

Transcriptome signatures preceding the induction of anti-stalk antibodies elicited by a chimeric hemagglutinin-based universal influenza virus vaccine

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Abstract

A phase 1 clinical trial to test the immunogenicity of a chimeric group 1 HA (cHA) universal influenza virus vaccine targeting the conserved stalk domain of the hemagglutinin of influenza viruses was carried out. Vaccination with adjuvanted-inactivated vaccines induced high anti-stalk antibody titers. We sought to identify gene expression signatures that correlate with such induction. Messenger-RNA sequencing in whole blood was performed on peripheral blood of 53 vaccinees, at early and late time points after priming and boosting. We generated longitudinal data on peripheral blood of 53 volunteers, at early (days 3 and 7) and late (28 days) time points after priming and boosting with cHAs. Differentially expressed gene analysis showed no differences between placebo and live-attenuated vaccine groups. However, an upregulation of genes involved in innate immune responses and type I interferon signaling was found at day 3 after vaccination with inactivated adjuvanted formulations. Cell type deconvolution analysis revealed a significant enrichment for monocytes and different subsets of dendritic cells as mediators for optimal B cell responses and significant increase of anti-stalk antibodies in sera. A significant upregulation of immunoglobulin related genes was only observed after administration of adjuvanted vaccines (either as primer or booster) with specific induction of anti-stalk *IGVH1-69*. This approach informed of specific immune signatures that correlate with robust anti-stalk antibody responses, while also helping to understand regulation of gene expression induced by cHA proteins under different vaccine regimens.

Significance: Antibodies targeting the conserved stalk domain of the hemagglutinin protein of influenza viruses can prevent and reduce the severity of influenza virus infection. We have developed a chimeric HA vaccination strategy targeting these conserved regions and showed that vaccination induced high anti-stalk antibody titers in humans. Here we showed that anti-stalk vaccines elicit robust gene expression signatures similar to those described for subtype-specific influenza vaccines with an early upregulation of genes involved in innate immune response and type I interferon (IFN) signaling needed for an optimal B cell activation and proliferation. The current study provides a better understanding of the mechanism of regulation of gene expression and the transcriptional pathways that must be activated to induce optimal vaccine responses in the context of different universal vaccine strategies.

Introduction

How influenza virus vaccines can be designed to induce strong broadly cross-reactive antibody responses is still debated and intensely studied¹. Most commonly used influenza virus vaccines contain four different vaccine strains: two influenza A virus components belonging to the H1N1 and H3N2 subtypes; and two influenza B virus components derived from the B/Victoria/2/87-like and B/Yamagata/16/88-like lineages. The vaccine strains are selected based on worldwide surveillance and prediction methods to determine the most likely circulating antigenic variants of the upcoming influenza season. However, circulating influenza virus strains are continuously evolving and surveillance efforts are not equally effective around the world and may result in mismatches between circulating strains and vaccine strains leading to reduced efficacy². Influenza virus vaccines are updated annually due to the rapid accumulation

of mutations in the haemagglutinin (HA) - and to a lesser extent - in the neuraminidase (NA) genes of the viruses. This phenomenon, known as antigenic drift, originates from the ability of influenza viruses to escape pre-existing immunity in humans. A second mechanism to evade immune recognition is known as antigenic shift based on the acquisition of genes coding for novel surface proteins. Antigenic shift is responsible for the emergence of pandemic influenza virus strains. While the current licensed seasonal influenza virus vaccines are still the best preventive measure against influenza virus infection^{3,4}, they are strain-specific and have a narrow coverage. We need improved strategies that can provide broad and long-lasting protection against multiple influenza virus strains, including pandemic influenza viruses. Additionally, the development of improved next-generation universal influenza virus vaccines would also prevent seasonal vaccine failure due to mismatches between the predicted strains and the circulating influenza viruses.

We have developed chimeric HA (cHA) vaccination strategies targeting conserved regions of the HA surface protein, particularly the stalk domain⁵⁻⁷. In contrast to the head of the HA, the stalk domain is relatively conserved^{8,9}. However immune responses are largely targeted against the head, which is immunodominant and rather permissive to mutations^{2,10}. To overcome this, the cHAs strategy exposes the immune system sequentially to constructs that share the same stalk domain (group 1, group 2 or influenza B HA stalks) but in combination with different head domains from exotic avian influenza virus subtypes^{6,7,11}. This leads to preferential induction of responses against the stalk through reactivation of memory B cells while the *de novo* response against the novel head domains which the immune system has never encountered before is relatively weak. We used this approach to perform an observer-blind, randomized, placebo-controlled phase I trial (NCT03300050) to assess safety and immunogenicity of chimeric hemagglutinin-based vaccines in adults. Results are published^{5,6} and showed that our cHA-based universal influenza virus vaccine approach is safe and elicited a strong response of broadly cross-reactive antibodies against the HA stalk. Additionally, the antibody response was durable, and anti-stalk antibodies lasted during the 1.5-year follow-up. These data are encouraging since antibodies directed against the stalk of the group 1 HA have demonstrated not only to provide protection against pandemic H1N1 virus infection¹², but also disease severity and development of lower respiratory infections¹³. Additionally, stalk antibodies have shown antibody-dependent cell cytotoxicity (ADCC) and phagocytosis (ADCP) activity, which can help to clear the virus after infection has been established in epithelial cells^{14,15}. Indeed, the induced antibody response by our cHA group 1 vaccines also showed strong activity in both ADCC and ADCP reporter bioassays⁶.

Here we expand on the safety and immunogenicity studies of our group 1 cHA-based universal influenza virus vaccine candidate⁶ by performing RNA sequencing (RNA-seq) of whole blood from vaccinees to identify gene expression signatures that correlate with the induction of group 1 stalk antibodies. We generated longitudinal unblinded RNA-seq data of 53 volunteers, at early (days 3 and 7) and late (28 days) time points after priming and boosting with the cHA vaccines and found that an early upregulation of genes involved in innate immune response and type I interferon (IFN) signaling was followed by upregulation of genes involved in B cell activation and proliferation in subjects with higher anti-stalk

antibody induction. Moreover, cell type deconvolution analysis revealed a significant enrichment for monocytes and different subsets of dendritic cell (DC) that mediated an optimal B cell response. The current study provides a better understanding of the mechanism of regulation of gene expression and the transcriptional pathways that must be activated to induce optimal vaccine responses after vaccination with cHA proteins, that are likely to also be involved in the induction of immunity by other vaccines.

Results

Significant changes in gene expression precede the robust induction of anti-stalk antibodies

This clinical trial was designed under the assumption that adult humans possess pre-existing immunity to the H1 HA including low levels of antibodies and memory B cells with specificity against the HA stalk domain. The objective was to redirect the immune response to the immunosubdominant stalk through sequential vaccination with cHA constructs that feature head domains from avian influenza virus subtypes but share the same H1 stalk domain (**Figure 1A**). Fifty-three volunteers were randomized into three different vaccine groups and two placebo control groups in a regimen of prime-booster vaccination: Group 1 (G1) received cH8/1N1 live attenuated influenza vaccine (LAIV) on day 1 followed by AS03A-adjuvanted cH5/1N1 inactivated influenza vaccine (IIV) on day 85 (LAIV8-IIV5/AS03). Group 2 (G2) received the same vaccination regimen but with nonadjuvanted booster vaccination (LAIV8-IIV5). Group 4 (G4) received adjuvanted cH8/1N1 IIV followed by adjuvanted cH5/1N1 IIV (IIV8/ AS03-IIV5/AS03). Group 5 (G5) served as outpatient placebo group and received PBS intramuscularly twice (PBS-PBS). The sequential vaccination strategy as well as the scheme of vaccination groups and blood collection timeline is shown in **Figure 1A-C**. A detailed description of the trial design, immunogenicity and safety of the experimental vaccines can be found in Nachbagauer *et al*⁶. Of note, an additional saline intranasally placebo group was enrolled (G3) but was not included for the present RNAseq analysis because it was considered equivalent to G5.

