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Abstract

Colonization of the upper respiratory tract by pneumococcus is important both as a determinant of disease and for transmission into the population. The immunological mechanisms that contain pneumococcus during colonization are well studied in mice but remain unclear in humans. Loss of this control of pneumococcus following infection with influenza virus is associated with secondary bacterial pneumonia. We used a human challenge model with type 6B pneumococcus to show that acquisition of pneumococcus induced early degranulation of resident neutrophils and recruitment of monocytes to the nose. Monocyte function was associated with the clearance of pneumococcus. Prior nasal infection with live attenuated influenza virus induced inflammation, impaired innate immune function and altered genome-wide nasal gene responses to the carriage of pneumococcus. Levels of the cytokine CXCL10, promoted by viral infection, at the time pneumococcus was encountered were positively associated with bacterial load.

Introduction

Pneumonia is a major global health problem; it kills more children under 5 years of age than any other disease [1]. The burden of disease is aggravated by old age, chronic lung disease, immunosuppression and viral co-infection. Secondary pneumonia following pandemic and seasonal influenza virus infection is a significant cause of mortality worldwide [2].

Nasopharyngeal colonization by *Streptococcus pneumoniae* (Spn, pneumococcus) is common with 40-95% of infants and 10-25% of adults colonised at any time [3]. Such pneumococcal carriage is important as the pre-requisite of infection [4], the primary reservoir for transmission [5] and the predominant source of immunizing exposure and immunological boosting in both children and adults [6,7].

Immune dysregulation caused by respiratory virus infection such as influenza leads to increased carriage load [8]. Increased carriage load has been associated with pneumonia incidence and severity, as well as with within-household Spn transmission [5,9-11]. The mechanisms and markers associated with this pathogen synergy have been difficult to study in human subjects due to the rapid nature of the disease.

One safe way to simulate influenza infection in the nose is using Live Attenuated Influenza Vaccine (LAIV), consisting of cold-adapted influenza viruses. LAIV has been shown to affect the subsequent susceptibility to Spn and to lead to increased carriage load in murine models of infection and in vaccinated children [12,13]. Furthermore, LAIV administration prior to Spn challenge led to 50% increase in Spn acquisition by molecular methods as well as 10-fold increase in nasopharyngeal bacterial load [14].

In murine models of pneumococcal carriage, T_H17-dependent recruitment of neutrophils and monocytes to the nasopharynx mediates immunological control and clearance [15-17]. Influenza virus infection promotes Type I interferons which interfere with recruitment of these phagocytes, although IFN-ɣ is postulated to impair phagocytosis by macrophages through downregulation of the scavenger receptor MARCO [18-20]. However, the precise immune mechanisms and gene regulators involved in the control and clearance of pneumococcal carriage in humans have not been revealed [21]. Moreover, how these mechanisms are altered during human influenza virus infection remains largely unknown.

Systems biology approaches have allowed for the identification of immune mechanisms associated with protection from infectious diseases and with robust immune responses during vaccination [22-28]. Here, we applied systems biology to nasal samples collected in the setting of human challenge with LAIV and Spn, to emulate nasal effects of influenza infection on Spn carriage. We identified for the first time in humans the key cellular mechanisms that control newly acquired pneumococcal carriage, and how they are disrupted following nasal influenza infection.

Results

LAIV-induced inflammation leads to increased pneumococcal carriage load and acquisition

In a double-blinded controlled randomized clinical trial, we administered LAIV (n=55) three days prior to Spn inoculation (day 0). To verify the requisite topical application for an effect on pneumococcal carriage, we administered tetravalent inactivated influenza vaccine (TIV) as a control (n=62). LAIV infection led to transiently increased pneumococcal acquisition at day 2 (60.0% vs. 40.3% by molecular methods in LAIV vs. control groups, respectively) [14]. LAIV also increased Spn carriage load in the first 14 days following pneumococcal inoculation (Supplementary Fig. 1 and [14]). We collected a series of nasal micro-biopsies and nasal lining fluid throughout the study to assess ongoing cellular and cytokine responses. Participants were grouped into those who did not become colonized following Spn challenge (carriage–) and those who did (carriage+), as determined by classical microbiology (Fig. 1a). To investigate whether LAIV-induced immune responses were associated with a predisposition to pneumococcal carriage, we measured concentrations of 30 cytokines, and proteins including IL-1RA, in nasal lining fluid (Fig. 1b). At day 0, directly prior to Spn inoculation, LAIV significantly increased concentrations of twenty cytokines after multiple testing correction, including CXCL10 (IP-10), TNF, IL-10, IFN-ɣ and IL-15 (Fig. 1b and Supplementary Table 1). In contrast, the control group did not show any significant increase in cytokine response at day 0. Following Spn inoculation, Spn carriage in the absence of LAIV was associated with increased concentration of epidermal growth factor (EGF) at day 2 and decreased concentrations of IL-1RA at day 9 post Spn inoculation compared to baseline, neither of which remained significant after multiple testing correction. No other cytokines, including IL-17A or CCL2, were significantly altered by carriage alone (Fig. 1b).

