Th17 responses to pneumococcus in blood and adenoidal cells in children

Oliver, E.; Pope, C.; Clarke, E.; Langton Hewer, C.; Ogunniyi, A. D.; Paton, J. C.; Mitchell, Tim; Malley, R.; Finn, A.

DOI:
10.1111/cei.13225

Full title

Th17 responses to pneumococcus in blood and adenoidal cells in children

Short Title

Th17 responses to pneumococcus in children

Authors

Elizabeth Oliver[1]

Caroline Pope[1]

Edward Clarke[2]

Chris Wright (to confirm)

Claire Langton Hewer[3]

A. David Ogunniyi[4]

Tim Mitchell[5]

Richard Malley[6]

Adam Finn[1]

[1] School of Cellular and Molecular Medicine, Biomedical Sciences Building, University of Bristol, Bristol, UK.

[2] Vaccines and Immunity Theme, MRC Unit The Gambia, Atlantic Road, Fajara, Gambia.

[3] Bristol Royal Hospital for Children, Bristol, BS2 8AE, UK.

[4] Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Adelaide, Australia.

[5] Institute of Microbiology and Infection, University of Birmingham, Birmingham, UK.

[6] Division of Infectious Diseases, Department of Medicine, Children's Hospital and Harvard Medical School, Boston, Massachusetts, USA.

Corresponding author

Elizabeth Oliver

School of Cellular and Molecular Medicine,

Biomedical Sciences Building,

University of Bristol,

University Walk,

Bristol,

BS8 1TD

Key words

Streptococcus pneumoniae

Th17 cells

Mucosal immunity

Vaccine

Children

List of abbreviations

PCV Pneumococcal conjugate vaccines

IL-17A Interleukin-17A

IL-22 Interleukin-22

WCA Whole Cell Antigen

CbpA Choline binding protein A

PsaA Pneumococcal surface adhesin A

PspA Pneumococcal surface protein A

PhtD Pneumococcal histidine triad protein D

AMNC Adenoidal mononuclear cells

PBMC Peripheral blood mononuclear cells

1. **Summary**

Pneumococcal infections cause a large global health burden and the search for serotype independent vaccines continues. Existing conjugate vaccines reduce nasopharyngeal colonisation with target serotypes. Such mucosal effects of novel antigens may be likewise important. CD4+ Th17 cell dependent antibody-independent reductions in colonisation and enhanced clearance has been described in mice. We here describe methods to evaluate Th17 cytokine responses to potential pneumococcal vaccine candidate antigens in a human cell culture system, using adenoidal and peripheral blood Ficoll-density gradient separated mononuclear cells. Optimal detection of IL-17A was at day 7 and of IL-22 at day 11 in these primary cell cultures, and MACS removal of CD45RO+ cells abolished these responses. Age-associated increases in magnitude of responses were only evident for IL-17A in adenoidal cells. There was strong evidence of correlation between individual IL-17A and IL-22 responses after pneumococcal antigen stimulation ($p<0.015$). Intra-cellular cytokine staining following PMA/Ionomycin stimulation demonstrated that >30% CD4 T cells positive for IL-22 express the innate markers γδT-cell receptor and/or CD56 with much lower proportions for IL-17A+ cells ($p<0.001$). Natural acquired responses to several vaccine candidate antigens were observed and notable for consistent absence, particularly in blood, to PhtD ($p<0.0001$), recently shown to lack impact on colonisation in a clinical trial of a PhtD-containing conjugate vaccine in infants. This approach has the potential to assist

vaccine antigen design aimed at reducing pneumococcal carriage and transmission.

2. Introduction

Streptococcus pneumoniae (pneumococcus) remains a global challenge and cause of much disease including pneumonia, meningitis, sepsis and otitis media and is a particular challenge in developing countries. Pneumococcus accounts for 11% of all deaths in children under 5 years of age resulting in up to a million childhood deaths every year (1, 2). Of growing global concern are the emergence of non-vaccine serotypes and antibiotic resistant strains of pneumococcus (3, 4).

Pneumococcus is a commensal of the human upper respiratory tract and there are more than 90 serotypes. Colonisation of the nasopharynx with pneumococcus is common in young children and decreases with age (5-7), it is not usually associated with significant illness whereas development of invasive pneumococcal disease is, relatively speaking, rare. Transmission between children and to other family members sustains the bacteria within a community (8). The introduction of multivalent pneumococcal conjugate vaccines (PCV) has resulted in overall decreases in the incidence of pneumococcal disease, but overall colonisation rates in children have changed little as non-vaccine

serotypes replace formerly dominant vaccine types and also now cause some disease undermining vaccine (9-11).

Thus, development of serotype-independent pneumococcal vaccines is a priority in the fight against pneumococcus. If, like PCVs, such vaccines are to impact on disease by reducing carriage and transmission, understanding naturally-acquired mucosal immune responses to pneumococcus and how they may affect pneumococcal colonisation could guide antigen selection and vaccine formulation. Antibody-independent CD4+ T cell dependent prevention of pneumococcal colonisation has been demonstrated in mice (12-14). A role for CD4+ Th17 cells ~~which~~ that can kill and clear pneumococci by recruiting neutrophils to the site of infection, has been proposed (15). Th17 cells exist in adults and children(15), and produce both Interleukin-17A (IL-17A) and Interleukin -22 (IL-22) (16, 17). The stimulation of production of IL-17A and IL-22 by candidate pneumococcal vaccine antigens could indicate their capacity to influence pneumococcal colonisation either by preventing acquisition or promoting clearance.

Here we describe optimisation of measurement and characteristics of Th17 responses in human primary cell cultures from blood and adenoidal tissue (nasal associated lymphoid tissue) of children, and preliminary use of this technique to screen potential pneumococcal vaccine candidate antigens. We show that, the case of one antigen, consistent lack of demonstrable responses

is associated with recently reported lack of efficacy in human trials against carriage.

3. Materials and methods

3.1 Subjects and samples

With informed consent, adenoids were collected from children aged 1-14 years (eligibility age range 0-15yrs), who were undergoing routine adenoidectomy or adenotonsillectomies at the Bristol Royal Hospital for Children. Up to 10ml of peripheral blood from each child were collected using 1% heparin (Sigma-Aldrich Company Ltd., UK) as the anti-coagulant in some cases. Children were healthy at the time of surgery and were excluded if they had any known immunodeficiency or if they had received treatment likely to cause immunosuppression within 2 weeks of surgery. Ethical approval was obtained from the North Somerset & South Bristol Research Ethics Committee and in some cases also from the research committee of PATH, one of the funders of this work.

