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Author/s:
Liu, H;Sarkar, S;Azzopardi, K;Day, S;Yeow, S;Ranganathan, S;Sutton, P

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PROF. PHILIP SUTTON (Orcid ID : 0000-0003-3129-1800)

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A bacterial stimulation assay for bronchoalveolar lavage immune cells from young children with cystic fibrosis

Haipei Liu,[1,2] Sohinee Sarkar,[1] Kristy Azzopardi,[1] Sophie Day,[1] Serene Yeow,[1] Sarath Ranganathan[1,3,4], Philip Sutton[1,3#]; on behalf of the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF)

[1] Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia

[2] Department of Pediatric Respiratory Medicine, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

[3] Department of Paediatrics, University of Melbourne, Parkville, Victoria 3010, Australia

[4] Department of Respiratory and Sleep Medicine, Royal Children's Hospital, Melbourne, Victoria, Australia.

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#Correspondence: Professor Philip Sutton, Mucosal Immunology, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia. phil.sutton@mcri.edu.au; Ph: +61 3 9936 6751

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Running Head: Bacterial stimulation of BAL cells from children with cystic fibrosis

Keywords: Lung; human, mucosal immunity; bacterial infection

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1 **ABSTRACT**

2 Cystic Fibrosis (CF) is primarily a progressive lung disease, characterised by chronic 3 pulmonary infections with opportunistic pathogens. Such infections typically commence 4 early in life, producing an inflammatory response marked by IL-8 chemokine production and 5 neutrophilic infiltration, major contributory factors in CF progression. Studying this 6 inflammation, especially early in life, is critical for developing new strategies for preventing 7 or slowing disruption to the structural integrity of the CF airways. However, evaluating the 8 immune responses of bronchoalveolar lavage (BAL) cells from children with CF faces 9 technical challenges, including contamination carried from the lung due to pre-existing 10 infections and low cell number availability. Here we describe a technique for preparing BAL 11 cells from young children with CF and using those cells in a bacterial stimulation assay. 12 Initial antibiotic treatment proved essential for preventing resident bacteria from overgrowing 13 BAL cell cultures, or non-specifically activating the cells. *ACTB*, identified as an optimal 14 reference gene, was validated for accurate analysis of gene expression in these cells. 15 *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used as bacterial stimulants to 16 evaluate the immune response of BAL cells from young children with CF. Addition of 17 gentamicin prevented bacterial overgrowth, although if added after 3 hours of culture an 18 extremely variable response resulted, with the bacteria causing a suppressive effect in some 19 cultures. Addition of gentamicin after 1 hour of culture completely prevented this suppressive 20 effect. This technique was then able to reproducibly measure the IL-8 response to stimulation 21 with *S. aureus* and *P. aeruginosa*, including co-stimulation with both bacteria.

22 **INTRODUCTION**

23 The common genetically inherited condition, cystic fibrosis (CF) arises from a mutation in 24 the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that encodes for a 25 chloride channel. CF is primarily a progressive lung disease, characterised by chronic 26 infection of the lung with a range of opportunistic pathogens. Pulmonary infections with 27 bacteria such as *Staphylococcus aureus* and non-typeable *Haemophilus influenzae* typically 28 commence early in life, with the major CF pathogen *Pseudomonas aeruginosa* becoming 29 more prevalent later in childhood.[1] A key hallmark of CF is the infiltration into the lung of 30 inflammatory immune cells, recruited by these pathogenic infections, which instigate a 31 chronic innate inflammatory response that is a major contributory factor in CF disease 32 progression. It has recently become evident that inflammatory responses that occur in the

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33 lung in the first few years of life of an individual with CF play a critical role in deciding the
34 long term consequences of disease progression.[2]

35 Dissecting early immune events that occur in the CF lung is therefore important for a full
36 understanding of the factors that contribute to or protect against disease progression. Our
37 ability to understand these events is currently limited by the paucity of studies which have
38 evaluated how immune cells derived from the CF lung respond to pathogens of relevance to
39 this disease. Functional studies on immune cells in CF have almost entirely been restricted to
40 the analysis of those found circulating in the blood, revealing that peripheral blood immune
41 cells can differ from those of control individuals. For example, circulating T cells from CF
42 patients express lower levels of the chemokine receptor CXCR1 which impacted on their
43 motility in a chemotaxis assay.[3] Additionally, peripheral blood T cells from young CF
44 patients produced lower levels of IL-8 than cells from age-matched controls, but contained
45 increased numbers of IL-2 secreting T cells.[4] This latter feature was only detectable upon
46 stimulation, demonstrating the importance of evaluating immune cells following activation.
47 These differences might indicate direct effects of *CFTR* mutations on immune cell function,
48 or alternatively arise from indirect downstream effects of CF disease. While studies on
49 circulating immune cells are valuable for gaining knowledge as to the overall effect of *CFTR*
50 mutations on immunity, the lung is the main site of pathology in CF, so insights into the
51 functionality of pulmonary immune cells would considerably increase our understanding of
52 how such cells respond to infectious challenge and contribute to disease progression. Such
53 studies are currently lacking, especially with respect to the response of lung immune cells to
54 direct activation with CF-relevant pathogens such as *S. aureus* and *P. aeruginosa*.

55 Another important feature of such analyses is that, where practical, they should measure the
56 response generated to exposure to live pathogens. It is common for immune cell stimulation
57 assays to use killed pathogens or fractions thereof. While informative, pathogens often
58 possess immunomodulatory activity that might play an important role *in vivo*, but be
59 potentially missed *in vitro* if killed pathogens are used. *Vibrio cholerae*, for example, induces
60 higher levels of IL-23 secretion when used to stimulate cells in culture, as compared to when
61 heat-inactivated.[5] The CF pathogen *S. aureus* possess immunomodulatory activity that at
62 least partially appears to require the active secretion of membrane vesicles.[6]

63 This study therefore aimed to develop a method for measuring the response, to live
64 pathogenic stimulation, of immune cells collected in bronchoalveolar lavage (BAL) fluid
65 from young children with CF. The predominant immune cell type present in BAL collected

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66 from the healthy human lung is the alveolar macrophage. In CF however, the production of
67 pro-inflammatory cytokines, in particular the chemokine IL-8, in response to chronic
68 infection can result in the recruitment of large numbers of other immune cells, in particular
69 neutrophils, that are believed to play a major role in CF pathogenesis.[7-9] Other immune cell
70 populations are also present in BAL from children with CF, including T cells, B cells, NK
71 cells and dendritic cells, albeit in lower numbers.[10] Such cells would be expected to play a
72 role in combatting infection, and potentially also contribute to the chronic inflammatory
73 response seen in CF.[3, 4] However very little is known about the role, if any, of these cells in
74 the progression of CF disease or how the immune cells present in the lungs of children with
75 CF respond to major pathogens.

