

Chronic infection by mucoid *Pseudomonas aeruginosa* associated with dysregulation in T cell immunity to OprF

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Abstract

Rationale. *Pseudomonas aeruginosa* (PA) is an environmental pathogen that commonly infects individuals with cystic fibrosis (CF) and non-CF bronchiectasis, impacting on morbidity and mortality. To understand the pathobiology of interactions between the bacterium and host adaptive immunity and to inform rational vaccine design, it is important to understand the adaptive immune correlates of disease.

Objectives. We characterized T cell immunity to the PA antigen, outer membrane porin F (OprF), analyzing immunodominant epitopes in relation to infection status.

Methods. Non-CF bronchiectasis patients were stratified by frequency of PA isolation. T cell IFN γ immunity to OprF and its immunodominant epitopes was characterized. Patterns of HLA-restriction of immunodominant epitopes were defined using HLA class II transgenic mice. Immunity was characterized with respect to cytokine and chemokine secretion, antibody response and T cell activation transcripts.

Measurements and main results. Patients were stratified according to whether PA was never, sometimes (<50%) or frequently ($\geq$ 50%) isolated from sputum. Patients with frequent PA sputum-positive isolates were more likely to be infected by mucoid PA, and showed a narrow T cell epitope response and a relative reduction in Th1 polarizing transcription factors, but enhanced immunity with respect to antibody production, innate cytokines and chemokines.

Conclusions. We have defined the immunodominant, HLA-restricted, T cell epitopes of OprF. Our observation that chronic infection is associated with a response of narrowed specificity, despite strong innate and antibody immunity, may help to explain susceptibility in these individuals and pave the way for better vaccine design to achieve protective immunity.

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Pseudomonas, Bronchiectasis, Adaptive Immunity, Epitopes T lymphocyte,

At a Glance Commentary

Scientific Knowledge on the Subject:

Non-CF bronchiectasis is a chronic progressive lung disease associated with impaired lung function, sputum production and recurrent infection. Chronic infection with the gram-negative bacteria *Pseudomonas aeruginosa* is associated with higher morbidity and mortality. It is unclear why, among susceptible patient groups, some individuals will develop chronic PA infections and others, do not. Chronic infection with PA is associated with transcriptional changes in bacterial gene expression, such as those associated with the development of a mucoid phenotype.

What This Study Adds to the Field:

In patients with non-CF bronchiectasis, we looked at immunological differences associated with differential susceptibility to chronic infection with PA. Patients from whom PA was frequently cultured from sputum tended to be infected with mucoid isolates and showed altered T cell immunity to an immunodominant PA antigen: T cell immunity was much more narrowly targeted on the PA protein, OprF despite strong innate and antibody immunity. These findings help to explain susceptibility to chronic infection and should prove valuable for vaccine design.

Pseudomonas aeruginosa (PA) is an environmental pathogen, able to exploit predisposing host conditions to cause chronic infection (1,2). Chronic respiratory PA colonization of non-CF bronchiectasis patients impacts on morbidity and mortality (3,4). Features of host-pathogen interplay during PA infection pose novel and important challenges. Chronic infection can be long term, with the pathogen locating in hypoxic masses in the airway lumen and an associated morphotypic shift to a biofilm state involving generation of a protective, alginate, exopolysaccharide coat (5,6). This state can persist in the face of high titers of neutralizing antibody (6). The biofilm state is an adaptation presumed to protect the pathogen from host immunity (7,8), but little is known about adaptive immunity to PA during long-term infection.

As with many other large, variable bacterial genomes, there is a paucity of data available on detailed mapping of functional, protective antigen targets; often there are clear data on serodominant targets, but ability to activate antibody can differ from the ability to elicit protective immunity (9). Various PA antigens have been considered in mouse, rat and non-human primate as well as trials in human CF or non-CF vaccinees (10-16). In a phase III vaccine trial of non-colonized CF patients, a flagellin vaccine roughly halved the number of PA infections in the recipient cohort (10). Studies have considered the lipopolysaccharide O antigens, flagellin and the outer membrane porins F and I (OprF and I). OprF is a major outer membrane protein that is conserved across clinical isolates (17-19). In comparison of aerobic and anaerobic PA biofilms, OprF is upregulated 39-fold during anaerobic (relative to aerobic) biofilm growth (6). Studies have considered the immunogenicity and protective efficacy of OprF (20-25), commonly focusing on OprF antibodies.

Non-CF bronchiectasis offers a highly relevant clinical group in whom to map the immune

events predisposing to chronic PA colonization. A disease of chronic lung inflammation, damage and infection, it is defined by a pathological endpoint: irreversible, abnormal, bronchial dilatation with chronic airway inflammation, reached through diverse etiologies (26,27). The pathobiology suggests interplay between immunogenetic susceptibility, immune dysregulation, lung damage and chronic bacterial infection (27-30). Damaged epithelium impairs mucus removal, allowing mucus accumulation that facilitates bacterial infection, promoting chronic colonization associated with chronic cough and sputum production, recurrent chest infections and airflow obstruction. Chronic infections can encompass *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* as well as non-tuberculous mycobacteria, and *Aspergillus fumigatus* (26,27). Genetic associations suggest involvement of genes controlling adaptive immunity and innate immunity (28-30). We here analyzed adaptive immunity to PA in a cohort of 57 bronchiectasis patients stratified according to the frequency with which PA was cultured from monthly, longitudinal sputum samples.

Materials and Methods

Patient cohort, clinical and microbiological investigations

57 unrelated clinically stable subjects (mean age=58y±29y(2SD);28%male) with a diagnosis of non-CF bronchiectasis were recruited from the Royal Brompton Hospital (Table 1). Patients were clinically diagnosed as per the BTS bronchiectasis guidelines (28,31). Sputum samples were collected monthly for 6 months and sent for microscopy and culture by standard microbiology. Peripheral blood samples were collected. Patients were stratified into three groups: never (53y±31y(2SD); 29%male), less than 50% (66y±17y(2SD); 8%male), and more than 50% (61y±26y(2SD); 43% male) culture positive for PA in sputum samples collected. The study was approved under ethics code 10/H0801/53 with full, informed, patient consent.

Production and purification of OprF protein and peptide panel

OprF protein was produced using the recombinant vector pSUMO-OprF as described previously, with minor modifications (25,32). The identity and purity of OprF protein was confirmed by SDS-PAGE and mass spectrometry. An OprF (PA1777) peptide library comprising 20-mer peptides overlapping by 10 amino acids (Table 2) was synthesized (GL Biochem Ltd, Shanghai, China).

HLA class II transgenic mice and mapping of HLA restricted T cell responses

HLA-DR1(DRA*0101/DRB1*0101)Aβ°, HLA-DR4(DRA*0101/DRB1*0401)Aβ° and HLA-DR15(DRA*0101/DRB1*1501)Aβ° transgenic mice described previously (33-36) aged 8-15 weeks were used. UK Home Office regulations for animal welfare were strictly observed. HLA-DR1, -DR4 and -DR15 transgenic mice were footpad immunized with 25µg OprF emulsified in Complete Freund's adjuvant. Popliteal draining lymph nodes were harvested at

day 10 and stimulated with 25µg/ml protein or individual peptides and cellular responses detected by murine IFN γ ELISpot (Gen-Probe Diaclone SAS, Besançon, France).

Human T cell responses to OprF protein and peptide panel. Quantification of immunological effector proteins and OprF IgG specific ELISA

PBMCs were stimulated with 50µg/ml protein or individual peptides and cellular responses detected using the ELISpot^{PRO} Human IFN γ Kit (Mabtech, Nacka Strand, Sweden) (Table 5B). Supernatants from PBMCs cultured with OprF protein in human IFN γ ELISpot assays were used to determine cytokine and chemokine levels (Table 5A) using the 30-plex Luminex assay (Invitrogen, Life Technologies Ltd UK) on the Bio-plex 200 system. OprF IgG antibody titers were determined in patient serum samples by ELISA (Table 5C).

Real-time PCR analysis of patient PBMC

PBMCS were stimulated using PMA/Ionomycin as a generic form of lymphoid activation to obtain a snapshot of activated transcription. RNA was prepared (Absolutely RNA microprep kit, Agilent Technologies, Wokingham, UK) and cDNA synthesized using SuperScript III reverse transcriptase (Invitrogen, Life Technologies, UK). Real time PCR reactions were carried out using GAPDH(PPH00150F), TBX21(PPH00396A), RORC(PPH05877A), Gata3(PPH02143A), S1PR1(PPH01350F)(RT² qPCR Primer Assays, Qiagen, UK) and GAPDH(Hs02758991_g1), FoxP3(Hs01085834_m1) and IL10(Hs00961622_m1)(Applied Biosystems, USA) primers. PCR reactions were run in triplicate and Ct values obtained using a MX3000P real-time PCR machine (Stratagene, USA). Variance RNA amount between samples was controlled by normalizing to GAPDH and relative levels of gene expression between samples were determined using the standard curve method (Table 5D)

Peptide/HLA-DR binding assays

HLA-DR heterodimers were purified and relative binding of the OprF peptide panel to the HLA-DR molecules HLA-DRB1*01:01, 03:01, 04:01, 07:01, 09:01, 11:01, 13:01, and 15:01 was measured by competitive ELISA, as previously described (33-36). Data were expressed as relative affinity: ratio of the IC₅₀ of the peptide to the IC₅₀ of the reference peptide.

