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The Split Virus Influenza Vaccine rapidly activates immune cells through Fcγ Receptors

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Abstract

Seasonal influenza vaccination is one of the most common medical procedures and yet the extent to which it activates the immune system beyond inducing antibody production is not well understood. In the United States, the most prevalent formulations of the vaccine consist of degraded or “split” viral particles distributed without any adjuvants. Based on previous reports we sought to determine whether the split influenza vaccine activates innate immune receptors—specifically Toll-like receptors. High-dimensional proteomic profiling of human whole-blood using Cytometry by Time-of-Flight (CyTOF) was used to compare signaling pathway activation and cytokine production between the split influenza vaccine and a prototypical TLR response *ex vivo*. This analysis revealed that the split vaccine rapidly and potently activates multiple immune cell types but yields a proteomic signature quite distinct from TLR activation. Importantly, vaccine induced activity was dependent upon the presence of human sera indicating that a serum factor was necessary for vaccine-dependent immune activation. We found this serum factor to be human antibodies specific for influenza proteins and therefore immediate immune activation by the split vaccine is immune-complex dependent. These studies demonstrate that influenza virus

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Authorship

Contribution: W.O.G designed the study, performed research, analyzed data, and wrote the manuscript; H.H, and Y.L.W contributed to study design, performed research, analyzed data, and aided in editing; M.M.D directed the project and aided in editing; K.L.D and C.L.D aided in sample acquisition; S.C.B, H.T.M, and M.D.L provided mass cytometry expertise; B.A.K aided analysis. Y.H.C aided study design. All authors approved the final article.

Conflict of interest statement:

No conflicts to declare.

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“splitting” inactivates any potential adjuvants endogenous to influenza, such as RNA, but in previously exposed individuals can elicit a potent immune response by facilitating the rapid formation of immune complexes.

Keywords

influenza; vaccine; Fcγ Receptors; immunology; mass cytometry

Introduction

Influenza is a viral respiratory pathogen that causes substantial morbidity and mortality worldwide. Each year, an estimated 250,000 to 500,000 people die from seasonal influenza infections and many more are hospitalized [1]. Vaccination is currently the main strategy used to limit morbidity and mortality [2].

The first influenza vaccine licensed in the United States consisted of formalin fixed whole-inactivated virus (WIV) particles [3]. The WIV formulation, while thought to be highly immunogenic, was also very reactogenic often resulting in inflammation at the sight of injection and febrile illness [4]. Due to these adverse reactions, the WIV formulation was replaced with the split virus (SV) vaccine in which viral particles are first inactivated and then disrupted with detergent or ether [5]. Influenza particle splitting increases vaccine safety but reduces immunogenicity particularly in individuals, principally children, who are immunologically naïve to influenza [6,7].

Currently, the split virus format is the most prevalent influenza vaccine prescribed for adults and is distributed in the United States without adjuvant. Clinical influenza vaccine research has primarily focused on quantitating adaptive immune responses that evolve over days or weeks. Basic research studies on innate immune responses to influenza infection typically concentrate on how infectious viral particles (not split vaccine particles) activate pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Therefore little is known about the exact mechanisms through which SV activates early or innate immune responses.

Innate immune activation is thought to be a key feature of successful human vaccines such as vaccinia and yellow fever virus [8,9]. We thus sought to evaluate the immediate response of immune cells to SV *ex vivo*. Previous studies conducted mainly in rodent models suggest that viral RNA is the primary pathogen associated molecular pattern generated by WIV and that particle splitting inactivates viral RNA [10–12]. Other investigators have challenged this conclusion and assert that viral RNA is active in the split vaccine [13]. In humans, Panda *et al.* identified an age-associated decrease in TLR function that can predict *in vivo* serological responses in SV immunized subjects [14]. It still remains unclear whether this association is causal or if the SV influenza vaccine can engage TLRs. A recent study has found that SV vaccination elicits transcriptional activity *in vivo* within hours of human vaccination [15]. This early transcriptional signature involves monocytes, NK cells, and interferons (IFNs). The initiating event(s) eliciting this early *in vivo* signature are unknown [15].