76 While studying the biology of these cells is clearly important for understanding CF disease
77 pathogenesis and progression, it has unique technical challenges. Firstly, samples available
78 for such research purposes are commonly residual aliquots collected from children with CF
79 for clinical evaluation; the cells available for analysis are therefore typically limited in
80 number. Secondly, these cells are standardly contaminated by the infections present in the
81 lungs of children with CF. Thirdly, there is a high degree of variability in the nature of these
82 infections, which might be bacterial (Gram positive and negative), fungal or viral in origin.
83 This has resulted in a paucity of studies assessing the functional response to pathogenic
84 stimulation of immune cells from the lungs of children with CF.

85 The aim of this study was to develop a protocol for facilitating the measurement of an
86 immune response of cells collected from the lung washings of children with CF to stimulation
87 with live pathogen. The model used to achieve this involved stimulating BAL immune cells
88 with the CF bacterial pathogens *P. aeruginosa* and *S. aureus*, measuring secretion of
89 important cytokines, including the chemokine IL-8, as a relevant read-out.

90

91 **MATERIALS AND METHODS**

92 ***Bacterial culture***

93 *P. aeruginosa* strain NCTC 10662 and *S. aureus* strain ATCC 29213 were grown overnight
94 in LB broth at 37°C with shaking at 200 rpm. Bacterial cultures were centrifuged at 3270 g
95 for 10 min then culture media decanted. Bacteria were washed twice with phosphate buffered
96 saline (PBS) and then resuspended in Assay Medium (RPMI-1640 (Roswell Park Memorial

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97 Institute medium 1640), containing 10% foetal calf serum (FCS) and 2 mM glutamine
98 (Gibco)) at 2 x 10^6 bacteria/mL.

99

100 ***BAL cell culture and bacterial stimulation***

101 Bronchoalveolar lavage (BAL) fluid was collected from children aged 1 to 6 years old, who
102 received a diagnosis of CF and who were participants in the Australian Respiratory Early
103 Surveillance Team for Cystic Fibrosis (AREST-CF) program conducted by the Royal
104 Children's Hospital, Melbourne. Samples were collected during routine clinical procedures
105 and were analysed with approval by the Human Research Ethics Committee of the Royal
106 Children's Hospital Melbourne. The method for collecting BAL was as described previously
107 in detail.[11] Briefly, three aliquots of normal saline (1 mL/Kg body weight) were lavaged into
108 the right middle lobe and one aliquot lavaged into the lingula. The first lavage from each lobe
109 was processed for detection of bacteria, viruses, or fungi as previously described.[12] The
110 second and third lavages from the right middle lobe were used for BAL cell culture.

111 BAL cells were centrifuged at 600 g for 10 min, supernatants discarded, cells washed twice
112 with PBS and then resuspended overnight in a 6-well culture plate (Nunc) in 3-5 ml of
113 antibiotic medium (RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 U/mL penicillin
114 (Gibco), 0.1 mg/mL streptomycin (Gibco), 2.5 µg/mL amphotericin B (Sigma) and 0.5
115 mmol/L EDTA). Cells (2x10^4-10^5/mL) were then stimulated with *P. aeruginosa* (multiplicity
116 of infection 5) in RPMI 1640 medium as above without antibiotics, at 37°C in 5% CO_2 for
117 either 1 or 3 hours, after which 100 µg/mL gentamicin (Sigma) was added to inhibit further
118 bacterial growth. After stimulation for different times as indicated (the total stimulation time
119 was 6 h, 12 h, 18 h or 24 h), cell cultures were centrifuged at 600 g for 10 min. Supernatants
120 were collected, centrifuged at 13000 g for 10 min to remove bacteria and cell debris, and
121 stored at -20°C for cytokine assessment.

122 ***Cytokine enzyme-linked immunosorbent assay***

123 IL-8 secretion into culture supernatant was adopted as a standard measurement of BAL cell
124 response to immune activation. The concentrations of IL-8 in the supernatants of BAL cell
125 cultures were determined by enzyme-linked immunosorbent assay (ELISA) after bacterial
126 stimulation for different times (6, 12, 18 or 24 hours). 96-well Maxisorp plates (Nunc) were
127 coated with mouse anti-human IL-8 (0.2 µg/well; R&D Systems) in PBS overnight at 4°C.
128 Plates were blocked with 1% (wt/vol) bovine serum albumin (BSA) in PBS (PBS-BSA) for 2

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129 h at room temperature (RT). Samples were diluted 1:10 in PBS-BSA, and 50 µL was added to
130 triplicate wells, before overnight incubation at 4°C. After washing, biotinylated goat anti-
131 human IL-8 (1 ng/well; R&D Systems) in PBS-BSA was added and incubated at RT for 2 h,
132 followed by 50 µL of horseradish peroxidase conjugated streptavidin per well (1:40 in PBS-
133 BSA; 30 min; R&D Systems). Colour was developed by addition of 3,3',5,5'-
134 tetramethylbenzidine (TMB) (Invitrogen) and the reaction was stopped by adding 100 µL 2
135 mol/L H_2SO_4. Absorbance was read at 450 nm, and IL-8 concentrations determined against a
136 standard curve of recombinant human IL-8 (R&D Systems). IL-6 was measured with the
137 same protocol using reagents from the Human IL-6 Duoset ELISA (R&D Systems).

