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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].

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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 $\mu\text{g/ml}$; and antigen-specific subclass and IgG-isotype activity as absorbance. IFN γ 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. FEV₁/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. FEV₁/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 $\mu\text{g/ml}$ (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 FEV₁/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 IFN γ

response was found with 1 in 100 dilution of PSA (Supplement.Fig.SF.7), and whilst minimal anti-PM T cell responses were detected, IFN γ 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 IFN γ responses did not differentiate PFL⁺ from PFL⁻ (Fig.2d). Furthermore, the IFN γ responses did not correlate with symptom score (Fig.2e). There was however, a strong negative correlation between the FEV₁/FVC%-predicted and anti-PSA IFN γ 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⁺ ($\alpha 4$ 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 IFN γ at 100pg/ml. Since IL-10 is a potent immunosuppressive cytokine, we investigated the relationship between IL-10 and IFN γ , 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 IFN γ 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 IFN γ , 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 IFN γ responses and anti-Rhinovirus IgG titre showed no correlation. Spearman correlation was carried out (n=28).

Figure 1

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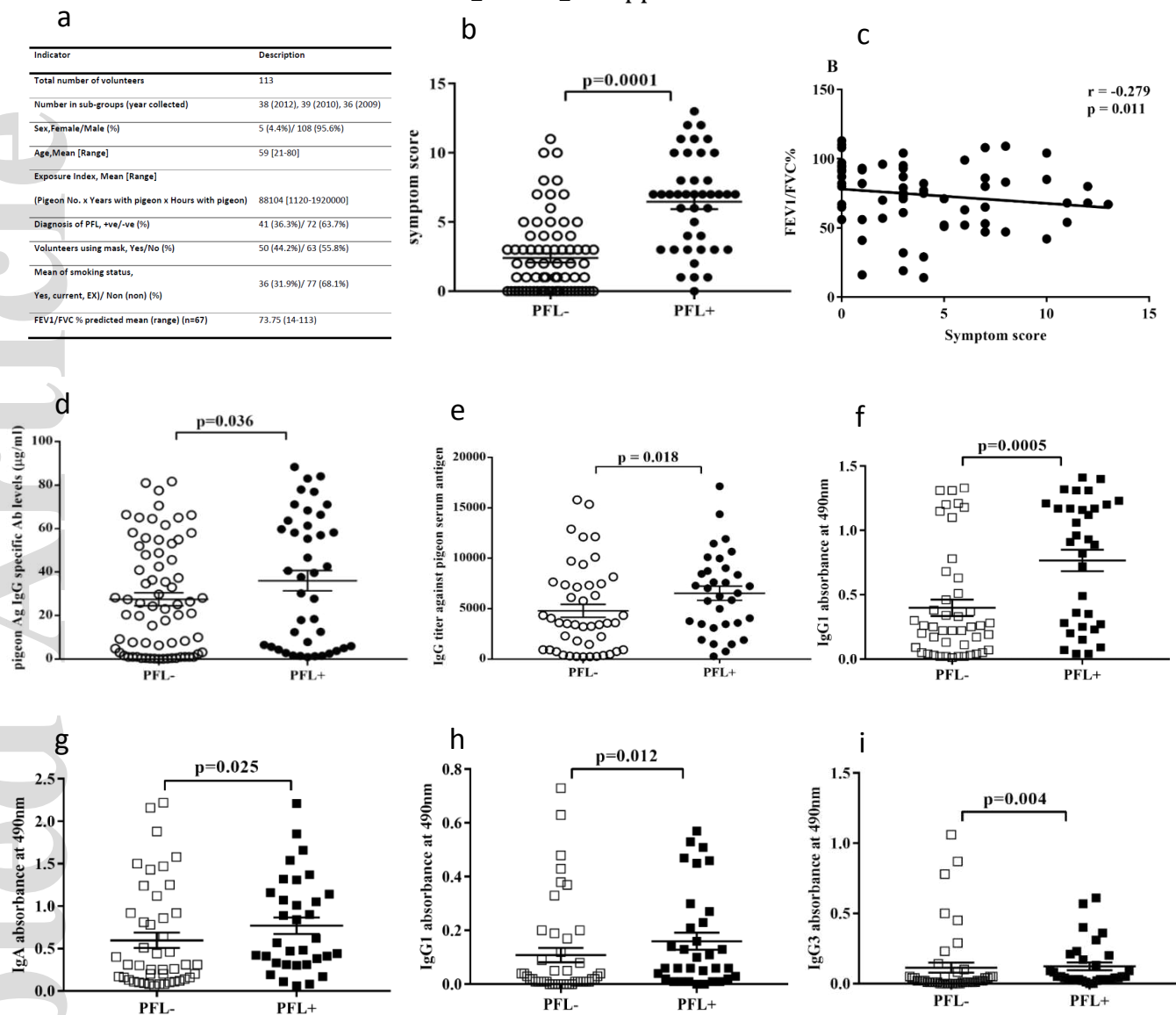
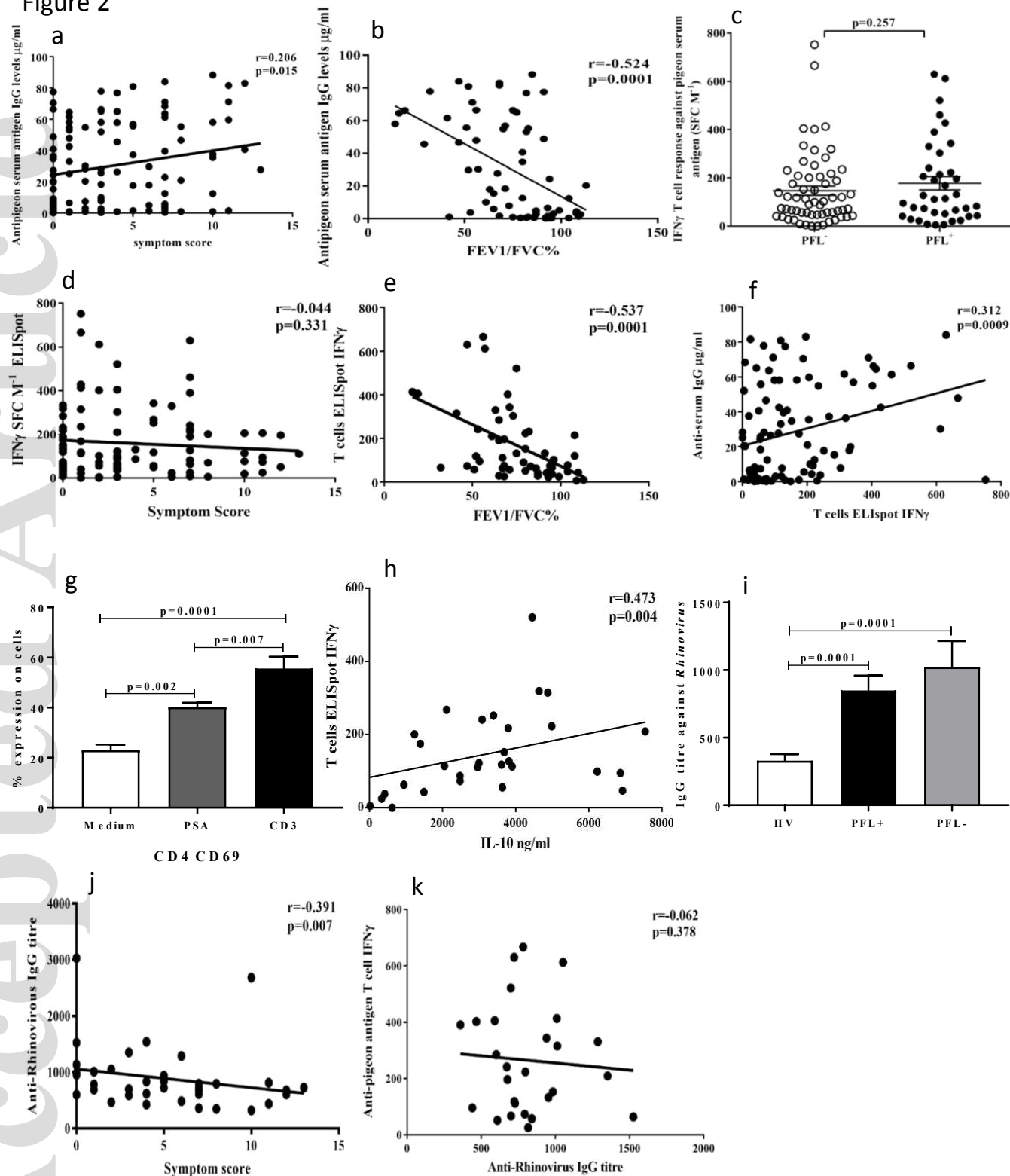


