npj | vaccines

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Published in partnership with the Sealy Institute for Vaccine Sciences

https://doi.org/10.1038/s41541-025-01072-6

Transcriptome analysis in human breast milk and blood in a randomized trial after inactivated or attenuated influenza immunization

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Transcriptomic signatures were identified in human peripheral blood mononuclear cells (PBMCs) and breast milk lymphocyte (BML) cells induced by trivalent inactivated influenza vaccine (TIV) or live attenuated influenza vaccine (LAIV) administered after delivery. We performed an RNA-Seq analysis on blood and breast milk samples from a subset of subjects enrolled in a randomized, double-blind controlled study in breastfeeding women who received either intranasal LAIV and intramuscular placebo, or intramuscular TIV and intranasal placebo (LAIV, $n = 10$ and TIV, $n = 6$). Differentially expressed genes, gene clusters, and enriched pathways were identified. We observed increased innate immune signaling responses in BML but not in PBMC at Day 28 for the LAIV group. We hypothesize that breastfeeding extended the innate response to LAIV via mucosal immunity. An association between an increased IgG antibody response in TIV vs. LAIV identified in the parent study using ELISA corresponded to *IGHG1* immunoglobulin gene expression in Day 28 PBMCs.

Influenza illness causes significant morbidity and mortality in young infants[1–3]. In some winters, as many as nine percent of all infants less than 6 months of age experience an influenza-related illness and require care in a clinic, emergency department or hospital[1]. Similarly, pregnant women are at increased risk of adverse outcomes related to influenza infection[4]. The 2009 H1N1 influenza pandemic disproportionately affected pregnant women[4], and seasonal influenza-related hospitalizations are increased in this population[5].

Influenza vaccination during pregnancy prevents adverse maternal and fetal outcomes of influenza infection, including increased maternal mortality and prematurity[6]. Maternal influenza vaccination has been shown to confer significant reductions in influenza illness among women and their infants, as well as decreased influenza-associated hospitalizations during pregnancy[7–11]. Maternal influenza vaccination also protects against adverse fetal and infant health outcomes, including prematurity, small for gestational age (SGA) births, and low birthweight[12–15].

Because of these benefits to mothers and infants, influenza vaccination is recommended for all pregnant women during any trimester of pregnancy[16]. Both TIV and LAIV are licensed to be administered postpartum to women who are breastfeeding an infant, and the safety of both vaccines has been demonstrated in postpartum women[17]. However, little information is available to guide decisions regarding the selection of one vaccine over the other in breastfeeding women[18].

Systems vaccinology has emerged as a novel, multi-disciplinary approach to analyzing vaccine responses. A systems biology approach can identify early gene signatures after vaccination that can be used to predict later protective immune responses to vaccination, including responses to both TIV and LAIV[19]. This approach has been used across multiple populations, including older adults and diabetics[20], as well as with multiple vaccines[21]. However, these systems analyses have not been used in pregnant or postpartum women to understand the unique contribution of maternal antibodies transferred through both the umbilical cord and human milk via breastfeeding after maternal vaccination.

Recently, we conducted a study to compare breast milk IgA and IgG immune responses in postpartum women who received LAIV to the response of those who received TIV after delivery[17]. Results showed that

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breast milk IgG, breast milk IgA (H1N1 only), serum hemagglutination inhibition (HAI) responses, and serum IgG responses as measured using ELISA were significantly higher following administration of TIV compared to LAIV. Both vaccines were safe in women and their infants. However, we suspect that immunologic responses to influenza vaccine are complex and unlikely to be limited to HAI and IgG antibody responses alone, as reported in our previous investigation of anti-influenza IgA in human milk after antepartum immunization[22].

In addition to maternal serum IgG antibody transferred through the umbilical cord prior to delivery, there may be potential protective effects from the oral transfer of maternal immune cells through breast milk when infants consume milk from immunized mothers. Our previous work demonstrated that greater exclusivity of breastfeeding in the first 6 months of life significantly decreased the expected number of respiratory-illness-with-fever episodes in infants of influenza-vaccinated mothers[22]. The sustained high levels of actively produced anti-influenza IgA in breast milk and the decreased infant episodes of respiratory illness with fever suggest that breastfeeding may provide local mucosal protection for the infant.

This study seeks to understand the immunologic mechanisms of breast milk mediated protection by analyzing mucosal immunity via sequencing of breast milk lymphocyte cell RNA when subjects are immunized postpartum with LAIV versus TIV. The primary goal of this study was to identify transcriptomic signatures using RNA-Seq in human peripheral blood mononuclear cells (PBMCs) and breast milk lymphocyte cells (BMLs) in response to TIV or LAIV after delivery. Little is known about the immunologic components of breast milk that affect influenza vaccine response, and systems vaccinology is a unique approach to understanding the immunologic mechanisms of both vaccines in breast milk compared to peripheral blood. We therefore compared the effects of two influenza vaccines on PBMC and BML responses by systemic or mucosal immunization in postpartum women.

Results

Comparisons of gene expression signatures in breast milk lymphocyte cells and PBMCs

Prior to differential gene analysis, we inspected global gene expression patterns to detect any strong outlying samples stratified by specimen type. The Day 0 sample for one subject (Subject B) in the TIV vaccine group showed a globally outlying gene expression pattern in BML samples (Supplemental Figures S21–S23). This sample was excluded from downstream analyses. Next, to assess differences in immune responses on the gene expression level, we first determined genes that were differentially expressed relative to pre-vaccination (FDR-adjusted p-value < 0.2 and a fold change of ≥1.5-fold) for each vaccine group, specimen type, and time point (Fig. 1, Supplemental Tables S10–S15). In BML, we identified a total of 382 DE genes in the LAIV group, most of which were up-regulated at Day 28 (Fig. 1A). For TIV, three DE genes were identified at two time points in BML, of which two up-regulated genes (*IL1A* and *IL1B*) overlapped with LAIV at Day 28. These genes, encoding for the Interleukin-1 alpha/beta cytokines, were strongly differentially up-regulated from pre-vaccination following administration of LAIV (10.9 and 18.9-fold, respectively) and TIV (11.8 and 9.2-fold, respectively). At Day 28, *MTRNR2L8, SIGLEC14, CCL4, ST18*, and *RNVU1-19* showed the strongest differential responses between vaccine groups with 2.8-to-5-fold higher fold change responses in the LAIV group relative to the TIV group.

