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Toll-like Receptor 3 L412F Polymorphism Promotes a Persistent Clinical Phenotype in Pulmonary Sarcoidosis

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Title: Toll-like receptor 3 L412F polymorphism promotes a persistent clinical phenotype in pulmonary sarcoidosis

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G.C., M.S. and E.H. performed experiments. I.K. collated patients' clinical data. L.M., A.T., C.O.R. and D.N.O.D. processed clinical samples. S.L.K. and D.R.M. provided patient samples. U.G.K., D.C.S., A.G.B. and P.G.F. contributed to experimental design. C.M.H. provided patient samples and contributed to experimental design. M.E.A conceived of, designed and performed experiments, analysed data and wrote the paper. S.C.D. conceived of experiments, analysed data and contributed to manuscript drafting.

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ABSTRACT

Background: Sarcoidosis is a multisystemic disorder of unknown etiology, characterised by the presence of non-caseating granulomas in target organs. In ninety percent of cases, there is thoracic involvement. Fifty to seventy percent of pulmonary sarcoidosis patients will experience acute, self-limiting disease. For the subgroup of patients who develop persistent disease, no targeted therapy is currently available.

Aim: To investigate the potential of the single nucleotide polymorphism (SNP), Toll-like receptor 3 Leu412Phe (*TLR3* L412F; rs3775291), as a causative factor in the development of, and in disease persistence in pulmonary sarcoidosis. To investigate the functionality of *TLR3* L412F *in vitro* in primary human lung fibroblasts from pulmonary sarcoidosis patients.

Methods: Cohorts of Irish sarcoidosis patients (n=228), healthy Irish controls (n = 263) and a secondary cohort of American sarcoidosis patients (n=123) were genotyped for *TLR3* L412F. Additionally, the effect of *TLR3* L412F in primary lung fibroblasts from pulmonary sarcoidosis patients was quantitated following TLR3 activation in the context of cytokine and type I interferon production, TLR3 expression, and apoptotic- and fibroproliferative-responses.

Results: We report a significant association between *TLR3* L412F and persistent clinical disease in two cohorts of Irish and American Caucasians with pulmonary sarcoidosis. Furthermore, activation of TLR3 in primary lung fibroblasts from 412F-homozygous pulmonary sarcoidosis patients resulted in reduced IFN- β and TLR3 expression, reduced apoptosis- and dysregulated fibroproliferative-responses compared with TLR3 wild-type patients.

Conclusions: This study identifies defective TLR3 function as a previously unidentified factor in persistent clinical disease in pulmonary sarcoidosis and reveals *TLR3* L412F as a candidate biomarker.

INTRODUCTION

Sarcoidosis is a multi-systemic disorder of unknown cause which is characterised by the presence of non-caseating granulomas in target organs. Ninety percent of sarcoidosis cases have thoracic involvement (1, 2). The highest annual incidence of sarcoidosis has been observed in northern European countries (5 to 40 cases per 100,000 people) (3). Phenotypically, sarcoidosis follows either an acute or chronic course. Up to seventy percent of patients present with acute sarcoidosis and experience self-limiting disease which will resolve within 1-2 years (4). In contrast, thirty to fifty percent of patients will develop persistent pulmonary sarcoidosis for which no approved treatments are currently available and corticosteroid use is the standard, non-specific treatment method (4).

Although mechanisms underlying the development of sarcoidosis are currently unknown, it is hypothesized to be caused by an aberrant host immune response to unknown environmental antigens in genetically predisposed individuals. A number of alterations in sarcoidosis patients' immune responses has been reported including, an exaggerated Th1 response, increased Th17 activity, attenuated regulatory T cell responses and oligoclonal expansion of CD4+ T cell responses, which is consistent with chronic antigenic stimulation (5-9).

To date, no specific pathogen has been identified as a causative factor in sarcoidosis. Several studies have a role for mycobacterial or propionibacterial organisms in the pathogenesis of sarcoidosis. Specifically, a meta-analysis of studies carried out between 1980 and 2006 demonstrated that 26% of all sarcoidosis tissues had evidence of mycobacterial nucleic acids (10). In the context of viral infection, seroepidemiological studies have demonstrated significant levels of antibodies to Epstein-Barr virus (EBV), rubella and parainfluenza 3 in sarcoidosis (11). However, no correlation could be made between viral antibody titre and stage of disease or activity (11).

In this study, we investigated the role of defective TLR3 in the development of persistent clinical disease in pulmonary sarcoidosis. TLR3 has previously been shown to bind dsRNA from viruses, bacteria and helminths, respectively, in addition to mRNA released from necrotic cells (2, 12-15). Specifically, here we investigated the role of the TLR3 polymorphism, Leu412Phe (*TLR3* Leu412Phe, L412F; rs3775291) as a causative factor in the development of and in disease persistence in pulmonary sarcoidosis, respectively. Previously, we identified a role for *TLR3* L412F in accelerated disease progression and increased risk of mortality in idiopathic pulmonary

fibrosis (IPF) (16). *TLR3* L412F has also been implicated as a causative factor in a number of autoimmune and inflammatory diseases such as diabetes, systemic lupus erythematosus (SLE) and rheumatoid arthritis (17-19), as well as a variety of cancers (20-24). *TLR3* L412F has also been demonstrated to have either a protective or pathogenic effect in viral infection (25, 26).

In this study, we report a significant association between development of a persistent clinical phenotype in pulmonary sarcoidosis and the *TLR3* L412F variant in cohorts of Irish and American Caucasians, respectively. Furthermore, activation of TLR3 *in vitro* in primary human lung fibroblasts from 412F-homozygous patients resulted in decreased TLR3 and IFN- β expression, reduced apoptosis and dysregulated proliferation, respectively, compared with fibroblasts from TLR3 wild-type patients. Our findings imply that defective TLR3 promotes a persistent disease phenotype in sarcoidosis and reveals *TLR3* L412F as a candidate prognostic biomarker in this interstitial lung disease.

MATERIALS AND METHODS

Study Subjects

A cohort of Irish Caucasian pulmonary sarcoidosis patients (n=228; **Table 1**) was recruited from St Vincent's University Hospital, Elm Park, Dublin 4 (SVUH). A cohort of Irish Caucasian healthy volunteers (n = 263) was additionally recruited as a control group. Genomic DNA was obtained from the American cohort of sarcoidosis patients attending the specialized Sarcoidosis Clinic at Johns Hopkins University School, Baltimore, MA, USA (n=123; **Table 1**).