High-dimensional proteomic profiling using mass-cytometry and luminex technology was used to quantify signaling pathway activation and cytokine production across all major immune cell populations in human whole-blood stimulated with SV *ex vivo*. As a positive control, we compared SV to prototypical RNA-based TLR activation through TLRs 7/8.

Unexpectedly, the SV rapidly and potently induced signaling responses across the immune system. Analyzing a subset of parameters, the SV induced immune activation was similar to TLR7/8 signaling; however, broader signaling pathway analysis demonstrated that SV elicits a proteomic signature overlapping with but very distinct from TLR activation. Subsequent studies found that the stimulatory activity of the vaccine was dependent on the presence of human sera since SV activity was apparent in human whole-blood but absent when human cells were cultured in cell media with bovine serum. Lastly, immunoglobulin G (IgG) antibodies specific for influenza proteins were found to be necessary for SV induced responses and thus immune complex formation and Fc receptor activation is the initial event in SV influenza vaccination.

These data suggest that viral RNA is most likely not relevant in human SV vaccination and also highlight the potential importance of immune complex formation in the context of influenza vaccination and in other vaccines that target previously exposed individuals. Conceptually this is an important insight for studies aimed at creating more effective influenza vaccines and at identifying predictive biomarkers of influenza vaccination efficacy in at-risk populations.

Material and Methods

Study participants

All donors were adults and enrolled in a study to analyze whole-blood or in a vaccination study conducted by the Stanford-LPCH Vaccine Program. Study participants were all adults in good health with an average age of 32. Subjects not in good health were excluded. 70% of participants were male 30% were female. Subjects were included regardless of vaccination history. Anti-SV IgG concentrations in donor plasma ranged from 25–500 µg/ml. Study protocols were approved by the Institutional Review Board of the Research Compliance Office at Stanford University. Informed consent was obtained from all subjects. Data were analyzed by either W.O.G or H.H.

Vaccines and viruses

Stimulation studies used the 2011–2012 and/or the 2012–2013 trivalent inactivated influenza vaccines (Fluzone; Sanofi Pasteur). Whole inactivated virus was prepared from purified attenuated influenza strains (Medimmune) that were treated with .02% formaldehyde at 37°C for 12 hours. The following strains were used: A/California/07/2009 H1N1, A/Victoria/210/2009 H3N2, A/Victoria/361/2011 H3N2, B/Brisbane/60/2008, B/Texas/6/2011. We calculated the total protein concentration of Fluzone to be 300ug/ml using the Bradford assay.

Cells and stimuli

Freshly isolated human whole-blood was collected into heparinized vacutainers (BD) and used intact or washed with room temperature RPMI 1640 media (Life Technologies) 2–3 times to generate plasma-depleted whole-blood. Washed WB cells were resuspended in unsupplemented RPMI media. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (Ficoll-Paque; GE healthcare) and cultured in RPMI 1640 + 10% FBS or AIM V media (Life Technologies). Cells were stimulated at various concentrations of SV for either 30mins or 6hrs and then fixed for 15mins at 37°C with Phosflow lyse/fix buffer (BD). In SV pre-incubation studies, SV was diluted 1:10 in 100% human plasma for 30min at 4°C and then added to washed WB or PBMCs at a 1:20 dilution. SV was complexed with protein A/G spin column (Pierce) purified polyclonal IgG at a ratio of .3ug of SV (1ul of undiluted vaccine) per 75ug of IgG for 30mins (at 7.5mg/ml) prior to adding SV+IgG to PBMCs at a concentration of .75–3ug/ml SV. HAG was generated by heating purified IgG at a 5ug/ml concentration for 20mins at 63°C. Recombinant H1N1 influenza hemagglutinin and self-proteins were purchased from Sino Biological. R848 was purchased from Invivogen. Polyclonal F(ab')₂ was prepared using a F(ab')₂ micro isolation kit (Pierce).