3.2 Antigens

The whole-cell killed unencapsulated pneumococcal antigen (WCA) was made as described in (18) following “Good manufacturing Practice” (GMP) (19) at a concentration of $1x10^6$cfu/ml as determined in previous experiments (data not shown).

Recombinant proteins Choline binding protein A (CbpA), Pneumococcal surface adhesin A (PsaA) and Pneumococcal surface protein A (PspA) were purified from recombinant *E.coli* expressing the respective cloned genes (20, 21). Recombinant protein Pneumococcal histidine triad protein D (PhtD) was produced as previously described (22). All proteins were used at a concentration of 8µg/ml to stimulate optimal CD4+ T cell proliferation as determined in previous experiments (data not shown).

Comment [TJM1]: Methods are correct as far as I am concerned

3.3 Cell isolation and culture

Adenoidal tissue was processed within 24h of collection and collected into Hanks' Balanced Salt Solution (HBSS)/ 2% Hepes (Thermo Scientific/Life Technologies, USA and Sigma-Aldrich Company Ltd., UK) with 10% Penicillin/Streptomycin (Sigma-Aldrich Company Ltd., UK). Peripheral blood was processed within 6h of collection and was prepared by diluting it 50:50 with HBSS/2% Hepes. Mononuclear cells from adenoids and peripheral blood were separated on Ficoll-density gradients as previously described (23). Cells were cultured in 48-well culture plates at $1x10^6$ cells/ml in a 1ml volume, in either complete RPMI media with 10% Foetal bovine serum (Sigma-Aldrich Company Ltd., UK) for cytokine analysis, or in complete RPMI media/2% human serum (Sigma-Aldrich Company Ltd., UK) for intra-cellular cytokine analysis.

3.4 Cell depletions

Memory T cells (CD45RO+) were depleted from the mononuclear cell population using positive selection magnetic-activated cell sorting (MACS) according to the manufacturer's guidelines (Miltenyi Biotech, Germany). A positive control ("add back") was made by mixing the depleted cells with the positively selected cells retained on the magnet during the cell separation procedure. The purity of these cell suspensions (CD45RO- and CD45RO+) was confirmed by immunofluorescence staining (CD4-APC, CD45RO-FITC and CD45RA-PE-Cy7 (BD Biosciences, UK)) and flow cytometry (FACS Canto II (BD Biosciences, UK; analysis with FlowJo (FlowJo, LLC, USA) and then re-mixed at a 1:1 ratio.

3.5 IL-17A and IL-22 immunoassays

Cells were stimulated with or without antigen, and IL-17A was measured in the cell supernatant collected on day 7 using a Human IL-17A ELISA Ready-Set-Go kit according to the manufacturer's instructions (Affymetrix eBiosceinces,USA). IL-22 was measured from cell supernatants mostly collected at day 11 using a Human IL-22 ELISA Ready-Set-Go kit according to the manufacturer's instructions (Affymetrix eBiosciences,USA). In most cases the same cell supernatants were used for both cytokine assays. Supernatants were stored at -20^{o}C for short term and at -80^{o}C for long term storage.

3.6 Intra-cellular cytokine production

Intra-cellular cytokine staining was conducted on day 7 after cell stimulation with or without antigen. Cells were re-stimulated with antigen late on day 6 to boost their cell specific cytokine responses. On day 7 PMA 0.05µg/ml, Ionomycin 1µg/ml (Sigma-Aldrich Company Ltd., UK) and Golgistop (BD Biosciences, UK) were added for 5 hours. Cell viability staining, cell surface staining and the intracellular cytokine staining processes were carried out using a BD Cytofix/Cytoperm Fixation/Permeabilization Kit according to the manufacturer's instructions (BD Biosciences, UK). Fixable viability dye eFluor780 ~~was~~ (Affymetrix eBiosceinces,USA) was used to assess cell viability. Cell surface antibody markers CD4 Alexa Fluor700 (BD Biosciences, UK), CD56 PE-Cy7/ Brilliant Violet 510 (BD Biosciences, UK/ Biolegend, USA), TCR γδ FITC/ PE-Cy5.5 (BD Biosciences, UK/ Beckman Coulter, USA) and intracellular antibodies IL-17A PE/ Brilliant Violet 605 (Affymetrix eBiosceinces,USA/ Biolegend, USA) and IL-22 eFluor660 (Affymetrix eBiosceinces,USA) were used. The fixed and stained cells were left overnight at 4^{o}C to reduce autofluorescence before being analysed on a LSR II flow cytometer (BD Biosciences, UK), where 20,000 live cells in the lymphocyte gate were collected per stimulation. Analysis was carried out using the software program FlowJo, and only live cells in the lymphocyte gate were analysed.

3.7 Statistical analysis

Significance of differences between groups was analysed using paired student t-tests. The relationship between age and cytokines responses ~~were~~ was

compared using linear regression analysis. Pearson correlation was used to investigate correlations between individual cytokines responses. Group mean cytokine responses to the panel of antigens were compared by repeated measures one-way ANOVA. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, USA).

4. Results

Identifying the optimal time detection point for Th17 cytokines IL-17A and IL-22 in response to WCA

In time course experiments using PBMC and in AMNC stimulated with WCA, Th17 cytokine IL-17A (Fig. 1a) and IL-22 (Fig. 1b) responses, when present, were maximal at days 7 and 11 respectively and these timings were used in subsequent experiments.

Memory T cells are essential for IL-17A production in blood

In two experiments using PBMC, depletion of memory T cells (CD45RO+) almost entirely eliminated detectable IL-17A responses (Fig 2.a), which were restored by their replacement (Fig 2.b). This finding was reconfirmed in four additional children whose un-depleted PBMC IL-17A responses were median (range) 40.5 (6.3-65.6) pg/ml on day 5, and CD45RO-depleted (99.83% purity) IL-17A responses 0 (0-0.4) pg/ml (data not shown).

Relationships between cytokine responses and age

Apart from IL-17A responses in adenoidal cells for which an association was apparent, there was little evidence that age predicted the size of IL-17A and IL-22 responses to WCA in the children studied (Fig 3).

Correlation between IL-17A and IL-22 responses

There was strong positive correlation in the size of PBMC and AMNC IL-17A and IL-22 responses in individual subjects both to WCA and to CbpA stimulation (Fig 4).

