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REPORT OF WORKING GROUP 2

Genetic predisposition and pathogenetic mechanisms of interstitial lung diseases of unknown origin

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Genetic predisposition and pathogenetic mechanisms of interstitial lung diseases of unknown origin. G.M. Verleden, R.M. du Bois, D. Bouros, M. Drent, A. Millar, J. Müller-Quernheim, G. Semenzato, S. Johnson, G. Sourvinos, D. Olivieri, A. Pietinalho, A. Xaubet. ©ERS Journals Ltd 2001.

ABSTRACT: Understanding of the cellular and cytokine interactions associated with inflammation and fibrosis in interstitial lung diseases (ILDs) has increased substantially during the past few years. Presently, many agents are known to have the ability to induce ILDs, although only a small percentage of exposed individuals will develop the disease. In addition, the majority of ILDs are of unknown origin and many are labelled "idiopathic". Therefore, host susceptibility, genetic factors and, possibly, environmental cofactors may be important for the clinical expression of ILDs.

The present review reports evidence of the genetic predisposition to develop ILDs of unknown origin, more specifically sarcoidosis, idiopathic pulmonary fibrosis (IPF), lymphangioleio-myomatosis and ILDs, in systemic sclerosis. For instance, for sarcoidosis and IPF several histocompatibility antigens have been associated with the development and/or the clinical presentation of the disease. Furthermore, there are also several types of ILD that are associated with inherited disorders, of which the tuberous sclerosis complex is only one example. This clearly indicates that pulmonary fibrosis can be influenced by genetic factors. Familial occurrence of sarcoidosis and IPF is also well known, although the exact modes of inheritance are debatable.

Several studies have shown that extrinsic factors, such as single or multiple fibrosing agents, probably contribute to the development of clinical ILDs of unknown origin. It is probable that some of these studies deal with patients who do not have classical IPF, as recently defined by the American Thoracic Society (ATS)/European Respiratory Society (ERS) consensus. Therefore, the true role of these extrinsic factors in the development of IPF, or even sarcoidosis, remains speculative.

With the help of animal studies and, more specifically, by using knock-out mice, it may be possible in the near future to unravel at least some of the genes that are responsible for the increased susceptibility of the development of interstitial lung diseases.

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Complex diseases are generally influenced by more than one gene or environmental factor, and, as a consequence, do not exhibit a simple mode of inheritance. Cancer, for instance, is a specific form of a complex genetic disease where several clonal mutations are required together with environmental cofactors [1]. Most of these mutations will have a somatic basis. In contrast, more is now known about the aetiology of many single-gene disorders. Although, for many diseases, the protein defect has been well identified, this has still not led to a cure for most of these disorders (*e.g.* cystic fibrosis).

Although for several diseases, such as interstitial lung diseases (ILDs) of unknown aetiology, the understanding of the cellular and cytokine interactions associated with inflammation and fibrosis has increased significantly, the aetiology of the majority of the ILDs is still unknown. Many agents are known to have the ability to induce lung fibrosis, but only a small percentage of the exposed individuals will develop ILDs. Therefore, host susceptibility, genetic factors and/or environmental cofactors may be important for the clinical expression of each disease.

How these factors interact is currently unknown. For the purposes of the present report, the working group members have addressed what they regarded as the five key questions.

What are the key pathogenetic mechanisms in ILDs of unknown origin? It is well known that extrinsic factors, such as single or multiple fibrosing agents on a background of genetic predisposition, probably contribute to the development of clinical disease. It is, however, largely unknown how these interactions occur, although central pathogenesis pathways are beginning to emerge. Therefore, further investigation is needed in order to define key pathogenic mechanisms

that produce the chronic inflammatory response and fibrotic response.

How can the exact phenotype be defined? Many ILDs have similar features. It is, however, crucial to define the exact phenotype of ILDs. As a consequence, definitions and criteria for the disease are required.

Which genes should be targeted? Candidate genes that predispose to ILD have to be identified. This requires familial or other clustering, if present, or targeting genes thought to be central to pathogenesis (*e.g.* immune response, early cytokine or fibrogenesis genes).

How can genetic associations be interpreted? If a specific genotype is present, what does this mean for the risk of disease development, for its severity, and for the prognosis of the disease?

What is the possible role of animal studies? Can animal models contribute to the unravelling of the pathogenetic mechanisms of ILD and are animal models representative of human disease?

This report aims to summarize some of the knowledge regarding these specific questions that has accumulated over recent years. Since most of the data are derived from sarcoidosis and idiopathic pulmonary fibrosis (IPF), a focus will be placed predominantly on these diseases as examples of the way the key questions have been approached. Possible genetic factors in lymphangioleiomyomatosis, and systemic sclerosis with ILD, will also be briefly discussed, as will the role of animal studies in the search for genetic mechanisms in ILD.

Sarcoidosis

General aspects

Sarcoidosis occurs throughout the world, affecting both sexes and all races and ages. The disease, however, shows a predilection for adults <40 yrs of age, especially seen in the 20–29 yrs age group [2]. In Scandinavian countries, there is a second peak incidence in females >50 yrs of age [3].

In a population-based incidence study in the USA, sarcoidosis was found in 5.9 males and 6.3 females per 100,000 person-yrs, suggesting that there is a slightly higher disease rate in females [4]. Racial differences have also been described. For example, Swedes, Danes and US African-Americans appear to have the highest prevalence rates in the world [5]. Several studies suggest that sarcoidosis in US African-Americans is not only more prevalent, but also more severe and chronic than in US Caucasians. Furthermore, some manifestations of sarcoidosis, such as extrathoracic disease, seem to occur more often in certain ethnic and racial groups, for instance cardiac and ocular involvement in Japanese patients [6] and erythema nodosum in Europeans [7].

