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Nasal pneumococcal density is associated with microaspiration and heightened human alveolar macrophage responsiveness to bacterial pathogens.

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1 **Abstract**

2 **Rationale:** Pneumococcal pneumonia remains a global health problem. Colonization of the
3 nasopharynx with *S.pneumoniae* (Spn), although, a prerequisite of infection, is the main
4 source of exposure and immunological boosting in children and adults. However, our
5 knowledge of how nasal colonization impacts on the lung cells, especially on the predominant
6 alveolar macrophage (AM) population, is limited.

7 **Objectives:** Using a Controlled Human Infection model to achieve nasal colonization with 6B
8 serotype, we investigated the effect of Spn colonization on lung cells.

9 **Methods:** We collected bronchoalveolar lavages from healthy pneumococcal challenged
10 participants aged 18-49 years. Confocal microscopy, molecular and classical microbiology
11 were used to investigate microaspiration and pneumococcal presence in the lower airways.
12 AM opsonophagocytic capacity was assessed by functional assays *in vitro*, whereas flow
13 cytometry and transcriptomic analysis were used to assess further changes on the lung
14 cellular populations.

15 **Measurements and Main Results:** AM from Spn-colonized exhibited increased
16 opsonophagocytosis to pneumococcus (11.4% median increase, $p=0.005$) for four months
17 after clearance of experimental pneumococcal colonization. AM had also increased responses
18 against other bacterial pathogens. Pneumococcal DNA detected in the BAL samples of Spn-
19 colonized were positively correlated with nasal pneumococcal density ($r=0.71$, $p=0.029$).
20 Similarly, AM heightened opsonophagocytic capacity was correlated with nasopharyngeal
21 pneumococcal density ($r=0.61$, $p=0.025$).

22 **Conclusions:** Our findings demonstrate that nasal colonization with pneumococcus and
23 microaspiration prime AM, leading to brisker responsiveness to both pneumococcus and
24 unrelated bacterial pathogens. The relative abundance of AM in the alveolar spaces, alongside

25 with their potential for non-specific protection, render them an attractive target for novel
26 vaccines.

27

28 **Keywords:**

29 *S. pneumoniae*, Nasopharyngeal colonisation, Controlled human infection, Micro-aspiration,
30 Alveolar macrophages, Innate cells, Lung immunity, Interferon- γ , CD4+ T cells

31 **Introduction**

32 *Streptococcus pneumoniae* (the pneumococcus, Spn) is a leading cause of severe infection,
33 responsible annually for the death of up to a million children worldwide (1). Pneumonia is the
34 most frequent manifestation of pneumococcal disease (2) and despite the current vaccination
35 strategies the burden of pneumococcal pneumonia remains very high globally (3), affecting
36 disproportionately the very young and very old throughout the world (4).

37 Despite its pathogenicity, *S. pneumoniae* commonly colonizes the human nasopharynx, a
38 state known as pneumococcal colonization or carriage (4). Pneumococcal colonization rates
39 in the absence of disease range from 40 to 95% in infants and approximately 10-25% among
40 adults (5, 6). In humans, exposure to pneumococcus through nasopharyngeal colonization is
41 an immunising event, as it elicits humoral and cellular immune-responses, both systemically
42 and in the nasal mucosa (7-9).

43 However, lung mucosal immune-responses to pneumococcus are not well understood in
44 humans. It is believed that protection against development of pneumonia relies on a
45 successful regulation of colonization in the nasopharynx and a brisk alveolar macrophage-
46 mediated immune response in the lung (10). The alveolar macrophage (AM) – an innate type
47 resident lung cell- is an integral component of lung immunity (11) and the first cell type to
48 combat pneumococci during early infection (12). It also plays a key role in shaping the
49 adaptive immunity through their effects on dendritic cells and T cells (13). In murine models,
50 it has been shown that AMs are mainly self-maintained, although during lung insult or as a
51 result of ageing (14) peripheral monocytes contributes to their replenishment (15, 16).

52 A clear understanding of the mechanisms that underlie brisk but controlled lung immune-
53 responses at the early stages of the infection is essential to inform us why high rates of
54 pneumonia persists in the high-risk groups (infants, elderly and immunocompromised). These
55 high-risk groups are characterized by underdeveloped or defective adaptive immunity.
56 Although, current immunization strategies to pneumococcal diseases target exclusively B cell

57 dependent immunity, the recently described memory properties of innate cells, including
58 natural killer (NK) cells and monocytes, indicate that innate immune cells could be considered
59 as a promising alternative or complementary vaccine target (17-19).

60 In this study, we investigated the effect of antecedent pneumococcal colonization on alveolar
61 macrophage function in healthy adults. We showed for the first time that nasal human
62 colonization in absence of disease leads to pneumococcal aspiration to the lower respiratory
63 tract, which enhanced AM opsonophagocytic capacity against a range of bacterial respiratory
64 pathogens likely through an interferon- γ (IFN- γ)-mediated mechanism.

65 **Methods**

66 **Study design and bronchoalveolar lavage collection**

67 Healthy, non-smoking, adult volunteers aged from 18-49 years, enrolled in one of the
68 Experimental Human Pneumococcal Challenge (EHPC) studies (20) between 2015-2018 (21,
69 22) underwent an one-off research bronchoscopy, as previously described (23, 24).
70 Experimental human pneumococcal challenge was conducted as previously described (7, 20)
71 and 80,000 colony-forming-units (CFU) of serotype 6B (strain BHN418) were instilled into
72 each nostril of participants. Pneumococcal colonization was detected by classical
73 microbiology methods and individuals were defined as Spn colonised, if any nasal wash
74 culture following experimental challenge grew *S. pneumoniae* serotype 6B. Bronchoalveolar
75 lavage samples were obtained from 29 to 203 days post the intranasal inoculation (Figure 1A).
76 Spn colonised individuals received 3 doses of amoxicillin at the end of the clinical trial (at day
77 14 or 27 or 29) prior to the bronchoscopy.

78

79 **Ethics statement**

80 All volunteers gave written informed consent and research was conducted in compliance with
81 all relevant ethical regulations. Ethical approval was given by the National Health Service

82 Research Ethics Committee (REC). Ethics Committee reference numbers: 15/NW/0146,
83 14/NW/1460 and 15/NW/0931 and Human Tissue Authority licensing number: 12548.

84

85 **Bronchoalveolar lavage processing and alveolar macrophage isolation**

86 Bronchoalveolar lavages (BAL) samples were processed (23, 24) and AMs were routinely
87 separated from other cell populations using adherence step, as previously described (25). In
88 the experiments that highly pure AM population was requested, AMs were purified from the
89 whole BAL sample through cell sorting (FACS ARIAIII), following seeding on 96-well plate and
90 overnight incubation at 37°C, 5% CO₂ (full details in supplementary methods).

91

92 **Alveolar macrophage opsonophagocytic killing (OPA)**

93 AMs opsonophagocytic capacity was evaluated as previously described with minor
94 modifications (26, 27) (full details in supplementary methods).

95

96 **Bacterial DNA extraction and quantification of pneumococcal DNA in BAL samples**

97 Extraction of bacterial DNA from the BAL samples was performed as previously described
98 with minor modifications (28). Presence of pneumococcal DNA in BAL samples was
99 determined using primers and probe specifically designed for 6B serotype, targeting on a
100 capsular polysaccharide gene known as wciP, the rhamnosyl transferase gene. The primers
101 and probe sequences were: forward primer 5'- GCTAGAGATGGTTCCTTCAGTTGAT- 3';
102 reverse primer 5'- CATACTCTAGTGCAAACCTTTGCAAAT- 3' and probe 5'- [FAM] ACT GTC
103 TCA TGA TAA TT [MGBEQ] -3' as previously published (29) (full details in supplementary
104 methods).

105

106 **Confocal microscopy**

107 Fresh BAL cells were washed and stained with anti-human CD14 texas-Red and CD45-
108 AlexaFluor647). Cells were permeabilized and incubated with 6B pneumococcal antisera

109 (Statens Serum Institute) for 30 minutes and then with secondary-conjugated antibody (anti-
110 rabbit 488) for another 30 minutes. After washing, cells were cytospun onto microscope slides.
111 DAPI solution was applied directly on the spun cells for 5 minutes. After washing, samples
112 were mounted using Aqua PolyMount (VWR International). Images were captured using an
113 inverted TissueFAXS Zeiss Confocal Microscope. Z stacks were recorded at 1µm intervals at
114 either 40x oil or 63x oil objectives.

115 In thawed BAL samples, fixed and permeabilized cells were incubated in blocking solution
116 (PBS-5% goat serum), following 1hour incubation with primary antibodies and 45 minutes with
117 secondary antibody solution, following rinsing and then mounting with DAPI. Anti-
118 pneumococcal capsule anti-6B serum (Statens Serum Institute) was used to stain bacteria,
119 whereas macrophages were labelled with anti-human CD169 (Thermoscientific).
120 Combinations of Alexafluor conjugated antibodies (Thermoscientific) were used as secondary
121 antibodies (488 and 568 with different host specificity). Images were acquired in Olympus
122 FV1000 confocal laser scanning microscope using 40x objectives. For the bacterial
123 localisation assays, Alexafluor 633-conjugated Wheat germ agglutinin was used prior to
124 membrane permeabilization. Z-stack was created from microscope images, elaborated using
125 Huygens Essential deconvolution software version 16 (Scientific Volume Imaging,
126 Netherlands) and viewed in Imaris 3D reconstruction software 9.4 (Bitplane, Switzerland).

127

128 **Flow cytometry assays**

129 In each flow cytometry assays, the corresponding cell population was stained with
130 predetermined optimal concentration of fluorochrome-conjugated monoclonal antibodies
131 against human cell surface proteins or intracellular cytokines (full details in supplementary
132 methods).

133

134 **Luminex analysis of Bronchoalveolar lavage fluid**

135 The acellular BAL fluid was collected post centrifugation of whole BAL sample (400g for 10min
136 at 4°C), divided to 1ml aliquots and stored at -80oC until analysis. On the day of the analysis
137 samples were concentrated x10 (1ml of BAL supernatant concentrated to 100ul using vacuum
138 concentrator RVC2-18), following acquisition using a 30-plex magnetic Luminex cytokine kit
139 (ThermoFisher) and analyzed on a LX200 with xPonent3.1 software following manufacturer's
140 instructions. Samples were analysed in duplicates and BAL samples with a CV > 50 % were
141 excluded.