138 ***Optimisation and validation of reference gene by qPCR***

139 To quantify gene expression by PCR (qPCR), RNA was extracted from BAL cells (cultured
140 overnight in either DMEM (Dulbecco's Modified Eagle Medium) or RPMI complete assay
141 medium) using Tri Reagent (Ambion). Fifty ng of total RNA from each sample was then
142 converted to cDNA with the GoScript™ Reverse Transcription System (Promega) in a total
143 reaction volume of 20 µL. Resulting cDNA was further diluted to a total volume of 100 µl.
144 For qPCR, duplicate reactions were set up (5 µl of diluted cDNA and 167 nM of each primer
145 per reaction) for reference gene optimisation and subsequent validation in an Mx3000P cycler
146 (Stratagene) using the GoTaq® qPCR Master Mix (Promega) under the following cycling
147 conditions: initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at
148 95°C for 30 s, annealing and extension at 60°C for 50 s. Melt-curve analysis was undertaken
149 immediately after to ensure that there was no non-specific amplification in any well. Primer
150 efficiencies were calculated with LinRegPCR.[13] mRNA levels of CXCL8 (IL-8) were
151 expressed as Primer efficiency ΔCt (cycle threshold), which was then normalised against the
152 reference gene for relative gene expression.

153 Primers: *HPRT1*-F, 5'-GCTATAAATTCTTTGCTGACCTGCTG-3', R, 5'-
154 AATTACTTTTATGTCCCCTGTTGACTGG-3';[14] *RPL32*-F, 5'-
155 CATCTCCTTCTCGGCATCA-3', R, 5'-ACCCTGTTGTCAATGCCTC-3';[15] *ACTB*-F, 5'-
156 GCACCACACCTTCTACAATGAGC-3', R, 5'- CATGATCTGGGTCATCTTCTCG-3';
157 *GAPDH*-F, 5'-GACATCAAGAAGGTGGTGAAGC-3', R, 5'-
158 GTCCAACCCTGTTGCTGTAG-3';[16] *CXCL8*-F, 5'- TTCTGCAGCTCTGTGTGAAG-3',
159 R, 5'- ACAGAGCTCTCTTCCATCAG-3'.

160 ***Differential cell counts***

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161 BAL cells counted using a haemocytometer were diluted to 10^6/mL in PBS then spun on to
162 slides using a Shandon Cytospin®4 Cytocentrifuge (Thermo Fisher Scientific). Slides were
163 stained with Wright-Giemsa (Hema-tek stain pack, Bayer Health Care) and percentages of
164 macrophages, neutrophils, lymphocytes and eosinophils counted by light microscopy.

165 ***Statistics***

166 Comparison of data from matched samples cultured under different conditions were made
167 using the Wilcoxon matched-pairs signed ranks test. Other data were log-transformed then
168 compared by either t test (2 samples) or ANOVA (3 or more samples) with Tukey's multiple
169 comparisons test. Statistical analyses were performed using GraphPad Prism 7 software.

170 **RESULTS**

171 **Optimisation of preparation of BAL cells from CF patients**

172 BAL samples obtained from CF patients (in our case typically 3-10 mL in volume after
173 aliquots for clinical analyses were removed) almost always contain live bacterial
174 contamination, due to infections present in the CF lung. In order to perform standardised
175 stimulation assays it is necessary to first inactivate and remove or at least minimise the effect
176 of this contamination. To do so, BAL cells were centrifuged at 600 g for 10 min, supernatants
177 discarded, cells washed twice with PBS and then resuspended in 3-5 ml of antibiotic medium.
178 Incubating the CF BAL cells overnight in antibiotic culture medium containing penicillin,
179 streptomycin and gentamicin was found to be effective at killing contaminating bacteria.
180 RPMI-1640 is the standard medium used for the culture of human BAL cells. To confirm this
181 for BAL cells collected from the lungs of children with CF, cells were cultured overnight in
182 different media, which indeed revealed a generally better yield when the same BAL cell
183 collections were cultured in RPMI-1640 rather than DMEM (Figure 1a).

184 **Selection of reference gene for qPCR analyses**

185 Analysing gene expression using quantitative PCR (qPCR) is an important tool for studying
186 the cellular response to infection. For this technique, the level of expression of target genes
187 are compared with reference genes; it is therefore critically important to identify the optimal
188 reference gene(s) for the analysis of gene expression levels in each cell population, as this can
189 vary. After overnight antibiotic treatment (as above), cells were thoroughly washed to remove
190 dead bacteria and antibiotics, and the antibiotic media replaced by Assay Medium for the *in*
191 *vitro* assays. For these assays, BAL cells were cultured for 6 hours in media alone, then the
192 expression of the reference genes Hypoxanthine-guanine phosphoribosyltransferase

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(*HPRT1*), ribosomal protein 32 (*RPL32*), ß-actin (*ACTB*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured by qPCR. Quantifying expression of four standard reference genes in BAL cells from children with CF, cultured in antibiotic media, indicated considerable variability in the expression of these genes. While *ACTB* was the highest expressing reference gene, *HPRT1* was virtually undetectable (Figure 1b). No difference in the expression of these reference genes was observed whether the CF BAL cells were cultured in DMEM or RPMI. There was little difference in deviation from the mean for values generated for these reference genes: for cells in DMEM, mean±SD were 36.52±1.97 (*RPL32*), 38.79±2.83 (*GAPDH*) and 31.93±2.13 (*ACTB*); for cells in RPMI, mean±SD were 37.25±1.18 (*RPL32*), 39.76±2.51 (*GAPDH*) and 31.82±1.41 (*ACTB*). Given this similarity, *ACTB* was selected as the optimal reference gene, based on being the only gene for which the Ct value was <35. From the improved cell yield shown in Figure 1a, RPMI-1640 was maintained as the medium of choice. The utility of *ACTB* as reference gene was confirmed by quantifying expression of the important chemokine, IL-8 (Figure 1c). For gene expression analyses, each sample yielded at least 50 ng of total RNA for 20 µl of cDNA synthesis; this was diluted to a total volume of 100 µL, of which only 5 µL was used for each qPCR replicate, meaning at least 9 gene targets plus 1 reference gene could be analysed using this technique.

Length of treatment with gentamicin is critical for measuring an *in vitro* response of BAL cells from the CF lung to *P. aeruginosa*

After optimising the protocol for preparing and culturing BAL cells from the CF lung, we tested its utility for measuring the response of BAL cells to simulation with live *P. aeruginosa*. In our initial studies we stimulated CF BAL cells with *P. aeruginosa* for 3 hours, before adding gentamicin to kill the bacteria and measuring the secretion of the key cytokine IL-8 at 18 hours post-infection. These initial studies provided extremely inconsistent results, with BAL cells from the CF lungs of only 3/10 patients with CF responding positively to the *P. aeruginosa* stimulation when gentamicin was added 3 hours after the bacteria (Figure 2a). Observation of the wells suggested bacterial overgrowth. We therefore modified the protocol to add gentamicin only 1 hour after addition of the bacteria to the BAL cell cultures; this prevented this overgrowth and facilitated the highly reliable detection of an innate immune response to stimulation with *P. aeruginosa* so a positive response was obtained from BAL cells from 10/10 patients (Figure 2b).