Serum samples for antibody quantification were collected at baseline (day 1), and day 29 post-prime; day 85 (pre-boost) and day 113 (day 29 post-boost) (**Figure 1C**). We used data from Nachbagauer *et al*⁶ to represent the anti-stalk antibody induction shown in **Figure 1D-E**. The group receiving the IIV8/AS03 priming (G4) had the higher increase in stalk antibodies while priming with LAIV8 did not induce specific antibodies against the stalk of the HA. Comparable results between the groups were found after the boost, with higher induction in subjects from G1 receiving IIV5/AS03 as a booster, followed by volunteers from Group 2 (IIV5 booster). To investigate early molecular changes after receiving the experimental vaccines, whole blood was subjected to RNA sequencing for transcriptional profiling and the correlation of these changes with the anti-stalk antibody responses found in Nachbagauer *et al*⁶ investigated. For this, blood was collected at baseline (day 1), day 3, day 7 and day 29 post prime; and day 85 (pre-boost), day 93 (day 7 post-boost) and day 113 (day 29 post-boost).

We first performed differential gene expression analysis to assess the dynamic changes in gene expression between each of the vaccination groups compared to placebo. A total of 227 genes were

differentially expressed between the vaccination groups and placebo for all the timepoints, with no significant differences at baseline. A heatmap of average gene expression changes for each comparison indicated that most changes occurred at day 3 and day 7 in the G4 group (IIV8/AS03 – IIV5/AS03), while significant differences were absent for the other vaccination groups after the prime (**Figure 2A**). These responses decreased by day 28 and pre-boost, at day 85. Additionally, we found significant differentially expressed genes (DEGs) for G1 seven days after receiving the booster dose on day 92. The top G4 induced genes at day 3 included genes related to innate immunity activation and IFN signaling while DGEs at day 7 were related to B cell proliferation signatures. Interestingly, the transcriptional profile was similar between these two groups after receiving the IIV/AS03 vaccine for the first time, marked by expression of *IGHG1*, *IGLV1-44* and B cell related genes at day 7 post- prime for G4, and day 7 post-boost in G1.

Next, we performed within-group longitudinal comparisons relative to baseline (day 1) levels. This approach allows not only to characterize the transcriptional programs that are being regulated for each vaccination group, but it also controls for interindividual differences in pre-existing transcript levels. When compared to baseline levels within each group, G4 vaccinees showed significant induction or downregulation of at day 3 (2321 genes) and 7 after priming (81 genes), while no changes were detected after the prime dose with LAIV or placebo (G1, G2 and G5, respectively) (**Figure 2B-C**). The longitudinal comparisons also confirmed that after the booster dose, only the subjects who received the IIV5-AS03 (G1) showed significant induction of gene expression (80 genes). Altogether significant transcriptional responses as early as day 3 after vaccination anticipated significant changes in stalk antibody levels measured 29 days later (**Figure 1C**).

IIV8/AS03 – IIV5/AS03 administration induced early activation of cell signaling and innate immune pathways associated with B cell proliferation and induction of anti-stalk antibodies

To better understand the relationship between anti-stalk antibody induction and the dynamic regulation of gene expression longitudinally, we expanded our previous analysis and investigated the functional pathways perturbed after prime - boost in G4 (IIV8/AS03 – IIV5/AS03) vaccinees. We first analyzed changes early after priming (day 3 and day 7). As shown in **Figure 3A**, significant differences were mostly found on day 3, with 1317 upregulated genes versus 1004 genes downregulated. The number of upregulated genes decreased on day 7 (80 genes) and only two genes were found to be downregulated. By day 29, no significant differences were found relative to the vaccine regimens suggesting that gene expression changes were back to baseline. We next used this analysis to perform gene ontology (GO) enrichment analysis (**Figure 3B**) and to build volcano plots (**Figure 3C-D**). The gene expression changes were associated with 59 (up-) and 33 (down-) biological processes (BP) categories on day 3 (**Supplemental Dataset 1**), which included innate immune responses and responses to virus infection. Particularly, the top-10 enriched GO categories of induced genes included cytokine signaling and immune cell activation (**Figure 3B**). Genes included in these pathways were classical genes induced in response to virus exposure such as *ISG15*, *IFIT3*, *OAS1*, *DDX58* (RIG-I) or *SERPING1*, among others (**Figure 3C** and **Table S1**). In contrast downregulated BP included chromatin organization and regulation of transcription

and histone acetylation, suggesting cell remodeling changes triggered by immune signaling. On day 7, the top-10 GO categories of upregulated genes transitioned from innate immunity to activation of adaptive immune cells (**Figure 3B**), including leukocyte migration, phagocytosis and recognition, and regulation of B cell activation. In total there was enrichment of 17 GO categories on day 7 post-prime (**Table S1**), aligned with a high upregulation of immunoglobulin transcripts and the *CD38* cell activation marker (**Figure 3D** and **Table S1**). When looking at the top upregulated immunoglobulin transcripts, we found that the response was dominated by a variety of clonotypes, with overrepresentation of *IGKV1-39* followed by *IGHG1* > *IGLV1-44* > *IGKV1D-39* > *IGHG3* (**Figure 3D**). As expected, by day 29 gene expression reverted to pre-vaccination levels and no significant differences were found when the peak of the antibody response was detected by serological methods. Similarly, no significant changes of the transcriptional profile were detected on day 85, before the booster dose, or on day 93 (post-boost), consistent with a minimal increase of anti-stalk antibodies in this group (**Figure 1C**).

The immune response is a complex biological process that requires multiple interactions among immune cells with heterogeneous differentiation states. We next decided to interrogate the function and relative cell-type composition of the cells circulating in blood after vaccination with the cHA influenza vaccine. For this we used transcriptional signatures obtained from healthy peripheral blood mononuclear cells (PBMCs) profiled by bimodal protein-RNA measurements with Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq) at the single-cell level¹⁶. These cell type-specific gene expression signatures consisted of 58 cell types and subtypes, including subsets of the T, B and myeloid cell compartment. To simplify cell type groups, subclusters were collapsed into 37 major cell types. We applied a false discovery rate (FDR) < 0.05 (adjusted p value) to deconvolute the bulk DEG data and determine cell type specific signatures enriched or depleted in the peripheral blood of G4 after priming with IIV8/AS03 on day 3 and 7 (**Figure 4A**). Fold-change gene signatures revealed a significant enrichment for monocytes, both classical-CD14+ and non-classical-CD16+, and different subsets of dendritic cells (DC) on day 3 post-prime. This was consistent with the biological processes found to be enriched in the GO analysis, as these populations are main mediators of cell signaling processes, innate immune responses and antigen presentation. Enrichments on day 7 suggested that relative cell type composition changed to an enrichment of B cells with different maturity state: memory > intermediate > naïve of the kappa and lambda chain expressing cells. At this timepoint a significant enrichment was also found for antibody-secreting cells: plasma cells > plasmablast confirming the presence of affinity matured long- and short-life antibody producing cells induced by the universal vaccine prototype. Additionally, plasmacytoid DCs (pDCs) were also induced at day 7. This is in contrast to conventional DC (cDCs) that were enriched earlier, on day 3 post-prime. **Figure 4B** shows the calculated fold-change for 29 cell types, including subsets from the main cell type lineages: CD4+ and CD8+ T cells, B cells, DCs, monocytes, and natural killers (NK) on day 3 and 7 post-prime.

Finally, we quantified the diversity of immunoglobulin (Ig) transcripts that were differentially expressed, as a proxy for the induction of antibodies in response to vaccination. The relative composition of enriched isotypes and subclasses, and the heavy, kappa light and lambda light chain (IGH, IGK and IGL)

loci usage for the variable (V) regions on day 7 after priming with IIV8/AS03 are shown in **Figures 4C** and **4D**. Enriched transcripts included members of all loci (**Figure 4C**). The majority of the response of the constant (C) region was dominated by IGHG and IGHM isotypes, while the most abundant subtypes were, as expected, *IGHG1* > *IGHG3* > *IGHG4* (**Figure 4D**). For the V regions, the majority of IGH genes were from the *IGHV3* (35%) and *IGHV4* (32%) subgroups followed by *IGHV1* > *IGHV5*. For the IGK locus, enriched transcripts were dominated by those from the *IGKV1* (51%) and *IGKV3* (24%) and *IGKV4* (23%) subgroups, while the IGL locus was dominated by *IGLV1* (36%) followed by *IGLV2* (33%) and *V3* (24%), and included transcripts from at least 6 different loci ¹⁷.