Mass & flow cytometry

Clone and conjugation information for all mAbs used are shown in Figure S1. Numbers associated with mAbs in Figure S1 represent the atomic masses of lanthanide isotopes that were chelated to polymers and then conjugated to the indicated mAbs. Cells were surface stained and then permeabilized with either ice-cold methanol for signaling analysis or Perm/Wash buffer I (BD) for cytokine analysis. For flow cytometry cells were analyzed on an LSRII (BD). For mass cytometry cells were treated with an iridium intercalator (DVS Sciences) prior to analysis on a CyTOF (DVS sciences). Data were analyzed using Flowjo (Treestar). Mass cytometry based signaling responses were quantified as previously described [16]. Mass cytometry based cytokine responses, and flow cytometry based responses were quantified by thresholding signaling responses based on 100 counts or the 99th percentile of PBS treated controls.

Luminex (capture-bead ELISA kits)

A human 51-plex luminex kit (Affymetrix) was used according to the manufacturer's recommendations. Cytokine responses were quantified by dividing the median fluorescent intensity (MFI) of capture beads from stimulated plasma by the MFI of beads from unstimulated plasma.

Results

Comparison of signaling network activation between the SV vaccine and a TLR7/8 agonist

SV and TLR activity was compared using mass cytometry to perform a systematic analysis of signaling pathways. In mass cytometry elemental tags are used to facilitate an unprecedented level of dimensionality in single-cell analysis [16,17]. 30 parameters were measured simultaneously to deconstruct the cellular complexity in human blood and monitor

key signaling pathways using phosphorylation specific monoclonal antibodies (mAbs). Utilizing this approach 8 cell populations were defined with 19 surface markers and probed with 11 mAbs specific to distinct signaling molecules (Figure S1). Specifically, the ERK/mTOR, histone, stress, NFκB, and STAT pathways were monitored in CD14hi monocytes, CD16hi monocytes, granulocytes, plasmacytoid dendritic cells (pDCs), conventional dendritic cells (cDCs), natural killer (NK) cells, B cells, and T cells. Whole-blood techniques were used because Ficoll density centrifugation was found to activate CD14hi monocytes (as previously described [18]).

Infectious influenza particles activate multiple PRRs [19]; however, virus inactivation via fixation limits immune detection to only TLR7 sensing of viral RNA in pDCs [10]. Virus splitting has been reported to inactivate viral RNAs in some studies [11,12] but a recent report suggests viral RNA is present in split vaccines and serves as an intrinsic adjuvant [13]. We utilized commercial formulations of the 2011–2012 and 2012–2013 influenza split vaccine (Fluzone; Sanofi Pasteur) to model clinical human exposure. As a positive control for RNA induced activation, we stimulated blood cells *ex vivo* with the small molecule agonist resiquimod (R848), which is specific for TLRs 7 & 8. Influenza RNA has been shown to activate human TLRs 7 and 8 [20].

Unexpectedly, the SV rapidly activated multiple signaling pathways across various cell populations but overall yielded a proteomic signature distinct from TLR7/8 stimulation (Figures 1A and 1B). In myeloid cells SV induced phosphorylation of ERK, the S6 ribosomal protein (S6), CREB, and Histone H3—molecules involved in MEK, PI3K, and mTOR signaling [21]. SV did not however stimulate stress kinases such as p38 and MAPKAPK2 or the NFκB pathway as indicated by a lack of total IκBα degradation. In contrast, R848 activated almost all signaling pathways in the myeloid lineage. Importantly, p38 phosphorylation is a hallmark of almost all TLR responses and was thus a key difference between these signaling profiles.

SV induced activity also differed from TLR7/8 stimulation in lymphocytes. NK cells were activated by the vaccine but did not respond to R848, which is consistent with a lack of TLR7/8 expression in these cells [22]. B-cells were activated by R848 but only weakly affected by SV. T-cells were not activated by either stimulus. Both SV and R848 also induced shedding of CD16 (FcγRIII) in monocytes and NK cells (data not shown). SV responses in monocytes were observed in all donors sampled while NK cell and dendritic cell responses were observed stably but only in particular donors. No early activity was observed in the STAT pathways in any cell type at 30mins (data not shown).

Next we sought to compare the sensitivity of human immune cells to SV and R848 in order to ensure that SV was active at low concentrations. Similar to R848, SV demonstrates potent activity at nanogram per milliliter concentrations – representing 1/500th of a single vaccine dose (Figure 2). To ensure SV activity was not due to non-influenza related components in the vaccine, we stimulated whole-blood with gelatin, octylphenol ethoxylate, and thimerosal and found no detectable activity (data not shown).