142

143 **AMs gene analysis using Nanostring platform**

144 Nanostring for AM gene analysis was used as previously described (21) (full details in
145 supplementary methods).

146

147 **Quantification and statistical analysis**

148 Statistical analyses were performed using GraphPad Prism (Version 6, GraphPad Software,
149 La Jolla, CA) and R software (version 3.5.1), including Bioconductor packages. P values are
150 two-tailed. For parametric groups comparisons, t test was used for unpaired and paired
151 groups. For non-parametric groups comparisons, a Mann-Whitney or Wilcoxon test was used
152 for unpaired and paired groups, respectively. For gene expression and Luminex analysis p
153 values were corrected by applying multiple correction testing (Benjamin-Hochberg). To
154 quantify association between groups, Pearson or Spearman correlation test was used for
155 parametric or non-parametric groups, respectively. Differences were considered significant at
156 $p \leq 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

157

158 **Results**

159 **Alveolar macrophages exhibit augmented responsiveness to bacteria over three**
160 **months after the clearance of experimental pneumococcal colonization.**

161 We coupled the experimental human pneumococcal challenge model with research
162 bronchoscopy to investigate whether and how nasopharyngeal pneumococcal colonization
163 affects alveolar macrophage function in healthy adults. BAL sample was collected from both
164 pneumococcal (Spn) colonised and non-colonized healthy adults (aged from 18-49yrs)
165 between one and seven months (29 to 203 days) post bacterial challenge (Figure 1A).
166 Antecedent pneumococcal colonization was associated with 11.4% increase in alveolar
167 macrophage capacity to take up pneumococci *in vitro*, ranged in non-colonized (carriage-)
168 from 58.5% to 81.6% and in Spn colonized (carriage+) group from 69.7% to 90.7% (p=0.005,
169 Figure 1B). The observed differential AM opsonophagocytic activity (OPA) was reproducible
170 between studies and persisted for 4 months following the intranasal pneumococcal inoculation
171 (Figure 1C). We also sought to examine whether this enhanced activity was specific to
172 pneumococcus or whether AM responses to other pathogens were similarly increased. AMs
173 from Spn colonized individuals had greater capacity to take up the respiratory pathogens
174 *Streptococcus pyogenes* and *Staphylococcus aureus* (increased by 18% and 11%,
175 respectively) when compared with AMs isolated from non-colonised individuals (p=0.009 and
176 p=0.038 respectively, Figure 1D). For the gram-negative bacterium *Escherichia coli* there was
177 a non-significant increase in AM OPA in the Spn colonized group (median: 20.6% increase,
178 p=0.067, Figure 1D).

179 ***S. pneumoniae* can be detected in the lung after clearance of nasal colonization**

180 To investigate whether pneumococcus is the stimulus of the enhanced AM responses in the
181 pulmonary mucosa post nasal colonization, we sought to find evidences of presence of the
182 pneumococcal challenge strain (Spn6B) in the alveolar spaces. For the detection of
183 pneumococcus in the BAL samples, we utilised both classical microbiology and molecular
184 methods targeting a capsular polysaccharide gene specific to Spn6B (*wciP*). Spn6B DNA was
185 detected in the BAL of 41% (9/22) of Spn colonized individuals (Table S1), 1 to 3 weeks
186 following the clearance of nasal colonization. None of the non-colonized individuals had

187 detectable Spn6B DNA in their BAL sample. Nasal pneumococcal density positively correlated
188 with the copies of pneumococcal DNA detected in BAL samples (Figure 2A). Spn colonized
189 individuals differed in both density and duration of the colonization episode (Figure 2B). In
190 addition, AMs capacity to take-up pneumococci correlated positively with nasal pneumococcal
191 density (Figure 2C). Utilising confocal microscopy and anti-sera against the Spn6B capsule,
192 we confirmed the relationship between nasal colonization and presence of pneumococcal
193 particles in the lung. Pneumococcal cells were found associated with the surface of AMs or
194 internalised by them, a phenomenon only observed in the Spn colonized group (Figure 2D-F
195 Video S1). These data suggest that during asymptomatic pneumococcal colonization of the
196 nasopharynx, aspiration of pneumococci can occur, modulating the pulmonary immunological
197 responses.

198 **CD4⁺ Th1 skewed responses rapidly prime AMs**

199 To investigate whether the observed augmented AM capacity to take up pneumococci *in vitro*
200 was dependent on lung lymphocytes, we co-incubated AMs with autologous CD3⁺CD4⁺ T cells
201 during *in vitro* infection with Spn6B. The presence of CD3⁺CD4⁺ T cells enhanced the basal
202 AM opsonophagocytic capacity in both non-colonized (1.6-fold, $p < 0.0001$) and Spn colonized
203 individuals (1.8-fold, $p < 0.0001$) (Figure 3A). Although, AM uptake capacity differed between
204 the two groups at baseline (prior to lung-derived autologous CD3⁺CD4⁺ T cell addition), the
205 presence of this cell subset further amplified the observed basal difference in AM
206 pneumococcal uptake (Figure 3A).

207 To elucidate the mechanism underlying this increased boosting of AM function by CD4⁺ T cells
208 from Spn colonized individuals, we stained lung lymphocytes intracellularly for T-box
209 transcription factor expressed in T-cells (T-bet), GATA-binding protein-3 (GATA-3) and
210 Forkhead box P3 (FoxP3) transcription factors (Figure S1). In the Spn colonized group the
211 levels of CD4⁺ T-bet expressing cells were twice as high than in the non-colonized group ($p =$
212 0.003), indicating Th1-polarisation (Figure 3B). There were no significant differences in the

213 levels of neither CD4⁺ GATA-3 expressing nor CD4⁺ FoxP3 expressing T cells between the
214 two groups (Figure 3B).

215 In parallel, lymphocytes from both Spn colonized and non-colonized volunteers were
216 stimulated with pneumococcal antigen (Heat Inactivated-Spn6B). Cytokine (IFN- γ , Tumour
217 necrosis factor α [TNF- α] or Interleukin 17A [IL-17A]) producing CD4⁺ T-cells were
218 subsequently detected by flow cytometry (Figure S2). AM OPA correlated with cytokine
219 producing CD4⁺ T cells, classified as spontaneous (unstimulated) or pneumococcal-
220 responding cells (Figure 4). Increased levels of IFN- γ producing CD4⁺ T cells, both
221 pneumococcal-specific and spontaneous responding, positively correlated with AM ability to
222 take up live pneumococci *in vitro*. (Figure 4A). On the other hand, AM OPA correlated
223 positively with only the pneumococcal-specific TNF- α producing CD4⁺ T cells (Figure 4B),
224 whereas IL-17A producing CD4⁺ T cells did not correlate with AM OPA in any condition (Figure
225 4C).

226 **Increased IFN- γ and GM-CSF levels are present in the alveolar spaces post nasal** 227 **pneumococcal colonization**

228 The alveolar microenvironment is crucial for cell signalling, shaping how local cells respond to
229 different stimuli (30). To assess alterations of the alveolar cytokine milieu induced by nasal
230 pneumococcal colonization, we measured levels of 30 cytokines in the BAL fluid retrieved from
231 both Spn colonized and non-colonized individuals (Figure 5A, Table S2). Three cytokines had
232 higher detectable levels in the BAL fluid of Spn colonized group: GM-CSF (p=0.03) and the
233 pro-inflammatory cytokines IFN- γ (p=0.047) and IFN- α (p=0.043) (Figure 5B). To address the
234 role of increased secretion of IFN- γ , a prototypic Th1 cytokine, in the pulmonary airspaces and
235 its effect on AM function, we stimulated AMs with 10-fold increasing concentrations of
236 exogenous IFN- γ . The lowest tested titers of IFN- γ (2 and 20ng/ml) augmented AMs OPA,
237 resulting both in 1.5-fold increase (1.5x median; IQR:1.2x- 2.1x) in AM pneumococcal uptake,
238 whereas no significant increase was seen with the highest used concentrations (200 and

239 2000ng/ml) (Figure 5C). These results were verified when AM response was assessed using
240 a flow cytometric cytokine production assay (Figure S3). AMs produced increased levels of
241 TNF- α in response to stimulation with HI-Spn6B only at the lower pre-stimulation doses of
242 IFN- γ (Figure 5D). The mechanism seems to have a threshold, as demonstrated by the data,
243 with IFN- γ signalling being beneficial for AM function at lower doses *in vitro*, but not at higher
244 concentration.

245 **Pneumococcal colonization may promote monocyte-to-macrophage differentiation in** 246 **the alveolar spaces**

247 Previously, we have demonstrated that AM phenotype is not altered by nasopharyngeal
248 pneumococcal colonization, as defined by classical monocyte polarisation surface markers
249 (23). However, given the increased capacity of AM to take up pneumococci, we extended our
250 assessment to other lung myeloid cell populations and neutrophils, in order to determine
251 whether recent pneumococcal carriage alters the distribution of these cells in the airway
252 (Figure S4). Spn colonized individuals displayed significantly greater AM levels (1.2-fold
253 increase, $p=0.04$) and higher AM/monocyte ratio (2.3-fold increase, $p=0.04$) in the lung
254 compared to non-colonized individuals (Figure 6A). On the other hand, monocyte levels, both
255 total and CD14^{hi}CD16^{lo} and CD14^{hi}CD16^{hi} subsets, had no significant difference between the
256 two groups, despite their trend for increased presence in the non-colonized group (Figure 6A-
257 B). Similarly, no difference in neutrophil levels was observed between the two groups (Figure
258 6B), indicating that nasal carriage in absence of disease does not lead to neutrophil
259 recruitment to the lung.

260 To test whether antecedent pneumococcal colonization led to monocyte differentiation and
261 AM activation, we sought to identify the differential gene signatures of Spn colonized and non-
262 colonized volunteers. We isolated AMs by cell sorting from a subset of BAL samples and
263 performed NanoString expression analysis of 594 immunological genes. The analysis
264 revealed 34 differentially expressed genes (DEG) between the two groups (Table S3). Gene

265 set enrichment analysis was performed on all genes, ranked from high to low expressed in the
266 Spn colonized compared to the non-colonized group, using published blood transcriptional
267 modules (31). Purified alveolar macrophages from Spn colonized individuals showed an
268 enrichment in pathways of cell differentiation and function, revealing under-presentation of
269 monocytes surface markers and over-presentation of antigen-presentation markers in the Spn
270 colonised group (Figure 6C). This finding complements the previous observation that nasal
271 Spn colonization may lead to monocyte-alveolar macrophage differentiation. When the AM
272 OPA per individual was compared with gene expression (log counts per million [CPM])
273 measured for each of the 594 genes, 34 genes were positively correlated with AM function to
274 take up the bacteria (Table S4). Only four genes were both significantly correlated with AM
275 OPA and significantly increased in Spn colonized individuals: T-box 21 (*TBX21*), ecto-5'-
276 nucleotidase (*NT5E*), Carcinoembryonic antigen-related cell adhesion molecule 6
277 (*CEACAM6*) and Toll like receptor 8 (*TLR8*) (Figure 6B).

278 **Discussion**

279 This study provides insights into the immune responses elicited at the human pulmonary
280 mucosa post a pneumococcal carriage episode. Using our experimental human
281 pneumococcal challenge model, we demonstrated that prior nasopharyngeal pneumococcal
282 colonization results in bacterial aspiration to the lower airspaces, leading to a brisker AM
283 opsonophagocytic capacity against both pneumococcus and other bacterial pathogens.
284 Aspirated pneumococci most likely act as the stimulus that leads to enhanced AM
285 responsiveness mediated by AM - CD4⁺ T cells cross-talk and Th1 cytokine secretion.

286 The lung mucosa is not the sterile environment previously thought (32, 33). By employing
287 classical microbiology, molecular and visualization methods, we demonstrated that
288 pneumococcal aspiration occurs during nasal pneumococcal colonization, a phenomenon that
289 was previously observed only in pneumonia cases (34, 35). The positive correlation between
290 AM opsonophagocytic activity and nasal pneumococcal density suggested pneumococcal cell

291 trafficking from the nasopharynx to the lung airways. The increased opsonophagocytic
292 capacity displayed by AM was a non-specific response to pneumococcal stimulus. AM
293 responded with equal efficacy to both Spn and other gram-positive respiratory pathogens *in*
294 *vitro*. By contrast, we did not see significant enhancement of AM opsonophagocytic activity
295 (OPA) against *E. coli*, although the small sample size used might have limited the detection of
296 a less pronounced difference between the two experimental groups.