Person-to-person transmission or shared exposure to a common environmental agent have been suggested in several studies. Forty per cent of sarcoidosis cases occurring in a cluster in the Isle of Man reported prior contact with a sarcoidosis patient, whereas in the control group this was only 1–2% [8]. Other reports mention husband/wife transmission and seasonal clustering of sarcoidosis in early winter and spring. There are numerous publications about familial clustering of sarcoidosis. Sarcoidosis is considered to be familial if any biologic relative of a patient is also diagnosed with the same disease. Familial sarcoidosis has been reported as early as 1923 [9], and since then, over 400 families have been described in the literature [5]. The prevalence of familial sarcoidosis varies according to the population studied. For example, in Finland the prevalence was 3.6–4.7%, whereas in Hokkaido (Japan) it was 2.9–4.3% [10]. Prevalence has been reported as high as 14% [11]. Factors associated with familial sarcoidosis include female sex (odds ratio (OR): 1.78), African-American origin (OR: 4.24) and active disease (OR: 1.64) [12]. Fewer familial cases have been described in Caucasians, suggesting possible aetiological differences among families [13]. Two family studies have proposed different modes of inheritance: one autosomal recessive [10] and the other multifactorial inheritance [14]. Because of this disagreement, it was suggested that African-Americans should be targeted for studies of sarcoidosis that focus on Mendelian hypotheses and genetic linkage [14].

In the USA, familial clusters occur more often in African-American than in Caucasian families (19% in affected African-American families and 5% in Caucasian families) [15], which may provide a basis for genetic studies.

The cause of sarcoidosis is still unknown, although various agents have been suggested to be involved (table 1) [16, 17]. Recently a multicentre case-control study has been started to investigate the aetiology of sarcoidosis, together with the socioeconomic status and the clinical course of patients with sarcoidosis in the USA. In this study, 720 newly diagnosed cases of sarcoidosis will be enrolled and compared to 720 age-, sex- and race-matched controls [18]. It is hoped that this study will define some of the aetiological factors important in the development of sarcoidosis.

Most of the aetiological factors that have been implied to date are speculative. For some, the evidence

Table 1. – Types and examples of agents suggested to be involved in the aetiology of sarcoidosis

Infectious	Inorganic	Organic
Viruses (herpes, Ebstein-Barr, coxsackie B virus, cytomegalovirus)	Aluminium	Pine tree pollen
Borrelia burgdorferi	Zirconium	Clay
Propionibacterium acnes	Talc	
Mycobacterium tuberculosis and other Mycobacteria	MMMF	
Mycoplasma	Titanium	
Fungi		
Spirochetes		
Tropheryma whippelii		
Corynebacteria		

Adapted from [16, 17]. MMMF: man-made mineral fibres.

is more compelling. For instance, the demonstration of tuberculostearic acid and muramyl dipeptide (components of the cell wall of mycobacteria) in sarcoid nodules, suggests the involvement of mycobacteria [19, 20]. Furthermore, antibodies to mycobacteria have been detected in 50–70% of sera of patients with sarcoidosis, whereas fewer of the controls were positive [21]. However, using sequence capture-polymerase chain reaction, which is a sensitive tool for the detection of mycobacterial deoxyribonucleic acid (DNA) in paucibacillary samples, VOKURKA *et al.* [22] were not able to detect DNA corresponding to two different sequences specific for organisms of the *Mycobacterium tuberculosis* complex in surgical biopsies from patients with sarcoidosis. This well-conducted study of large biopsy samples suggests that *M. tuberculosis* does not play a pathogenic role in sarcoidosis.

All these epidemiological studies, therefore, suggest that sarcoidosis results from exposure of genetically susceptible hosts (racial and familiar clustering) to specific environmental factors (husband/wife transmission, geographical distribution, occupational risk factors).

How should gene targets in sarcoidosis be chosen?

Since the inflammation in sarcoidosis is characterized by activated macrophages and CD4 T-helper (Th) lymphocytes, it is presumed that candidate genes predisposing an individual to develop sarcoidosis reside in loci that influence T-cell function, the regulation of antigen recognition, processing, and presentation or regulation of matrix deposition that favour granuloma formation and fibrosis [16]. These loci have been identified close to the human leukocyte antigen (HLA) histocompatibility genes. Therefore, the role of HLA polymorphism has been investigated extensively in sarcoidosis.

Other possibilities have been suggested by VASSILAKIS *et al.* [23], who examined sputum cytological specimens from 30 patients with sarcoidosis and 30 healthy, matched individuals, using 10 highly polymorphic microsatellite markers located at several chromosomal arms. They found that 14 (47%) of the sarcoidosis patients showed genetic alterations, either microsatellite instability (MSI) or loss of heterozygosity (LOH). Six patients (20%) exhibited MSI and 9 (30%) exhibited LOH in at least one microsatellite marker. One of the patients exhibited MSI in two microsatellite markers and 3 (10%) showed LOH in more than one marker. In one patient, a complete deletion of the chromosomal arm 17q11.2-q21 was found. This locus was the most frequently affected in this series. None of the healthy subjects exhibited any genetic alteration in the studied markers.