Figure 2



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

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Supplement section

Methods (M)

Study Participants, Ethics and Spirometry (M1)

This was a community-based study undertaken at pigeon shows where pigeon breeders were invited to attend the stand run by British Pigeon Fancier's Medical Research. Approximately 1000 pigeon breeders attended a show. Those volunteering underwent an interview with a doctor using a structured questionnaire, documenting details of age, sex, previous medical illnesses, smoking, and symptoms occurring in relation to contact with pigeons. Circumstances of exposure to pigeons were assessed by documenting the number of pigeons kept, average number of hours per week spent in the loft and duration of exposure in years. They were given advice about any symptoms, the nature of pigeon fancier's lung, and the need to consult their general practitioner.

The Medical Ethics Committee of Stobhill Hospital, Glasgow, approved the study (ref. 00/44) as well as the University of Glasgow College of Medical, Veterinary & Life Sciences Ethics Committee (Project No: 200130054). All subjects provided signed informed consent which included a doctor-supervised structured questionnaire collecting demography, indices of exposure to pigeons, acute respiratory symptoms associated with pigeon exposure, cigarette smoking history and any relevant comorbidities. A 25ml blood sample was taken. Lung function was measured using a spirometer (Draeger Medical UK Ltd., Hemel Hempstead, UK). Spirometry procedure gave forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) from which the FEV₁/FVC%-predicted was obtained.

Blood processing (M2)

Heparinized venous blood samples were taken from volunteers and were processed by layering onto Lymphoprep (Axis-Shield) solution in a Leukoprep tube (Greiner), and centrifugation at 2100 rpm for 12 minutes. Separated plasma was aliquoted and stored at 4°C short term or frozen at -20°C for

longer term storage before use in ELISA. Resulting peripheral blood mononuclear cells (PBMC) were washed in RPMI 1640 and frozen in 90% Foetal Calf Serum / 10% Dimethylsulphoxide at -150°C.

Bacterial Lysate Production (M3)

A panel of reference bacterial strains were provided by the Microbiology Department, Freeman Hospital, for the production of antigen lysates (Supplement Table S1). These strains were cultured from a single plated colony in 10 ml of broth, and incubated with shaking at 200 rpm overnight at 37°C. Then, 1 ml of this starting culture was transferred into broth in a 200 ml flask and incubated with further shaking for approximately 2-3h at 37°C until the optical density (OD) of the culture reached 0.8, indicating mid-log growth. The bacteria were pelleted by centrifugation at 4000 rpm for 15 min at 4°C and then washed 3 times with 50 ml of phosphate buffered saline (PBS). The final pellet was suspended in 5 ml of PBS and sonicated using a Soniprep 150 (MSE) in 30 second pulses on ice. The lysate was decanted into microfuge tubes and centrifuged to clarify at 13000 rpm at 4°C and then the soluble lysate was aliquoted and stored at -20°C. The protein content was confirmed by SDS-PAGE and quantified using a Bradford assay compared to an albumin standard curve. The LPS content of the lysates was measured using the Limulus Amebocyte Lysate (LAL) kit (Thermo Scientific) and a modified in-house Mono Mac 6/Interleukin-6 *in vitro* pyrogen test [1].

ELISA for antibodies (M4)

96-well ELISA plates (Nunc Maxisorp, Sigma-Aldrich, UK) were coated with selected antigen optimally diluted in PBS, and incubated overnight at 4°C. The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) then blocked with 100 µl of 5% milk powder in PBST for 1 hour. After 3 washes, 100 µl of diluted sera in PBST containing 0.5% milk powder (diluent) was added and plates incubated for 2 h. After 6 washes, the plates were incubated for 1 h at RT with 50 µl of anti-human IgG/HRP (polyclonal Rabbit P0214 Dako, Ely, Cambridgeshire) 1 in 5000 in diluent, and then washed 6 times, and developed with OPD (Sigma) substrate solution. The reaction was stopped with 25µl 2M H₂SO₄ and absorbance was read at 490 nm. Antigen-specific antibody classes and isotypes were measured using class- or isotype-specific biotinylated monoclonal antibodies followed by avidin-peroxidase (Sigma). Initial screening of sera at a 1 in 25 dilution was carried out to establish the starting dilution of the sera based on the mean of absorbance obtained. A 4-step dilution series for each serum sample obtained an end-point titre. Inhibition ELISA was carried out to determine cross reactivity between antigens and hence specificity of the ELISA. Sera were pre-incubated with a dilution series of antigens before proceeding with the regular ELISA procedure. Measurement of Ig subclasses was performed by ELISA with the secondary detector antibody substituted to allow detection of the subclasses, including IgG1, IgG2, IgG3 and IgG4, as well as the other classes, IgA and IgM. In some instances, in which the secondary antibody was biotin-conjugated, an extra step was

required, adding a dilution of Avidin–Peroxidase at 1 in 40000 and incubating for 1h at RT prior to adding substrate. The absorbance (minus blank) value at a fixed serum concentration of 1 in 25 was used as the result for the Ig subclasses.