Early and strong induction of the antiviral response correlates with high anti-stalk antibody titers

Next, we asked whether individual differences of the induction of specific antibodies targeting the stalk of the HA of group 1 influenza viruses after vaccination with IIV8/AS03 – IIV5/AS03 (G4) could be linked to specific and unique transcriptomic signatures. First, we looked at individual cH6/1 IgG antibody responses before (pre-) and 29 days (post-) after prime. All vaccinees showed similar antibody titers post-prime (**Figure 5A**). We next calculated the fold rise on cH6/1 antibodies as the ratio between post-antibody value to pre- levels for each vaccinee. We then computed the geometric mean rise (GMR) by taking the exponent (log₁₀) of the mean fold rise of all individuals. As shown in **Figure 5B**, GMR (95% CI) was 12.1 (6.9-21.2). We then set a GMR value of 10 as a threshold of higher (>GMR, above the geometric mean) versus lower (\leq GMR, below the geometric mean) vaccine responders. Comparison of both low (n = 6) and high (n = 9) responders at baseline showed no pre-existing differences that could explain the variability in the magnitude of the antibody response after vaccination. We next investigated differences on gene expression on day 3 and 7 post-vaccination by comparing each group to their respective baselines (day 0). A total of 469 (390 up and 79 down) and 942 (620 up and 322 down) genes were differentially expressed for the low and high responders, respectively, on day 3. Among these genes, 310 were upregulated in both groups, all of them related to canonical immune response processes (**Figure 5C-D**). Gene ontology analysis of genes that were exclusively up (n=80) in the low responders returned no significant GO annotated processes, whereas those that were exclusively up in the high responders included additional genes mainly related to innate immune response and cytokine mediated signaling (**Figure 5D**, top 10 categories). In addition, processes exclusively upregulated in high responders included mitochondrial organization, respiratory chain and upregulation of transcription factor activity (**Supplemental Dataset 2**). For the downregulated genes, those that were common or exclusive to either group did not return relevant GO categories implicated in immune activation (**Supplemental Dataset 2**). We then compared levels of the top-10 expressed genes between individuals with low and high antibody titers. Results showed that, while non-significant, expression across the top DEGs was lower in individuals with fold induction of anti-stalk antibodies below the geometric mean (**Figure 5E**). While differences in group size (low, n=6 vs high n=9) could affect the power to detect DEGs, the differences in absolute expression levels in each group suggest that the overall differences between low and high responders was due to the magnitude of the induction rather than the expression of specific gene programs. On day 7, the transcriptional response for both low- and higher- responders converged in the expression of an

adaptive immune response signature, including complement activation, leukocyte migration and B cell activation (**Figure 5D**). Notably, the levels of expression for Ig genes on day 7 were similar regardless of the magnitude of the measured antibody response (**Figure 5F**).

G1 and G4 induce similar functional patterns but different immunoglobulin repertoires

As shown before, priming with LAIV8 did not induce specific antibodies against the stalk of the HA protein (**Figure 1C**). However, LAIV-primed individuals showed a significant increase of antibody titers after boosting with IIV5/AS03, or IIV5 only, in the G1 and G2 groups, respectively. We aimed to characterize the functional pathways perturbed by the booster regimen. We interrogated G1 first since antibody responses on day 113 (29 post-boost) were higher compared to the other vaccination groups after the booster dose. Longitudinal changes of gene expression were determined after normalization by baseline or day 85 (pre-boost) as indicated in **Figure 6A**: day 85, 92 and 113 using baseline as a reference; or day 92 and 113 using day 85 as a reference. No significant differences were found between baseline before priming (day 1) and pre-boost (day 85), and DEGs at day 92 showed comparable transcriptomic profiles independent of the reference used. Enrichment of 72 genes related to B cell activation and immunoglobulin transcription were identified: *IGHG1* > *IGLV1-44* > *IGKV2D-29* > *IGHV1-69* > *IGKV2-28*. Remarkably, we found preferential usage of *IGHV1-69* among the top-10 DEGs. Additionally, *IGHV1-69-2*, and *IGVH1-18* were also found among the top-25 genes induced after the boost. Since anti-HA stalk antibodies are preferentially encoded by immunoglobulin heavy chain V region gene VH1-69 and VH1-18¹⁸, usage of these clonotypes indicated that the vaccine used in this trial induced specific and not generic immune responses against the stalk of group 1 HA protein. Intersection of the top-10 most significant biological processes, and top-10 significant genes at day 92 are shown in **Figure 6B-C**. Finally, gene expression levels at day 113 (day 29 post-boost) were restored to baseline levels before vaccination, similar to those on day 29 after prime.

Before we showed that G1 (LAIV8-IIV5/AS03) and G4 (IIV8-IIV5/AS03) induced similar antibody levels and functional patterns after receiving the inactivated adjuvanted cHA vaccine for the first time, either after boosting or priming, respectively (**Figure 2A**). Since G1 vaccinees were primed with LAIV8, we asked whether relative cell type composition and relative Ig repertoire would also be the same. Cell type deconvolution (shown in **Figure 6D**) indicated similar enrichment of cell types enriched in G1 after boost compared to G4 after prime (**Figure 4A**). However, some differences were found when comparing the Ig transcripts encoding for the C region of immunoglobulin heavy chains. Ig alpha (IgA) is the major immunoglobulin class in body secretions and can be found on linings of the respiratory tract, digestive system, and saliva¹⁹. It was expected that the administration of LAIV8 would induce mucosal antibodies at the mucosal surfaces. However, saliva and serum samples analyzed by enzyme-linked immunosorbent assays (ELISAs) for anti-stalk IgA and secretory IgA (sIgA) showed no such antibodies in G1 or G2 after LAIV administration⁶. Contrary to this, when we look at the relative abundance of the Ig repertoire transcripts (**Figure 6E-F**), we found that it was still dominated by IGHG, but vaccinees from the G1 showed also an enrichment of the IGHA subclass (19%). This contrasted with G4, in which the response was dominated by IGHG followed by IGHM and no IGHA transcripts were detected (**Figure 4C**). Except for the

presence of IGHA1 transcripts, the composition of transcripts from the C region in G1 (**Figure 6F**) was similar to G4 (**Figure 4D**). However, the diversity and the relative proportion of Ig transcripts at the V chains from the IGH, IGK, and IGL loci was also different from those elicited by the G4. Subgroups of encoded transcripts for G1 included *IGHV3* (46% each), *IGHV1* (35%), and *IGHV4* (19%) only, whereas the IGK responses were dominated by the *IGKV3* subgroup (*IGKV3* and *IGKV3D* accounted for 45% of *IGKV* detected transcripts) followed by the *IGKV3* (29%) and *IGKV2* (6%). Lastly, the induced IGL transcripts were less diverse compared to G4, with only *IGLV1* (57%), *IGLV3* (23%), and *IGLV2* (20%) subgroups being expressed. The immunoglobulin repertoire on day 7 after boost with IIV5/AS03 across the differentiation spectrum is shown in **Figure 6E**. The observed differences in the repertoire of Ig transcripts and detection of IgA expression shows that the intramuscular adjuvanted vaccine effectively boosted previously undetectable mucosal immunity induced by the LAIV8 prime. This in contrast with G2 for which the only difference in vaccine regime was the presence of adjuvant in the booster dose.

Finally, similar to G4, we looked at expression levels across individuals that developed low (\leq GMR) or high ($>$ GMR) cH6/1 antibody titers, as measured by ELISA. Both groups showed similar levels of gene expression on day 7 post-boost, regardless of measured antibody titers at the late timepoint post-boost, on day 113 (**Figure 6G**). This was independent of pre-existing immunity as Ig transcript levels before priming or boosting showed no correlation with induction of antibodies levels.

Differences of vaccine induced antibody responses attributed to the adjuvant

Because we observed that higher IgG responses in serum were associated with the use of the AS03 adjuvant after boosting with the inactivated formulation, we interrogated the specific contribution of the adjuvant to the gene expression signatures detected. For this, we compared DEGs between G1 (IIV5/AS03) versus G2 (IIV5) on day 7 post-boost in parallel to DEGs between G1 (IIV5/AS03) and G5 (PBS) at the same time point. Analysis showed 93 (up-) and 28 (down-) genes for the G1 vs. G2 comparison; and 29 (up-) and 10 (down-) for the G1 vs. placebo (**Figure 7A-B**). When we compared the GO categories enriched, we determined that both comparisons shared categories such as complement activation (classical pathway), leukocyte migration, and regulation of B cell activation. In contrast, some categories were unique for the G1 vs. G2 including phagocytosis (recognition and engulfment), immune-activating cell surface receptor signaling pathway or protein folding (**Figure 7C**). Finally, we considered that the comparison of G1 versus G2 assessed the contribution of the adjuvant independently (although in the context) of IIV5, while the comparison of G1 versus G5 – placebo – assessed the contribution of both IIV5 and AS03. If we then intersect the datasets obtained, the induction or depletion of those genes in common should be driven by the use of the AS03 adjuvant independently. As shown in the Venn-diagram (in **Figure 7D**), 27 of the induced genes were shared between both datasets. While 12 of them were directly related to transcription of Ig: *IGHG1*, *IGHG3*, *IGKV1D-39*, or *JCHAIN* among others; 15 referred to other processes such as regulation of innate and adaptive immune responses, including complement activation, response to stress, and B cell development: *IRF4*, *XBP1*, *ITM2C*, *MZB1*, *PDIA4*, *POU2AF1*, *STT3A*, *TNFRSF17* (**Supplemental Table 1**).