Even before bacterial inoculation, nasal inflammatory responses to LAIV differed between those who went on to become carrier and those who were protected from carriage (Fig. 1c). In particular, IL-10

was significantly increased in LAIV-vaccinated subjects who did not acquire Spn following inoculation (5.8-fold increase), but not in those who became carriers following inoculation (2.0-fold increase). In contrast, CXCL10 was significantly increased in subjects who went on to become carriers (2.4-fold increase), but not in those who remained carriage-negative (1.5-fold increase). Moreover, subjects with increased concentrations of CXCL10 before inoculation displayed higher pneumococcal load following Spn inoculation (Fig. 1d). This suggests that differences in the response to influenza virus are associated with secondary susceptibility to Spn. To test whether this was specific for LAIV infection, we measured CXCL10 in nasal washes from an independent cohort in which a subset of subjects had asymptomatic viral upper respiratory tract infection the week before Spn inoculation, which did not progress to symptomatic infection. These comprised rhinovirus (n=12), coronavirus (n=5), respiratory syncytial virus (n=2) and parainfluenzavirus (n=1) [29]. The predominant virus, rhinovirus, was recently shown to associate with increased pneumococcal acquisition and transmission [30]. In these virus-infected subjects, CXCL10 concentration was increased (Supplementary Fig. 2), and baseline CXCL10 concentration correlated with increased pneumococcal load also in this second cohort (Fig. 1e). Unfortunately, sample sizes were too small to further investigate in depth the effect of infection by the different viruses, which are likely to have divergent effects. Although the correlation was modest in this validation cohort suggesting that other host and environmental factors are involved, this is the first time a biomarker predicting Spn load has been identified.

Early neutrophil degranulation in response to carriage is impaired by LAIV infection

In murine models, neutrophil recruitment after onset of carriage contributes to control of the bacteria [15]. We observed pre-existing high numbers of neutrophils in the human nasal lining and pneumococcal carriage did not lead to significant further recruitment of neutrophils (Supplementary Fig. 3a,b). To investigate whether luminal neutrophils were involved in the early control of carriage, we measured myeloperoxidase (MPO) concentrations, a marker for neutrophil degranulation [31], in

nasal washes. Concentrations were increased (2.2-fold) at 2 days after challenge in control carriage+ but not carriage– individuals (Fig. 2a). This neutrophil activation was impaired in the LAIV group, who displayed high carriage load during early carriage and had increased acquisition compared to controls. Together, this suggests that neutrophil degranulation is important for the initial control of carriage. To investigate whether neutrophils were also impaired systemically following LAIV as reported during wild-type influenza infection [32], we isolated blood neutrophils before, and at three days after, LAIV administration from a subset of subjects. We confirmed that opsonophagocytic (OPK) killing of Spn by blood neutrophils was decreased following LAIV (Fig. 2b). This effect could be mimicked by the addition of TNF, but not CXCL10, to neutrophils from healthy donors in vitro, decreasing killing capacity in a dose-dependent manner (Fig. 2c,d). Nanostring expression analysis of 594 genes revealed 10 differentially expressed genes in blood neutrophils 3 days post LAIV (Supplementary Table 2). Among those were *MAP4K2* (3.2-fold increase), which acts on the TNF signal transduction pathway [33], and the co-inhibitory receptor *TIGIT* (3.6-fold increase, Fig. 2e). *TIGIT* expression levels also negatively correlated with neutrophil killing capacity (r=-0.73, Fig. 2f). TIGIT is an "immune checkpoint" protein, which has been described to promote regulatory T cell (T_{reg} cell) function [34], but its expression on neutrophils has not been previously appreciated. Incubation of whole blood with recombinant TNF increased TIGIT levels on neutrophil surface within 30 minutes in a dose-dependent manner (Fig. 2g).

Taken together, inflammation following LAIV impaired the response of nasal and systemic neutrophils to pneumococcus, which could be mimicked by addition of exogenous TNF to neutrophils and associated with an upregulation of *TIGIT*.