297 Our observation shares some similarities with the findings of emerging studies on “trained
298 immunity” (or innate immune memory) (19, 36), which reported increased responsiveness of
299 innate immune cells to microbial stimuli, caused by epigenetic changes, post their activation
300 by varying stimuli (e.g. Bacille Calmette-Guerin [BCG] or measles vaccination). Similarly to
301 our observation, this augmented functional state persisted for weeks to months, and
302 additionally conferred resistance to reinfection or heterologous infection (17, 18, 37, 38).
303 Further controlled human infection studies, including pre- and post- pneumococcal challenge
304 BAL sampling and focusing on AM epigenetic and metabolic changes, will be able to address
305 whether human alveolar macrophages acquire a “trained immunity” phenotype as response
306 to pneumococcal exposure. It will also enable comparisons of immune-responses pre- and
307 post- colonization on individual level.

308 Our findings on CD4⁺ Th1 skewed responses and exogenous IFN- γ effect on AM antimicrobial
309 function supported the idea that Th1 type responses and interferons are crucial in controlling
310 bacteria at the early stages of infection. Increased rates of pneumococcal colonization in
311 children and clinical cases of pneumonia in adults have been associated with a reduction in
312 systemic circulating Th-1 (IFN- γ secreting) CD4⁺ T-cells (39, 40). Polymorphisms in the
313 adaptor MAL, which regulates IFN- γ signalling (41), have been associated with altered
314 susceptibility to a number of infectious diseases including severe pneumococcal disease(42).
315 Moreover, we observed a rapid priming of AMs when co-cultured with autologous lung derived
316 CD4⁺ T cells *in vitro*. A very recent study in mice described a similar mechanistic link between

317 adaptive and innate immune system, suggesting that effector CD8⁺ T cells, in the context of
318 respiratory adenoviral infection, are able to prime AM and render innate memory via IFN- γ (43).

319 Our study highlighted that IFN- γ has a dose-dependent effect on human AM function, which
320 offers an explanation to the contradictory reports around this topic. For instance, in murine
321 models high production of IFN- γ during influenza infection impaired phagocytosis and killing
322 of *S. pneumoniae* by alveolar macrophages (44, 45). In contrast, many other evidences
323 suggest that induction of IFN- γ secretion, related to non-acute viral infection, is beneficial for
324 innate immune cells, promoting a range of antimicrobial functions, plus macrophage
325 polarization and activation (43, 46, 47). The dose-dependent effect of IFN- γ on AM OPA could
326 also explain why HIV-infected adults are still at increased risk of developing pneumococcal
327 pneumonia, despite the preserved Th1 responses against *S. pneumoniae* (48).

328 By assessing AM gene expression levels, we found that AM population derived from Spn
329 colonized individuals was characterized by increased antigen-presentation and decreased
330 monocytes surface markers signature. Our flow-based data corroborated this result by
331 showing greater AM levels and increased AM to monocyte ratio in the Spn colonized
332 individuals. The positive correlation of AM OPA with genes such as *NT5E* (or *CD73*) (49) and
333 *TBX21* (a master regulator of Th1 responses) indicate at some degree that monocyte-to-
334 macrophage differentiation and AM polarisation to a more active functional state occur in the
335 human lung, after interacting with the pneumococcus. Studies on human
336 monocytes/macrophages have reported detectable expression of *CD73* in only M(LPS-TNF)
337 polarized cells and increased of *T-bet* mRNA displayed by M1 polarized macrophages (50,
338 51). Also, our finding on increased expression of TLR8 in the Spn colonized group might be
339 the readout of IFN- γ signalling, as TLR8 is responsive to interferons. Increase in TLR8 levels
340 might lead to enhanced viral sensing and thus have a beneficial effect upon viral infection,
341 such as influenza.

342 In conclusion, this study emphasizes the effect that nasopharyngeal pneumococcal
343 colonization has upon the pulmonary innate immune system. The seeding of human lung with
344 activated AM that exert prolonged and enhanced opsonophagocytic properties has potential
345 implications for vaccine development. Pneumococcal vaccines that focus solely on inducing a
346 robust Th17 response may not be the best strategy for vaccine targeting serotype-independent
347 protection against pneumonia. On the other hand, such a non-specific boosting of innate lung
348 immunity may be an alternative strategy to successful pneumonia prevention, especially for
349 the new-borns, whose immune system is still developing, or for the elderly, whose acquired
350 immunity is beginning to wear off. In particular the elderly, who have been described as the
351 age group with the lowest pneumococcal colonization rates and high incidence of community-
352 acquired pneumonia cases, would benefit from the boosting effect that mucosal stimulation
353 with whole cell pneumococcus confers to the lung immune cells. These results, in combination
354 with our previous finding on increased frequency of pneumococcal-specific CD4⁺ Th-17 cell in
355 human lung post nasal colonization (25), suggest that a nasally administered live-attenuated
356 pneumococcal vaccine could augment the pulmonary immune-responses and confer
357 serotype-independent protection against development of pneumococcal pneumonia.

358

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369

370 **Author contributions**

371 EM contributed to conceiving, designing, conducting and analysing experiments, design of the
372 study and writing of the paper. BC, JReine, EN, CS, SP and MDSC contributed to conducting
373 and analysing experiments. SS, VC, AC, HH and AHW contributed to sample collection. ASS,
374 JRylance, AS, KJ, MO and SG contributed to designing and analysing experiments. SJ and
375 DF contributed to conceiving, designing and analysing experiments, design of the study and
376 writing of the paper. All authors have read and approved the manuscript.

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378 **Competing Interest**

379 The authors declare no competing interests.

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Figures

Figure 1: AMs display an increased opsonophagocytic activity (OPA) against bacterial pathogens for a prolonged period post nasal pneumococcal carriage. A) Defined time-

period of BAL samples collection from Spn colonised (carriage+) and non-colonised (carriage-) individuals within three independent Experimental Human Pneumococcal Challenge (EHPC) studies. Individuals were purposively sampled according to colonization state. After the final nasal wash (day 27, 17 or 29 based on study), Spn colonised individuals received a three-day course of antibiotics. **B)** Percentage of pneumococcal uptake by AM post in vitro infection in carriage- (n=35) and carriage+ (n=37) group. **p=0.005 by Mann-Whitney test. Multiplicity of infection (MOI) used was 1: 100. **C)** Chronological representation of all BAL samples (n=72) collected from one to six months post intranasal pneumococcal inoculation divided into three consecutive time periods. T1: p= 0.001, T2: p= 0.003 and T3: p= 0.82 by Mann-Whitney test. **D)** Percentage of bacterial uptake by AM post in vitro infection with Spn6B or *S. aureus* or *S. pyogenes* or *E. coli*. **p= 0.009, **p= 0.009, *p= 0.038 and p=0.067, respectively by Mann-Whitney test. Boxplots and individual subjects are depicted with Carriage- in black dots and Carriage+ in red dots.

Figure 2: Evidence of pneumococcal presence in the lung of nasopharyngeal Spn colonized individuals. **A)** Positive correlation between the nasal pneumococcal density, expressed as the Area Under the Curve (log AUC) and the copies of pneumococcal DNA (Spn6B) detected in the BAL fluid of carriage+ individuals. $r= 0.71$, *p=0.029 by Pearson correlation test. **B)** Duration and density of nasal colonization per individual with detected Spn6B DNA in the BAL fluid (9 in 22 Spn colonized). The end of each coloured line indicates the time point that the individual cleared colonization, assessed by classical microbiology. **C)** Positive correlation between the nasal pneumococcal density (log AUC) and sorted AMs opsonophagocytic activity (n=13). Pearson correlation test results and linear regression line with 95% confidence interval are shown. **D-F)** Representative images taken by confocal microscope showing: **D)** pneumococci around AMs and **E)** internalised pneumococci by AMs derived from Spn colonized individuals. CD14-red, nucleus-blue (DAPI) and Spn6 capsule-green. Scale bar= 2 μ m. **F)** 3D reconstruction of deconvolved Z-stack confocal images of

alveolar macrophages. The samples were stained with either wheat germ agglutinin (on the left; magenta) or anti-CD169 monoclonal antibody (on the right; red). Spn6 in green and nuclei in blue (DAPI). Video images of the 3D reconstructions are available as a supplementary file. A scale bar is shown on the images.

Figure 3: AM cross-talk and priming by autologous CD4+ T subsets. A) Comparison of phagocytic activity between sorted AM and sorted AM plus autologous BAL isolated CD4+ T cells from both carriage- (n=11) and carriage+ (n= 13). MOI=1:20. AM and CD4+ T cells were used in a 10:1 ratio. ****p< 0.0001 in both groups by paired t-test. Comparison of AM basal opsonophagocytic activity between the two groups. *p= 0.018 by unpaired t-test with Welch's correction. Comparison of AM opsonophagocytic activity in the presence of lung CD4+ T cells between carriage- and carriage+ group. **p= 0.001 by unpaired t-test with Welch's corrections. Boxplots and individual subjects are depicted with Carriage- in black Carriage+ in red, with paired samples connected by dashed line. **B)** Intracellular staining of CD4+ T cells for T-bet, GATA-3 and FoxP3 transcription factors, expressed as percentage of CD3+CD4+ BAL lymphocytes. **p= 0.003, p= 0.85, p= 0.33 respectively by unpaired t-test with Welch correction test. Boxplots and individual subjects are depicted with carriage- in black dots and carriage+ in red dots.

Figure 4: Correlations of AM opsonophagocytic activity with CD4+ Th1 and Th17 responses. A) From left to right are illustrated significant correlations between the levels of IFN- γ expressing CD4+ T cells at baseline (non-stimulated), total IFN- γ expressing CD4+ T cells post stimulation with Heat Inactivated (HI) Spn6B and the Spn-specific responding CD4+ T cells (non-stimulated condition subtracted from Spn-stimulated condition) with alveolar macrophage OPA. Spearman Rho and p values are shown. **B)** Significant correlation of Spn-specific, TNF- α expressing CD4+ T cells with AM OPA. Spearman Rho and p value are shown. **C)** From left to right are illustrated the levels of IL-17A expressing CD4+ T cells at baseline

and the levels of total and Spn-specific, IL-17A expressing CD4+ T cells in association with AM OPA. No significant correlations. Spearman correlation test results and linear regression line with 95% confidence interval (purple shading) interval and are shown.

Figure 5: Lung cytokine milieu, alterations post nasal colonization and the effect of IFN- γ on AM opsonophagocytic function. **A)** Heatmap of the 30 cytokines levels, expressed as log₁₀ median (pg/mL), measured in the BAL fluid (carriage-; n=20 and carriage+; n=22). **B)** Levels of significantly different cytokines between the two groups, expressed as pg/ml. GM-CSF, IFN- γ and IFN- α with *p= 0.032, *p=0.047 and *p=0.043 respectively, analysed by Mann-Whitney test. Boxplots and individual subjects are depicted with carriage- in black dots and carriage+ in red dots. **C)** The effect of 10-fold increasing doses of exogenous IFN- γ (2-2000ng/ml) on the capacity of AM to uptake pneumococcus (live Spn6B used, MOI= 1:100). AM isolated from 6 non-challenged subjects. Individuals samples are depicted and connected by dashed lines. ** p< 0.01 by Friedman test followed by Dunn's multiple comparison. **D)** TNF- α production from AMs, pre-treated or not with exogenous IFN- γ (2-2000ng/ml), following stimulation with HI-Spn6B. AM isolated from 4 non-challenged subjects. Individuals samples are depicted and connected by dashed lines. *p< 0.05, ** p< 0.01 by Friedman test followed by Dunn's multiple comparison.