Diagnosis of pulmonary sarcoidosis and classification of persistent disease

Irish and American pulmonary sarcoidosis patients were diagnosed at initial hospital presentation by the same physician (S.C.D. and D.R.M), respectively, and followed-up for at least 2 years (see **Supplementary Methods**). Patients at 2 years follow-up were classified as having either “persistent” disease or “non-persistent” disease based on a modification of a system previously described (2)(see **Supplementary Methods**).

***TLR3* L412F genotyping**

TLR3 L412F genotyping was carried out as described by us previously in a parallel study investigating the role of the *TLR3* L412F in IPF (16)(see **Supplementary Methods**).

Analysis of *TLR3* L412F functionality in primary human lung fibroblasts from pulmonary sarcoidosis patients

Primary fibroblast cell lines were isolated from lung biopsies of sarcoidosis patients (supplied by SVUH) and cultured as described previously (27) (see **Supplementary Methods**). Methodology pertaining to the analysis of the effects of *TLR3* L412F on fibroblast-apoptosis, -proliferation and -cytokine/interferon production, respectively, is detailed in the **Supplementary Methods** section.

Statistical analysis

All statistical analyses were carried out using GraphPad Instat Software (GraphPad Software Inc. CA, USA). Statistical analyses of genotype and allele frequencies were performed using two-tailed χ^2 tests (3 x 2 χ^2 tests for independence and trend, respectively, or 2 x 2 χ^2 test where

appropriate) or 2 x 2 Fisher's exact tests if the χ^2 test was inappropriate. Forward, stepwise logistic regression analysis was carried out to obtain corrected *P* values for appropriate confounders. One-way analysis of variance (ANOVA) was used to test for statistical significance (two-tailed analysis) between experimental groups of three. Multiple comparisons between groups were then assessed using the Tukey-Kramer *post-hoc* test (for parametric analysis) or Dunn's *post-hoc* test (for non-parametric analysis). Statistical significance was recorded at $p < 0.05$.

RESULTS

***TLR3* L412F (rs3775291) is not associated with development of pulmonary sarcoidosis in Irish patients**

In this study, we tested for an association between *TLR3* L412F and development of pulmonary sarcoidosis in an Irish case-control study of 263 control subjects and 228 sarcoidosis cases (**Table 2**). L412F genotype frequencies in the Irish control group and Irish sarcoidosis group were determined to be consistent with Hardy-Weinberg Equilibrium (HWE; controls: $P=0.32$; cases: $P=0.72$). No significant association was found between development of sarcoidosis and the L412F genotypes [$P=0.6326$; Odds ratio (OR) for 412F (Phe) carriers: 1.105 (95% C.I.: 0.774-1.578); OR for Phe/Phe homozygotes (Phe/Phe): 0.801 (C.I.: 0.387-1.660); **Table 2**] or allele frequency [$P=0.8942$; OR: 1.003 (C.I.: 0.774-1.372); **Table 2**]. This indicates that the variant allele does not strongly promote development of the disease in an Irish sarcoidosis population.

***TLR3* L412F promotes a persistent clinical phenotype in Irish patients with pulmonary sarcoidosis**

We then tested for an association between *TLR3* L412F and disease persistence in sarcoidosis. Patients were defined as having either “persistent” ($n=104$) or “non-persistent” ($n=124$) disease at 2 years post-diagnosis according to established criteria (2) (**Table 2**). We observed a significant association between development of a persistent disease phenotype in Irish sarcoidosis patients and 412F homozygosity [$P=0.0095$; OR for Phe/Phe homozygotes: 5.762 (C.I.: 1.594-20.284)], **Table 2**) and F allele frequency [$P=0.0166$; OR: 1.713 (C.I.: 1.122-2.617); **Table 2**]. This suggests that *TLR3* 412F homozygosity may be a useful prognostic biomarker in sarcoidosis.

***TLR3* L412F is associated with disease persistence in American Caucasians with sarcoidosis**

To test for replication of the association between *TLR3* L412F and disease persistence in Irish sarcoidosis patients, we carried out a validation study in an American cohort of sarcoidosis patients. We genotyped 123 genomic DNA samples from patients attending a tertiary referral centre for sarcoidosis (**Table 1**). We found a significant association between L412F heterozygosity [$P=0.0432$; OR for Phe carriers: 3.535 (C.I.: 1.246-10.029); **Table 3**) and F allele

frequency [$P=0.0221$; OR: 2.836 (C.I.: 1.129-7.122); **Table 3**], respectively, and development of persistent disease in the overall American population. Further analysis of individual Caucasian American and African American cohorts revealed a significant association between disease persistence and 412F heterozygote genotype [$P=0.0114$, OR for Phe carriers: 4.4 (CI: 1.411-13.717); **Table 3**] and allele frequency [$P=0.0205$, OR: 3.175 (C.I.: 1.207-8.351); **Table 3**], respectively, in Caucasian Americans but not African Americans.

Primary lung fibroblasts from *TLR3* L412F homozygote sarcoidosis patients produce reduced IL-8 and IFN- β , and have reduced *TLR3* expression

In order to elucidate the mechanisms underlying the association between development of persistent disease and *TLR3* 412F, we investigated *TLR3* function in primary human fibroblasts from *TLR3* 412F wild-type (Leu/Leu) versus homozygous (Phe/Phe) sarcoidosis patients with a persistent disease phenotype. Following *TLR3* activation by Poly(I:C) treatment, variant Phe/Phe fibroblasts had significantly reduced IL-8 production (**Fig. 1A**; NF- κ B-readout) and IFN- β expression (**Fig. 1B**; IRF3-readout), respectively. These findings conferred with the authors who first described the polymorphism and who reported that it resulted in defective *TLR3* function via reduced NF- κ B and IRF3 signaling (28). In addition, following *TLR3* activation by Poly(I:C) treatment, variant Phe/Phe fibroblasts also had blunted *TLR3* mRNA expression compared with wild-type fibroblasts (**Fig. 1C**). We have also shown additionally using FACS that the upregulation of extracellular *TLR3* following Poly(I:C) treatment on Phe/Phe primary lung fibroblasts was blunted compared with wild-type cells (**Supplementary Fig. S1A**). In contrast, levels of intracellular *TLR3* expression on wild-type and Phe/Phe fibroblasts were comparable following Poly(I:C) treatment (**Supplementary Fig. S1B**).

