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1 Screening for Th17-dependent pneumococcal vaccine antigens: comparison of murine

2 and human cellular immune responses

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Abstract

Conjugate vaccines against *Streptococcus pneumoniae* have significantly reduced diseases caused by serotypes included in those vaccines; however, there is still need for vaccines that confer serotype-independent protection. In the current study, we have constructed a library of conserved surface proteins from *S. pneumoniae* and screened for IL-17A and IL-22 production in human immune cells obtained from adenoidal/tonsillar tissue of children and IL-17A production from splenocytes from mice that were immunized with a killed whole cell vaccine or previously exposed to pneumococcus. A positive correlation was found between rankings of proteins from human IL-17A and IL-22 screens, but not between human and mouse screens. All proteins were tested for protection against colonization, from which we identified protective antigens that are IL-17A dependent. We found that the likelihood of finding a protective antigen is significantly higher from groups of proteins ranked in the top 50% of all three screens than for groups of proteins ranked in the bottom 50% of all three. The results thus confirmed the value of such screens to identify Th17 antigens. Further, these experiments have evaluated and compared the breadth of human and mouse Th17 responses to pneumococcal colonization and enabled the identification of potential vaccine candidates based on immunological responses in mouse and human cells.

Introduction

Streptococcus pneumoniae, or pneumococcus, is a Gram-positive bacterium that is a major cause of morbidity and mortality in infants, toddlers and the elderly in both developed and developing countries. Introduction of the first conjugate vaccine PCV7, followed by PCV10 and PCV13, has greatly reduced the incidence of invasive disease and colonization caused by the serotypes included in these vaccines (1). Despite the success of these vaccines, serotypes that are not included in these vaccines pose an emerging threat. Indeed, there are at least 97 identified pneumococcal serotypes with variability in regional distribution of predominant serotypes; furthermore, the phenomenon of serotype replacement has reduced the impact of these vaccines in many settings (2).

While it is well established that anticapsular antibodies are sufficient to prevent invasive disease such as pneumonia, meningitis and sepsis, other mechanisms of immunity may also play important roles. Either live exposure to pneumococcus or immunization with killed whole cell vaccine (SPWCV) can induce CD4+ Th17 dependent protection against nasopharyngeal colonization in mice (3-5). The reduction in colonization in mice is dependent on generating pneumococcus-specific Th17 immunity and recruiting neutrophils to the mucosal site (3).

Evidence is accumulating to suggest that Th17-based immunity is alsounity important for protection against pneumococcus in humans. The existence of pneumococcal-specific Th17-type T cells was demonstrated in both children and adults by analysing Th17 responses to a killed preparation of pneumococcus (pneumococcal whole cell antigen, WCA) or individual pneumococcal antigens (6). Furthermore, the frequency of pneumococcal-specific Th1 and Th17 CD4+ T cells within mucosal lymphoid tissue demonstrates age-dependent increases, likely due to cumulative exposure to pneumococcus (7). In an experimental human challenge model, lung IL-

66 17A-secreting CD4+ memory T-cells were detected following intentional pneumococcal
67 carriage (8). Furthermore, patients with autosomal dominant hyper-IgE syndrome (Job's
68 syndrome), who lack the ability to generate memory Th17 cells due to mutations in the
69 *stat3* gene, are highly susceptible to recurrent pneumococcal infections (9, 10). More
70 recently two studies provided support for a role of IL-17A in pneumococcal carriage in
71 children: a specific polymorphism of the IL-17 gene (G-152A) in Finnish children (11)
72 and decreased IL-17A secretion in Fijian children (12) were associated with increased
73 risk of pneumococcal carriage.

74 In addition to Th17 cells, antibodies are also likely to play an important role in
75 protection against pneumococcal disease. A SPWCV consisting of WCA and aluminium
76 adjuvant given subcutaneously or intramuscularly elicits anti-protein antibody-mediated
77 protection against pneumococcal pneumonia and sepsis in mice, which can also be
78 reproduced by passive transfer of antibodies obtained from rabbits immunized with
79 SPWCV (13). Naturally acquired protection against pneumococcal disease in humans
80 was recently shown to depend largely on antibody to protein antigens rather than to
81 capsular polysaccharides (14).

82 As a consequence, there has been a concerted effort over many years to identify
83 protective protein antigens against pneumococcal disease (15, 16). Traditional methods,
84 such as protein separation by two-dimensional gel electrophoresis and identification by
85 mass spectroscopy, have been used to identify proteins from the cell wall fraction of
86 pneumococcus that interact with convalescent sera from patients (17). As another
87 approach, libraries of purified surface proteins have been used to identify antigens that
88 may induce protection against pneumococcal invasive disease (18). A display library
89 expressing 15-150 amino acid fragments of the pneumococcal proteome was used to
90 identify proteins that interact with serum from infected individuals, leading to selection of
91 StkP and PcsB as candidate antigens (19).

Using similar approaches, other investigators have used pneumococcal antigens to identify potentially protective T-cell antigens. Putative Th17-eliciting antigens were identified from the soluble fraction of the WCA using preparative SDS gel electrophoresis followed by mass spectroscopy (20). In a more comprehensive approach using the ATLAS© system, an expression library containing >96% of predicted pneumococcal proteins was used to identify antigens recognized by Th17 cells from SPWCV-immunized mice (21) and from human volunteers naturally exposed to pneumococcus (22).

The studies cited above used either mouse splenocytes or human peripheral blood mononuclear cells (PBMCs) as a source of immune cells. There have been some studies using arguably a more relevant source of immune cells, the human mucosal lymphoid tissue, probed for Th17 responses following stimulation with a small number of pneumococcal proteins (23-25), but a more comprehensive analysis of the range of Th17 responses to the pneumococcal proteome has not been performed. Here, we constructed a protein library consisting of genetically conserved pneumococcal surface proteins. We used this library to screen human adenoidal cells for Th17-cytokine-inducing antigens and compared these human responses to those observed from splenocytes from mice immunized with SPWCV or exposed to pneumococcus. Positive correlations between two human screens but no correlations between human and murine screens were observed. Several antigens were identified that subsequently showed IL-17A-dependent protection against pneumococcal colonization in mice.

Results

Age-dependent immune response to WCA

We obtained 35 adenoidal samples from children undergoing adenoidectomy and analyzed IL-17A and IL-22 production in response to stimulation with WCA in 33 of these

119 samples. As shown in Figure 1, there was an age-dependent, significant increase in IL-
120 17A responses (p=0.0235, r=0.3996) and IL-22 (p=0.0429, r=0.3786). The finding that
121 immune responses to pneumococcus in adenoids in children tend to increase with age
122 supports the hypothesis that these adenoidal cells may represent a good source of
123 responsive immune cells for antigen discovery.