Figure 6: Pneumococcal colonization may promote monocyte-to-macrophage differentiation. **A)** Levels of monocytes and AMs in the BAL of carriage- (n=8) and carriage+ (n=9), expressed as percentage of CD45+ cells. Significant comparison of AM levels and AM: Monocytes ratio between the two study groups, * p=0.046 by Mann-Whitney test. Boxplots and individual subjects are depicted with Carriage- in black and Carriage+ in red. **B)** Monocytes and neutrophils analysed based on their CD14, CD16 expression. In monocytes, CD16 expressional levels divided them to two subsets, CD14hiCD16lo and CD14hiCD16hi. Boxplots and individual subjects are depicted with carriage- in black and carriage+ in red. **C)**

Top pathways after gene set enrichment analysis for pathways and function applied on 2logFC (n= 5 subjects per group). NES presented in gradient colour. Red shades indicate pathways over-presented, whereas blue shades pathways under-presented in the carriage positive group. 100% Significance scored the pathways with **p <0.001, 60% pathways with *p<0.05 and 20% pathways with p>0.05. **D)** Correlations between alveolar macrophage OPA and 2log CPM of TBX21, NT5E, TLR8 and CEACAM6. Spearman correlation test results and linear regression line with 95% confidence interval (purple shading) interval and are shown.

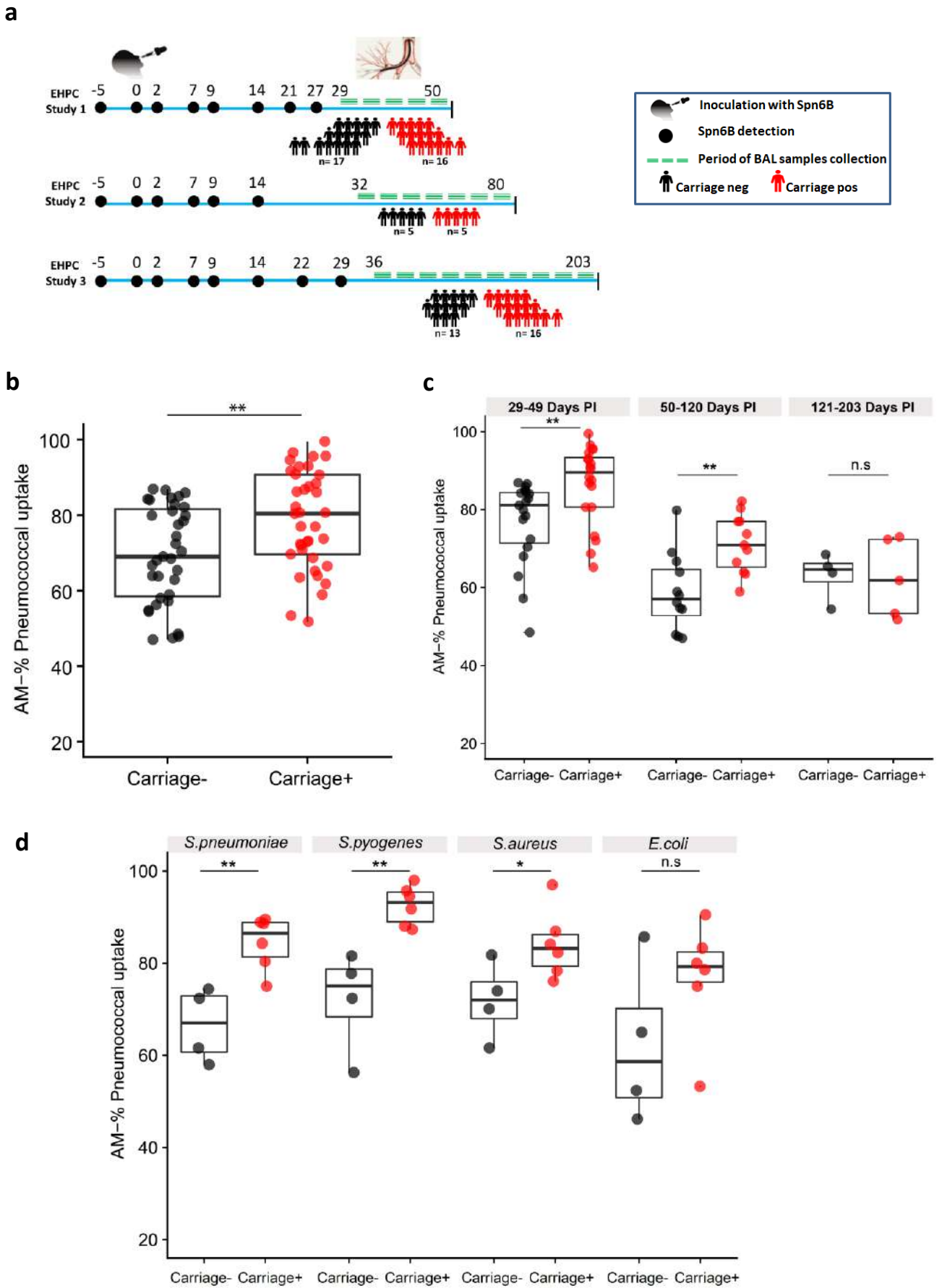


Figure 1

SUPPLEMENTARY MATERIALS

Material and Methods

Experimental human pneumococcal challenge (EHPC)

Experimental human pneumococcal challenge was conducted in Liverpool as previously described (1, 2). Briefly, mid-log-growth vegetative culture of *Streptococcus pneumoniae* serotype 6B (strain BHN418) was prepared and stored at -80°C, and independently tested by Public Health England for purity and antibiotic sensitivity. 80,000 colony-forming-units (CFU) were instilled into each nostril of participants. Pneumococcal colonization was detected by classical microbiology methods and individuals were defined as Spn colonised (carriage+) if any nasal wash culture following experimental challenge grew *S. pneumoniae* serotype 6B.

Bronchoalveolar lavage processing

Bronchoalveolar lavages (BAL) samples were processed as previously described (3, 4). Briefly, the BAL fluid was filtered using sterile gauze and centrifuged at 400g for 10 min at 4°C. The supernatant was removed, the cell pellet was resuspended and washed with PBS. The centrifugation step was repeated once, and the cell pellet was resuspended in cold RPMI medium (Gibco™ RPMI 1640 Medium) containing antibiotics (Penicillin, Neomycin and Streptomycin, Sigma-Aldrich, Sigma Chemical Co) (hereafter referred to as complete RPMI). Cell counts in each BAL sample were performed using a haemocytometer.

Alveolar macrophages isolation

AMs were routinely separated from other cell populations by seeding and adherence on 24-well plate (Greiner Bio-One, Kremsmünster, Austria), as previously described (5). After 4h adherence step, the non-adherent fraction was removed, and the AMs were washed with complete RPMI, following overnight incubation at 37°C with 5% CO₂. In the experiments that highly pure AM population was requested, AMs were purified from the whole BAL sample

through cell sorting (FACS ARIAIII), following seeding on 96-well plate and overnight incubation at 37°C, 5% CO₂.

Alveolar macrophage opsonophagocytic killing (OPA)

AMs opsonophagocytic capacity was evaluated as previously described with minor modifications (6). Briefly, live *S. pneumoniae* serotype 6B (inoculation strain) or *S. pyogenes* or *S. aureus* or *E. coli* were opsonized in a 1:16 final dilution of human intravenous immunoglobulin (IVIG, Gamunex, Grifols Inc, Spain) in HBSS +/- (with Ca²⁺ Mg²⁺) at 37°C for 15min. AMs were washed twice with RPMI without antibiotics, and incubated with an opsonised bacterial strain in Opsonisation Buffer B (HBSS +/- plus 1% gelatine solution and 5% FBS) and baby rabbit complement (Mast Group) at 37°C on a shaking rotor for 60min. Multiplicity of infection (MOI) used was 1 :100 for all the gram-positive bacteria. Opsonophagocytic killing assay for the gram-negative (*E. coli*) was modified as described elsewhere (MOI= 1:20 for 30min) (7). In the assays where isolated by cell sorting AMs were infected with opsonised Spn6B, the MOI was modified to 1:20 due to increased loss of cells during the high-throughput cell sorting. In some experiments AMs were stimulated with 2ng/ml, 20ng/ml, 200ng/ml and 2,000ng/ml of recombinant IFN- γ (Bio-technie).

Bacterial DNA extraction and quantification of pneumococcal DNA in BAL samples

Extraction of bacterial DNA from the BAL samples was performed as previously described with minor modifications (8). Briefly, 15mls of BAL sample was centrifuged at 1000g for 15min. Following centrifugation, the supernatant was discarded, and DNA was extracted from the pellet using the Agowa kit for bacterial DNA extraction. The extracted DNA was eluted in a volume of 63ul of elution buffer. DNA purity and quality were assessed by a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific).

Presence of pneumococcal DNA in BAL samples was determined using primers and probe specifically designed for 6B serotype, targeting on a capsular polysaccharide gene known as wciP, the rhamnosyl transferase gene. The primers and probe sequences were: forward

primer 5'- GCTAGAGATGGTTCCTTCAGTTGAT- 3'; reverse primer 5'- CATACTCTAGTGCAAACCTTTGCAAAT- 3' and probe 5'- [FAM] ACT GTC TCA TGA TAA TT [MGBEQ] -3' as previously published (9). Primers and probe used in their optimised concentrations, 900nM primers and 200nM TaqMan MGB probe per reaction. A non-template control and a negative control per DNA extraction, were included in every run. DNA was amplified with the real-time PCR System (Agilent Technologies, Statagene Mx3005P) by using the following cycling parameters: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A standard curve of a 10-fold dilution series of genomic DNA extracted from Spn6B was used. The genomic DNA was extracted using the Qiagen Genomic-tip 20/G Kit (Qiagen) and quantified by nanodrop. The conversion from weight pneumococcal DNA to number of DNA copies *S. pneumoniae* was based on the weight of one genome copy TIGR4 calculated by the genome length in base pairs times the weight of a DNA base pair (650 Dalton). The lower limit of detection (LLOD) of the method was set at 40 cycles. Amplification values > 40. A sample was considered positive if at least two of three yielded a positive result within the < 40-cycle cut-off. Data was analysing using MxPro software.

Flow cytometry assays

AM and monocyte immunophenotyping: Myeloid lineage cells were immunophenotyped using monoclonal antibodies for key surface markers. In brief, whole BAL cells (1×10^6 cells) were stained with Aqua Viability dye (LIVE/DEAD® Fixable Dead Cell Stain kit, Invitrogen, UK), anti-CD45 FITC, anti-CD80 APC-H7, anti-CD86 PE, anti-CD206 PE-CF594, anti-CD14 PerCP Cy5.5, anti-CD16 PE Cy7, anti-CD163 APC, anti-CD11b AF700, anti-CD11C PB, anti-CD64 BV605 and anti-HLADR BV785. All the samples were acquired on a FACS ARIA III sorter/cytometer (BD Biosciences) and analyzed using Flowjo version 10 (Treestar). BAL samples with macroscopically visual red blood cell contamination were excluded from the analysis.

AM stimulation with HI-Spn6B and IFN- γ : 1 million of BAL cells per condition, resuspended in complete RPMI, were added in 24-well plate and incubated overnight at 37°C, 5% CO₂. Non-adherent cells were removed, and AMs were washed 3x with pre-warmed plain RPMI, following stimulation with 10x increased concentration of IFN- γ (2ng/ml, 20ng/ml, 200ng/ml and 2,000ng/ml) for 30min. Post the cytokines stimulation, cells received 5 μ g/ml of heat-inactivated (HI) Spn6B and were incubated for 2 hours. Non-cytokine/non-Spn treated and non-cytokine/Spn treated controls were included per volunteer. Cytokines were retained within the cells by the addition of GolgiPlug (BD Biosciences) and stimulation for 2 more hours. Post incubation time, AMs were washed with PBS and detached from the wells by adding of 2.5mM EDTA solution. Cells were collected in FACS tubes and pelleted (400g for 10 min centrifugation), following staining for human AM surface markers - anti-CD14 PerCpP5.5, anti-CD169-PE, CD206 PE-CF594 and CD45 Pacific Orange and anti-TNF- α BV605 (BD Biosciences).

