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Article type : Letter to the Editor

Antibody and T cell responses against avian and microbial antigens associate with hypersensitivity pneumonitis disease parameters in pigeon breeders

To the Editor,

Pigeon Fancier's Lung (PFL) is a form of Hypersensitivity Pneumonitis where lung pathology occurs due to an immune reaction to repeated inhalation of large amounts of feather-derived and droppings-derived dust [1]. The 1μm dust particles penetrate the lower respiratory tract, initiating characteristic granulomatous inflammation in distal airways and alveoli. The particles, which carry pigeon antigens, cause irritation, and likely contain microbial components, both of which enhance immunogenicity. PFL affects ~10% of pigeon breeders in the UK, with acute manifestations of antigen exposure including fever, sweating, headaches, coughing and dyspnoea, beginning 4-8 hours following exposure to dust, and resolving 24-48 hours after removal. Sustained antigenic exposure may lead to chronic disease comprising interstitial lung disease with fibrosis, that reduces lung capacity and compliance, measurable by lung function tests, and presents as ground-glass opacities on X-ray. Chronic disease can develop with intermittent acute episodes, and determinants of both aspects are difficult to disentangle. Pigeon breeders typically possess high levels of serum IgG antibodies specific for pigeon antigens, but these are seldom directly associated with disease presence or severity. A proposed antibody-mediated disease pathway involves the formation of immune complexes between inhaled pigeon antigens and alveolar IgG, which activate complement and phagocytes in a local type-III hypersensitivity reaction. Another mechanism of immunopathogenesis is type-IV hypersensitivity involving pigeon-antigen-reactive T cells of the T_{h1} (IFNγ-secreting) phenotype. Histological findings and bronchoalveolar lavage confirm lymphocytic infiltration (including $CD4^+$ and $CD8^+$ T cells) and high pro-inflammatory cytokine concentrations [1-3]. Pigeon-antigen-specific proliferative T cell responses are not associated with disease status [4] despite genetic association of HLA-DR with PFL suggesting $CD4^+$ T cell involvement [5]. Detailed analysis of reactive T cell phenotypes in PFL is limited, whilst T_{reg} appear reduced in PFL [6]. A role of lung microbial infections in exacerbating PFL and other lung conditions is suggested [7].

In this study we hypothesised that measurable specific immune responses would associate with PFL disease parameters, and so we aimed to measure these and to perform correlative and group-based analyses. At community-based pigeon shows, and with ethical approval (Supplement.M1), 113 pigeon breeder volunteers (Fig.1a) completed a questionnaire regarding nature and frequency of symptoms (generating an acute symptoms score), donated a blood sample, and underwent spirometry (Supplement M1). An attending respiratory physician categorized breeders into PFL^+ or PFL^- based on the presence of at least: one classic respiratory symptom and one classic systemic symptom; occurring repeatedly after pigeon contact. ELISA (Supplement.M4,Fig.SF3) measured plasma antigen-specific (pigeon-serum-antigen [PSA] or pigeon mucin [PM]), and microbe-specific, IgG antibody as titre or μg/ml; and antigen-specific subclass and IgG-isotype activity as absorbance. IFNγ ELIspot [8] measured blood T cell responses against antigens, as did flow cytometry for: activation markers and CFSE dilution; and multiplex assays for secreted cytokines. We found that symptom score was significantly greater in PFL^+ compared to PFL^- (Fig.1b), but was a subjective self-reported measurement, therefore it was tested for its relationship to objective spirometry. FEV_1/FVC%-predicted, a measure of obstructive and restrictive lung disease, correlated negatively with symptom score (Fig.1c), and was used in further analyses. Symptom score showed significant correlation (r=0.357,p=0.015) with plasma CC16 levels (Club cell activation Supplement.M7), indicating acute lung epithelial damage. FEV_1/FVC%-predicted correlated negatively with impulse-oscillometry DX5 expiratory flow limitation (r=-0.468, p<0.001), suggestive of interstitial lung disease [10]. Comparing the IgG antibody levels between PFL^+ and PFL^- breeders showed a significantly higher level in PFL^+ for both the μg/ml (Fig.1d) and the end-point titres (Fig.1e), but these were unable to differentiate disease because of inter-group overlap. Titres against PM showed no difference between the groups (Supplement.Fig.SF2a). An association of the antibody response with CRP (r=0.348, p=0.017) supports its relevance for acute inflammatory disease. IgG affinity/avidity against PSA and PM was not significantly different between PFL^+ and PFL^- (Supplement.Fig.SF4) nor were the low IgE levels (Supplement.Fig.SF2b,c). There was a differing pattern of antibody isotype response (Supplement.Fig.SF.5) against PSA and PM, with IgG1 predominating for PSA, and IgA for PM; and the levels of anti-PSA IgG1 (Fig.1f) and IgA (Fig.1g) were significantly higher in PFL^+ than PFL^-. For PM, significantly increased levels in PFL^+ over PFL^- were seen for IgG1 (Fig.1h) and IgG3 (Fig.1i). These antibody levels were tested in parallel by precipitin formation or immunofluorescence (Supplement.Fig.SF.1) and all correlated with each other (p<0.001). PSA and PM antigens showed limited cross-reactivity (Supplement.Fig.FS.6). The anti-PSA antibody values correlated albeit weakly with the symptom score (r=0.206, p=0.015)(Fig.2a), but correlated strongly negatively with FEV_1/FVC%-predicted (r=-0.524,p=0.0001)(Fig.2b). No such relationships were seen with the anti-mucin response (Supplement.Fig.SF.2d). The optimal T cell IFNγ