Results

Strong T cell responses to PA protein, OprF in patients with non-CF bronchiectasis

We initially compared the T cell responses to the major PA immunogen, OprF, in three groups of bronchiectasis patients, stratified according to whether PA was never, sometimes (<50%) or frequently ($\geq$ 50%) cultured from monthly sputum samples collected over 6 months (Tables 1 and 5B). Patients showed a strong IFN γ response to the protein antigen. Individuals that were never sputum culture positive for PA or sputum culture positive < 50% of the time had mean frequencies of 158 (+/- 377) and 202 (+/- 484) spot forming cells (SFC) /10⁶ respectively, suggestive of robust immune memory devoted to the host PA response (Figure 1A). However, patients with evidence of chronic PA infection that were sputum culture positive for PA $\geq$ 50% of the time, showed reduced responsiveness to OprF, with a mean response of 119 (+/- 262) SFC (Figure 1A). Patients with chronic PA infection were more likely to be infected by mucoid isolates (Chi-Square Test 5.42; p=0.0199, Pearson). In the group that were <50% sputum sample culture positive for PA, 4/12 (25%) individuals grew mucoid isolates and in the $\geq$ 50% group, 11/14 (79%) individuals grew mucoid isolates (Supplementary Figure 1A). Figure 1B shows the patient ELIspot response to OprF in terms of infection by mucoid or non-mucoid PA isolates.

Strong antibody responses to PA protein, OprF in patients with non-CF bronchiectasis patients with evidence of chronic PA infection.

Next, we measured serum antibody responses to OprF in bronchiectasis patients (Table 1 & 5C), stratified according to whether PA was never, sometimes (<50%) or frequently ($\geq$ 50%) cultured from monthly sputum samples (Figure 1C) and according to the type of PA isolate, no PA, non-mucoid or mucoid PA (Figure 1D). Individuals that were sputum culture positive

for mucoid and non-mucoid PA made significantly stronger antibody responses to OprF than those that were consistently sputum culture negative for PA (mean + 2SD for mucoid PA (1692 ± 3059 ; $p < 0.001$) and non-mucoid PA (947 ± 2355 ; $p < 0.01$)) compared to culture negative for PA (325 ± 457). Individuals with non-CF bronchiectasis with evidence of chronic PA infection that were frequently culture positive for PA ($\geq 50\%$) had significantly higher serum antibody responses to OprF (mean $\pm$ 2SD for $\geq 50\%$ for PA (2082 ± 3131 ; $p < 0.001$), $< 50\%$ PA (414 ± 330 ; $p < 0.01$) and no PA (325 ± 457)).

OprF sequence contains strong HLA-DR binding epitopes

Since OprF is immunogenic and protective in experimental pseudomonas [20-25] and seroreactive in non-CF bronchiectasis patients with chronic PA infection (37), we further investigated the candidacy of OprF as a CD4 T cell antigen. A synthetic peptide panel of 20mers overlapping by 10 amino acids was generated, covering the full coding sequence (Table 2) and binding affinities determined for the HLA-DR alleles, HLA-DR1, -DR3, -DR4, -DR7, -DR9, -DR11, -DR13, and -DR1501 (Table 3). Of the 34 peptides in the panel, over 60% were able to bind one or more of the HLA alleles tested with moderate to high relative affinity, showing that OprF contains peptides that can bind across many HLA class II alleles.

Narrowed OprF T cell epitope responses in non-CF bronchiectasis patients with chronic PA infection

Patient T cell responses to OprF were epitope mapped by screening responses to an overlapping peptide panel spanning the full-length sequence (Tables 1 and 5B). Responses in non-CF bronchiectasis patients from whose sputum PA was never cultured show broad immune responses to multiple T cell epitopes within OprF (Figure 2A). The spread of epitope

responses appeared somewhat similar in the patients who were sometimes culture positive (<50%) for PA. However, in the individuals with evidence of chronic PA infection, the response focused on a significantly smaller number of epitopes (mean $\pm 2SD$; 9 ± 13 in the never-sputum PA culture-positive group, compared to 3 ± 10 in the >50% sputum PA culture positive group; $p < 0.01$). For clarity, this relationship is displayed in Figure 2B, with patients stratified by mucoid morphotype of PA isolates (see also Supplementary Figure 1B). Representative examples of patient T cell responses to the peptide panel are shown for the 'never PA culture positive' group (Figure 2C), sometimes culture-positive group (<50% of sputum samples) (Figure 2D) and frequently culture positive group ($\geq 50\%$ of sputum samples) (Figure 2E). The T cell response to OprF in non-CF bronchiectasis patients with evidence of chronic PA infection is focused on fewer epitopes within the antigen. Since it was theoretically possible that breadth of epitope recognition was simply a correlate of overall magnitude of response to the protein, we checked to see if such a correlation could be determined, but found none (Supplementary Figure 2). The notion that breadth of epitope recognition is not a simple correlate of magnitude of response to the whole antigen is further supported by analysis of those specific epitopes most commonly recognized by T cells from the patients in each group (Figure 2F); while some epitopes were recognized by some donors from each of the groups, others were more commonly recognized by individuals from one group or another.

CD4 T cell epitope responses to OprF peptide panel in HLA class II transgenic mice

Because of the difficulties inherent in any attempt to impute patterns of peptide-HLA class II restriction in human populations where most individuals will be heterozygotes across the HLA region and, furthermore, each antigen presenting cell (APC) will express multiple, different HLA class II heterodimers, we compared patterns of CD4 OprF epitope recognition from

patients to the pattern of responses in HLA transgenic mice (Figure 3). For this, we used a panel of mice that carry a knockout for endogenous mouse H2-A β and utilize human HLA class II heterodimers, HLA-DR1, DR4 and DR15. As can be seen in Figure 3, screening of OprF epitopes in the context of individual HLA-DR molecules allows for unequivocal attribution of peptides presented in this context: the response of HLA-DR1 transgenics focuses on p4, of DR4 transgenics on p6, 14 and 15 and of DR15 on p11. While the complexities of human T cell repertoire selection and immunogenetics make it challenging to juxtapose findings in HLA transgenics and in humans, comparison of the response patterns offers some insights as to peptide-HLA complexes involved in the PA response.

We next looked at to what extent p11, the sole immunodominant epitope presented to CD4 T cells by HLA-DR15 in transgenic mice, features in the T cell response of patients who carry one or two HLA-DR15 alleles. It is one of the more common epitopes in HLA-DR15⁺ patients, and 7/14 (50%) individuals in the group from which PA was never cultured respond to p11. However, for individuals with evidence of PA infection in the sputum, this epitope was recognized in only 1/9 (11%) of HLA-DR15⁺ patients (Fisher Exact Probability Test: two tailed, $p=0.0858$). The number / percentage of individuals carrying the HLA-DR1501 allele did not significantly differ between groups [$n=14/31$ (44%) for never PA culture positive vs $n=6/14$ (43%) for $\geq 50\%$ culture positive]. Thus, the altered disease state to chronic infection may be associated not just with a quantitative diminution in T cell response but in a qualitative shift in the recognition of immunodominant epitopes.

Enhanced cytokine and chemokine responses in non-CF bronchiectasis patients with evidence of chronic PA infection

Despite a substantially reduced antigen and peptide specific IFN γ response to PA OprF, the patients with evidence of chronic PA infection showed an enhanced response with respect to a number of innate cytokines and chemokines when compared to responses in patients from whom PA was never isolated (Table 5A and Figure 4). This increased response encompassed significantly raised levels of IL-12, IL-6, MIP-1 α (CCL3) and MIP-1 β , collectively suggesting increased chemotaxis for neutrophils, monocytes and NK cells. IL-8 was also increased, although this difference did not reach significance. A small, but significant increase in secreted IL-4 may indicate a Th2-skewed program in these individuals.

Lymphocyte transcriptional changes in non-CF bronchiectasis patients with evidence of chronic PA infection

PBMC responses to OprF suggested an immune profile in chronically infected individuals whereby antigen specific IFN γ T cell effector responses are narrowed in epitope specificity, but the innate/inflammatory and antibody response is enhanced. We therefore investigated whether there was evidence for any intrinsic difference between patient groups in terms of transcripts associated with T cell adaptive immunity programs (Table 5D & Figure 5). We looked at the hallmark transcription factors of Th1, Th17 and Th2 cells, that is, TBX21, RORC and Gata3. We also looked at S1P receptor 1 (S1pr1) transcription, a molecule implicated in T cell trafficking between tissues and lymph nodes. Transcripts for TBX21 were significantly reduced in PBMC samples from patients that were frequently ($\geq 50\%$) sputum culture positive for PA compared to those that were never sputum culture positive for PA (Figure 5A). This is suggestive of a relative reduction in Th1 programmes in non-CF bronchiectasis patients with evidence of chronic PA infection. No change was seen with respect to transcription of Gata3, the Th2 transcription factor (Figure 5D) and RORC the Th17

transcription factor is reduced, but this did not achieve significance (Figure 5C). We found no evidence that these changes may have been mediated through a systemic bias to a more regulated phenotype: transcription of FoxP3 and IL10 was also reduced, though this did not reach statistical significance (Figure 5 E,F). Transcription of S1pr1 was significantly reduced in PBMC of the chronic PA carriers relative to PBMC from individuals who were never culture positive for PA (Figure 5B).

Discussion

OprF has received attention as an immunogen that confers protection in murine challenge studies and trialled for immunogenicity as a PA vaccine in humans (11-13,15,16, 22-25). It is an abundantly expressed outer membrane protein of PA, initially characterized with respect to anchoring to the peptidoglycan layer (38) and with a structural role in adhesion to eukaryotic cells (39). It is upregulated during biofilm formation under anaerobic conditions and the airway mucus of some CF patients contains cleaved OprF protein. OprF antibody titer is regarded a potential biomarker of biofilm infection (6). OprF binds IFN γ , leading to stimulation of the quorum sensing network (40). Oprf deletion mutants define a role of OprF in virulence, partly through modulation of quorum-sensing (41).