In PBMCs, at Day 28, 10 (2 up and 8 down-regulated) DE genes were identified for TIV (Fig. 1B) while one up-regulated gene was identified for LAIV but not TIV (*MTRNR2L8*) (Fig. 1A). This gene was also identified in BML LAIV at Day 28 (Fig. 1A). Up-regulation of *MTRNR2L8* was 17.1-fold for the LAIV group and 1.04-fold for the TIV group compared to pre-vaccination in PBMCs as well as 5-fold higher in BML at Day 28 for LAIV (14.1-fold) vs. TIV (2.8-fold). The two up-regulated genes for TIV encoded for immunoglobulin proteins (*IGHG1* and *IGHG3*). Both encoded for immunoglobulin (Ig gamma-1 and gamma-3 chain C region) proteins with 6.7-fold and 2.2-fold higher gene expression responses induced by TIV compared to LAIV vaccination, respectively.

Heatmap analysis of BML DE gene fold changes showed some initial up-regulation from baseline for DE genes at Day 2 and the strongest signal at Day 28 for the LAIV group with the majority of subjects showing increased responses for most of the DE genes (Fig. 1B). There was evidence of a weak Day 2 vaccine group effect, with LAIV subjects tending to have a higher fraction of subjects with up-regulated genes compared to subjects in the TIV group (Fig. 1B). At Day 8, a TIV vaccine effect was observed with 4 of 6 subjects showing a consistent upregulation of most DE genes (Fig. 1B). By Day 28, most subjects showed an up-regulation trend for most of the DE genes, but this pattern was stronger for LAIV compared to TIV with the highest consistent up-regulation identified in LAIV BML samples (Fig. 1B). In contrast, the Day 28 signal in PBMCs did not indicate strong overall effects for either vaccine (Fig. 1C).

Breast milk lymphocyte cells showed increased expression of interferon-inducible antiviral genes following LAIV vaccination

Next, to identify DE genes with similar responses following vaccination, we executed gene clustering analysis using $\log_2$ fold changes of DE genes as input (Fig. 2, Supplementary Tables S17–S20 and Figure S12). Inspection of mean $\log_2$ fold change time trends (Day 2, 8, 28) for 36 co-expressed DE gene clusters in BML showed that TIV generally induced responses ahead of LAIV (Day 8 vs. Day 28), albeit not as strongly, i.e., the peak was lower at Day 8 for TIV compared to that of LAIV at Day 28. A cluster of co-expressed interferon-inducible antiviral genes (*IFIT3, OAS3, IFI44L, MX1, OAS2, IFIT1, IFI6*) showed a statistically significant increase from pre-vaccination at Day 2 and an even stronger increase at Day 28 for the LAIV group. In both cases, the lower bound of the 95% bootstrap CI confidence interval (CI) was above 0 $\log_2$ fold change. In contrast, the TIV group did not exceed pre-vaccination levels for this cluster in BLMC at any post-vaccination time-point (Fig. 2). Interestingly, the Day 28 signal was reversed in PBMC samples with the TIV group showing statistically significantly higher than pre-vaccination levels, while the LAIV group remained at pre-vaccination levels.

LAIV activated genes involved in innate immune signaling pathways in breast milk lymphocyte cells

Next, to assess the functional context of DE genes, we carried out pathway enrichment analysis (Supplementary Tables S20–S34). Two days following LAIV vaccination, several innate immune signaling pathways were significantly enriched in DE genes in BMLs at Day 2 including *IL1 signaling* and *Signaling by interleukins* as well as *NF-kappa B signaling*, *Cytokine-cytokine receptor interaction*, *Toll-like receptor signaling*, and *TNF signaling* pathways. Two of the top three enriched MSigDB immunologic gene sets were from a TIV/LAIV vaccine study by Nakaya et al. (PMCID:PMC3140559) (*GSE29618 MONOCYTE VS MDC UP, MONOCYTE VS MDC DAY7 FLU VACCINE UP*)[19]. Similar innate immune signaling pathways were enriched at Day 28 following LAIV vaccination. The top significantly enriched pathways included *Osteoclast differentiation*, *Cytokine-cytokine receptor interaction*, *TNF signaling*, *NF-kappa B signaling*, and *Toll-like receptor signaling* pathways. In contrast to Day 2, *Influenza A*, *Cytokine signaling in immune system*, *Signaling by interleukins*, *Interferon signaling*, and more specifically *Interferon alpha-beta signaling* were significantly enriched at Day 28. The association of DE genes and cell signaling was further confirmed by a significant enrichment of cell membrane-associated proteins (74 of 382 (19%) DE genes, source: GO Cellular Components). At Day 28, the best overlap with the LAIV/TIV study by Nakaya et al. was observed for *GSE29618 MONOCYTE VS MDC UP*, *GSE29618 MONOCYTE VS PDC UP*, and *GSE29618 MONOCYTE VS MDC DAY7 FLU VACCINE UP* with 41–47 overlapping up-regulated DE genes[19]. For the TIV group at Day 28, IL1 SIGNALING (Reactome) was significantly enriched in up-regulated DE genes (2 DE genes: *IL1A* and *IL1B*). Time trends of average pathway fold change responses and associated 95% confidence intervals confirmed significant activation of key innate immune