ELIsport (M5)

Sterile 96-well ELIsport plates (Millipore, Watford.UK) were coated with 10 µg/ml of capture anti-human IFN γ (Mabtech, Nacka Strand, Sweden) in bicarbonate buffer overnight at 4°C. The plate was washed 3x with sterile PBS and blocked with 100 µl of RPMI 1640 supplemented with penicillin, streptomycin, glutamine and 10% foetal calf serum (R10) for 1h at RT. PBMC were thawed and counted using a CASY[®] cell counter (Roche Diagnostics GmbH, Mannheim, Germany), and adjusted to 8×10^6 /ml. Following decanting of the R10 block, 50 µl of stimulus and 50 µl of PBMCs were added to the plate. Stimuli comprised medium-only negative control, positive controls of anti-CD3 and/or phytohaemagglutinin (PHA), pigeon antigens and bacterial antigens (Table S1). The plate was incubated for 20 h at 37°C in a 5% CO₂ incubator. The plate was then washed 6 times before adding 50 µl of biotinylated-anti-IFN γ detector antibody at 1 µg/ml (Mabtech). The plate was incubated for 2h at RT and was then washed, and 50 µl of streptavidin-ALP (Mabtech) at 1 µg/ml was added followed by 1 hour's incubation. Finally, AP Conjugate Substrate kit 170-6432 (Bio-Rad Laboratories Inc), 50 µl/well, was used to develop the plate for 10 minutes, and the reaction was stopped by washing the plate thoroughly with tap water. The plate was dried overnight and spot-forming cells were counted using an Immuno-Spot reader (Auto-immun Diagnostika GmbH, Strassberg, Germany). The frequency of IFN γ -producing cells was calculated from the number of spots in the wells after background subtraction and multiplied by the cell concentration factor (2.5 for 8×10^6). The results are expressed as spot-forming cells (SFC)/ 10^6 (M) PBMC.

Cell activation and surface staining and Flow Cytometry (M6)

Frozen PBMC samples were thawed, washed and resuspended in R10 and counted. 250 µl of 8×10^6 PBMCs were incubated with 250 µl of medium, polyclonal anti-CD3mAb (Mabtech) as positive control, or pigeon serum antigen (PSA). The cultures were incubated for 20h at 37°C, 5% CO₂. The cells were washed with PBS and incubated (15 min, at RT, in the dark) with the antibody combinations shown in Table S2, together with 50 µl of Brilliant Stain Buffer (BD Horizon[™]). The stained cells were washed with 2 ml of PBS and re-suspended in 300 µl of PBS prior to immediate acquisition on the flow cytometer. A BD FACSCanto[™] II flow cytometer (BD Biosciences, Oxford, UK) with 8 channels was used with BD FACSDiva[™] software.

Cytokine assays (M7)

For each panel, 1 ml of diluent-1 was added to 1 g of blocker-B. Serial dilutions of 1 in 100 were made for each standard for both panels. Then, 25 µl of these standards were added to the appropriate 96-well plate (Meso Scale Diagnostic, LLC, Gaithersburg, USA). 25 µl of culture supernatant samples from previously stimulated PBMCs were added to each well of the 96-well plates. The plates were covered with an adhesive cover (Thermo Scientific, UK) and incubated for 2h at RT on a plate shaker (100 rpm). 25 µl of detector antibodies were added in pro-inflammatory panel-1 (human calibrator blend) including anti-human IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF α . 25 µl of detector antibodies was also added to cytokine panel-2 (human calibrator blend): anti-human IL-5 and IL-17A. These detection antibodies (Sulfo-TAG) were diluted in diluent 100 (MSD). The plates were incubated for 2h at RT on a plate shaker (100 rpm). The plates were washed 6 times with 150 µl of PBS 0.05% Tween-20. Then, 150 µl of read buffer 4X with surfactant (MSD) diluted in sterilized water (50:50) was added to each well of the plates. The plates were read on MSD sector imager. Data were analysed in MSD Software and Excel. Cytokine detection levels were 0 – 10ng/ml. Measurement of CC16, a measure of Club Cell activation, and found in diseased alveolar epithelium [7], was carried out using the Human Uteroglobin DuoSet ELISA (Cat no DY4218) from R&D Systems (Abingdon, UK).

Statistical Analysis (M8)

Data was first determined as being normally distributed or not. Groups were compared for significance using the Mann-Whitney U test. Associations between data were examined using Spearman correlation. A p value of <0.05 was considered significant. Effect size (Cohen's d) was calculated thus: $d = (\text{mean for group1} - \text{mean for group2}) / \text{pooled standard deviation}$; large is >0.8, medium is >0.5, small is >0.2.

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Table ST1. Protein antigens used in current study

NO.	Microorganism/ antigen	source of antigens
1	<i>Alternaria alternata</i>	ALK-Abello, Reading, UK
2	<i>Aspergillus fumigatus</i>	ALK-Abello, Reading, UK
3	<i>Chlamydia trachomatis</i>	The native antigen company, Oxfordshire, UK
4	<i>Haemophilus influenzae</i>	In-house
5	<i>Mycobacterium avium</i>	NIBSC, Potters Bar, UK
6	<i>Penicillium notatum</i>	ALK-Abello, Reading, UK
7	<i>Pseudomonas aeruginosa</i>	In-house
8	<i>Rhinovirus</i>	Gift from A. Fisher, Newcastle University
9	Respiratory syncytial virus	Gift from G. Toms, Newcastle university
10	Pigeon-serum-antigen (PSA)	From C.McSharry [5]
11	Pigeon Mucin (PM)	From K. Walker [6]

Protein concentrations ranged between 1 and 16.2mg/ml and LPS was $\leq 0.2\text{ng/ml}$. Strains were either obtained from the National Collection of Typed Cultures (NCTC), Laboratory of Microbiology, University of Ghent (LMG) Belgium, or were fully identified wild in-house isolated and characterized strains.

Table ST2. Antibodies used to determine the markers expressed by T cells.

Fluorochromes	Cell markers (Antibodies)	Function	Clone	Laser (nm) Excitation	EM _{max} ^a (nm) (detection)	Volume added
FITC	OX40 ^b	Co-stimulation	ACT35	488	525	2 μ l
PE	CD49d ^b	Homing	9F10	488	575	5 μ l
APC	PD-1 ^b	Energy marker	eBioJ105	633	660	2 μ l
APC-Cy7	CCR5 ^c	Chemokine receptor (inflammatory)	2D7/CCR5	633	785	2 μ l
BV421	CD69 ^c	Activation marker	KN50	405	421	2 μ l
BV510	CD4 ^c	Th cell marker	SK3	405	510	2 μ l

^aMaximum emission wavelength; ^b eBioscience (Affymetrix-eBioscience, Hatfield, UK); ^c BD Biosciences (Oxford, UK).