Pneumococcal carriage-induced monocytes recruitment to the nose is impaired by LAIV infection

Immunophenotyping revealed a significant recruitment of monocytes to the nose following establishment of carriage (Fig. 3a and Supplementary Fig. 4). Monocyte numbers increased as early

as 2 days following Spn inoculation, peaked at 9 days (median 4.8x increase) and remained elevated 29 days post Spn inoculation. In contrast, there was no recruitment of $CD3^+$ T cells to the nose (Supplementary Fig. 4b). LAIV infection prior to pneumococcal carriage impaired the recruitment of monocytes to the nose (Fig. 3a). Moreover, peak pneumococcal load associated with increased monocyte recruitment in the control group, but not the LAIV group (Fig. 3b,c). Indeed, for subjects in the control group with very low carriage densities, which were only detectable by molecular methods, no monocyte recruitment was observed (Supplementary Fig. 4c). This suggests that a minimum Spn load is required for sensing and monocyte recruitment and that LAIV infection interferes with this process. Although CCL2 (MCP-1) was not substantially induced following Spn carriage, its concentration correlated with numbers of monocytes at all timepoints (Supplementary Fig. 5a). Furthermore, stratification of individuals showed that those with increased CCL2 concentration at day 2 post Spn inoculation exhibit increased monocyte recruitment (Supplementary Fig. 5b). Concentrations of IL-6, IFN-γ and TNF also correlated with numbers of monocytes at each time point, but stratification of individuals did not reveal a differential recruitment of monocytes (Supplementary Fig. 5a,b). In a second, independent cohort that did not receive any vaccine, monocytes were increased at day 9 following Spn inoculation, which correlated with an increased CCL2 concentration in nasal fluid, validating these results (Supplementary Table 3 and Supplementary Fig. 5c).

Thus, acquisition of pneumococcal carriage led to a recruitment of monocytes to the nasopharynx, a process that associated with pneumococcal load, CCL2 induction and that was inhibited by LAIV infection.

Nasal responses associated with pneumococcal clearance are impaired by LAIV

To assess anti-pneumococcal responses induced by carriage, we collected nasal cells 29 days post Spn inoculation and stimulated *in vitro* with heat-killed Spn and measured concentrations of 30 cytokines in supernatant. An increased production (fold-change (FC) > 2 and q < 0.05 to unstimulated control) of TNF, MIP-1α, IL-10, IL-6 and GM-CSF upon restimulation was observed in the control carriage+ group (Fig. 4a and Supplementary Fig. 6a). In the LAIV carriage+ group,

however, this boosting of anti-pneumococcal cytokine responses by re-challenge was absent (Fig. 4a and Supplementary Fig. 6a). The production of the above five cytokines correlated with decreased pneumococcal load at day 29 post Spn inoculation, suggesting these responses are involved in Spn clearance (Fig. 4b). To test whether monocytes/macrophages were the source of these cytokines we compared the cytokine signature from whole nasal cells with that from alveolar macrophages exposed to Spn in vitro (Fig. 4c). Relative cytokine production highly correlated between the two cell populations, suggesting that nasal monocytes/macrophages could be the source of these cytokines. This is supported by the observation that in carriers with low carriage load (only detectable by molecular methods), absence of monocyte recruitment associated with absent Spn-specific responses (Supplementary Fig. 6b).

In conclusion, carriage led to increased responses of nasal cells to pneumococcal stimulation, which was potentially due to the infiltration of monocytes. This was impaired by prior LAIV infection and correlated with clearance of pneumococcal carriage (Supplementary Fig. 7).

LAIV alters nasal gene expression responses to carriage

To identify gene signatures associated with the observed responses to pneumococcal carriage and infection with LAIV, we performed RNA-sequencing on whole nasal cells at days -5, 2 and 9 from Spn inoculation (Fig. 5 and Supplementary Table 4). Carriage without LAIV induced 834 and 176 differentially expressed genes (DEG) at day 2 and day 9, respectively (Fig. 5a). These genes were enriched for pathways associated with Gap junction trafficking and regulation (including *GJA1*, *TJP1* and multiple *GJB*) and degradation of the extracellular matrix (including *COL17A1*, *COL12A1*, *LAMA3*, *KLK7*). In the carriage– group, a smaller number of DEG was observed (161 and 248 at day 2 and day 9, respectively).

In the LAIV carriage+ group, 936 and 711 DEG were observed at day 2 and day 9, respectively. Surprisingly, despite the high concentrations of inflammatory cytokines observed in the LAIV carriage– group, only a relatively small number of DEG were observed at days 2 and 9 (126 and

153, respectively). DEG of carriage+ subjects receiving LAIV and DEG of carriage+ without LAIV showed very little overlap with only 38 DEG at day 2 and 2 DEG at day 9 in common. Very little overlap was observed at the pathway level between these groups, indicating LAIV alters the natural responses to pneumococcus (Fig. 5b and Supplementary Table 5). This could reflect transcriptome kinetics, such as observed in altered differentiation and cellular activation, or changes in cell migration to the nasal mucosa.