124 **Screening antigens using human adenoidal cells**

125 Fifty-six recombinant proteins out of 81 genetic constructs were successfully expressed
126 in *E. coli* and purified using Ni-NTA chromatography. These proteins were further
127 purified by gel filtration before being used in stimulation experiments with adenoidal cells.
128 Due to the limited number of cells obtained from each adenoid, we were able to screen
129 all 56 proteins in 13 samples and only a subgroup of these proteins with the other 22
130 human samples. Both we and others have showed that IL-17A production in human in
131 response to pneumococcal whole cell or purified pneumococcal proteins are mostly due
132 to stimulation of memory T cells (26, 27). The results of IL-17A and IL-22 production in
133 these 13 samples after subtracting the baseline responses following incubation with
134 medium alone are shown in Figure 2. IL-17A responses from each protein were
135 generally in the range of 50-100 pg/ml (mean 157 pg/ml), with WCA having the highest
136 responses (far right) (Figure 2A). IL-22 responses were generally higher than IL-17A,
137 mostly ranging from 200-600 pg/ml (Figure 2B).

138 **Screening antigens using murine immune cells**

139 While colonization and infection models in mice are widely used to test candidate
140 pneumococcal vaccines, the extent to which murine immune responses mimic human
141 responses is still unclear. A comparison of the responses of murine and human immune
142 cells following stimulation with proteins from the same pneumococcal library may
143 improve our understanding of the differences between and common features of these
144 two species. To this end, murine splenocytes were obtained from mice either

145 immunized with SPWCV or previously colonized with a serotype 6B pneumococcal strain
146 for 4 weeks (the latter model may more closely mimic pneumococcal exposure in
147 humans). Both methods result in the generation of protective and antigen-specific
148 memory CD4+ T cell responses against nasopharyngeal colonization in mice (3, 13, 28,
149 29), and we have confirmed that these mice were protected from nasal colonization
150 (data not shown). IL-17A responses following stimulation of splenocytes from these two
151 mice models with the library of proteins are shown in Figure 3. Stimulation with SPWCA
152 and proteins in splenocytes from naïve mice did not generate any IL-17A (data not
153 shown). Responses in SPWCV immunized mice, most of which were between 50 and
154 200 pg/ml (Figure 3A) were generally lower than those in pneumococcus-exposed mice,
155 most of which were between 100 and 500 pg/ml (Figure 3B). SPWCA induced the
156 largest responses in both mouse models (far right data points).
157 **Evaluation of antigens in colonization models**. Next, we tested the ability of the
158 library of proteins to provide protection against colonization in mice when used as
159 immunogens. Mice were immunized intranasally twice at a one-week interval with one of
160 the top 5 murine proteins (10 μg/dose) and 1μg of CT; control mice received 1 μg of CT
161 alone. The whole library was tested in six different experiments, as shown in Figure 4. A
162 total of 18 constructs (16 antigens) was found to be protective in this model; their
163 identities and predicted functions are listed in table 1.
164 **Protection against colonization is IL-17A dependent**
165 We have shown previously that protection against colonization is dependent on CD4+
166 Th17 cells whereas antibodies directed against antigens contained in the whole cell
167 vaccine were not protective in the colonization model (3, 13, 28). We then tested
168 whether the protection afforded by these antigens is dependent on IL-17A production. To
169 facilitate the experiment, we chose combinations of the three antigens which induced
170 greatest responses either in murine screens (SP0648-3, SP0757 and SP1500) or in the

171 human IL-17A screen (SP0648-1, SP0662-1 and SP0742) and tested the dependence of
172 protection on IL-17A. Mice were immunized intranasally and were given either anti-IL17A
173 antibody or an isotype control antibody both one day before and 3 days after challenge
174 inoculation with *S. pneumoniae.* As shown in Figure 5, immunization with both antigen
175 combinations (Figure 5A & B) protected mice from colonization while protection was
176 abolished in immunized mice treated with antibody directed against IL-17A, thus
177 confirming the role of this cytokine in this model.

178 **Ranking of antigens and correlation between screens**

179 Proteins were ranked in each screen as explained in the methods section. We compared
180 the ranking results of SPWCV-immunized mice, pneumococcal exposed mice and
181 human samples with respect to IL-17A and IL-22. There was a weak correlation between
182 the results from immune cells in the two different mouse models (Figure S1A) while
183 there was a strong positive correlation between the IL-17A and IL-22 screens using
184 human cells (Figure 6). No statistically significant correlation was found between the IL-
185 17A screen results using human cells and cells from SPWCV-immunized mice (Figure
186 S1B) or mice previously exposed to pneumococcus (Figure S1C), nor was any
187 correlation between human cell IL-22 production and either mouse screen found (data
188 not shown).

189 **Validation of the screening method**

190 We analyzed how well each screen predicted protection against colonization. Overall,
191 the rate of protection among the proteins in the library is quite high (18/56, 32.1%; Figure
192 4) comparing to regular screening with whole genome (21), which probably reflects the
193 way the library was designed. Antigens were categorized as belonging either to the top
194 or bottom 50%, based on their rankings in each screen. We then compared the "hit rate"
195 (i.e. identifying an antigen that is protective in the colonization model) in the top 50%
196 responders versus the bottom 50% responders in the two murine and one human IL-17A

197 screens. While the hit rates were higher in the top 50% responders, none of these
198 differences was significant (table 2). In contrast, when we identified antigens that were
199 highly ranked either in two screens or amongst all three screens, the hit rates were
200 significantly higher: for example, for antigens that were in the top 50% rank in all three
201 screens, the hit rate was 61.5%, significantly higher than the 11.1% hit rate for antigens
202 that fall into the bottom 50% in all three screens (p=0.0306 by Fisher's exact test), and
203 about twice the hit rate for the whole library, which trended towards significance (p=0.06,
204 by Fisher's). When the same analysis was done with two murine and human IL-22
205 screen, a similar result was obtained. The hit rate in the top 50% rank in all three
206 screens was 58.5%, comparing to 8.3% in the bottom of all three screens (p=0.009).

207 **Discussion**

208 Using a conserved pneumococcal surface protein library, we stimulated human
209 and murine immune cells in order to identify antigens that elicit IL-17A and IL-22
210 responses and tested which of them confer protection against pneumococcal
211 colonization following intranasal immunization in mice. Overall, we found that antigens
212 that more consistently induced cytokine responses across both murine and human cell
213 screens were more likely to be protective . In addition, we identified many antigens that
214 showed IL-17A-dependent protection against pneumococcal colonization in mice.

215 One goal of this study was to investigate whether the immune responses to
216 pneumococcus in murine and human cells are correlated and whether murine screens
217 could predict the results of the human screen. IL-17A and IL-22 responses of human
218 cells were highly correlated to each other, which confirms our previous findings that IL-
219 17A and IL-22 responses to WCA in children's PBMC are highly correlated (6). However,
220 there was no correlation between the rankings of the IL-17A responses to our protein
221 library in murine and human screens, suggesting that there are differences in responses
222 that are both host- (mouse vs. human) and exposure-dependent (live vs. killed bacteria).

223 The lack of correlation between exposed mouse and human cells could be
224 explained by many factors. First, there are clear differences between mouse and human
225 immune systems (30). Furthermore, the local environment for pneumococcal
226 colonization might not be the same in mice and human, given growing evidence of the
227 influence of microbiota on immune development (31). Thirdly, most children may have
228 been exposed to pneumococcus many times, after which they may have generated high
229 Th17 response to nonprotective proteins whereas the mice were only exposed to
230 pneumococcus or pneumococcal antigens for a short defined period in our experiment.
231 Lastly, in addition to intrinsic differences between mouse and human immune cells, we
232 are comparing human adenoidal mucosal cells to murine splenocytes, cells from very
233 different compartments.