Supplementary Results

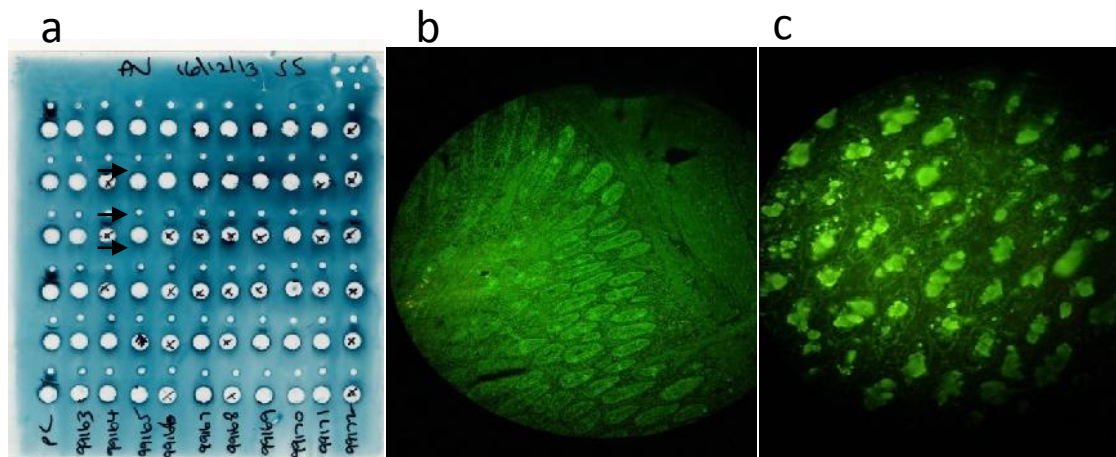


Figure SF.1 a.Countercurrent electrophoresis gels for pigeon/avian precipitins. Small wells contain antigens and larger wells contain the pigeon breeder serum. Antibodies and antigen migrate out of their wells towards each other to form a precipitin line indicating antibody reactivity. Arrows indicate rows with pigeon feathers, faeces and serum. Each column is one breeder's serum. Immunofluorescence (IF) of a patient's sera with pigeon gut. b.Indirect IF for negative control (no primary antibody), c.Indirect IF for positive control showing serum antibody reactivity with pigeon gut crypts where mucin antigen is shown.

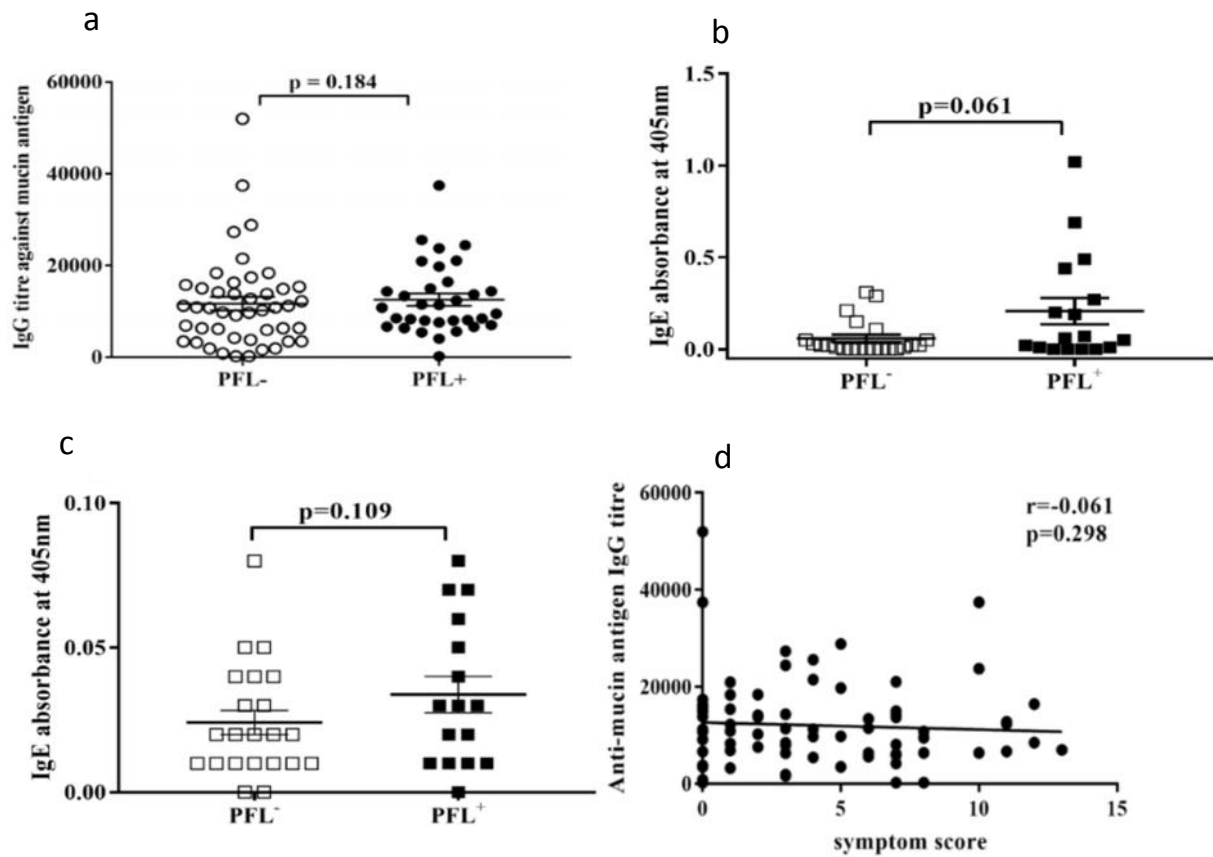


Figure SF.2 Antibodies and symptoms in pigeon breeders

Comparison between antibody levels for PFL⁺ and PFL⁻ pigeon breeders: a. not significant for PM-specific IgG antibody titres; not significant for PSA-specific IgE levels against PSA (b) or PM (c). d. non significant relationship between anti-PM titre and symptom score. Mean $\pm$ SEM is given. PSA=pigeon-serum-antigen; PM=pigeon mucin.

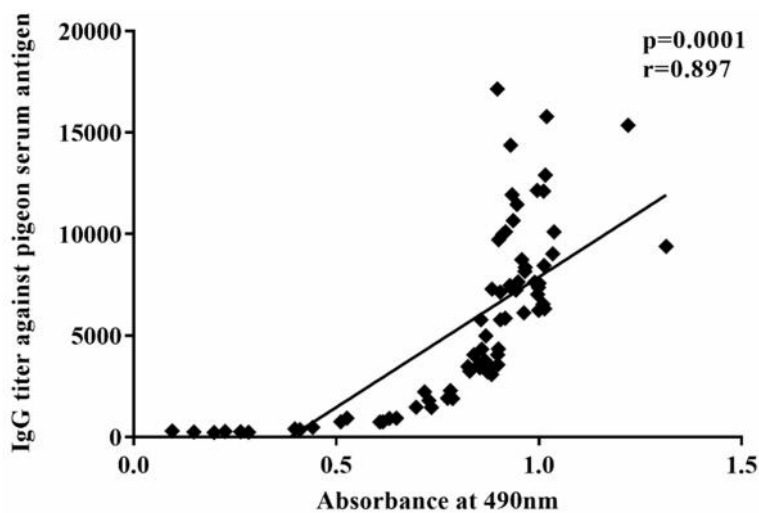


Figure SF.3 The relationship between IgG titre and the absorbance for the screening against pigeon serum antigen. Anti-pigeon serum IgG absorbance for sera samples screened at 1 in 25 dilution was plotted against the end point titre for PFL volunteers to find the association between the two data. The relationship was assessed using Spearman correlation; the two variables were strongly correlated $r = 0.897$, $p = 0.0001$.

