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ORIGINAL INVESTIGATION

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Major histocompatibility complex and alveolar epithelial apoptosis in idiopathic pulmonary fibrosis

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Abstract Idiopathic pulmonary fibrosis (IPF) is a chronic disease characterized by fibroblast expansion, and tissue remodeling. It is considered a multifactorial disease but the possible involved genes are largely unknown. Interestingly, studies regarding the possible role of major histocompatibility complex (MHC) are scanty and show contradictory results. In this study, we evaluated the polymorphisms of the MHC, locus HLA-B, -DRB1, and -DQB1 in a cohort of 75 IPF patients and 95 controls by using PCR and hybridization with sequence-specific oligonucleotide probes. In addition, we examined the effect of bronchoalveolar lavage (BAL) from IPF patients with different MHC haplotypes on alveolar epithelial growth rate by WST-1 cell viability assay and on epithelial apoptosis by flow cytometry and by cleaved caspase-3 in cell homogenates. Three haplotypes were significantly increased in IPF: (1) HLA-B*15-DRB1*0101-DQB1*0501 (OR = 10.72, CI = 1.43–459.6; pC = 0.011); (2) HLA-B*52-DRB1*1402-DQB1*0301 (OR = 4.42, CI = 1.21–24.1; pC = 0.024); and (3) HLA-B*35-DRB1*0407-DQB1*0302 (OR = 4.73, CI = 1.53–19.5; pC = 0.005). BAL from patients with the later haplotype significantly reduced epithelial growth rate (~30%) and caused epithelial cell apoptosis assayed by cleaved caspase-3 (351.7 ± 16.5 pg/10^6 cells versus 264 ± 24 from controls, and 274 ± 36.8 and 256.5 ± 10.7 from the other haplotypes; $P < 0.05$), and DNA breaks labeling by flow cytometry ($23.7 \pm 6.9\%$ versus $3.1 \pm 0.7\%$ from controls, and $6.5 \pm 0.6\%$ and $7.6 \pm 1.2\%$ from the other two haplotypes; $P < 0.01$). These findings suggest that some MHC polymorphisms confer susceptibility to IPF, which might be related with the induction of epithelial cell apoptosis, a critical process in the development of the disease.

Keywords MHC · HLA system · Pulmonary fibrosis · Epithelial · Apoptosis

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and usually lethal disease characterized by epithelial damage/activation, fibroblastic/myofibroblastic foci formation and aberrant extracellular matrix remodeling (Selman et al. 2001). Susceptibility to IPF is probably determined by both genetic and environmental factors, although to date, the putative gene(s) implicated remain elusive (Du Bois 2002; Pardo and Selman 2002; Thannickal et al. 2004). Several gene polymorphisms have been studied in IPF patients but results have shown weak association or no association at all (Whyte et al. 2000; Pantelidis et al. 2001; Hutyrova et al. 2002; Selman et al. 2003; Zorzetto et al. 2003; Xaubet et al. 2003; Hodgson et al. 2005). Interestingly, studies regarding the possible role of major histocompatibility complex (MHC) in susceptibility are scanty, old, and show contradictory results (Fulmer et al. 1978; Turton et al. 1978; Varpela et al. 1979; Libby et al. 1983). Moreover, these studies were performed before the new morphological classification of the idiopathic interstitial pneumonias that allow a more precise diagnosis of IPF (American Thoracic Society 2002), and with the use of serologic techniques that have been replaced by the more precise polymerase chain reaction (PCR)-based MHC typing.

The MHC maps to the short arm of chromosome 6 and contains the most polymorphic loci in humans, the human leukocyte antigens (HLA) class I and class II genes. HLA class I molecules comprise the classical HLA-A, -B, and -C antigens which are expressed on the surface of most mammalian cells (Rhodes and Trowsdale 1999; McCluskey and Peh 1999; Holling et al. 2004). The HLA class II subtypes HLA-DR, DQ and DP regulate T cell dependent immune responses and are expressed by specific cell types, primarily on antigen processing cells such as dendritic cells and macrophages, although they can be expressed by other cell types, including fibroblasts and epithelial cells. The MHC has as its primary function the presentation of peptides to T-cell receptors and plays an essential role in the immune system (Rhodes and Trowsdale 1999; McCluskey and Peh 1999; Holling et al. 2004).

MHC has been widely studied in human diseases, and it has been associated mostly with degenerative, autoimmune and infectious disorders; and we hypothesized that some haplotypes of the MHC could be associated with IPF.

With this aim we typed the HLA-B, DRB1, and DQB1 locus using polymerase chain reaction and hybridization with sequence-specific oligonucleotide probes. Our results indicate that three haplotypes: HLA-B*15-DRB1*0101-DQB1*0501, HLA-B*52-DRB1*1402-DQB1*0301 and HLA-B*35-DRB1*0407-DQB1*0302 were increased in the IPF group. Then we explored some possible phenotypic differences between these haplotypes. Since alveolar epithelial cell apoptosis is a critical event in the pathogenesis of IPF (Uhal et al. 1998; Barbas-Filho et al. 2001; Maeyama et al. 2001), and a number of genes related to apoptosis are located close to MHC loci (Rebhan et al. 2005), we evaluated programmed cell death induced by BAL fluids of IPF patients with the different haplotypes. Our results show that one of the increased haplotypes is associated with increased epithelial apoptosis in vitro.

Patients and methods

Study population

Seventy-five unrelated IPF patients were included in this study. Diagnosis of IPF (49 males, 26 females, 63 ± 9.5 years) was supported by history, physical examination, pulmonary function studies, HRCT, and bronchoalveolar lavage (BAL) findings (American Thoracic Society 2000). In 41% of the patients diagnosis was confirmed by morphology (open lung biopsy) based on typical microscopic findings of usual interstitial pneumonia (Katzenstein and Myers 1998). In the absence of biopsy, patients had to fulfill the criteria of the ATS/ERS international consensus (American Thoracic Society 2000). Patients with known causes of interstitial lung disease (i.e. collagen vascular disease, drug toxicity, environmental exposure), were excluded.