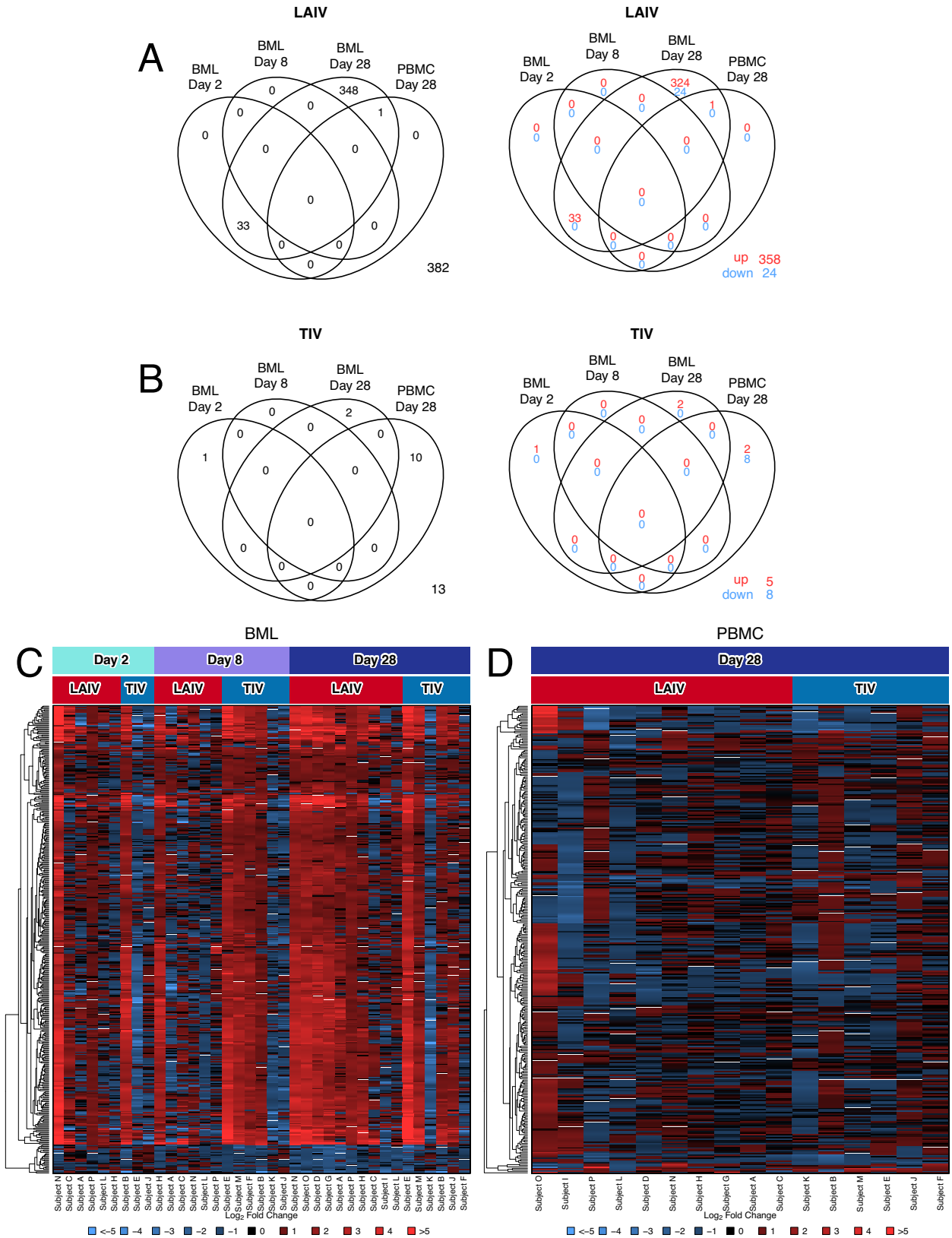

Fig. 1 | Differential gene expression signatures in breast milk and PBMCs following TIV and LAIV vaccination. In panels (**A**, **B**), the Venn diagrams summarize differentially expressed (DE) genes for each time point post-vaccination versus pre-vaccination, the overall number of DE genes (left side), and both up-regulated (red font) and down-regulated (blue font) genes (right side) for each vaccine group. In (**C**, **D**), the heatmap summarizes subject-level $\log_2$ fold changes for DE genes separately for each specimen type. DE differentially expressed, LAIV live attenuated influenza vaccine, TIV trivalent influenza vaccine, BML breast milk lymphocytes, PBMC peripheral blood mononuclear cells.

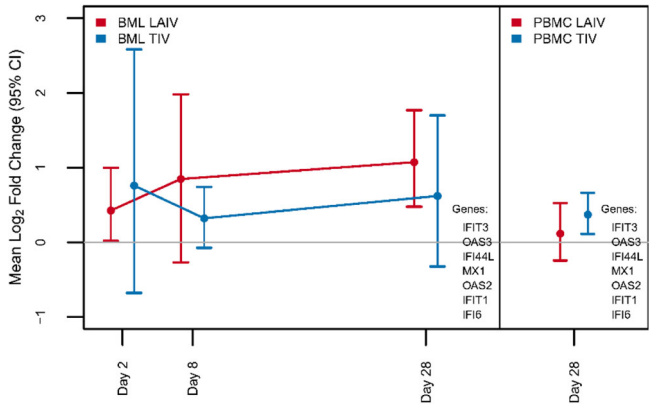

Fig. 2 | Time trend of cluster of interferon-inducible antiviral genes. The mean log_2 fold change from baseline and associated 95% bootstrap CIs for a co-expressed DE gene cluster of interferon-inducible genes (*IFIT3*, *OAS3*, *IFI44L*, *MX1*, *OAS2*, *IFIT1*, *IFI6*) are demonstrated by post-vaccination day, specimen type, and vaccine group. CI confidence interval, BML breast milk lymphocytes, LAIV live attenuated influenza vaccine, TIV trivalent influenza vaccine, PBMC peripheral blood mononuclear cells.

signaling pathways at Day 2 and then later at Day 28 in BML following LAIV vaccination and activation of IL1 SIGNALING at Day 28 following TIV vaccination (Fig. 3). Pathway maps for enriched KEGG pathways color-coded by vaccine effect are included in the Supplemental Text (Figures S28–S80).