Comparison of cytokine production between the SV vaccine and a TLR7/8 agonist

Signaling pathway activation in immune cells frequently causes the production of cytokines that mediate intercellular communication. A mass cytometry based staining panel capable of measuring pan-immune cytokine production was used to compare SV to TLR7/8 stimulation. This intracellular cytokine staining (ICS) panel consists of the surface markers previously mentioned as well as mAbs against IL-1 β , IL-1RA, IL-2, IL-4, IL-6, IL-12 (p40), IL-17A, MCP1 (CCL2), TNF α , IFN γ , IFN α , Perforin, and GM-CSF. Whole-blood was stimulated with either SV or R848 for 6hrs prior to red-blood cell (RBC) lysis and fixation. Both brefeldin and monensin were added to blood at the beginning of assays or after 2–3hrs of stimulation.

Systematic profiling of cytokine production revealed that SV and R848 induce different patterns of cytokine expression (Figure 3A). In CD14^{hi} monocytes both stimuli induced production of the chemokine MCP1—a molecule capable of recruiting monocytes and dendritic cells to sites of inflammation [23]. IL-1RA, an anti-inflammatory cytokine, was also induced by both stimuli but far more potently by R848. Importantly, the major pro-inflammatory cytokines (IL-1 β , IL-6, TNF α , and IL-12) surveyed in monocytes were induced only by TLR7/8 stimulation and not by SV. In NK cells, SV induced production of TNF α as well as IFN γ . While R848 did not activate signaling pathways in NK cells, it did cause some cytokine production, most likely induced by a paracrine mechanism. IFN α production in pDCs is the hallmark response of TLR7 activation [10] and was elicited by R848 as expected. In contrast, SV did not cause IFN α production, which is consistent with the lack of SV dependent p38 activation in pDCs. Figure 3B depicts the variance in these responses over 3 different donors. Dose kinetics for intracellular cytokine production are shown in Figure S2A.

Intracellular cytokine staining assays are limited in that they assess cytokine production but not secretion due to the obligatory need for secretion inhibitors. In order to verify that MCP1 was secreted by SV activated monocytes and to detect cytokines not included in our mass cytometry panel we used luminex technology to quantitate the secretion of 51 cytokines in human plasma [24]. Whole-blood was collected from 15 adult volunteers and stimulated *ex vivo* with SV for 6hrs. Cytokines demonstrating at least a 5X increase in median fluorescence signal after all 15 subjects were averaged are shown in Figure S2B. Specifically, Mip1 α (CCL3), IP-10 (CXCL10), IL-8, TNF α , Mip1 β (CCL4), MCP1, and IL-1RA were secreted at high levels in most subjects.

Influenza protein derived immune complexes are responsible for SV induced activity *ex vivo*

While investigating the mechanisms through which SV stimulates human immune cells, it was observed that SV could not stimulate peripheral blood mononuclear cells (PBMCs) under standard tissue culture conditions (Figure 4A). Subsequently, we hypothesized that a factor in human plasma was necessary for SV induced immune activation. To evaluate this hypothesis, SV was first pre-incubated with plasma and then added to either plasma depleted whole-blood or PBMCs to determine whether this rescued SV non-responsiveness. Since the ribosomal protein S6 is a near-terminal response element in ERK/mTOR signaling [25], we

monitored its phosphorylation as a general activation marker for a variety of cell types. Remarkably, SV pre-incubated with plasma could stimulate CD16hi monocytes (Figure 4B). Thus a human serum factor in addition to SV is necessary to activate immune cells.