In light of this evidence for OprF immunogenicity and hyper-expression during biofilm formation, it might be predicted that patients undergoing such infection would show enhanced OprF adaptive immunity: it has previously been shown by others and confirmed by us that chronic infection is associated with raised antibody titers (6,37). Studies in a mouse infection model allowing comparison of PA that was either alginate wild-type, hyper-expressing or null, showed that alginate over-expression was associated with poor bacterial clearance and exacerbated lung pathology, yet with heightened pro-inflammatory cytokines – that is, raised IFN γ and IL-12 but lower IL-10 in lung homogenates (7). However, in other studies, mucoid or biofilm phenotypes are associated with reduced innate immunity (8,42). The present study shows that patients with chronic PA infection have reduced T cell immunity to OprF, although with enhanced innate immunity with respect to several innate cytokines and chemokines. OprF has intrinsic ability to stimulate release of diverse innate cytokines from dendritic cells (43); while this clearly would not have contributed to the differences in epitope focus we observed in peptide-specific CD4 responses, it may be that in chronically PA infected

patients showing up-regulated OprF, this up-regulation may stimulate increased systemic IL-12 and TNF α . From our study we cannot be certain whether the altered T cell response pattern we observed is cause or effect of a heightened susceptibility to PA infection. It is likely that polymorphisms in pathways underpinning both innate and adaptive immunity to PA contribute to susceptibility (27-30). Certainly the enhanced innate inflammatory response will contribute to the progressive lung damage, deterioration in lung function and associated increased morbidity and mortality seen in non-CF bronchiectasis patients with chronic PA infection (4)

A key finding here was the narrowed repertoire of T cell OprF epitopes during chronic PA infection. This raises the question whether functional clearance of PA by adaptive immunity benefits most from a highly focused or a more diffuse response. The issue of epitope focus versus breadth has been considered most extensively in relation to viral infections, including HIV, CMV and HCV. SIV and HIV studies have argued variously that either a very broad or a very focused repertoire response can be correlated with better control of infection and improved outcome (44-46). In the case of PA, curated sequences for OprF are largely conserved and indeed, the epitopes we have characterized in the present study are highly conserved across diverse *Pseudomonas* family members, including *Pseudomonas alcaligenes*, *indica*, *citronellolis* and *stutzeri*. Thus, in principle, a T cell response targeted at any epitope should be able to support a protective response. This suggests that the correlation of multiple epitopes with effective host immunity may lie not in the breadth of epitope responses *per se* but in the cumulative frequency of responding cells.

This study showed a strong correlation between chronic PA infection and mucoid isolates, making it difficult to establish whether the driving correlate of altered adaptive immunity in

chronic infection is the altered morphotype/transcriptome itself, or some other parameter of chronic infection (for example, the altered inflammatory environment). Changes in the bacterial transcriptome with shift to a mucoid morphotype are considerable, with likely impacts on innate recognition, phagocytosis and uptake, and accessibility to proteolytic enzymes for antigen processing. It is noteworthy, however, that the change in T cell epitope recognition in chronically infected patients is not just a matter of 'missing responses'. For example, a response to p22 is seen most commonly in the chronic infection group (data not shown).

At the same time as analyzing antigen specific changes at the level of epitope recognition, we analyzed systemic changes in adaptive immunity programs at the level of T cell transcription. Individuals from whom PA was never isolated in sputum show heightened PBMC transcription of the Th1-controlling transcription factor, Tbet and the S1P1 receptor, implicated in both CNS and lung lymphocyte trafficking (47). A relative reduction in Tbet transcription in chronic infection may indicate an intrinsic defect in (or, at least, program bias away from) Th1 polarization. Related observations have focused on the corollary of this: a switch to Th2 immunity (48). Tbet was first defined as a hallmark transcription factor for differentiation of Th1 cells, and since then, has often been taken as a biomarker of Th1 immunity (49); in Tbet knockout mice the key phenotype relates to Th1 defects (50,51). However, alterations in Tbet transcription may encompass programmatic changes in other cell types - CD8 cells, NK cells, ILCs, dendritic cells and B cells (52-56). The lipid second messenger, S1P and its receptor, S1PR1, control lymphoid organ egress, trafficking and T cell subset determination (57). Our finding that patients with chronic PA infection have an intrinsic reduction in S1PR1 lymphocyte expression may help explain their abnormal immunity to infection, suggesting the potential for therapy via modulation of this this pathway.

With relatively large numbers of multiple sclerosis patients receiving Fingolimod (FTY720, S1P analog) as a new therapeutic of choice, there is a case for increased clinical vigilance for possible breakdown of lung surveillance of respiratory infections (58). The reduction in RORC transcription in chronic PA patients did not achieve significance, but the findings appear compatible with a trend to reduced systemic Th17 immunity. The importance of Th17 immunity is highlighted by a study from Chan and colleagues, showing enhanced antigen specific Th17 responses in DLNs of explanted lungs of CF patients, suggesting that this subset may be important for lung immunity (59). Patients chronically sputum PA culture positive show a trend to reduced systemic FoxP3 and IL10 transcription, compatible with the notion that defects in immune regulation may contribute to an inflammatory environment that facilitates PA lung infection (60).

It has become apparent that chronic immune stimulation, commonly by tumor antigens or viral antigens, can lead to an 'exhausted' T cell phenotype, characterized by raised expression of PD-1 (61). Since the example of chronic PA infection encompasses protracted immune exposure to enhanced antigen expression and yet results in lowered rather than heightened T cell immunity, it is interesting to speculate that this may constitute a bacterial-driven example of exhaustion. Alternatively, other lung co-infections could be contributing to an altered immune environment in the lung. The lung is a complex immunological environment, with interplay between innate and adaptive receptors and signals from diverse microbial species (27). This encompasses not just co-infections identified in sputum by standard microbiology, but also the microbiota species which are far from immunologically silent. A fully integrated analysis of the interplay between immune and microbial repertoires is both necessary and timely. We have previously reported observations on the immunogenetics of susceptibility to idiopathic bronchiectasis in terms of HLA-C and DR

(28,29). Determining within this patient group the genetic factors driving susceptibility to chronic PA infection is an important goal, but a much larger cohort would need to be enrolled into a study dedicated to specifically address this question.

While a number of PA vaccines have been trialed including those utilizing OprF, OprI and Flagellin (10-16, 20-25), studies such as this one raise an additional possibility of exploiting the potency and flexibility of incorporating a large number of stimulatory T and B cell epitopes into epitope string vaccines. In other contexts, such as *P. falciparum* vaccination, these can be successfully administered in a 'prime-boost' regime through incorporation into an adenoviral vector followed by DNA boost (62). The appeal of the approach to a patient group such as this would be the ability to tailor a polyvalent construct to cover several different pathogens, including PA.

Table 1. Study subjects

Underlying cause of non-CF bronchiectasis	Patient ID	Age (years)	Sex (M/F)	HLA-Cw		HLA-DR		Percent of sputum samples culture positive for <i>Pseudomonas aeruginosa</i> (0%, <50%, >50%)	Other pathogens isolated in sputum
PCD	B01	57	F	6	7	11	15	0%	<i>H. influenza</i> , <i>S. pneumonia</i>
CVID	B03	66	F	7	8	1	17	0%	<i>H. influenza</i> , <i>S. pneumoniae</i> , <i>M. catarrhalis</i>
Post Infective	B10	71	F	10	16	4	7	0%	URT flora, <i>S. aureus</i>
Idiopathic	B09	62	F	7	7	15	17	0%	-
Post Infective	B18	76	M	6	10	13	14	0%	-
Idiopathic	B19	40	M	1	4	4	12	0%	-
Idiopathic	B25	75	M	4	7	1	1	0%	-
Idiopathic	B26	77	F	12	14	4	10	0%	URT flora
Idiopathic	B28	64	F	5	7	17	4	0%	URT flora
Post Infective	B29	31	F	4	15	14	15	0%	-
Idiopathic	B33	42	M	5	7	4	13	0%	URT flora
PCD	B35	40	F	7	7	1	15	0%	<i>S. aureus</i>
PCD	B36	59	F	9	7	11	13	0%	URT flora, <i>S. maltophilia</i>
PCD	B38	22	F	6	12	17	11	0%	<i>H. influenza</i> , <i>S. pneumoniae</i> , <i>M. catarrhalis</i> , <i>S. aureus</i>
Post Infective	B39	51	F	4	5	1	14	0%	URT flora, <i>S. aureus</i>
Youngs	B45	59	M	1	4	4	15	0%	<i>H. influenza</i>
Post Infective	B46	29	F	8	10	4	7	0%	<i>S. pneumonia</i>
ABPA	B27	56	F	10	6	17	13	0%	URT flora, <i>Aspergillus fumigatus</i>
ABPA	B55	60	M	10	10	7	15	0%	URT flora
CVID	B56	41	F	1	5	1	11	0%	URT flora, <i>H. influenza</i>
Post Infective	B54	59	F	6	10	7	13	0%	URT flora, <i>H. influenza</i>
ABPA	B63	27	F	7	7	4	11	0%	URT flora, <i>S. aureus</i>
Idiopathic	B77	64	M	7	12	15	17	0%	URT flora, <i>M. catarrhalis</i>
PCD	B79	33	F	2	12	4	13	0%	URT flora, <i>M. catarrhalis</i> , <i>H. influenza</i>
Idiopathic	B83	55	M	5	7	7	15	0%	URT flora
Post Infective	B73	60	M	7	7	4	15	0%	URT flora, <i>S. pneumoniae</i> , Coliform
Youngs	B64	62	F	5	7	15	17	0%	URT flora, <i>Candida albicans</i>
PCD	B72	29	F	7	16	11	15	0%	URT flora, <i>S. aureus</i> , <i>S. pneumonia</i>
Idiopathic	B74	61	F	7	7	4	15	0%	URT flora, <i>S. aureus</i>
Post Infective	B75	64	F	7	6	1	15	0%	URT flora, <i>H. influenza</i>
Post Infective	B62	53	F	7	7	15	15	0%	<i>H. influenza</i>
ABPA	B12	67	M	4	7	1	14	<50% 3/7 non-mucoid	<i>S. aureus</i>
Post Infective	B17	72	F	1	7	1	15	<50% 1/6 non-mucoid	URT flora
Idiopathic	B31	72	F	7	14	1	17	<50% 2/6 mucoid;	URT flora, <i>P. vulgaris</i>
Idiopathic	B43	61	F	1	12	1	12	<50% 3/8 mucoid	URT flora

