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Proteomics show antigen presentation processes in human immune cells after AS03-H5N1 vaccination

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Abstract

Adjuvants enhance immunity elicited by vaccines through mechanisms that are poorly understood. Using a systems biology approach, we investigated temporal protein expression changes in five primary human immune cell populations: neutrophils, monocytes, natural killer (NK) cells, T-cells, and B-cells after administration of either an Adjuvant System 03 (AS03)-adjuvanted or unadjuvanted split-virus H5N1 influenza vaccine. Monocytes demonstrated the strongest differential signal between vaccine groups. On day 3 post-vaccination, several antigen presentation-related pathways, including MHC class I-mediated antigen processing and presentation, were enriched in monocytes and neutrophils and expression of HLA Class I proteins was increased in the AS03 group. We identified several protein families whose proteomic responses predicted seroprotective antibody responses (>1:40 Hemagglutination inhibition titer), including inflammation and oxidative stress proteins at day 1 as well as immunoproteasome subunit (PSME1 and PSME2) and HLA Class I proteins at day 3 in monocytes. While comparison between temporal proteomic and transcriptomic results showed little overlap overall, enrichment of the MHC Class I antigen processing and presentation pathway in monocytes and neutrophils was confirmed by both approaches.

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Conflict of Interest

The authors have declared no conflict of interest.

Keywords

AS03; Avian influenza; Immune response; Quantitative Proteomics; Vaccine

Developing vaccines against highly pathogenic avian influenza H5N1 is an important public health priority due to the high morbidity and mortality associated with infection [1]. However, vaccine development has been hindered by poorer immunogenicity of the H5N1 hemagglutinin (HA) protein when compared with seasonal influenza vaccines [2]. Oil-in-water emulsion adjuvants have demonstrated great promise in enhancing antibody responses at low antigen doses [3, 4]. Adjuvant system 03 (AS03) [5, 6], an α -tocopherol-based oil-in-water emulsion adjuvant, has been shown to markedly enhance antibody responses, increase the frequency of H5N1-specific memory B cells and CD4+ T cells, and to induce both cross-reactive antibody and CD4+ T cell responses when administered with an H5N1 vaccine [7]. In mouse models, AS03 increased production of monocyte- and neutrophil-recruiting chemokines, promoted the migration of antigen presenting cells to the draining lymph nodes, and enhanced antigen uptake by monocytes [8]. Recently, it was reported that injection of AS03 in rabbits induced a transient inflammatory response as measured by an increase in neutrophil number in the blood [9]. While AS03 enhances the immune response to H5N1 vaccine antigens, the underlying molecular mechanisms are still poorly understood.

Systems vaccinology allows the human immune response to be comprehensively studied by combining -omics analysis with immune responses to increase our understanding of the mechanisms by which vaccines and adjuvants confer protection [10–14]. Such studies typically focus only on transcriptome analysis. A comprehensive systems vaccinology approach that includes both transcriptomics and proteomics would enhance the functional and mechanistic understanding of vaccine responses.

We developed a systems biology approach to study the immune response following vaccination with a seasonal, inactivated influenza vaccine (trivalent) at the individual primary immune cell type-level [15]. Using the same immune cell-based approach, we conducted a randomized, double-blinded, controlled, Phase I clinical trial to assess the temporal molecular immune responses of an intramuscular split-virus (SV), influenza A/H5N1 (A/Indonesia/05/2005) vaccine given with or without AS03 adjuvant [20]. Here we assess changes in the immune cell proteome in response to vaccination (clinical and transcriptomic responses are discussed in [16]).

We performed quantitative proteomics on five primary immune cell types (monocytes, neutrophils, NK-cells, B-cells, and T-cells) isolated from peripheral blood of adult subjects at days –28, –14, and 0 pre-vaccination and days 1, 3, 7, and 28 post-vaccination. After assembling proteins into unique protein groups based on shared peptide identifications and retaining the top protein per group, we uniquely quantified the relative abundance of 3,247 proteins from 1,580 protein families (based on 50% sequence identity) compared to an immune cell common standard [15]. We detected 1,252 (monocytes), 860 (neutrophils), 813 (B-cells), 665 (NK-cells), and 533 (T-cells) protein families (Table S1).

Many proteins were only quantifiable in a subset of samples, and we observed different levels of quantification sensitivity across cell types with best sample coverage for monocytes and neutrophils and poorest coverage for T-cells (Fig. S1). This observation could be due to low amounts of starting protein material, highly abundant proteins diluting the low abundance signals, rapidly changing protein abundances in immune cells, or the common phenomenon of missing peptides in iTRAQ experiments [17, 18]. Due to the high degree of missing observations, T-cells were excluded from downstream analysis. For cell types with the best coverage (monocytes and neutrophils), we generated additional “imputed protein family baseline response profiles” using proteins with 90% non-missing responses per vaccine group.