Given the abundance of antibodies against influenza proteins, particularly hemagglutinin (HA), in human plasma it seemed probable that SV activity could be due to the formation of immune complexes and Fc receptor activation. Consistent with this prediction pre-incubation of SV with purified polyclonal human IgG could also rescue SV activity in serum depleted whole-blood monocytes (Figure 4B). Similar results were obtained in NK cells stimulated in the PBMC format, as well as in enriched NK cell and CD16hi monocyte populations (Figure S3). At the concentrations used to rescue SV activity neither IgG or plasma alone activated S6 (data not shown). IgG was the only plasma factor necessary for SV activity since IgG-depleted plasma had no activity when pre-incubated with SV (data not shown). Figure S4 demonstrates that cytokine production in response to SV was also dependent on the presence of IgG. These experiments strongly implicated influenza protein derived immune complexes as responsible for SV induced signaling pathway activation and cytokine production. CD16hi monocyte data is shown because S6 phosphorylation in CD14hi monocytes can be activated by centrifugation but CD16hi monocytes are not.

To demonstrate that this activity was influenza protein dependent, we stimulated intact whole-blood and plasma-depleted whole-blood with recombinant HA from the H1N1 strain. Similar to SV, HA only showed activity in monocytes in the presence of human plasma (Figure 4B). Recombinant human proteins (LAMP1 & HER2) produced under the same conditions were used as negative controls and did not stimulate whole-blood (Data not shown). Based on this evidence Fc γ receptor (Fc γ R) triggering by SV immune complexes was the most plausible explanation for SV activity.

Fc γ Rs are expressed throughout the immune system in a manner consistent with the observed stimulation patterns of SV (Figure S5) [26]. Specifically, CD64 (Fc γ RI), CD32 (Fc γ RII), and CD16 (Fc γ RIII), are all differentially expressed within distinct monocyte populations. CD16 is expressed by the majority of NK cells. B cells only express CD32 and most T cells do not express any Fc γ Rs. We next sought to determine if SV activity is dependent on the Fc portion of IgG. Polyclonal F(ab')₂ proteins were produced and as expected were unable to rescue SV's lack of activity in human serum free conditions (Figure 5A). To confirm that F(ab')₂ proteins could still bind to SV, immobilized vaccine was incubated with F(ab')₂ and an anti-light chain Ab was used to quantitate binding via ELISA. Polyclonal F(ab')₂ bound to SV, confirming that pepsin treatment had not rendered the F(ab')₂ proteins incapable of reacting with SV (data not shown). Importantly, blocking mAbs against Fc γ Rs were capable of specifically inhibiting S6 ribosomal protein phosphorylation in response to SV immune complexes (Figure 5B). SV immune complex stimulation of CD16hi monocytes and NK cells (PBMC format) was blocked by mAbs targeting CD16 and CD32 but not by isotype control mAbs. Thus SV activity in these cell types is dependent upon an intact IgG Fc region as well as CD16 and CD32.

To independently assess whether the SV induced proteomic signature is Fc γ R dependent, we analyzed the effects of heat-aggregated IgG (HAG) on human immune cells. HAG, like

influenza-derived immune complexes, selectively stimulated ERK/mTOR signaling but not the p38 or NF κ B pathways in myeloid cells (Figure S6). HAG also caused monocytic production of MCP1 in human whole-blood and NK cell production of IFN γ and TNF α in PBMCs (data not shown).

Influenza vaccine formulations vary in proteomic responses

We evaluated if SV induced cytokine secretion was also dependent on human serum using luminex (Figure 6A). Similarly to S6 ribosomal protein phosphorylation, SV dependent cytokine secretion only occurred in the whole-blood format. In contrast, the whole inactivated virus had activity in both whole-blood and PBMCs. In general, WIV induced similar cytokines as SV except for IL-8 and IFN α . WIV dependent IFN α production was expected based on previous studies which found this activity to be RNA dependent [10]. We also found that treating WIV with RNase reduced IFN α production (data not shown).

Lastly, we sought to determine whether the split vaccine induced more rapid and potent Fc γ R responses than intact viral particles. SV was compared to Live-Attenuated Influenza Virus (LAIV) in signaling assays (Figure 6B). At equivalent concentrations of HA, SV was found to be a far more potent inducer of immediate signaling responses than LAIV. However, at later time points (3hrs) LAIV elicited appreciable activity but still less robustly than SV (data not shown).

