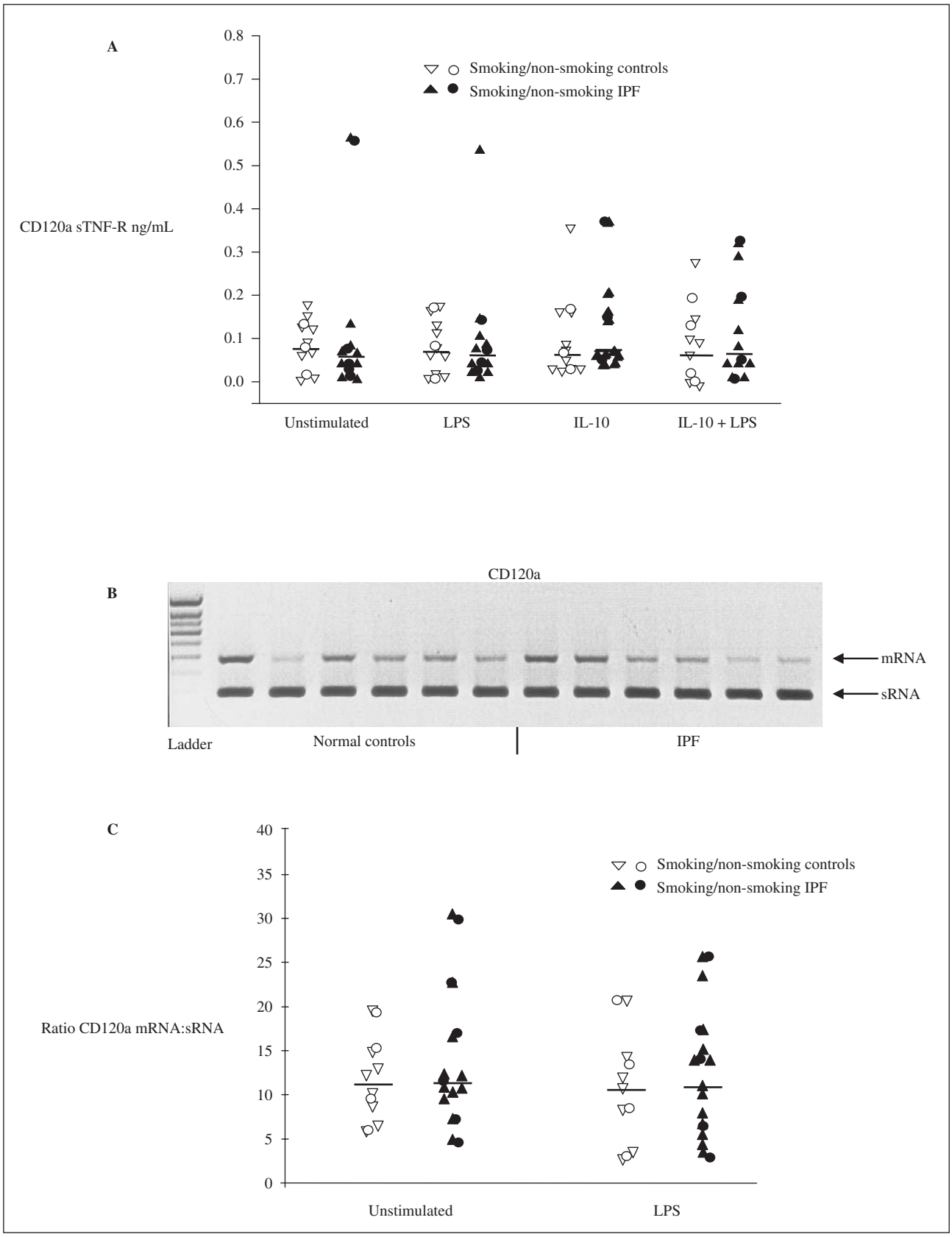

Figure 2

Expression of CD120a in AM. **A**) Soluble CD120a detected by ELISA. AM were cultured in medium alone (unstimulated), with addition of LPS, IL-10 or both IL-10 + LPS. **B**) Representative agarose gel of CD120a RT-PCR products from normal controls and patients with IPF, showing co-amplified mRNA band and shorter control sRNA band. **C**) CD120a mRNA expression from AM in 12 normal controls and 21 patients with IPF. AM were cultured in medium alone (unstimulated) or with LPS.