Discussion

Universal influenza virus vaccines are urgently needed. We have carried out a phase 1 clinical trial to test the immunogenicity of a chimeric HA-based (cHA) group 1 universal influenza virus vaccine and showed that the cHA vaccine was safe and induced high anti-stalk antibody titers with broad activity against group 1 HAs (as reported in Nachbagauer *et al*⁶. In particular, subjects vaccinated with adjuvanted, inactivated cHA vaccines at either prime or boost -IIV8/AS03 or IIV5/AS03, respectively- induced high anti-stalk antibody titers⁶. To further our understanding, we used a systems biology approach and performed longitudinal transcriptomics analyses after both prime and boost with the universal influenza virus vaccine prototype.

While the transcriptional changes that occur during influenza vaccination and infection have been previously described^{20,21} some of the early studies were performed with microarrays (using predefined sets of immune related transcripts), in the context of conventional subtype/strain specific vaccines, or enriched for specific cell types²²⁻²⁴. For this study we generated global transcription profiles from whole-blood bulk RNAseq data and show that gene expression changes associated with two universal vaccine regimes (IIV5/AS03-IIV5/AS03 and LAIV8-IIV5/AS03) results in induction of cell composition changes and gene programs that correlate with robust antibody responses, in this case to anti-stalk epitopes.

This study complements and adds on our previous profiling of serological and mucosal anti-stalk vaccine responses²⁵ by dissecting the cellular composition of the immune response induced by the different vaccination schemes. As shown by Nachbagauer *et al*, immunogenicity for G1 and G4 (IIV5/AS03-IIV5/AS03 and LAIV8-IIV5/AS03) was similar at the protein level, however, differences in diversity observed for the Ig transcripts between these groups suggest that these two groups may have developed different Ig repertoires with yet unknown roles in response to infection.

Our study also provides the transcriptional context of immune responses that lead to the development of broadly neutralizing antibodies specific to the HA stalk in the same individuals²⁵. When we compared transcriptional responses according to vaccination groups, our data demonstrated that subjects with optimal stalk antibody responses showed an early upregulation of genes involved in innate immune response and type I IFN signaling. As expected, triggering the innate immune compartment corresponded to a robust upregulation of genes involved in B cell activation and proliferation in subjects from G4. Additionally, we found a number of consistently enriched blood cell type specific signatures associated with these processes. An early enrichment of monocytes, and different subsets of dendritic cell (DC) cells was especially marked on day 3 reflecting innate immune activation, while cell type composition transitioned to B cell responses on day 7. Importantly, our analyses suggest the transient enrichment of long-lived plasma cells in peripheral blood post vaccination, which is a desirable quality for any vaccine^{26,27}. However, the accurate identification of cell type-signatures in our RNAseq data is subject to the accuracy of the reference data set used to deconvolute this data, and the presence of the different cell subtypes should be confirmed by alternative methods. While not surprising, these results support the inclusion of immunomodulatory components as a means to improve vaccine performance and even in to

reinforce mucosal immunity²⁸⁻³⁰. Adjuvanted-vaccine recipient groups were not only the ones that show significant changes in gene expression in response to vaccine administration, but we also found that differences of transcriptional programs were highly dependent on the use of the adjuvant. In particular, vaccinees boosted with IIV5/AS03 (G1) showed a unique and distinct upregulation of biological processes directly related to activation and developmental programs of the immune system when compared to IIV5 only (G2). For example, XBP1 and IRF4 were correlated with the use of the adjuvanted vaccine. These two transcription factors are essential in the differentiation of activated B cells into plasma cells³¹⁻³⁴. XBP1, in particular, is part of the endoplasmic reticulum (ER) stress response, the unfolded protein response (UPR), and well known for its role not only on B-cell expansion, maturation, and class switching of antibodies for an optimal immunoglobulin production, but also almost every immune cell type³⁵⁻³⁸. In contrast IRF4 is essential for plasma cell survival³⁹. The apparent differences found in the induction of such factors in the adjuvanted vaccine group compared to IIV5 only, reflects the direct activation of immune compartments at multiple cell levels by the AS03-formulated vaccines, which is likely to be related to the molecular mechanisms associated with AS03 adjuvancy.

In addition, we also investigated the gene signatures associated with the magnitude of the antibody responses. This hasn't been previously shown for universal influenza vaccines, and it is important since a stalk antibody titer of 10^3 and 10^4 can suffice to prevent and reduce the severity of influenza virus infection, respectively^{12,13}. Importantly, the anti-stalk antibody titer after vaccination with the cHA in this trial exceeded those levels. After comparison of high (> GMR) versus low (\leq GMR) responders in G4, we found that individuals with greater induction did not have an independent transcriptomic profile, but rather higher levels of early-induced signatures of inter-related biological pathways on day 3, which promoted an optimal immune response. On the other hand, levels of expression for immunoglobulin genes were similar regardless of the magnitude of the measured antibody response. While this study was not designed to address within vaccine-group differences, these findings, suggest that more granular analyses and fine-tuning of early responses (day 1 to 3 post-vaccination) might be crucial to define and understand vaccine-correlates of protection. Nonetheless, both high and low responders showed specific and not generic usage of immunoglobulin transcripts such as *IGHV1-69* or *IGKV1-39* frequently used by stalk-binding heterosubtypic antibodies^{18,40-42}. Finally, our study also allowed for the simultaneous detection of the overall transcriptional responses of immunoglobulins within the transcriptome of the vaccinated volunteers. Profiling of the Ig repertoire in G4 after vaccination showed primary and secondary responses with induction of both IgG and IgM isotypes. More importantly, different subtypes could be distinguished, with high frequency of IGHG1 and IGHG3, classically related to higher potency of antibody mediated functions, such as ADCC or complement activation. In contrast, *IGHG2*, typically related to response to bacterial infections, was not induced. Another key observation was the detection and observed enrichment of IGHA transcripts, particularly *IGHA1*, after boosting with IIV5/AS03 (G1) in subjects primed with LAIV. This is relevant since IgA responses were not detectable in serum or on mucosal surfaces after any of the vaccine doses and demonstrates that system biology approaches, such as transcriptomics, can provide a comprehensive picture of how the immune system responds to vaccination. Further studies will help to understand the necessary pathways that need to be triggered to

induce anti-stalk antibodies after vaccination with cHA influenza vaccines in combination with different vaccine regimes. Such insight can be exploited to develop effective universal influenza virus vaccines.

Materials And Methods

Study design

A randomized, placebo-controlled, observer-blind, phase I clinical study was conducted at Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA) and Duke Early Phase Clinical Research Unit (Durham, NC, USA). This clinical trial was designed to assess the safety and immunogenicity of a chimeric hemagglutinin-based vaccine approach; and its ability to elicit broadly cross-reactive antibodies against the hemagglutinin stalk domain of influenza viruses. The trial design has been previously published^{5,6}. Briefly, 66 participants were block-randomized in each site and received LAIV8-IIV5/AS03 (Group 1, G1), LAIV8-IIV5 (Group 2, G2), SALINE-PBS (Group 3, G3), IIV8/AS03-IIV5/AS03 (Group 4, G4) or PBS-PBS (Group 5, G5). The Cincinnati Children's Hospital Medical Center Institutional Review Board (IRB) served as the central IRB of record for review, approval and oversight of this study on behalf of the Icahn School of Medicine at Mount Sinai IRB, Duke IRB and PATH Research Ethics Committee. All patients provided written informed consent prior to participation. ClinicalTrials.gov identifier NCT03300050. De-identified samples were made available for this analysis (Icahn School of Medicine at Mount Sinai IRB approval #IRB-17-01779).