Discussion

Blood transcriptional activity induced by *in vivo* SV immunization is detectable within hours and peaks a day after vaccination [15]. Modular analysis of this activity indicates that pathways involved in monocyte, NK cell, and interferon biology are perturbed in the early phases of influenza vaccination. However, these transcriptomic approaches have not as of yet shown what mechanisms elicit these responses. Our analyses demonstrate that the influenza vaccine activates monocytes and NK cells through Fc γ Rs and causes the production of IFN γ but not Type I IFNs. As previously reported [10–12], we also found no evidence to suggest that RNA in the SV vaccine is capable of activating TLR receptors. Thus, immune complex formation and Fc γ R activation is most likely the immediate immune response to SV vaccination in individuals with pre-existing IgG Abs against influenza proteins.

The question of whether viral particle splitting inactivates TLR activity is important because highly successful vaccines such as the Yellow Fever vaccine (YFV) and vaccinia have been shown to activate multiple PRRs [8,9]. Smallpox and YFV immunization campaigns sought to induce immunity in subjects that were largely immunologically naïve to these pathogens. In contrast, influenza vaccination in adults typically occurs in the presence of pre-existing immunological memory caused by natural exposure to influenza. As such we found the immediate effect of adding SV to human blood was immune complex formation. This raises the important question of how pre-existing influenza specific Abs affect SV immunization. Due to the *ex vivo* nature of these studies caution must be taken in assuming that myeloid and NK cell activation by SV immune complexes will necessarily enhance vaccination. Evidence exists that IgG binding and immune complex formation can both enhance [27–30]

and inhibit [31–35] serological responses to different forms of antigen. If Fc receptor activation can enhance vaccination responses then it is significant that SV had far more immediate activity in signaling assays than LAIV (Figure 6B). Detergent-induced dissociation of influenza particles will likely expose additional viral proteins (particularly matrix proteins) that are masked by egg-derived lipid bilayers and thus make for a more potent Fc receptor mediated immune response in previously exposed hosts.

In the context of influenza vaccination one of the most predictive metrics of a poor serological response is high pre-vaccination levels of Abs against influenza hemagglutinin [36–38]. Thus it is possible that pre-existing Abs against SV proteins actively suppress immunization responses through feedback inhibition. The extent to which such a suppressive mechanism would require Fc γ R activation is not entirely clear but it has been shown that Fc γ RIIb binding can suppress B cell responses to viral antigens bound by IgG [33]. More research is necessary to understand how pre-existing Abs influence influenza vaccination responses and whether particular adjuvants can overcome immune complex based feedback inhibition.

A recent meta-analysis of clinical trials has suggested that, based on serological responses, SV is more efficacious in adults than in children and conversely LAIV is more efficacious in children than in adults [39]. Our observations may explain this difference in that the lack of PRR ligands on SV would make it a weak primer for initiating immune responses in children, but it would be more effective in adults because mature memory responses are far less dependent on innate immune activation [10]. The key question is how influenza reactive or cross-reactive memory T cells, memory B cells, and pre-existing Abs influence subsequent SV or LAIV vaccination. Poor serological responses in adults vaccinated with LAIV may be explained by IgA neutralization of viral particles in the respiratory mucosa prior to viral replication. Overall, immune complex formation is most likely the essential event in understanding adult influenza vaccination.

Conclusions

These studies provide new insights into how the SV vaccine contributes to the immune response and illustrates how any vaccine administered to previously exposed individuals will likely trigger a similar Fc γ R-mediated early response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The split influenza vaccine rapidly activates signaling pathways and cytokine production in immune cells
- Mass cytometric analysis showed that activation was IgG dependent and activated monocytes at low concentrations of vaccine
- Many signaling pathways were induced and are dependent on immune complex formation and Fc γ receptor activation

Figure 1.