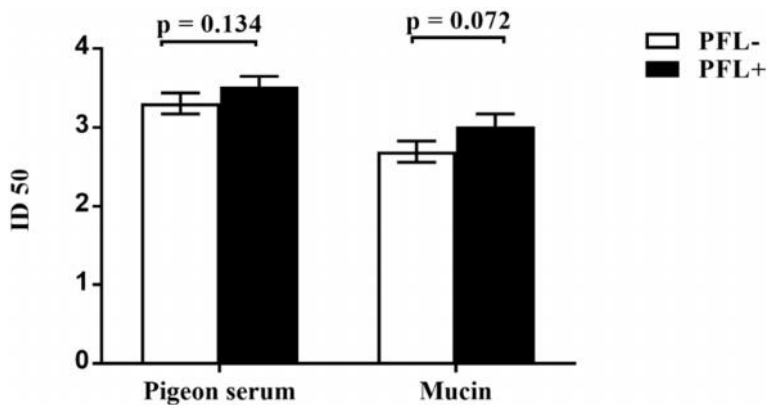


Figure SF.4 Antibody affinity for pigeon serum and mucin antigens in PFL⁻ and PFL⁺. ID 50 indicates the amount of Na thiocyanate needed to reduce the total antibody binding by 50%. There were no significant differences in the affinity for both antigens; for PM $t = -1.125$, $p = 0.072$ (Mean $\pm$ SEM = 3.307 ± 0.133), for PSA $t = -1.492$, $p = 0.134$ (Mean $\pm$ SEM = 2.692 ± 0.135), using t test.

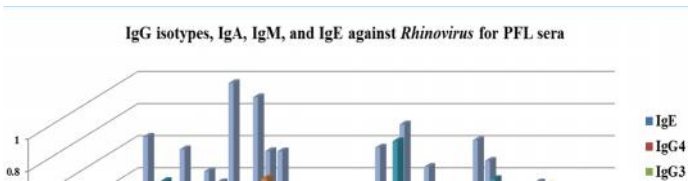
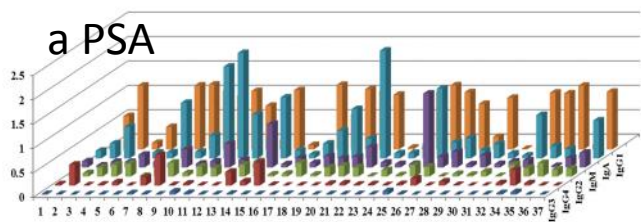


Figure SF.5 PFL volunteers' IgG Isotypes, IgA and IgM against PSA, PM and Rhinovirus. Y axis is absorbance at 490nm and x axis represents the id number of pigeon breeders.

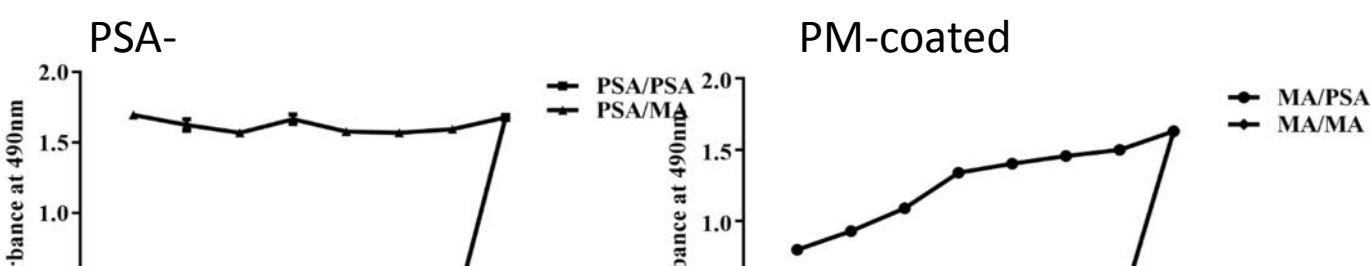


Figure SF.6 Specificity Inhibition. Antibody specificity against pigeon antigens was examined by inhibition ELISA. Recognition of PSA-coated ELISA plate was inhibited by PSA antigen but not by pigeon mucin. Recognition of MA-coated ELISA plate was inhibited by MA antigen and by 50% by pigeon serum antigen.

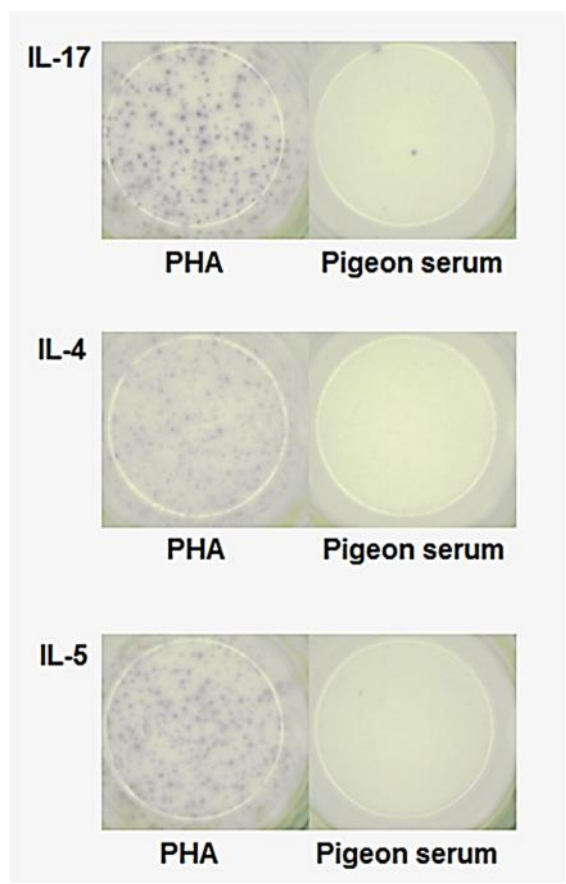
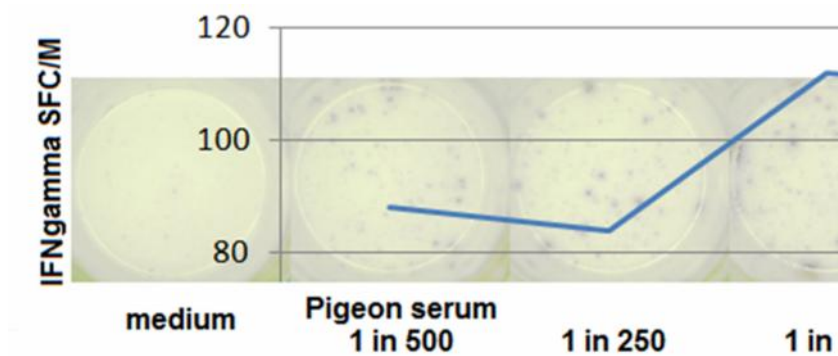


Figure SF.7 T cell IFN γ responses against PSA showed optimal response at 1 in 100 PSA dilution, hence this dilution was used throughout.