234 Despite these differences, we were able to identify several protective candidates
235 by combining the three screens, which suggests that screening for cytokine production
236 using cells from different sources may be a useful method to identify cytokine-mediated
237 protection. Indeed, we found that the protective hit rate was highest in the pool of
238 proteins that induced higher responses in all the screens. The 16 protective antigens
239 that we identified have not previously been reported as conferring protection, with the
240 single exception of SP1683 which was recently reported as a Th17-dependent antigen
241 which protects against colonization while this manuscript was being preparared (32).
242 While protection against colonization by any single antigen did not approach that of
243 SPWCV in mouse models (Figure 4), a combination of three antigens significantly
244 improved the protective efficacy of any individual protein antigen (Figure 5). This
245 suggests that a candidate protein-based vaccine should likely comprise several antigens,
246 to maximize protection and coverage.

247 Another important implication of our work is that immune responses
248 demonstrable in mouse models do not accurately predict those of humans. In light of

249 the recent failure of at least two different protein vaccine formulations(33, 34) to provide
250 protection against colonization, one is left wondering whether excessive reliance on
251 mouse models may be responsible. Clearly,murine models are much more convenient
252 and practical for large scale screening and identification of potential candidates, as were
253 performed here. However, prior to performing expensive and time-consuming proof-of-
254 concept studies of impact on colonization in toddler or infant subjects, perhaps other
255 approaches that can serve to de-risk the process may be useful. One approach would
256 be to try to establish correlates of protection, by performing longitudinal studies of
257 colonization and systemic T cell responses in children. Another approach may be to test
258 promising candidates in intentional challenge studies in humans (8, 35). A potential
259 caveat to this strategy is that intentional challenge studies are performed in adults,
260 whereas the intended target population of these vaccines is generally toddlers and
261 infants. However, such studies could still be helpful in providing a gating strategy:
262 impact on carriage density or duration of carriage in a properly powered study of
263 intentionally challenged adult volunteers could be used to decide whether or not to
264 pursue studies of the candidate vaccine in younger subjects.

265 A potential limitation of our study is that children undergoing adenoidectomy, who
266 are the only practical and ethically acceptable source of pediatric NALT, may be
267 immunologically distinct from a randomly selected sample of healthy children.
268 Nevertheless, previous studies have confirmed a very similar pattern of acquisition of
269 serum antibodies with age in both these children and healthy controls. Furthermore,
270 rates of pneumococcal colonization at the time of surgery are also similar to those seen
271 in healthy children of comparable age (36). Another potential limitation is that some
272 children may have had limited prior pneumococcal exposure at the time of surgery.
273 However previous studies have shown that the majority of such children have mucosal
274 specific immune responses to pneumococcal protein antigens (37).

275 In summary, we report here the results of screening of human and mouse cells
276 following exposure to pneumococcus using a library of conserved pneumococcal
277 proteins. We believe the use of human and murine cells for this type of screening can
278 inform the selection of potential candidates worthy of further study. This approach could
279 also be applied to the identification of other important mucosal pathogens whose route of
280 entry begins in the nasopharynx.

281 **Materials and Methods:**

282 **Materials**: Cholera toxin was purchased from List Biological Laboratories. Ni-NTA resin
283 was purchased from Qiagen. CloneEZ PCR cloning kits were obtained from Genscript
284 Inc. All other reagents were obtained from Sigma or Thermo Fisher Scientific.

285 **Selection of protein candidates by bioinformatic analysis *in silico***

286 We chose 42 *S. pneumoniae* sequences (including some finished and others in draft
287 form) from the integrated microbial genomes website (http://img.jgi.doe.gov/cgi-
288 bin/w/main.cgi). Beginning with the TIGR4 strain, we identified 335 proteins with a
289 secretion signal peptide and 15 proteins with possible cell wall anchor motifs. The
290 protein library was then narrowed down to 76 proteins based on the following
291 parameters chosen *a priori*:

292 a. Conservation across all 42 sequences defined as >90% identity at the amino acid
293 level (reduced to 203).

294 b. Exclusion of proteins with >40% homology with proteins in the human genome
295 (reduced to 160).

296 c. Exclusion of proteins containing an extracellular domain smaller than 100 amino
297 acids (to focus on proteins more likely to be accessible to antibodies in the presence
298 of polysaccharide capsule; reduced to 88)

299 We specifically excluded previously-studied antigens (including PsaA (38), SP2018 and
300 SP0148 (21), StkP and PcsB (19), Pht family proteins (39)) in order to focus on novel

301 antigens. The breakdown of the 76 proteins (see table S1) is as follows: 23 hypothetical
302 proteins, 17 proteins proposed to play roles in substrate binding and transportation, 17
303 proteins with predicted enzymatic activity and 19 others with unknown or hypothetical
304 functions. Only extracellular domains without signal peptides or transmembrane regions
305 were cloned.

306 **Construction of the pneumococcal expression library.**

307 The extracellular domains of selected proteins were amplified by PCR using TIGR4
308 genomic DNA as template and then integrated into pET21b expression vectors using the
309 CloneEZ PCR cloning kit. Two large (>250 kDa) proteins (SP0648 and SP1154) proved
310 difficult to purify full-length. We divided each amino acid sequence into 3 parts based on
311 predictions of their secondary structure by BCL::Jufo (http://meilerlab.org/index.php/
312 servers/show?s_id=5), making truncations in unconserved sequence areas, and purified
313 each fragment separately. The two possible extracellular domains of one protein,
314 SP0662, were both cloned and designated SP0662-1 and SP0662-2. Thus the final
315 protein library consists of 81 proteins and peptides. Plasmid inserts were sequenced by
316 Genewiz Inc. for confirmation.

317 **Protein Purification**. *E. coli* transformants containing the cloned proteins were grown to
318 OD_{600}=0.6 and protein expression induced overnight with 0.2 mM IPTG at 16°C. Cells
319 were spun down and pellets resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl,
320 pH8.0) and lysed by sonication. The proteins of interest were purified from supernatants
321 over a Ni-NTA column and eluted in imidazole buffer. Elutions containing each protein
322 were combined and purified over a gel-filtration column in 20 mM Tris-HCl, 150 mM
323 NaCl, pH 8.0.

324 **Stimulation of mouse immune cells and human adenoidal cells**

325 Splenocytes were harvested from mice that were either immunized with SPWCV
326 intranasally or colonized intranasally with *S. pneumoniae* strain 0603 (6B serotype) (29).