***TLR3* L412F inhibits apoptosis and dysregulates proliferation in primary lung fibroblasts from homozygote sarcoidosis patients**

Here we assessed the induction of *TLR3*-induced apoptosis in Leu/Leu and Phe/Phe fibroblasts and found a significantly reduced ability of Phe/Phe fibroblasts to undergo late-phase apoptosis compared with wild-type cells (**Fig. 2A, B**). Furthermore, we also observed a decreased ability of Phe/Phe cells to reduce their proliferation in response to Poly(I:C) compared with Leu/Leu cells (**Fig. 3A**). Interestingly, a comparable level of reduction in fibroproliferation was observed

following treatment of primary human lung fibroblasts for 24 h with 1000 I.U./ml IFN- β in cells from Leu/Leu and Phe/Phe patients (**Fig. 3B**). This result suggests that the dysregulated fibroproliferation seen in TLR3 defective, Phe/Phe fibroblasts may be due to their reduced ability to produce IFN- β .

DISCUSSION

In this study, we investigated the role of defective TLR3 in the pathogenesis of pulmonary sarcoidosis. Specifically, we investigated the role of the TLR3 polymorphism, *TLR3* L412F (rs3775291), as a causative factor in the development of, and in disease persistence in pulmonary sarcoidosis, respectively. Previously, we identified a role for *TLR3* L412F in accelerated disease progression and increased risk of mortality in idiopathic pulmonary fibrosis (IPF) (16). In this study, we established that *TLR3* L412F was not associated with development of pulmonary sarcoidosis but was significantly associated with disease persistence. Irish patients who presented with sarcoidosis, and who were *TLR3* 412F-homozygous, were significantly more likely to develop persistent disease. Therefore, these findings suggest that *TLR3* L412F plays a broader role in interstitial lung disease and that its pathogenic effects are not limited to IPF.

In this study, the association between *TLR3* L412F and disease persistence in Irish pulmonary sarcoidosis patients was validated in a modest-sized American cohort of patients, attending a tertiary referral centre. American pulmonary sarcoidosis patients who had one copy of the variant allele were almost five times more likely to develop persistent disease. Interestingly, there was also a significant association between disease persistence and race. When Caucasian American and African American populations were analysed individually, a significant association was found between the 412F allele and disease persistence in Caucasian Americans but not in African Americans. This may reflect specific genetic backgrounds in Caucasian Americans compared to African Americans which results in different spectrums of disease presentations in both populations. These novel results merit further investigation using larger cohort-size.

Here, we also report that primary lung fibroblasts from *TLR3* 412F-homozygous pulmonary sarcoidosis patients had reduced IL-8, IFN- β and TLR3 production or expression, reduced fibroblast apoptosis and dysregulated fibroproliferative responses compared with cells from wild-type patients, following TLR3-activation. Our findings imply that defective TLR3 promotes a persistent disease phenotype in sarcoidosis by dysregulating apoptotic and fibroproliferative processes via an IFN- β -dependent process. Thus, this study identifies defective TLR3 function as a previously undescribed factor in the development of persistent clinical disease in pulmonary sarcoidosis and reveals the TLR3 signaling pathway as a novel therapeutic target in its treatment. (see **Supplementary Fig. S2** for schematic of proposed mechanism). The

strength of this study lies in its exploration of the role of the change in primary lung fibroblast function in disease persistence in pulmonary sarcoidosis patients, in the context of the *TLR3* L412F polymorphism. In this study, primary lung fibroblasts from three patients from each respective genotype were utilised (i.e. *TLR3* Leu/Leu wild-type and *TLR3* Phe/Phe homozygote patients). In future studies to investigate the role of *TLR3* L412F further in the pathogenesis of persistent pulmonary sarcoidosis, and as a candidate prognostic marker, additional lung fibroblasts from wild-type and homozygote patients will be recruited. To date, no specific pathogen has been identified as a causative factor in sarcoidosis. Several studies have a role for mycobacterial or propionibacterial organisms in the pathogenesis of sarcoidosis. Previously, a human herpes 8 (HHV-8) open reading frame DNA was detected in a significantly higher proportions of sarcoid- compared with non-sarcoid lung tissue (29). However, the role of these viruses, and of EBV particularly, in the etiology of sarcoidosis remains speculative (11, 29). *TLR3* was originally identified as an anti-viral receptor and was shown to bind viral dsRNA (12). More recently, the role for *TLR3* in microbial infection has been expanded. *TLR3* is now known to additionally bind dsRNA from bacteria and helminths (13, 14). It has also been shown to bind mRNA which has been released from necrotic cells during infection and inflammation (15). In this study, the defective function associated with the effects of *TLR3* L412F in cells from 412F-homozygous patients would provide a mechanism by which bacterial or viral infection could promote a persistent clinical phenotype in sarcoidosis.

In this study, we also observed that reduced IFN- β expression or production is one of the mechanisms by which *TLR3* L412F mediates its effects. In the context of IFN- β , this has been shown to directly induce apoptosis in cells following *TLR3* activation in an autocrine manner (30). Interestingly, the addition of IFN- β to *in vitro* cultures of our 412F-homozygous cells in our study resulted in a restoration to a wild-type proliferative phenotype. With the current therapeutic use of IFN- β in a variety of cancers and autoimmune disorders, our data would support the therapeutic targeting of sarcoidosis patients exhibiting the 412F-homozygous genotype with IFN- β . Therefore, we suggest that treatment of 412F-homozygous sarcoidosis patients with persistent clinical disease with recombinant IFN- β may represent a novel treatment regimen. Other authors have reported the induction of IFN- α -induced sarcoidosis in a patient being treated for hepatitis C and the development of sarcoidosis in multiple sclerosis and myeloma patients, respectively, following recombinant IFN- β treatment (31-34). However,

previously, Charlier *et al.* examined a series of four patients with sarcoidosis, treated by IFN- α or IFN- β for viral hepatitis or multiple sclerosis. Interestingly, no recurrence or exacerbation of sarcoidosis had occurred at 4 years of follow-up. This study series suggests that type I IFNs do not exacerbate sarcoidosis in remission and this makes their use possible if indicated. However, their effect in persistent forms of the disease needs further evaluation (35).