Figure SF.8 Images of IL-17, IL-4, and IL-5 response against pigeon serum. PBMCs were tested for their ability to produce IL-17, IL-4 and IL-5 cytokines against PSA. It can be seen that minimal if any response for these cytokines against pigeon serum was found compared with positive control (PHA). One representative responding individual's result is shown.

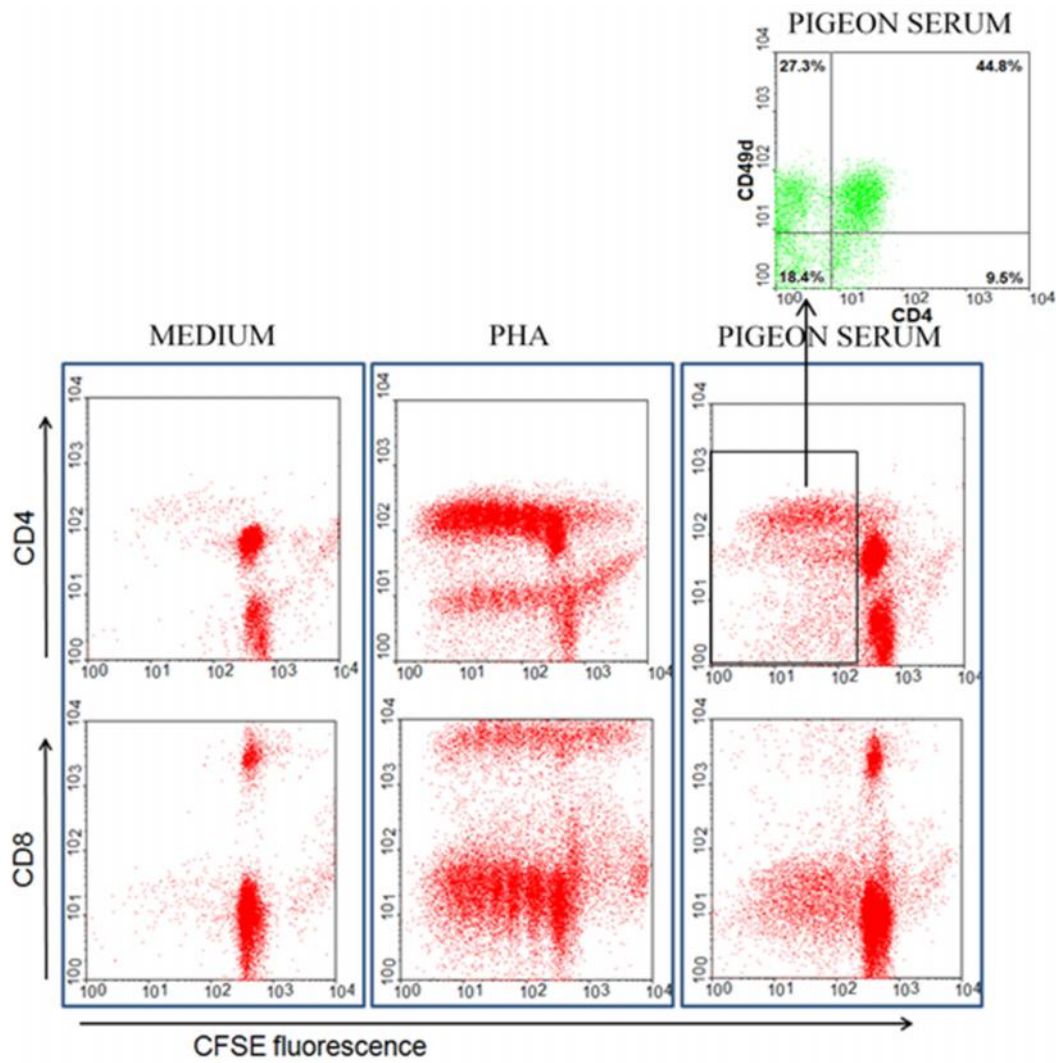


Figure SF.9 T cell proliferation CFSE dilution demonstrated proliferation after 5 days of incubation of CD4⁺ cells in response to PSA, that also possessed CD49d (green cells in panel indicated by arrow). Proliferation of T cell response to PHA was a positive control showing proliferation of CD4⁺ and CD8⁺ T cells. A representative of 3 experiments is shown. b.Stimulation of PBMC with PSA showed significant expression of activation marker CD69.

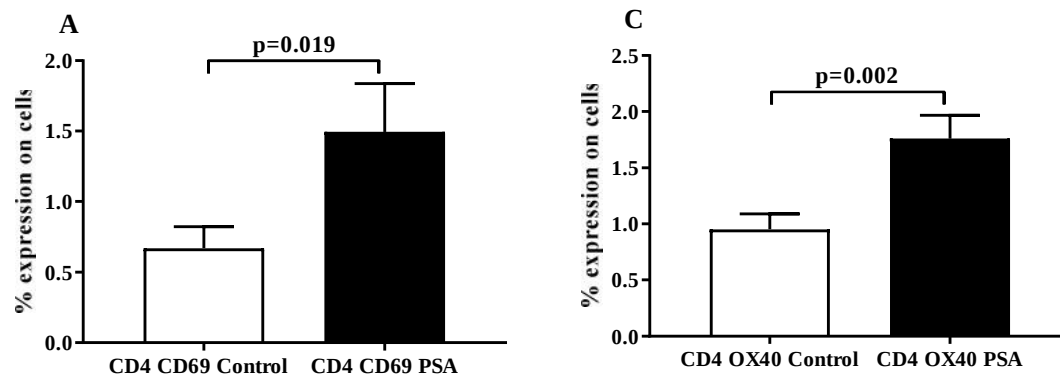


Figure SF.10 T cell activation following stimulation with PSA. Following activation with PSA CD4⁺ T cells were gated and investigated for the expression of (a) CD69 or (b) OX40. Mean + SEM is given. T-test was carried out.