Correlation between ELISA IgG/IgA and IgG/IgA gene expression responses

Next, we assessed whether IgG immunoglobulin heavy chain gene expression (*IGHG1* and *IGHG3*) in PBMCs could serve as a marker for serologic response. In our cohort, as for the main study[17], the directionality of the geometric mean titers (GMTs) was the same for all 5 antigens tested with higher GMTs for the TIV group (Table 1). Antibody responses to TIV at Day 28 were approximately three times higher than the antibody responses to LAIV, with the exception of the vaccine antigen for B/Brisbane/60/2008 (2011/2012 strain), in which there was a minimal difference between vaccine groups. In our study, a significant increase in gene expression from pre-vaccination for *IGHG1* and *IGHG3* genes in PBMCs was observed in the TIV group with the lower bounds of the CIs exceeding 0 log_2 fold change (Fig. 4A). The effect was stronger for *IGHG1* which encodes the constant region of IgG1. The Day 28 increase in *IGHG1* gene expression in PBMCs showed weak positive correlations with fold changes in ELISA IgG titers against 4 vaccine antigens: A/H3N2 (Perth and Victoria), A/H1N1 (California), and influenza B (Brisbane) (Supplemental Figures S2–S5). However, we observed a moderate positive association between *IGHG1* gene expression and IgG responses for the influenza B antigen – Wisconsin, which is presented in Fig. 4B ($r = 0.483$, $p = 0.0584$). Sensitivity analysis following the removal of one strongly outlying observation (highlighted in red) resulted in a statistically significant correlation of $r = 0.805$ ($p < 0.0003$).

Following these results, we assessed whether immunoglobulin heavy constant alpha 1 and 2 gene expression (*IGHA1* and *IGHA2*) in breast milk lymphocyte cells could serve as a marker for IgA response in breast milk. We observed a moderate positive association for the influenza B Wisconsin ($r = 0.406$, $p = 0.1191$) and the Swine H1N1 (0.486, $p = 0.0565$) antigens, which is presented in Supplemental Figure S9, S10, respectively; however, neither correlation was statistically significant.

Discussion

The main goal of this systems vaccinology analysis was to characterize gene expression signatures in PBMCs and in BMLs in response to one dose of TIV ($n = 6$) or LAIV ($n = 10$) administered to postpartum women. Our results demonstrated increased gene expression of two genes encoding for Immunoglobulin G (IgG) in PBMCs in the TIV group which corresponded with findings of increased Day 28 serum IgG geometric mean titers (GMTs) in the TIV group compared to the LAIV group in the parent study[17]. This gene expression signal for increased adaptive immunity was absent from the LAIV group – a fact that was in line with the parent study, which showed that HAI and ELISA IgG titers were significantly higher following administration of TIV compared to LAIV. The breast milk IgA response to H1N1 was also higher in TIV recipients compared to LAIV recipients over both seasons in the parent study ($p = 0.003$)[17]. Serum IgG GMTs were measured by both ELISA and hemagglutination inhibition (HAI) assay in the parent study, but ELISA more directly assesses the IgG concentration than HAI assay. Indeed, we found that the Day 28 increase in *IGHG1* gene expression levels in TIV group PBMCs correlated well with corresponding increases in serum ELISA IgG titers for the influenza B - Wisconsin antigen, suggesting that this gene could serve as a potential biomarker of ELISA response.

Global fold change responses following vaccination were more pronounced in BML compared to PBMC, with more genes passing the minimum fold change cut-off compared to PBMC. In the LAIV vaccine group, 94% of the 382 differentially expressed genes in BMLs were upregulated compared to pre-vaccination, and 91% were differentially expressed at Day 28. This high number of DE genes contrasted with the relatively small number of up-regulated DE genes identified in the TIV group ($n = 3$). However, DE genes numbers in the TIV group were likely negatively impacted by the smaller sample size ($N = 6$ vs. $N = 10$). In a more quantitative way, we compared groups using heatmap analysis and time trends of gene clusters based on the union of all DE genes observed. Using this approach, we identified (1) a delay in the overall peak DE response for LAIV vs. TIV (Day 28 vs. 8) in BML and (2) BML LAIV-specific gene expression signatures in BML at Day 28 that were absent or less pronounced in PBMCs.

The innate mucosal immunity associated with LAIV may be associated with the upregulation in innate signaling responses in BML, particularly at Day 28. Most of these significantly enriched pathways, including *Cytokine-cytokine receptor interaction*, *TNF signaling*, *NF-kappa B signaling*, and *Toll-like receptor signaling* pathways were significantly enriched at both Day 2 and Day 28, suggesting an immediate and sustained innate immune response in human milk, particularly after LAIV. Toll-like receptors (TLRs) play a crucial role in the innate immune system by recognizing pathogen-associated molecular patterns, and tumor necrosis factor (TNF) and NF-kappa B induce expression of various pro-inflammatory genes, including those encoding cytokines and chemokines. The combination of a mucosal vaccine (LAIV) and a mucosal immune mechanism (breastfeeding) at the time of vaccination may lead to more profound innate immune responses that persist for a longer duration, potentially influencing anti-influenza vaccine immune responses in a way that is not captured by the typically measured HAI titers. Indeed, LAIV requires replication of the live attenuated virus in the mucosa of the upper airways to induce protection, so LAIV mimics natural infection and elicits both humoral and cellular immune responses. Another previous study supported this finding by demonstrating higher levels of anti-influenza specific IgA and of adjusted specific IgA in influenza vaccinees through 6 months and significantly lower numbers of respiratory illness with fever episodes in infants of influenza-vaccinated mothers[22].

Two genes (*IL1A* and *IL1B*) encoding for the Interleukin-1 alpha/beta cytokines were strongly differentially up-regulated in the BMLs from pre-vaccination following both LAIV and TIV at Day 28. This response correlated with these cytokines' essential role in regulating immune and inflammatory responses and is expected in both types of vaccines, as cytokines are presumably involved in BML responses to both types of vaccines. The interleukin-1 beta cytokine is primarily induced under disease conditions[23], which may explain why *IL1B* was more strongly differentially up-regulated from pre-vaccination than *IL1A* following administration of LAIV, a vaccine that more closely mimics infection than TIV. Interleukin-1 beta production also increases after in vitro stimulation with