The control group comprised of 95 unrelated healthy subjects (48 men and 47 women). All patients and control subjects were informed of the purpose of the study and their consent obtained. The protocol was approved by the Ethics Committee of the National Institute of Respiratory Diseases, México. Patients and controls were integrated by individuals with the same ethnic and geographic origin and with a minimum of second generation born in Mexico. To confirm the presence of inferred haplotypes in linkage disequilibrium, a family-based study was additionally made in the IPF group. For this purpose, three or four first degree relatives of patients carrying out these putative haplotypes were examined.

Molecular typing for HLA-B, HLA-DQB1* and HLA-DRB1* loci

Genomic DNA was extracted from 20 ml of peripheral blood using the BD genomic DNA isolation kit (Maxim Biotech, San Francisco CA). Molecular typing was done by PCR sequence-specific primers (PCR-SSP) technique using the Fastype HLA-DNA SSP Typing System (Biosynthesis Inc., Dallas TX), which have been certified by the American Society for Histocompatibility and Immunogenetics (ASHI). Briefly, 47 primer pairs were used to determine the 45 major HLA-B group-specificities (from B7 up to B81) in low resolution modality; For HLA-DQB1* typing, 24 primer pairs were used. DQB1 high-resolution kit was used to achieve DQB1 amplification, which includes 55 alleles for DQ6, DQ3, DQ4, DQ5 and DQ2.

HLA-DRB1 typing was performed by low resolution modality, which includes DRB1, DRB3, DRB4, and DRB5 alleles in 24 independent reactions. For DR4 it covers up to DRB1*0422, for DR11 up to DRB1*1122, for DR13 up to DRB1*1322, for DR14 up to DRB1*1421 and for DR8 up to DRB1*0811. Allelic discrimination was done by DRB1* high resolution, where subtyping for DR3, DR4, DR8, DR11, DR13 and DR14 were performed with a panel of 16 primer pairs for each one. DR1, DR7, DR12, DR15 and DR16 subtyping included a panel of 8 primer pairs in each case. In the study of the three loci a 410 bp genomic fragment of the human G3PDH gene was coamplified as an internal control for amplification: The last well included a DNA-free reaction as a contamination control. The information for primer design was achieved from IMGT/HLA database (Robinson et al. 2000).

All PCR amplifications were performed on 75 ng of genomic DNA in a 10 µl reaction volume containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl2; 60 µM of each dNTP's (Olerup et al. 1993; Zetterquist et al. 1997). Samples were subjected to 20 cycles 94°C 20 s. for denaturing, 61°C 50 s. for annealing, and 72°C 30 s. for extension using an automated thermal cycler (GeneAmp PCR system 9700, PE Applied Biosystems. Foster City CA). An additional hold of 94°C 20 s, 65°C

1 min by 10 cycles, were added to the denaturing step before the first cycle, and an additional 5 min was added to the extension step of the after last cycle. In all cases, amplifications was performed using recombinant Taq DNA polymerase (Gibco BRL, Rockville MD). Amplified PCR-SSP products were then electrophoresed in submarine 2% agarose gels containing 0.2 μg/ml ethidium bromide for 40 min (30 V/cm) and amplified bands were visualized in a dual intensity UV light transilluminator (UVP Inc. Upland, CA), and then analyzed and stored in EDAS290 fotodocumentation system (Kodak, Rochester, NY). The results were interpreted following the instructions of typing sheets from the procedure guide.

Bronchoalveolar lavage

BAL was performed through flexible fiberoptic bronchoscopy under local anesthesia (Pardo et al. 2000). Eight healthy individuals were studied for apoptosis induced by BAL fluids (two current smokers, two former smokers and four that never smoked). All had normal chest X rays and spirometries. Briefly, 300 ml of normal saline were instilled in 50-ml aliquots, with an average recovery of 60–70%. In IPF patients the bronchoscope was wedged in a subsegment of the right or left lower lobe according to the extent of the lesion based on HRCT appearances. Controls were also lavaged in the lower lobes. The recovered BAL fluid was centrifuged at 250 *g* for 10 min at 4°C. The cell pellet was resuspended in 1 ml phosphate buffer saline and an aliquot was used to evaluate the total number of cells. Other aliquots were fixed in carbowax, stained with hematoxylin and eosin and used for differential cell count. Supernatants were kept at −70°C until use.

Growth rate assay

Human lung epithelial cells (A549 cell-line from ATCC, Rockville, MD) were trypsinized, harvested, resuspended in DMEM (Dulbecco's modified eagle's medium, Sigma) supplemented with 10% FBS, (GIBCO, BRL, Grand Island, NY) counted, plated in 96-well culture plates at a concentration of 2.5×10^3 cells/well, and incubated at 37°C in 5% CO_2-95% air. After 24 h, culture medium was replaced by 1% FBS-supplemented medium plus 25% (4:1 v/v) of two-fold concentrated BAL fluids. Cell number was examined at 48 h with the cell proliferation reagent WST-1 (Roche Applies Science, Indianapolis, IN, USA) as previously described (Vazquez de Lara et al. 2000). The medium of corresponding wells was replaced with fresh medium, and the dye solution was added to each well according to the manufacturer's instructions. Absorbance was read on an ELISA plate reader at a wavelength of 450 nm, with a reference wavelength of 620 nm.

Apoptosis assays

Detection of DNA strand breaks by flow cytometry

To detect DNA breaks, 1×10^6 A549 epithelial cells were cultured for 48 h in serum-free F12 HAM medium in the presence of BAL supernatants (25% total volume) from patients with different haplotypes and healthy controls. Cells stimulated 24 h with cisplatin (20 μg/ml; Bristol-Myers, Puerto Rico) were used as positive controls. Cells were resuspended in 5 ml PBS with 1% paraformaldehyde, (ice, 15 min), washed with PBS, and stored in ice-cold 70% ethanol. Then an APO-BRDU kit (PharMingen, San Diego, CA) was used as described by the manufacturer. Briefly, cells were washed twice with Wash Buffer and supernatant was discharged by centrifugation. Freshly prepared DNA labeling solution (containing TdT and Br-dUTP) was added to the cell pellet and incubated for 1 h at 37°C with occasional shaking. Cells were labeled by FITC-conjugated mAbs to BrdU, washed again, and resuspended in staining solution containing PI and RNAse. Cells were incubated for 30 min at room temperature and immediately analyzed using a FACSAria flow cytometer (Becton Dickinson). The percentage of green fluorescent-positive cells with DNA strand breaks was calculated using the FACS Diva V.4.1 software.