What do these gene targets mean?

In line with these findings, it remains to be elucidated what these gene targets really mean in terms of disease development and severity, response to treatment and prognosis.

The identification of MSI and LOH in sarcoidosis, although a frequent finding, does not seem to relate to disease severity. The precise significance remains unknown, but it is speculated that the relatively high mutational rate in sarcoidosis patients, as reflected in the instability of the microsatellite sequences, indicates a destabilization of the genome that may affect other genes, resulting in the dysregulation of the cells harbouring these mutations [23].

The histocompatibility antigens HLA-B8 and -DR5 have been associated with the development of sarcoidosis, although those associations have not been found consistently [24]. In the Czech Republic and Italy, an association has been found between certain disease manifestations and the presence of HLA-A1, -B8 and -DR3. By contrast, a negative association was found for HLA-B12 and -DR4. Amongst the Italians, however, there was an association between disseminated systemic disease and HLA-B22, which was not present in the Czech Republic patients [25]. In a Scandinavian population, the presence of HLA-DR17 is associated with a favourable prognosis, whereas the presence of HLA-DR14 and -15 points to a more protracted disease course [26]. HLA-DRw52-positive Japanese patients with sarcoidosis were likely to have limited-stage disease without ophthalmic involvement, whereas HLA-DR5J-positive patients often experienced a poorly resolving disease [27, 28].

A recent study of chronic beryllium disease has identified an association between beryllium disease susceptibility and the presence of a glutamine (Glu) residue at position 69 (Glu69+) of the B1 chain of the HLA-DPB molecule [29]. Since beryllium disease is pathologically very similar to sarcoidosis, it was suggested that this might also be the case in sarcoidosis. In a subsequent study, a significant increase in the frequency of HLA-DPB1*Glu69+ was found in patients with sarcoidosis compared to controls [30], which was, however, not confirmed in a follow-up study [31]. MALIARIK *et al.* [32] reported that HLA-DPB1 Glu69 was not associated with sarcoidosis in African-Americans, whilst Valine (Val)36 and Aspartate (Asp)55 were associated with an increased risk for sarcoidosis (ORs: 2.3 and 2.03, respectively). SCHÜRMANN *et al.* [33] found an over-representation of HLA-DPB*0201 (a Gly69 allele) among familial sarcoidosis patients, but the finding remained inconclusive since sibling triplets suffering from sarcoidosis did not share the identified alleles. In another report, a glutamine residue at position 55 of the HLA-DBP1 chain was identified, which, however, only occurred in patients with beryllium disease susceptibility [34].

Another recent study identified a region on chromosome 6 close to the HLA class II genes and next to the microsatellite marker D6S291, which disclosed a significantly elevated score for nonparametric linkage [35]. This means that this region on this chromosome may include a gene that predisposes to the development of sarcoidosis.

As a consequence, a clear linkage of class II histocompatibility antigens and sarcoidosis has not yet been found. These contradictory results suggest that the genetic predisposition is more complex and

that multiple genes and external stimuli are probably involved.

Several other polymorphisms have been examined in sarcoidosis. Serum levels of angiotensin II converting enzyme (ACE) have been demonstrated to have potential value in confirming the diagnosis and in estimating the activity of sarcoidosis [36]. It is thought that ACE is produced by epithelioid cells and other components of the sarcoid granuloma, and is likely to be important in mediating and modulating inflammation [37]. The prevalence of increased serum ACE levels in active sarcoidosis, however, ranges from 33–88% and it is also reported that serum ACE levels are higher in African-Americans than in Caucasians with sarcoidosis [38].

These findings suggest the existence of genetic or racial differences in serum ACE levels in sarcoidosis patients. Even in normal subjects, serum ACE may differ between individuals [39]. In addition, familial clustering of normal and raised serum ACE levels has been reported in healthy families, suggesting the possibility of genetic control of serum ACE levels [40]. It has recently been shown that a polymorphism exists both at the ACE gene locus and in the angiotensin II type 1 receptor [41, 42]. In the ACE gene, three genotypes have been described: DD (homozygous deletion), DI (heterozygous deletion/insertion) and II (homozygous insertion) [43]. ACE gene polymorphism has been studied in different populations with sarcoidosis. In a group of 341 unrelated healthy controls and 103 consecutive patients with sarcoidosis, it was demonstrated that ACE gene polymorphism was associated with serum ACE levels in both groups, although it could not be concluded from this study that the presence of the D allele in the ACE gene correlated with an increased risk to develop sarcoidosis [43]. ARBUSTINI *et al.* [44] reported similar findings in a group of 61 sarcoidosis patients and 80 healthy controls. In a further study, looking at three patient groups (sarcoidosis, tuberculosis and controls), no difference in allele frequency of the angiotensin II type 1 receptor and the angiotensin II type 2 receptor was found amongst the three groups. By contrast, serum ACE levels were higher in the patients with sarcoidosis with the angiotensin II type 1 receptor A/C (A to C translocation) genotype than in others, which could be one of the explanations for the increased serum ACE activity in sarcoidosis [45]. Looking at ACE gene polymorphism and the association with clinical manifestations, TOMITA *et al.* [46] found no significant differences in the percentage of eye, skin or heart involvement between the three ACE genotypes. They also found no correlation with the prognosis of sarcoidosis. The frequency of the DD genotype was, however, significantly increased in sarcoidosis patients with autoimmune manifestations and major granuloma mass (radiograph stage III), indicating that the DD genotype may confer susceptibility for autoimmune manifestations in sarcoidosis [47].