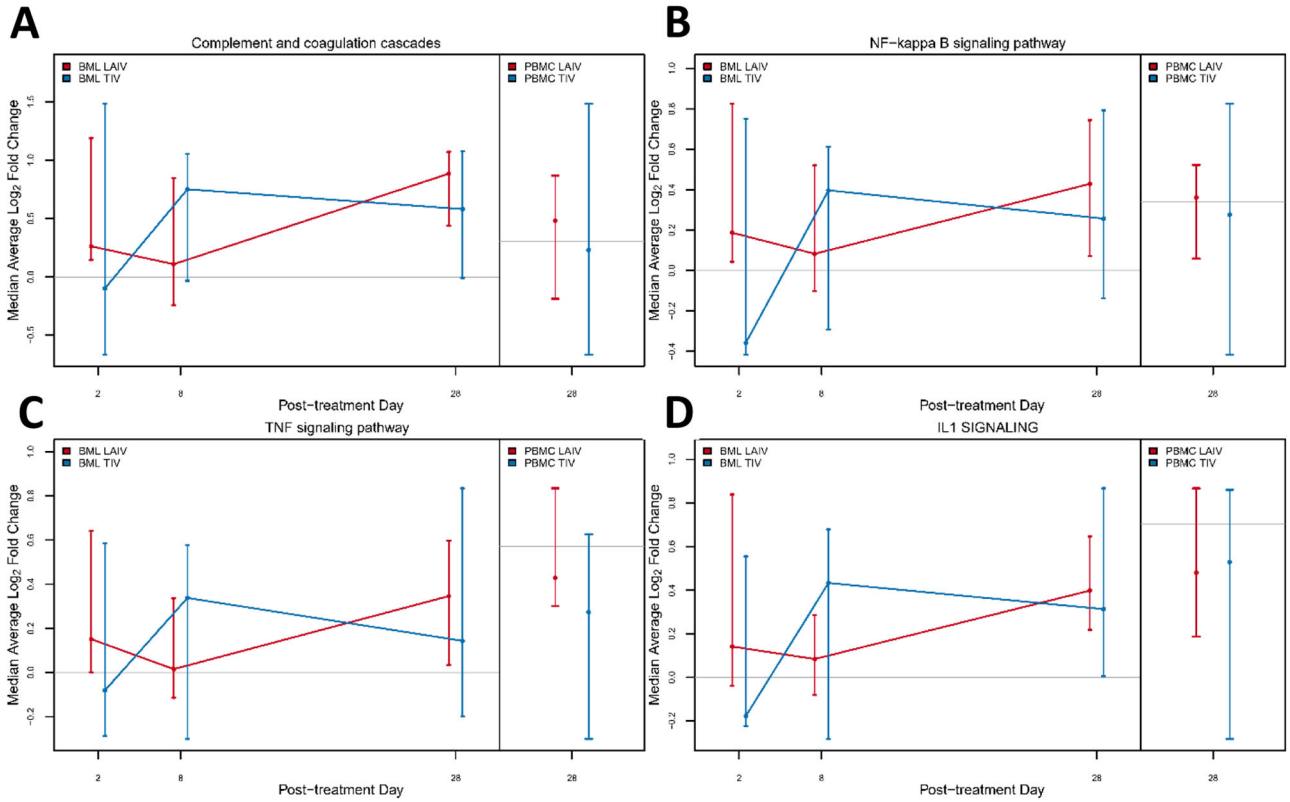

Fig. 3 | Activation of innate immune signaling pathways over time. The median average *log*$_2$ fold change from baseline and associated 95% bootstrap Cis for a subset of enriched KEGG and Reactome pathways is demonstrated by post-vaccination (post-treatment) day, specimen type, and vaccine group. Each panel demonstrates the median average Log_2 fold change in a pathway over time: complement and coagulation cascades (**A**), NF-kappa B signaling (**B**), TNF signaling pathway (**C**), and IL-1 signaling pathway (**D**). LAIV live attenuated influenza vaccine, TIV trivalent influenza vaccine, BML breast milk lymphocytes, PBMC peripheral blood mononuclear cells, TNF tumor necrosis factor, IL1 interleukin 1.

Table 1 | Geometric mean titers (GMTs) by antigen, maternal vaccination group, and study day

	Maternal LAIV				Maternal TIV			
	Study Day				Study Day			
	Day 0		Day 28		Day 0		Day 28	
Antigen	***N***	**GMT (95% CI)**	***N***	**GMT (95% CI)**	***N***	**GMT (95% CI)**	***N***	**GMT (95% CI)**
A/California/7/2009 (H1N1) - 2011/2012 and 2012/2013 Strain	10	32.5 (14.9, 70.7)	10	33.6 (16.5, 68.6)	6	75.5 (22.2, 257.4)	6	119.9 (57.1, 251.8)
A/Perth/16/2009 (H3N2) - 2011/2012 Strain	10	24.6 (10.3, 59.1)	10	30.3 (14.4, 64.0)	6	44.9 (15.4, 131.0)	6	95.1 (38.2, 237.0)
A/Victoria/361/2011 (H3N2) - 2012/2013 Strain	10	37.3 (18.9, 73.6)	10	56.6 (33.1, 96.6)	6	63.5 (19.4, 208.3)	6	179.6 (76.7, 420.3)
B/Brisbane/60/2008–2011/2012 Strain	10	21.4 (8.6, 53.7)	10	27.3 (11.5, 64.7)	6	15.9 (7.5, 33.6)	6	22.4 (11.0, 45.9)
B/Wisconsin/1/2010–2012/2013 Strain	10	7.6 (5.0, 11.5)	10	10.0 (5.9, 16.9)	6	8.4 (5.4, 13.1)	6	37.8 (11.6, 123.1)

lipopolysaccharide, demonstrating the capacity to respond to stimulation after leaving the breast[24].

The *MTRNR2L8* gene was strongly up-regulated from baseline in PBMC and BML following LAIV at Day 28, but not TIV vaccination. This anti-apoptotic gene may represent a marker for differentiating between LAIV and TIV response at 28 days following vaccination, irrespective of specimen type. It is unclear why an anti-apoptotic gene would be more involved in LAIV compared to TIV vaccination, but this gene may be more influential for mucosal immunity associated with LAIV. The replication elicited by the live-attenuated vaccine may benefit from these anti-apoptotic properties[25].

Combining gene cluster time trends and heatmap response profiles with pathway enrichment analysis results, our data showed that, while LAIV generally induced an up-regulation of innate immune signaling pathway genes in BML earlier (Day 2), TIV induced peak innate immune signaling gene responses ahead of LAIV (Day 8 vs. 28), albeit not as strongly. In contrast, a group of interferon-alpha/beta-inducible genes showed slightly higher responses at Day 2 for the TIV group with the LAIV group reaching similar levels by Day 8 and stronger peak levels by Day 28. This suggested that some level of interferon-signaling occurred earlier in the TIV group and that interferon-signaling increased over time for the LAIV group. In PBMCs, the TIV group showed increased responses for this cluster at Day 28 compared to pre-vaccination.