Cleaved-caspase 3 protein

Cleaved caspase-3 (Asp175) was analyzed by Sandwich ELISA Kit (Cell Signalling, Technology Beverly, MA) according to the manufacturer's instructions. Alveolar epithelial cells (A549) were stimulated with the BAL fluids from patients with different haplotypes during 6 hours, and levels of cleaved caspase-3 were spectrophometric determined reading absorbance at 450 nm.

Statistical analysis

Differences between groups were evaluated through the Mantel-Haenszel chi-square test with Yates correction, or the Fisher exact test when appropriate, by using the Epi Info v5.0 statistical program (Stone Mountain, GA). Odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. Results of growth rate and apoptosis were expressed as mean ± SD and analyzed by Tukey's and Dunnett's tests. Statistical significance was set at $P < 0.05$.

Results

Baseline characteristics of the IPF patients

Demographic data, pulmonary function tests, and BAL differential cell counts are summarized in Table 1. All

Table 1 Baseline demographic, clinical, physiologic and BAL characteristics of the IPF patients

Variable	IPF ($n=75$)
Age	63.0 ± 9.5
Sex (male/female)	49/26
Duration of symptoms before diagnosis[a]	29.6 ± 23.4
Smoking status (Never)	36
Former	33
Current	6
Clubbing (Yes/No)	59/75
FVC % predicted	66 ± 23
FVC/FEV_1 %	91.6 ± 10.3
PaO_2mmHg[b]	49 ± 10
BAL Macrophages %	81.6 ± 11.6
BAL Lymphocytes %	14.0 ± 11.0
BAL Neutrophils %	4.3 ± 3.5
BAL Eosinophils %	1.0 ± 2.0

BAL differential cell in controls: Macrophages 85–90%; Lymphocytes 10–15%
FVC, forced vital capacity; FEV_1, flow expiratory volume in 1 s
[a] Months
[b] Normal values at Mexico City altitude: 67 ± 3 mmHg

patients exhibited clinical, radiological and functional evidence suggestive of IPF, with variable degrees of dyspnea, decreased lung capacities, and hypoxemia at rest that worsened during exercise. High resolution computed tomography, BAL cell profile exhibiting macrophages predominance with moderate increase of neutrophils and eosinophils, and histopathology in the cases with biopsy, corroborated diagnosis of IPF.

HLA-DRB1-DQB1 allele frequencies and haplotypes in IPF patients and controls

Tables 2 and 3 illustrate the frequencies for HLA-DR and HLA-DQ alleles among IPF patients and control subjects. Regarding HLA-DRB1* a significant increase of the alleles HLA-DRB1*-0407 and −1402 was observed in the IPF group. The allele HLA-DRB1-0802 showed a tendency to increase in the control group, although it did not reach statistical significance (Table 2). Likewise, a significant increase of the allele HLA-DQB1*-0301 was observed in the IPF group (Table 3).

Table 4 shows the HLA-DR/HLA-DQ haplotype frequencies in both groups. Inferred haplotypes were corroborated by family study which included 3-4 first degree relatives. Three haplotypes DRB1*0407-DQB1*0302 (OR = 2.49, IC = 1.28-4.95; $P=0.0057$), DRB1*1402-DQB1*0301 (OR = 5.48, IC = 2.22-15.36; $P=0.00005$), and DRB1*0101-DQB1*0501 (OR = 3.66, IC = 1.31-11.69; $P=0.01$) were significantly associated with increased risk to IPF. The first two haplotypes have been identified in Amerindian populations (Alaez et al. 2001, 2002; Vargas-Alarcon et al. 2003). To further determine the ethnic origin and racial contribution to the different haplotypes, we evaluated the HLA-class I locus B. Results are shown in Table 5. The haplotype DRB1*0101-DQB1*0501 was associated to HLA-B*15, (OR = 10.7, IC = 1.4-459.6; $P=0.011$). Likewise, the haplotype DRB1*1402-DQB1*0301, was associated to allele HLA-B*52, (OR = 4.42, IC = 1.2-24.1; $P=0.024$) and the haplotype HLA DRB1*0407-DQB1*0302 was found mainly associated to HLA-B*35 (OR = 4.73, IC = 1.5-19.5; $P=0.005$). HLA-B*35 has been associated with Hispanic-American populations.

BAL from patients with the HLA-B*35 DRB1*0407-DQB1*0302 haplotype reduced epithelial growth rate

To investigate whether BAL fluids obtained from IPF patients with different haplotypes affect cell growth, alveolar epithelial cells were exposed to twofold concentrated fluids for 48 h. As shown in Fig. 1, BAL fluids obtained from patients carrying the haplotype HLA-B*35 DRB1*0407-DQB1*0302 induced a significant loss of cell density as measured by the WST-1 cell viability

Table 2 Gene frequency of HLA-DRB1* in IPF patients and controls subjects

HLA DRB1*	Patients ($n=75$) (alleles = 150)		Controls ($n=95$) (alleles = 190)		p^C	OR	CI
	n	gf	*n*	gf			
0407	32	0.213	19	0.100	0.0059	2.44	1.27–4.78
1402	26	0.173	8	0.042	0.00013	4.77	2.01–12.55
0101	17	0.113	7	0.036	NS		
0701	16	0.106	21	0.117	NS		
0802	13	0.086	31	0.163	NS	0.49	0.22–1.00
1602	8	0.053	3	0.015	NS		
1101	7	0.046	11	0.057	NS		
1302	7	0.046	4	0.021	NS		
0301	5	0.033	9	0.047	NS		
1301	4	0.026	4	0.021	NS		
1503	4	0.026	3	0.015	NS		
1501	3	0.020	9	0.047	NS		
1201	3	0.020	1	0.005	NS		
1407	1	0.006	7	0.036	NS		
1104	1	0.006	3	0.015	NS		
Others	3	< 0.006	50	< 0.015	NS		

NS not significant; *HLA*, human leukocyte antigen; *gf* genotype frequency; *OR*, odds ratio; *CI*, confidence interval

assay ($32 \pm 5\%$; $P < 0.01$), suggesting that these fluids provoked cell death. BAL fluids from normal subjects induced a modest increase of epithelial cell growth. The comparison of IPF patients with the different haplotypes against controls by Dunnett's test showed that the haplotype HLA-B*15-DRB1*0101-DQB1*0501 also caused a modest but significant decrease of epithelial growth rate ($P < 0.05$).