Intracellular cytokine detection of IL-17A and IL-22 in response to antigen stimulation

For intracellular cytokine staining work, IL-22 signals were sought after 7 days rather than the optimal day 11 (Fig 5) as there were limited available cells. Live lymphocyte gating (based on forward and side scatter parameters) in flow cytometric staining for intracellular IL-17A and IL-22 showed strong evidence for increases of 2% or more above the background following stimulation with the recombinant pneumococcal antigen CbpA (Fig 5.a).

50% or more IL-17A+ live lymphocytes expressed the cell surface marker CD4+, with a somewhat lower percentage of IL-22+ AMNC expressing CD4 (Fig 5. b). Following stimulation with the antigen WCA there was very strong

evidence of an increase in the proportion of IL-17A+ AMNC expressing CD4 compared to cultured cells that were stimulated with only PMA/Ionomycin.

Expression of innate surface markers by CD4+ AMNC expressing IL-17A and IL-22.

There was very strong evidence that a much higher proportion of IL-22+ CD4+ AMNC expressed one, the other or both of the innate cellular markers CD56 and TCR-γδ, than IL-17A+ CD4+ AMNC both before and after antigen stimulation (Fig 6).

IL-17A and IL-22 responses to a panel of pneumococcal antigens

Cytokine release by peripheral blood and AMNC following stimulation with pneumococcal antigens including 3 additional proteins previously investigated as candidate vaccines were measured. Results showed significant variation between antigens (Fig 7), and in particular there was little apparent response to the surface protein PhtD in PBMC, while responses to the additional proteins PsaA and PspA were weak in AMNC.

5. Discussion

We defined IL-17A and IL-22 responses to pneumococcal antigens in adenoidal and peripheral blood mononuclear cells aiming to develop tools with which to evaluate Th17 cell immune responses to candidate pneumococcal vaccine

antigens. This may permit elucidation of their effects on colonisation and thus preventing transmission.

Previous Th17 experiments in mice found day 3 to be optimal to detect IL-17A responses, and IL-17A can also be detected from human pharyngeal tonsil mononuclear cells after 3 days (15). However, we have previously shown that CD4+ cell proliferation in response to pneumococcal antigens in children are optimal after 7 days of stimulation in culture (24) and likewise in the experiments presented here, 7 days of culture were optimal for IL-17A detection while 11 days was the best time for IL-22 detection (figure 1). We have previously demonstrated clearer and more reliable mucosal responses in adenoidal than tonsillar cells (25). Although these relatively slow response rises might suggest that these are not memory responses following previous exposure, depletion of CD45RO cells resulted in their almost complete disappearance (figure 2), confirming that they are anamnestic albeit not extremely rapid in concordance with previous studies of other aspects of these mucosal cellular immune responses (24).

Both rates of colonisation and of invasive disease due to pneumococcus drop rapidly with increasing age in young children (5-7), suggesting progressive rises in specific mucosal immunity either in response to exposure, or through immune maturation or both. Both we (5) and others have shown evidence of emergence of specific B cell immunity to pneumococcal antigens particularly during the

second year of life (7, 26). IL-17A responses to pneumococcal antigens in blood leukocytes are higher in adults than children and vary between children in different populations, possibly due to different levels of exposure (15, 27). In the results presented here, upward trends in Th17 responses with age were not convincingly demonstrated in blood although there was evidence of rises in adenoidal cellular elaboration of IL-17A in response to pneumococcal stimulation (figure 3). It is possible that clearer age-dependency was not seen in this study owing to other potentially confounding factors including ~~variable recency~~timing of recent ~~of~~ exposure and colonisation. Alternatively, or in addition, much of any cytokine increase may occur by the end of the second year of life as seen for antibodies to pneumococcal antigens (5) while the children studied here we nearly all aged 2 years or older.

The classic Th17 pathway shows production of IL-17A and IL-22 to be from CD4+ T cells under the influence of TGF-β, IL-6 and IL-23 (15, 28-30). Given existing evidence that mucosal immunity to colonisation by pneumococcus can be antibody independent and CD4+ T cell dependent in mice (14, 15), and that CD4+ T cells may be important in protection of humans against pneumococcal colonisation and disease, as for example in HIV-infected individuals with reduced numbers of CD4+ T cells and high risk of this infection (31), the question arises whether CD4+ T cells are an important cellular source of IL-17A and IL-22. While it was clear that both cytokines were elaborated by cells falling within the lymphocyte scatter gate, our results suggest that CD4+ T cells are a source of IL-17A following stimulation with pneumococcal antigens, this was not

clearly demonstrated for IL-22 under the conditions we used (figure 5). Nevertheless, we did show evidence of strong correlation at an individual level between the size of IL-17A and IL-22 responses measured after pneumococcal antigen stimulation both in AMNC and PBMC (figure 4). Further characterisation of CD4+ T cells expressing the two cytokines both before and after pneumococcal antigen stimulation showed that those producing IL-22 were much more likely to be expressing innate cell phenotypes (figure 6). Both innate NK T cells (32, 33) and TCR- Yδ cells (34, 35) have previously been shown to be sources of IL-17A and IL-22, and both these cell types have been shown to recruit neutrophils to the pneumococcal infection site (36, 37). Our observations extend these observations to include CD4+ T cells expressing innate markers as a potential source of IL-22, particularly in the human upper respiratory tract.

We and others have previously investigated mucosal and systemic B cell and T cell CD4+ responses to several pneumococcal antigens including WCA, CbpA, PsaA and PspA (5, 12, 23, 24, 38). These antigens have also been shown to have protective effects in murine models of pneumococcal colonisation and infection (39-41). There is recent interest in PhtD as a vaccine candidate antigen (22). The IL-17A and IL-22 responses we demonstrate here to these antigens in primary human cell cultures, notwithstanding wide inter-individual variation, demonstrate significance differences between antigens as well (figure 7). The relatively larger responses seen in blood than adenoidal cells occur in the context of much lower background cytokine production by un-stimulated

cultures and a known lower T-regulatory environment (42). Of particular note ~~are~~ were the relative lack of responses to PhtD, an antigen which has recently been shown to lack efficacy against pneumococcal colonisation in children (43). Although it has been proposed that this and related pneumococcal proteins, which are released extra-cellularly by the bacterium in large quantities, might act as a sink for potentially opsonophagocytosing antibodies (44), our data suggest PhtD may also fail to induce mucosal cellular immune responses. ~~, in addition.~~

We here describe Th17 responses to pneumococcal antigens in human cell cultures in detail. This approach not only allows detailed description of the immunological responses to pneumococcus in the upper respiratory tract of young children, but also has potential to guide antigen design and selection for candidate vaccines aiming to impact upon carriage and transmission. Future studies should seek to elucidate whether such responses reliably predict protection against acquisition or clearance of carriage in children having repeated evaluation of colonisation over time.