Study participants

The study was carried out between 10 October 2017 to 9 August 2019. General inclusion criteria were male or non-pregnant female between, 18 and 39 years at the time of the first vaccination. Exclusion criteria included previous history of Guillain-Barré syndrome, immunosuppression, history of influenza virus vaccination within 6 months prior to study enrollment or use of any other investigational drug or vaccine other than in the present study, among others. A complete list of inclusion and exclusion criteria is provided at <https://clinicaltrials.gov/ct2/show/NCT03300050>.

Declarations

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Author contributions: TA and AGR are co-first authors since they both supervised the RNAseq study with the help of AGS, FK, PP and HVB. TA and AGR analyzed data and prepared the figures. TA wrote the manuscript with help of AGR. Order was assigned based on involvement on conception, design, analysis and writing. DS provided data on the antibody response. MAA, VN and SS performed RNA extractions and library prep. CM provided administrative support. All the authors reviewed and edited the manuscript.

Competing interests: The Icahn School of Medicine at Mount Sinai (ISMMS) has issued patents and filed patent applications covering the use of chimeric hemagglutinin antigens as vaccines. A.G.-S., P.P. and F.K. are named as inventors on these patents and applications. The ISMMS and the inventors have received payments as consideration for these rights. The laboratories of A.G.-S., P.P. and F.K. were also engaged in a research program that was funded by GlaxoSmithKline Biologicals SA. All other authors declare no competing interests. The A.G.-S. laboratory has received research support from Pfizer, Senhwa Biosciences, Kenall Manufacturing, Avimex, Johnson & Johnson, Dynavax, 7Hills Pharma, Pharmamar, ImmunityBio, Accurius, Nanocomposix, Hexamer, N-fold LLC, Model Medicines, Atea Pharma and Merck, outside of the reported work. A.G.-S. has consulting agreements for the following companies involving cash and/or stock: Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Vaxalto, Pagoda, Accurius, Esperovax, Farmak, Applied Biological Laboratories, Pharmamar, Paratus, CureLab Oncology, CureLab Veterinary and Pfizer, outside of the reported work.

Data availability and sharing: All data is available in the manuscript or the supplementary materials.

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Figures

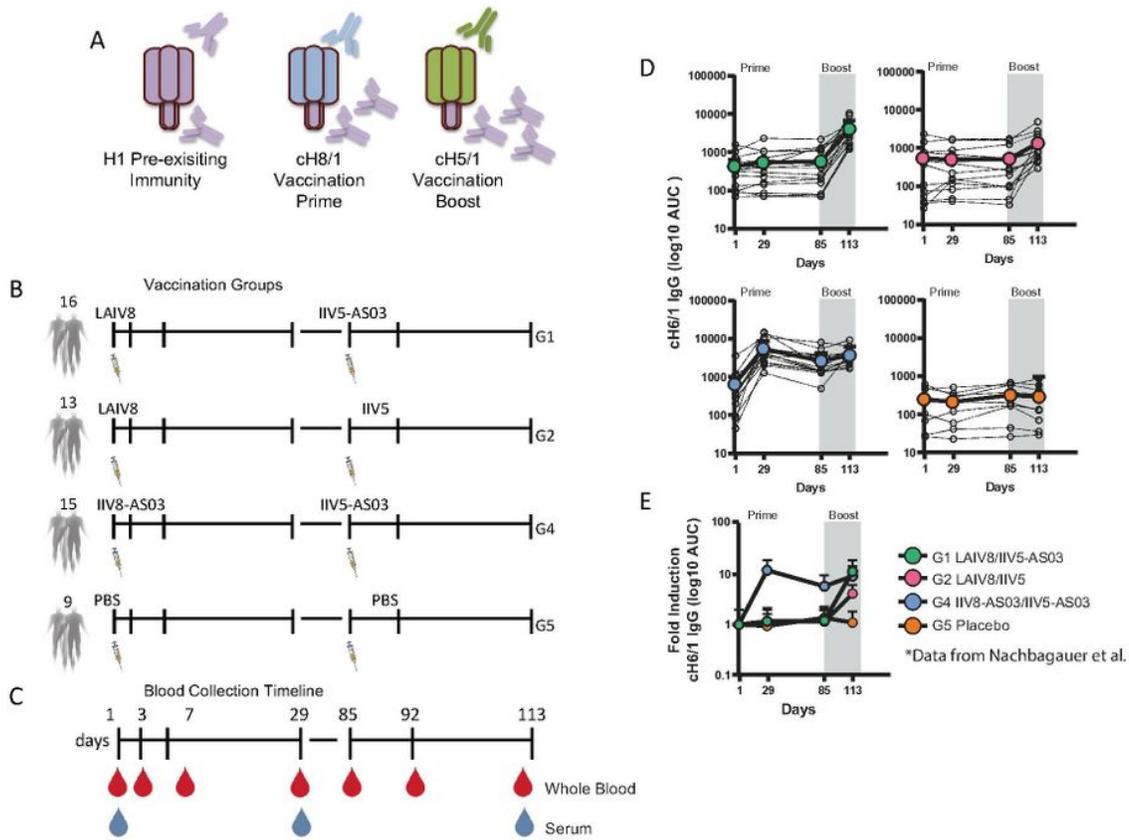


Figure 1. Overview of design and experimental approach (CVIA057; ClinicalTrials.gov identifier NCT03300050). A) Sequential cHA vaccination strategy and the different vaccination groups. B) Study design including vaccination and blood collection timeline. Blood for transcriptomic profiling was collected (indicated in red) at baseline (day 1), days 3, 7 and 29 post prime; and on day 85 (pre- boost), day 93 (day 7 post- boost), and day 113 (day 29 post- boost). Paired samples for antibody quantification (light blue) were collected only on days 1, 29, 85 and 113. Three different vaccination regimens as well as placebo groups were included in the trial. Group 1 received

Figure 1

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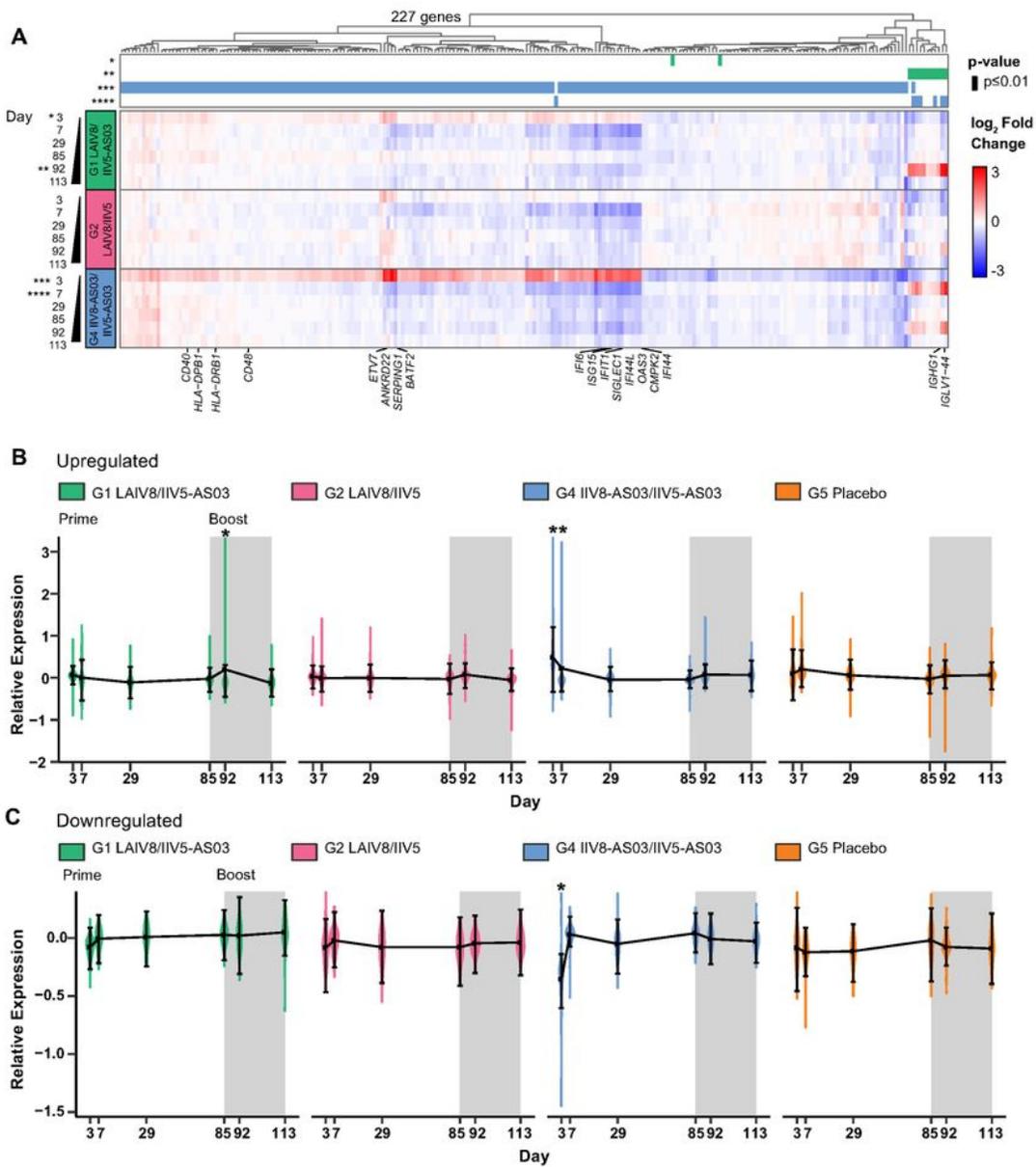