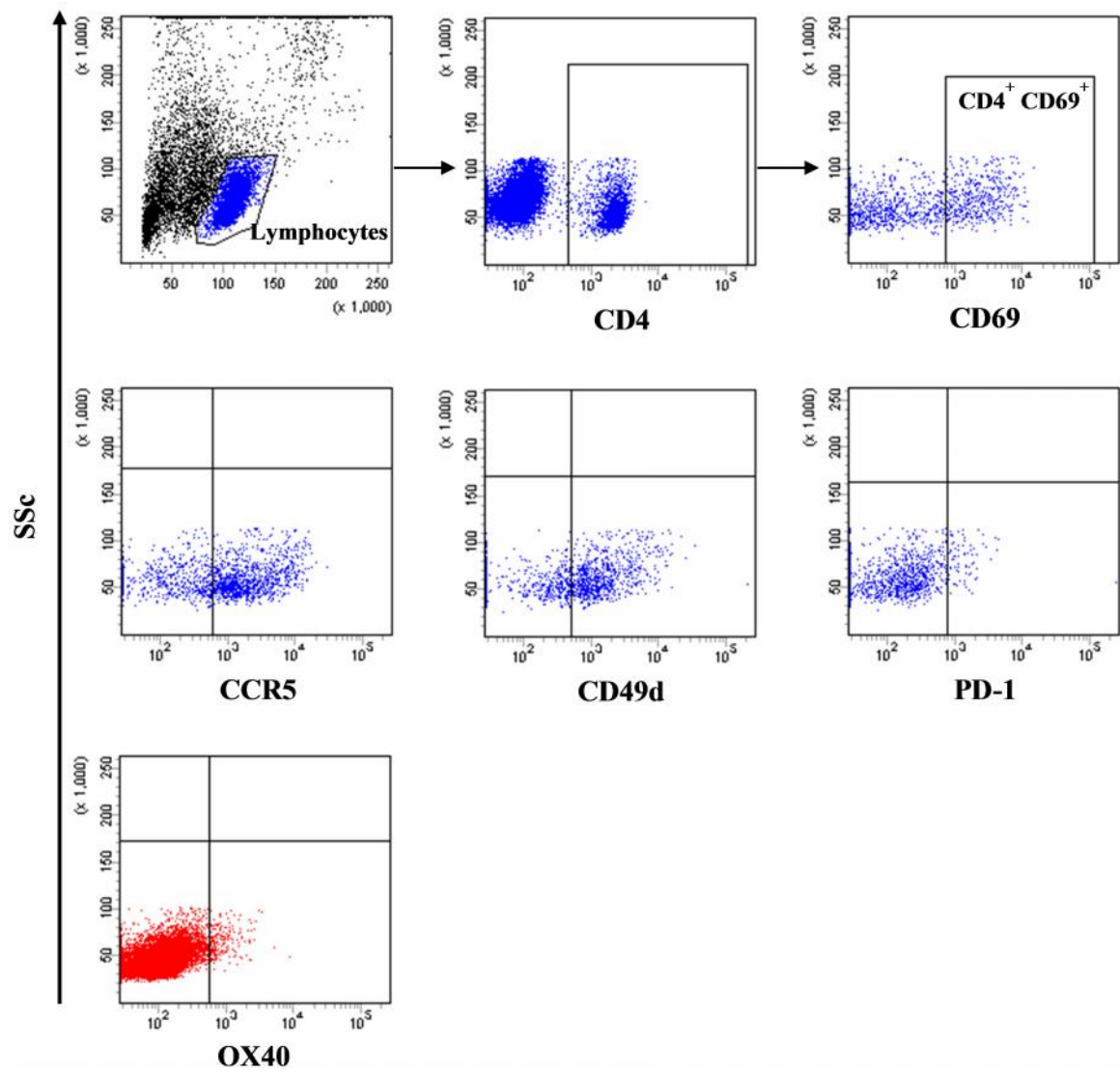
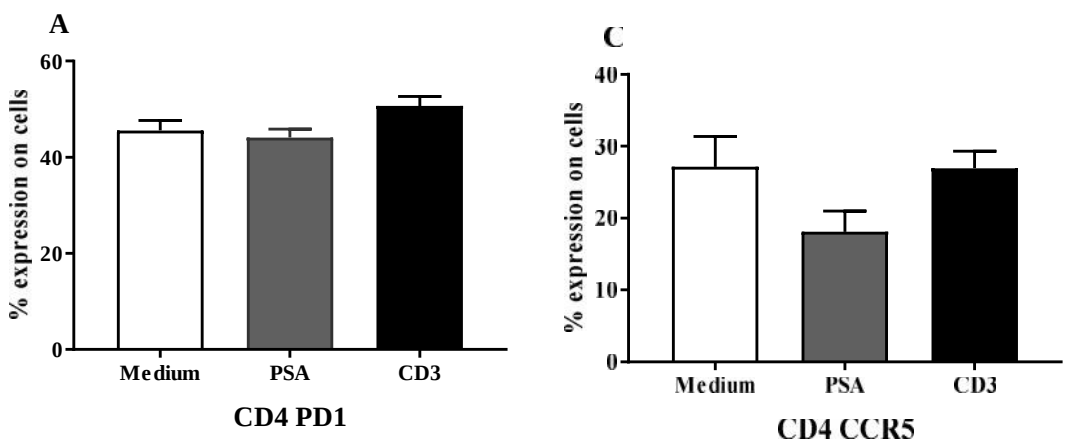


Figure SF.11 Phenotypic markers on CD4⁺ T cells Lymphocytes occupy the gated region beneath monocytes. Lymphocytes are gated CD4⁺ T cells, then those that possess CD69; followed by CCR5, CD49d, PD-1, and OX40, individually. SSc side-scatter



A

B

Figure SF.12 Marker-positive CD4⁺ and CD8⁺ T cells.

ANOVA test showed there were no significant difference among the groups; (a) CD4⁺ PD1, (b) CD4⁺ CCR5. Mean + SEM is shown. The stimuli were medium as negative control, pigeon serum as test stimulus and anti-CD3 as positive control, n=6.