6. Acknowledgments

EO, CP and CW conducted the experiments. AF, EC, CP, CW and EO designed the experiments. CLH assisted providing samples. AO, TM and RM provided the antigens. EO and AF wrote the paper.

We would like to thank our study funder PATH for providing us with a grant to carry out this work. We thank all the children who donated samples to us, the staff at the Bristol Royal Hospital for Children, and our nurses Phoebe Moulsdale, Clare Harrison and Jo Jenkins for recruiting the children to the study. We also acknowledge the assistance of Dr. Andrew Herman and the University of Bristol Faculty of Biomedical Sciences Flow Cytometry Facility.

7. Conflict of interest

The authors have no conflict of interest to disclose.

8. References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet. 2009;374(9693):893-902.
2. Pneumococcal conjugate vaccine for childhood immunization--WHO position paper. Wkly Epidemiol Rec. 2007;82(12):93-104.
3. Reinert RR. The antimicrobial resistance profile of Streptococcus pneumoniae. Clin Microbiol Infect. 2009;15 Suppl 3:7-11.
4. Kim L, McGee L, Tomczyk S, Beall B. Biological and Epidemiological Features of Antibiotic-Resistant Streptococcus pneumoniae in Pre- and Post-Conjugate Vaccine Eras: a United States Perspective. Clin Microbiol Rev. 2016;29(3):525-52.
5. Zhang Q, Bernatoniene J, Bagrade L, Pollard AJ, Mitchell TJ, Paton JC, et al. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. Eur J Immunol. 2006;36(1):46-57.
6. Hogberg L, Geli P, Ringberg H, Melander E, Lipsitch M, Ekdahl K. Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci. J Clin Microbiol. 2007;45(3):948-52.
7. Lipsitch M, Whitney CG, Zell E, Kaijalainen T, Dagan R, Malley R. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoS Med. 2005;2(1):e15.
8. Bogaert D, De Groot R, Hermans PW. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis. 2004;4(3):144-54.

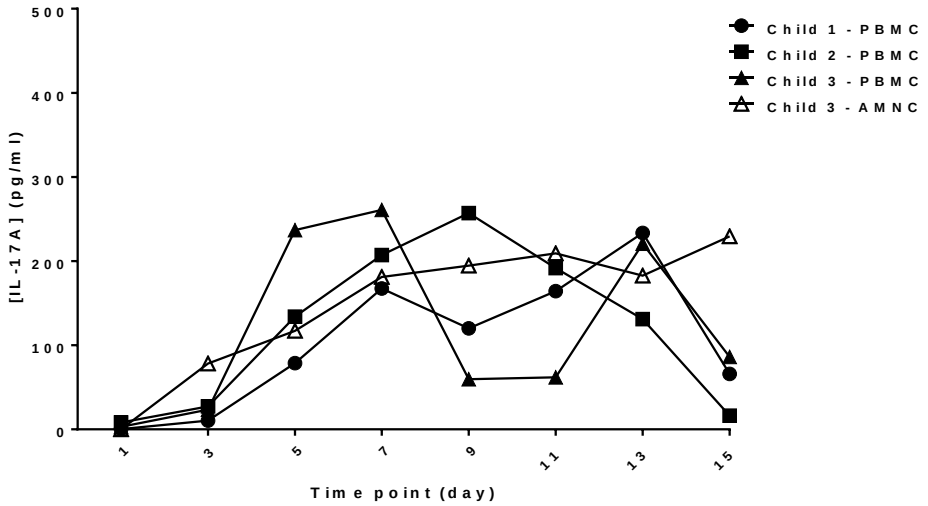
9. Waight PA, Andrews NJ, Ladhani NJ, Sheppard CL, Slack MP, Miller E. Effect of the 13-valent pneumococcal conjugate vaccine on invasive pneumococcal disease in England and Wales 4 years after its introduction: an observational cohort study. Lancet Infect Dis. 2015;15(6):629.
10. PHE. Pneumococcal disease infections caused by serotypes not in Prevenar 13 vaccine 2017 [updated 27th March 2017. Available from: https://www.gov.uk/government/publications/pneumococcal-disease-caused-by-strains-not-covered-by-prevenar-13-vaccine/pneumococcal-disease-infections-caused-by-serotypes-not-in-prevenar-13-vaccine.
11. Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. Lancet. 2011;378(9807):1962-73.
12. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc Natl Acad Sci U S A. 2005;102(13):4848-53.
13. Malley R, Srivastava A, Lipsitch M, Thompson CM, Watkins C, Tzianabos A, et al. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infection and immunity. 2006;74(4):2187-95.
14. Basset A, Thompson CM, Hollingshead SK, Briles DE, Ades EW, Lipsitch M, et al. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. Infect Immun. 2007;75(11):5460-4.
15. Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, Zhang Q, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathog. 2008;4(9):e1000159.
16. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6(11):1123-32.
17. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med. 2006;203(10):2271-9.
18. Malley R, Lipsitch M, Stack A, Saladino R, Fleisher G, Pelton S, et al. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect Immun. 2001;69(8):4870-3.
19. Lu YJ, Leite L, Goncalves VM, Dias Wde O, Liberman C, Fratelli F, et al. GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. Vaccine. 2010;28(47):7468-75.
20. Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with Streptococcus pneumoniae. Infect Immun. 2000;68(5):3028-33.
21. Ogunniyi AD, Woodrow MC, Poolman JT, Paton JC. Protection against Streptococcus pneumoniae elicited by immunization with pneumolysin and CbpA. Infect Immun. 2001;69(10):5997-6003.
22. Rioux S, Neyt C, Di Paolo E, Turpin L, Charland N, Labbe S, et al. Transcriptional regulation, occurrence and putative role of the Pht family of Streptococcus pneumoniae. Microbiology. 2011;157(Pt 2):336-48.
23. Zhang Q, Choo S, Finn A. Immune responses to novel pneumococcal proteins pneumolysin, PspA, PsaA, and CbpA in adenoidal B cells from children. Infect Immun. 2002;70(10):5363-9.