BAL from patients with the HLA-B*35 DRB1*0407-DQB1*0302 haplotype induced epithelial cell apoptosis

To determine whether programmed cell death was playing a role in the effect of BAL on epithelial cell growth, apoptosis was determined by the laser scanning cytometry APO-BRDU assay, a two-color staining method for labeling DNA strand breaks and cellular DNA content. The percentage of apoptotic cells, was significantly increased after treatment of A549 alveolar epithelial cells with BAL fluids obtained from patients with the HLA-B*35-DRB1*0407-DQB1*0302 haplotype ($23.7 \pm 6.9\%$ versus $3.1 \pm 0.7\%$ from controls, and $6.5 \pm 0.6\%$ and $7.6 \pm 1.2\%$ from the other two increased haplotypes; $P < 0.01$). One representative flow citometry result is illustrated in Fig. 2.

To further examine the apoptotic effect of the BAL fluids, we conducted an assay of cleaved caspase-3. Consistent with the flow cytometric assay, we found that alveolar epithelial cells treated with BAL fluids from HLA-B*35-DRB1*0407-DQB1*0302 haplotype showed a significant increase in caspase-3-like activity (351.7 ± 16.5 versus 264 ± 24 from controls; $P < 0.05$; Fig. 3). BAL fluids obtained from patients with other haplotypes showed no effect and were similar to control BAL (Fig. 3).

Clinical behavior and HLA haplotypes

No differences in pulmonary function tests, radiology, BAL cell profile, and smoking were found between IPF patients with the different MHC haplotypes. We have

Table 3 Gene frequency of HLA-DQB1* in IPF patients and controls subjects

HLA DQB1*	Patients ($n = 75$) (alleles = 150)		Controls ($n = 95$) (alleles = 190)		p^C	OR	CI
	n	gf	*n*	gf			
0301	40	0.266	27	0.153	0.0063	2.20	1.23–3.95
0501	17	0.113	10	0.054	NS		
0302	32	0.213	38	0.225	NS		
0201	21	0.133	30	0.172	NS		
0402	13	0.093	27	0.153	NS		
0602	8	0.053	15	0.082	NS		
0502	8	0.053	5	0.026	NS		
0604	6	0.040	3	0.015	NS		
0603	2	0.013	3	0.015	NS		
0503	1	0.006	8	0.043	NS		
Others	2	0.006	24	< 0.015	NS		

NS not significant; *HLA*, human leukocyte antigen; *gf* genotype frequency; *OR*, odds ratio; *CI*, confidence interval

Table 4 Haplotype frequency of HLA-DRB1*-DQB1* in IPF patients and control subjects

Haplotype HLADRB1*-DQB1*	Patients ($n = 75$) (haplotypes = 150)		Controls ($n = 95$) (haplotypes = 190)		p^C	OR	CI
	n	hf	*n*	hf			
0407–0302	31	0.234	18	0.099	0.0057	2.49	1.28–4.95
1402–0301	26	0.191	7	0.037	0.00005	5.48	2.22–15.36
0101–0501	16	0.113	6	0.032	0.01	3.66	1.31–11.69
0701–0201	16	0.113	19	0.105	NS		
0802–0402	13	0.090	25	0.141	NS		
1101–0301	8	0.054	10	0.054	NS		
1302–0604	6	0.040	3	0.015	NS		
0301–0201	5	0.033	6	0.032	NS		
1602–0502	5	0.033	2	0.010	NS		
1501–0602	3	0.020	9	0.048	NS		
1201–0301	3	0.020	1	0.005	NS		
1301–0502	3	0.020	1	0.005	NS		
1503–0602	3	0.020	1	0.005	NS		
1407–0503	1	0.006	7	0.037	NS		
Others	11	< 0.012	75	< 0.025	NS		

NS not significant; *HLA*, human leukocyte antigen; *hf*, haplotype frequency; *OR*, odds ratio; *CI*, confidence interval

Table 5 Haplotype frequency of HLA-B*-DRB1*-DQB1* in IPF patients and control subjects

Haplotype HLA B*-DRB1*-DQB1*	Patients ($n = 75$) (haplotypes = 150)		Controls ($n = 57$) (haplotypes = 114)		p^C	OR	IC
	n	hf	*n*	hf			
35-0407-0302	22	0.146	4	0.035	0.005	4.73	1.53–19.53
52-1402-0301	16	0.106	3	0.026	0.024	4.42	1.21–24.15
15-0101-0501	13	0.086	1	0.008	0.011	10.72	1.43–459.63

HLA, human leukocyte antigen; *hf*, haplotype frequency; *OR*, odds ratio; *CI*, confidence interval