Post Infective	B48	81	F	10	15	4	9	<50% 1/6 non-mucoid	URT flora
Idiopathic	B50	64	F	7	5	7	15	<50% 1/7 non-mucoid	URT flora, <i>M. catarrhalis</i> , <i>S. maltophilia</i>
Idiopathic	B52	52	F	7	7	1	7	<50% 1/9 non-mucoid	URT flora, Coliform, <i>S. maltophilia</i> , <i>S. aureus</i>
Idiopathic	B76	67	F	7	8	1	17	<50% 1/9 non-mucoid	URT flora, β - Haemo Streptococcus group C
Post Infective	B78	72	F	1	1	8	14	<50% 2/7 mucoid	URT flora, <i>Candida albicans</i>
Post Infective	B81	63	F	7	9	17	7	<50% 2/7 non-mucoid	URT flora, <i>H. influenza</i> , <i>S. aureus</i>
PCD	B68	53	F	4	12	15	4	<50% 3/8 non-mucoid	URT flora
Post Infective	B71	68	F	2	14	1	4	<50% 1/7 mucoid	<i>S. aureus</i>
Idiopathic	B02	78	F	2	7	4	17	$\geq$ 50% 4/8 mucoid	<i>M. catarrhalis</i>
Post Infective	B13	70	M	6	12	11	14	$\geq$ 50% 7/7 mucoid	-
Post Infective	B14	65	M	14	16	7	9	$\geq$ 50% 7/7 mucoid	-
ABPA	B16	64	M	7	10	1	7	$\geq$ 50% 5/7 mucoid	<i>S. aureus</i> , <i>H. influenza</i>
Post Infective	B21	62	F	9	7	7	15	$\geq$ 50% 7/7 mucoid	-
PCD	B23	30	M	7	7	4	15	$\geq$ 50% 1/1 mucoid	-
ABPA	B40	64	M	4	7	1	13	$\geq$ 50% 5/7 mucoid	<i>Candida albicans</i>
Post Infective	B47	72	F	7	8	4	14	$\geq$ 50% 4/4 non-mucoid	-
Idiopathic	B57	76	F	4	12	4	15	$\geq$ 50% 4/7 mucoid	-
ABPA	B82	60	M	7	12	13	17	$\geq$ 50% 6/7 mucoid	-
Post Infective	B67	66	F	17	4	1	7	$\geq$ 50% 4/7 non-mucoid	URT flora, <i>Aspergillus fumigatus</i>
ABPA	B70	55	F	5	7	4	15	$\geq$ 50% 7/7 mucoid	-
Idiopathic	B61	57	F	7	12	1	15	$\geq$ 50% 5/7 mucoid	URT flora
PCD	B85	39	F	7	10	4	15	$\geq$ 50% 6/6 non-mucoid	

^aSputum samples were collected monthly for six months and analysed by microscopy and culture using standard microbiology techniques. Patients were divided into three groups defined by the % of sputum cultures collected that were positive for *P. aeruginosa* (0%, <50%, and $\geq$ 50%). The column also shows the number of sputum samples positive for mucoid isolates over the total number of sputum samples collected and analyzed. Where no mucoid *P. aeruginosa* was isolated the number of sputum samples positive for non-mucoid isolates is given over the total number of sputum samples collected.

Table 2. OprF peptide panel

An OprF peptide library comprising 20-mer peptides overlapping by 10 amino acids was synthesized from the sequence of OprF, PA1777 (GL Biochem Ltd, Shanghai, China).

Peptide number	Peptide name	Peptide sequence
1	PA1777 [1-20]	MKLKNTLGVVIGSLVAASAM
2	PA1777 [11-30]	IGSLVAASAMNAFAQGQNSV
3	PA1777 [21-40]	NAFAQGQNSVEIEAFGKRYF
4	PA1777 [31-50]	EIEAFGKRYFTDSVRNMKNA
5	PA1777 [41-60]	TDSVRNMKNADLYGGSIGYF
6	PA1777 [51-70]	DLYGGSIGYFLTDDVELALS
7	PA1777 [61-80]	LTDDVELALSYGEYHDVRGT
8	PA1777 [71-90]	YGEYHDVRGTYETGNKKVHG
9	PA1777 [81-100]	YETGNKKVHGNLTSLDAIYH
10	PA1777 [91-110]	NLTSLDAIYHFGTPGVGLRP
11	PA1777 [101-120]	FGTPGVGLRPYVSAGLAHQN
12	PA1777 [111-130]	YVSAGLAHQNITNINSDSQG
13	PA1777 [121-140]	ITNINSDSQGRQQMTMANIG
14	PA1777 [131-150]	RQQMTMANIGAGLKYYFTEN
15	PA1777 [141-160]	AGLKYYFTENFFAKASLDGQ
16	PA1777 [151-170]	FFAKASLDGQYGLEKRDNGH
17	PA1777 [161-180]	YGLEKRDNGHQGEWMAGLGV
18	PA1777 [171-190]	QGEWMAGLGVGFNFGGSKAA
19	PA1777 [181-200]	GFNFGGSKAAPAPEPVADV
20	PA1777 [191-210]	PAPEPVADVCSDSNDGVCD
21	PA1777 [201-220]	SDSDNDGVCDNVDKCPDTPA
22	PA1777 [211-230]	NVDKCPDTPANVTVDANGCP
23	PA1777 [221-240]	NVTVDANGCPAVAEVVRVQL
24	PA1777 [231-250]	AVAEVVRVQLDVKFDFDKSK
25	PA1777 [241-260]	DVKFDFDKSKVKENSYADIK
26	PA1777 [251-270]	VKENSYADIKNLADFMKQYP
27	PA1777 [261-280]	NLADFMKQYPSTSTTVEGHT
28	PA1777 [271-290]	STSTTVEGHTDSVGTDAYNQ
29	PA1777 [281-300]	DSVGTDAYNQKLSERRANAV
30	PA1777 [291-310]	KLSERRANAVRDVLVNEYGV
31	PA1777 [301-320]	RDVLVNEYGVEGGRVNAVGY
32	PA1777 [311-330]	EGGRVNAVGYGESRPVADNA
33	PA1777 [321-340]	GESRPVADNATAEGRAINRR
34	PA1777 [331-350]	TAEGRAINRRVEAEVEAEAK

Table 3. OprF peptide relative binding affinity to HLA-DR molecules

OprF peptide	DR1	DR3	DR4	DR7	DR9	DR11	DR13	DR1501
p1	1	>1 060	3	2	10	50	>309	4
p2	2	>1 060	3	4	3	11	>309	3
p3	13	>1 060	350	9	23	756	2	94
p4	1,134	224	10	964	420	75	3	173
p5	15	>1 060	249	1,420	122	1,500	>309	3
p6	36	21	11	10	52	354	>309	49
p7	1,793	>1 060	>2 968	534	37	>4 297	>309	81
p8	1,120	>1 060	>2 968	>5 056	>1 642	537	>309	>1 119
p9	15	533	2,000	1,412	>1 642	1,333	>309	1,771
p10	18	447	753	91	42	129	>309	8
p11	112	>1 060	67	67	39	548	200	0.5
p12	8,571	>1 060	>2 968	>5 056	>1 642	>4 297	>309	>1 119
p13	11,429	26	1,333	355	345	1,500	>309	417
p14	11	23	283	49	13	707	2	67
p15	8	>1 060	5	>5 056	13	22	>309	20
p16	2,673	>1 060	>2 968	9,412	574	149	>309	1,458
p17	4,286	>1 060	>2 968	1,765	548	4,167	>309	>1 119
p18	66	>1 060	460	127	57	420	>309	123
p19	212	>1 060	4,333	241	177	8,333	>309	1,250
p20	28,571	>1 060	6,000	11,765	>1 642	>4 297	>309	>1 119
p21	20,000	>1 060	>2 968	>5 056	>1 642	6,667	>309	>1 119
p22	3,714	683	>2 968	7,059	>1 642	>4 297	>309	>1 119
p23	94	>1 060	>2 968	>5 056	>1 642	>4 297	>309	>1 119
p24	>11 262	>1 060	>2 968	>5 056	>1 642	>4 297	>309	>1 119
p25	2,857	13	1,467	1,412	237	2,083	>309	833
p26	822	>1 060	258	1,065	1,200	335	>309	70
p27	71	>1 060	103	80	85	866	169	4
p28	>11 262	>1 060	>2 968	>5 056	>1 642	>4 297	>309	>1 119
p29	1,225	>1 060	>2 968	1,412	600	342	>309	1,042
p30	949	>1 060	467	142	63	1,667	>309	38
p31	90	>1 060	161	290	64	130	>309	33
p32	35	>1 060	>2 968	183	31	391	>309	15
p33	14,286	>1 060	2,667	>5 056	3,600	2,917	>309	>1 119
p34	>11 262	>1 060	>2 968	>5 056	>1 642	7,500	>309	>1 119

*Results are expressed as a relative binding ratio obtained by dividing the IC₅₀ of each peptide by that of a reference peptide that binds strongly to the HLA molecule tested. Lower numbers correspond to a higher binding affinity. Numbers in red (ratio of 20 or less) = high affinity binding; numbers in green (ratio 20-100) = moderate binding affinity. Each peptide - MHC combination was evaluated in 2 independent experiments.