24. Zhang Q, Bagrade L, Bernatoniene J, Clarke E, Paton JC, Mitchell TJ, et al. Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. J Infect Dis. 2007;195(8):1194-202.
25. Pope C, Oliver EH, Ma J, Langton Hewer C, Mitchell TJ, Finn A. Genetic conjugation of components in two pneumococcal fusion protein vaccines enhances paediatric mucosal immune responses. Vaccine. 2015;33(14):1711-8.
26. Rapola S, Jantti V, Haikala R, Syrjanen R, Carlone GM, Sampson JS, et al. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. J Infect Dis. 2000;182(4):1146-52.
27. Lundgren A, Bhuiyan TR, Novak D, Kaim J, Reske A, Lu YJ, et al. Characterization of Th17 responses to Streptococcus pneumoniae in humans: comparisons between adults and children in a developed and a developing country. Vaccine. 2012;30(26):3897-907.
28. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med. 1996;183(6):2593-603.
29. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006;441(7090):231-4.
30. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.
31. Klugman KP, Madhi SA, Feldman C. HIV and pneumococcal disease. Curr Opin Infect Dis. 2007;20(1):11-5.
32. Rachitskaya AV, Hansen AM, Horai R, Li Z, Villasmil R, Luger D, et al. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. J Immunol. 2008;180(8):5167-71.
33. Megumi Gotoa MM, Kumiko Kadoshima-Yamaokaa, Yoshitaka Tanakaa, Kazuhiro Nagahiraa, Yoshiaki Fukudaa and Takashi Nishimura. Murine NKT cells produce Th17 cytokine interleukin-22. Cellular Immunology. 2009;254(2):81-4.
34. Euan Lockhart AMGaJLF. IL-17 Production Is Dominated by γδ T Cells rather than CD4 T Cells during Mycobacterium tuberculosis Infection. The Jounral of Immunology. 2006;177(7):4662-9.
35. Kristin J. Ness-Schwickerath CJaCTM. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. The Journal of Immunology. 2010;184(12):7268-80.
36. Nakasone C, Yamamoto N, Nakamatsu M, Kinjo T, Miyagi K, Uezu K, et al. Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. Microbes Infect. 2007;9(3):251-8.
37. Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. Eur J Immunol. 2003;33(12):3322-30.
38. Zhang Q, Bernatoniene J, Bagrade L, Paton JC, Mitchell TJ, Hammerschmidt S, et al. Regulation of production of mucosal antibody to pneumococcal protein antigens by T-cell-derived gamma interferon and interleukin-10 in children. Infect Immun. 2006;74(8):4735-43.
39. Briles DE, Hollingshead SK, Nabors GS, Paton JC, Brooks-Walter A. The potential for using protein vaccines to protect against otitis media caused by Streptococcus pneumoniae. Vaccine. 2000;19 Suppl 1:S87-95.
40. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly

protective against nasopharyngeal carriage of Streptococcus pneumoniae. Infect Immun. 2000;68(2):796-800.
41. Trzcinski K, Thompson C, Malley R, Lipsitch M. Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. Infect Immun. 2005;73(10):7043-6.
42. Zhang Q, Leong SC, McNamara PS, Mubarak A, Malley R, Finn A. Characterisation of regulatory T cells in nasal associated lymphoid tissue in children: relationships with pneumococcal colonization. PLoS Pathog. 2011;7(8):e1002175.
43. Odutola A, Ota MO, Antonio M, Ogundare EO, Saidu Y, Foster-Nyarko E, et al. Efficacy of a novel, protein-based pneumococcal vaccine against nasopharyngeal carriage of Streptococcus pneumoniae in infants: A phase 2, randomized, controlled, observer-blind study. Vaccine. 2017;35(19):2531-42.
44. Plumptre CD, Ogunniyi AD, Paton JC. Surface association of Pht proteins of Streptococcus pneumoniae. Infect Immun. 2013;81(10):3644-51.

Figures and figure legends

(a)



(b)

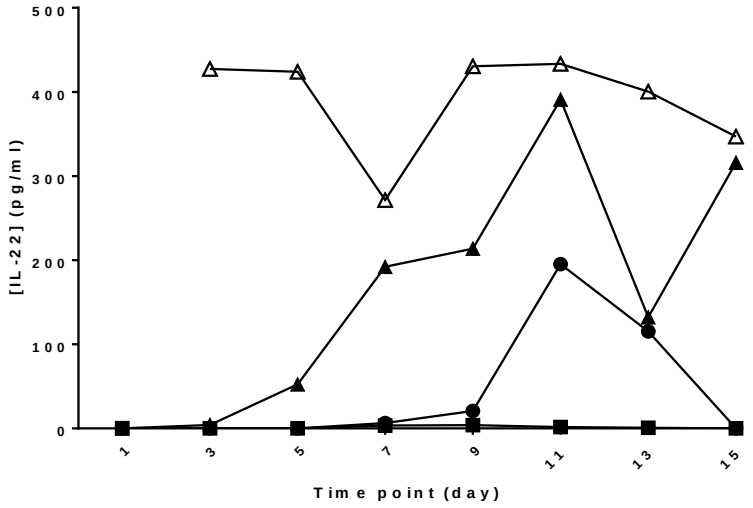
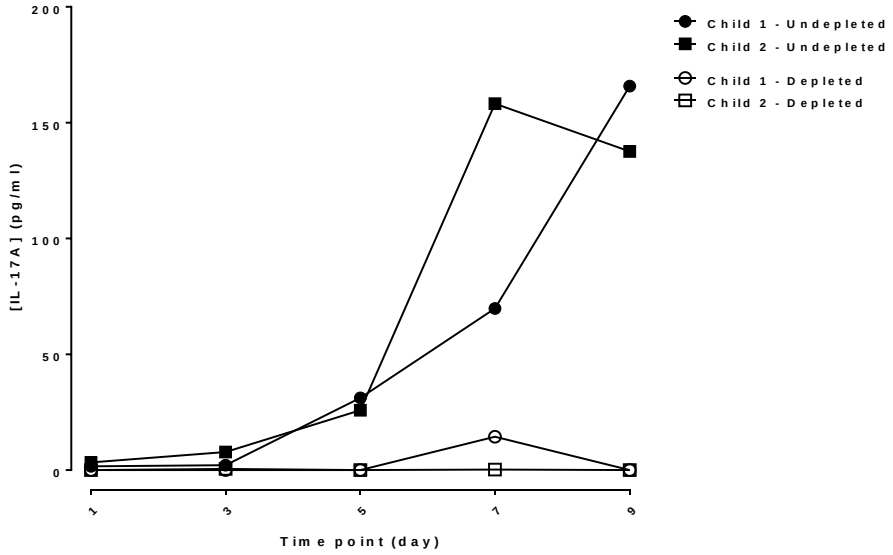


Figure 1. Detection of Th17 cytokines (a) IL-17A and (b) IL-22 in three children's PBMC, and in one child's AMNC after WCA stimulation over a 15 day

time course. Each data point represents a child's cytokine response and the background has been subtracted. Each supernatant was taken from a different well of cells at each time point.