Transcription factors analysis: 1 million of BAL cells were washed with 3 mL of PBS and stained with Aqua Viability dye (LIVE/DEAD Fixable Dead Cell Stain kit, Invitrogen, UK) and the surface markers CD3-APC.cy7, CD4-PerCP5.5, CD8-AF700, CD69-BV650, anti-CD25-PE-TexasRed and CD45-BV711 (Biolegend, San Diego, CA). For permeabilization and fixation, Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, San Diego, CA) was used as per the manufacturer's instructions, following intracellular staining with T-bet-APC, Gata-3-PE and Foxp3-FITC. All samples were acquired on a LSRII flow cytometer (BD Biosciences).

INF- γ , TNF- α and IL-17 producing CD4⁺ T cells post stimulation with HI-Spn6B: Cells were harvested, stained and analysed as previously described, with minor modifications(6, 10). In brief, non-adherent cells were collected from the BAL samples post an adherence step, centrifuged at 400g for 5min, resuspended in complete RPMI and seeded in 96-well plates at equal concentrations of 600,000 to 1 million cells per condition. Cells were stimulated with 5 μ g/ml of HI-Spn6B and incubated for 2 hours at 37°C, following addition of GolgiPlug (BD Biosciences) and overnight incubation at 37°C, 5% CO₂. A non-stimulated with Spn6B (mock)

cell condition was included per volunteer. After 16 hours, the cells were washed with PBS and stained with Violet Viability dye (LIVE/DEAD Fixable Dead Cell Stain kit, Invitrogen, UK) and anti-CD3-APCH7, TCR- $\gamma\delta$ -PECy7 (BD Biosciences, USA), anti-CD4-PerCP5.5, anti-CD8-AF700, anti-CD69-BV650, anti-CD25-PE.TxsRed, anti-CD103-BV605, anti-CD49a-APC (Biolegend, San Diego, CA). For the assessment of intracellular cytokine production, after permeabilization and fixation, the cells were stained with the following markers: anti-IFN- γ -PE, anti-IL17A-BV510 and TNF- α -BV711 (BD Biosciences). All samples were acquired on a LSRII flow cytometer (BD Biosciences).

AMs gene analysis using Nanostring platform

Nanostring was used as previously described (11). Briefly, AMs were sorted by FACS ARIALL cell sorter and stored in RLT buffer (Qiagen) with 1% 2-mercaptoethanol (Sigma) at -80C until RNA extraction. Extraction was performed using the RNEasy micro kit (Qiagen) with on column DNA digestion. Extracted RNA was quantified by qPCR targeting B2M gene (Bioanalyzer, Agilent). The single cell immunology v2 kit (Nanostring) was used with 20 pre-amp cycles for all samples. Hybridized samples were prepared on a Prep Station and scanned on a nCounter® MAX (Nanostring). Raw counts were analysed using the R/Bioconductor package DESeq2 for internal normalization, which gave lower variance than normalizing to included housekeeping genes. DEG were identified using a model matrix correcting for repeated individual measurements. Log CPM from raw counts were calculated using the 'edgeR' package. 2logFold chances were further analysed by the 'fgsea' package, through BMT pathways gene set enrichment analysis.

Results

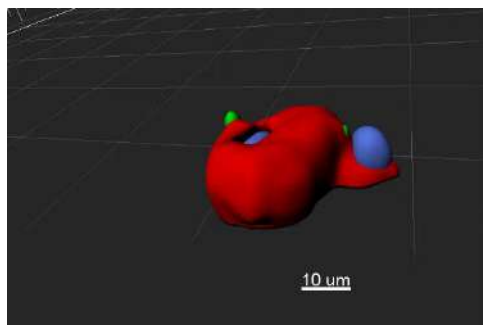
Methods of spn6B DNA detection in BAL and NP	Carriage pos.	Carriage neg.
Spn6B detected in the BAL by qPCR	9/22 (41%)	0/21 (0%)

Live Spn6B detected in BAL by culturing	2/16 (12.5%)	0/10 (0%)
Live Spn6B detected in NP swabs by culturing	0/12 (0%)	0/10 (0%)

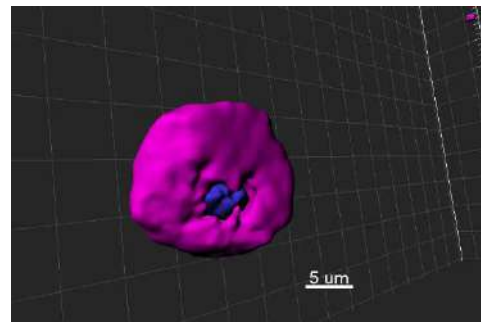
Table S1. Methods of Spn6B detection in lung and nose the day of research bronchoscopy.

Spn6B DNA was detected in 41% of carriage positive volunteers (9 in 22 carriers) by qPCR targeting a Spn6B specific capsular polysaccharide gene. Spn6B DNA was not detected in any (0/21) non-colonised subjects. BAL samples were plated on blood agar plate and pneumococcal growth was observed in 12.5% (2/16) *Spn*-colonised volunteers, whereas there was no growth for any the carriage negatives (0/10). Nasopharyngeal (NP) swabs were taken prior to the bronchoscopy from 22 participants. No live SPN6B was detected in any NP sample after culturing.

a



b



Supplementary video 1: Spn6 pneumococci internalised by alveolar macrophages.

Video images of 3D reconstruction of deconvolved Z-stack confocal images of human alveolar macrophages. The samples were stained with anti-CD169 monoclonal antibody (panel A; red) or wheat germ agglutinin (panel B; magenta). Spn6 polysaccharide capsule was stained in green and nuclei are shown in blue (DAPI).

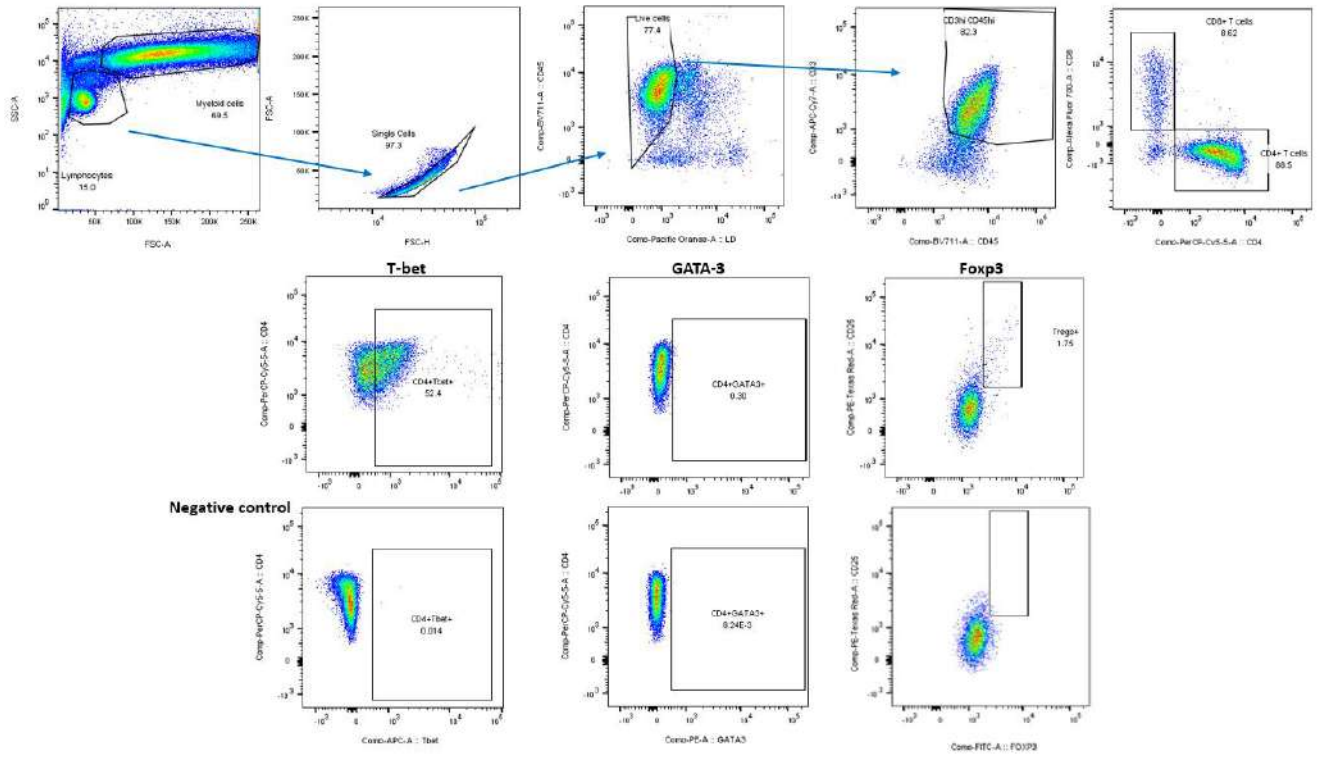


Figure S1: Gating strategy of C3+CD4+ T cells for transcriptions factors – T-bet, GATA-3 and FoxP3 - expression for one representative volunteer.

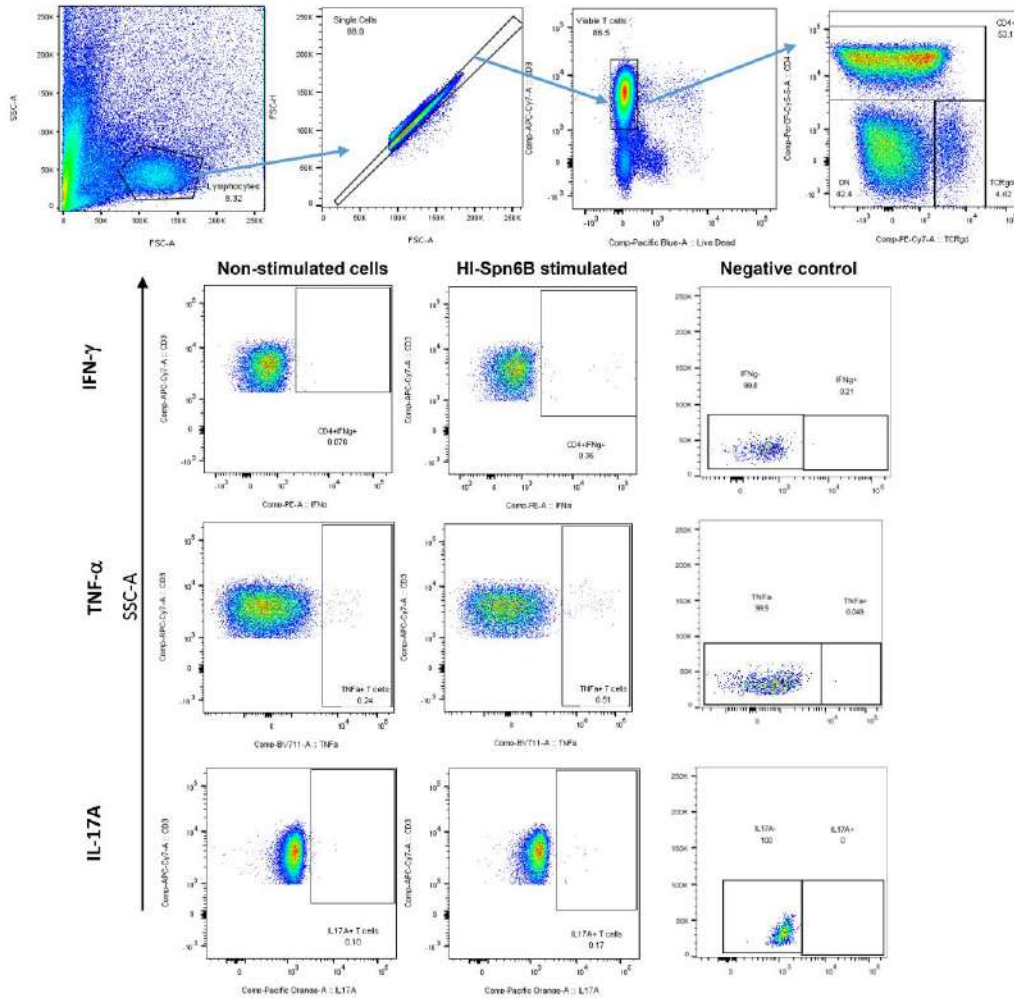


Figure S2: Gating strategy of cytokine (INF- γ , TNF- α and IL-17A) producing T cells following overnight stimulation with HI-Spn6B or not. Gates from one representative volunteer are shown.