Table 4. Cytokine and chemokine responses in non-CF bronchiectasis.

Immunological protein		Never culture positive (pg/ml) ¹	Culture positive >50% (pg/ml) ²	P value
Cytokines	GM-CSF	136 ± 25	240 ± 50	
	G-CSF	348 ± 77	779 ± 258	
	TNFα	517 ± 96	1027 ± 319	
	IFNα	3.9 ± 2	10.7 ± 6	
	IL-1β	1671 ± 175	2315 ± 276	
	IL-2	0.2 ± 0.05	0.2 ± 0.1	
	IL-4	1.2 ± 0.4	3.6 ± 0.9	**
	IL-5	0.5 ± 0.1	0.8 ± 0.3	
	IL-6	5339.1 ± 1508	12772 ± 3401	*
	IL-7	15.1 ± 2	21.6 ± 5.8	
	IL-8	35230 ± 17683	95584 ± 34319	
	IL-10	88.3 ± 21	159 ± 68	
	IL-12	12.4 ± 2	24.9 ± 5	*
	IL-13	1.1 ± 0.3	2.2 ± 0.7	
	IL-15	52.8 ± 10	60.6 ± 28	
	IL-17	0.1 ± 0.05	0.3 ± 0.2	
	IL-1RA	0	0	
	IL-2R	53.8 ± 11	83.5 ± 25	
Chemokines	MIP-1^α	7338 ± 2465	18963 ± 4145	*
	MIP-1β	1215 ± 230	4038 ± 1040	**
	RANTES	161 ± 36	202 ± 46	
	EOTAXIN	0.05 ± 0.02	0.05 ± 0.02	
	IP-10	1.9 ± 0.7	2.7 ± 2	
	MCP1	0	0	
	MIG	2.5 ± 1	2.3 ± 2	
Growth Factors	VEGF	72.1 ± 13	78.3 ± 32	
	EGF	1.3 ± 0.3	0.6 ± 0.3	
	HGF	3.1 ± 1	3.0 ± 2	
	FGF	6.6 ± 1	7.8 ± 2	

¹Never (0%) (n=13 for IL-6, Mip1^α and IL-8; n=24 for MIP-1^β and IL-12) or ²frequently (>50%) (n=12 for IL-6, Mip1^α and IL-8; n=8 for MIP-1^β and IL-12) culture positive for PA. Data is presented ±SEM. Statistical significance was determined using the Mann-Whitney U test (*, p<0.05; **, p<0.01).

Table 5. Subjects used in T cell functional analysis**A. Luminex**

Group Classification	Luminex protein	Age ($\pm 2SD$)	Sex	n number
Never culture positive for PA	IL-6/IL-8/MIP-1 α	52 $\pm$ 33	11F/2M	13
	All other proteins	51 $\pm$ 30	18F/6M	24
>50 % cultures positive for PA	IL-6/IL-8/MIP-1 α	64 $\pm$ 21	7F/5M	12
	All other proteins	55 $\pm$ 28	4M/4F	8

B. ELISpot

Group Classification	Age ($\pm 2SD$)	Sex	n number
Never sputum culture positive for PA	53 $\pm$ 31	22F/9M	31
<50 % of sputum cultures positive for PA	66 $\pm$ 17	11F/1M	12
>50 % of sputum cultures positive for PA	61 $\pm$ 26	8F/6M	14

C. ELISA

Group Classification	Age ($\pm 2SD$)	Sex	n number
Never sputum culture positive for PA	54 $\pm$ 31	33F/12M	45
<50 % of sputum cultures positive for PA	64 $\pm$ 23	14F/2M	16
>50 % of sputum cultures positive for PA	61 $\pm$ 22	10F/10M	20

D. Real-time PCR

Group Classification	Age ($\pm 2SD$)	Sex	n number
Never culture positive for PA	47 $\pm$ 32	6F/1M	7
>50 % of sputum cultures positive for PA	58 $\pm$ 26	2F/2M	4

Legends

Figure1

T cell and serum antibody responses to PA protein, OprF in patients with non-CF bronchiectasis.

(A) The magnitude of the T cell response to OprF and control anti-CD3 in the 3 bronchiectasis groups classified according to the PA infection status were determined (Never (0%) n=31; sometimes (<50%) n=12 and frequent ($\geq$ 50%) n=14 culture positive for PA). In the group that were <50% sputum sample culture positive for PA, 4/12 (25%) individuals grew mucoid isolates and in the $\geq$ 50% group, 11/14 (79%) individuals grew mucoid isolates. (Chi-Square Test 5.42; p=0.0199, Pearson). (B) The magnitude of the T cell response to OprF and control anti-CD3 dependant of PA isolate cultured (Never (0%) n=31, non-mucoid n=11 or mucoid n=15). (C) The IgG OprF specific antibody titers in the 3 bronchiectasis groups classified according to the PA infection status were determined (Never (0%) n=45; sometimes (<50%) n=16 and frequent ($\geq$ 50%) n=21). (D) The IgG OprF specific antibody titers dependant on PA isolate cultured (Never (0%) n=45, non-mucoid n=17 or mucoid n=19). Data is presented $\pm$ SEM. Statistical significance was determined using the Kruskal wallis test followed by a post Dunn's test. (*, p<0.05; **, p<0.01, ***p<0.001).

Figure 2. T cell epitope responses to OprF peptide panel in non-CF bronchiectasis patients with evidence of chronic PA infection.

Epitope mapping of patient T cell responses to OprF were determined by screening responses to an overlapping peptide panel of OprF in (A) for PA infection status: Never (0%) n=31; sometimes (<50%) n=12 and frequent ($\geq$ 50%) n=14 culture positive or (B) PA isolate: never PA positive n=31, non-mucoid n=11 or mucoid n=15. Representative examples of patient T cell responses to the peptide panel are shown for the never (0%) (C), sometimes (<50%) (D) and frequently ($\geq$ 50%)(E) culture positive. (F) The overlapping relationships of epitopes identified in bronchiectasis patients with PA isolates classified as never PA culture positive (n=31), non-mucoid (n=11) and mucoid (n=15) positive cultures visualized as a Venn diagram. Data is presented $\pm$ SEM. Statistical significance was determined using the Kruskal Wallis Test followed by a post Dunn's test. (*, p<0.05, **, p<0.01)

Figure 3. CD4 T cell epitope responses to OprF peptide panel in HLA class II transgenic mice

Draining lymph nodes from transgenic mice for DRB1*0101, n=15 (A), DRB1*0401, n=9 (B) and DRB1*1501, n=10 (C) were harvested 10 days post immunization with OprF protein in CFA. Recall peptide responses were determined using the overlapping peptide panel for OprF. Responses were considered positive if the response was >2SD above the mean of the negative control.

Figure 4. Cytokine and chemokine responses to OprF protein in non-CF bronchiectasis with evidence of chronic PA infection.

Pro-inflammatory cytokine concentrations for (A) IL-6, (B) Mip1 α , (C) Mip1 β , (D) IL-12 and (E) IL-8 were determined from the supernatant of PBMCs stimulated with OprF. Concentrations were measured using the 30-plex Luminex assay in bronchiectasis patients classified as never (0%) (n=13 for IL-6, Mip1 α and IL-8; n=24 for MIP-1 β and IL-12) or frequently ($\geq$ 50%) (n=12 for IL-6, Mip1 α and IL-8; n=8 for MIP-1 β and IL-12) culture positive for PA. Data is presented $\pm$ SEM. Statistical significance was determined using the Mann-Whitney U test (*, p<0.05; **, p<0.01).

Figure 5. Lymphocyte transcriptional changes in non-CF bronchiectasis patients with evidence of chronic PA infection.

The expression of (A) TBX21, (B) S1pr1, (C) RORc α , (D) Gata3, (E) FoxP3 and (F) IL10 were determined by qRT-PCR. PBMCs were stimulated with PMA/Ionomycin from which RNA was isolated and converted to cDNA. Patients with bronchiectasis were classified as never (0%) (n=7) or frequently (>50%) (n=4) culture positive for PA. Data is presented $\pm$ SEM. Statistical significance was determined using the Mann-Whitney U test (*, p<0.05).

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Competing interests

Dr. Wilson reports personal fees from Bayer PLC, outside the submitted work. The rest of the authors have declared that no competing interests exist.