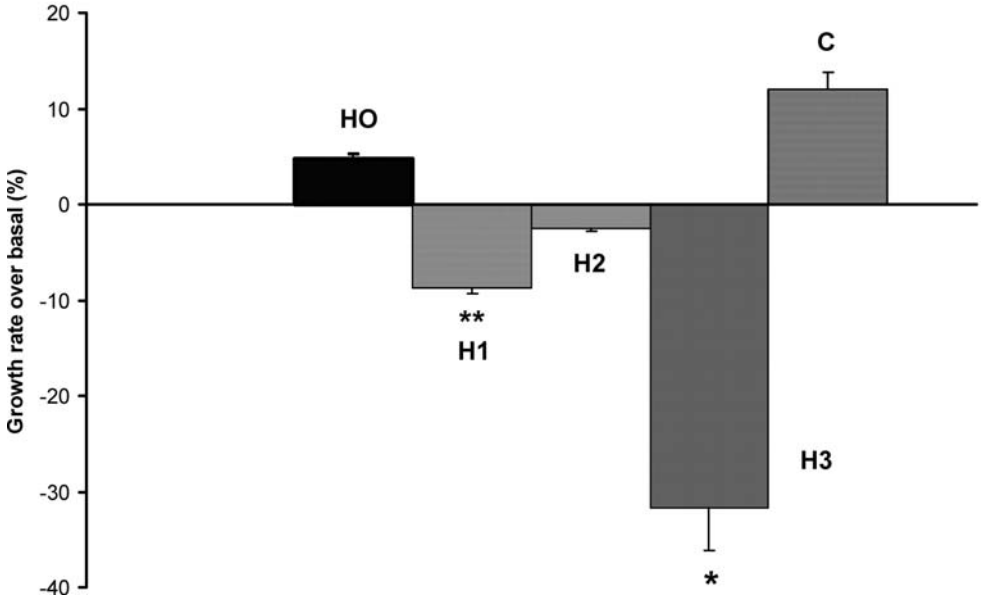

Fig. 1 Effect of BAL fluids on epithelial cell growth rate. A549 epithelial cells were exposed during 48 h to BAL supernatants of patients with the different HLA haplotypes: HO = IPF patients with other haplotypes; *H1*, Patients with the haplotype HLA-B*15-DRB1*0101-DQB1*0501; *H2*, Patients with the haplotype HLA-B*52-DRB1*1402-DQB1*0301; *H3*, Patients with the haplotype HLA-B*35-DRB1*0407-DQB1*0302; *C* = Control individuals. Each bar represents the mean ± SD from 7 to 8 subjects and the experiments were done by triplicate. * $P < 0.01$ shows significant difference comparing with all the other groups. ** $P < 0.05$ compared with controls

recently found that some IPF patients may develop an accelerated form of IPF, usually consulting few months after the beginning of symptoms, with severe lung damage (Selman et al. 2005). Interestingly, 32% of the patients with the haplotype HLA-B*35-DRB1*0407-DQB1*0302 exhibited this type of accelerated course of the disease compared with 11.7% of the patients with the other haplotypes (Fisher exact test = 0.033).

Discussion

Studies on gene polymorphisms and susceptibility to IPF have been mostly focused on genes related to cytokine pathways and most results have been negative or have shown a weak association (Avila et al. 1999; Whyte et al. 2000; Pantelidis et al. 2001; Hutyrova et al. 2002; Latsi et al. 2003; Xaubet et al. 2003; Zorzetto et al. 2003; Hodgson et al. 2005).

In the present study, we examined the possible contribution of the MHC class I and II regions (HLA-B*-DRB1*-DQB1*) to IPF susceptibility in patients who met the stricter recommended criteria for diagnosis. Our results showed that three haplotypes were significantly increased in the IPF group suggesting that the MHC may be involved, at least partially, in the genetic susceptibility for this disorder. Two of these haplotypes: HLA-B*52-DRB1*1402-DQB1*0301 and HLA-B*35-DRB1*0407-DQB1*0302 have been identified in Amerindian populations (Alaez et al. 2001, 2002; Vargas-Alarcon et al. 2003).

The mechanisms by which these haplotypes may enhance the development of IPF need to be elucidated. In other diseases, primarily autoimmune diseases such as rheumatoid arthritis, it has been suggested that some MHC haplotypes predispose to alterations in the T-cell regulation and consequently to an increased susceptibility (Zanelli et al. 2000). However, an association with some HLA alleles has been also described in epithelial-driven fibrotic diseases that do not seem to have an autoimmune/inflammatory background, such as idiopathic focal segmental glomerulosclerosis. Interestingly, this non-inflammatory fibrotic disease characterized by podocytes (glomerular visceral epithelial cells) dysfunction that evolves to segmental glomerular scars and nephrotic syndrome has been also associated to HLA-DR4 (Glicklich et al. 1988; Gerbase-DeLima et al. 1998). In these cases, the effect may be related with other neighboring genes from this region that is characterized by high linkage disequilibrium.

Recently, it has been proposed that a key pathogenic event in the development of IPF is the damage/activation of the alveolar epithelial cells which is followed by fibroblast migration/proliferation and an aberrant tissue remodeling (Selman et al. 2001; Pardo and Selman 2002). In this context, several studies support the notion that apoptosis of alveolar epithelial cells is a critical step in the pathogenesis of this disorder (Uhal et al. 1998; Barbas-Filho et al. 2001; Maeyama et al. 2001). Moreover, it has been found by in situ end labeling of fragmented DNA and electron microscopy the presence of numerous apoptotic cells in an otherwise normal alveoli with only few in fibrotic remodeled areas of IPF lungs suggesting that this process can be an early event in this disease (Barbas-Filho et al. 2001). However, the possible mechanisms implicated in this pathological process are unclear.

Interestingly, some recent studies suggest that HLA class II molecules, in addition to participate in antigen presentation by professional antigen-presenting cells, can also activate intracellular signaling pathways often associated with programmed cell death mainly of B-lymphocytes (Drenou et al. 1999). The mechanisms of HLA-class II associated with lymphocyte apoptosis have not been elucidated. It has been proposed that CD95 activation recruits the adapter molecule Fas-associated death domain protein providing a scaffold for procaspase-8 activation (Blancheteau et al. 2002). Other studies however, have suggested that HLA-DR-mediated apoptosis does not involve caspases activation (Drenou et al. 1999).

However, the possible relationship between some HLA haplotypes and epithelial apoptosis has not been previously described. Importantly, the expression of MHC class II molecules is actively regulated upon various cellular stimuli, and it has been demonstrated that alveolar epithelial cells from IPF lungs express HLA class II molecules (Kallenberg et al. 1987). Moreover, some experimental work has shown that class II MHC antigens is expressed early on the type II alveolar epithelium and before any inflammatory cellular infiltration (Nakayama et al. 1992).