The LAIV carriage+ group showed an enrichment for genes in the Toll-like receptor 3 (TLR3) signalling cascade, RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways and IFN-gamma signalling, which is in agreement with the induction of antiviral responses following LAIV vaccination [35]. Moreover, TLR4 signalling was also enriched in this group. The pneumococcal protein pneumolysin is sensed by TLR4 [36] and it is possible that the increased pneumococcal load following LAIV vaccination led to increased pneumolysin sensing. O-linked glycosylation of mucins, which are used by Spn as a carbohydrate source for growth [37], was also enriched in the LAIV carriage+ group (including genes *ST3GAL4*, *GALNT7*, *GCNT3*, *B4GALT5)*. *ST3GAL4* is a sialyl transferase and cleavage of sialic acids by the influenza neuraminidase has previously been shown to promote pneumococcal growth [38]. This finding supports a LAIV-mediated effect on pneumococcal growth through alterations of host factors. Common genes and pathways between the LAIV-vaccinated and control carriers include “Innate immune system” and “Signaling by interleukins” (*IL1B*, *CLEC4E*, *CD55*, *IL1RN*). In conclusion, the genome-wide transcriptomic response to pneumococcal carriage was substantially altered on both the gene and the pathway level by LAIV.

Gene modules associated with recruitment of monocytes

To identify sets of co-expressed genes post LAIV and carriage, we used CEMiTool on the baseline-normalized data of LAIV and control groups, separately [39]. This modular expression analysis revealed the genes that may act together or are similarly regulated during the immune responses to carriage and infection.

Genes in the control cohort were grouped into four co-expression modules, of which three were significantly enriched for known Reactome pathways (Supplementary information 1). Module M1 was enriched in carriage+ at day 9 post Spn inoculation (Fig. 6a). Numbers of monocytes correlated with the average fold change count in this module, suggesting that these genes reflect the infiltration of monocytes (Fig. 6b). To further investigate these monocytes, we performed gene set enrichment analysis on the Module M1 genes using list of genes from distinct monocyte subsets (Fig. 6c) [40]. These genes were enriched for classical $CD14^{+}CD16^{-}$ monocytes and not for other monocyte subsets. Moreover, this module was enriched for genes related to "chemokine receptors bind chemokines" and "interferon α/β signalling" (Fig. 6d). Type I interferon has been shown to be required for the clearance of pneumococcal carriage in murine models [41] and these findings suggest that their activity in monocytes might be critical for this. CEMiTool also integrates co-expression analysis with protein-protein interaction data. Expression of *CXCL6* and its receptor *CXCR2* were identified as hubs in this module M1 (Fig. 6e and Supp html file 1). CXCR2 engagement has been shown to induce attachment of monocytes to the endothelial layer, initiating chemotaxis, which suggests this interaction could contribute to monocyte recruitment [42]. Module M3 was enriched in genes related to "extracellular matrix organization" and "collagen formation" (Fig. 7).

For LAIV, we identified six distinct co-expression modules (Supplementary information 2), which were strongly enriched in genes related to "Diseases associated with O-glycosylation of proteins" (module M1), "Immunoregulatory interactions between a lymphoid and a non-Lymphoid cell" (module M3), "chemokine receptors bind chemokines" (module M4), as well as "interferon signalling" (module M5, Fig. 8). Indeed, the hubs of module M5 are well known type I interferon-related genes, such as *ISG15*, *OAS1*, *OASL*, *IFIT1-3*, and *IFITM1*. Altogether, our findings reveal that a strong local antiviral response is elicited in response to LAIV infection.

Discussion

This study addresses fundamental questions about the immune responses that control and clear Spn carriage and how influenza infection can alter this control. By using for the first time a double experimental human challenge model with LAIV and Spn, we revealed that Spn carriage led to a quick degranulation of pre-existent nasal neutrophils in the human nose and recruitment of monocytes, promoting bacterial clearance. LAIV infection impaired these immune responses following carriage. LAIV is an attenuated influenza strain and wild-type influenza viruses might have even more pronounced effects on the host response to pneumococcus. Carriage in the absence of LAIV was associated with only limited inflammation, corroborating the view of Spn as a commensal bacterium that can asymptomatically colonize healthy adults [43]. In contrast, robust pro-inflammatory cytokine responses were measured following LAIV at both the protein and gene expression level. Altogether, these results provide explanation for our previous report that LAIV increased acquisition of Spn and carriage load [14].

In addition, our findings that LAIV led to impaired blood neutrophil killing capacity and that the addition of TNF, which was increased following LAIV, to neutrophils in vitro impaired their activity, highlights their crucial roles in susceptibility to secondary bacterial infection [44]. The association of TIGIT in this impaired neutrophil function following influenza infection warrants further investigation as TIGIT-blocking therapeutics are currently being developed for cancer and HIV treatment [45].