Comparison of signaling network activation induced by either SV or a TLR7/8 agonist. (**A and B**) Freshly isolated human whole-blood was stimulated with PBS, SV (15ug/ml), or R848 (10ug/ml) for 30mins prior to RBC lysis and fixation. Cells were then stained with isotope labeled mAbs against surface proteins and signaling proteins and prepared for 31-parameter mass cytometric analysis. Cell populations were identified as CD11c⁺ CD33⁺ HLADR⁺ CD14hi Monocytes, CD11c⁺ CD33⁺ HLADR⁺ CD16hi Monocytes, CD66⁺ Granulocytes, HLADR⁺ CD123⁺ pDCs, HLADR⁺ CD1c⁺ cDCs, CD3⁻ CD7⁺ NK cells, CD19⁺ CD20⁺ B cells, and CD3⁺ T cells. See Figure S1 for detailed gating strategy. Signaling induction was calculated as the difference of arcsinh median intensity compared

with PBS control. A representative experiment is shown from 4 independent mass cytometry experiments conducted on 7 adult donors.

Figure 2.

Dose response dynamics of SV versus TLR7/8 stimulation. Whole-blood was stimulated at varying concentrations of either SV or R848 and monitored for S6 phosphorylation using 10-parameter flow cytometry. Cell populations were defined as CD33⁺ HLADR⁺ CD14hi Monocytes, CD33⁺ HLADR⁺ CD16hi Monocytes, CD66⁺ Granulocytes, CD33⁺ HLADR⁺ CD14⁻ CD16⁻ cDCs, CD56⁺ NK cells, CD20⁺ B cells, and CD3⁺ T cells. Signaling induction was calculated as the % of cells showing greater than basal S6 phosphorylation. Mean data points of replicates from 1 donor are shown.

Figure 3.

Comparison of cytokine production between SV and a TLR7/8 agonist. **(A)** Freshly isolated human whole-blood was stimulated with PBS, 15ug/ml SV, or 5ug/ml R848 for 6hrs prior to RBC lysis and fixation. Secretion inhibitors were added for either the entirety of stimulation (SV) or after 2hrs (R848). Fixed cells were then prepared for 34-parameter mass cytometric intracellular cytokine staining (ICS) analysis. Cytokine positive monocytes and pDCs were defined as cells showing signal greater than 10^2 counts. Cytokine positive NK cells were defined as cells showing signal greater than the 99th percentile of unstimulated cells. SV also did not induce IFN α in pDCs when secretion inhibitors were added after 2hrs. One representative experiment is shown. **(B)** Quantitation of the variance in cytokine production based on 5 independent experiments conducted on 3 adult donors. Bar graphs show mean $\pm$ SD.

Figure 4.

SV signaling activity is induced by influenza protein derived immune complexes. **(A)** Whole-blood and PBMCs prepared via Ficoll density centrifugation were stimulated with either PBS, SV, or R848 at 10ug/ml for 30mins prior to fixation and flow cytometric analysis as in Figure 2. Representative data depicting CD16hi monocytes from 3 experiments (2 donors) are shown. **(B)** Intact whole-blood or plasma depleted whole-blood was stimulated with either SV, SV pre-incubated with autologous plasma, SV pre-incubated with autologous purified polyclonal human IgG, or recombinant H1N1 hemagglutinin (5ug/ml) for 30mins prior to fixation and flow cytometric analysis. See methods section for detailed information on pre-incubation. Representative data from 7 experiments are shown.

Figure 5.

SV activation is Fc and FcγR dependent. **(A)** Plasma depleted whole-blood was stimulated with PBS, SV preincubated with polyclonal F(ab')₂, or SV preincubated with intact IgG. Representative data from 2 experiments are shown. **(B)** PBMCs treated with blocking mAbs against CD16 and CD32 or isotype control mAbs were stimulated with .75–3μg/ml of SV + IgG complexes for 30mins prior to fixation and S6 phosphorylation analysis. Representative data from 3 experiments conducted on 2 donors are shown.

Figure 6.

Comparison of different vaccine formulations. **(A)** Whole-blood or PBMCs were stimulated with 15 μ g/ml SV or WIV for 6hrs prior to centrifugation and plasma or supernatant isolation. MFI fold change was calculated as in Fig. S2B. Representative data from 4 independent experiments are shown; bar graphs show mean $\pm$ SD from 3 donors. **(B)** Whole-blood was stimulated (.3 μ g/ml HA) with either split or live attenuated viral particles for 40mins and assayed for signaling pathway activation as in Figure 1. Mean arcsinh difference $\pm$ SD from 3 donors is shown.