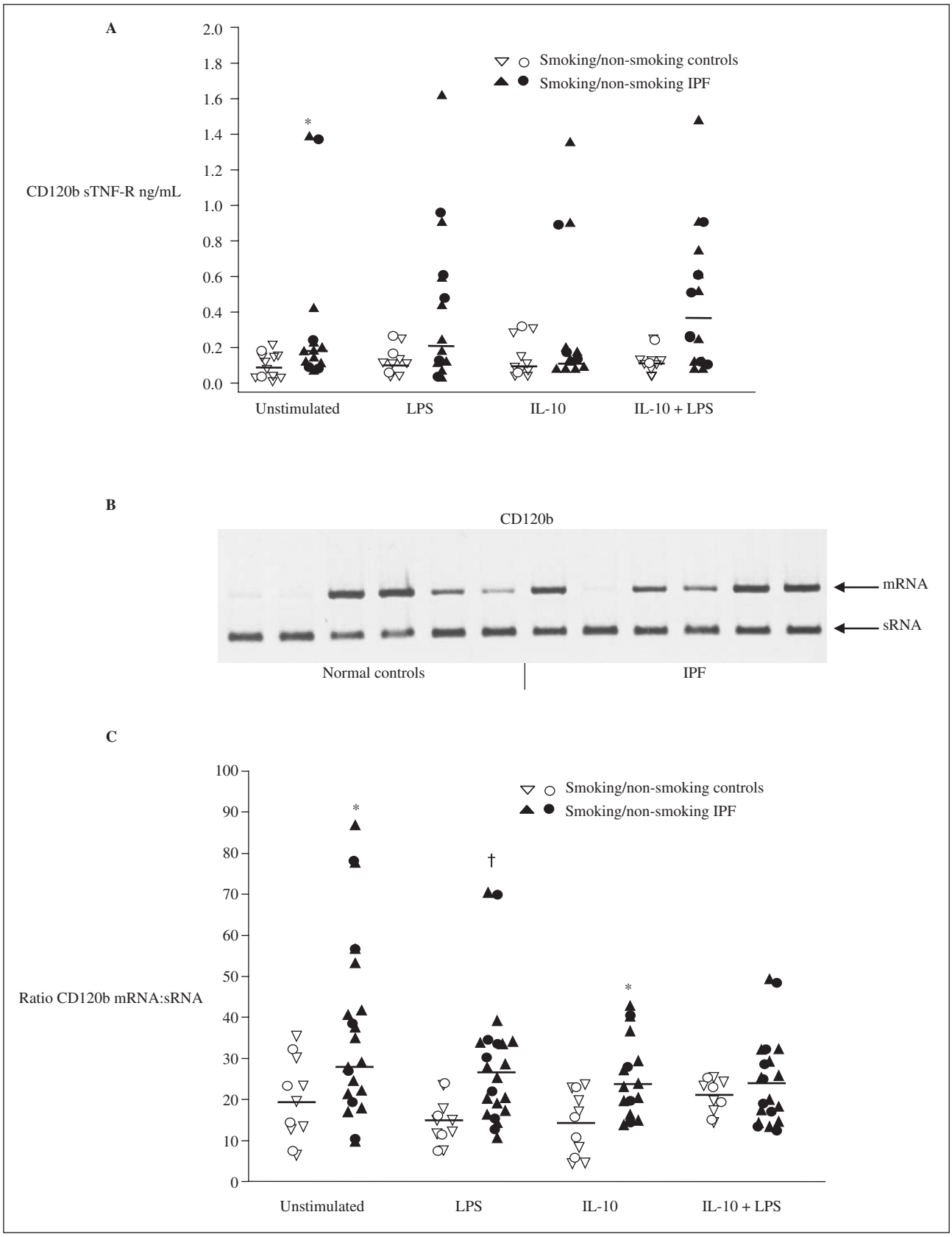

Figure 3

Expression of CD120b in AM. **A**) Soluble CD120b detected by ELISA. **P* = 0.02 normal *versus* IPF. **B**) Representative agarose gel of CD120b RT-PCR products from normal controls and patients with IPF, showing co-amplified mRNA band and shorter control sRNA band. **C**) Comparison of CD120b mRNA expression from AM in 12 normal controls and 21 patients with IPF. **P* = 0.02 normal *versus* IPF unstimulated, †*P* = 0.01 normal *versus* IPF with LPS, **P* = 0.02 normal *versus* IPF with IL-10.