In another case-control study, comparing African-Americans and Caucasians, it was found that the ACE gene polymorphism did not differ between Caucasian sarcoidosis patients and controls, but a marked difference in genotypic distribution was observed between African-Americans and controls. In African-Americans, the risk to develop sarcoidosis was 1.3 for ID heterozygotes and 3.17 for DD homozygotes [48].

Comparing 101 sarcoidosis patients with 216 controls, SEITZER *et al.* [49] demonstrated a significant shift to the tumour necrosis factor (TNF)-α2 allele in Löfgren′s syndrome. There was also a significant shift towards the TNF-α2 allele in Löfgren′s syndrome, when compared with nonacute sarcoidosis. Therefore, it was concluded from this study that the TNF-α2 allele seemed to predict a more favourable prognosis [49]. However, in another study, Lipopolysaccharide (LPS)-stimulated bronchoalveolar lavage (BAL) cells and blood mononuclear cells from sarcoidosis patients with the TNF-α2 allele did not produce a statistically different amount of TNF-α, compared to sarcoidosis patients with another TNF polymorphism (TNF-α1, TNF-β1 and TNF-β2), which suggests that the TNF-α2 locus does not define a phenotype [50].

Polymorphism of the Vitamin D receptor (VDR) has recently also been demonstrated. Three genotypes have been identified: homozygous for the digestive allele "bb", homozygous for the undigestive allele "BB" and heterozygous for "Bb" [51]. Polymorphisms of the VDR have been investigated in 101 patients with sarcoidosis and in 105 healthy controls. In the sarcoidosis patients, the frequency of BB, Bb and bb genotypes were 1%, 37.6% and 61.4% respectively, whereas in the healthy control subjects the figures were 1%, 20% and 79%, respectively. The difference in the genotype distribution between patients and controls was significant, with the frequency of the B allele being increased. Therefore, it was concluded that in VDR polymorphism the B allele might be a genetic risk factor for sarcoidosis [52].

Polymorphisms of the transporter associated with antigen processing (TAP) have also been investigated in two European, ethnic-independent populations with sarcoidosis, and compared with controls from the same two countries (UK and Poland). There were significant differences in TAP2 between the UK control and patient group, as well as between the Polish control and patient groups. Comparing the UK and Polish control groups, there was a difference in TAP1. These results suggest that TAP polymorphism might be involved in determining susceptibility to develop sarcoidosis, and that such susceptibilities may differ between two ethnic-independent populations [31].

At the moment it is not quite clear whether these gene polymorphisms have an effect on the risk of developing sarcoidosis (except perhaps for the ACE gene polymorphism in African-Americans) or whether they influence the clinical course or prognosis of sarcoidosis. Further studies are required to clarify the exact role of gene polymorphisms in sarcoidosis.

What are the key pathogenetic mechanisms?

Sarcoidosis is characterized by a granulomatous process, involving a number of cytokines, predominantly early cytokine and Th1 cytokines, which

perpetuate the inflammatory process. TNF-α has been studied extensively.

Cytokines

TNF-α is a cytokine which, by virtue of its ability to promote the release of pro-inflammatory mediators, vascular endothelial permeability and fibrogenesis, has been implicated in *in vitro*, animal and *in vivo* studies, to have a crucial role in the pathogenesis of a large number of inflammatory processes [53–56]. This potential to inflict damage is limited by a number of different homeostatic regulatory mechanisms. These include the production of anti-inflammatory cytokines, such as interleukin (IL)-10 and the cleavage of membrane receptors, which both reduce the signalling capabilities of TNF-α and bind excess TNF-α in biological fluids, thereby restricting its *in vivo* activity [57]. The biological activity of TNF-α is triggered when it binds to specific cell-surface receptors, which can result in both the paracrine and autocrine effects of TNF-α. Antigenic stimulation increases the shedding of these proteins into tissue fluids [58]. The shed receptors act as physiological inhibitors of TNF-α, but in some circumstances they may also act as carrier proteins (especially CD120b) and may alter monocyte T-cell interactions and prolong the half-life of TNF-α bioactivity [59]. Cytokines considered to have an anti-inflammatory effect, such as IL-10 and TGF-β, also play a regulatory role by decreasing TNF-α production and activity [60]. Conversely, IL-10 also increases TNF-receptor expression and shedding [61]. Furthermore, TNF-α itself has been shown to stimulate IL-10 production [62]. Thus, TNF-α induces at least one molecule that affects its own production. These observations give insight into the complex homeostatic mechanisms designed to control the inflammatory response, which may be crucial in the development of ILD.

The role of TNF-α in the pathogenesis of sarcoidosis remains controversial, with variable findings reported in studies of BAL fluid (BALF) and alveolar macrophage (AM) culture supernatants [63–67], although the majority suggest increased TNF-α production. This exemplifies one of the practical problems in this area of research. The detection of TNF-α by an enzyme-linked immunosorbent assay (ELISA) or immunoassay may not discriminate between bound and unbound cytokine, although in sarcoid BAL cell supernatants, a good correlation has been demonstrated [66]. The presence of soluble TNF receptors and total TNF-α in subjects with stage I and stage II/III sarcoidosis has been compared to healthy controls in one recent study [55]. A significant increase in TNF-α bioactivity was not observed in either the BALF or AM supernatants derived from subjects with stage I or stage II/III sarcoidosis, compared to healthy subjects, although the highest bioactive TNF-α levels were detected in the stage II/III population. The stage I population had significantly reduced TNF-α bioactivity compared to the other two groups. This demonstrated that in the lungs of subjects with sarcoidosis, much of the TNF-α is in the biologically inactive form. In a previous study, an alternate bioassay (using the L929 cell line) was used to measure TNF-α in active and inactive sarcoidosis [67]. No increase in TNF-α bioactivity in the BALF or AM supernatants was found, with the exception of AMs from active sarcoidosis subjects stimulated with 10 $\mu g \cdot mL^{-1}$ LPS, where a small significant elevation was observed. These studies, which use similar methods, are largely comparable and highlight the importance of using bioassays whenever possible when implicating a cytokine in a disease process.