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225 In these first studies comparing the effect of gentamicin addition either 1 or 3 hours after the
226 addition of live *P. aeruginosa*, each BAL cell culture from each participant only received one
227 of the antibiotic treatments. The results generated could therefore have been influenced by a
228 number of variables including patient genetic differences, infection status, or variability in the
229 bacterial culture used to stimulate the cells. Therefore, we confirmed this observation by
230 treating matching wells from an additional six BAL cell cultures where each received both
231 treatments (gentamicin for either 1 or 3 hours). This direct comparison, while controlling for
232 the aforementioned variables, confirmed the initial observation, as treating BAL cells from
233 CF patients gentamicin for 1 hour produced a 100% (6/6) response to *P. aeruginosa*
234 stimulation, while 3 hour gentamicin treatment of matching wells containing the same BAL
235 cell preparation, stimulated with the same bacterial culture, was only occasionally effective
236 (33%, 2/6) (Figure 2c).

237 Expanding on these analyses we examined the time course of the IL-8 response of BAL cells
238 collected from children with CF following stimulation with *P. aeruginosa*. As previously, no
239 meaningful response was observed when gentamicin was added 3 hours after addition of *P.*
240 *aeruginosa*. This time course analysis revealed that the peak response occurred 18-24 hours
241 post-bacterial stimulation, when gentamicin was added 1 hour after addition of the bacterial
242 pathogen (Figure 3a). Analysis of BAL cell viability at the end of the cultures surprisingly
243 revealed no difference in cell viability between BAL cells that responded to bacterial
244 stimulation when gentamicin was added after 1 hour, and those for which gentamicin was
245 added after 3 hours and which did not respond to this stimulation. All cultures had good
246 viability as measured by trypan blue exclusion of around 80% (Figure 3b). The lack of
247 response of these CF BAL cells was therefore not associated with overt BAL cell death, but
248 appeared instead to be the result of a suppressive activity of the increased bacterial cell
249 numbers.

250 **Co-stimulation of BAL cells from children with CF with *P. aeruginosa* and *S. aureus***

251 Using this optimised assay, we examined the cytokine (IL-8 and IL-6) response of BAL cells
252 from children with CF to combined stimulation with *P. aeruginosa* and another important CF
253 pathogenic bacterium, *S. aureus*. Interactions between these bacteria are known to be
254 important in the pathogenesis in respiratory diseases, and especially in CF where one third of
255 patients are infected with both pathogens.[17] Cells were obtained from children aged
256 approximately 1 to 5 years of age, with variable cellular compositions including normal

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257 profiles comprised predominantly of macrophages, with others exhibiting considerable
258 neutrophil infiltration (Table 1).

259 This analysis revealed a synergistic response when BAL cells were stimulated with both
260 bacterial species (Figure 4). Despite the observed variations in cellular composition, of the
261 ten patient samples examined, all had extremely similar patterns of IL-8 response to
262 stimulation, with significant production of IL-8 following stimulation with *P. aeruginosa*
263 alone (10/10), *S. aureus* alone (7/10) and both together (10/10) (Figure 4). The response to *P.*
264 *aeruginosa* was consistently greater than to *S. aureus* for all ten samples examined and the
265 combined stimulation had a significant synergistic response in 7/10 samples. This synergistic
266 effect occurred irrespective of the infection status of the CF patient from whom the cells were
267 collected (Table 2). The lung infection status was, as would be expected for children with CF,
268 highly variable. While one patient (#9) had an active *P. aeruginosa* infection, and two (#3
269 and #5) were infected with *S. aureus*, this did not appear to have a major effect on their
270 response to stimulation with these bacteria.
271 The IL-6 response to these bacterial stimulations were generally very similar to that of IL-8
272 for the majority (seven) of the patients. However, there was a complete absence of IL-6
273 response in three individuals (#3, #6, #8).
274

275 **DISCUSSION**

276 Inflammation in the lung occurs soon after diagnosis in CF,[18-20] and appears to lead to the
277 development of early structural lung damage.[2] Further studies of drivers of this inflammation,
278 especially early in life, are critical for the development of new strategies aimed at preventing
279 or slowing the associated disruption to the structural integrity of the early CF airways. For
280 obvious reasons, there is limited availability, especially for research purposes, of immune
281 cells from the lungs of young children with CF when the critical events of disease
282 development are occurring.

283 Infections play an important role in inflammation found in the CF lung and disease
284 progression, so it is particularly informative and valuable to be able to study the response of
285 immune cells from these organs to infectious stimuli. CF is a complex disease in which the
286 lungs of infected individuals are often colonised by several pathogens simultaneously, as
287 shown in Table 2. While an analysis of the *in vivo* response to a specific pathogenic infection
288 would be preferable, the presence of such complex combinations of lung infections in CF
289 make this extremely difficult. Moreover, many of the organisms infecting the CF lung are not

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290 identified, making interpretation difficult. An alternative approach and potentially useful tool
291 for studying the response of immune cells from the lungs of children with CF is to do ex vivo
292 stimulation experiments in controlled defined conditions with specific pathogens. In so doing,
293 information can be learnt about the type and variability of the immune response mounted by
294 such cells to different pathogenic organisms of relevance to CF.

295 However, the application of stimulation assays to evaluate the immune responses of these
296 cells to external stimulation encounters particular challenges. The most significant of these is
297 likely the intrinsic contamination carried from the lung due to pre-existing infections,
298 including bacteria. Such contaminants typically contain or release Pathogen-Associated
299 Molecular Patterns (PAMPs) that can activate innate immune receptors and thus inevitably
300 have the potential to influence the response of BAL cells to experimental stimulation in
301 culture.

302 The immune cell composition of BAL cells collected from such patients is also quite
303 variable, with the proportions of macrophages, neutrophils, lymphocytes and other cells
304 differing with infection and inflammation status. Commonly there is low cell number
305 availability, as such cells are usually only obtainable opportunistically from excess samples
306 collected during routine clinical investigations of the CF patient. Moreover, BAL cells
307 isolated from the CF lung are often under considerable stress, or pre-activated, before being
308 placed in cell culture, both from the inflammatory environment in which they previously
309 resided and/or the bronchoscopy procedure and storage of BAL sample prior to culture. All
310 these factors, combined, produce a highly heterogeneous mixed cell population that, when
311 examined in culture, presents a variable immune baseline. It is against this background that
312 we attempted to measure a specific immune response following exposure to individual or
313 combined bacterial pathogens of relevance to CF.