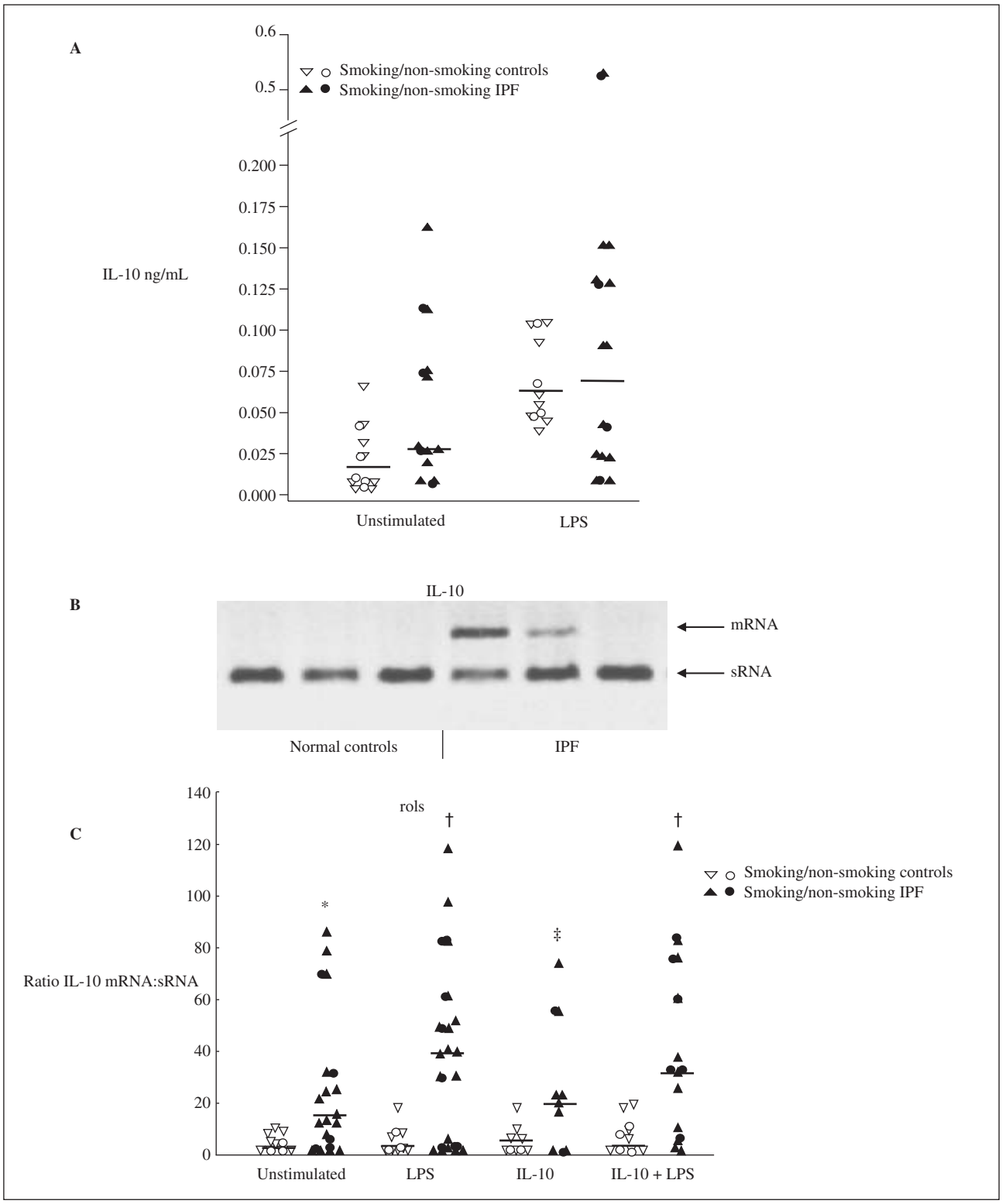

Figure 4

Expression of IL-10 in AM. **A**) IL-10 protein detected by ELISA. Cells were cultured in medium alone (unstimulated) or with addition of LPS (LPS). **B**) Representative agarose gel of IL-10 RT-PCR products from normal controls and patients with IPF, showing co-amplified mRNA band and shorter control sRNA band. **C**) IL-10 mRNA expression from AM in 12 normal controls and 21 patients with IPF. **P* = 0.02 normal *versus* IPF unstimulated, †*P* = 0.01 normal *versus* IPF with LPS, ‡*P* = 0.03 normal *versus* IPF with IL-10 and †*P* = 0.01 normal *versus* IPF with IL-10 + LPS.

this study we investigated these mechanisms in subjects with IPF. We initially confirmed that significantly more LPS-stimulated, bioactive TNF-α was produced by AM from patients with IPF than normal subjects as previously determined by Zhang [29] and Martinez [20]. We found no such increase in TNF-α mRNA, which was in agreement with Martinez but in contrast to the findings of Zhang. This may be due to the use of a different method and time course. It is also possible that other cells in the lung may be sources of IL-10 as suggested by Bonfield *et al.*, looking at