Sarcoidosis can follow a variable clinical course. Historically, it is well recognised that presentation with erythema nodosum and bilateral hilar adenopathy on chest radiograph has a better prognosis (15% risk of chronicity and progression) compared to presentation with bilateral chest radiograph infiltrates (40% chance of progression). However, we are unable to stratify, with a high degree of accuracy, the prognosis of individual patients at presentation. This is a significant clinical unmet need in clinical practice and in clinical trials design. It would be of significant advantage in clinical trials if we could enrich patient recruitment favoring a more aggressive clinical phenotype. This would offer the best opportunity of assessing whether specific, proposed therapies are clinically efficacious or not. This study reveals *TLR3* L412F as a candidate prognostic biomarker in pulmonary sarcoidosis.

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TABLE 1. Study Demographics for Irish and American Sarcoidosis Cohorts, and *TLR3* L412F (rs3775291) Genotypes.

	Irish Sarcoidosis cases			American Sarcoidosis Cases		
No.	228			123		
Sex, M/F	109/119			53/70		
Median age, year range	32 (16-66)			42 (18-72)		
	Leu/Leu	Leu/Phe	Phe/Phe	Leu/Leu	Leu/Phe	Phe/Phe
No.	127	85	16	91	28	4
Sex, M/F	53/74	48/37	8/8	37/54	13/15	3/1
Median age, year range	30 (17-65)	32 (16-63)	36 (24-66)	42 (18-65)	44 (28-75)	35 (23-38)

TABLE 2. *TLR3* L412F (rs3775291) Polymorphism Frequencies in Pulmonary Sarcoidosis: Irish Case-Control and Disease Persistence Studies.

Irish Case-Control Study				Irish Disease Persistence Study			
Status	Leu/Leu	Leu/Phe	Phe/Phe	Status	Leu/Leu	Leu/Phe	Phe/Phe
Control Subjects (n=263)	140 (0.53)	108 (0.41)	15 (0.06)	Persistent (n=104)	52 (0.50)	39 (0.37)	13 (0.13)
Irish Sarcoidosis Cases (n=228)	127 (0.56)	85 (0.37)	16 (0.07)	Non-Persistent (n=124)	75 (0.61)	46 (0.37)	3 (0.02)
<u>P values</u>				<u>P values</u>			
Genotype	^a 0.6326			Genotype	^f 0.0095 ^g (0.0157)		
Trend	^b 0.8350			Trend	^h 0.0133		
Allele	^c 0.8942			Allele	ⁱ 0.0166		
<u>Odds ratio (95% C.I.)</u>				<u>Odds ratio (95% C.I.)</u>			
Phe carrier	^d 1.105 (0.774-1.578)			Phe carrier	^j 1.531(0.904-2.592)		
Phe/Phe homozygote	^e 0.801 (0.387-1.660)			Phe/Phe homozygote	^k 5.762 (1.594-20.284)		

Irish Case-Control Study: L412F genotype frequencies did not differ significantly between control subjects and sarcoidosis cases: ^a χ^2 test for independence (2 d.f.) and ^b χ^2 test for trend (1 d.f.). Odds ratio (OR) and 95% confidence interval (C.I.) for ^dPhe carriers (Leu/Phe and Phe/Phe) and ^ePhe/Phe homozygotes. ^cAllele frequencies did not differ significantly between subjects and cases [χ^2 test: $P=0.8942$; OR: 1.003 (C.I.: 0.774-1.372)]. Irish Disease Persistence Study: a significant association was observed between *TLR3* L412F and disease persistence in Irish sarcoidosis cases. L412F genotypes were compared using: ^f χ^2 test for independence and ^h χ^2 test for trend, respectively. ^gLogistic regression analysis was performed to calculate the adjusted *P* value for the confounding factors: age at diagnosis, gender and erythema nodosum positivity. OR (C.I.) for ^jPhe carriers and ^kPhe/Phe homozygotes. ⁱAllele frequencies were compared using a χ^2 test [OR: 1.713 (C.I.: 1.122-2.617)].

TABLE 3. Replication study: *TLR3* L412F (rs3775291) Genotype and Disease Persistence in American Pulmonary Sarcoidosis cases.

Status	Leu/Leu	Leu/Phe	Phe/Phe
Persistent (n=82)	55 (0.67)	24 (0.29)	3 (0.04)
Non-persistent (n=41)	36 (0.88)	4 (0.10)	1 (0.02)
<u>P values (All U.S. cases)</u>		<u>Odds ratio (95% C.I.; All U.S. cases)</u>	
Genotype	^a 0.0432 ^b (0.0077)	Phe carrier	^c 3.535 (1.246-10.029)
Trend	^c 0.0278	Phe/Phe homozygote	^f 1.519 (0.153-15.082)
Allele	^d 0.0221		
<u>P values (Ethnicity)</u>	<u>Caucasians (n=71)</u>	<u>African Americans (n=52)</u>	
Genotype	^g 0.0114	^h 0.3070	
Allele	ⁱ 0.0205	^j 0.3204	
<u>Odds ratio (95% C.I.)</u>	<u>Caucasians</u>	<u>African Americans</u>	
Phe carrier	^k 4.400 (1.411-13.717)	^l 4.761 (0.2466-91.939)	
Phe/Phe homozygote	^m 1.902 (0.188-19.290)	ⁿ 4.761 (0.2466-91.939)	

A significant association was detected between *TLR3* L412F and disease persistence in an American cohort of sarcoidosis patients attending a tertiary referral centre. L412F genotypes were compared within the overall American (U.S.) cohort using: ^a3x2 χ^2 test for independence (2 d.f.) and ^c3x2 χ^2 test for trend (1 d.f.), respectively. OR (95% C.I.) for ^ePhe carriers and ^fPhe/Phe homozygotes in overall U.S. cohort. ^dAllele frequencies were compared using a 2x2 Fisher's Exact test (OR: 2.836; 95% CI: 1.129-7.122). ^bLogistic regression analysis was performed to calculate the adjusted *P* value, using the confounding factors: age at diagnosis, gender and race. A significant risk of disease persistence was conferred by L412F genotype ^b(*P*=0.0077). However, a significant risk of disease persistence was also conferred by Caucasian ancestry (*p*=0.0295) compared with African American ancestry. Therefore, further analysis was carried out for individual Caucasian and African American populations, respectively. A significant association was found between disease persistence and genotype in Caucasians but not African Americans: ^gGenotype frequencies and ^{l,j}allele frequencies were compared using 2x2 Fisher's Exact tests. OR (95% C.I.) for ^{k,l}Phe carriers and ^{m,n}Phe/Phe homozygotes for Caucasians and African Americans, respectively.