314 We here describe an optimised technique for the preparation of BAL cells obtained from
315 young children with CF and the use of those cells in *in vitro* bacterial stimulation assays. To
316 achieve this it is important, as far as possible, to optimise and standardise the culture and
317 stimulation assay to maximise cell survival and health, and minimise the effects of
318 contaminant carry-over. The results reported here represent several years of experience,
319 optimisation and refinement that now describe methodology allowing for the reproducible
320 measurement of the immune response of BAL cells from the lungs of young children with CF
321 to bacterial stimulation. A critical feature of this study was the examination of BAL cells

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322 from young children, as information on the optimization of co-stimulation studies obtained in
323 different samples-types, and from older subjects, are likely not applicable to BAL
324 inflammatory cells obtained much earlier in the CF pulmonary disease course.

325 As BAL samples from even young children with CF usually harbour infection, an initial
326 antibiotic treatment step is essential to prevent bacteria resident in some patients from either
327 overgrowing BAL cell cultures or, as a minimum, activating the cells and thereby
328 invalidating the stimulation assay. After confirming the optimal culture media to use to
329 support BAL cells we investigated the choice of reference gene necessary for qPCR analysis.
330 This analysis demonstrated considerable variability in the expression of reference genes,
331 indicating the choice of reference gene is critically important for accurate analysis of gene
332 expression in these cells. *ACTB* was identified as the highest expressing reference gene, of
333 four tested, in CF BAL cells. The use of *ACTB* for in gene expression studies was validated
334 by analysing the expression of mRNA of the key CF chemokine, *IL-8*.

335 Optimisation of the culture step then allowed for an evaluation of the response to bacterial
336 stimulation. For this, live *P. aeruginosa* and *S. aureus* were used as model bacterial
337 stimulants, due to their relevance as two important CF pathogenic bacteria. The use of live
338 bacteria potentially provides a more physiological measurement of the BAL cell response to
339 such pathogens, like that which occurs in the lung. Although few if any studies have
340 measured the BAL cell response to bacterial pathogens in CF, some have been performed for
341 other disease states. In those cases, many have involved stimulating BAL cells with proteins,
342 heat-treated bacteria or bacterial lysate,[21-23] with exceptions often being those involving slow
343 growing bacteria such as mycobacteria.[24] While informative, lysing or heat-treating bacteria
344 could release PAMPs, normally contained within the structure of the cell (i.e. LPS,
345 peptidoglycan, lipopeptides, DNA, RNA)[25] possibly facilitating increased TLR ligand
346 activation as compared to live bacteria. Hence such stimulations might not necessarily trigger
347 the same activation pathways *in vitro*, as occur with live pathogenic bacteria, as in the lung.
348 An issue with using live bacteria in stimulation assays is that they can multiply rapidly in
349 culture media. However, this study showed that this can be controlled by adding an antibiotic
350 such as gentamicin to prevent bacterial overgrowth. However, we noted that cell cultures in
351 which gentamicin was added after 3 hours gave an extremely variable response to bacterial
352 stimulation. This was not related to a loss of BAL cell viability, but due to an unidentified
353 suppressive effect induced by the increasing bacteria numbers. Critically, we identified that
354 addition of gentamicin after 1 hour of culture completely prevented this suppressive effect,

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355 allowing the added stimulating bacteria to induce a highly consistent response in immune
356 cells from the CF lung, without any inhibition of the cellular response.

357 Previous studies have used epithelial cells to examine how co-cultures of *P. aeruginosa* and
358 *S. aureus* interact; for example, CF bronchial epithelial cells have been used to show that,
359 over long-term co-culture, *P. aeruginosa* can reduce the viability of *S. aureus*,[26] while
360 immortalised epithelial cells were used to show that filtrate from *S. aureus* can downregulate
361 the IL-8 response of these cells to filtrate from *P. aeruginosa*.[27] However, to our knowledge
362 no such studies have previously been done with immune cells from the CF lung. Using this
363 optimised technique, we were able to measure the IL-8 and IL-6 response to live bacterial
364 stimulation. The levels of IL-8 and IL-6 secreted in response to *P. aeruginosa* and *S. aureus*
365 in this study, was generally similar to that produced by BAL cells from healthy control
366 children or those with chronic bronchitis, stimulated with LPS.[28] However we found that the
367 IL-8, and in many cases the IL-6 response of BAL immune cells to co-stimulation with both
368 *S. aureus* and *P. aeruginosa* was greater than stimulation with either bacteria alone, in
369 contrast with the reported result using immortalised epithelial cells.[27] Individually, although
370 the pro-inflammatory response to stimulation with *P. aeruginosa* appeared greater, *S. aureus*
371 also resulted in a demonstrable inflammatory response assayed *in vitro*. As only a single
372 strain of each bacterial species was compared in these methodological studies, the effect of
373 strain variation and impact of variability in bacterial virulence factors remains to be
374 determined.
375 Notably, some variation was observed in the IL-8 and IL-6 response in this study. While all
376 BAL cell samples responded to the bacterial stimuli with secretion of IL-8, cells from three
377 individuals produced IL-8 but no IL-6. This did not appear to be pathogen-related, as the
378 infection profiles of these three patients were all different with no common organism
379 identified (Table 2). Rather, the lack of IL-6 response was associated with a reduced
380 proportion of macrophages present in the BAL cell composition in at least two of the three
381 relevant samples, although data from the third was unavailable (Table 1). As individual #3
382 (16% macrophages) presented with a neutrophil infiltration and #6 (18% macrophages) a
383 lymphocyte infiltration, the loss of IL-6 production in the assay appeared to be due to a
384 reduction in the proportion of macrophages rather than the effect of infiltrating cells. This
385 does not necessarily indicate reduced numbers of macrophages in the lungs from which the
386 samples were obtained, as these cells might well have been diluted in relative proportion, by
387 increased numbers of the other cell types indicated. However, it does suggest that

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388 macrophages were the primary source of IL-6 in the BAL cell response to *P. aeruginosa* and
389 *S. aureus*. In contrast, IL-8 was still produced by BAL cells from patients #3, #6 and #8,
390 albeit at a relatively low level compared to most other patients. This indicates macrophages
391 are not the only cell to secrete cytokine into these cultures, but that they might contribute.
392 In summary, this study provides a method for evaluating the response of CF BAL immune
393 cells, in particular involving cells from young children with early stage disease, to stimulation
394 with pathogenic stimulation. The measurement of IL-8 response to bacterial stimulation was
395 reliably and reproducibly demonstrated, paving the way for future studies aimed at measuring
396 the response to infection of BAL cells in the early formative stages of CF.