Cytokine	Median concentration Carriage neg.	Median concentration Carriage pos.	p-value	adjusted p-value
IL2	0.17	0.17	9.36E-01	9.36E-01
IL17	0.30	0.30	1.42E-01	6.34E-01
TNF α	0.42	0.42	7.41E-01	9.31E-01
FGF Basic	0.65	0.65	1.83E-01	6.34E-01
GM-CSF	0.49	0.96	3.41E-02	4.84E-01
EGF	1.01	1.01	6.19E-01	9.12E-01
IL 10	0.92	1.11	4.43E-01	8.31E-01
IL 1 β	1.09	1.09	2.90E-01	7.25E-01

IL 4	1.25	1.24	6.17E-01	9.12E-01
Eotaxin	1.19	1.33	9.14E-01	9.36E-01
RANTES	1.79	1.79	9.03E-01	9.36E-01
IL 5	2.30	2.78	3.61E-01	8.31E-01
IFN- γ	1.89	3.20	4.84E-02	4.84E-01
IFN α	2.96	3.61	4.43E-02	4.84E-01
MIG	2.07	7.37	1.11E-01	6.34E-01
IL 13	4.87	6.03	1.00E-01	6.34E-01
IL 12	8.13	7.07	8.84E-01	9.36E-01
MIP 1 α	9.35	9.57	4.29E-01	8.31E-01
MIP 1 β	11.00	11.42	5.03E-01	8.87E-01
IL 15	16.56	11.29	2.32E-01	6.34E-01
IL 6	13.49	16.22	2.03E-01	6.34E-01
IL 2R	17.62	22.65	4.06E-01	8.31E-01
IL 7	17.58	25.34	1.77E-01	6.34E-01
IP 10	23.07	29.58	2.12E-01	6.34E-01
G-CSF	33.03	43.81	7.33E-01	9.31E-01
MCP 1	51.34	48.13	8.95E-01	9.36E-01
VEGF	56.40	61.40	6.38E-01	9.12E-01
HGF	79.20	73.13	8.00E-01	9.36E-01
IL 8	61.92	90.55	5.38E-01	8.97E-01
IL 1RA	959.97	775.27	7.45E-01	9.31E-01

Table S2. Levels of 30 cytokines and chemokines measured in the BAL fluid of carriage negative (n=20) and carriage positive (n=22) volunteers, who underwent research bronchoscopy up to 50 days post the pneumococcal inoculation. Levels are expressed as pg/ml and are ordered from low to high values. Median per group, p-values by Mann-Whitney test and p-values corrected by multiple-comparison testing (Benjamini-Hochberg) are displayed.

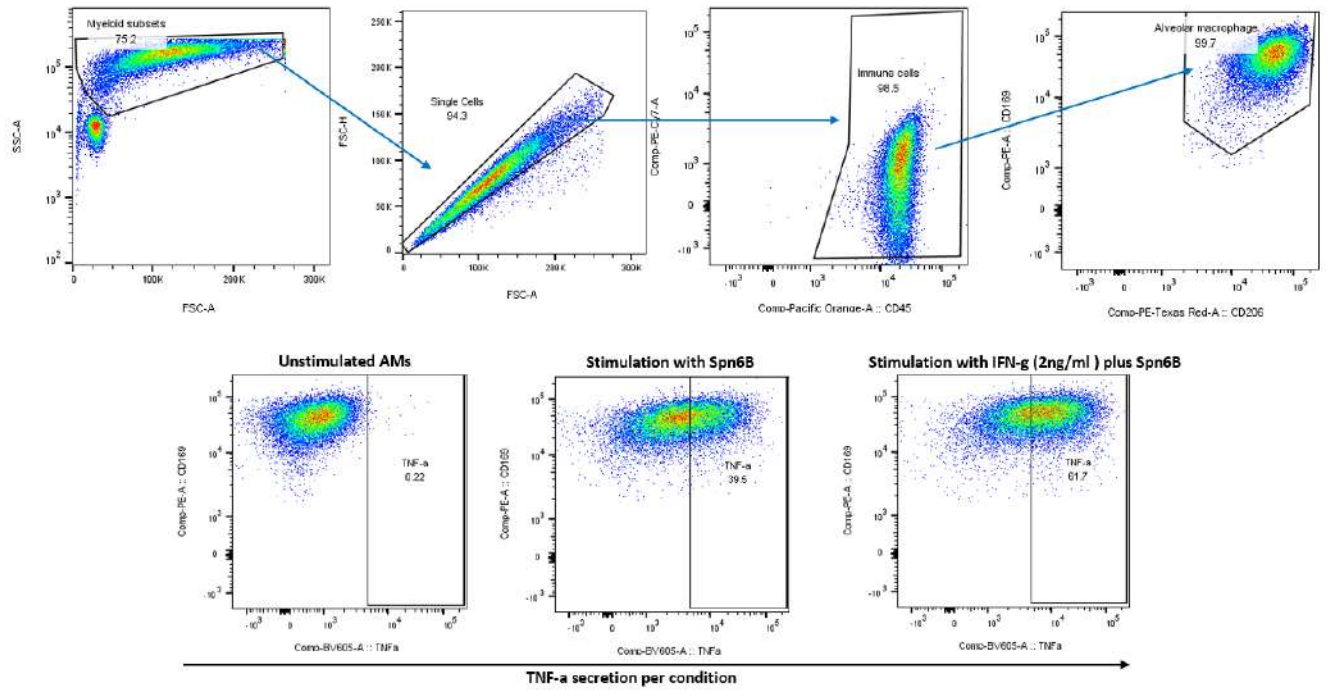


Figure S3: Intracellular cytokine staining gating strategy of TNF- α production. AMs post treatment with IFN- γ and stimulation with HI-Spn6B. Gates from one representative volunteer are shown.

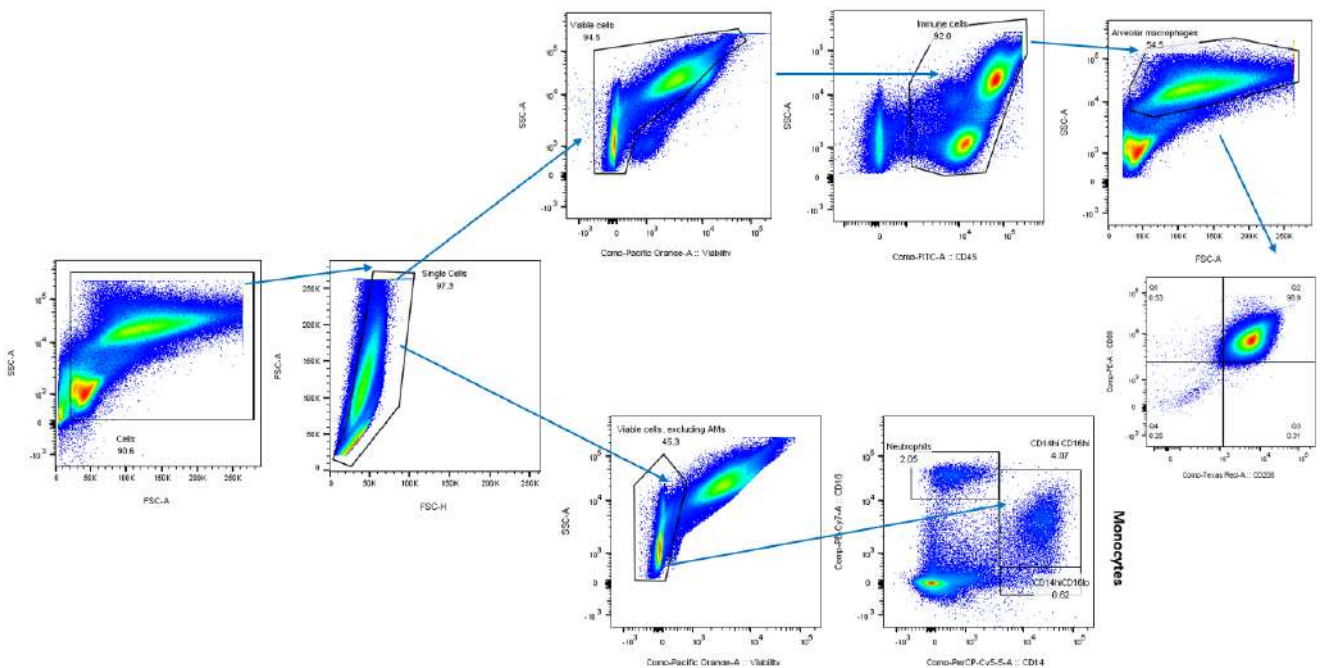


Figure S4: Gating strategy of monocyte analysis for one representative volunteer.

Gene	log2FoldChange	p-value	adjusted p-value
<i>C1QA</i>	-1.73	1.17E-03	4.25E-01
<i>CD14</i>	-1.86	3.95E-03	5.00E-01
<i>CSF1R</i>	-1.84	7.82E-03	5.00E-01
<i>IRF4</i>	1.77	8.43E-03	5.00E-01
<i>C1QB</i>	-1.66	9.23E-03	5.00E-01
<i>CXCL11</i>	1.96	1.05E-02	5.00E-01
<i>GPI</i>	-1.27	1.13E-02	5.00E-01
<i>NT5E</i>	1.38	1.81E-02	5.00E-01
<i>CCND3</i>	-1.45	1.83E-02	5.00E-01
<i>CLEC7A</i>	2.37	2.10E-02	5.00E-01
<i>CEACAM6</i>	1.29	2.16E-02	5.00E-01
<i>LY96</i>	2.19	2.24E-02	5.00E-01
<i>TAPBP</i>	-1.34	2.41E-02	5.00E-01
<i>TNFSF4</i>	1.32	2.61E-02	5.00E-01
<i>HLA-DRB3</i>	-3.36	2.82E-02	5.00E-01
<i>ITGAX</i>	-1.83	2.85E-02	5.00E-01
<i>IL13</i>	1.18	2.98E-02	5.00E-01
<i>FCGRT</i>	-1.32	3.18E-02	5.00E-01
<i>CMKLR1</i>	-1.55	3.28E-02	5.00E-01
<i>TNFSF13B</i>	1.78	3.37E-02	5.00E-01
<i>CD164</i>	2.01	3.48E-02	5.00E-01
<i>S100A8</i>	-1.46	3.52E-02	5.00E-01
<i>CXCL2</i>	2.08	3.62E-02	5.00E-01
<i>PYCARD</i>	-1.02	3.62E-02	5.00E-01
<i>TBX21</i>	1.42	3.67E-02	5.00E-01
<i>TAGAP</i>	1.08	3.72E-02	5.00E-01
<i>KLRC4</i>	1.24	3.78E-02	5.00E-01
<i>CCRL1</i>	1.27	3.85E-02	5.00E-01
<i>GAPDH</i>	-1.42	4.05E-02	5.08E-01
<i>IL10RA</i>	-1.14	4.48E-02	5.43E-01
<i>TLR8</i>	1.56	4.72E-02	5.45E-01
<i>KIR3DL2</i>	1.25	4.84E-02	5.45E-01
<i>ITGB2</i>	-1.41	4.94E-02	5.45E-01

Table S3. List of differentially expressed genes (DEG with $p < 0.05$) in sorted AMs on the day of the bronchoscopy (36 to 115 days post intranasal inoculation), compared *Spn* colonised (n=5) to non-colonised (n=5) individuals. Log2fold change (carriage positive over carriage negative), p-values by Mann-Whitney test and corrected p-values by using Benjamini-Hochberg procedure are displayed.