(a)


200
150
100
50
0
[IL-17A] (pg/ml)
1
3
5
7
9
Time point (day)
Child 1 - Undepleted
Child 2 - Undepleted
Child 1 - Depleted
Child 2 - Depleted

(b)

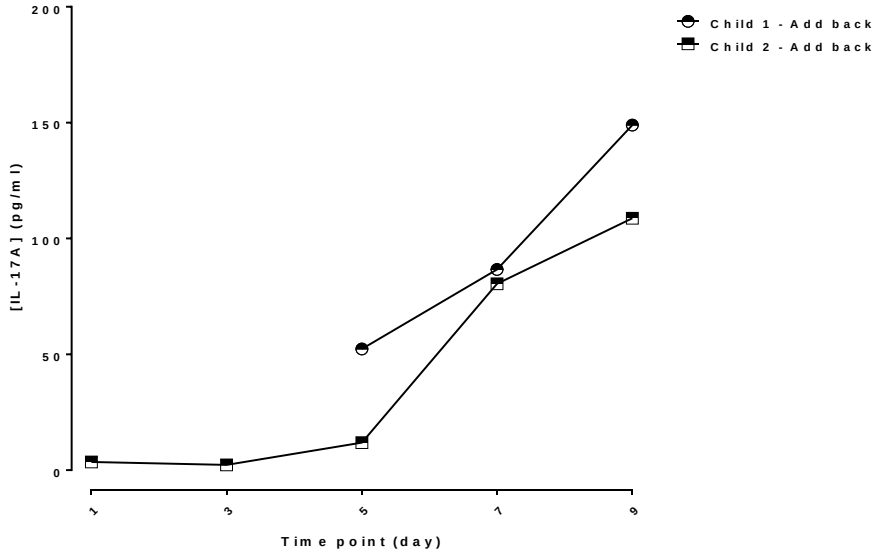

200
150
100
50
0
[IL-17A] (pg/ml)
1
3
5
7
9
Time point (day)
Child 1 - Add back
Child 2 - Add back

Figure 2. The effect of depleting memory T cells (CD45RO) from PBMC had on IL-17A responses to WCA**.** Each data point represents a child's IL-17A response to WCA over a 9 day time course in (a) undepleted PBMC and in memory T cell depleted PBMC, and in (b) the add back PBMC population of memory T cells and naïve T cells (CD45RA). The purity of the CD45RO depleted population in child one was 99.93%, and in child two it was 99.78% (data not shown). Due to a limitation with the number of cells available for child 1 the add back experiment could only be conducted between days 5-9. At each time point the background has been subtracted, and the supernatant was taken from a different well of cells.

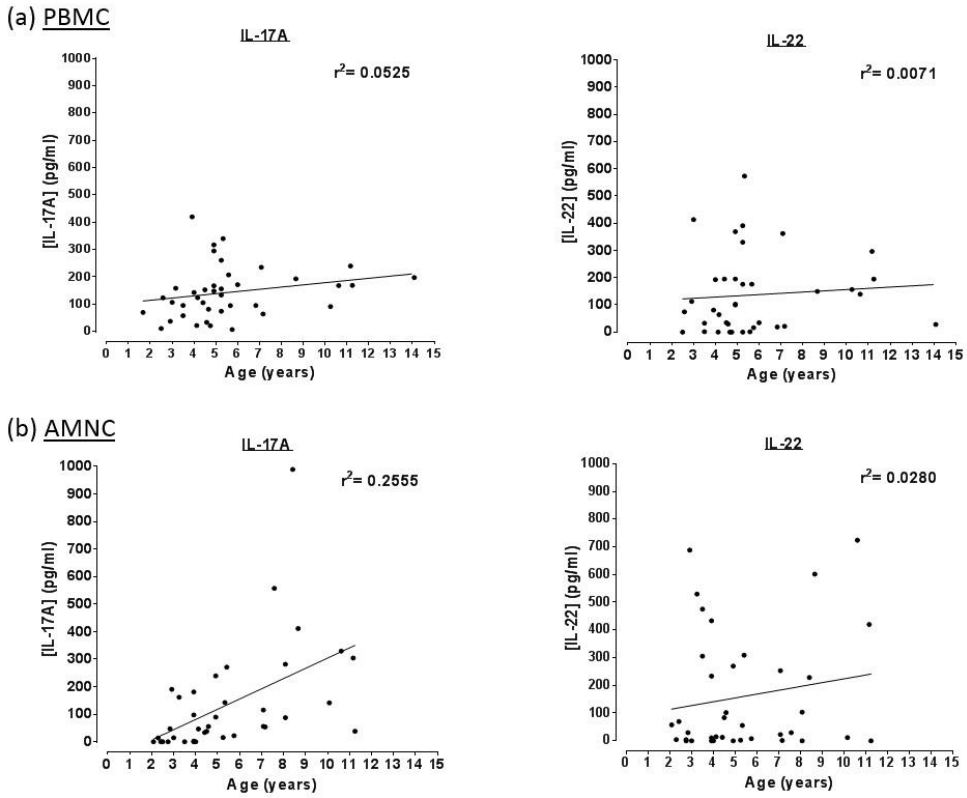


Figure 3. The association between age and IL-17A and IL-22 responses (above background) to WCA in (a) PBMC and (b) AMNC. Each data point represents a child's IL-17A or IL-22 response at day 7 or 11 respectively to WCA. n = 37-39. Linear regression lines are shown with the r^2 values.