327 Stimulations were carried out for 3 days with 5 µg/ml of each protein at 37ºC with 5%
328 CO_2. Plates were then spun to pellet the cells, after which the supernatants were
329 collected and assayed for IL-17A using a mouse IL-17A ELISA kit (R&D Systems, Inc).
330 Human adenoidal mononuclear cells from children undergoing adenoidectomy were
331 separated on Ficoll gradients and cultured at $1x10^6$ cells per well in 48 well plates. Cells
332 were stimulated with 8 µg/ml of recombinant proteins or SPWCA at a concentration
333 representing $1x10^6$ cfu/ml of killed bacteria for 7 days and supernatants assayed for IL-
334 17A and IL-22 concentrations using human IL-17A and IL-22 ELISA kits (eBioscience).
335 **Ranking of antigens.** Cytokine responses to each protein were ranked based on the
336 average of the rankings among all responses to all the proteins in the cells from each
337 donor (child or mouse) in each screen, instead of their actual values, to minimize the
338 effects of variation in the responsiveness of the cells of each individual. In each
339 experiment, the protein with the greatest cytokine response was allocated the highest
340 rank and each subsequent protein was assigned a chronological rank in the decreasing
341 order of their responses. Proteins that had responses lower than that of unstimulated
342 cells were all numbered "1". The ranking numbers from each experiment were averaged
343 to calculate the final rank of each protein.
344 **Immunization and challenge of mice.** Female C57BL/6J mice (Jackson lab) were used
345 in all experiments. All animal studies were approved by our local animal ethics
346 committee. The age at time of first immunization was 4-6 weeks. Intranasal (i.n.)
347 immunization was done by instilling 20 µl of saline with 1 µg of cholera toxin (CT) as a
348 control, or CT mixed with 10 µg of antigen as specified, atraumatically into
349 unanesthetized mice twice at a one-week interval. Blood was drawn 3 weeks after the
350 last immunization, and assayed for IL-17A production upon stimulation with 5 µg/ml of
351 the corresponding protein for 6 days. Nasopharyngeal colonization with the clinical
352 pneumococcal isolate 0603 (serotype 6B) was carried out as previously described (29).

353 **IL-17A depletion.** Anti-IL-17A monoclonal (clone 17F3) and matching isotype-control

354 antibodies were purchased from BioXcell. Mice were injected intraperitoneally with a

355 dose of 150 µg per mouse 24 hours before and 3 days after infection.

356 **Statistical analysis.** Correlation was analyzed by the nonparametric Spearman method.

357 NP colonization densities were compared by the Mann-Whitney *U* test. Both were done

358 using PRISM (version 7.0a, GraphPad Software, Inc).

359

360

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364

365 References:

366 1. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, Reingold A,
367 Thomas A, Schaffner W, Craig AS, Smith PJ, Beall BW, Whitney CG, Moore MR.
368 2010. Sustained reductions in invasive pneumococcal disease in the era of
369 conjugate vaccine. J Infect Dis 201:32-41.
370 2. Balsells E, Guillot L, Nair H, Kyaw MH. 2017. Serotype distribution of
371 Streptococcus pneumoniae causing invasive disease in children in the post-PCV
372 era: A systematic review and meta-analysis. PLoS One 12:e0177113.
373 3. Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, Zhang Q, Kolls JK, Srivastava A,
374 Lundgren A, Forte S, Thompson CM, Harney KF, Anderson PW, Lipsitch M,
375 Malley R. 2008. Interleukin-17A mediates acquired immunity to pneumococcal
376 colonization. PLoS Pathog 4:e1000159.
377 4. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M.
378 2005. CD4+ T cells mediate antibody-independent acquired immunity to
379 pneumococcal colonization. Proc Natl Acad Sci U S A 102:4848-53.
380 5. Zhang Z, Clarke TB, Weiser JN. 2009. Cellular effectors mediating Th17-
381 dependent clearance of pneumococcal colonization in mice. J Clin Invest
382 119:1899-909.
383 6. Lundgren A, Bhuiyan TR, Novak D, Kaim J, Reske A, Lu YJ, Qadri F, Malley R.
384 2012. Characterization of Th17 responses to Streptococcus pneumoniae in
385 humans: comparisons between adults and children in a developed and a
386 developing country. Vaccine 30:3897-907.
387 7. Pido-Lopez J, Kwok WW, Mitchell TJ, Heyderman RS, Williams NA. 2011.
388 Acquisition of pneumococci specific effector and regulatory Cd4+ T cells
389 localising within human upper respiratory-tract mucosal lymphoid tissue. PLoS
390 Pathog 7:e1002396.
391 8. Wright AK, Bangert M, Gritzfeld JF, Ferreira DM, Jambo KC, Wright AD, Collins
392 AM, Gordon SB. 2013. Experimental human pneumococcal carriage augments
393 IL-17A-dependent T-cell defence of the lung. PLoS Pathog 9:e1003274.
394 9. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher
395 DA, Tangye SG, Cook MC. 2008. Deficiency of Th17 cells in hyper IgE syndrome
396 due to mutations in STAT3. J Exp Med 205:1551-7.
397 10. Minegishi Y, Saito M, Nagasawa M, Takada H, Hara T, Tsuchiya S, Agematsu K,
398 Yamada M, Kawamura N, Ariga T, Tsuge I, Karasuyama H. 2009. Molecular
399 explanation for the contradiction between systemic Th17 defect and localized
400 bacterial infection in hyper-IgE syndrome. J Exp Med 206:1291-301.
401 11. Vuononvirta J, Peltola V, Ilonen J, Mertsola J, He Q. 2015. The Gene
402 Polymorphism of IL-17 G-152A is Associated with Increased Colonization of
403 Streptococcus pneumoniae in Young Finnish Children. Pediatr Infect Dis J
404 34:928-32.
405 12. Hoe E, Boelsen LK, Toh ZQ, Sun GW, Koo GC, Balloch A, Marimla R, Dunne
406 EM, Tikoduadua L, Russell FM, Satzke C, Mulholland EK, Licciardi PV. 2015.
407 Reduced IL-17A Secretion Is Associated with High Levels of Pneumococcal
408 Nasopharyngeal Carriage in Fijian Children. PLoS One 10:e0129199.
409 13. Lu YJ, Leite L, Goncalves VM, Dias Wde O, Liberman C, Fratelli F, Alderson M,
410 Tate A, Maisonneuve JF, Robertson G, Graca R, Sayeed S, Thompson CM,
411 Anderson P, Malley R. 2010. GMP-grade pneumococcal whole-cell vaccine
412 injected subcutaneously protects mice from nasopharyngeal colonization and
413 fatal aspiration-sepsis. Vaccine 28:7468-75.