Our findings indicate that some specific haplotypes of the MHC, specifically in this cohort the HLA-B*35-DRB1*0407-DQB1*0302, may be associated with a phenotype characterized by the increase of signals in the lung microenvironment that enhance the apoptosis of the alveolar epithelial cells. Thus, when human lung

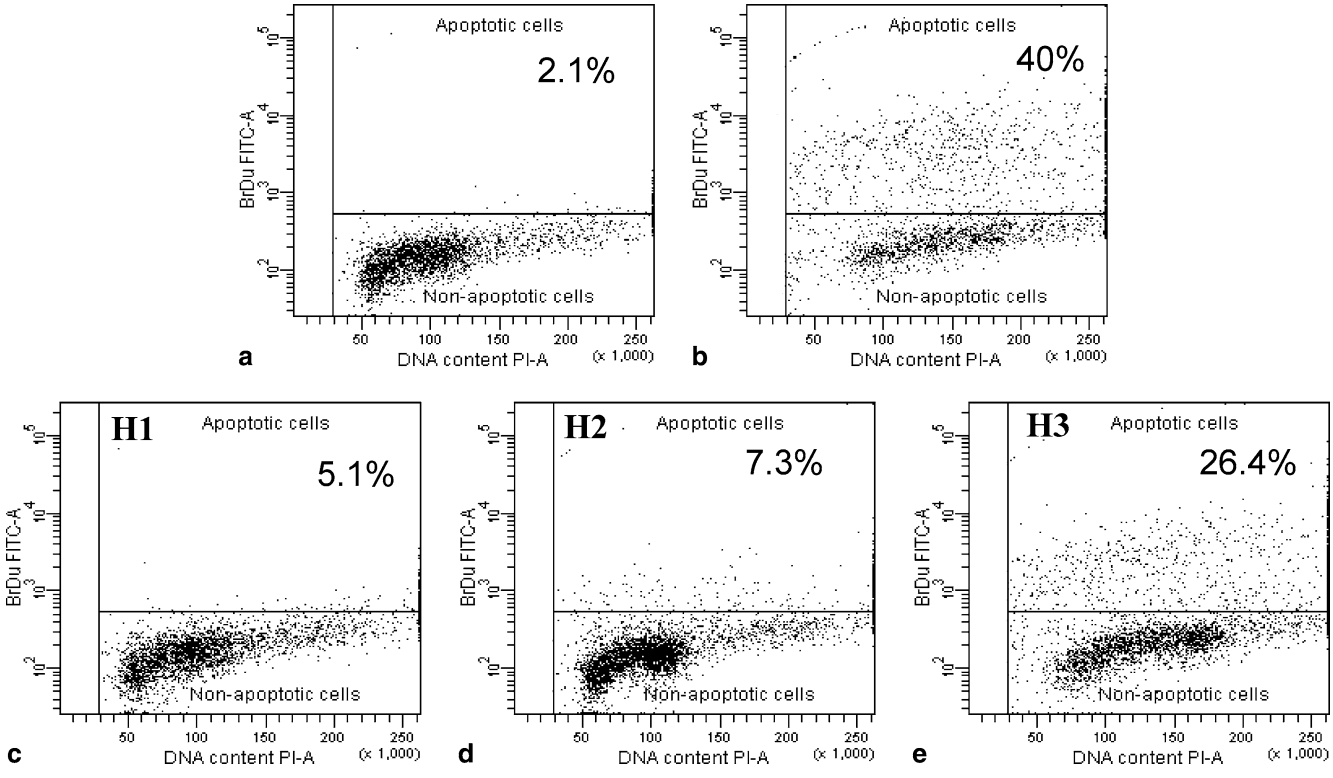

Fig. 2 Detection of DNA strand breaks by flow cytometry. A549 epithelial cells were harvested at 48 hours after BAL stimulation and processed for flow cytometric analysis. A typical experiment is shown. Cells were labeled by FITC-conjugated mAb to BrdU and resuspended in staining solution containing PI/RNAse. The percentage of green fluorescent-positive cells with DNA strand breaks was calculated using the FACS Diva V.4.1 software. Panel A: BAL fluid from a control individual. Panel B: Positive control stimulating the cells with cisplatin. Panel C: BAL fluid from an IPF patient with the haplotype HLA-B*15-DRB1*0101-DQB1*0501 (H1). Panel D: BAL fluid from an IPF patient with the haplotype HLA-B*52-DRB1*1402-DQB1*0301 (H2); Panel E: BAL fluid from an IPF patient with the haplotype HLA-B*35-DRB1*0407-DQB1*0302 (H3). Flow cytometry was performed in 4 controls and 7-8 patients with the different haplotypes

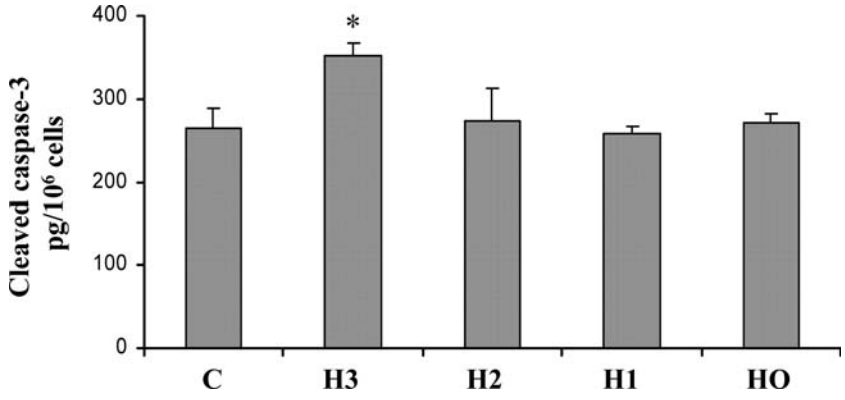

Fig. 3 Effect of BAL fluids on cleaved caspase-3. A549 epithelial cells were stimulated with the BAL fluids from patients with different haplotypes during 6 h and cleaved caspase-3 was analyzed by ELISA. Equal amount of protein of the cell lysates was applied for the assay. *HO*, IPF patients with other haplotypes; *H1*, Patients with the haplotype HLA-B*15-DRB1*0101-DQB1*0501; *H2*, Patients with the haplotype HLA-B*52-DRB1*1402-DQB1*0301; *H3* patients with the haplotype HLA-B*35-DRB1*0407-DQB1*0302; *C*, Control individuals. *Each bar* represents the mean ± SD from 7 to 8 subjects and the experiments were done by duplicate. * $P < 0.05$ shows significant difference comparing with all the other groups

epithelial cells were exposed to the BAL fluids from patients with this haplotype, apoptotic death determined by BrdU-staining of DNA strand breaks was detected in ~25% of the treated cells. Similarly, an increase of the effector caspase-3-dependent apoptosis was also observed.