A distinction can be made between stage I disease, which often has a self-limiting clinical course requiring no treatment, and stage II and III disease, where there may be progressive lung involvement and impairment of lung function. The data reported suggests that in stage I subjects, TNF-α bioactivity is restricted, and this may be an important factor in the prevention of the development of interstitial changes, despite the presence of a mononuclear cell alveolitis and granulomata.

A possible explanation for reduced bioactivity in stage I disease is the inhibitory activity of soluble TNF-R, especially CD120a. Significantly elevated levels of CD120a and CD120b were found in the BALF obtained from stage I and stage II/III subjects, compared to controls, as was enhanced production of CD120a and CD120b by stage I and stage II/III AMs, respectively, *in vitro*.

It is widely accepted that TNF-Rs are successful inhibitors of TNF-α bioactivity *in vivo* [68, 69], and that they are part of the regulatory response in acute diseases, such as meningitis [70] and sepsis [71, 72], as well as in response to LPS challenge in experimental conditions [73]. However, in one study on sepsis patients [73], elevated TNF-R was found to correlate with mortality. Infusion of low levels of TNF-R has resulted in an increased half-life for TNF-α in plasma in animal models of endotoxaemia, suggesting their presence can be associated with increased injury, rather than protection [74]. This implies that limiting TNF-α bioactivity in the acute inflammatory response may lead to slow resolution and possibly, chronicity. Interestingly, serum levels of both soluble TNF-α receptors are elevated in sarcoidosis and correlate with clinically evaluated disease activity, which suggests that these molecules have an important role in the regulation of systemic inflammatory responses in sarcoidosis [75]. Since the aetiology of sarcoidosis is presently unknown, this theory cannot be applied directly, but it does demonstrate how so-called beneficial mediators could be deleterious in certain circumstances. Whilst it must be appreciated that BALF and BAL-derived AMs do not necessarily reflect the inflammatory processes within the sarcoid granuloma itself, they could provide information about an important homeostatic mechanism, which may prevent the development of interstitial lung changes that are more characteristic of stage II/III sarcoidosis.

Chemokines

Recently, the pathogenetic role of the chemoattractant molecules, and in particular CXCL10 (interferon

(IFN)-γ-inducible protein, IP-10) and its receptor CXCR3, in initiating and maintaining T-cell alveolitis in different ILDs characterized by CD4 or CD8 cell alveolitis has been demonstrated. The expression of chemokines (*e.g.* CXCL10, macrophage inflammatory protein-1α) has been identified in the lungs of patients with sarcoidosis, fibrosis [76, 77] and in human bronchial epithelial cells [78]. There are also data indicating that CXCL10 is expressed and released by pulmonary macrophages and alveolar cells *in vivo*. In particular, it has been shown that AMs, purified from patients with ILD, release CXCR3 ligands that exert chemotactic activity on a CXCR3+ T-cell line [76].

CXCL9, CXCL10, CXCL11 chemokines and their receptor CXCR3 represent an exception in the complex world of chemokines. CXCR3 is expressed by activated T-lymphocytes of the Th1 phenotype and, in turn, its ligands are specifically chemotactic for T-cells. These molecules keenly regulate the migration of Th1 cells to sites of pulmonary inflammation in different ILDs. It has been shown that an upregulated expression of CXCL10/CXCR3 occurs in the pulmonary milieu, and that CXCL10/CXCR3 interaction is crucial in favouring the trafficking of disease-causing Th1 cells. Since the expression of chemokines and chemokine receptors is under genomic control, studies are in development to evaluate whether the more or less rapid resolution of ILD is to some extent related to a genetically determined alteration of the CXCL10/CXCR3 interactions. If this were the case, it would be expected that the administration of biological response modifiers, capable of inhibiting the CXCR3-mediated recruitment of T-cells, might be crucial in patients with ILD who are unresponsive to immunosuppressants and evolve toward pulmonary fibrosis. The application of factors blocking CXCL10/CXCR3 interactions as therapeutics could be central since the outcome of ILDs is clearly related to the extent and persistence of lung inflammation.