FIGURE LEGENDS

Figure 1. Effect of *TLR3* L412F on TLR3-induced IL-8, IFN- β mRNA and TLR3 mRNA in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients. *TLR3* L412F attenuates Poly(I:C)-driven (A) IL-8 production, (B) IFN- β mRNA and (C) TLR3 mRNA expression in primary human lung fibroblasts from Phe/Phe sarcoidosis patients compared with Leu/Leu patients at 24h post-treatment, as quantitated by ELISA and QPCR analysis, respectively. (A-C) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: Poly(I:C) 100 $\mu\text{g/ml}$ compared with Medium-only at 24h post-treatment. ++ $p < 0.01$: Poly(I:C) 100 $\mu\text{g/ml}$ in fibroblasts from Phe/Phe ($n=3$) compared with Leu/Leu patients ($n=3$). Results shown are the mean \pm S.E.M. of (B, C) three or (A) six replicates from a representative of (B) two or (A, C) three separate experiments.

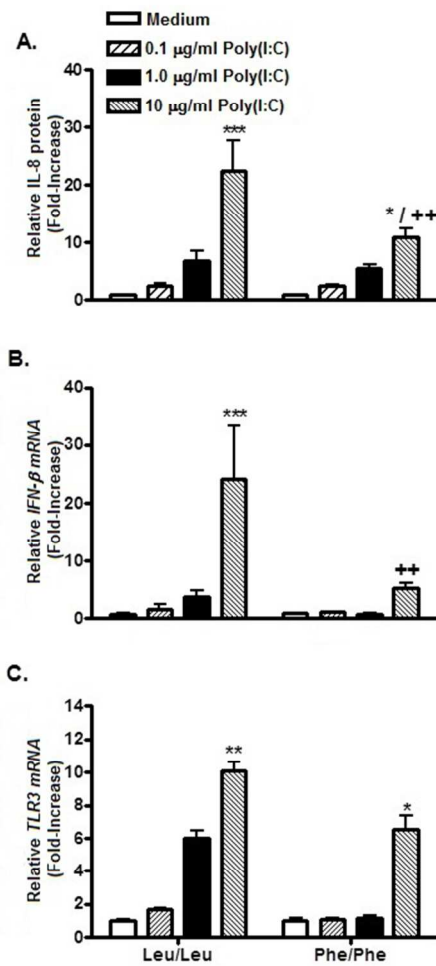
Figure 2. Effect of *TLR3* L412F on TLR3-induced apoptotic responses in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients.

A significant increase in Poly(I:C)-induced (A, B) late-phase apoptosis in Leu/Leu, but not Phe/Phe, fibroblasts following 24h treatment, as quantitated by Annexin V/Propidium Iodide staining using FACS analysis. (B) * $p < 0.05$, Poly(I:C) 100 $\mu\text{g/ml}$ compared with Medium-only. ++ $p < 0.01$: Treatment with 100 $\mu\text{g/ml}$ Poly(I:C) in fibroblasts from Phe/Phe ($n=3$) compared with Leu/Leu ($n=3$) Results shown are the mean \pm S.E.M. of (B) six or (A) five replicates from a representative of (A, B) three separate experiments.

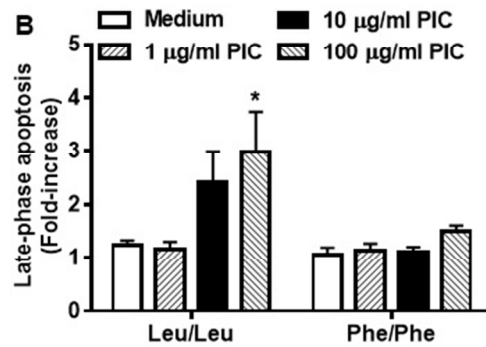
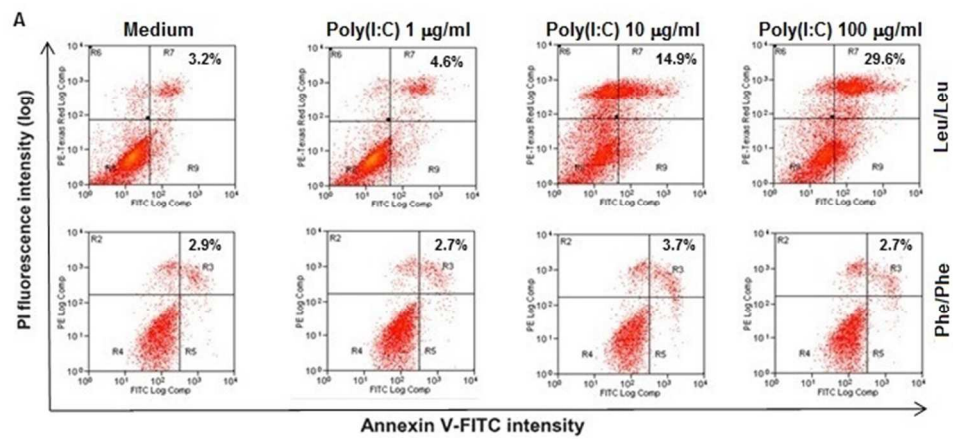
Figure 3. Effect of *TLR3* L412F on TLR3-induced proliferative responses in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients.