397

398 **CONFLICTS OF INTEREST**

399 The authors declare that there is no conflict of interests regarding the publication of this
400 paper.

401

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406

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478

479 **FIGURE LEGENDS**

480 **Figure 1**

481 **a)** BAL cells from children with CF (n=9) were incubated overnight in 6-well culture plates
482 in antibiotic medium, comprising either RPMI-1640 or DMEM (supplemented with FCS,
483 glutamine, penicillin, streptomycin, amphotericin B, gentamicin and EDTA). Data show the

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484 live cell counts recovered after the overnight culture, from paired samples of cells from each
485 individual, cultured in either RPMI-1640 or DMEM. Live cells recovered following
486 overnight culture in RPMI-1640 was significantly greater than in DMEM (Wilcoxon
487 matched-pairs signed ranks test). **b)** Expression levels of reference genes in BAL cells
488 collected from children with CF (n=3), cultured separately overnight in antibiotic media as in
489 (a), determined by qPCR. The expression of *ß-actin* (*ACTB*) was significantly greater than
490 that of the other reference genes measured, *HPRT1*, *RPL32* and *GAPDH* (*** $p<0.0001$;
491 ANOVA). N.D. = Not detectable (>45 cycles). **c)** Expression of *IL-8* mRNA in BAL cells
492 collected from children with CF (n=3), cultured separately overnight in antibiotic media as in
493 (a), was determined by qPCR relative to the reference gene *ACTB*.
494
495 **Figure 2**
496 BAL cells from children with CF (1-6 years old) were cultured in antibiotic free complete
497 media with or without *P. aeruginosa* (multiplicity of infection of 5). **a)** Gentamicin was
498 added 3 hours after the addition of *P. aeruginosa* to BAL cell cultures from ten individual
499 children (1-10). **b)** Additional BAL cell cultures from ten different children (11-20) received
500 gentamicin 1 hour after the addition of the same bacteria. **c)** To allow a direct comparison of
501 these time points on the same cell populations, gentamicin was added either 1 or 3 hours after
502 the addition of *P. aeruginosa* to identical BAL cell cultures from six children with CF,
503 analysed individually. IL-8 levels in supernatants collected 18 hours post-stimulation were
504 quantified by ELISA. Pairs of bars (clear unstimulated; shaded stimulated) present IL-8
505 levels measured in supernatants from BAL cells cultured from individual children (each
506 generated from technical replicates of between 3 and 6 wells depending on cell availability).
507 Bars present mean + SD (*$p<0.05$; ANOVA).
508
509 **Figure 3**
510 **a)** BAL cells from children with CF were cultured in antibiotic free complete media with or
511 without *P. aeruginosa* (PA; multiplicity of infection of 5). Gentamicin was added 1 (n=2
512 children) or 3 (n=2 children) hours after addition of PA and supernatants collected for
513 analysis at 6, 12, 18 and 24 hours (triplicate wells at each time point). PA stimulation
514 significantly induced IL-8 secretion, quantified by ELISA, where marked (*$p<0.05$,
515 **$p<0.01$, ***$p<0.001$; t test). **b)** Cell viability in the same triplicate wells at each time-point
516 was determined by trypan blue staining and light microscopy. Bars present mean + SD.

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517 Neither *P. aeruginosa* stimulation nor gentamicin treatment had any significant effect on cell
518 viability as measurable by trypan blue exclusion.
519
520 **Figure 4**
521 BAL cells from children with CF (n=10) were cultured in antibiotic free complete media with
522 or without stimulation with *P. aeruginosa* (PA; multiplicity of infection of 5) alone, *S. aureus*
523 (SA) alone, or both *P. aeruginosa* and *S. aureus*. Gentamicin was added after 1 hour and IL-8
524 and IL-6 levels in 18-hour culture supernatants were determined by ELISA. Co-culture with
525 both bacteria produced an increased IL-8 and in some cases IL-6 response (*significantly
526 greater than unstimulated control; $p<0.05$, ANOVA with Tukey's multiple comparisons test).

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Patient	Patient age	Total cell count (x10^6)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)
#1	4.92	0.89	n/a	n/a	n/a	n/a
#2	4.89	0.38	91	7	2	0
#3	1.89	16.65	16	84	0	0
#4	3.09	0.54	96	4	0.3	0
#5	5.86	1.93	73	27	0	0
#6	4.08	3.34	18	38	43	0
#7	0.98	2.07	n/a	n/a	n/a	n/a
#8	0.95	1.00	n/a	n/a	n/a	n/a
#9	2.98	4.00	n/a	n/a	n/a	n/a
#10	5.08	5.48	92	5	2	0

TABLE 1: Differential Counts of BAL Cell Samples from children with CF

Differential counts of cytospins of BAL cells, stained with Wright-Giemsa stain, and analysed under light microscopy. n/a: data not available.

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Patient	Pulmonary Infections
#1	Upper respiratory tract flora
#2	Haemophilus species, Upper respiratory tract flora, fungus
#3	S. aureus, H. parainfluenzae, E. coli
#4	Haemophilus species, Upper respiratory tract flora
#5	S. aureus, H. influenzae, S. pneumoniae, Upper respiratory tract flora
#6	H. influenzae, S. pneumoniae, Upper respiratory tract flora
#7	Upper respiratory tract flora
#8	Upper respiratory tract flora
#9	P. aeruginosa, H. influenzae, Upper respiratory tract flora
#10	Upper respiratory tract flora