Interestingly enough, a number of genes encoding for proteins involved in apoptosis are located in the short arm of the chromosome 6, relatively close to the MHC complex (Rebhan et al. 2005). These include among others, the cyclin-dependent kinase inhibitor 1A (CDKN1A), the BCL2-antagonist/killer 1 (BAK1), the death-associated protein 6 (DAXX), the immediate early response 3 (IER3), the sex determining region Y-box 4 (SOX4), lymphotoxin alpha [which is part of the MHC class IV, (Beck and Trowsdale 2000)], the high mobility group AT-hook 1 (HMGA1), and the presenilin-associated protein. Regarding the latter, it has been reported that presenilin-2 homozygous deficient mice spontaneously develop with age, and as the only pathologic phenotype, a considerable thickening of alveolar walls by fibrosis (Herreman et al. 1999). Furthermore, in situ experiments using several techniques also revealed cells undergoing programmed cell death scattered throughout the parenchyma and within the bronchial epithelium (Herreman et al. 1999). Another apoptotic related gene is the HLA-B associated transcript 3 (BAT3) which has a canonical caspase-3 cleavage site (Wu et al. 2004). There is evidence indicating that activated caspase-3 acts on BAT3 releases a C-terminal fragment designated CTF-131 which in turn induces apoptosis (Wu et al. 2004). Whether this or other of the apoptosis related genes are upregulated in association with determined HLA haplotypes is presently unknown and will require further studies.

An additional clinical observation was the increased number of patients with the HLA-B*35-DRB1*0407-DQB1*0302 haplotype who request medical assistance after few months of rapidly progressive symptoms. We have recently found that patients with this clinical behavior often show an accelerated form of the disease with the worst survival rate (Selman et al. 2005).

In summary, our results suggest a role for a genetic susceptibility associated to MHC in IPF and provide support to the notion that susceptibility should be visualized through a model involving several genes, including those of the MHC. One particular haplotype, HLA-B*35-DRB1*0407-DQB1*0302, is associated with a phenotype characterized by the presence of lung factor(s) that induce alveolar epithelial cell apoptosis in vitro.

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References

Alaez C, Vazquez-Garcia MN, Gorodezky C (2001) DQA1 and DQB1 promoter diversity and linkage disequilibrium with class II haplotypes in Mexican Mestizo population. Genes Immun 2:216–221

Alaez C, Infante E, Pujol J, Duran C, Navarro JL, Gorodezky C (2002) Molecular analysis of HLA-DRB1, DQA1, DQB1, DQ promoter polymorphism and extended class I/class II haplotypes in the Seri Indians from Northwest Mexico. Tissue Antigens 59:388–396

American Thoracic Society (2000) Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med 161:646–664

American Thoracic Society/European Respiratory Society (2002) International multidisciplinary classification of the idiopathic interstitial pneumonias. Am J Respir Crit Care Med 165:277–304

Avila JJ, Lympany PA, Pantelidis P, Welsh KI, Black CM, du Bois RM (1999) Fibronectin gene polymorphisms associated with fibrosing alveolitis in systemic sclerosis. Am J Respir Cell Mol Biol 20:106–112

Barbas-Filho JV, Ferreira MA, Sesso A, Kairalla RA, Carvalho CR, Capelozzi VL (2001) Evidence of type II pneumocyte apoptosis in the pathogenesis of IPF/usual interstitial pneumonia (UIP). J Clin Pathol 54:132–138

Beck S, Trowsdale J (2000) The human major histocompatability complex: lessons from the DNA sequence. Annu Rev Genomics Hum Genet 1:117–137

Blancheteau V, Charron D, Mooney N (2002) HLA class II signals sensitize B lymphocytes to apoptosis via Fas/CD95 by increasing FADD recruitment to activated Fas and activation of caspases. Hum Immunol 63:375–383

Drenou B, Blancheteau V, Burgess DH, Fauchet R, Charron DJ, Mooney NA (1999). A caspase-independent pathway of MHC class II antigen-mediated apoptosis of human B lymphocytes. J. Immunol 163:4115–4124

Du Bois RM (2002) The genetic predisposition to interstitial lung disease: functional relevance. Chest 121:14S–20S

Fulmer JD, Sposovska MS, von Gal ER, Crystal RG, Mittal KK (1978) Distribution of HLA antigens in idiopathic pulmonary fibrosis. Am Rev Respir Dis 118:141–147

Gerbase-DeLima M, Pereira-Santos A, Sesso R, Temin J, Aragao ES, Ajzen H (1998) Idiopathic focal segmental glomerulosclerosis and HLA antigens. Braz J Med Biol Res 31:387–389

Glicklich D, Haskell L, Weiss RA (1988) Possible genetic predisposition to idiopathic focal segmental glomerulosclerosis. Am J Kidney Dis 12:26–30

Herreman A, Hartmann D, Annaert W, Saftig P, Craessaerts K, Serneels L, Umans L, Schrijvers V, Checler F, Vanderstichele H, Baekelandt V, Dressel R, Cupers P, Huylebroeck D, Zwijsen A, Van Leuven F, De Strooper B (1999) Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc Natl Acad Sci U S A 96:11872–11877

Hodgson U, Tukiainen P, Laitinen T (2005) The polymorphism C5507G of complement receptor 1 does not explain idiopathic pulmonary fibrosis among the Finns. Respir Med 99:265–267

Holling TM, Schooten E, van Den Elsen PJ (2004) Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. Hum Immunol 65:282–290

Hutyrova B, Pantelidis P, Drabek J, Zurkova M, Kolek V, Lenhart K, Welsh KI, Du Bois RM, Petrek M (2002) Interleukin-1 gene cluster polymorphisms in sarcoidosis and idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 165:148–151

Kallenberg CG, Schilizzi BM, Beaumont F, De Leij L, Poppema S, The TH (1987). Expression of class II MHC antigens on alveolar epithelium in interstitial lung disease: relevance to pathogenesis of idiopathic pulmonary fibrosis. J Clin Pathol 40:725–733

Katzenstein ALA, Myers JL (1998) Idiopathic pulmonary fibrosis. Clinical relevance of pathological classification. Am J Respir Crit Care Med 157:1301–1315

Latsi P, Pantelidis P, Vassilakis D, Sato H, Welsh KI, Du Bois RM (2003) Analysis of IL-12 p40 subunit gene and IFN-gamma G5644A polymorphisms in Idiopathic Pulmonary Fibrosis. Respir Res 4(1):6