(A) Poly(I:C) treatment significantly reduces proliferation of Leu/Leu fibroblasts following 24h treatment with 1-100 $\mu\text{g/ml}$ Poly(I:C), as quantitated using ^3H -thymidine incorporation. The fold-decrease in Poly(I:C)-induced proliferation at 100 $\mu\text{g/ml}$ at 24h is significantly more in Leu/Leu compared with Phe/Phe fibroblasts, as quantitated using ^3H -thymidine incorporation. (B) Reconstitution of Phe/Phe cells with recombinant human IFN- β (1000 I.U./ml) leads to an equivalent reduction in cell proliferation in Leu/Leu and Phe/Phe cells. (A, B) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: Poly(I:C) 100 $\mu\text{g/ml}$ compared with Medium-only. ++ $p < 0.01$: Treatment

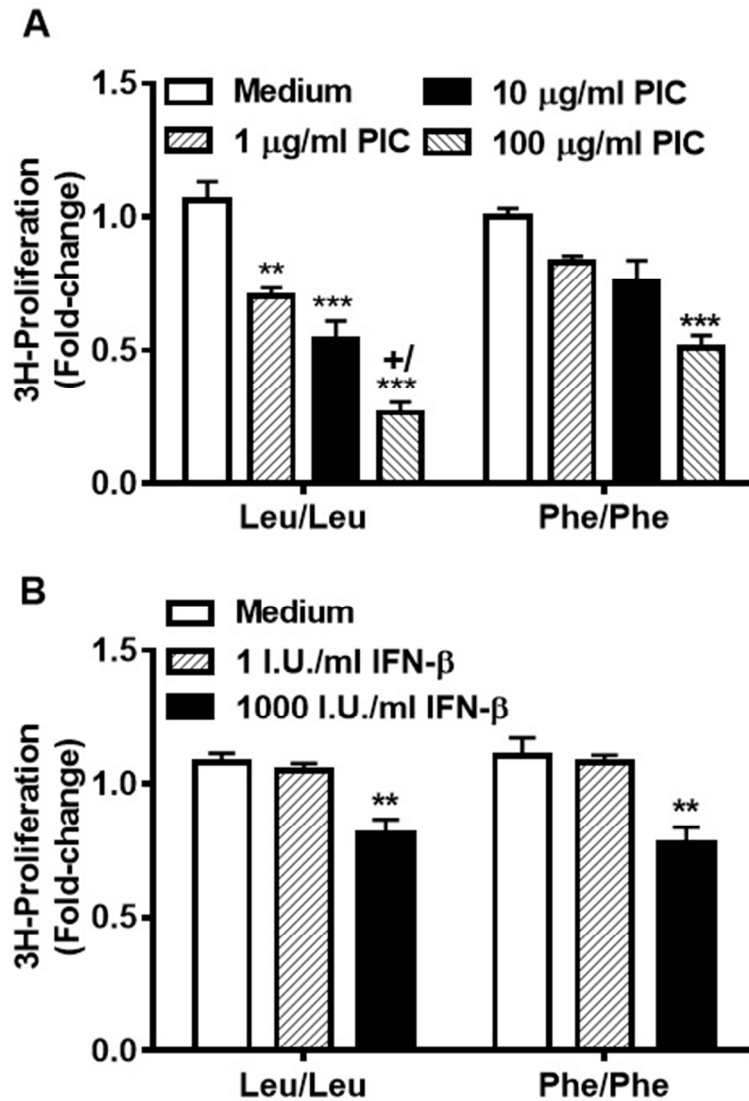
with 100 µg/ml Poly(I:C) in fibroblasts from Phe/Phe (n=3) compared with Leu/Leu patients (n=3). Results shown are the mean \pm S.E.M. of **(B)** six or **(A)** five replicates from a representative of **(A, B)** three separate experiments.



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ONLINE SUPPLEMENTARY MATERIAL

Title: Toll-like receptor 3 L412F polymorphism promotes a persistent clinical phenotype in pulmonary sarcoidosis

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MATERIALS AND METHODS

Diagnosis of pulmonary sarcoidosis in Irish and American cohorts

All Irish and American patients were assessed at initial hospital presentation by the same physician, respectively (Ireland: S.C.D, SVUH; US: D.R.M., JHU) and followed up for at last 2 years. A tissue diagnosis was obtained in 90% of patients via transbronchial / bronchial / skin / mediastinal or video assisted thoracoscopic (VATS) biopsies. The remaining patients presented with a compatible clinical presentation, associated with supportive radiological and bronchoalveolar (BAL) findings (i.e. CD4/CD8 ratio > 3.5). Disease severity in sarcoidosis was assessed using chest radiographic staging. Chest radiographs (staged 0–IV, *i.e.* normal to fibrosis), and/or high-resolution computed tomography, determined the presence or absence of a pulmonary infiltrate or fibrosis. All patients were classified at presentation on chest radiograph based on the Scadding staging system (1). Forced expiratory volume in one second, forced vital capacity and transfer factor were also measured at recruitment and expressed as % predicted, according to international guidelines (2).

Classification of persistent or non-persistent pulmonary sarcoidosis at 2 years post-diagnosis

Irish and American pulmonary sarcoidosis patients at 2 years follow-up were classified as having either “persistent” disease or “non-persistent” disease based on a modification of a system previously described (2). Briefly, persistent disease at 2 years follow-up was defined as: (a) patients who were Scadding stage II or III on chest radiograph with associated abnormal pulmonary function parameters (FVC and/or total lung capacity and or transfer factor < 80% of predicted values), (b) Patients who were Scadding stage IV on chest radiograph, (c) patients who were prescribed corticosteroids. Corticosteroids were prescribed for patients exhibiting a significant deterioration in pulmonary physiological parameters (DLCO decrease > 15%) associated with radiographic progression (2).

Culturing of primary fibroblasts from lung biopsies from patients

Primary fibroblast cell lines were isolated from lung biopsies of sarcoidosis patients (supplied by SVUH) as follows. Briefly, lung tissue was mechanically separated into single-cell preparation and contaminating red blood cells (RBC) were lysed with hypotonic buffer (150 mM NH₄Cl, 10

mM NaHCO₃, 1 mM EDTA) for 2 min at 4°C. The remaining cells were added to 175 ml tissue culture flasks. These cells were grown at 37°C in a humidified CO₂ incubator and fed DMEM containing 1% (v/v) antibiotic-antimycotic and 15% (v/v) FBS twice weekly. All fibroblast cell lines were isolated by serial passaging (up to five times) until a >99% fibroblast population was determined using morphological and immunohistochemical staining as previously described, to ensure the relative absence of other contaminating cell types such as epithelial cells and myofibroblasts. For *in vitro* studies, all cell lines were used between passages 3 and 8.