Figure 2. Global transcriptional profile in the CVIA 057 clinical trial. A) Heatmap of differentially expressed genes (DEG) in the groups receiving the experimental vaccine compared to placebo (G5: PBS/PBS): group 1 (G1: LAIV8-IIV5/AS03), group 2 (G2: LAIV8-IIV5) and group 4 (G4: IIV8/ AS03-IIV5/AS03). Average log₂ fold changes (FC) DEG in each group against placebo are shown at the indicated time points (adjusted p value ≤ 0.01). B-C) Temporal expression changes of induced (>0) and downregulated genes (<0) relative to baseline (day 1) levels in each of the vaccination groups. Asterisk indicates adjusted p value ≤ 0.01 .

Figure 2

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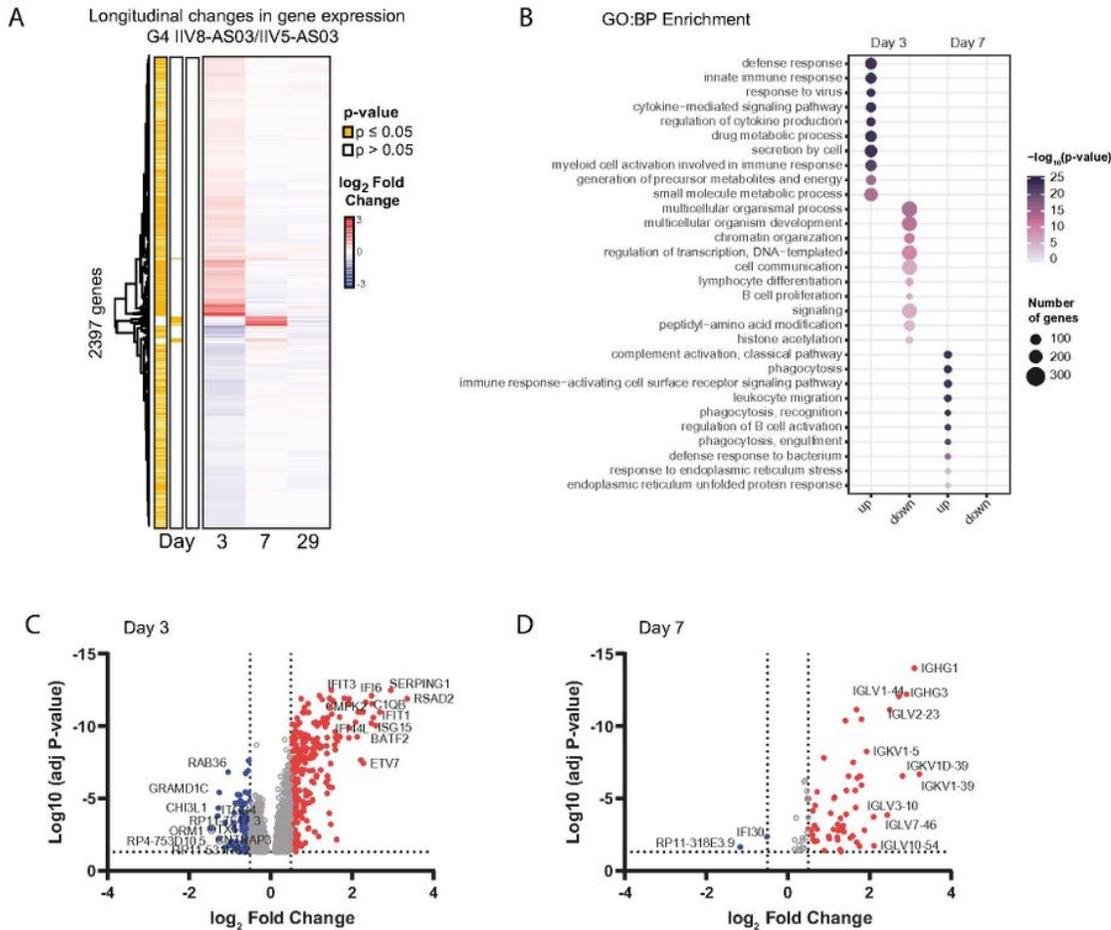


Figure 3. Longitudinal transcriptional profile of IIV8/AS03-IIV5/AS03 vaccination group (G4). A) Heatmap of DEG changes on day 3, 7 and 29 of subjects of G4. Average fold change relative to baseline levels is shown and statistically significant differences are highlighted in yellow (adjusted p value < 0.05). B) Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) on day 3 and 7 priming with IIV8/AS03. Diagram shows the top 10 enriched biological processes. Gene ontologies are ranked by their significance and dot size indicates overlap on number of genes. C-D) Volcano plots of upregulated (red) and downregulated (blue) genes. Expression differences > 1.5-fold change (FC) are highlighted ($\log_2FC \leq 0.5$, vertical dotted lines). The names of the top 10 genes are shown.

Figure 3

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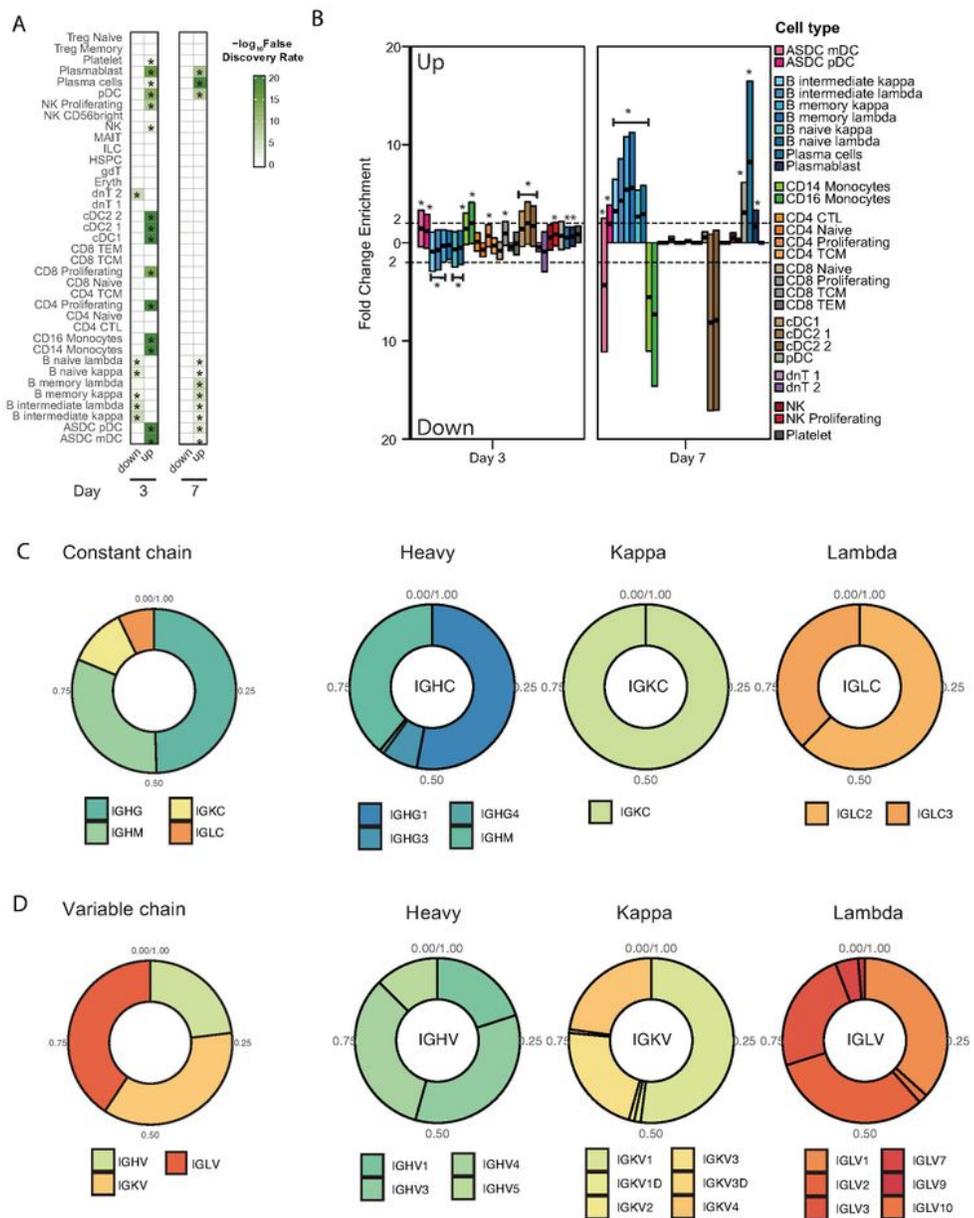