Gene	p value	Rho
<i>KLRD1</i>	0.007	0.818
<i>SLAMF1</i>	0.008	0.806
<i>IL13RA1</i>	0.011	0.760
<i>CCL15</i>	0.016	0.758
<i>KIR3DL1</i>	0.018	0.745
<i>KLRAP1</i>	0.018	0.745
<i>IL16</i>	0.021	0.733
<i>PRDM1</i>	0.024	0.721
<i>CCR10</i>	0.028	0.709
<i>LAG3</i>	0.028	0.709
<i>TRAF4</i>	0.028	0.709
<i>IRF8</i>	0.030	0.681
<i>EDNRB</i>	0.035	0.669
<i>KLRK1</i>	0.035	0.669
<i>IL6R</i>	0.035	0.685
<i>NT5E</i>	0.035	0.685
<i>ZAP70</i>	0.035	0.685
<i>DPP4</i>	0.039	0.657
<i>CD7</i>	0.039	0.673
<i>CEACAM6</i>	0.039	0.673
<i>FCER1A</i>	0.039	0.673
<i>LILRA4</i>	0.039	0.673
<i>IL12A</i>	0.042	0.650
<i>BCL2</i>	0.044	0.661
<i>MASP2</i>	0.044	0.661
<i>TBX21</i>	0.044	0.661
<i>TNFRSF9</i>	0.044	0.661
<i>HLA.DOB</i>	0.049	0.648
<i>IRF5</i>	0.049	0.648
<i>LILRA3</i>	0.049	0.648
<i>LILRA5</i>	0.049	0.648
<i>SELL</i>	0.049	0.648

<i>TLR8</i>	0.049	0.648
<i>TNFRSF14</i>	0.049	0.648

Table S4. List of genes for which expression significantly positively correlates with AM opsonophagocytic activity.

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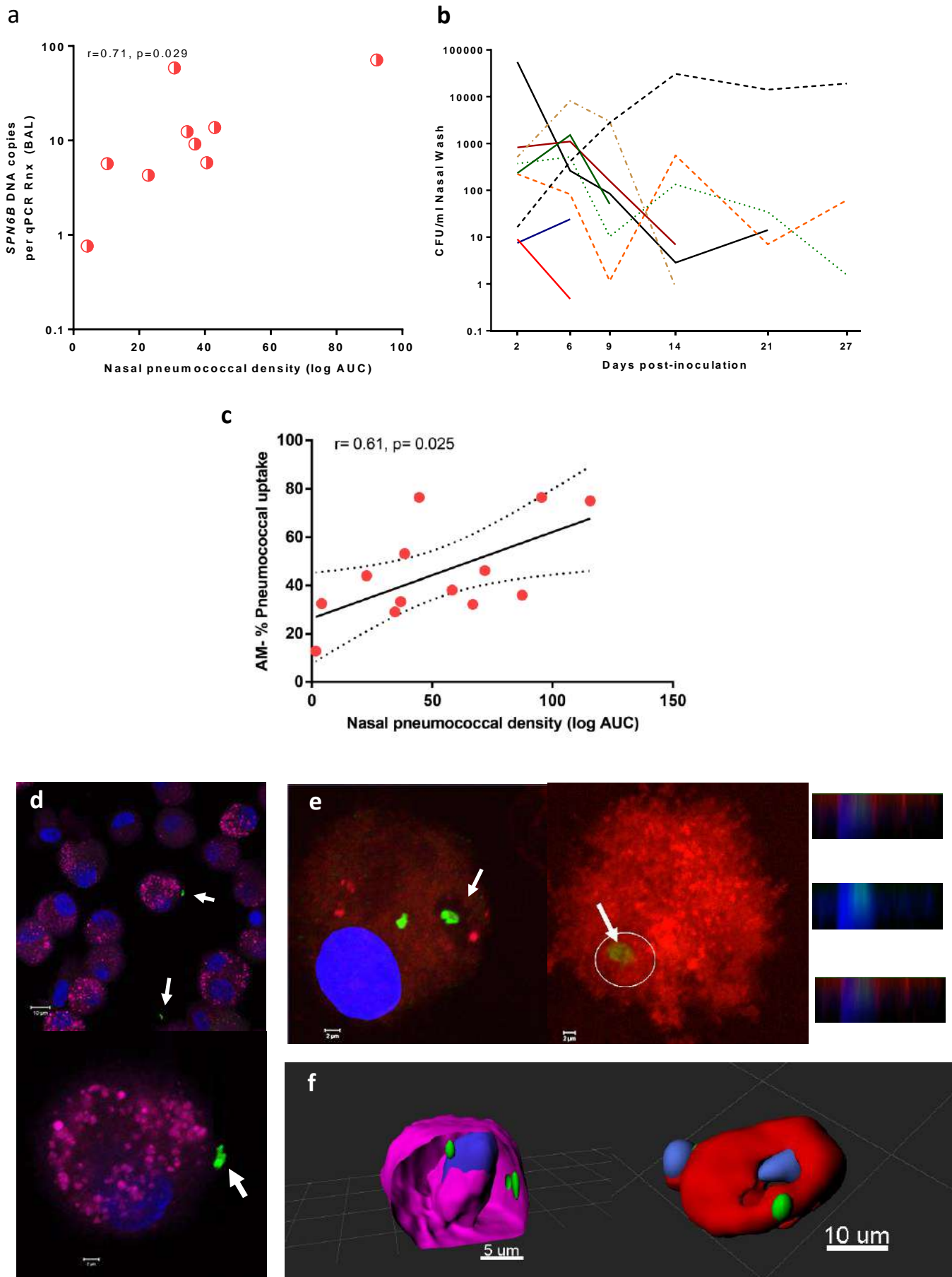


Figure 2

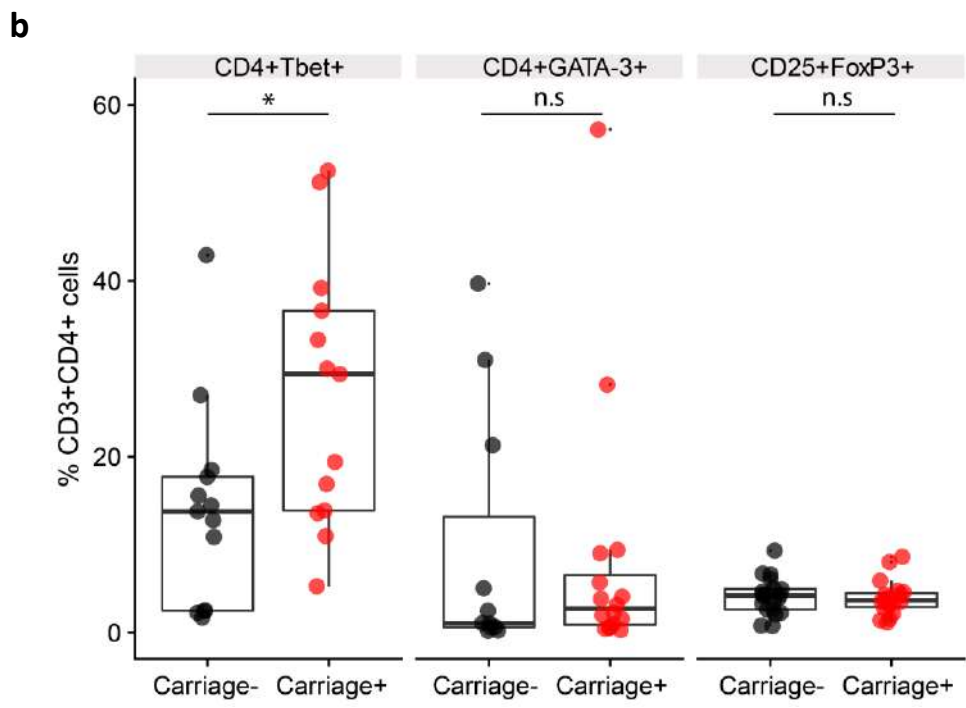
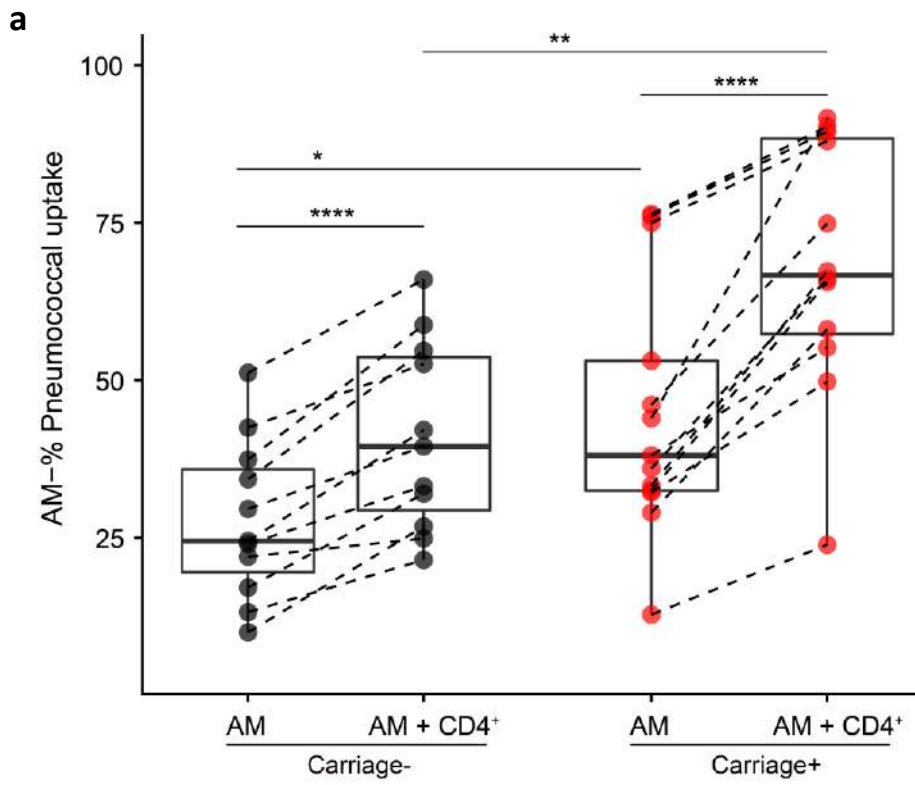