***TLR3* L412F genotyping**

Genomic DNA was extracted from (i) anticoagulated whole blood collected in EDTA from cases and controls and (ii) primary human lung fibroblasts from patients with pulmonary sarcoidosis, respectively, using Qiagen Genomic DNA extraction Kits (QIAGEN Ltd, Crawley, UK). 10 ng/μl DNA samples were genotyped for *TLR3* L412F using an Applied Biosciences Genotyping Assays at Medical Solutions Plc (Source BioScience, Nottingham, U.K.) according to manufacturer's instructions and as described by us previously (3).

Annexin V/Propidium Iodide Staining

Primary human lung fibroblasts were plated out at 100,000 cells/wells in a 6-well plate overnight. Following treatment with 1-100 μg/ml Poly(I:C) for 24h, cells were harvested and were prepared for Annexin V/Propidium Iodide staining [Annexin V-FITC Apoptosis Detection Kit II (Calbiochem); Propidium Iodide (Merck Chemicals Ltd. Nottingham, U.K.)] as described previously (4). Stained samples were analyzed by FACS (BD Facscan Flow Cytometer, BD Bioscience, Oxford, U.K.).

Proliferation assays

Primary human lung fibroblasts were plated out at 100,000 cells/well in a 24-well plate overnight. Cells were then treated for 24h with 1-100 μg/ml Poly(I:C). After 16h of this treatment, 0.5 μCi/well [³H] thymidine (Amersham Biosciences, Buckinghamshire, U.K.) was added for an additional 8 h of culture. Cells were then harvested and proliferation was assessed by [³H]thymidine incorporation as described previously (5). Fibroblasts were treated with 1000 I.U./ml recombinant human IFN-β for 24h where described.

Determination of cytokine levels by ELISA

Concentrations of IL-8 and RANTES were determined in cell supernatants by ELISA (DuoSet; R&D Systems, MN, USA) according to manufacturer's instructions, as carried out previously (6).

Quantification of IFN- β mRNA and TLR3 mRNA expression by quantitative real-time PCR (QPCR)

For QPCR analysis, primary human lung fibroblasts were plated out at 100,000 cells/well in a 24-well plate overnight. Cells were then treated for 24h with 0.1-10 μ g/ml Poly(I:C) before harvesting. Three wells of a 24-well plate were pooled per sample for total RNA extraction per treatment using TRI REAGENT™ (Sigma) according to manufacturer's instructions. First-strand cDNA synthesis and subsequent QPCR for IFN- β mRNA expression was performed (Stratagene MX3000P Real-Time PCR System; Stratagene, CA, USA) as described previously (5).

Quantitation of intracellular and extracellular TLR3 protein expression on in primary pulmonary fibroblasts by flow cytometry analysis.

TLR3 protein surface expression was examined by flow cytometry analysis using a Beckman Coulter Cyan™ADP flow cytometer. In brief, cells were cultured in 24 well culture plates and treated with incremental doses of poly(I:C) for 24 h. Cells were washed with sterile PBS and dissociated using 200 μ l of Trypsin-EDTA at 37°C. DMEM was added to neutralise Trypsin-EDTA. Two wells were pooled for an approximate total of 100,000 cells/ml. Cells were centrifuged at 1000rpm to pellet cells. The supernatant was discarded and the washing process was repeated twice more. For extracellular TLR3 protein staining the cell pellet was suspended in binding buffer to an approximate final concentration of $1-5 \times 10^6$ cells/ml. This consisted of 5% fetal calf serum solution in PBS at 4°C. Subsequently, 199 μ l of cell suspension was transferred to a clean tube and add 1 μ l TLR3 antibody labelled with FITC Mouse anti human (ab45053). Then samples were mixed gently and incubated for 20 min at room temperature. The cells were washed with 1X binding buffer and centrifuge at 1000 rpm to pellet the cells. The supernatant was discarded and cells were re-suspended gently in 300 μ l and analysed by flow cytometry or fluorescence microscopy. Software utilised for analysis was Summit 4.3 (Daco).

FIGURE LEGENDS

Figure S1. Effect of *TLR3* L412F on Poly(I:C) induced intracellular and extracellular TLR3 expression in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients. (A) A significant increase in Poly(I:C)-induced extracellular TLR3 expression was observed in Leu/Leu, but not Phe/Phe, fibroblasts following 24h treatment, as quantitated using FACS analysis. (B) Increased Poly(I:C)-induced intracellular TLR3 expression in Leu/Leu and Phe/Phe fibroblasts following 24h treatment, as quantitated using FACS analysis and expressed as fold-increase in mean channel fluorescence (MCF) in treated cells compared with medium-only cells. ** $p < 0.01$, *** $p < 0.001$: Poly(I:C) 10 and 100 $\mu\text{g/ml}$ compared with Medium-only, respectively. Results shown are the mean \pm S.E.M. of three replicates from a representative of two separate experiments.

Figure S2. Schematic of the putative effects of the *TLR3* L412F polymorphism in the pathogenesis of self-limiting and persistent pulmonary sarcoidosis via an-IFN- β dependent mechanism. The results from this study have led us to the hypothesis that when pulmonary sarcoidosis is caused in *TLR3* L412F wild-type patients ($\text{TLR3}^{\text{Leu/Leu}}$) by an unknown TLR3-dependent antigen (e.g. viral or bacterial dsRNA, or mRNA released from necrotic cells during inflammatory damage), this leads to the activation of TLR3 and subsequent production of IFN- β , increased fibroapoptosis and decreased fibroproliferation, respectively. This stabilizes the fibroblast pool and results in a self-limiting pulmonary sarcoidosis in *TLR3* L412F wild-type patients. In contrast, the induction of pulmonary sarcoidosis by an unknown TLR3-dependent antigen in *TLR3* L412F defective patients ($\text{TLR3}^{\text{Phe/Phe}}$) leads to significantly lower activation of the TLR3 signalling pathway, with a subsequent production of low levels of IFN- β production, a failure to induce fibroapoptosis and to reduce fibroproliferation, respectively. This results in the development of a persistent clinical phenotype in *TLR3* L412F defective patients and progressive pulmonary sarcoidosis.