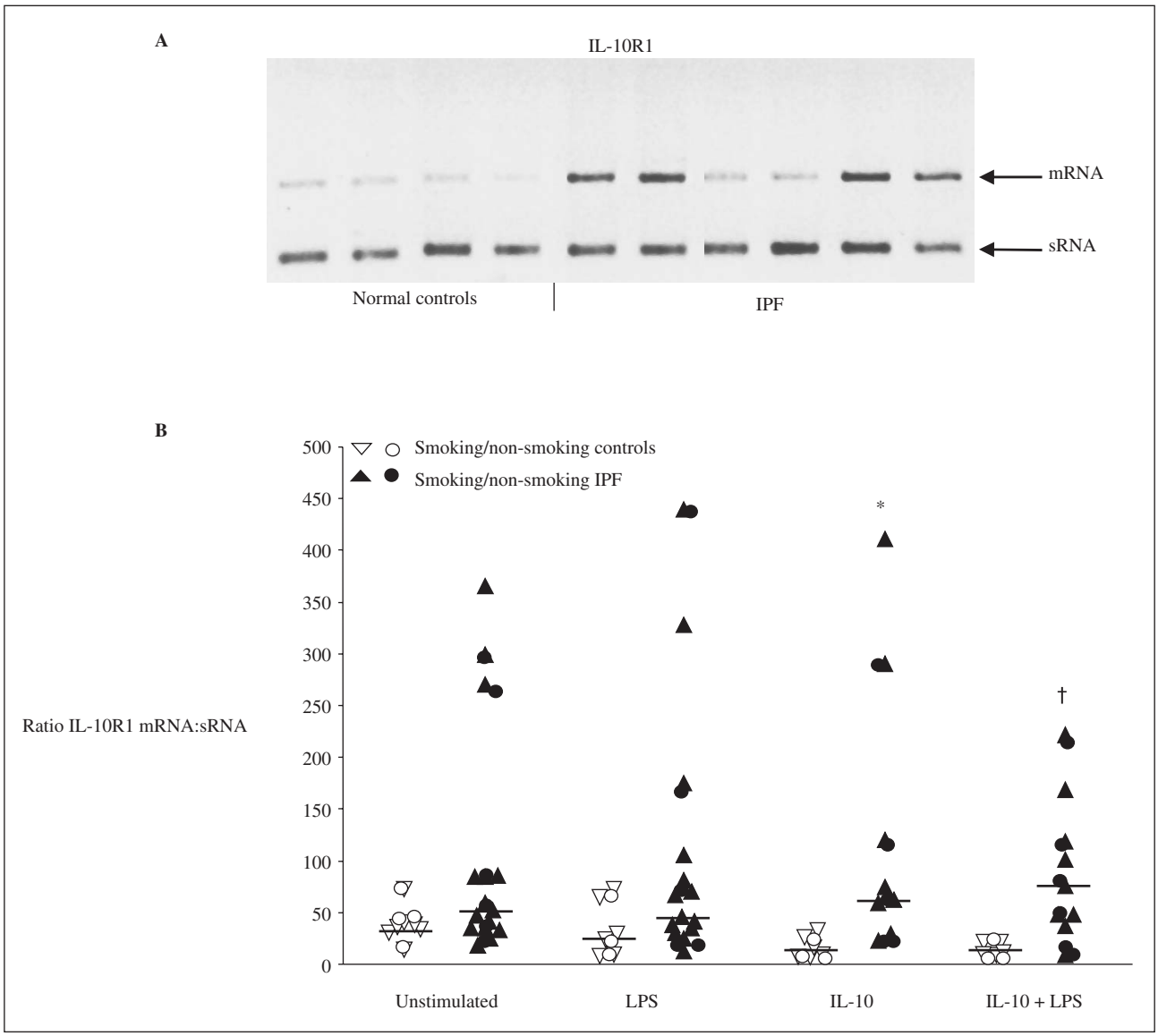

Figure 5

Expression of IL-10R1 mRNA in AM. **A**) Representative agarose gel of IL-10R1 RT-PCR products from normal controls and patients with IPF, showing co-amplified mRNA band and shorter control sRNA band. **B**) IL-10R1 mRNA expression from AM in 12 normal controls and 21 patients with IPF. *$P < 0.01$ normal *versus* IPF with IL-10 and †$P = 0.02$ normal *versus* IPF with IL-10 + LPS.

patients with cystic fibrosis [30]. However, other workers studying both normal and asthmatic subjects suggest that alveolar macrophages are the major source [31]. Our findings and those of Martinez suggest potential changes in mRNA stability or post-transcriptional regulation. In the normal pulmonary macrophage, the initial response following TNF-α upregulation is increased secretion and cleavage of the TNF receptors, CD120a and b [8], followed by upregulation of IL-10 which is involved in a feedback loop to regulate CD120a and b expression. We considered the possibility that the changes observed between the IPF and control subjects were due to age differences, but we had undertaken additional experiments which showed no evidence of age-related changes in the parameters measured (data not shown). There is also the issue that a proportion of both the patients and controls were smokers. This reflects the clinical situation and is represented in the data.

In the current study, we initially investigated the function of the TNF receptors and the effect of IL-10 upon their regulation. No significant difference was seen between the groups as regards the amount of soluble CD120a in the AM culture supernatant, or in the expression of CD120a at the mRNA level. However, more than twice the amount of soluble CD120b was detected in unstimulated AM cultures from patients with IPF (0.167 ng/mL) compared to healthy controls (0.071 ng/mL). This increase in CD120b in IPF was also evident in PCR analysis, which revealed an increased amount of CD120b mRNA in the IPF patients compared to controls under all culture conditions. Although it has been demonstrated that in lower concentration soluble CD120b may act to stabilise the activity of TNF-α and augment some of its effects [9], soluble CD120b has also been shown to compete for TNF-α with cell surface receptors, inhibiting TNF-α action and down-regulating the inflammatory response [32, 33]. This seems the more likely scenario in this case. As both soluble TNF receptors are derived by proteolytic cleavage from membrane bound receptor, the PCR primers used to measure CD120a and CD120b mRNA levels are unable to distin-