Glucose-6-phosphate dehydrogenase pathways

Glucose-6-phosphate dehydrogenase (G6PD) has a key role in the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH), utilized by glutathione reductase to maintain gluthathione (GSH) in the reduced form. GSH plays an important role in the homeostasis of a cell as it protects cells against oxidative challenge and detoxifies xenobiotics by conjugation. In G6PD-deficient tissues, a low level of NADPH leads to a decrease of intracellular GSH, which in turn increases the vulnerability of the cell to reactive oxygen species formed in the aerobic metabolism [79–81]. So far, little attention has been paid to the role of G6PD-deficiency in causing pulmonary damage [81–86]. G6PD-deficiency has recently been linked with more advanced diseases in sarcoidosis [87], and, therefore, it was hypothesized that there might be a link between the declined regulatory elements of the oxidative cascade, including G6PD, and the development or the severity of sarcoidosis. As a consequence, the prevalence of G6PD-deficiency was evaluated by measuring G6PD and glutathione reductase levels in a Dutch female sarcoidosis patient population (n=31) [88]. Twelve cases (38.7%) were classified as functional G6PD-deficient, of whom only two cases appeared to have a confirmed genetic G6PD-deficiency. The prevalence of a relative G6PD-deficiency in this sarcoidosis patient population was unexpectedly high. This might point to functional impairment of G6PD as a potential cofactor for the risk of developing sarcoidosis [87]. However, it is not clear whether a correlation between homozygous G6PD and the development of ILD exists. Nonetheless, it seems obvious that a decrease in antioxidant capacity due to a G6PD-deficiency or functional impairment of the G6PD protein, accounts for clinical problems facilitated by oxidative stressors [89]. Therefore, studies are needed to explore the prevalence of insufficient regulation of G6PD expression in oxidative stress. Additionally, the logic of antioxidant treatment needs to be explored in clinical trials to establish their true therapeutic role and clinical usefulness in ILD.

Idiopathic pulmonary fibrosis

General aspects

IPF is a worldwide disease, without specific predilection by race or ethnicity. Environmental (*e.g.* asbestos, silica, metal dust, wood dust) and pharmaceutical agents (*e.g.* bleomycin, methotrexate, cyclophosphamide, nitrofurantoin, antidepressants) have been identified as capable of producing pulmonary fibrosis. Cigarette smoking has been reported to be a potential risk factor in the development of IPF; the OR of developing IPF was 2.3 in those who had a smoking history of 21–40 pack-yrs [90], but smoking appears to reduce the risk of developing immune granulomatous diseases such as sarcoidosis and extrinsic allergic alveolitis.

The role of occult environmental agents in the development of IPF remains unknown, although four case-control epidemiological studies have investigated possible environmental causes of IPF [90–93]. The results of these studies are summarized in table 2. Subsequently, a meta-analysis was performed when relevant data were available from at least two of these

Table 2. – Environmental and occupational risk factors for developing interstitial pulmonary fibrosis

Exposure	Ref.			
	[89]	[90]	[91]	[92]
Livestock	2.65	10.89		
Farming	1.50			3.01
Wood dust		2.94	1.71	
Textile dust	1.89	0.90	1.80	
Stone/sand	3.90	1.59	1.76	
Metal dust	10.82	0.97	1.68	1.34
Smoking	1.60	1.11	1.57	2.94
Wood fires	0.83	12.55		

Data are presented as odds ratios.

four studies, which showed three significant occupational and environmental exposures: smoking (OR=1.5), work with livestock (OR 2.2) and wood dust (OR 1.8) [94].

Numerous viruses have also been implicated in the pathogenesis of IPF. Epstein Barr virus (EBV) capsid antigen has been found in lung tissue of IPF patients [95]. Other authors have also suggested an association between EBV and IPF [96]. A higher incidence of influenza [97], cytomegalovirus [98] and hepatitis C [99] infection have also been reported in IPF patients, although the prevalence of the latter in patients with IPF is comparable to that in other medical conditions [100].

The main problem in the interpretation of these data on environmental and possible infectious aetiologies of IPF, is the high reliance on a clinical diagnosis of IPF, without confirmation by lung biopsy or high-resolution computed tomography (HRCT). In order to overcome this problem, further studies will have to include patients with precisely defined clinical phenotypes.

How to define the phenotype

Recently, a new classification of ILD has been proposed that may facilitate phenotype definition and from which IPF has been redefined as requiring surgical biopsy confirmation or HRCT and BAL or transbronchial biopsy features, which exclude other diseases [101] (see Review by DU BOIS and WELLS [102] in this Supplement). A definite diagnosis of IPF in the presence of a surgical biopsy demonstrating usual interstitial pneumonia includes the following criteria: 1) exclusion of other known causes of ILD such as drug toxicities, environmental exposures and collagen vascular diseases; 2) abnormal pulmonary function studies that include evidence of restriction and/or impaired gas exchange; and 3) abnormalities on conventional chest radiographs or HRCT. If no biopsy is available, the presence of specific major and minor criteria (outlined in the Review by DU BOIS and WELLS [102] in this Supplement) strongly supports a diagnosis of IPF in the immunocompetent patient [101].

Most of the studies explaining genetic risk, environmental exposure and familial disease have rarely used these criteria to establish a definite diagnosis of IPF, which makes interpretation of these particular studies difficult.

Defining the right target genes in idiopathic pulmonary fibrosis

In 1990, JOHNSTON *et al.* [103] demonstrated that death from IPF is more common in males and increases with age. Although environmental factors might be important, it is clear that the majority of individuals sharing such an environment do not develop IPF. This suggests a genetic predisposition, although the precise mode of inheritance has hitherto not been identified [104] and requires more definitive studies.

A potential role for genes located on chromosome 14 has been suggested and positive associations between IPF and α_1-antitrypsin inhibitor alleles have been reported. An increase in the frequency of the MZ phenotype (α1-antitrypsin deficiency) has been demonstrated in patients with IPF, compared to controls [105]. Several potential associations between IPF and major histocompatibility genes have been suggested, including HLA-B15, -B8, -B12, -DR2 and -Dw6, although clear evidence for these associations is still lacking [104].