We identified CXCL10 as a marker for increased susceptibility to Spn and propose this should be further investigated as a potential therapeutic target for secondary bacterial infections associated with virus infections. Our data showed that individuals with higher concentrations of CXCL10 prior to Spn inoculation had higher bacterial load. In a previous study, children with pneumonia with viral and bacterial (predominantly pneumococcal) co-infection had increased amounts of CXCL10 compared to children with just viral or bacterial pneumonia, which associated with disease severity [46]. Murine data suggests that CXCL10 plays a direct role during pneumonia. Mice with genetic ablation of CXCR3, the receptor for CXCL10, CXCL9 and CXCL11, showed increased survival,

decreased lung inflammation and less invasion following infection, depending on pneumococcal inoculation strain used [47]. Moreover, addition of exogenous CXCL10 prior to infection of mice with influenza or respiratory syncytial virus (RSV) increased pneumonia severity [48].

Our results support previous findings from murine models showing that CCL2 signalling and monocyte recruitment are key mediators of pneumococcal carriage clearance [16]. However, contrary to key mechanisms described in murine models, we did not observe any production of IL-17A or neutrophil recruitment to the nose following carriage or associated with carriage clearance [15-17], underlining the importance of confirmation of murine findings by human data.

One limitation of this study is that only one pneumococcal serotype 6B isolate was used, future studies using other isolates with a more or less invasive phenotype will be able to answer how generalizable these findings across pneumococcal isolates are. Nonetheless, the observation that carriage load and duration declines in parallel for all serotypes following repeated exposure, suggests that immunological control of newly-acquired Spn is mediated by similar mechanisms independent of the colonizing serotype [49].

In conclusion, this study highlighted the importance of innate immunity in the control of carriage load and clearance of Spn, which was impaired by pre-existing viral infections. Secondary bacterial infection following viral respiratory tract infection has a large burden of disease worldwide and disrupting viral-bacterial synergy through host-directed therapy could prove an attractive addition to current therapeutic and vaccination options [50].

Accession Codes

Raw RNA-sequencing data have been deposited in the GEO repository, accession number GSE117580.

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Author contributions

SPJ contributed to conceiving, designing, performing and analysing experiments and writing of the paper. FM and HIN contributed analysing experiments and writing of the paper. BFC, MH, EM, ES, JFG, CS, J. Reiné, SP, EN, ELG, WAASP, DB contributed to conducting and analysing experiments. AHW, HH, CH, HA, SZ, VC, J. Rylance, SBG contributed to sample collection and/or design of the study. DMF contributed to conceiving, designing and analysing experiments, design of the study and writing of the paper. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Figure Legends

Figure 1. LAIV-pneumococcus co-infection leads to excessive pro-inflammatory responses that associate with increased pneumococcal load and impaired monocyte recruitment. a) Experimental design of the study. LAIV = live attenuated influenza vaccine, Spn = *Streptococcus pneumoniae*. Analysed timepoints are indicated by black circles. b) Heatmap showing for each cytokine the median log2 fold-change compared to baseline for the timepoints 0/2/7/9, n = 19 per group. c) The delta in median log_2 fold change (FC) following LAIV vaccination just prior to inoculation with Spn for subjects becoming carriage+ or carriage– (excluding subjects becoming positive by PCR only, who resemble subjects that become carriage+ by culture as well). The colour of each bar represents the median induction in the entire LAIV group. ** P = 0.0097 by two-tailed Wilcoxon test for LAIV

carriage– subjects comparing IL-10 day 0 to baseline, p = 0.073 for the LAIV carriage+ group. *** P = 0.0008 by two-tailed Wilcoxon test for LAIV carriage+ subjects comparing CXCL10 day 0 to baseline, p = 0.051 for the LAIV carriage– group. d) Pneumococcal load (median and interquartile range of CFU/mL nasal wash shown) for all carriage+ subjects with high (top quartile, n = 9) or low (all subjects below top quartile, n = 28) CXCL10 concentrations at day 0. P = 0.019 by two-tailed Mann-Whitney of AUC of log-transformed load over time. e) Scatter plot showing correlation of CXCL10 concentration at baseline with Spn load for a second validation cohort (n = 52) with an asymptomatic upper respiratory tract virus infection (n=15) or not. Spearman correlation test results and linear regression line with 95% confidence interval (grey shading) are shown.