Figure 1

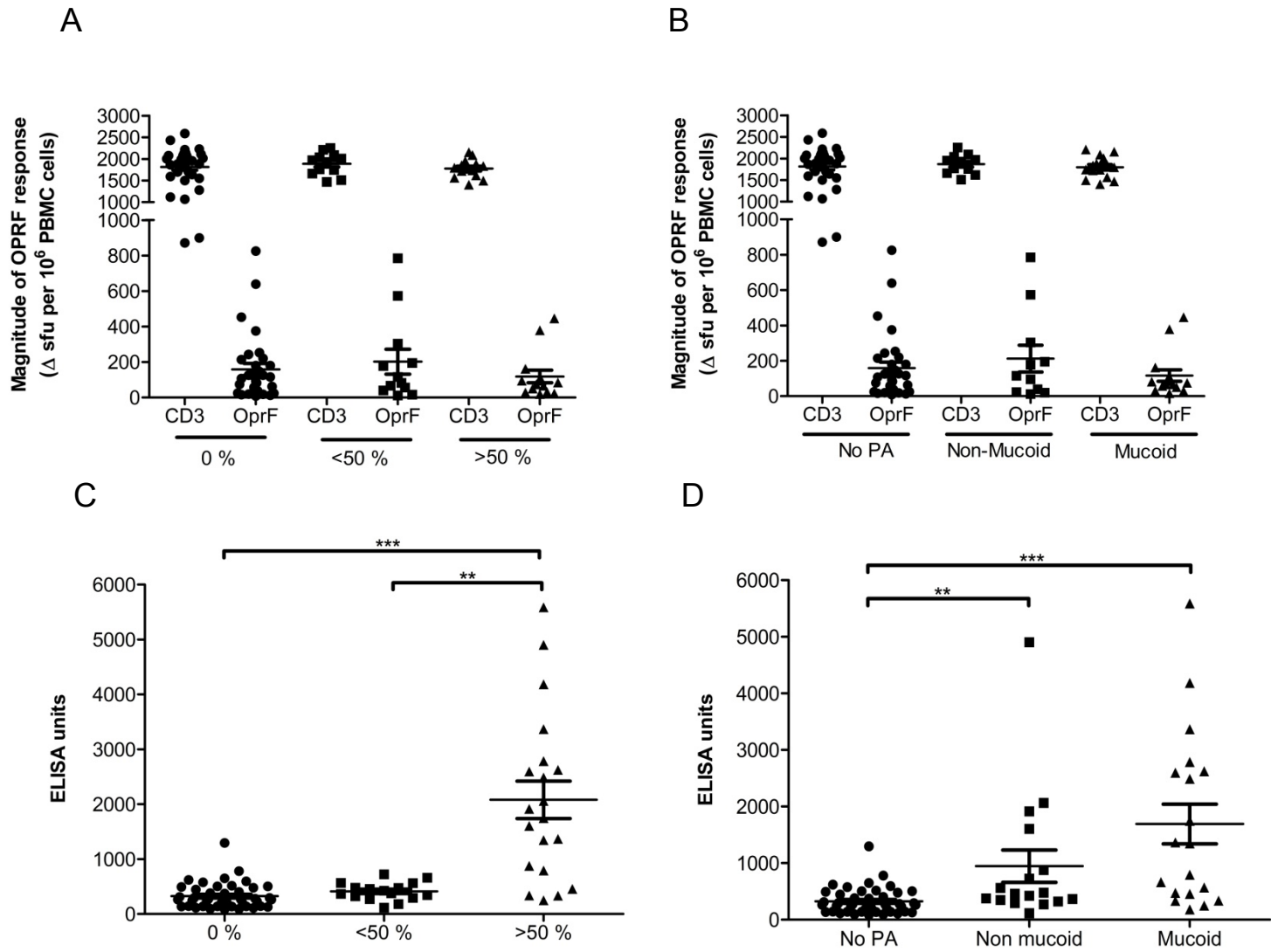


Figure 2

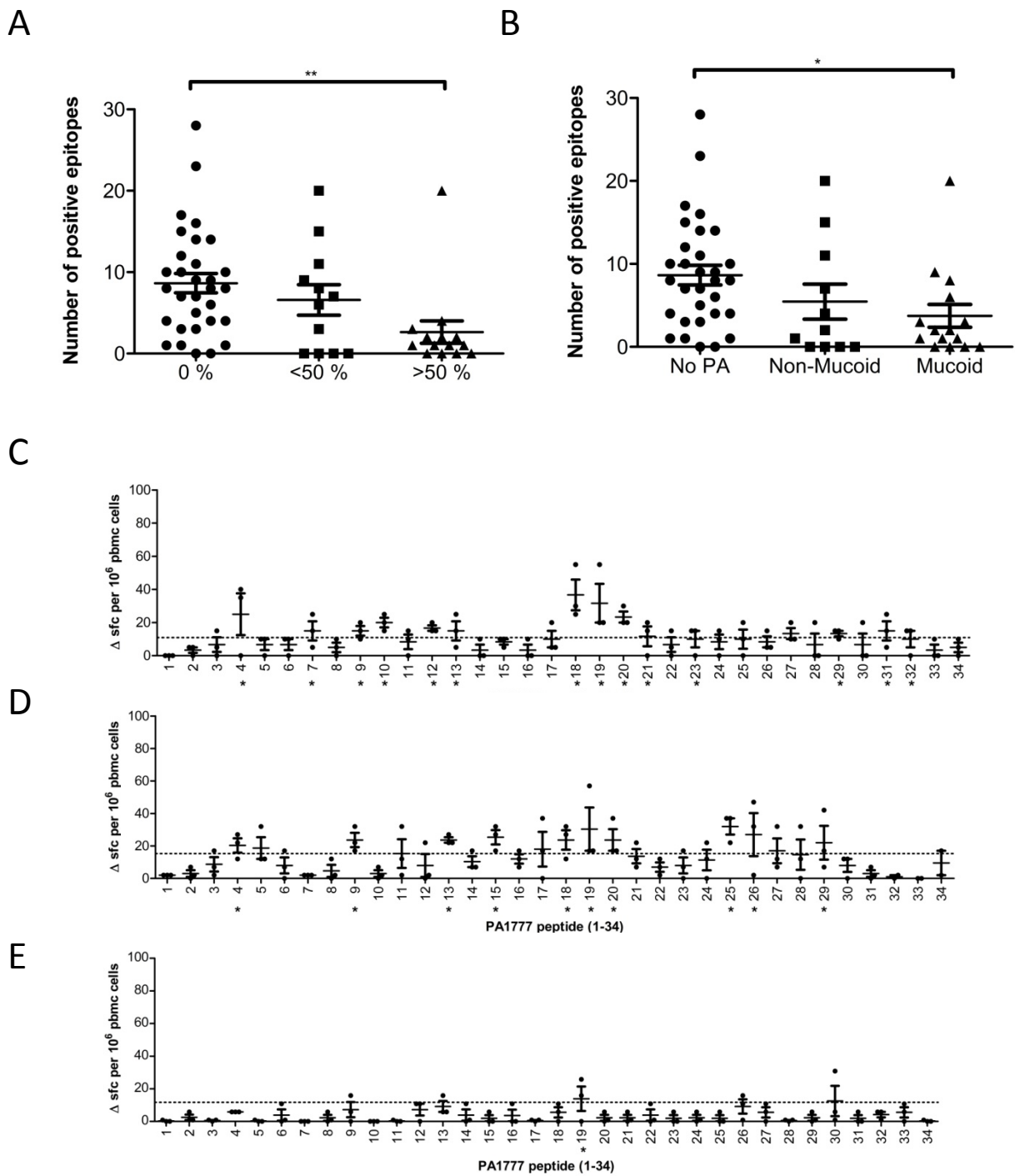


Figure 2F