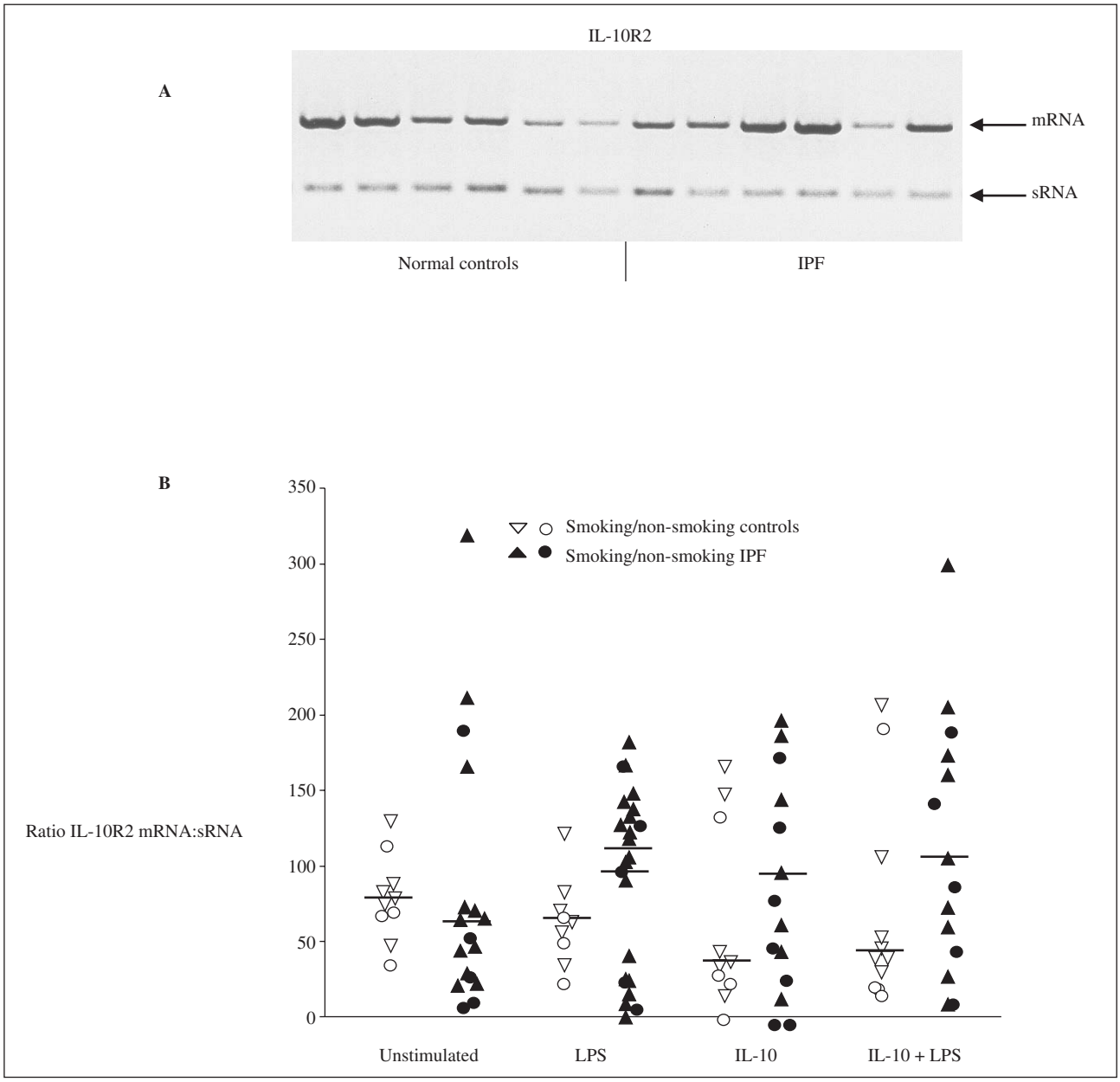

Figure 6

Expression of IL-10R2 (CRF4B) in AM. **A)** Agarose gel of IL-10R2 RT-PCR products from normal controls and patients with IPF, showing co-amplified mRNA band and shorter control sRNA band. **B)** IL-10R2 mRNA expression from AM in 12 normal controls and 21 patients with IPF. AM were cultured in medium alone (unstimulated) or with addition of LPS, IL-10 or both IL-10 + LPS.

guish between soluble and membrane-bound forms. Therefore, it is unclear whether the cell surface expression of either TNF receptor is significantly increased in IPF. IL-10 has been reported to regulate the cleavage of TNF receptors from monocytes and macrophages [34]. In this study, culture of AM with IL-10 alone did not appear to influence shedding of CD120b, but culture of AM with IL-10 and LPS resulted in increased shedding of CD120b in IPF patients compared to both unstimulated IPF AM and normal controls stimulated with IL-10 and LPS. Increased mRNA transcription and increased cleavage due to elevated IL-10 levels are therefore two possible mechanism for the increased soluble CD120b seen in IPF.