Libby DM, Gibofsky A, Fotino M, Waters SJ, Smith JP (1983) Immunogenetic and clinical findings in idiopathic pulmonary fibrosis. Association with the B-cell alloantigen HLA-DR2. Am Rev Respir Dis 127:618–622

Maeyama T, Kuwano K, Kawasaki M, Kunitake R, Hagimoto N, Matsuba T, Yoshimi M, Inoshima I, Yoshida K, Hara N (2001) Upregulation of Fas-signalling molecules in lung epithelial cells from patients with idiopathic pulmonary fibrosis. Eur Respir J 17:180–189

McCluskey J, Peh CA (1999) The human leukocyte antigens and clinical medicine: an overview. Rev Immunogenet 1:3–20

Nakayama A, Nagura H, Yokoi T, Ito M, Asai J (1992) Immunocytochemical observation of paraquat-induced alveolitis with special reference to class II MHC antigens. Virchows Arch B Cell Pathol Incl Mol Pathol 61:389–396

Olerup O, Aldener A, Fogdell A (1993) HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Tissue Antigens 41:119–134

Pantelidis P, Fanning GC, Wells AU, Welsh KI, Du Bois RM (2001) Analysis of tumor necrosis factor-alpha, lymphotoxin-alpha, tumor necrosis factor receptor II, and interleukin-6 polymorphisms in patients with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 163:1432–1436

Pardo A, Selman M (2002) Molecular mechanisms of pulmonary fibrosis. Front Bioscience 7, d1743–1761, August 1

Pardo A, Barrios R, Gaxiola M, Segura L, Carrillo G, Estrada A, Mejía M, Selman M (2000) Increase of lung neutrophils in hypersensitivity pneumonitis is associated with lung fibrosis. Am J Respir Crit Care Med 161:1698–1704

Rebhan M, Chalifa-Caspi V, Prilusky J, Lancet D (1997) GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science, Bioinformatics Unit and Genome Center (Rehovot, Israel), World Wide Web URL: http://bioinformatics.weizmann.ac.il/cards, last updated: January 11, 2005

Rhodes DA, Trowsdale J (1999) Genetics and molecular genetics of the MHC. Rev Immunogenet 1:21–31

Robinson J, Malik A, Parham P, Bodmer JG, Marsh SG (2000) IMGT/HLA database[em]a sequence database for the human major histocompatibility complex. Tissue Antigens 55:280–287

Selman M, King TE, Pardo A (2001) Idiopathic pulmonary fibrosis: prevaling and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med 134:136–151

Selman M, Lin HM, Montaño M, Jenkins AL, Estrada A, Lin Z, Wang G, DiAngelo SL, Guo X, Pardo A, Phelps DS, Floros J (2003) Surfactant protein A and B genetic variants predispose to idiopathic pulmonary fibrosis. Hum Genet 113:542–550

Selman M, Estrada A, Mejía M, Gaxiola M, Suárez T, Alonso D, Navarro C, Pardo A, Carrillo G (2005) Accelerated idiopathic pulmonary fibrosis represents a distinct subtype of IPF. Proc Am Thorac Soc 2:A243

Thannickal VJ, Toews GB, White ES, Lynch JP III, Martinez FJ (2004) Mechanisms of pulmonary fibrosis. Annu Rev Med 55:395–417

Turton CW, Morris LM, Lawler SD, Turner-Warwick M (1978) HLA in cryptogenic fibrosing alveolitis. Lancet 1:507–508

Uhal BD, Joshi I, Hughes WF, Ramos C, Pardo A, Selman M (1998) Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. Am J Physiol 275:L1192–L1199

Vargas-Alarcon G, Hernandez-Pacheco G, Zuniga J, Rodriguez-Perez JM, Perez-Hernandez N, Rangel C, Villarreal-Garza C, Martinez-Laso J, Granados J, Arnaiz-Villena A (2003) Distribution of HLA-B alleles in Mexican Amerindian populations. Immunogenetics 54:756–760

Varpela E, Tiilikainen A, Varpela M, Tukiainen P (1979) High prevalences of HLA-B15 and HLA-Dw6 in patients with cryptogenic fibrosing alveolitis. Tissue Antigens 14:68–71

Vazquez de Lara L, Becerril C, Montano M, Ramos C, Maldonado V, Melendez J, Phelps DS, Pardo A, Selman M (2000) Surfactant components modulate fibroblast apoptosis and type I collagen and collagenase-1 expression. Am J Physiol 279:L950–L957

Whyte M, Hubbard R, Meliconi R, Whidborne M, Eaton V, Bingle C, Timms J, Duff G, Facchini A, Pacilli A, Fabbri M, Hall I, Britton J, Johnston I, Di Giovine F (2000) Increased risk of fibrosing alveolitis associated with interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphisms. Am J Respir Crit Care Med 162:755–758

Wu YH, Shih SF, Lin JY (2004) Ricin triggers apoptotic morphological changes through caspase-3 cleavage of BAT3. J Biol Chem 279:19264–19275

Xaubet A, Marin-Arguedas A, Lario S, Ancochea J, Morell F, Ruiz-Manzano J, Rodriguez-Becerra E, Rodriguez-Arias JM, Inigo P, Sanz S, Campistol JM, Mullol J, Picado C (2003)

Transforming growth factor-beta1 gene polymorphisms are associated with disease progression in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 168:431–435

Zanelli E, Breedveld FC, de Vries RRP (2000) HLA association with autoimmune disease: a failure to protect? Rheumatology 39:1060–1066

Zetterquist H, Bengtsson M, Backstrom G, Egle-Jansson I, Ekdahl AM, Grunnet N, Gustafsson I, Knutsen I, Kuhle A, Rydberg L, Spurkland A, Steffensen R, Storgards M, Szojmer E, Soderholm G, Thuresson B, Turesson H, Olerup O (1997) Report from the HLA class II typing by PCR-SSP Multicentre Study. Eur J Immunogenet 24:191–199

Zorzetto M, Ferrarotti I, Trisolini R, Agli LL, Scabini R, Novo M, De Silvestri A, Patelli M, Martinetti M, Cuccia M, Poletti V, Pozzi E, Luisetti M (2003) Complement receptor 1 gene polymorphisms are associated with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 168:330–334