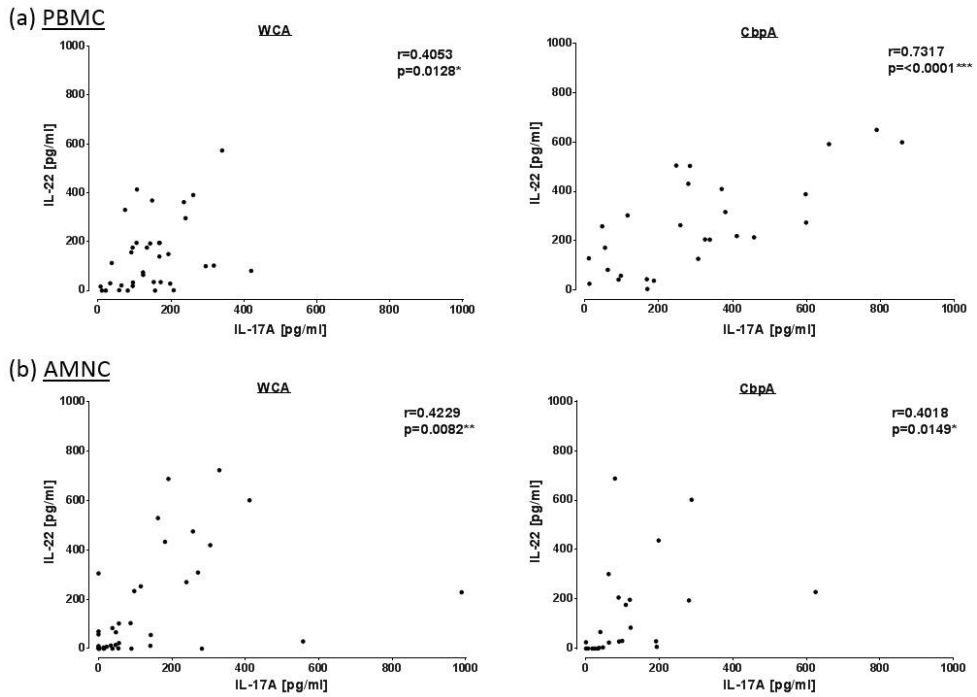


Figure 4. Correlation in children of their IL-17A and IL-22 responses in (a) PBMC and in (b) AMNC to WCA and to CbpA. Each data point represents a child's IL-17A and IL-22 response to WCA at day 7 or day 11 respectively, and the background has been subtracted. The n values range from 26-38. Pearson correlation was used to generate the correlation coefficient value, r, and a two-tailed P value.

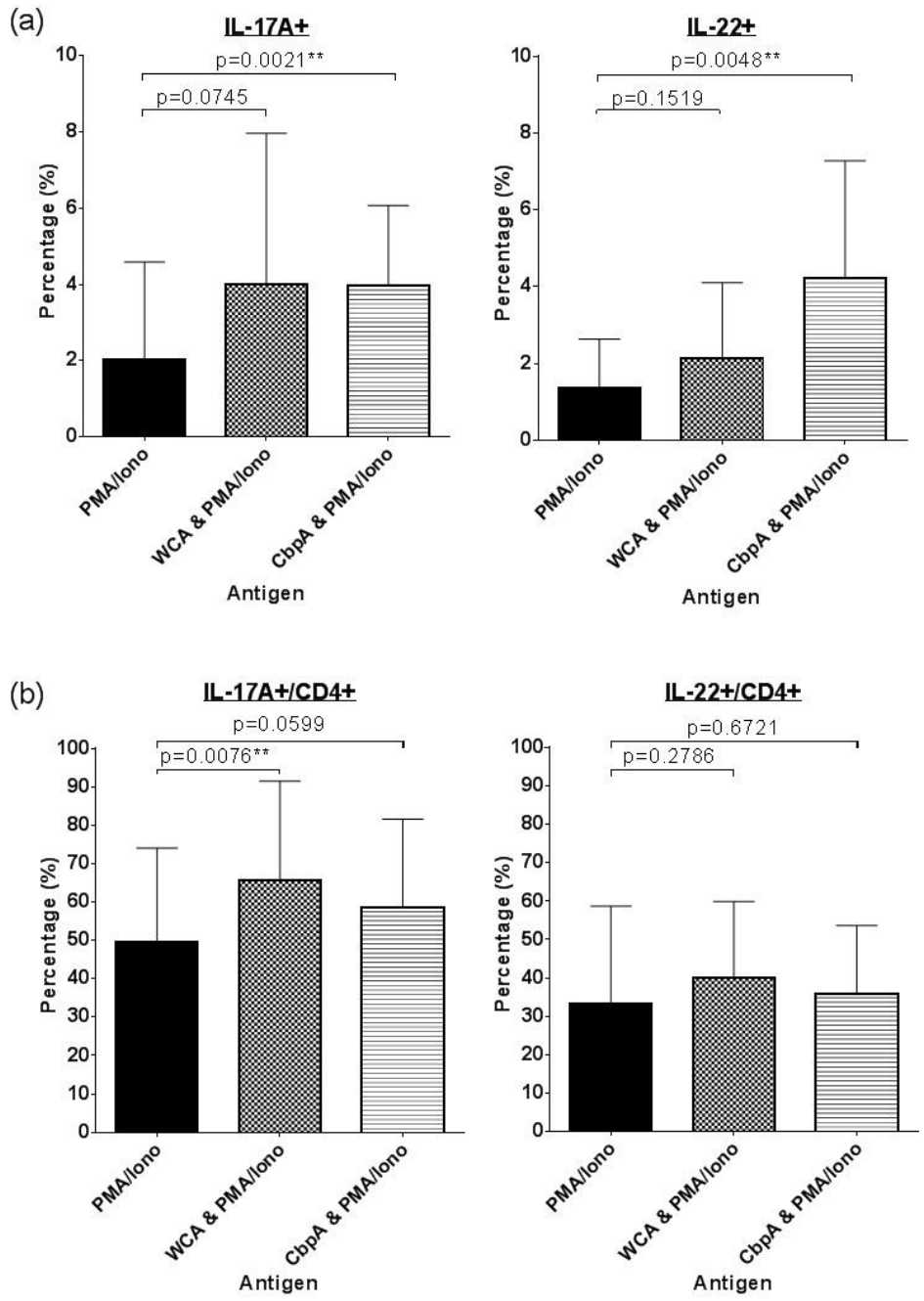


Figure 5. Intracellular cytokine staining analysis to identify IL-17A+ and IL-22+ expressing AMNC in response to WCA and CbpA at day 7, and to determine

their CD4+ expression level. (a) The percentage of live lymphocytes producing IL-17A or IL-22. The average percentage of live cells in the lymphocyte gate was 59.5%, n=14 (data not shown). (b) Comparison of the percentage of cytokine producing live lymphocytes expressing CD4+ in response to WCA and CbpA. Statistical analysis was conducted using a paired t-test with the p values shown.