To further assess this in PBMCs, we plotted responses for the innate immune signaling cluster (*IFIT3*, *OAS3*, *IFI44L*, *MX1*, *OAS2*, *IFIT1*, *IFI6*) based on RNA extracted from PBMCs from two prior vaccine studies. One study by Henn et al. assessed RNA-seq for 11 timepoints following TIV vaccination in 6 subjects[26]. Results indicated statistically significant increases for this cluster at Day 1, 2, 3 and Day 7 post-TIV vaccination in PBMCs with peak responses at Day 1 and 7

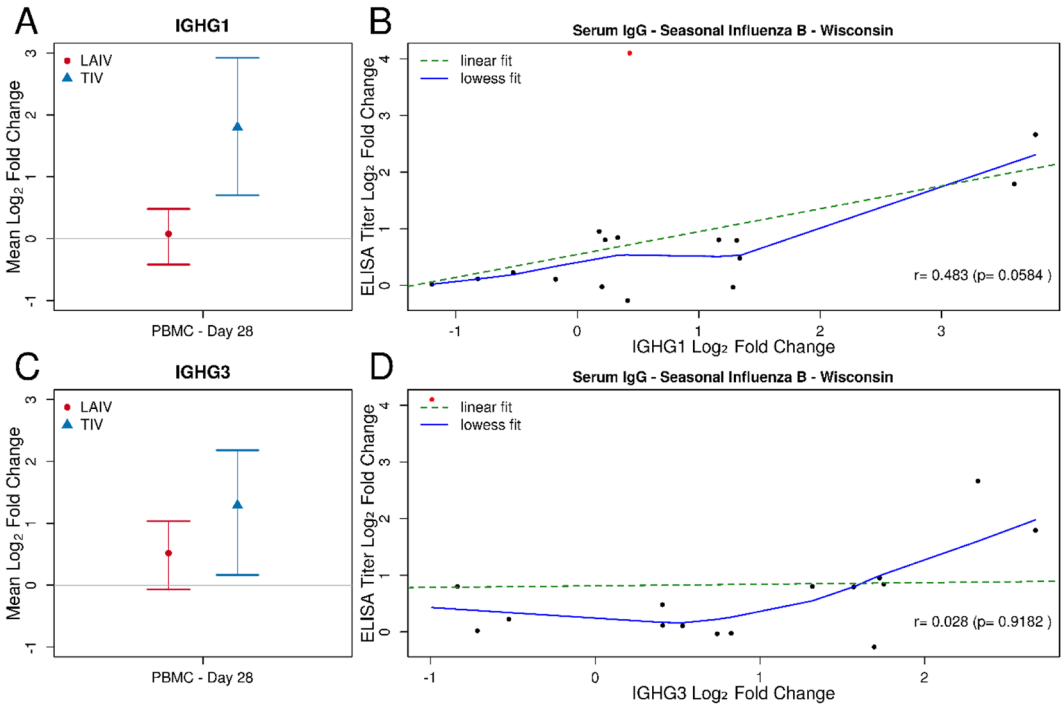

Fig. 4 | Assessment of IgG gene expression in PBMCs and correlation with ELISA IgG titer. In (**A**, **C**), the mean log_2 fold change from baseline and associated 95% bootstrap CIs for co-expressed IgG genes (*IGHG1* and *IGHG3*) in PBMC at Day 28 is demonstrated by vaccine group. For BML, gene expression levels for these two genes did not pass the low expression cut off for both vaccine groups. In (**B**, **D**), the scatterplot displays per-subject log_2 fold change in PBMC *IGHG1* and *IGHG3* gene expression versus log_2 fold change in ELISA IgG titers for the influenza B antigen (Wisconsin) 28 days following TIV or LAIV vaccination ($n = 16$, r: Pearson correlation, in green: linear regression fit, in blue: locally weighted regression fit). Sensitivity analysis following the removal of the outlying observation highlighted in red resulted in a correlation of r = 0.805 and $p = 0.0003$ for (**B**). LAIV live attenuated influenza vaccine, TIV trivalent influenza vaccine, PBMC peripheral blood mononuclear cells, ELISA enzyme-linked immunosorbent assay.

(Supplementary Figure S11). The other study from Nakaya et al. performed a microarray study at Day 0, Day 3, and Day 7 following TIV and LAIV vaccination[19]. Results showed that neither group achieved significant increase compared to pre-vaccination (Supplemental Figure S11). Cao et al. found that "TIV induced expression of IFN genes on day 1 after vaccination in all age groups, and LAIV induced expression of IFN genes on day 7 after vaccination but only in children < 5 years old."[27]

However, as we did not sample early timepoints for BML, we cannot confirm whether these signals observed in PBMCs would translate to BML. Our data did demonstrate that gene expression for these genes remained strongly upregulated in BML at Day 28 following LAIV but not TIV, while the reverse was seen in PBMCs with higher increase in expression of these genes following TIV at Day 28. Cao et al. concluded that early induction of IFN appears to be important for development of antibody responses. The relevance of the later and persistent interferon signaling response we observed in BML for modulating the transmission of immunity to infants would need to be assessed in future studies. While IgA titer in the breast milk was moderately positively correlated with *IGHA1* gene expression in BML for IgA responses against influenza B antigen – Wisconsin and Swine H1N1, the association did not reach statistical significance in our cohort.

There are several limitations to our study. We were primarily limited by the small number of subjects and samples available from the parent study at the time of enrollment. Because the parent study was nearing completion when this study began, and sub-study participants were newly consented prior to enrollment in this sub-study, half of the baseline samples and several Day 2 and 8 samples were missing when we enrolled participants in the sub-study. We accounted for this in the DE gene analysis using an unpaired version of the test and in the time trend visualizations by imputing missing baseline values using the average gene expression of available samples. To make sure we did not introduce a strong bias, we compared values for average-imputed vs. original values for those subjects that had baseline ($N = 8$ for PBMC and $N = 7$ for BMC) using scatterplots (Supplementary Figure S13). Results did not show a strong bias with a Pearson $r > 0.95$ for all samples and specimens. Nevertheless, this negatively impacted statistical power and our ability to detect DE genes overall. In addition, the number of timepoints we sampled was very limited, reducing the resolution of gene expression signals over time. It is also important to note that the LAIV vaccination effect is probably most acute and effective on the nasal mucosa, mimicking natural infection and influencing innate immunity. Thus, comparing the two results in PBMCs and breast milk cells may be a biased toward the vaccine that is given systemically.