Figure 2. Neutrophil function is impaired following LAIV administration. a) Concentrations of myeloperoxidase (MPO) in nasal wash (NW) of volunteers before or 2 days post Spn inoculation. Median and interquartile range are shown (for n=9 LAIV carriage– and LAIV carriage+ and for n=10 control carriage– and control carriage+ subjects). * P = 0.014 by two-tailed Wilcoxon paired test. b) Spn opsonophagocytic killing (OPK) capacity of blood neutrophils before and 3 days following LAIV (n=6) or control (TIV or no, n=7) vaccination. Individual subjects are shown and connected by lines. *P = 0.031 by two-tailed Wilcoxon paired test. c) Effect of exogenous TNF (n=10) and d) CXCL10 (n=8) on OPK activity of blood neutrophils of healthy volunteers. ** P = 1.15×10^{-5} by Friedman test. Neutrophils from 6 subjects were used in 3 independent experiments. Individual samples are depicted and connected by dashed lines. e) Normalized *MAP4K2* and *TIGIT* counts on sorted neutrophils before LAIV or in control arm (n=6, red) and following LAIV (n=4, blue). Individual samples are shown and paired samples are connected by black lines. ** P = 0.008 and *** p = 3.2×10^{-5} two-tailed unadjusted p-values using negative binomial generalized linear model (DESeq2). f) Correlation between OPK activity and TIGIT counts (n=10). Spearman rho and p-value are shown. Regression line and 95% confidence intervals (shaded area) are shown. g) Levels of TIGIT on blood neutrophil surface measured by flow cytometry after a 30 minute incubation without or with 1ng/mL

TNF or 100ng/mL TNF (n=4). *P = 0.042 by Friedman test. Individual subjects are depicted by dots and connected by lines.

Figure 3. Monocyte recruitment following pneumococcal colonization is impaired during LAIV co-infection. a) Median and interquartile range of nasal monocyte numbers normalized to epithelial cell numbers are shown for control carriage+ (n=24), control carriage– (n=37), LAIV carriage+ (n=25) and LAIV carriage– (n=30) groups. The dashed green line shows the baseline level in the control carriage+ group. *P = 0.038 at day 2 and p = 0.030 at day 29, **p = 0.002 by two-tailed Wilcoxon paired non-parametric test. Levels of maximum pneumococcal (Spn) load are shown for the b) Control (n=22) and c) LAIV group (n=23) and correlated with the maximum monocyte recruitment (fold change to baseline). Individual subjects are shown and Spearman correlation analysis is shown.

Figure 4. Pneumococcus-specific responses are induced following colonization, which is impaired by LAIV co-infection. a) Whole nasal cells were collected 28 days post-inoculation and stimulated for 18 hours with heat-killed Spn for 48 subjects. Supernatant was collected and concentrations of 30 cytokines were measured by multiplex ELISA. The median and interquartile range for cytokines induced at least 2-fold in at least one condition are displayed. b) Correlations between cytokine production following Spn stimulation and pneumococcal load are shown (n=22). Spearman non-parametric correlation test results and regression lines with shaded 95% confidence intervals are shown per cytokine. c) The cytokine profile from alveolar macrophages (median for 6 volunteers shown) exposed to Spn for 18 hours was compared with that of stimulated whole nasal cells (median of control carriage+ group shown). Spearman non-parametric correlation test results and a regression lines with shaded 95% confidence intervals are shown.

Figure 5. Nasal transcriptomics following LAIV-Spn co-infection (n=35). a) The number of differentially expressed genes (DEGs) between each time point and the baseline for each group are shown. Upregulated and downregulated genes are depicted in red and blue, respectively. Connections between bars show the number of common genes between LAIV and control conditions

where colors reflect distinct pathways. b) Circular representation of DEG and Gene Set Enrichment Analysis (GSEA) for LAIV carriage+ and control carriage+ groups at day 2 and day 9 to Spn inoculation. The individual log2-fold changes (baseline-normalized values) values were used as ranks in a single sample GSEA analysis to identify consistently enriched pathways among subjects. Genes and pathways are connected by lines.

Figure 6. CEMiTool applied to control cohort – Module 1. Raw counts were normalized using log counts per million (CPM) and log2-fold change were calculated for each timepoint against the baseline after which co-expression modules were extracted. a) Gene Set Enrichment Analyses showing the module activity on each timepoint for carriage+ and carriage– groups. b) Correlation with average fold change counts of all M1 genes at day 9 with paired numbers of monocytes from the volunteer's other nostril. Individual subjects, regression line with 95% confidence interval and Spearman correlation analysis are shown (n=13). c) The genes of module M1 present with genes highly expressed in $CD14^{+}CD16^{-}$ (578 genes), $CD14^{+}CD16^{+}$ (108 genes), $CD14^{-}CD16^{+}$ (162 genes), showing the overlapping number of genes between M1 and monocyte subsets in parentheses. The overlap for significance we analysed using the Chi-square test. d) Over Representation Analysis of module M1 using gene sets from the Reactome Pathway database. e) Interaction plot for M1, with gene nodes highlighted.