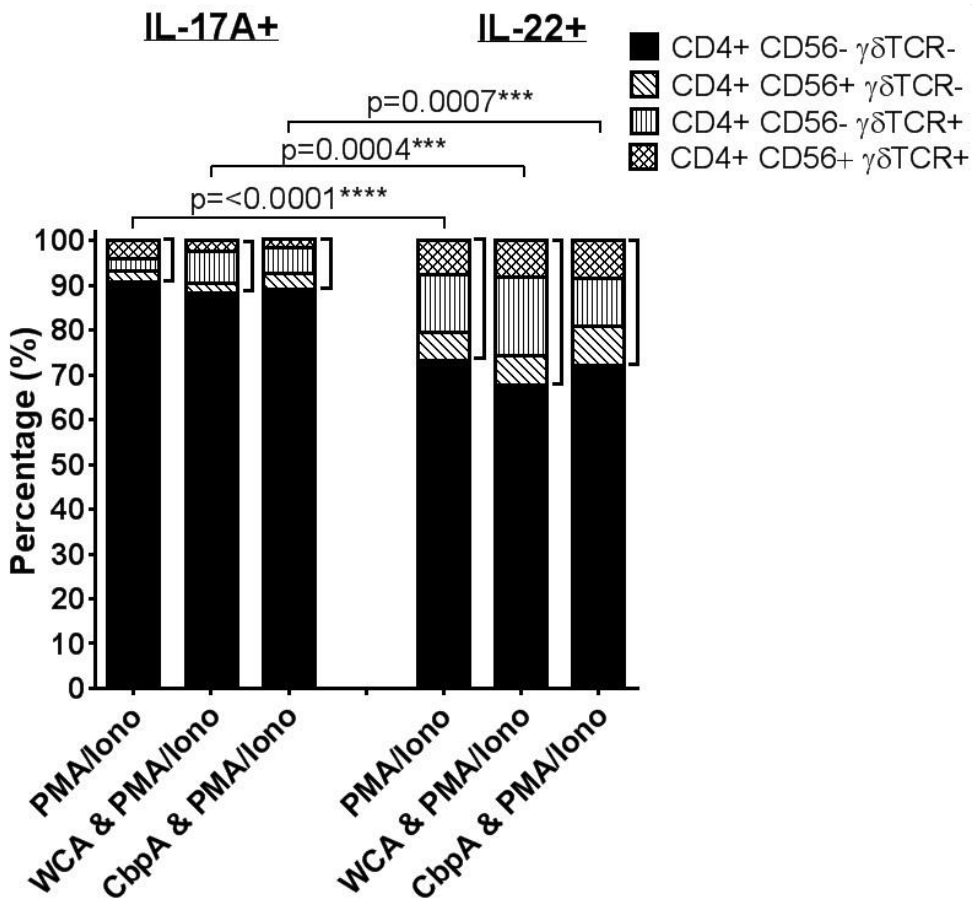


Figure 6. Expression of innate cellular markers on CD4+ IL-17A+ and IL-22+ AMNC. Each bar represents the mean percentage of live CD4+ cytokine producing cells expressing combinations of the cell surface markers CD56 and TCR-γδ in response to WCA and to CbpA. n=14 and a paired t-test was used to compare the IL-17A+ cells expressing combinations of the innate cellular markers, with live IL-22+ cells expressing combinations of the innate cellular markers, the p values are shown.

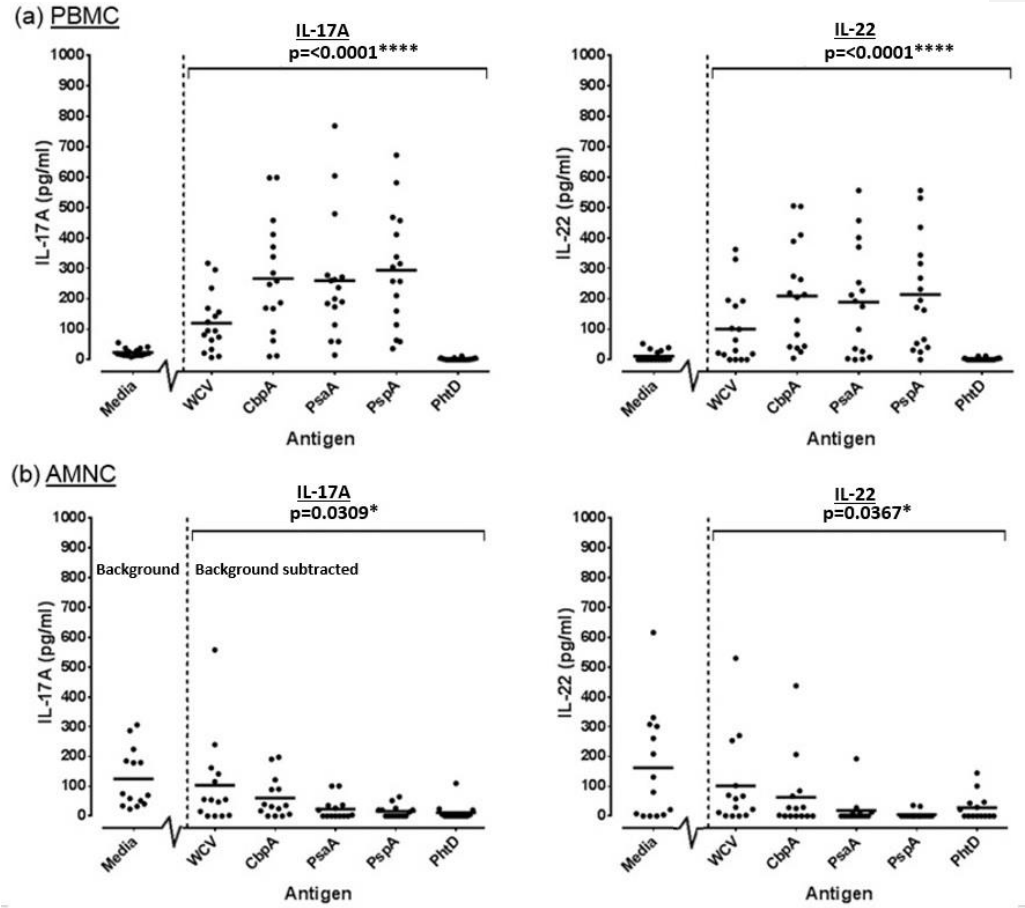


Figure 7. IL-17A and IL-22 responses to WCA and to a panel of pneumococcal antigens – CbpA, PsaA, PspA and PhtD - in (a) PBMC and (b) AMNC. Each data point represents a child's IL-17A or IL-22 response. The background is shown (media) for reference, however the background has been subtracted from the data points showing the responses to each of the stimulations. PBMC n=16 and AMNC n=14. The bar represents the mean. Comparing group means (excluding the media background) was conducted by repeated measures one-way ANOVA with the p values shown.