TABLE 2: Lung Infection Status

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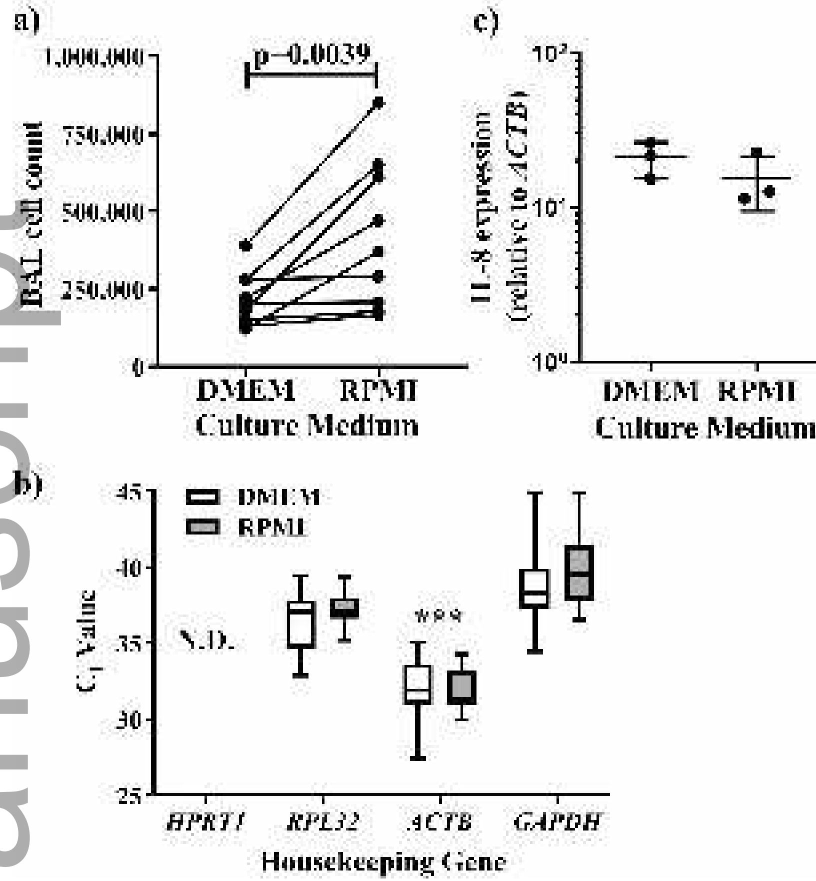

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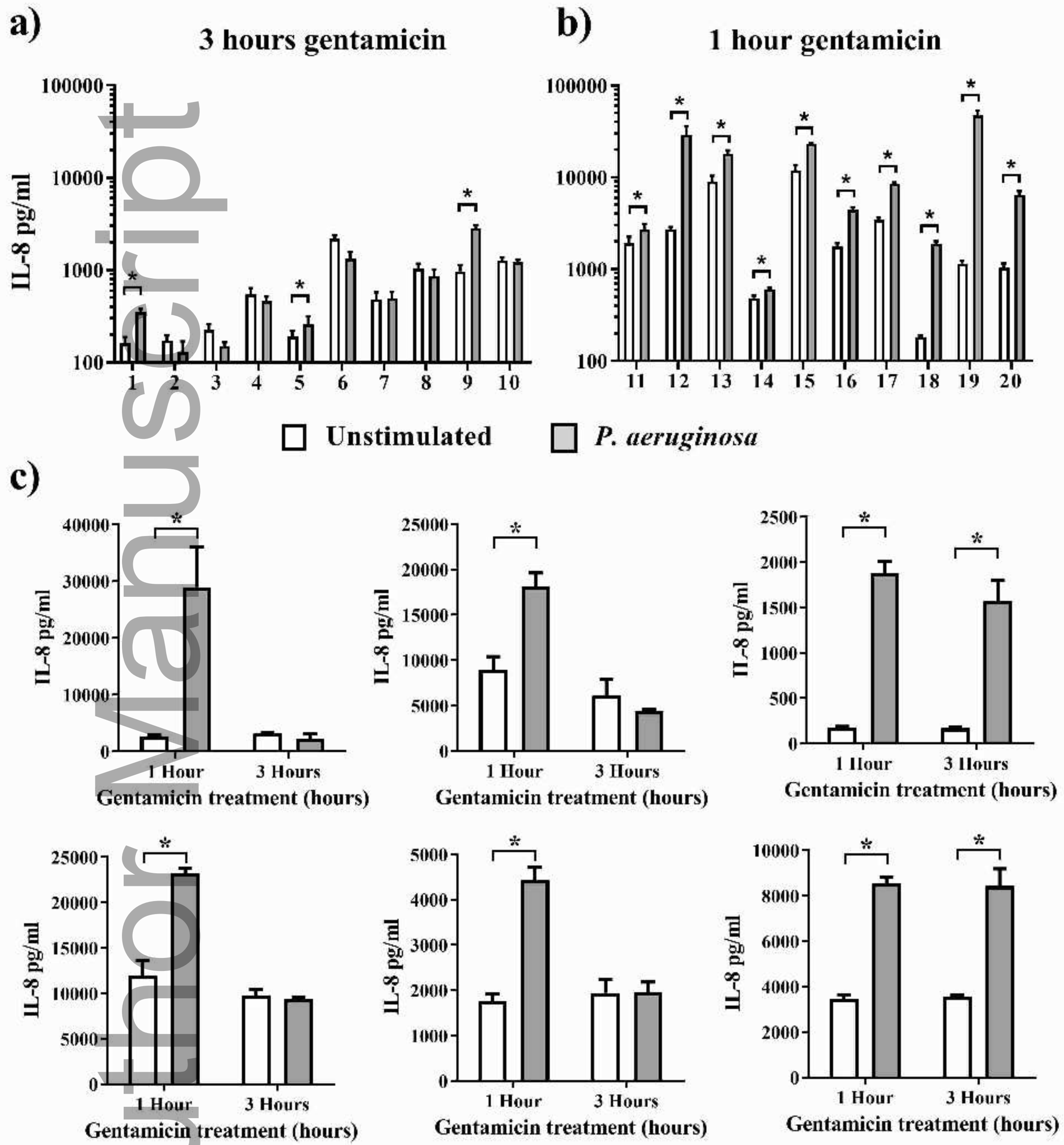