An increased risk of IPF has also been associated with IL-1 receptor antagonists and TNF-α gene polymorphisms [106].

Microsatellite DNA instability has also been demonstrated in IPF. In one study, 50% of the IPF patients showed genetic alterations, either microsatellite instability (MI) or loss of heterozygosity. No correlation was found between these genetic alterations and disease severity [107].

Familial IPF was first described in 1907 [108] and several clusters of familial IPF have been reported since [109–112]. Although IPF is still considered a rare disease, careful evaluation of family members of affected patients might demonstrate an increased prevalence of IPF. BAL in unaffected family members of an IPF patient may reveal evidence of lung inflammation [113]. However, the risk of developing IPF in unaffected family members is, at present, unknown. It is also unclear whether the inheritance is autosomal recessive, dominant with incomplete and variable penetrance [114], or polygenic, which is the most likely option. Some cases may be inherited in a Mendelian pattern, since the disease has been reported in identical twins who lived apart for 25 yrs [115, 116]. In a family with three siblings affected by IPF in combination with hypocalciuric hypercalcaemia and defective granulocyte function, prospective examination of 40 family members revealed an inheritance pattern to be autosomal dominant, with a variable penetrance, but this disease is probably different from IPF [117].

Key pathogenetic factors in idiopathic pulmonary fibrosis

As in other ILDs, cytokines have been identified that are pro- or anti-inflammatory and pro- or anti-fibrogenic. In the local milieu, the balance between these factors is likely to be important. It is beyond the scope of this review to discuss them all, so the discussion on cytokines will be limited to some new insights into the role of TNF. However, current knowledge on the role of other cytokines and receptors involved in the development of pulmonary fibrosis in experimental models is outlined in table 3.

Several groups have shown that significantly more constitutive and LPS-stimulated bioactive TNF-α is produced by AMs from patients with IPF [118–120]. Measurement at the messenger ribonucleic acid (mRNA) level is more controversial and subject to differences in time course and techniques.

Production of IL-10 is increased several-fold, both

Table 3. – Role of cytokines and receptors in the development of pulmonary fibrosis in animal models

Cytokine/receptor/gene	Effect on pulmonary fibrosis
Plasminogen-activator inhibitor-1	+
TNF-α	+
TNF-α receptor	+
IL-5	+
IL-4	+
IL-10	-
TGF-α	+
TGF-β	+
Platelet derived growth factor	+
Insulin-like growth factor	+
Endothelin-1	+
RANTES	+
Macrophage inflammatory protein-2	+
SPARC	+
Smad7	-
IFN-γ	-
Prostaglandin E2	-
CXCL10/CXCR3	- (attracts and activates T-helper 1 lymphocytes)

Adapted from [130, 131]. TNF-α: tumour necrosis factor-α; IL: interleukin; TGF-α: transforming growth factor-α; TGF-β: transforming growth factor-β; RANTES: regulated on activation, normal T-cell expressed and secreted; SPARC: secreted protein acidic and rich in cystein; IFN: interferon. +: enhances pulmonary fibrosis; -: inhibits pulmonary fibrosis.

constitutively and following LPS stimulation in patients with IPF. Addition of IL-10 to IPF AM cultures inhibits LPS-induced TNF-α protein production by almost 94%, compared to 63% inhibition seen in normal subjects [121]. The study of MARTINEZ [120] showed increased IL-10 mRNA in IPF AMs compared to healthy controls, and this was similar to the situation seen in the lungs of transgenic mice, which overexpress TNF-α, where the mRNA level of IL-10 was also constantly enhanced [122]. However, when MARTINEZ [120] examined BAL from patients with IPF and normal subjects, less IL-10 protein was found in the IPF patients, compared to controls [120]. 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 AMs. Immunohistochemical studies suggested increased levels of IL-10 receptors on the AMs of patients with IPF, whilst results from PCR analysis showed increased levels of IL-10R1 and IL-10R2 mRNA in the IPF patients compared to the normal controls, with significant increases most evident after culture of AMs with IL-10.

The effect of TNF receptors and the effect of IL-10 upon their regulation have also been studied. Differences between IPF and normal subjects were only detected in CD120b, which was increased in IPF subjects at both the protein and mRNA levels [121]. Soluble TNF receptors are derived by proteolytic cleavage from membrane bound receptors, so the PCR primers used to measure CD120a and CD120b mRNA levels are unable to distinguish 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 [123]. Culture of AMs with IL-10 alone did not appear to influence shedding of CD120b. However, culture of AMs with IL-10 and LPS resulted in increased shedding of CD120b in IPF patients, compared to both unstimulated IPF AMs 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 mechanisms for the increased soluble CD120b seen in IPF.

Lymphangioleiomyomatosis

Lymphangioleiomyomatosis (LAM), like other ILDs, probably results from the interaction of genetic abnormalities and environmental factors. Certain features of LAM require discussion: the relationship of LAM to tuberous sclerosis; the increased incidence of LAM in patients with tuberous sclerosis complex (TSC); the fact that the disease only occurs in females; and the presence of an unusual smooth muscle cell phenotype in LAM and angiomyolipoma.