response was found with 1 in 100 dilution of PSA (Supplement.Fig.SF.7), and whilst minimal anti-PM T cell responses were detected, IFNγ responses against PSA were detected in the majority of breeders, but no IL-4, IL-5 or IL-17 ELIspot responses (Supplement.Fig.SF.8). The anti-PSA IFNγ responses did not differentiate PFL^+ from PFL^- (Fig.2d). Furthermore, the IFNγ responses did not correlate with symptom score (Fig.2e). There was however, a strong negative correlation between the FEV_1/FVC%-predicted and anti-PSA IFNγ T cell response (r= -0.537,p=0.0001)(Fig.2f), suggesting T cells worsen lung function. A moderate positive relationship was found between the anti-PSA antibody response and T cell responses (r=0.312,p=0.0009)(Fig.2g) suggesting inter-dependence of magnitude (T cell help for antibodies). T cell proliferation against PSA was measured by CFSE-dye-dilution following stimulation with PSA over 5 days. Proliferating cells were $CD4^+$, not $CD8^+$, the majority being $CD49d^+$ (α4 integrin, associated with lung homing)(Supplement.Fig.SF.9,11). $CD4^+$ T cell predominance is consistent with the antigen source being exogenous to antigen-presenting cells and presented by MHC Class II. T cells stimulated with PSA significantly upregulated CD69 (Fig.2h) and OX40 (Supplement.Fig.SF.10,11) compared to unstimulated $CD4^+$ T cells, but their phenotypes did not differ (CCR5, PD-1; Supplement.Fig.SF.11,12). Supernatants of PSA-activated PBMCs showed significant secretion of multiple cytokines, except for IL-4 (Supplement.Fig.SF.13). The highest was IL-10, at 3ng/ml, followed by IFNγ at 100pg/ml. Since IL-10 is a potent immunosuppressive cytokine, we investigated the relationship between IL-10 and IFNγ, and a positive relationship was seen (r=0.473,p=0.004)(Fig.2i) suggesting co-expression rather than mutual exclusion. Immune responses against a range of lung microbes were investigated (Supplement.Fig.SF.14). Of these, only the IgG response to Rhinovirus (a potential marker of exposure and severity [9]) was significantly higher in pigeon breeders than in healthy volunteers (Fig.2j), with a trend for increase in PFL^+ over PFL^-. The anti-rhinovirus antibody levels correlated negatively with symptom score (r= -0.391,p=0.007) (Fig.2k), suggesting that exposure to Rhinovirus is protective against acute PFL (potentially through T_{h2} promotion), but no relationship between anti-rhinovirus antibody and anti-PSA T cells response was seen (Fig.2l). Whilst chest x-ray would have aided disease definition, and is a limitation of this study; in PFL, PSA-specific antibodies correlated with acute disease (increased symptoms), whilst both antibodies and Th1 cells correlated with chronic disease (reduced lung function). Rhinovirus-exposure appears protective against acute disease. Thus antigen-specific immune responses are potential candidate biomarkers and treatment targets in PFL disease.

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Conflict of interest

The authors have no conflicts of interest in relation to this work.

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Figure Legends.

Figure 1. Lung function, symptoms and antibodies in pigeon breeders

a.Pigeon breeder information and demographics. b.Symptom score was significantly higher in PFL^+ than in PFL^- pigeon breeders (n=113,d=1.12). c.A weak but significant correlation was found between symptom score and FEV1/FVC%-predicted (n=67). Comparison between antibody levels for PFL^+ and PFL^- pigeon breeders: d.significant for PSA-specific IgG antibody levels (μg/ml)(n=111,d=0.32), e.significant for PSA-specific IgG titre (n=78,d=0.42). f.significant for PSA-specific IgG1 (n=76,d=0,65) and g.IgA (n=76,d=0.28). h.significant for PM-specific IgG1 (n=76,d=0.33) and i.IgG3 (n=76,d=0.042). Mean + SEM is given. PSA=pigeon-serum-antigen; PM=pigeon mucin. d=Cohen's effect size.