To determine significantly differentially abundant (DA) proteins at day 1, 3, 7, and 28 post-vaccination, we compared cell type-specific protein $\log_2$ fold changes from baseline between the AS03-adjuvanted (SV-AS03) and unadjuvanted (SV-PBS) H5N1 vaccine groups, using a permutation test ($p < 0.05$) and an effect size cut off (1.2-fold difference between mean vaccine group responses). An individual cutoff was applied because multiple testing FDR-adjustment was too conservative (see methods). The number of DA proteins (and protein families) identified for any post-vaccination day was: monocytes: 172 (108), neutrophils: 100 (55), B cells: 109 (50), and NK cells: 80 (40) (Table S2–S17). Little overlap of DA proteins between post-vaccination days within the same cell type was observed (Fig. S2–3), which while unexpected, could be due to different subsets of proteins being activated at different times in the immune response [19]. Of the 184 unique DA protein families, 54 (29%) were reported for at least two cell types, with HLA Class I and TUBB3 family-related proteins being DA in all four cell types (Table S18).

Overall, the observed AS03 effect was strongest in monocytes at day 1 and 3 with the majority of protein families showing an increased baseline response for subjects in the SV-AS03 group most of which clustered together based on their response profiles (Fig. 1A,B). The effect was much less pronounced for days 7 and 28 (Fig. 1C,D). Supporting previous reports, these results indicate that AS03 modulated early responses in monocytes [8, 20]. Heatmaps for the other cell types are provided in Fig. S4–S17.

To functionally characterize DA proteins, we performed gene set enrichment analysis. Genes encoding for DA proteins were involved in a range of biological processes including protein metabolism, tubulin folding, platelet activation, and immune system-related processes (Tables S19–S28). At day 3, *Signaling by the B-cell receptor BCR* was significantly enriched in neutrophils and monocytes. Genes encoding DA proteins in this pathway included *LYN*, *RPS27A*, *CALM1*, *CALM3*, *PSME1*, *PSME2*, and *UBA52* all of which play a role in multiple pathways. In addition, pathways related to antigen processing and presentation were significantly enriched in monocytes and neutrophils at day 3, including *Antigen processing and cross presentation*, *ER phagosome*, *Interferon Signaling*, and *Class I MHC-mediated antigen processing and presentation* (Fig. 2). *Antigen presentation folding assembly and peptide loading of Class I MHC* was significantly enriched in neutrophils at day 28 (Table S25).

We further investigated this signal by inspecting HLA Class I protein family responses. As most DA HLA proteins in this family tended to cluster together based on their baseline fold changes (e.g. Fig. 1B), we used the mean $\log_2$ fold change to summarize HLA responses on the protein family level. For all post-vaccination days, higher HLA responses for the SV-AS03 compared to the SV-PBS vaccine group in monocytes and neutrophils were observed (Fig. 3A). For both cell types, an initial peak response was observed at day 3 for the SV-AS03 group with responses returning to near-baseline levels by day 7. Neutrophils showed an additional increase in responses for the SV-AS03 group at day 28. Time trends for the other cell types and HLA subgroup-specific responses are provided in Fig. S18–22.

To interpret peak differential HLA responses in a broader functional context, we inspected KEGG MHC class I sub pathway maps color-coded by difference in $\log_2$ fold change between vaccine groups and time points (Fig 3B, S23 and S24). In addition to HLA Class I, baseline responses for *PA28*, *HSP70*, and *CALR* gene products were increased in the SV-AS03 relative to the SV-PBS group in monocytes at day 3. While responses for HLA Class I gene products were significantly increased for the SV-AS03 group in neutrophils at day 28, responses related to up-stream mechanisms to process and chaperone cytosolic antigens (*PA28*, *HSP70*, and *HSP90*) were decreased, indicating that active antigen presentation was likely reduced at day 28.