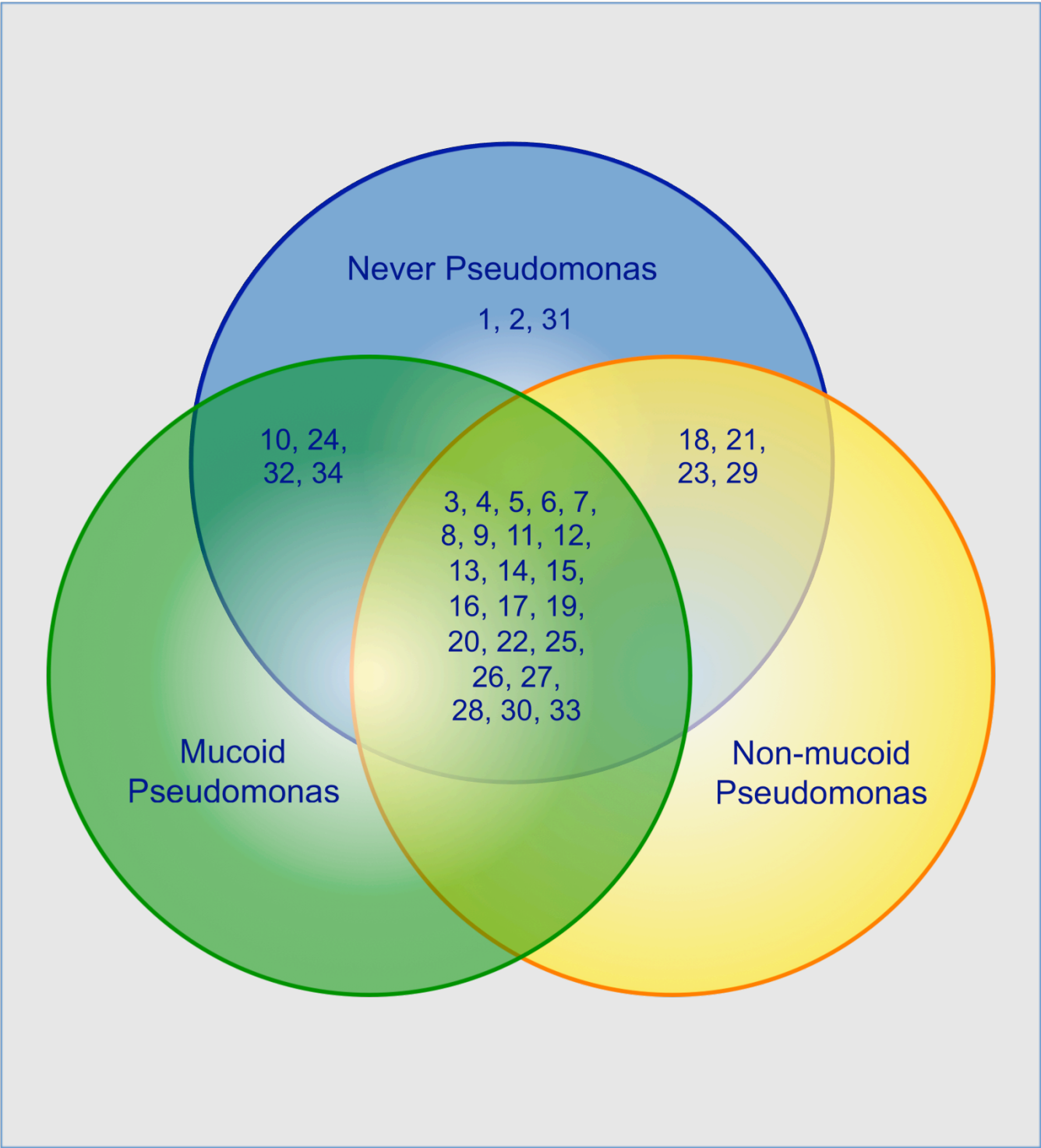


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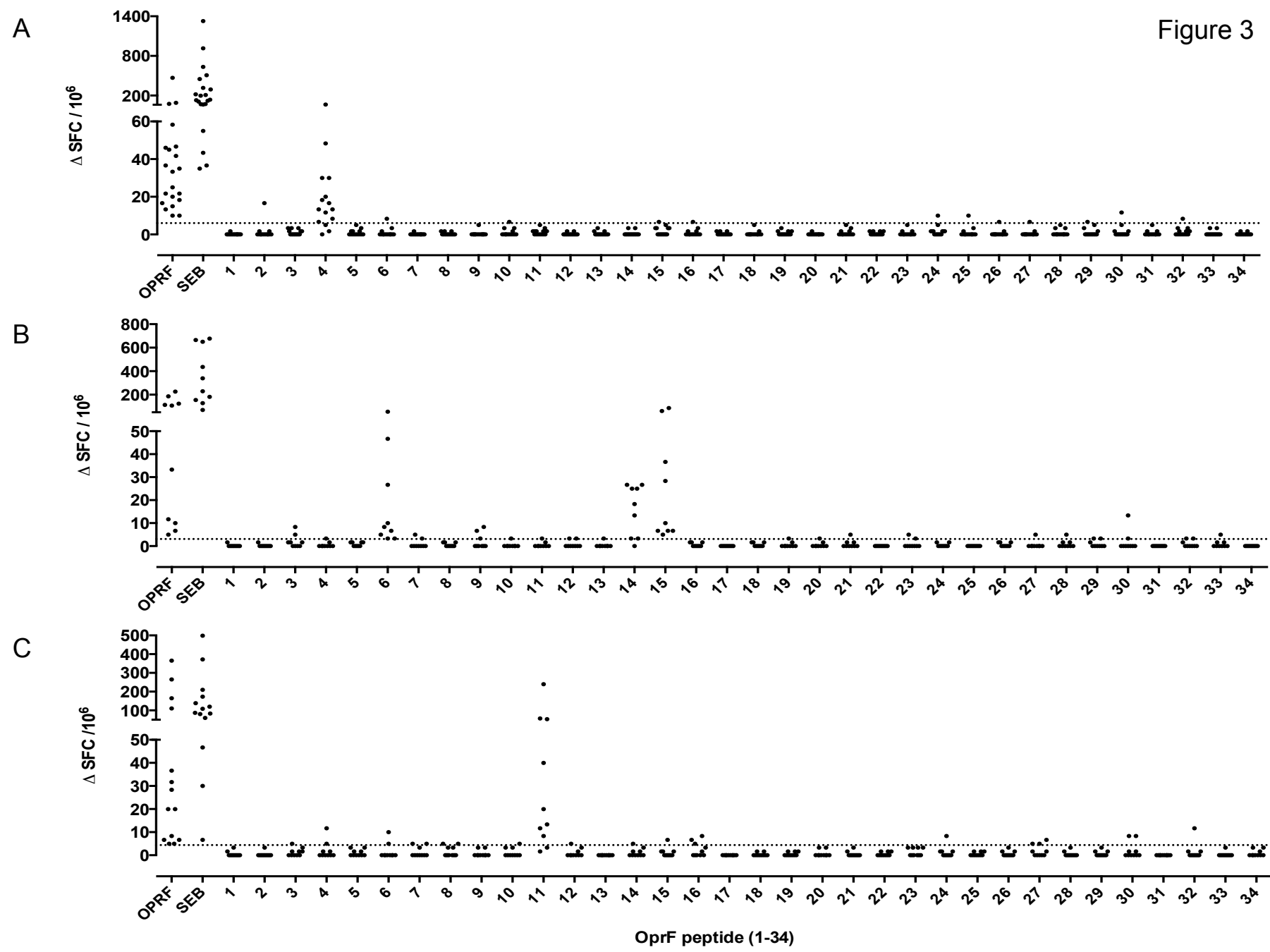