Figure 2. Antibodies and T cell responses against pigeon antigens and Rhinovirus

a.Anti-PSA antibody levels weakly correlated with symptom score (n=111), but showed a strong negative correlation (b) with FEV1/FVC%-predicted (n=67). c.T cell IFNγ responses showed no difference between PFL^+ and PFL^- (n=99). d.No correlation was seen between T cell response and symptom score (n=99). However, e.strong negative correlation was seen between T cell response and FEV1/FVC%-predicted (n=67). f.A moderate positive correlation was seen between T cell response and anti-PSA IgG antibody response (n=66). g.expression of CD69 was significantly raised on T cells stimulated with PSA (n=18). h. The relationship between IL-10 ng/ml (secreted from T cell) and T cell ELISpot IFNγ, both in response to PSA (n=30). i.IgG antibody responses against *Rhinovirus* were greater in PFL^+ and PFL^- than in healthy volunteers (HV)(n=34). Mean + SEM is shown. j. A negative correlation was seen between symptom score and anti-rhinovirus IgG titre in pigeon breeders (n=39). k.The relationship between anti-pigeon T cell IFNγ responses and anti-Rhinovirus IgG titre showed no correlation. Spearman correlation was carried out (n=28).

Figure 1

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Indicator	Description
Total number of volunteers	113
Number in sub-groups (year collected)	38 (2012), 39 (2010), 36 (2009)
Sex,Female/Male (%)	5 (4.4%)/ 108 (95.6%)
Age,Mean [Range]	59 [21-80]
Exposure Index, Mean [Range] (Pigeon No. x Years with pigeon x Hours with pigeon)	88104 [1120-1920000]
Diagnosis of PFL, +ve/-ve (%)	41 (36.3%)/ 72 (63.7%)
Volunteers using mask, Yes/No (%)	50 (44.2%)/ 63 (55.8%)
Mean of smoking status, Yes, current, EX)/ Non (non) (%)	36 (31.9%)/ 77 (68.1%)
FEV1/FVC % predicted mean (range) (n=67)	73.75 (14-113)

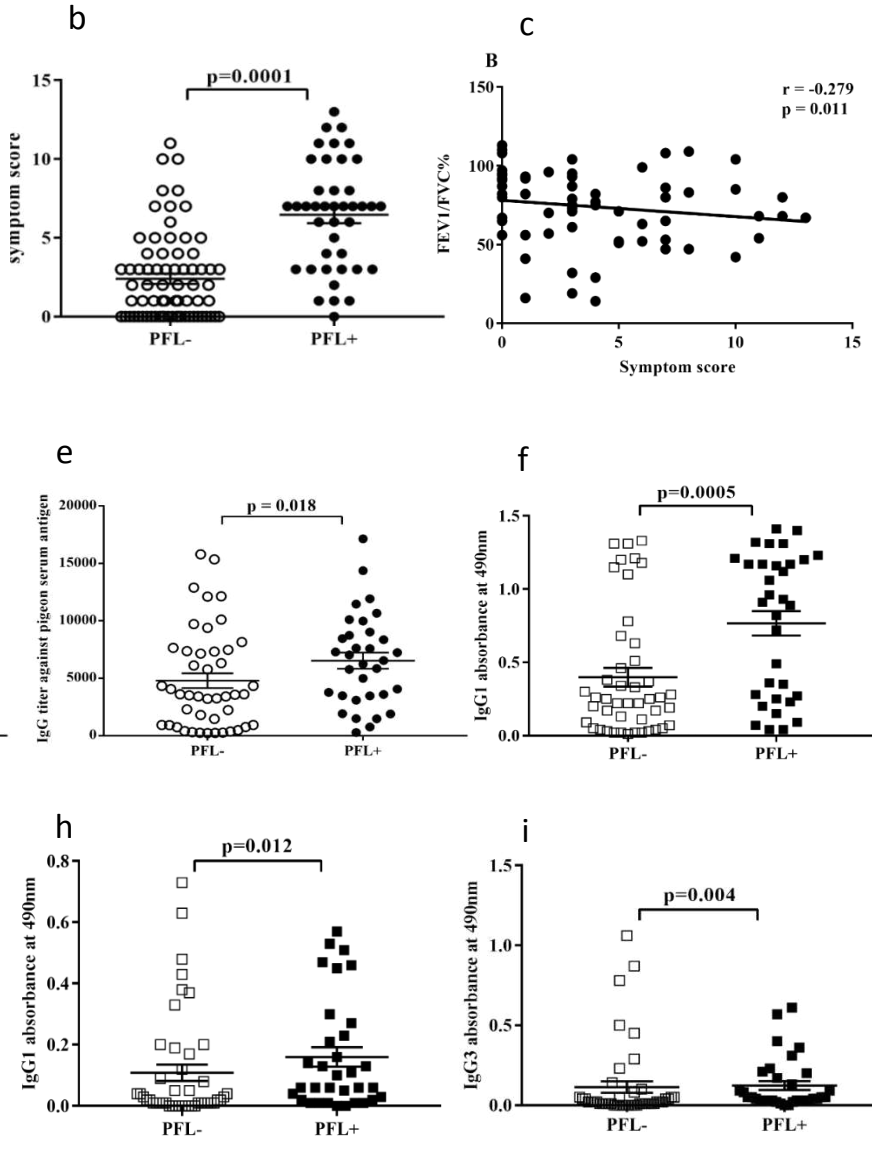

Figure 2