414 14. Wilson R, Cohen JM, Reglinski M, Jose RJ, Chan WY, Marshall H, de Vogel C,
415 Gordon S, Goldblatt D, Petersen FC, Baxendale H, Brown JS. 2017. Naturally
416 Acquired Human Immunity to Pneumococcus Is Dependent on Antibody to
417 Protein Antigens. PLoS Pathog 13:e1006137.
418 15. Darrieux M, Goulart C, Briles D, Leite LC. 2015. Current status and perspectives
419 on protein-based pneumococcal vaccines. Crit Rev Microbiol 41:190-200.
420 16. Moffitt K, Malley R. 2016. Rationale and prospects for novel pneumococcal
421 vaccines. Hum Vaccin Immunother 12:383-92.
422 17. Ling E, Feldman G, Portnoi M, Dagan R, Overweg K, Mulholland F, Chalifa-
423 Caspi V, Wells J, Mizrachi-Nebenzahl Y. 2004. Glycolytic enzymes associated
424 with the cell surface of Streptococcus pneumoniae are antigenic in humans and
425 elicit protective immune responses in the mouse. Clin Exp Immunol 138:290-8.
426 18. Wizemann TM, Heinrichs JH, Adamou JE, Erwin AL, Kunsch C, Choi GH, Barash
427 SC, Rosen CA, Masure HR, Tuomanen E, Gayle A, Brewah YA, Walsh W,
428 Barren P, Lathigra R, Hanson M, Langermann S, Johnson S, Koenig S. 2001.
429 Use of a whole genome approach to identify vaccine molecules affording
430 protection against Streptococcus pneumoniae infection. Infect Immun 69:1593-8.
431 19. Giefing C, Meinke AL, Hanner M, Henics T, Bui MD, Gelbmann D, Lundberg U,
432 Senn BM, Schunn M, Habel A, Henriques-Normark B, Ortqvist A, Kalin M, von
433 Gabain A, Nagy E. 2008. Discovery of a novel class of highly conserved vaccine
434 antigens using genomic scale antigenic fingerprinting of pneumococcus with
435 human antibodies. J Exp Med 205:117-31.
436 20. Moffitt KL, Malley R, Lu YJ. 2012. Identification of protective pneumococcal
437 T(H)17 antigens from the soluble fraction of a killed whole cell vaccine. PLoS
438 One 7:e43445.
439 21. Moffitt KL, Gierahn TM, Lu YJ, Gouveia P, Alderson M, Flechtner JB, Higgins DE,
440 Malley R. 2011. T(H)17-based vaccine design for prevention of Streptococcus
441 pneumoniae colonization. Cell Host Microbe 9:158-65.
442 22. Li Y, Gierahn T, Thompson CM, Trzcinski K, Ford CB, Croucher N, Gouveia P,
443 Flechtner JB, Malley R, Lipsitch M. 2012. Distinct effects on diversifying selection
444 by two mechanisms of immunity against Streptococcus pneumoniae. PLoS
445 Pathog 8:e1002989.
446 23. Pope C, Oliver EH, Ma J, Langton Hewer C, Mitchell TJ, Finn A. 2015. Genetic
447 conjugation of components in two pneumococcal fusion protein vaccines
448 enhances paediatric mucosal immune responses. Vaccine 33:1711-8.
449 24. Ahmed MS, Derbyshire S, Flanagan B, Loh C, McCormick M, Barocchi M,
450 Masignani V, Finn A, Zhang Q. 2014. Immune responses to pneumococcal pilus
451 RrgA and RrgB antigens and their relationship with pneumococcal carriage in
452 humans. J Infect 68:562-71.
453 25. Zhang Q, Leong SC, McNamara PS, Mubarak A, Malley R, Finn A. 2011.
454 Characterisation of regulatory T cells in nasal associated lymphoid tissue in
455 children: relationships with pneumococcal colonization. PLoS Pathog
456 7:e1002175.
457 26. Gray C, Ahmed MS, Mubarak A, Kasbekar AV, Derbyshire S, McCormick MS,
458 Mughal MK, McNamara PS, Mitchell T, Zhang Q. 2014. Activation of memory
459 Th17 cells by domain 4 pneumolysin in human nasopharynx-associated lymphoid
460 tissue and its association with pneumococcal carriage. Mucosal Immunol 7:705-
461 17.
462 27. Oliver EP, C.; Malley, R.; Ogunniyi, A.; Mitchell, T.; Morales-Aza, B.; Sikora-
463 Liszka P.; and Finn A. 2016. TH17 responses to pneumococcal antigens in

464 blood and adenoidal cells in children. The 10th International Symposium on
465 Pneumococci and Pneumococcal Diseases, Glasgow, UK,
466 28. Trzcinski K, Thompson CM, Srivastava A, Basset A, Malley R, Lipsitch M. 2008.
467 Protection against nasopharyngeal colonization by Streptococcus pneumoniae is
468 mediated by antigen-specific CD4+ T cells. Infect Immun 76:2678-84.
469 29. Malley R, Lipsitch M, Stack A, Saladino R, Fleisher G, Pelton S, Thompson C,
470 Briles D, Anderson P. 2001. Intranasal immunization with killed unencapsulated
471 whole cells prevents colonization and invasive disease by capsulated
472 pneumococci. Infect Immun 69:4870-3.
473 30. Mestas J, Hughes CC. 2004. Of mice and not men: differences between mouse
474 and human immunology. J Immunol 172(5):2731-8.
475 31. Lee YK, Mazmanian SK. 2010. Has the microbiota played a critical role in the
476 evolution of the adaptive immune system? Science 330:1768-73.
477 32. Moens L, Hermand P, Wellens T, Wuyts G, Derua R, Waelkens E, Ysebaert C,
478 Godfroid F, Bossuyt X. 2018. Identification of SP1683 as a pneumococcal protein
479 that is protective against nasopharyngeal colonization. Hum Vaccin Immunother
480 doi:10.1080/21645515.2018.1430541:1-9.
481 33. Odutola A, Ota MOC, Antonio M, Ogundare EO, Saidu Y, Foster-Nyarko E,
482 Owiafe PK, Ceesay F, Worwui A, Idoko OT, Owolabi O, Bojang A, Jarju S,
483 Drammeh I, Kampmann B, Greenwood BM, Alderson M, Traskine M, Devos N,
484 Schoonbroodt S, Swinnen K, Verlant V, Dobbelaere K, Borys D. 2017. Efficacy of
485 a novel, protein-based pneumococcal vaccine against nasopharyngeal carriage
486 of Streptococcus pneumoniae in infants: A phase 2, randomized, controlled,
487 observer-blind study. Vaccine 35:2531-2542.
488 34. Chang LB, W.A.; Bruyn, G.; Bologa, M.; Hopfer, R.; Kirby, D.; Sheng, X.; Neveu,
489 D.; Menezes, J.; Ochs, M.; Visan, L.; Dacosta, X.; Yuan, T.; Hinds, J.; and
490 Jordanov, E. 2014. A multi-compponent pneumococcal protein vaccine is safe
491 and immunogenic in a phase I randomized, placebo-controlled study. The 9th
492 International Symposium on Pneumococci and Pneumococcal Diseases,
493 Hyderabad, India,
494 35. Wright AK, Ferreira DM, Gritzfeld JF, Wright AD, Armitage K, Jambo KC, Bate E,
495 El Batrawy S, Collins A, Gordon SB. 2012. Human nasal challenge with
496 Streptococcus pneumoniae is immunising in the absence of carriage. PLoS
497 Pathog 8:e1002622.
498 36. Zhang Q, Bernatoniene J, Bagrade L, Pollard AJ, Mitchell TJ, Paton JC, Finn A.
499 2006. Serum and mucosal antibody responses to pneumococcal protein antigens
500 in children: relationships with carriage status. Eur J Immunol 36:46-57.
501 37. Zhang Q, Bagrade L, Clarke E, Paton JC, Nunez DA, Finn A. 2010. Bacterial
502 lipoproteins differentially regulate human primary and memory CD4+ T and B cell
503 responses to pneumococcal protein antigens through Toll-like receptor 2. J Infect
504 Dis 201:1753-63.
505 38. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, Virolainen
506 A, Swiatlo E, Hollingshead SK. 2000. Intranasal immunization of mice with a
507 mixture of the pneumococcal proteins PsaA and PspA is highly protective against
508 nasopharyngeal carriage of Streptococcus pneumoniae. Infect Immun 68:796-
509 800.
510 39. Godfroid F, Hermand P, Verlant V, Denoel P, Poolman JT. 2011. Preclinical
511 evaluation of the Pht proteins as potential cross-protective pneumococcal
512 vaccine antigens. Infect Immun 79:238-45.
513