Our study identified gene expression signatures that were vaccine and specimen-type specific some of which correlated with later immune responses after vaccination. Importantly, we observed increased innate immune signaling-related gene expression in BML but not in PBMC at Day 28 in the LAIV group. This signal was absent in subjects that received TIV. We hypothesize that breastfeeding extended the duration of the innate response to LAIV via mucosal immunity. Additional larger studies are required to test the interaction between LAIV and the duration of the innate response and its role in transmitting immunity to newborns.

Methods

Analysis population

We performed a randomized, double-blind study in breastfeeding women at Cincinnati Children's Hospital Medical Center who received either LAIV and intramuscular (IM) placebo, or TIV and intranasal (IN) placebo 28–116 days after delivery during the 2011–12 and 2012–13 influenza seasons (Clinicaltrials.gov NLM identifier: NCT01181323)[17]. The study time points all refer to date of vaccination, so all maternal subjects were vaccinated at least 28 days after delivery. The vaccination date (Day 0) coincides with the first date of human milk collection. Licensed TIV

(Fluzone®, Sanofi Pasteur, Swiftwater, PA) and LAIV (FluMist®, MedImmune, Gaithersburg, MD) were used as recommended each year. For the 2011–2012 season, the vaccines included hemagglutinin (in TIV) or fluorescent focus units of live attenuated influenza virus reassortants (in LAIV) of A/California/07/2009 (H1N1)-like virus, A/Perth/16/2009 (H3N2)-like virus, and B/Brisbane/60/2008-like virus. For the 2012–2013 season, TIV included hemagglutinin of A/California/07/2009 NYMC X-179A (H1N1)-like virus, A/Victoria/361/2011 IVR-165 (H3N2)-like virus, and B/Texas/6/2011 (a B/Wisconsin/1/2010-like virus). LAIV contained fluorescent focus units of live attenuated influenza virus reassortants of A/California/07/2009 (H1N1)-like virus, A/Victoria/361/2011 (H3N2)-like virus, and B/Wisconsin/1/2010-like virus. A subset of subjects who agreed to future use, met all inclusion and exclusion criteria, completed the informed consent process for this sub-study, and for whom at least one BML or PBMC specimen was collected (LAIV, $n = 10$ and TIV, $n = 6$) was used for this study (Fig. 5).

Human milk was collected on Days 0, 2, 8, and 28, and blood samples were collected on Days 0 and 28 (Table 2). Mononuclear cells were extracted from approximately 10 mL of each breast milk and blood sample and stored in a −80 degree C freezer. Demographic characteristics of each maternal vaccine group are outlined in Table 3. PBMC and BML RNA was extracted from each sample for analysis. Among the 16 subjects, half (TIV, $N = 4$; LAIV, $N = 4$) had baseline samples (Day 0) available for RNA-Seq testing. We imputed Day 0 values for subjects without available Day 0 samples (see Statistical Analysis).

The protocol and consent forms were approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board (IRB00000231, FWA00002988). Participants provided written, informed consent for themselves and their infants.

RNA-sequencing and data processing

The integrity of all RNA samples was checked using an Agilent 2100 Bioanalyzer. RNA-seq libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Prep Kit and sequenced to a depth of at least 20 million reads per sample on an Illumina HiSeq 2500. Sequencing reads were 75 nucleotides in length and generated in the paired-end configuration. Paired-end reads were mapped against the human reference genome (GRCh38) using TopHat2 (Version 2.1.1). Human gene models and annotations were obtained from ENSEMBL v84, (March 2016). Gene expression quantification was conducted on the gene level using the function featureCounts within the *Subread* v1.4.6 software counting mapped paired-reads to obtain fragment counts per gene. Fragments that overlapped with multiple genes or had multiple mapping locations on the reference genome were excluded.

Statistical analysis

The statistical methods are described below, but the supplementary text includes additional detail. *edgeR* (Version 3.14.0) was used to correct systematic sample differences in fragment counts using TMM normalization[24] to calculate moderated $\log_2$ fragment counts per million (LCPM), and to model fragment counts using a discrete probability distribution. LCPMs were used as input for multivariate analyses to detect outliers, to filter out lowly expressed genes, and for gene clustering analysis. Log_2 fold changes from baseline were calculated for each subject and immune cell type by subtracting the mean of the $\log_2$ baseline values from each of the subject's post-baseline days. For subjects without a baseline sample (8 of 16 subjects), the average LCPM of all available baseline samples for the respective specimen type were used in the fold change calculation to impute missing baseline values. Differentially expressed (DE) genes were identified for each post-vaccination day, vaccine group and specimen type by fitting negative binomial generalized linear models as implemented in *edgeR* under the assumption that discrete fragment counts are negative binomial distributed. Each model included a fixed effect for Day (0:baseline, 1:post-vaccination). For each gene, statistical significance of a contrast comparing the average $\log_2$ post-vaccination counts for a certain vaccine group to the average $\log_2$ baseline counts of all baseline samples for the respective specimen type was evaluated using a likelihood ratio test. We chose this approach over a paired analysis as only 8 of 16 (50%) of subjects had baseline samples available. To control for testing multiple genes, the false-discovery rate (FDR) based on the Benjamini-Hochberg procedure[25] as implemented in the *p.adjust* R function was applied. Genes with an FDR-adjusted p-value < 0.2 and a fold change of ≥1.5-fold were considered DE. To identify robust clusters of