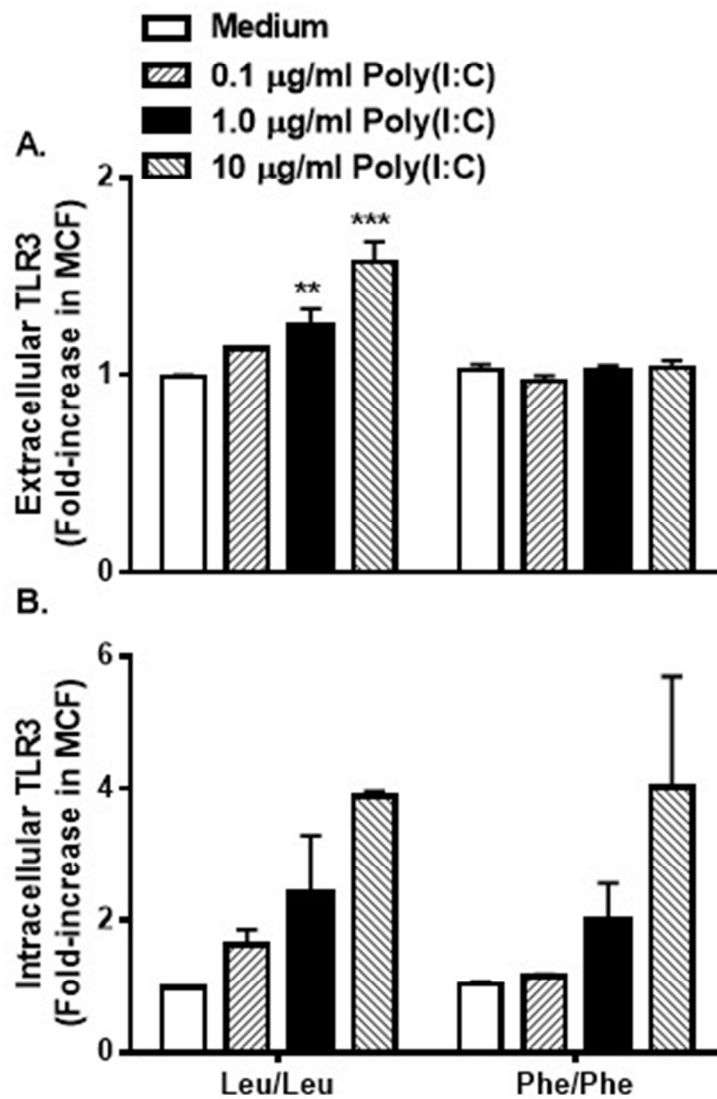


Fig. S1

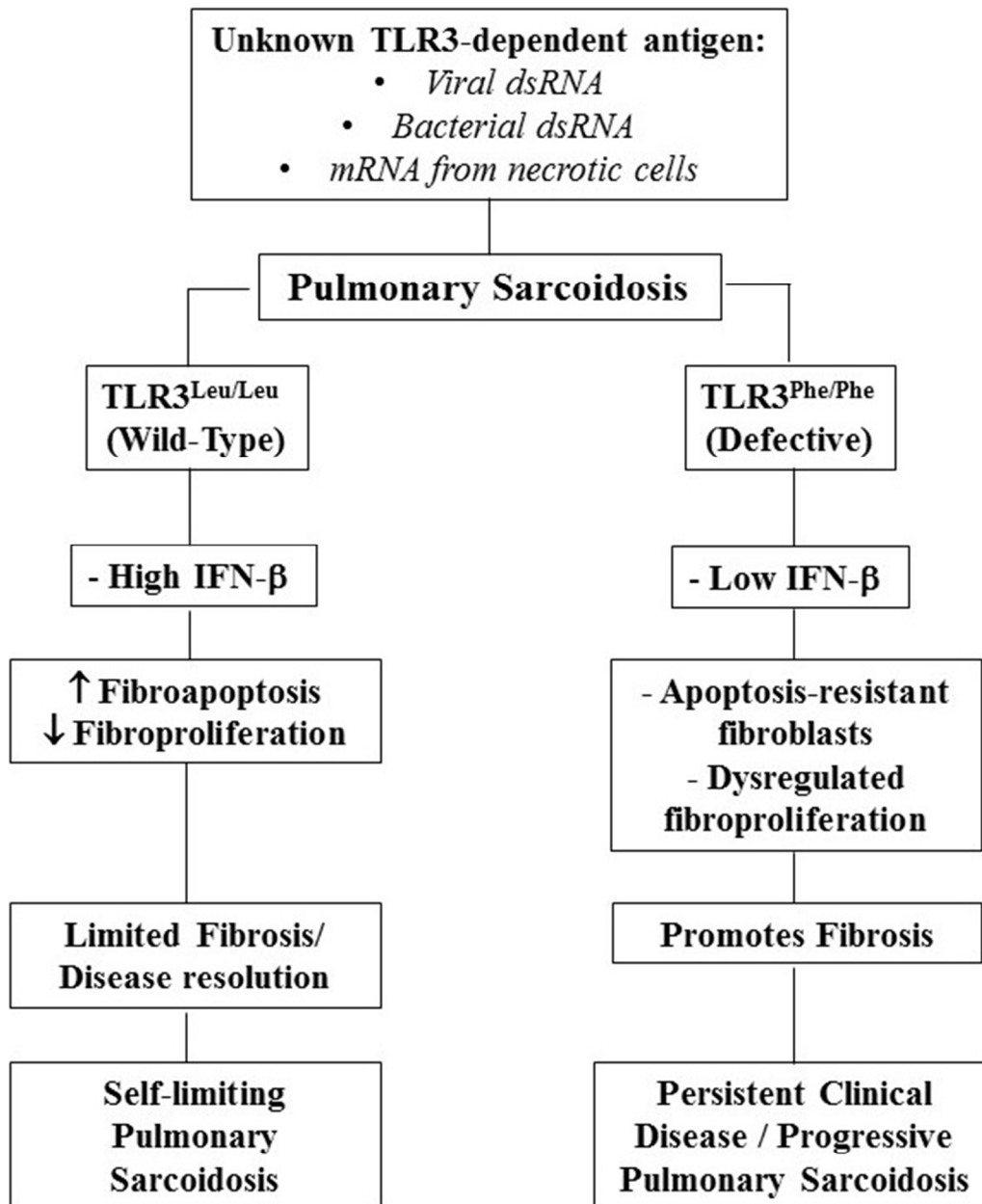


Fig. S2

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