To identify protein families that best predicted seroprotection status (HAI titer $\geq 1:40$ at day 56, i.e. 28 days post-second dose), we carried out regularized logistic regression analysis (Table S29 – 32). To reduce the impact of missing observations, we restricted this analysis to monocytes and neutrophils using imputed protein family baseline response profiles as predictors. Myeloperoxidase (MPO) and Superoxide dismutase 2 (SOD2) protein families, which are involved in inflammation and oxidative stress responses, were among those identified as predictors of seroprotection in monocytes at day 1. Similar inflammation and oxidative stress-related protein families, including Neutrophil cytosolic factor 4 (NCF4) and Granzyme B (GZMB), were identified in neutrophils at day 1. Four of the 12 selected protein families in monocytes at day 3 were related to *Class I MHC-mediated antigen processing and presentation*, including HLA Class I, PSME1 and PSME2 (PA28 alpha/beta proteasome activator subunit families), as well as ITGB5 (integrin, beta 5 family). For all these protein families, an increase in fold change from baseline resulted in an increased likelihood of seroprotection. Changes in PSME2 had the highest impact. The monocyte day 3 protein-protein interaction network (Fig. 4) visualizes these and other DA protein responses in the context of their known interaction partners. While ITGB5 was indirectly connected to several membrane proteins and proteasome family proteins, DA HLA proteins formed a distinct sub network, which was not connected to any of these proteins. Interestingly, one prominent hub protein (HNRNPK) with significantly increased responses in the SV-AS03 group is known to be targeted by multiple influenza virus proteins (NS and NS1).

We compared our proteomics results to differentially expressed (DE) genes (SV-AS03 vs. SV-PBS) identified in our transcriptomics study conducted in parallel [16]. We identified 22 DA protein families associated with DE genes identified for any cell type-time point combination (Table S33). Interestingly, increased PSME1 and PSME2 proteomic responses

for the SV-AS03 group in monocytes at day 3 corresponded with increased transcriptomic changes in the genes encoding these proteins in monocytes at day 1. Changes in PSME2 were significant in both the proteomic and transcriptomic studies. While HLA Class I protein encoding genes were slightly up-regulated but not significantly DE in the SV-AS03 group in monocytes, the *Class I MHC-mediated antigen processing and presentation* pathway was significantly enriched in DE genes up-regulated in the SV-AS03 group at day 1 [16]. Together these findings imply a delayed Class I-related antigen presentation response at the protein level (day 3) following an initial transcriptomics response at 24h post-vaccination. A post-transcriptional regulation mechanism may be modulating the expression of antigen processing and presentation proteins [21].

As anticipated, very few of the differential protein families (12%, 22 of 184) were identified in the transcriptomics analysis for any cell type-time point combination. This lack of shared responses has been seen previously [22, 23] and likely stems from translational control of gene expression, degradation of mRNA prior to translation, post-translational modifications of proteins, bias towards highly abundant proteins, small sample size, and general noise in the data [24]. Nevertheless, our proteomic analysis on the pathway level confirmed the enrichment of the *Class I antigen processing and presentation* pathway for monocytes and neutrophils, albeit two days after the transcriptomic signal (day 3 vs. day 1).

Further studies will be needed to corroborate the observed immune cell-specific responses. The small sample size of 10 subjects per group limits generalizability of our results. In addition, improved methods (MS3 [25] and MultiNotch MS3 [26]) for iTRAQ experiments that reduce iTRAQ reporter ratio distortion and missing observations have been published since this clinical trial was conducted. Nevertheless, our cell-based proteomics approach allowed for a more granular assessment of immune responses as compared to analyzing mixed responses in whole blood or PBMCs. In summary, our results demonstrated a strong early but transient proteomic response in monocytes and revealed details of the timing and protein composition of antigen processing and presentation signals triggered in response to AS03-adjuvanted influenza vaccine in monocytes and neutrophils. In addition, we detected several cell-specific markers that predicted later seroprotective antibody titers serving as potential candidates for future biomarker studies.

Experimental Procedures

Twenty healthy adult volunteers received two 0.5 mL intramuscular injection doses of 3.75 mcg split-virus vaccine (Sanofi Pasteur) with either PBS or AS03 adjuvant (GSK) 28 days apart. Peripheral blood samples (100 mL) were collected on days -28, -24, and 0, prior to the first dose, and days 1, 3, 7, and 28 post-vaccination. Purified immune cell populations were isolated from whole blood samples as previously described [15]. Protein extracts from purified immune cells (1×10^6 cells) were prepared using a modified lysis buffer (50% Trifluoroethanol 50mM HEPES). For each subject and cell type, 10 μ g of reduced, alkylated, and trypsinized protein extracts obtained for visit days were labeled with 7 distinct iTRAQ tags (AB Sciex), pooled, and analyzed by MudPIT using an Eksigent 2-D nanoLC pump coupled to a nanoESI-LTQ-OrbitrapXL mass spectrometer (Thermo Scientific). An immune cell common standard (ICCS) composed of protein extracts from PBMC and CD15+ cells

(80% and 20%, respectively) was included as a reference in each of the iTRAQ experiments. Precursor ions were analyzed in the Orbitrap followed by 4 CID fragment ion scans in the ion trap to identify peptides. The precursor ions were then fragmented by HCD to measure reporter ion intensities in the Orbitrap.