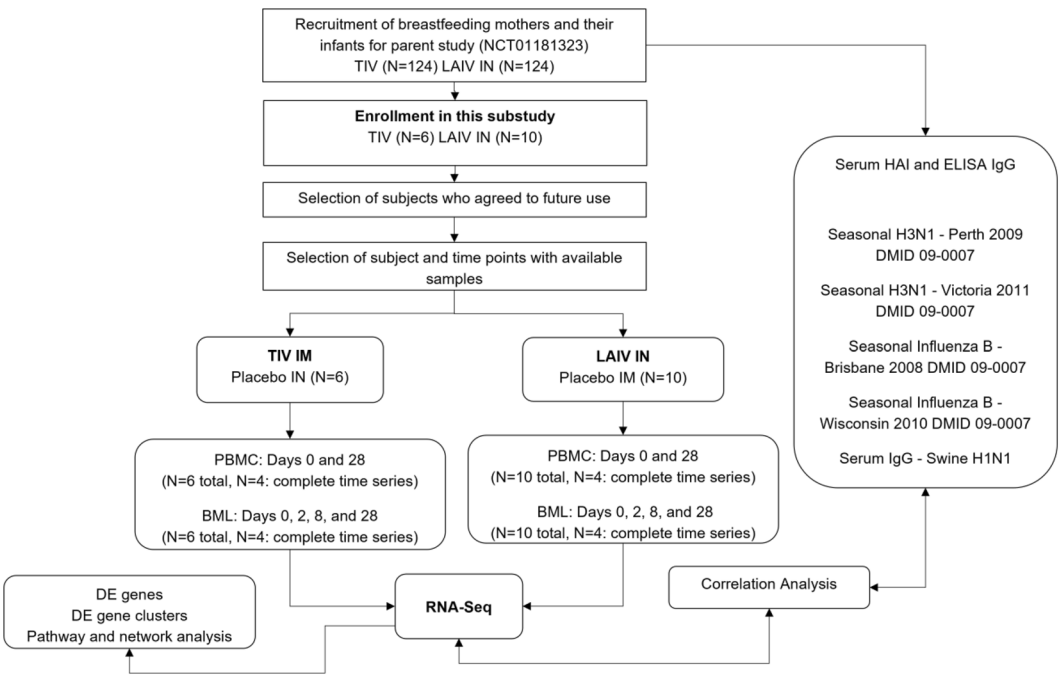

Fig. 5 | Overview of study design. TIV trivalent influenza vaccine, LAIV live-attenuated influenza vaccine, IN intranasal, IM intramuscular, PBMC peripheral blood mononuclear cells, BML breast milk lymphocytes, DE differentially expressed, HAI hemagglutination inhibition, DMID Department of Microbiology and Infectious Diseases.

Table 2 | Summary of study population by vaccine group, specimen type, and time point

Vaccine Group	Breast Milk	Breast Milk	Breast Milk	Breast Milk	Whole Blood PBMC	Whole Blood PBMC	Whole Blood PBMC	Whole Blood PBMC
Subjects	Day 0	Day 2	Day 8	Day 28	Day 0	Day 2	Day 8	Day 28
Subject E	X	X	X	X	X			X
Subject B	X	X	X	X	X			X
Subject J	X	X	X	X	X			X
Subject F	X	X	X	X	X			X
Subject M			X	X				X
Subject K			X	X				X
TIV Total	**4**	**3**	**6**	**6**	**4**			**6**
Subject P	X	X	X	X	X			X
Subject L	X	X	X	X	X			X
Subject C	X	X	X	X	X			X
Subject A	X	X	X	X	X			X
Subject H		X	X	X				X
Subject N		X	X	X				X
Subject G				X				X
Subject O				X				X
Subject I				X				X
Subject D				X				X
LAIV Total	**4**	**6**	**6**	**10**	**4**			**10**

Table 3 | Characteristics of study population by vaccine group

	All Maternal (*N* = 16)	Maternal LAIV (*N* = 10)	Maternal TIV (*N* = 6)
Gender – *n* (%)			
Female	16 (100)	10 (100)	6 (100)
Ethnicity – *n* (%)			
Non-Hispanic	15 (94)	9 (90)	6 (100)
Hispanic	1 (6)	1 (10)	0
Race – *n* (%)			
American Indian/Alaskan Native	0	0	0
Asian	0	0	0
Hawaiian/Pacific Islander	0	0	0
Black/African American	1 (6)	0	1 (17)
White	15 (94)	10 (100)	5 (83)
Multi-Racial	0	0	0
Other/Unknown	0	0	0
Age (Years)			
Mean (STD)	32.0 (3.6)	31.4 (3.0)	32.9 (4.6)
Median	31.5	30.2	33.3
Min, Max	(27.6, 39.1)	(28.0, 38.1)	(27.6, 39.1)

co-expressed DE genes based on $\log_2$ fold changes, multiscale bootstrapping was carried out using the *pvclust R* package[26] (Version 2.0.0). Pathway enrichment analysis to identify significantly enriched KEGG (Version 79.0, 07/16/2016) and MSigDB (Version 5.1, 01/19/2016) pathways was carried out using the *goseq* R package[27] (v1.12.0) correcting for gene length bias and multiple testing (FDR-adjusted p-value < 0.1). We assessed associations between $\log_2$ fold change in ELISA IgG/IgA titers for the 5 vaccine strains and $\log_2$ IgG / IgA gene expression fold changes using Pearson correlation. Analysis was carried out using the R statistical software (Version 3.2.5).

Data availability

Data that support the findings of this study have been deposited in GEO (Accession GSE281592).

Received: 13 September 2023; Accepted: 12 January 2025;
Published online: 20 March 2025

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Acknowledgements

This study was supported by the National Institute of Allergy and Infectious Diseases Division of Microbiology and Infectious Diseases. We would like to thank all of the subjects who participated in the study. We would also like to thank the John Hauck Foundation, Fifth Third Bank, and John W. Hauck, Co.-Trustees for their support.

Author contributions

E.P.S., T.L.J., and J.B.G. wrote the manuscript; E.P.S., M.C.S., and D.I.B. designed the study; J.B.G. conceptualized the analysis; J.B.G. and T.L.J. analyzed the data; P.J.D. performed the experiments; M.C.S. and D.I.B. consulted on the analysis and results; E.P.S. and C.P. curated the clinical data; E.P.S. and C.E.G. reviewed results; and all authors reviewed the manuscript.

Competing interests

Dr. Schlaudecker has received funding from Pfizer and the Centers for Disease Control and Prevention to conduct clinical research studies. She has received support from Sanofi Pasteur as a member of an advisory committee. All other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41541-025-01072-6.

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