514 Table 1 Protective antigens against pneumococcal colonization

515

	Gene name	Annotation
1	SP0321 [b]	PTS system, IIA component
2	SP0648 [a,b,c,e]	Beta-galactosidase
3	SP0662 [a,b,c]	Sensor histidine kinase
4	SP0742 [a]	Hypothetical protein
5	SP0757[a,b,c]	Cell division protein FtsX
6	SP0785 [a,b,c]	Hypothetical protein
7	SP0787 [a,c]	Hypothetical protein
8	SP0878 [b]	SpoE family protein
9	SP0899 [b]	Hypothetical protein
10	SP1032 [a,b,c]	Iron-compound ABC transporter
11	SP1069[d]	Hypothetical protein
12	SP1154-2 [a]	IgA1-specific metallopeptidase
13	SP1386 [a,c]	Spermidine/putrescine ABC transporter
14	SP1479 [b]	Peptidoglycan N-acetylglucosamine deacetylase A
15	SP1500 [b,c]	Amino acid ABC transporter substrate-binding protein
16	SP1683 [a,b,c]	Carbohydrate ABC transporter substrate-binding protein

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517 a, top 50% in human IL-17A screen; b, top 50% in murine WCV screen; c, top 50% in
518 exposed murine screen; d, not in any top 50% screen, e, SP0648 consisted of three
519 separate constructs, all of which were in the top 50% of all screens.

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524 Table 2 Hit rate in the top 50% responders in different screens

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Screens	Hit rate in top 50%	Hit rate in bottom 50%
All three	8/13 (61.5%)	1/9 (11.1%)
Human/WCV	8/14 (57.1%)	1/13 (7.7%)
Human/Exposed	10/21 (47.6%)	4/18 (22.2%)
WCV/Exposed	9/16 (56.3%)	3/17 (17.6%)
Human	12/27 (44.4%)	6/27 (22.2%)
WCV	12/28 (42.9%)	6/28 (21.4%)
Exposed	11/28 (39.3%)	7/28 (25%)

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536 Top 50% responders from ranked screen results of immunized mice (WCV),

537 exposed mice (exposed) and human IL-17A (human). Each screen was ranked and then

538 the top 50% or bottom 50% of proteins in the rankings were selected and overlap

539 between these antigens was shown here.

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543 **Figure legends**

544 Figure 1. IL-17A and IL-22 responses to SPWCV are positively correlated with donors'
545 ages. Adenoidal cells were stimulated with SPWCV and IL-17A and IL-22 were
546 measured from the cell supernatant. The correlations were evaluated using the
547 Spearman test.

548 Figure 2. Responses of human adenoidal cells to stimulation with each protein or with
549 whole cell vaccine. Human adenoidal mononuclear cells were stimulated with 8 μg/ml of
550 recombinant proteins or SPWCA at a concentration representing $1x10^6$ cfu/ml of killed
551 bacteria for 7 days and cytokines were measured with ELISA. The graph is presented as
552 mean±SEM. The antigens used for screening are: SP0079, SP0084, SP0092, SP0098,
553 SP0127, SP0149, SP0191, SP0198, SP0249, SP0346, SP0435, SP0453, SP0564,
554 SP0582, SP0601, SP0604, SP0617, SP0620, SP0629, SP0648-1, SP0648-2, SP0648-3,
555 SP0659, SP0662-1, SP0662-2, SP0678, SP0724, SP0742, SP0757, SP0785, SP0787,
556 SP0878, SP0899, SP1002, SP1032, SP1069, SP1154-2, SP1386, SP1404, SP1479,
557 SP1500, SP1534, SP1545, SP1560, SP1652, SP1683, SP1826, SP1872, SP1942,
558 SP2070, SP2083, SP2145, SP2192, SP2197, SP2207, SP2218 and SPWCV (from left
559 to right). SP0321 was toxic to human cells and was not used in human screens.

560 Figure 3. Responses of murine splenocytes to stimulation with each protein or with
561 whole cell vaccine. Mice splenocytes were stimulated with 5 μg/ml of recombinant
562 proteins or SPWCA at a concentration representing $1x10^6$ cfu/ml of killed bacteria for 3
563 days and IL-17A was measured with ELISA. The graph is presented as mean±SEM. The
564 antigens used for screening are: SP0079, SP0084, SP0092, SP0098, SP0127, SP0149,
565 SP0191, SP0198, SP0249, SP0321, SP0346, SP0435, SP0453, SP0564, SP0582,
566 SP0601, SP0604, SP0617, SP0620, SP0629, SP0648-1, SP0648-2, SP0648-3, SP0659,
567 SP0662-1, SP0662-2, SP0678, SP0724, SP0742, SP0757, SP0785, SP0787, SP0878,
568 SP0899, SP1002, SP1032, SP1069, SP1154-2, SP1386, SP1404, SP1479, SP1500,

569 SP1534, SP1545, SP1560, SP1652, SP1683, SP1826, SP1872, SP1942, SP2070,
570 SP2083, SP2145, SP2192, SP2197, SP2207, SP2218 and SPWCV (from left to right).
571 (A) IL-17A levels following stimulation of splenocytes from SPWCV immunized mice. (B)
572 IL-17A levels following stimulation of splenocytes from pneumococcus-exposed mice.
573 Figure 4. Protection against colonization by antigens from the surface protein library.
574 Mice were immunized with 10 μg of each protein and 1 μg of cholera toxin twice weekly
575 and challenged 4 weeks after the last immunization with a serotype 6B clinical strain.
576 Nasal wash was collected 7 days later and bacterial CFU was determined by plating.
577 Each dot represents CFU recovered from one mouse and the lines for each column
578 represent geometric means. Colonization rates in mice receiving antigen plus adjuvant
579 CT immunization were compared to those in control mice (receiving CT alone) using the
580 unpaired nonparametric Mann-Whitney test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.
581 Figure 5. Protection against nasal colonization conferred by proteins is IL-17A
582 dependent. Mice were immunized with top-ranked antigens identified via the human
583 screen (SP0648-1, SP0662-1 and SP0742) or via the mouse screen (SP0648-3,
584 SP0757 and SP1500) intranasally. Mice were immunized with 10 μg of each protein and
585 1 μg of cholera toxin twice weekly and challenged 4 weeks after the last immunization
586 with a serotype 6B clinical strain. Mice received either anti-IL-17A or isotype control
587 antibody one day prior to and 3 days after the infection. Statistical analysis was
588 performed with unpaired nonparametric Mann-Whitney test. n.s, not significant; *,
589 $p<0.05$; ****, $p<0.0001$.
590 Figure 6. Correlation between human IL-17A and IL-22 screens. The ranking of each
591 antigen was analyzed for their correlation between each screen using nonparametric
592 Spearman method.
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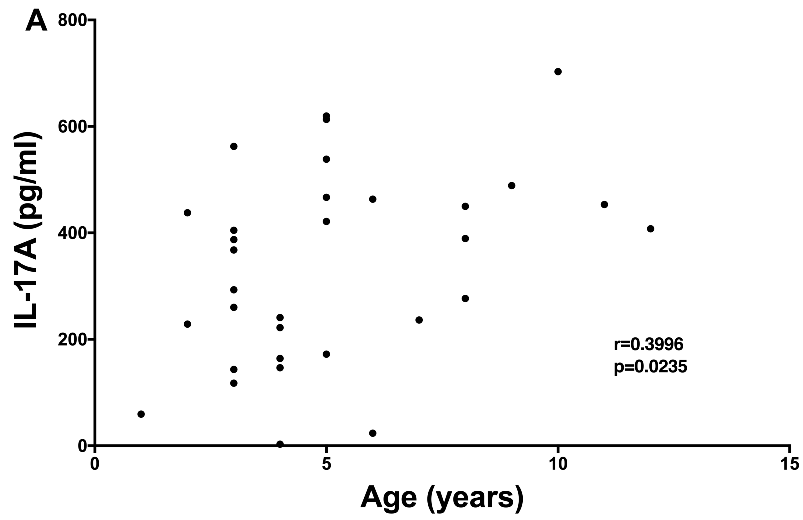