Figure 4. Cell type deconvolution and induction of immunoglobulin genes in G4. A) Main cell types enriched or depleted in the peripheral blood of patients after priming with IIV8/AS03 compared to baseline (day 1) on day 3 and 7. Asterisk indicates FDR < 0.05(16). B) Fold change enrichment of intersected gene sets of G4 after cell type deconvolution on day 3 and 7. Bar indicates fold change enrichment (up) or depletion (down) for each cell type on day 3 and 7. C-D) Relative abundance of immunoglobulin transcripts on day 7 after priming with IIV8/AS03. Relative proportion of enriched isotypes and subclass transcripts corresponding to VJ recombination is shown for the constant (panel C) and the variable (panel D) heavy, kappa and lambda chains (IGH, IGK, and IGL).

Figure 4

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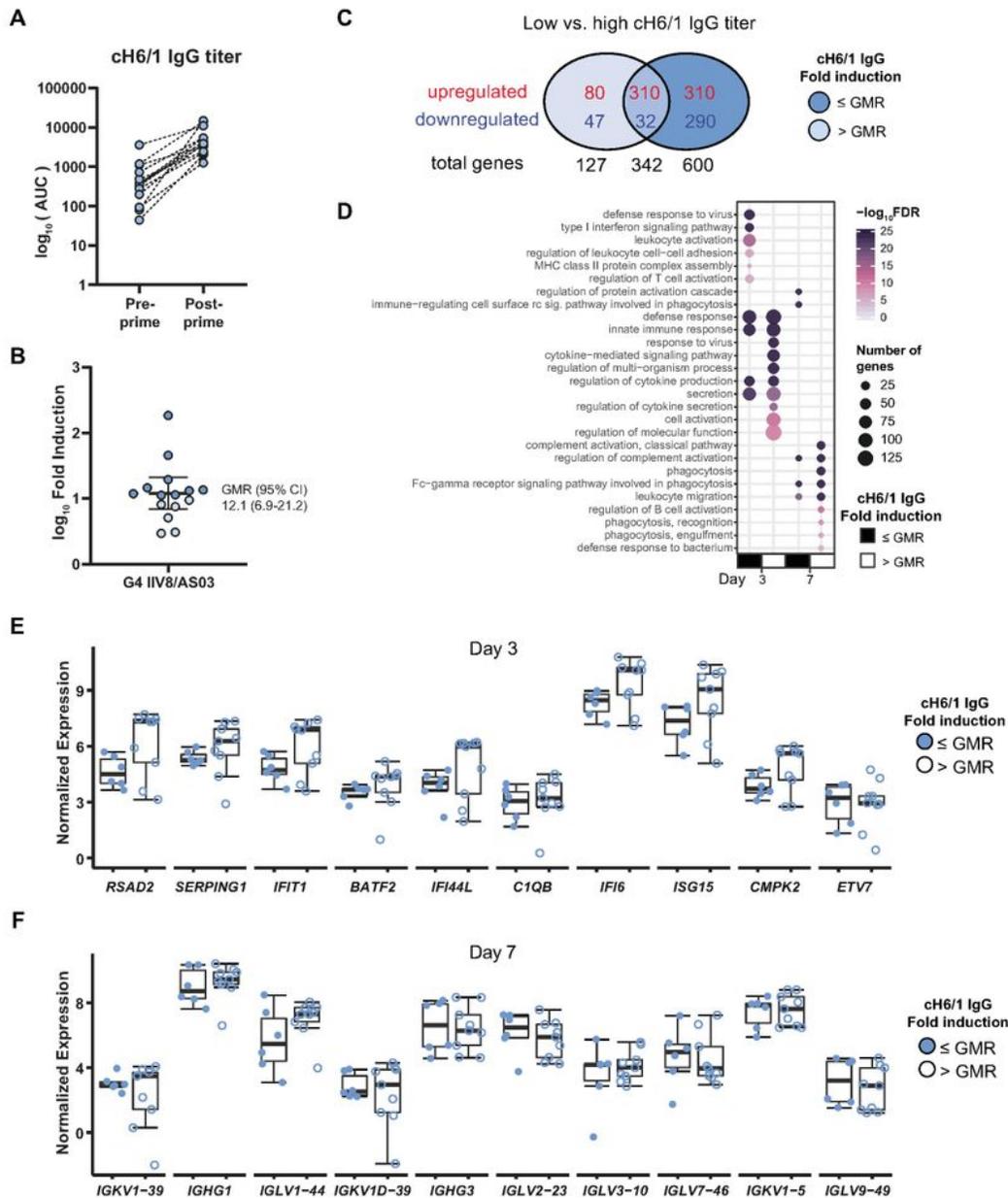


Figure 5. Transcriptional changes in subjects with high induction of anti-stalk antibodies in G4 after priming with IIV8/ASO3. A) IgG titers against cH6/1 in group one subjects after prime. Figure shows titers at baseline (day 1) and day 29 after vaccination with IIV8/ASO3. B) Fold induction after vaccination is also shown. Geometric mean rise (GMR) titers were calculated and GMR (95% CI) is indicated. C) Venn diagram showing the number of DEGs between subjects with low (\leq GMR, light blue) or high ($>$ GMR, dark blue) fold induction of anti-stalk antibodies at day 3 and 7 after prime. D) Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) at day 3 and 7 after priming with IIV8/ASO3 in subjects with \leq GMR fold induction compared to subjects with fold induction $>$ GMR. The top 10 enriched GO:biological processes are shown ranked by their significance, and dot size indicates overlap on number of genes. E-F) Normalized expression of top 10 genes at the indicated time points in subjects according to anti-stalk antibody induction relative to the GMR titers.