Spectra for each precursor ion were merged and searched against a forward and reverse concatenated human Ensembl database (v74) containing 169,816 sequence using *Sequest* in *Proteome Discoverer v1.3* (Thermo Scientific)[27, 28]. For more details, see “Materials and Methods” section in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AS03	Adjuvant System 03
ICCS	Immune Cell Common Standard
SV	Split-virus
DA	Differentially abundant
DE	Differentially expressed
HAI	Hemagglutination inhibition
HLA	Human leukocyte antigen/major histocompatibility complex
MHC	major histocompatibility complex
TUBB3	Tubulin, beta 3 class III
PSME	Proteasome activator subunit
MPO	Myeloperoxidase
SOD2	Superoxide dismutase 2
NCF4	Neutrophil cytosolic factor 4
GZMB	Granzyme B

ITGB5 Integrin, beta 5**References**

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Significance of the Study

Oil-in-water emulsion adjuvants, including AS03, have shown promise in enhancing immune responses to H5N1 vaccines. However, the molecular mechanisms by which AS03 enhances immunogenicity are not well understood. In the context of a Phase I clinical trial, we performed quantitative proteomics on five primary immune cell types isolated at multiple time points (days -28, -14, and 0 pre-vaccination and days 1, 3, 7, and 28 post-vaccination) from subjects receiving AS03-adjuvanted and unadjuvanted split-virus H5N1 vaccine. Our results demonstrated a strong early but transient proteomic response in monocytes and revealed details of the timing and protein composition of antigen processing and presentation signals triggered in response to AS03-adjuvanted influenza vaccine in monocytes and neutrophils. These results contribute to a better understanding of immune cell-specific temporal responses to AS03 when administered with a split-virus H5N1 influenza vaccine. In addition, we detected several cell-specific markers that predicted later seroprotection serving as potential candidates for future biomarker studies.

Fig 1. Heatmaps of DA protein family and protein baseline log₂ fold changes in monocytes by post-vaccination day

(A) day 1; (B) day 3; (C) day 7; and (D) day 28. Protein family baseline log₂ fold changes (top panels) represent mean responses for protein family members with non-missing fold changes for 90% of subjects in each vaccine group. Any missing protein family fold changes were imputed using the k-nearest neighbors algorithm. Protein families were filtered for families with at least one DA protein member. For individual proteins (bottom panels), a log₂ fold change close to 0 (10⁻⁶) was imputed for missing observations. Colored in red: increased from baseline; colored in green: decreased from baseline. Dendrograms were obtained using complete linkage clustering of uncentered pairwise Pearson correlation distances between log₂ fold changes. The dendrogram to the left is color-coded by protein family. The dendrogram at the top is color-coded by vaccine group.

Reactome Gene Set Enrichment

Fig 2. Reactome pathway enrichment by post-vaccination day
Monocytes (left panel); neutrophils (right panel). Pathways that were significantly enriched in both cell types or for two time points within the same cell type are shown in the form of spokes. The length of each spoke represents the enrichment score defined as $ES = -1 \times \log_{10}(\text{FDR-adjusted p-value})$. The area colored in grey marks the enrichment score below the statistical significance threshold (FDR-adjusted p-value < 0.05).

Fig 3. HLA Class I family proteins are upregulated after AS03-adjuvanted H5N1 vaccination
(A) Mean log₂ fold change from baseline and associated 95% bootstrap CI for DA HLA Class I family proteins by vaccine group (monocytes and neutrophils, day 1–28). DA HLA proteins are listed as part of the figure legend **(B)** KEGG MHC Class I sub pathway maps color-coded by difference in log₂ fold change between vaccine groups. In red: increased in the SV-AS03 group compared to the SV-PBS group, in green: decreased in the SV-AS03 group compared to the SV-PBS group. Complete KEGG *Antigen processing and presentation* pathway maps and additional caption information are provided in Fig. S23–S24.

Fig 4. Protein-protein interaction network analysis in monocytes at day 3
Nodes represent unique UniProt proteins. Color and size of individual nodes indicates the degree of mean log₂ fold change difference between vaccine groups. Edges colored in black represent experimentally determined interactions edges colored in grey represent protein family relationships (proteins with 50% protein sequence identity). Further details are provided as part of the legend within the figure.