In the normal human lung, AM are crucial mediators of the host defence response [35, 36]. Recent AM depletion studies have confirmed the requirement for their dual nature, both activating the inflammatory response when appropriate, whilst preventing an excess response which may interfere with gas exchange [37, 38]. Normal AM constitutively produce low levels of IL-10, and this protein is upregulated by both LPS stimulation and TNF-α itself [21, 22]. The role of IL-10 is predominantly anti-inflammatory; in IPF there are minimal data on the host-defence mechanisms against pathogens, probably due to the potential effects of concomitant therapy [39]. We considered the hypothesis that IL-10 may not be present or effective in subjects with IPF. In contrast, there was a non-significant trend to an increase in both constitutive and LPS-stimulated IL-10 as previously suggested by Bergeron *et al.* [2].

There is also *in vitro* evidence that fibroblasts from IPF subjects may modulate IL-10 activity [14]. We examined the possibility that AM from patients with IPF were insensitive to IL-10. Addition of IL-10 to AM cultures was able to inhibit LPS-induced TNF-α protein production by al-

most 94% compared to 63% inhibition seen in normal subjects. The inhibition of TNF-α protein levels by IL-10 was not reflected at the mRNA level in the cultured AM. Greater inhibition by IL-10 of LPS-induced TNF-α protein production in IPF may be explained by the greater expression of IL-10 receptors on the AM. Immunocytochemical studies, although not quanatitive, suggested increased levels of IL-10 receptors on the AM of patients with IPF (data not shown), while results from PCR analysis showed increased levels of IL-10R1 and IL-10R2 mRNA in the IPF patients compared to the normal controls. The most significant increases occurred after culture of AM with IL-10. This suggested that IL-10 activity *in vivo* may be compromised in IPF.

Analysis of the IPF AM supernatants showed a non-significant increase in IL-10 protein compared to controls, but this did not reflect the greater differences found in IL-10 mRNA expression. These differences were constitutively present but greatly increased after LPS stimulation. Martinez showed increased IL-10 mRNA in IPF AM compared to healthy controls, which is in agreement with the results found in our study and is similar to the situation seen in the lungs of transgenic mice that over-express TNF-α, where the mRNA level of IL-10 was constantly enhanced [20]. However, in the Zhang study, when BAL from patients with IPF and healthy individuals was examined, less IL-10 protein was found in the IPF patients compared to the healthy subjects [28]. Although we showed increased IL-10 protein in the AM supernatant in the patient with IPF compared to controls, the values did not reflect the large differences seen at the mRNA level in these patients. Bergeron also showed increased IL-10mRNA and protein in whole IPF lung tissue, but this was not quantified.

A potential explanation for these observations may be changes in the post-transcriptional regulation of IL-10 expression, which has been observed previously in cultured syno*via*l fibroblasts from subjects with rheumatoid arthritis [40]. It has been suggested that sequence changes in the 3'-untranslated region of IL-10 may be a mechanism by which this occurs [41]. In other culture systems, differences in LPS-induced IL-10 secretion are related to corresponding differences in mRNA production, implicating transcription as the principle mechanism in variable IL-10 production [42, 43]. TNF-α secretion does not relate solely to inter-individual differences in IL-10 production as there are other mechanisms involved in its complex regulation [28, 44].

In patients with IPF, increased bioactive, LPS-induced TNF-α is produced by AM despite an increased production of the natural inhibitors, soluble CD120b and IL-10. AM in patients with IPF are more responsive to exogenous IL-10, which may be due to the increased expression of IL-10 receptors. There is moderately increased expression of IL-10 protein in IPF, but this does not reflect the very significant increases at the mRNA level. These data lead us to suggest that in patients with IPF, the normal homeostatic control of TNF-α activity by IL-10 is ineffectual. There are several potential explanations for these observations, which include inhibition or neutralisation of endogenous IL-10 protein produced by AM in patients with IPF, altered translation of IL-10 mRNA and/or changes in mRNA stability. Further studies are required to elucidate the precise mechanisms involved.

Acknowledgements. *Dr R. Freeburn was funded by the British Lung Foundation and Dr L. Armstrong was funded by Action Research. We would also like to thank Mrs S. Standen for secretarial support.*

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