Patients with TSC have a germ line mutation in one of two tumour suppressor genes (TSC-1 and -2). A second mutation causing loss of the gene product may result in a hamartoma. Patients with LAM do not carry germ line mutations in these genes, but some patients do have loss of heterozygosity for TSC-2 in their angiomyolipomas and lymph nodes [124]. This suggests that TSC-2 is likely to be involved in LAM in some patients, perhaps with functionally related genes important in others. As LAM occurs almost exclusively in pre-menopausal females, and may be slowed by the menopause and progesterone [125], it is thought to be hormone-dependent. In addition, LAM cells express oestrogen and progesterone receptors in many cases [126]. The association of oestrogen receptors with the antiapoptotic protein Bcl-2 within LAM cells, suggests that oestrogen may inhibit apoptosis in these cells, leading to increased LAM cell proliferation and, therefore, disease progression [127]. Thus, hormonal factors may act as cofactors to an underlying primary cellular defect, possibly related to TSC-2. LAM cells express proteins normally seen in cells of melanoma lineage, and this and other common ultra-structural features suggest that LAM cells belong to a family of lesions, including angiomyolipoma and clear cell tumour of the lung, which are characterized by the presence of LAM cell-like perivascular epithelioid cells. Although the function of these melanocyte-related proteins in perivascular epithelioid cells is unknown, their expression within a population of LAM cells is inversely related to that of proliferating-cell nuclear antibody (PCNA), a marker of cellular proliferation, suggesting that these proteins may somehow be associated with proliferation [127]. The inclusion of LAM in this family of tumours and hamartomas suggests a basic defect in the control of

cellular proliferation to be present. Therefore, LAM probably arises from a defect in a pathway related to one of the TSC genes, but is then likely to require other external stimuli, some of which may be hormonal.

Genetic predisposition to systemic sclerosis with diffuse lung disease

There have been a number of studies that have confirmed that there is probably a significant genetic predisposition to systemic sclerosis [128] (table 4). The majority of these have been associated with the major histocompatibility complex (MHC) class I and II loci, with different associations reported internationally. This is almost certainly due to differences in ethnic gene pools, but it remains clear that systemic sclerosis is not to be considered as a Mendelian inherited disease [12]. Despite the discordance, there have been some studies showing similar HLA associations in different racial groups [129]. Recent studies have shown a strong association between the HLA-DPB1 locus and the presence of the anti-DNA topoisomerase 1 autoantibody, Scl-70, and this in turn is strongly associated with lung fibrosis. Fibronectin is a growth factor known to play a crucial role in lung fibrosis, and recently, fibronectin gene polymorphisms have been associated with fibrosing alveolitis in systemic sclerosis [130].

What is the role of animal studies in the search for genetic mechanisms in interstitial lung disease?

In an attempt to understand the pathogenesis of pulmonary fibrosis, several agents have been used to induce pulmonary fibrosis in animals. Examples include irradiation, bleomycin, cyclophosphamide, silica and asbestos exposure, and intratracheal instillation of fluorescein isothyocyanate.

During the last few years, several cytokines that induce fibrosis have been identified, whilst other cytokines are thought to be protective against the development of pulmonary fibrosis (table 3) [131, 132].

Studies of knock-out mice enabled the examination of the effect of transgenic mice and models of cytokine-overexpression in individual cytokines in the pathogenesis of pulmonary fibrosis. It has been demonstrated that the receptor for TNF-α is essential for the development of bleomycin-induced fibrosis [133] and that TNF-α receptor knock-out mice are protected against the fibroproliferative effects of inhaled asbestos fibres [134]. Furthermore, in a rat model of TNF-α overexpression, it was demonstrated that local overexpression resulted in severe pulmonary inflammation, with resulting fibrogenesis. The onset of fibrogenesis coincided with accumulation of myofibroblasts and upregulation of transforming growth factor-β1 [135]. Mice that lack the murine plasminogen activator inhibitor-1 gene (PAI-1) develop much less collagen accumulation after bleomycin challenge than mice that overexpress this gene, suggesting that a correlation exists between the genetically determined level of the PAI-1 gene and the extent of fibrosis [136]. In another inbred strain of mice, it was demonstrated that the susceptibility to bleomycin-induced pulmonary fibrosis was a heritable trait controlled by only a few genetic loci [137].

It remains to be established, however, whether these animal studies really represent a model for ILD, and especially IPF, or acute lung injury.

Table 4. – Suggested association between major histocompatibility complex (MHC) alleles and systemic sclerosis (scleroderma)

MHC Class I	MHC Class II
B8	DR3
A1	DR5
	DR3/DR52a (systemic sclerosis with pulmonary fibrosis)
	DQ7
	DQ5
A1/B3/DR4 (fibrosis)	DPB1*1301

*: position at which the change happened.

Conclusion

Although much data are already available in the literature, the key questions discussed above remain unanswered. The role of genetics and the environment is no longer disputed, but how these two factors interact is largely unknown.

New techniques in screening the genome for mutations (microsatellites and loss of heterozygosity) and candidate gene studies are being applied, although the search for the meaning and significance of these findings, especially regarding disease development, severity, progression, response to treatment and prognosis, remains challenging. The way environmental antigens and genetic factors interact to trigger pathogenetic inflammatory mechanisms requires further study and pathological responses need to be distinguished from physiological responses to the pathological process.

The role of animal studies is also unclear, since several animal models represent acute and not chronic inflammation, which characterizes most interstitial lung diseases.

The authors believe that the study of ILD is flourishing; basic and clinical research is of an increasingly high quality and new therapies are already emerging. Hopefully the use of animal models will help to resolve many unanswered questions.

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