Figure 7. CEMiTool applied to control group – M3. Raw counts were normalized using logCPM and log2-fold change were calculated for each timepoint against the baseline after which co-expression modules were extracted. a) Over Representation Analysis of module M3 of the control group using gene sets from the Reactome Pathway database. e) Interaction plot for M3, with gene nodes highlighted.

Figure 8. CEMiTool applied to LAIV. Raw counts were normalized using logCPM and log2-fold change were calculated for each timepoint against the baseline after which co-expression modules were extracted. a) Gene Set Enrichment Analyses showing the module activity on each timepoint for carriage+ and carriage– LAIV groups. b) Over Representation Analysis of module M5 of the

LAIV group using gene sets from the Reactome Pathway database. e) Interaction plot for M5, with gene nodes highlighted.

Methods

Additional information on data reporting can be found in the Life Sciences Reporting Summary.

Study design and sample collection

Healthy adult volunteers were 1:1 randomized to receive either intranasally LAIV (2015/2016 Fluenz Tetra or FluMist Tetra, AstraZeneca, UK) or intramuscular Quadrivalent Inactivated Influenza Vaccination (Fluarix Tetra, GlaxoSmithKline, UK) as described previously [14]. The control group also received a nasal saline spray, while the LAIV group also received a intramuscular saline injection. Three days post vaccination all subjects were inoculated with 80,000 CFU per nostril of 6B type Spn as described [6,51]. Nasal microbiopsies (ASL Rhino-Pro©, Arlington Scientific) and nasal lining fluid (Nasosorption™, Hunt Developments) samples were collected and stored at -80C as previously described [52].

Clinical Trial details

The double-blinded randomized clinical LAIV-EHPC trial was registered on EudraCT (Number 2014-004634-26) on 28th April 2015 and ISRCTN (Number 16993271) on 2nd Sep 2015 and was co-sponsored by the Royal Liverpool University Hospital and the Liverpool School of Tropical Medicine. Key eligibility criteria included: capacity to give informed consent, no immunocompromised state or contact with susceptible individuals, no pneumococcal or influenza vaccine or infection in the last two years and not having taken part in EHPC studies in the past three years. The primary endpoint was the occurrence of pneumococcal colonisation determined by the presence of pneumococcus in nasal wash samples (NW) at any time point post inoculation up to and including day 29, detected

using classical microbiology or *lytA* qPCR as described [6,51,53]. In this study, 130 volunteers were inoculated with pneumococcus, giving an 80% power to identify a 50% increase in carriage acquisition. Of 130 vaccinated volunteers, five were natural pneumococcal carriers (two in LAIV arm and three in control arm) and were excluded from further analysis. Another 8 subjects in the LAIV arm were excluded following a systematic LAIV dispensing error by a single practitioner, as recommended by the trial steering group. This resulted in a final 55 subjects analysed in the LAIV arm and 62 subjects in the control arm. Key secondary endpoints included the load of pneumococcal colonisation in NW at each time point following pneumococcal inoculation (days 2, 7, 9, 14, 22 and 29), detected using classical microbiology, the area under the curve of pneumococcal colonisation load following pneumococcal inoculation (days 2, 7, 9, 14 , 22 and 29), detected using classical microbiology or by molecular methods (*lytA*), and the immunological mechanisms associated with altered susceptibility to pneumococcus following LAIV. The outcomes reported in this manuscript were a priori included in the study protocol.

Ethics statement

All volunteers gave written informed consent and research was conducted in compliance with all relevant ethical regulations. Ethical approval was given by the East Liverpool NHS Research and Ethics Committee (REC)/Liverpool School of Tropical Medicine (LSTM) REC, reference numbers: 15/NW/0146 and 14/NW/1460 and Human Tissue Authority licensing number 12548.

Flow cytometry analysis

Immunophenotyping of nasal cells obtained by curettes was performed as described [52]. In brief, cells were dislodged from curettes and stained with LIVE/DEAD® Fixable Violet Dead Cell Stain (ThermoFisher) and an antibody cocktail containing Epcam-PE (9C4), HLADR-PECy7 (L243), CD16-APC (3G8), CD66b-FITC (G10F5, all Biolegend), CD3-APCH7 (SK7), CD14-PercpCy5.5

(MφP9, both BD Biosciences) and CD45-PACOrange (HI30, ThermoFisher). Whole blood was stained for 15 min at room temperature with TIGIT-PECy7 (A15153G, Biolegend) and CD16-APC, followed by 2x 10 min incubation steps with FACSLysis buffer (BD Biosciences) to remove erythrocytes. Samples were acquired on a LSRII flow cytometer (BD) and analysed using Flowjo X (Treestar). Fluorescent minus one controls for each of the included antibodies were used to validate results. For the LAIV and control cohorts, but not the additional validation cohort (Supplementary Fig. 5C), 84/553 samples (15.2%) with less than 500 immune cells or 250 epithelial cells were excluded from further analysis.