Figure 3

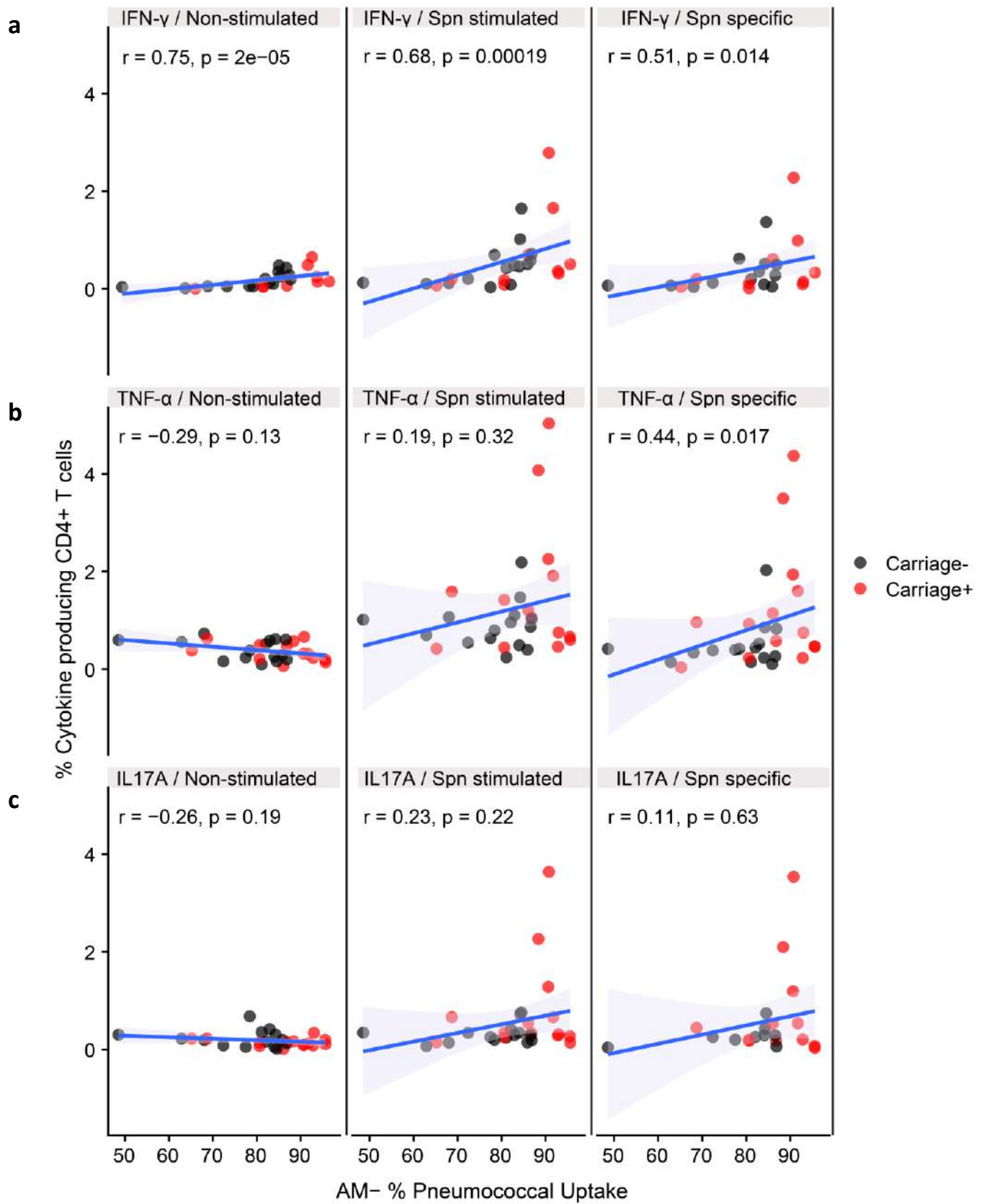


Figure 4

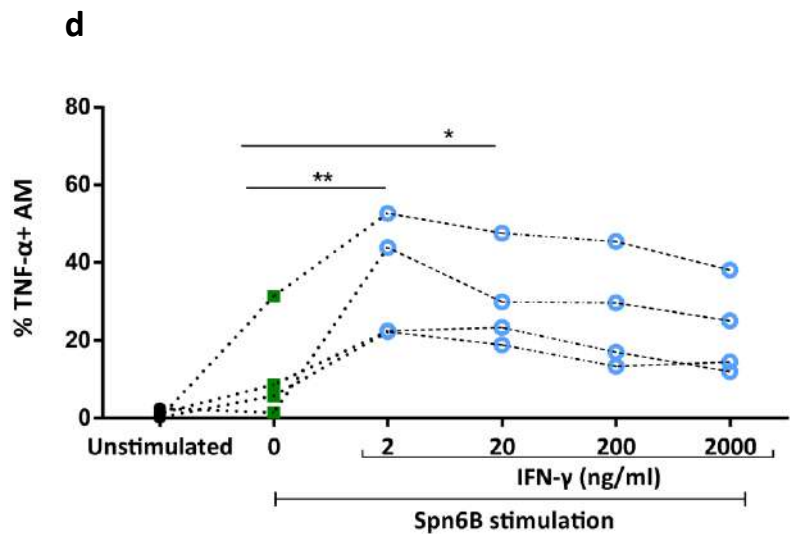
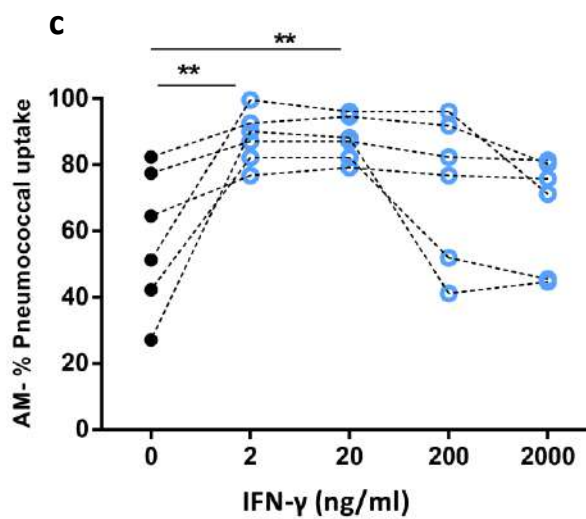
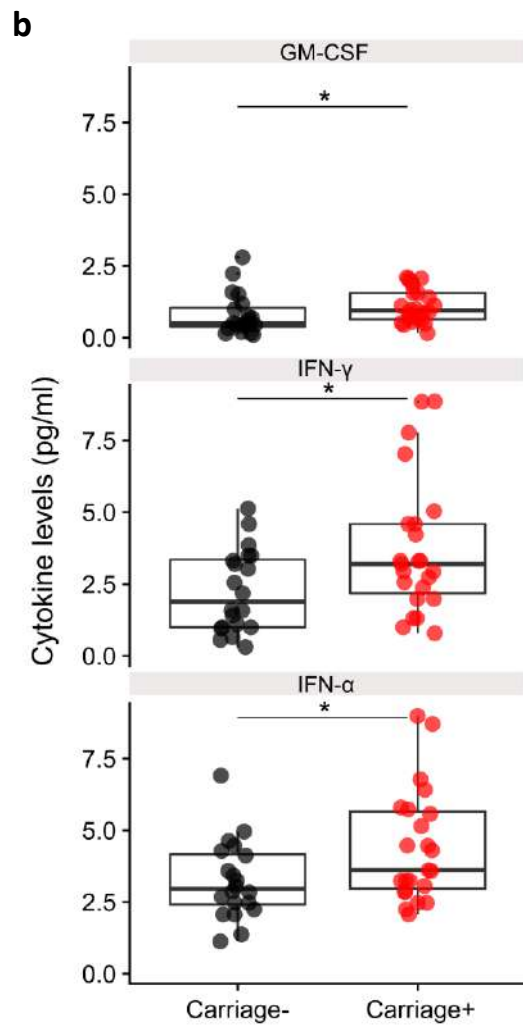


Figure 5

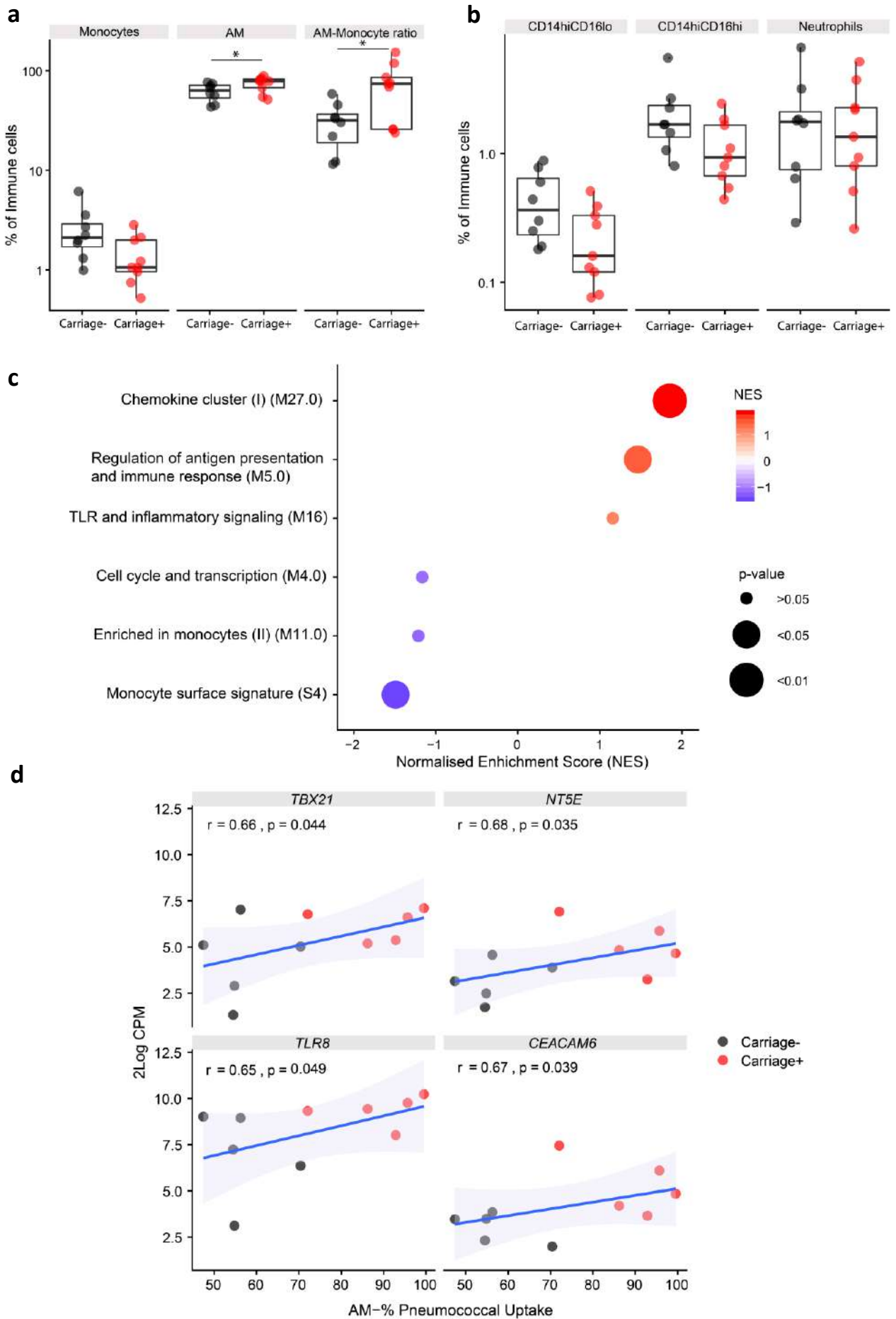


Figure 6