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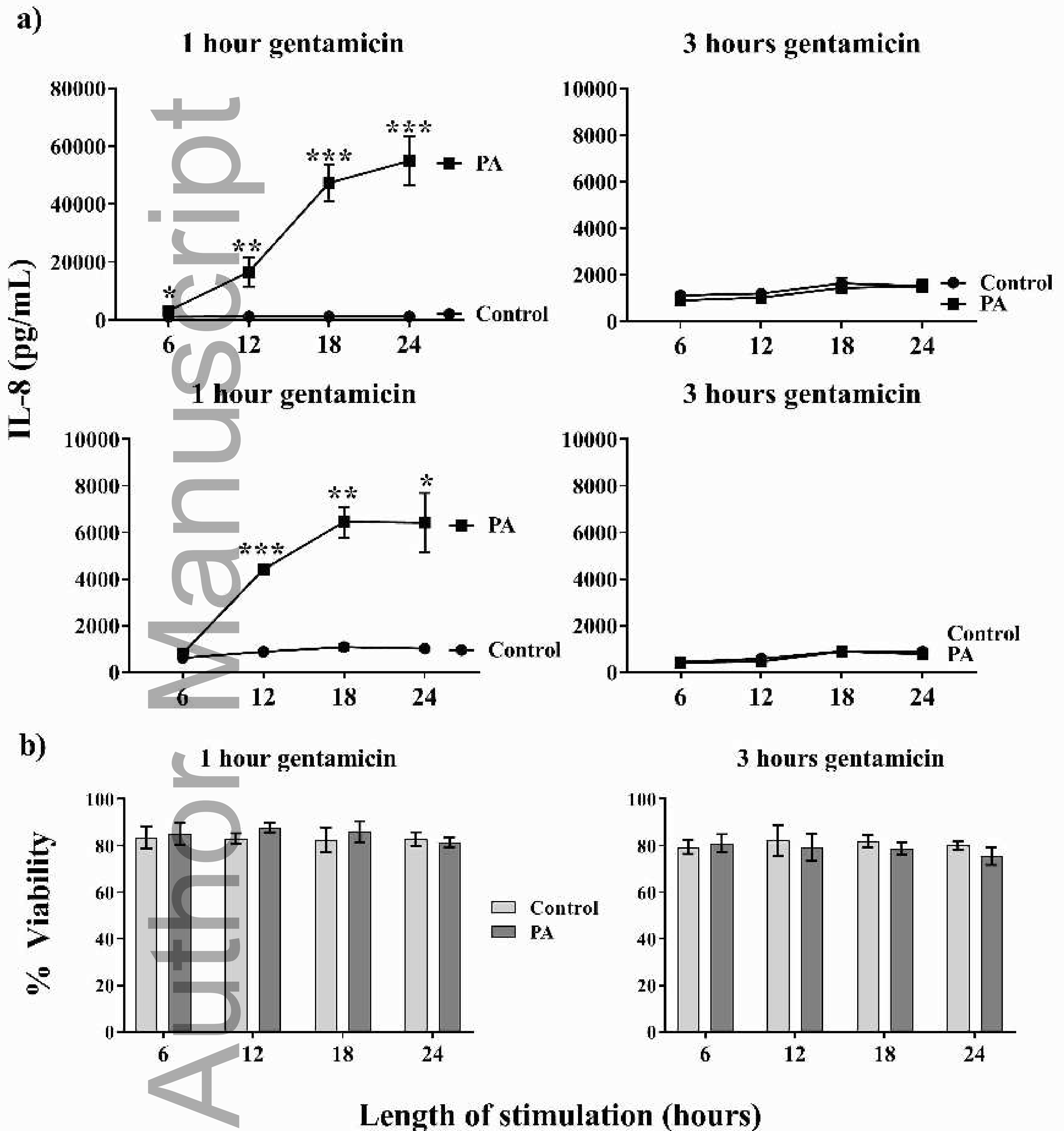

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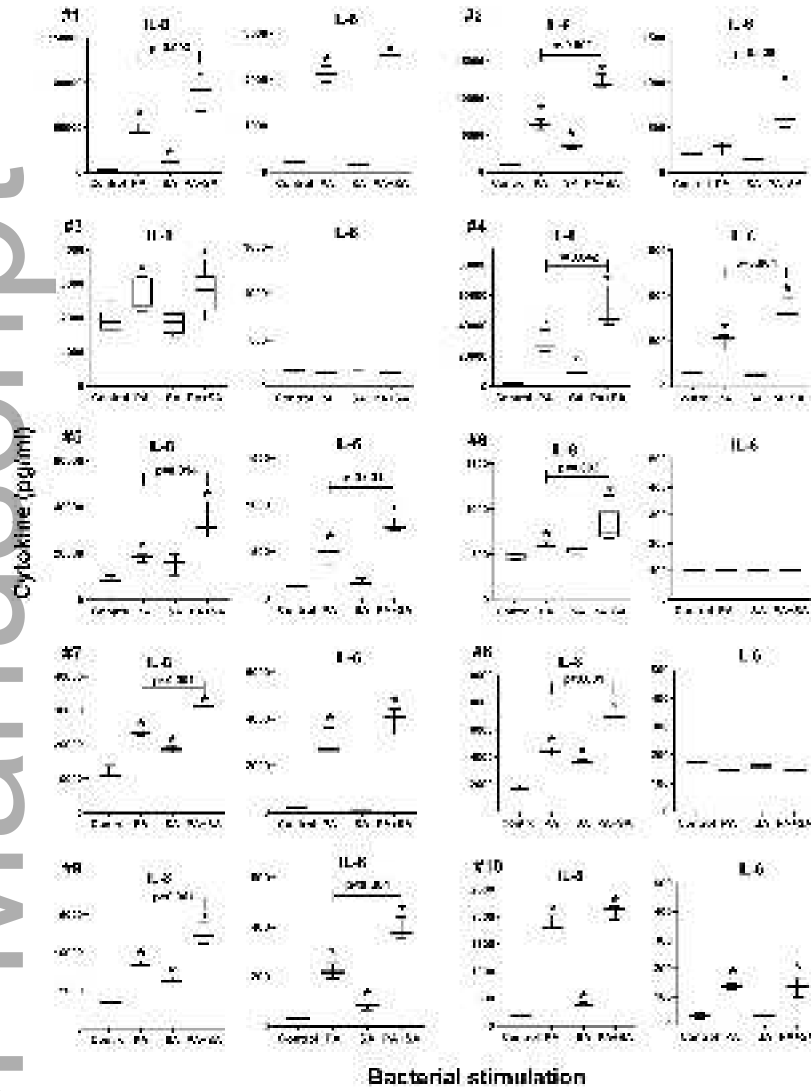
FIGURE 4



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