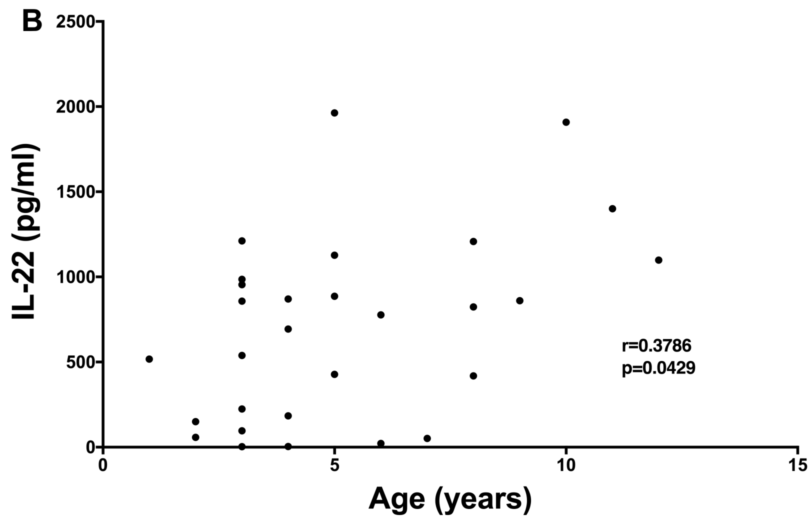

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596 Figure 1

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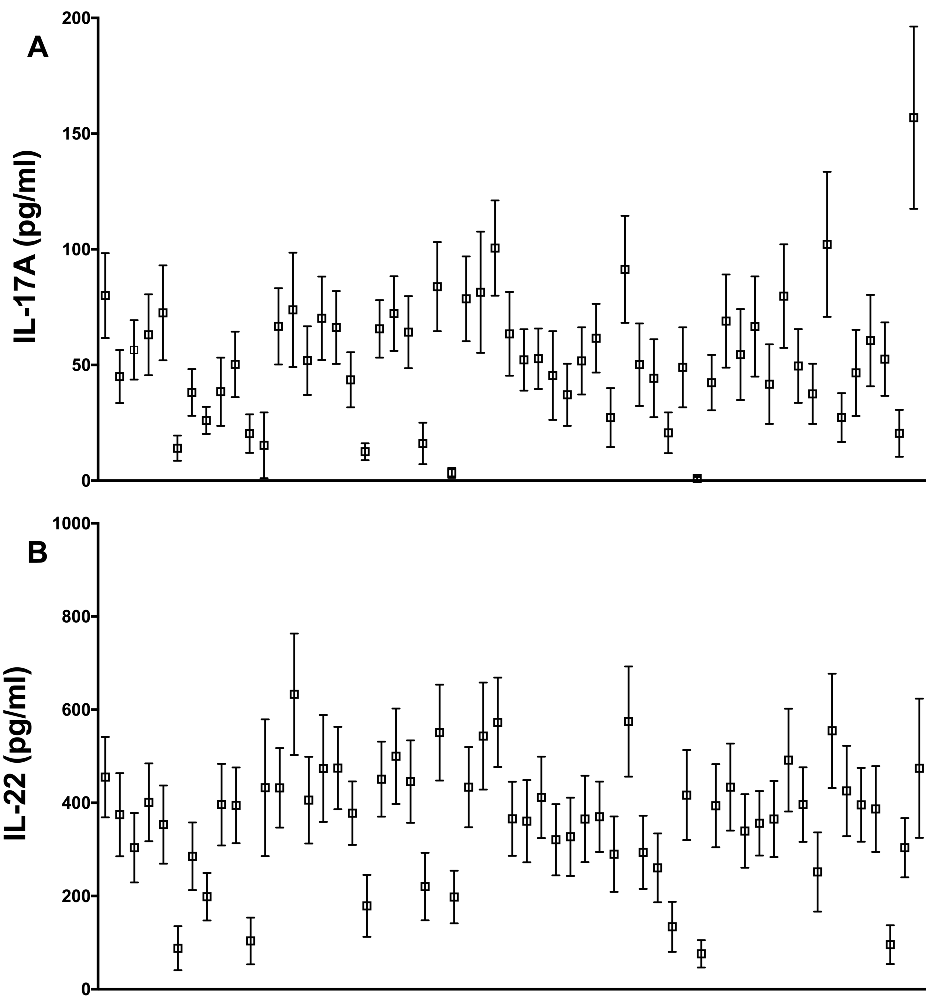

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602 Figure 2

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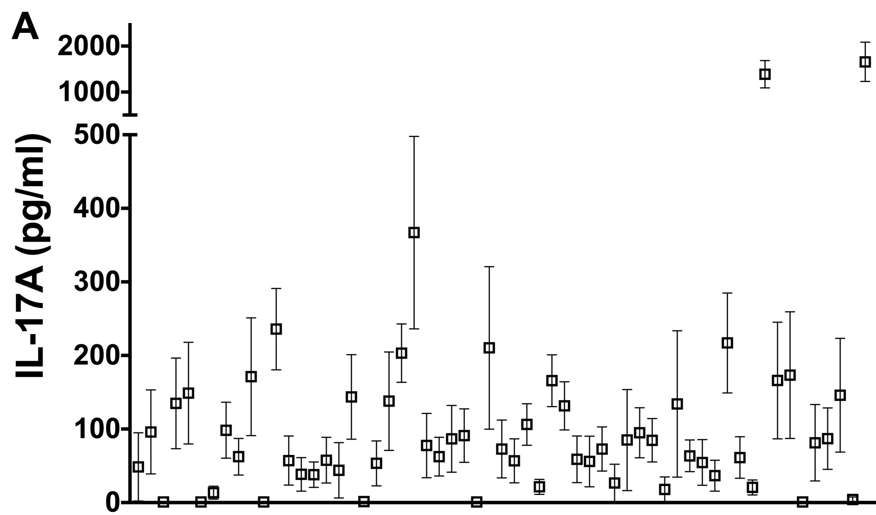