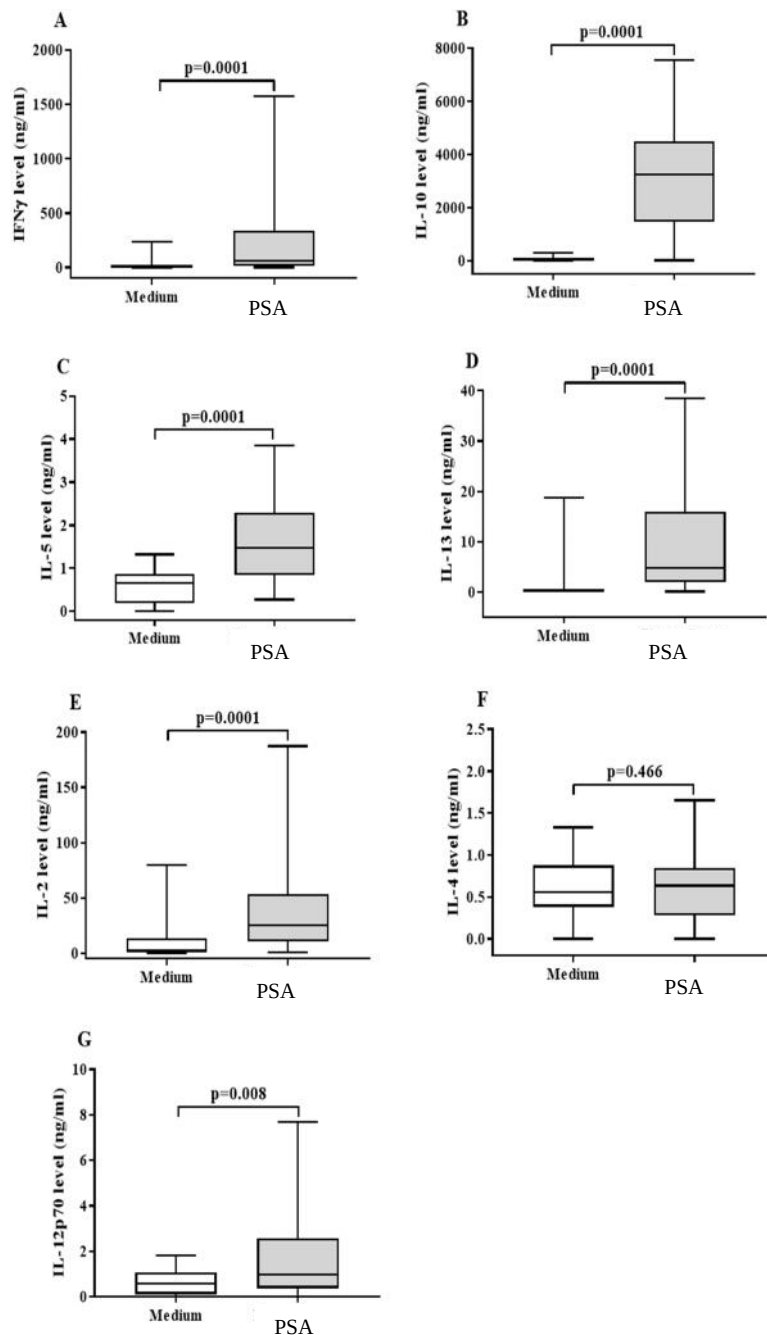


Figure SF.13 Cytokines Secretion. Cytokines secreted by PBMC from pigeon breeders stimulated for 48 hours with PSA. Median +IQR is shown. Cytokine levels (ng/ml) for 30 high pigeon breeders found previously to be high responders in ELISA and ELISpot assays. a. IFN γ , b. IL-10, c. IL-5, d. IL-13, e. IL-2, f. IL-4 g. IL-12.

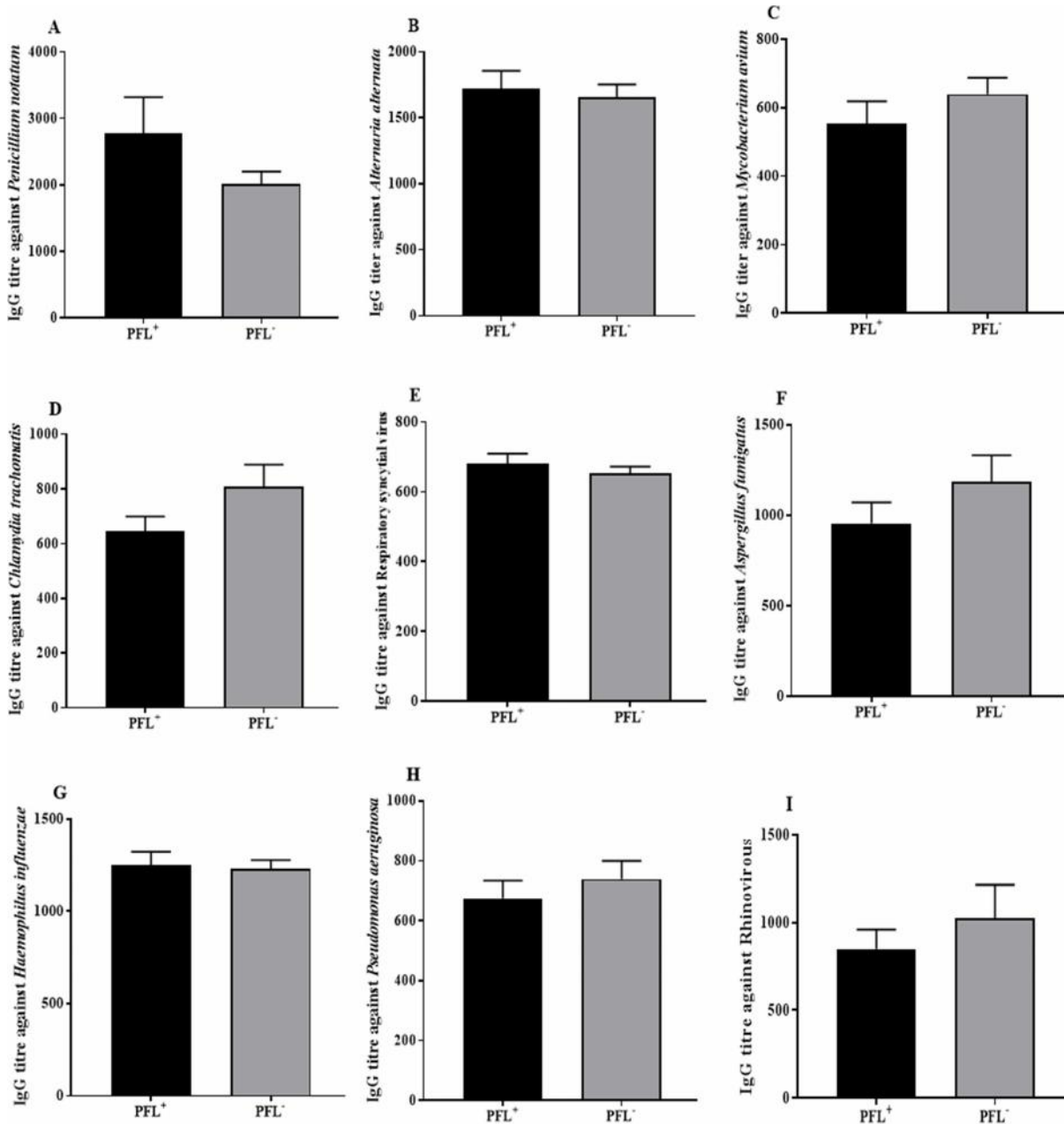


Figure SF.14 Anti-microbial IgG titres against microbes in pigeon breeders (A-H).

From the graph it can be seen that higher levels of antibodies were seen against *P. notatum* in PFL⁺ volunteers compared with PFL⁻ (A) but the difference in antibody responses between the two groups did not hit significance by Mann-Whitney test. IgG antibodies against Rhinovirus were higher in PFL⁻ rather than PFL⁺ (I) but again this difference did not hit significance. Mean $\pm$ SEM is shown.