Figure 5

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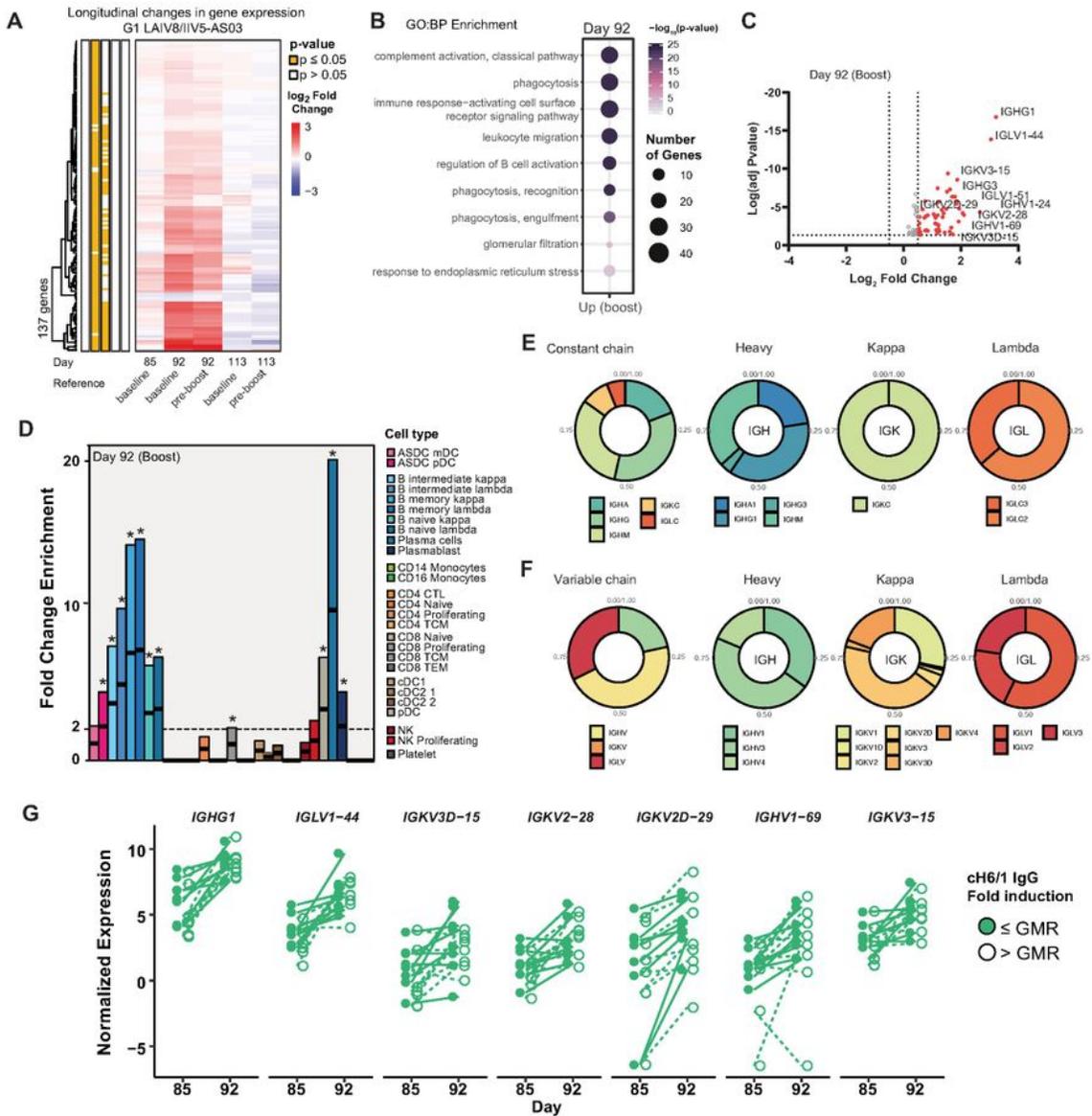


Figure 6. Transcriptomic profile of LAIV8-IIV5/AS03 vaccination group (G1) on day 92 (7 days after the booster dose). A) Heatmap of longitudinal DEG changes at day 92 (day 7 after boost) and day 113 (day 29 after the boost) of subjects in G1 compared to day 1 before priming with LAIV87 and after priming on day 85. Average log₂ fold change from baseline and on day 85 are shown and statistical differences are highlighted in yellow on the left. B) Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) on day 7 after boost. Diagram shows top 10 enriched biological processes ranked by their significance, and dot size indicates overlap on number of genes. C) Volcano plots of upregulated (red) and downregulated (blue) genes. Differences > 1.5 -fold are highlighted (vertical dotted lines). The names of the top 10 genes are shown. D) Fold change enrichment for cell-type specific gene sets in G1 at day 7 after boost with IIV5/AS03. Bar indicates fold change enrichment (up) or depletion (down) for each cell type after deconvolution. E-F) shows relative proportion of enriched isotypes and subclass transcripts corresponding to VJ recombination is shown for the constant (panel E) and the variable (panel F) heavy, kappa and lambda chains (IGH, IGK, and IGL). G) Dotplots of expression of the top 10 upregulated genes in G1 from day 85 to 92 according to fold induction of anti-stalk antibodies in serum (empty dot $>$ than GMR, filled dot \leq GMR). GMR: Geometric mean rise.

Figure 6

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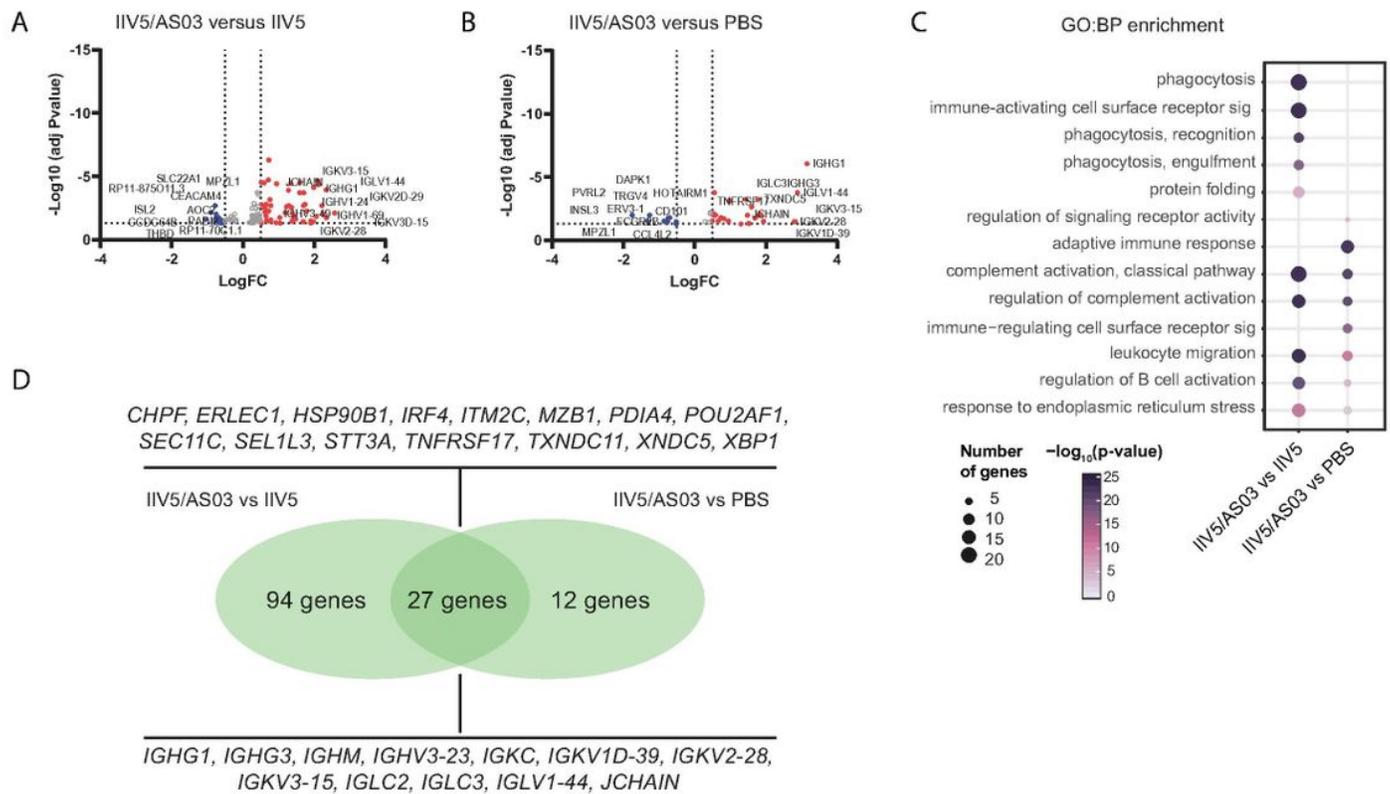


Figure 7. Contribution of AS03 adjuvant to gene expression changes. A) Volcano plots of upregulated (red) and downregulated (blue) genes in adjuvanted G1 (IIV5/AS03 booster) vs non-adjuvanted G2 (IIV5) post boost. Differences higher than 1.5-fold are highlighted and names for the top 10 genes are shown. B) Volcano plots of upregulated (red) and downregulated (blue) genes in adjuvanted G1 vs placebo (G5) groups. C) Venn diagram of DEGs for the comparison between G1 (IIV5/AS03 booster) versus G2 (IIV5), and G1 (IIV5/AS03 booster) versus G5 (placebo) at day 92. D) Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) at day 7 after boost for the indicated comparisons. Diagram shows top 10 enriched biological processes. Gene ontologies are ranked by their significance and dot size indicates overlap on number of genes.

Figure 7

See image above for figure legend.

Supplementary Files

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