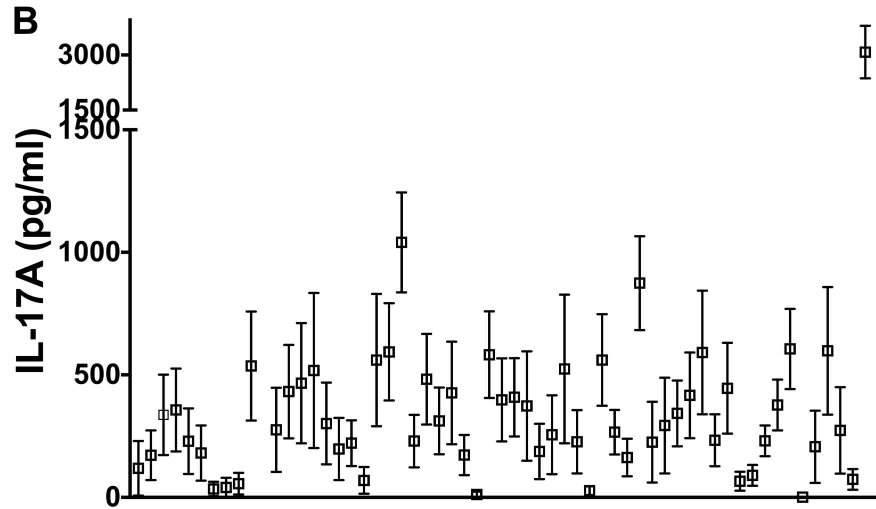

604 Figure 3

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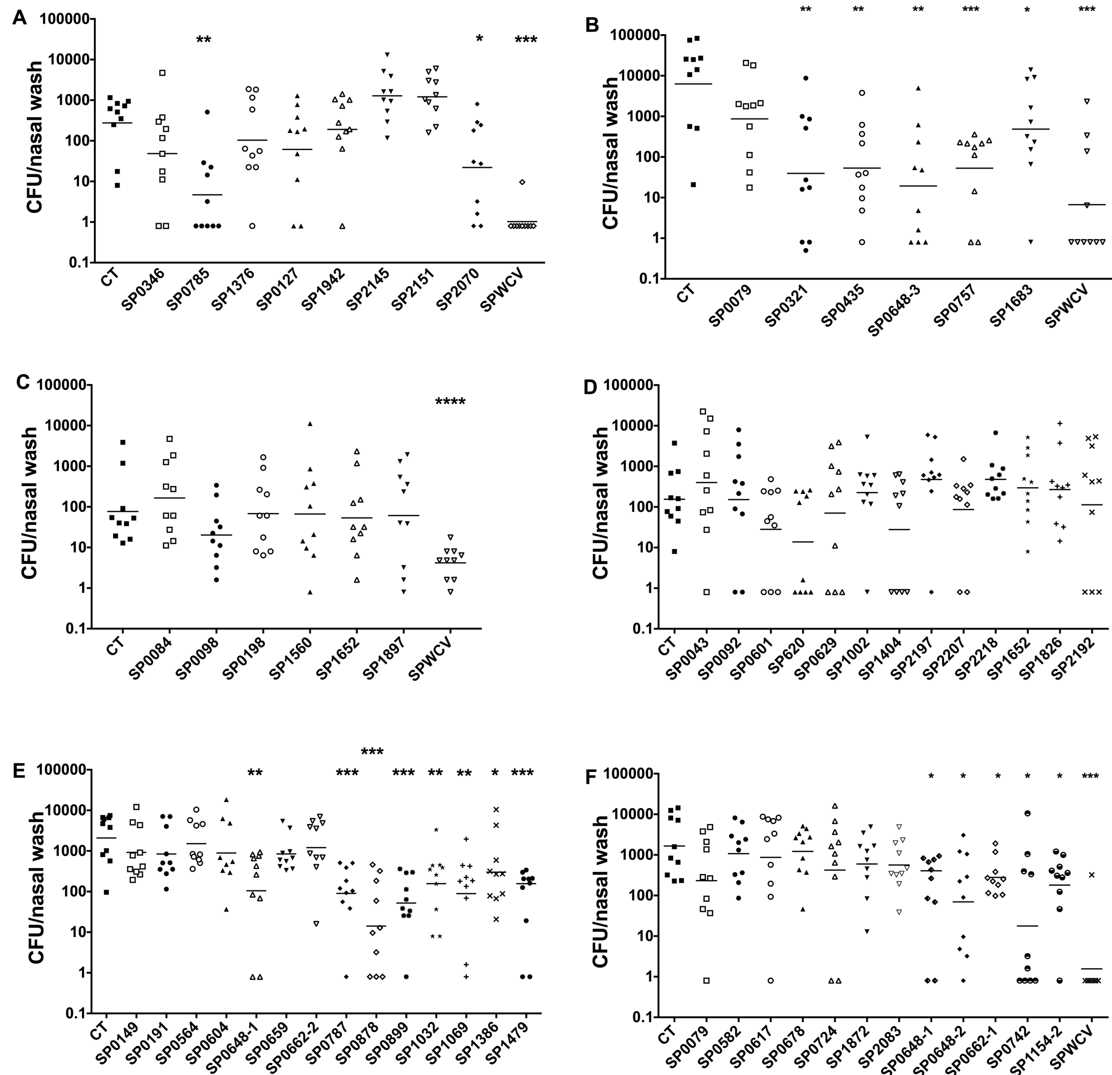

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608 Figure 4

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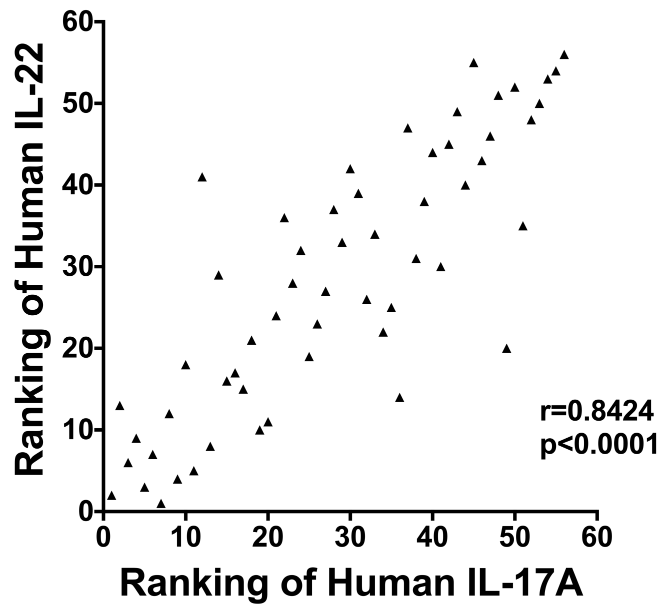
Figure 5



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617 Figure 6