Neutrophil opsonophagocytic killing

Neutrophil killing capacity was evaluated as previously described with minor modifications [54]. Briefly, neutrophils were isolated through density gradient centrifugation, followed by 45 min incubation with serotype 6B pneumococci (inoculation strain, MOI 100:1), baby rabbit complement (Mast Group, Bootle, UK) and human intravenous immunoglobulin (IVIG; Gamunex, Grifols Inc, Spain). In some experiments, recombinant TNF or CXCL10 (Biotechne) was added.

Luminex analysis of nasal lining fluid or stimulated nasal cells

Nasal cells collected in RPMI containing 1% penicillin/streptomycin/neomycin (ThermoFisher) and 10% heat-inactivated FBS (ThermoFisher) were incubated with 50ug/mL DNAse I (Sigma Aldrich) at room temperature for 20 min and filtered over a 70um filter (ThermoFisher). Cells were spun down at 440xg for 5 min, resuspended, counted and incubated at 250,000 cells/mL in 96-wells or 384-wells plates (ThermoFisher). Heat-killed Spn inoculation strain was added at a concentration of 5 µg/mL of total protein (corresponding to 4.3x10^7 CFU/mL) and cells were incubated for 18 h. Bacterial protein concentration was measured by Bradford assay, using bovine serum albumin as standard and titration experiments were performed to determine dose. Supernatant was collected

and stored at -80C until analysis. For nasosorption filters, cytokines were eluted from stored filters using 100 uL of assay buffer (ThermoFisher) by centrifugation, then the eluate was cleared by further centrifugation at 16,000g. Prior to analysis, samples were centrifuged for 10 min at 16,000xg to clear samples. These were acquired on a LX200 using a 30-plex magnetic human Luminex cytokine kit (ThermoFisher) and analysed with xPonent3.1 software following manufacturer's instructions. Samples were analysed in duplicates and nasosorption samples with a CV > 25% were excluded.

RNA extraction and sequencing

Nasal cells were collected in RNALater (ThermoFisher) at -80C until extraction. Extraction was performed using the RNEasy micro kit (Qiagen) with on column DNA digestion. Extracted RNA was quantified using a Qubit™ (ThermoFisher). Sample integrity assessment (Bioanalyzer, Agilent), library preparation and RNA-sequencing (Illumina Hiseq4000, 20M reads, 100 paired-end reads) were performed at the Beijing Genome Institute (China).

Nanostring

Purified blood neutrophils were stored in RLT buffer (Qiagen) with 1% 2-mercaptoethanol (Sigma) at -80C until RNA extraction as above. 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 DESeq2 using internal normalization, which gave lower variance than normalizing to included housekeeping genes. DEG were identified using a model matrix correcting for repeated individual measurements.

RNA sequencing analysis

Quality control of raw sequencing data was done using fastQC. Mapping to a human reference genome assembly (GRCh38) was done using STAR 2.5.0a [55]. Read counts from the resulting BAM alignment files were obtained with featureCounts using a GTF gene annotation from the Ensembl database [56,57]. The R/Bioconductor package DESeq2 was used to identify differentially expressed genes among the samples, after removing absent features (zero counts in more than 75% of samples) [58]. Genes with an FDR value < 0.1 and an absolute fold change (FC) > 1.5 were identified as differentially expressed.

Co-expression analysis

For co-expression analysis, counts were normalized using log CPM and the log2 fold change was calculated for each time point in a subject-wise manner. The co-expression analysis was performed separately for each group (control and LAIV) using the CEMiTool package developed by our group and available at Bioconductor (https://bioconductor.org/packages/release/bioc/html/CEMiTool.html) [39]. This package unifies the discovery and the analysis of coexpression gene modules, evaluating if modules contain genes that are over-represented by specific pathways or that are altered in a specific sample group. A p-value = 0.05 was applied for filtering lowly expressed genes.

Statistical analysis

All experiments were performed randomised and blinded. Two-tailed statistical tests are used throughout the study. When log-normalized data was not normally distributed, non-parametric tests were performed and multiple correction testing (Benjamin-Hochberg) was applied for gene expression and Luminex analysis.

Data availability statement

Raw RNA-sequencing data have been deposited in the GEO repository, accession number GSE117580. All other underlying data is provided in the manuscript.

Methods only references

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