Figure 4

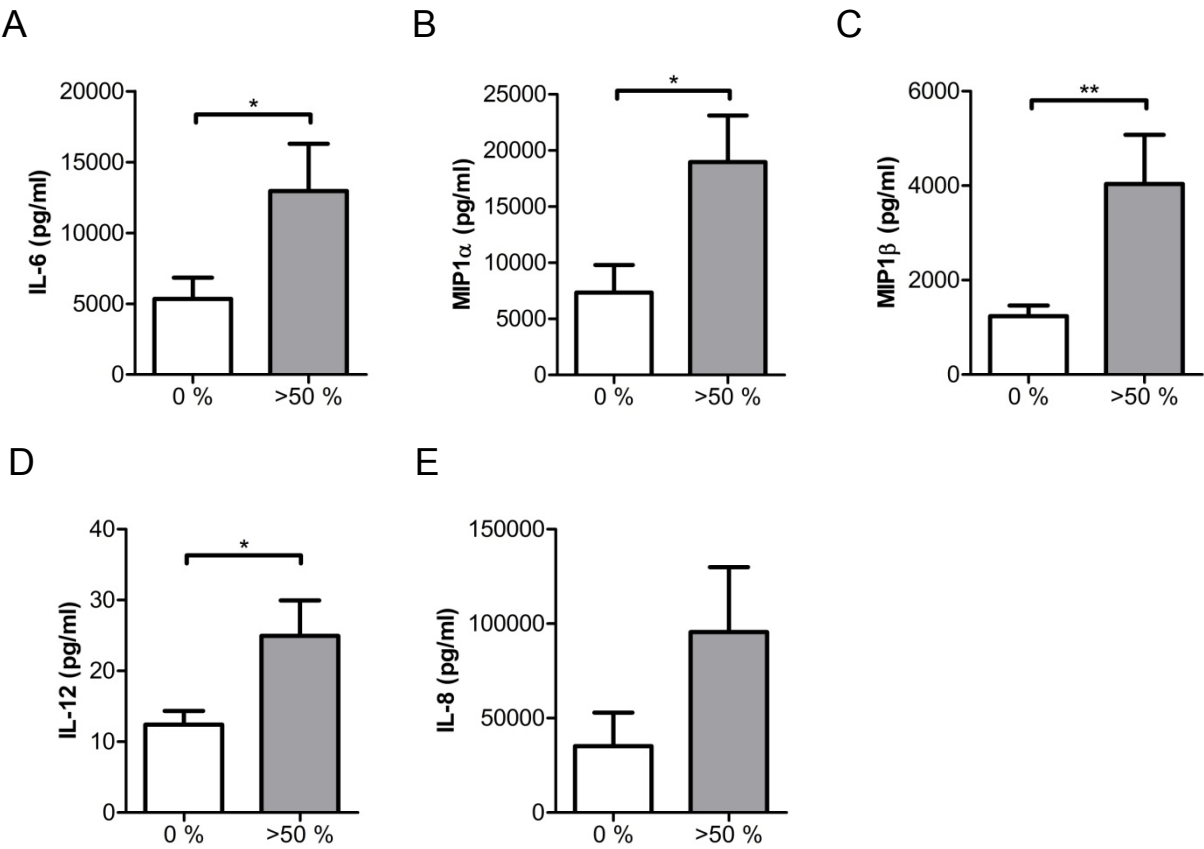
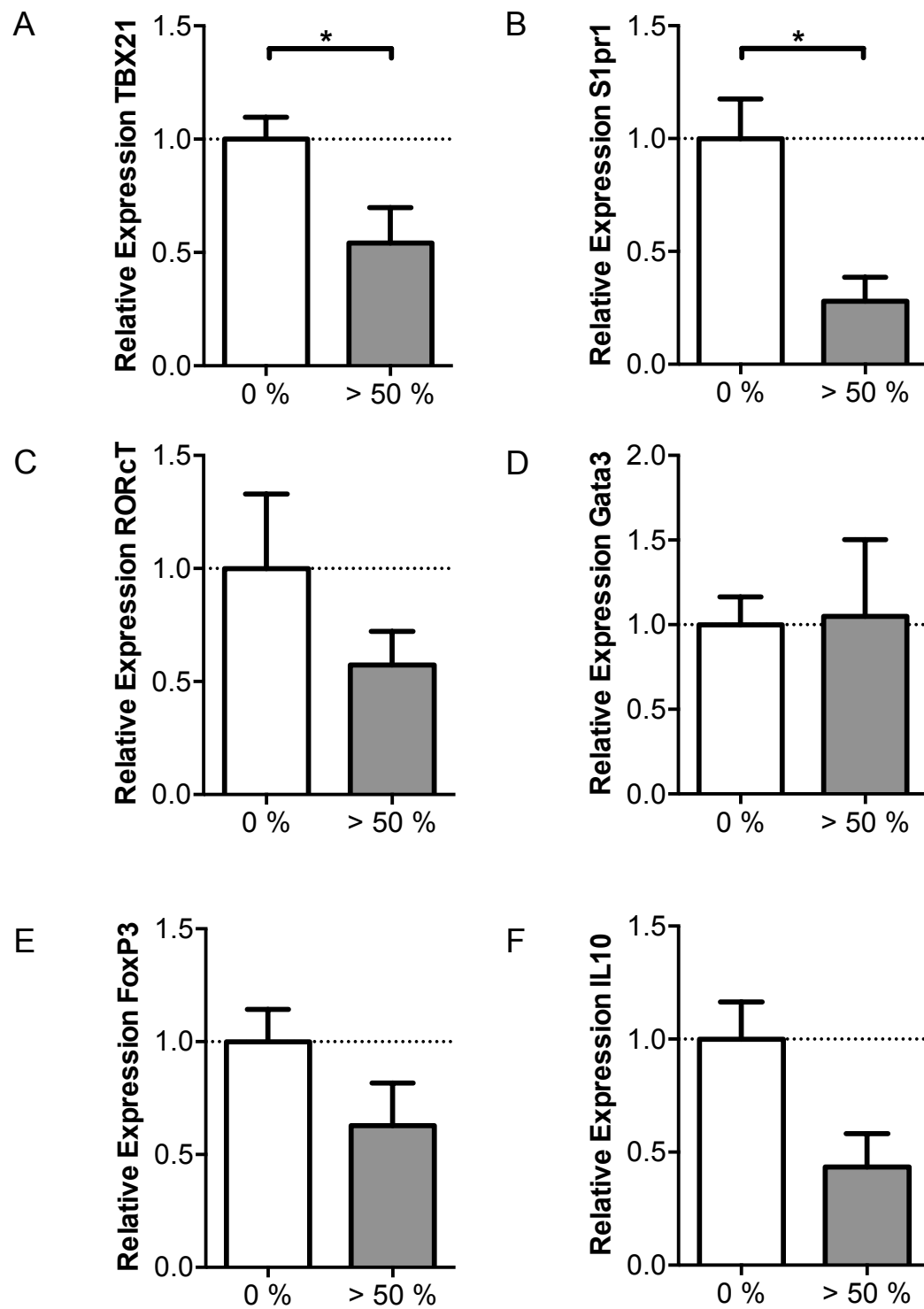
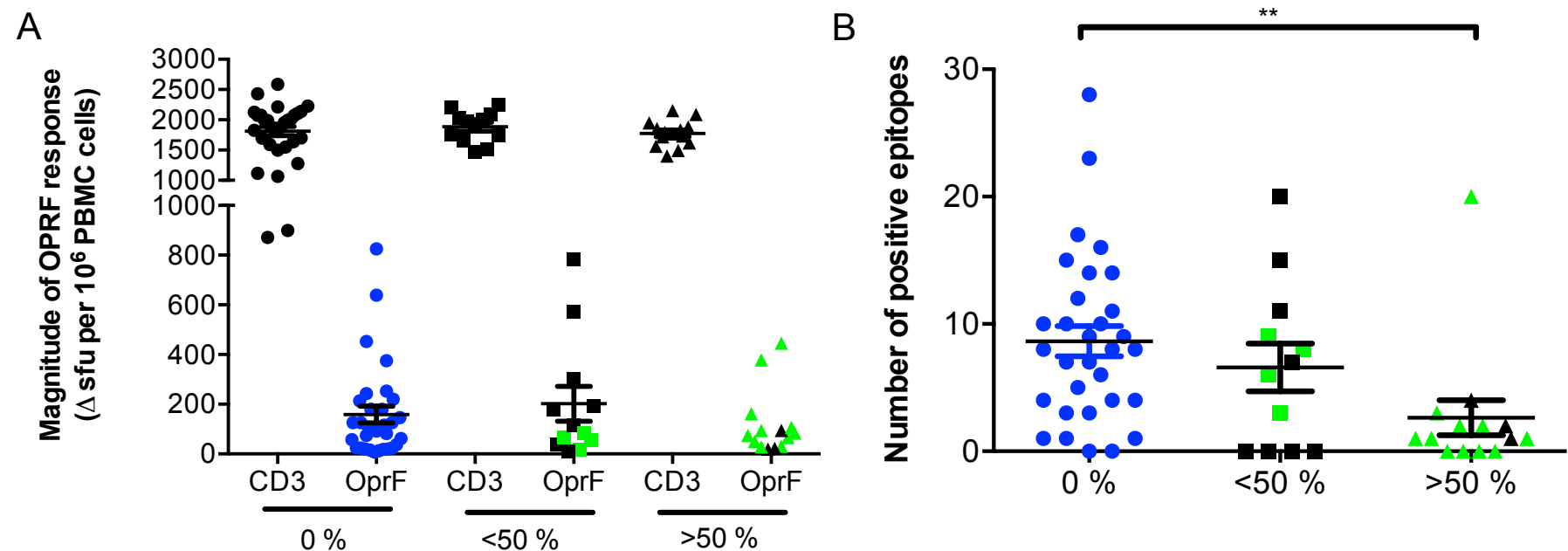


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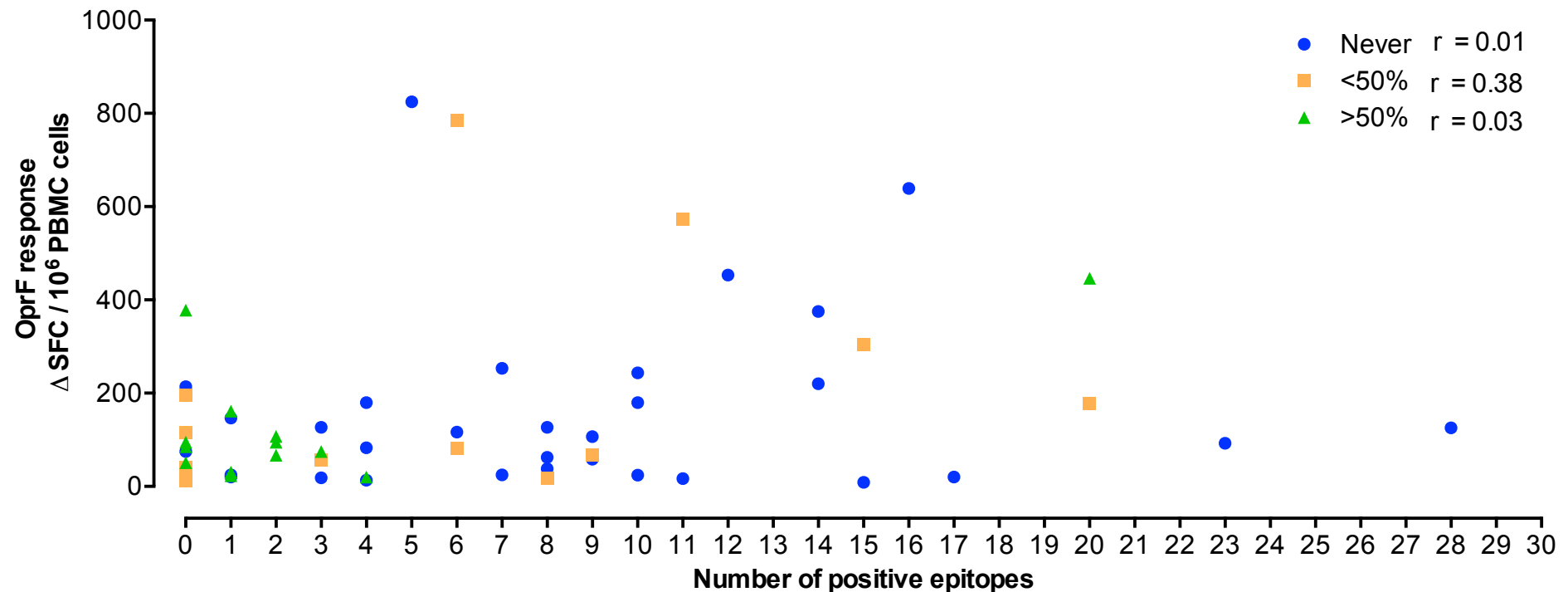




Supplementary Figure 1. T cell responses to the PA protein, OprF and number of T cell epitope responses to OprF peptide panel in patients with non-CF bronchiectasis.

(A) The magnitude of the T cell response to OprF and control anti-CD3; and (B) the number of T cell epitopes identified in the 3 bronchiectasis groups classified according to the PA infection status. Never (0%, circle) n=31; sometimes (<50%, square) n=12 and frequent (>=50%, triangle) n=14 culture positive for PA). Blue indicates no PA; Black indicates non-mucoid PA; and green indicates mucoid PA. Data is presented ±SEM. Statistical significance was determined using the Kruskal wallis test followed by a post Dunn's test. (**, p<0.01). In the group that was <50% sputum sample culture positive for PA, 4/12 (25%) individuals grew mucoid isolates and in the >=50% group, 11/14 (79%) individuals grew mucoid isolates. (Chi-Square Test 5.42; p=0.0199, Pearson).

Supplementary Figure 2



Supplementary Figure 2. Magnitude of OprF T cell response does not correlate with number of OprF epitopes recognized across this non-CF bronchiectasis cohort.

There is no correlation between the magnitude of the T cell response to OprF and the number of responding peptides determined for each of the 3 non-CF bronchiectasis cohorts classified according to the PA infection status (Never (0%) n=31; sometimes (<50%) n=12 and frequent (>50%) n=14 PA culture positive). Statistical significance was determined using the Spearman's rank correlation. Never (blue circle; r=0.01), sometimes <50% (orange square; r=0.38), and frequently >50% (green triangle; r=0.38) sputum culture positive for PA.