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The parasite-derived rOv-ASP-1 is an effective antigen-sparing CD4⁺ T cell-dependent adjuvant for the trivalent inactivated influenza vaccine, and functions in the absence of MyD88 pathway

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Abstract

Vaccination remains the most cost-effective biomedical approach for controlling influenza disease.

In times of pandemics, however, these vaccines cannot be produced in sufficient quantities for worldwide use by the current manufacturing capacities and practices. What is needed is the

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Authorship

HS, SH, MEB and SL contributed to the conception and design of the study; SJ, PJG, WD, JK, JJ, JL, ZL, JW, KP, and BZ contributed to acquisition of data; SJ, PJG, JK, JL, BZ, MEB, HS and SL contributed to analysis and interpretation of data; SJ, PJG, KJ, JL, and BZ drafted the article; and HS, MEB and SL edited the manuscript.

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Conflict of interest statement

The authors have declared that no competing interests exist.

development of adjuvanted vaccines capable of inducing an adequate or better immune response at a decreased antigen dose. Previously we showed that the protein adjuvant rOv-ASP-1 augments influenza-specific antibody titers and survival after virus challenge in both young adult and old-age mice when administered with the trivalent inactivated influenza vaccine (IIV3). In this study we show that a reduced amount of rOv-ASP-1, with 40-times less IIV3 can also induce protection. Apparently the potency of the rOv-ASP-1 adjuvanted IIV3 vaccine is independent of the IIV3-specific Th1/Th2 associated antibody responses, and independent of the presence of HAI antibodies. However, CD4⁺ T helper cells were indispensable for the protection. Further, rOv-ASP-1 with or without IIV3 elicited the increased level of various chemokines, which are known chemoattractant for immune cells, into the muscle 4 hours after immunization, and significantly induced the recruitment of monocytes, macrophages and neutrophils into the muscles. The recruited monocytes had higher expression of the activation marker MHCII on their surface as well as CXCR3 and CCR2; receptors for IP-10 and MCP-1, respectively. These results show that the rOv-ASP-1 adjuvant allows substantial antigen sparing of IIV3 by stimulating at the site of injection the accumulation of chemokines and the recruitment of immune cells that can augment the activation of CD4⁺ T cell immune responses, essential for the production of antibody responses. Protection elicited by the rOv-ASP-1 adjuvanted IIV3 vaccine also appears to function in the absence of MyD88-signaling. Future studies will attempt to delineate the precise mechanisms by which the rOv-ASP-1 adjuvanted IIV3 vaccine works.

Keywords

Adjuvant; immune response; antigen-sparing; antibody isotypes; chemokines; cell recruitment

Introduction

Influenza is a serious infectious disease which causes 250,000 to 500,000 annual deaths worldwide in mostly the elderly (>65 years); vaccination remains the major preventive measure against influenza infection and associated disease (1–4). Current seasonal influenza vaccines have their own limitations like not inducing an optimal vaccine effectiveness in young children and the elderly (5–8), not able to cross-protect against the diverse antigenic subtypes of the circulating virus due to antigenic shift, and the requirement of large quantities of vaccine antigens much ahead of the influenza season. Although there are many alternatives available like baculovirus based expression system, mammalian cell based recombinant vaccine antigens, and synthetic antigens for cost-effective and fast production of influenza vaccine, still in most parts of the world the influenza vaccines used are produced by a tedious and long process using eggs, which at times limits their availability in large quantities during sudden outbreak of pandemic disease (9). Such situation occurred during the 2009 H1N1 influenza pandemic disease when the need for vaccine antigen was large and the circulating virus was antigenically different than the already prepared vaccines (10–13). The use of adjuvants in a vaccine, however, may improve influenza vaccine potency (8, 14, 15), and induce a more effective cross-protection for diverse strains of influenza virus (16–18), which is important at times when circulating virus strain is different from the ones used in the seasonal vaccine formulation. In November 2015, FDA approved the usage of MF59, an oil-in-water adjuvant, in an influenza vaccine formulation for the

elderly in the United States (<https://www.cdc.gov/flu/protect/vaccine/adjuvant.htm>). Adjuvants can also overcome the limitation of virus availability in large quantities during sudden outbreak of pandemic disease by enhancing vaccine efficiency with low antigen dose allowing antigen sparing so that the stocked vaccines can be used for larger population (19).

In general, adjuvants conventionally act by activating innate immune system using either one or combination of the following mechanisms: antigen depot effect; recruitment of innate immune cells such as neutrophils, monocytes and macrophages; activation of several signaling pathways including inflammasome and pattern recognition receptor pathways; and enhancement of antigen presentation (20, 21). Activated innate immune system further stimulates adaptive T and B cells and a long-lasting protective immunity against the infection (22, 23).

Recently we have shown that a novel adjuvant, the recombinant *Onchocerca volvulus* (rOv-ASP-1) protein can protect against influenza infection when administered in an aqueous formulation with the trivalent inactivated influenza vaccine (IIV3) in both young adult and aged mice (18, 24), and that the adjuvanted IIV3 can also induce cross-protection in the mice. The rOv-ASP-1 adjuvant is a parasite-derived protein secreted by the helminth parasite *Onchocerca volvulus*; it drives balanced Th1/Th2 antibody responses against various bystander antigens (25), and in some vaccine models it also stimulated Th1 biased responses (26, 27). The mode of action of rOv-ASP-1 adjuvanticity has not been studied in detail yet. This protein is known to bind to naïve human monocytes and B cells in peripheral blood, elicit cytokine secretions from naïve human peripheral blood mononuclear cells, and stimulate *in vitro* human monocytes derived dendritic cells to secrete cytokines and matures them to induce activation of naïve T cells (27).

In the present study we further explored rOv-ASP-1 adjuvanticity in regard to its ability to support IIV3 antigen sparing and initiated studies to explore the mode of action of the rOv-ASP-1 adjuvanted IIV3 antigen spared vaccine.

Materials and methods

Mice:

6–8 week old female C57BL/6 WT mice, IFN γ ^{-/-} (B6.129S7-*Ifng*^{tm1Ts}/J), IL-4^{-/-} (B6.129P2-*Il4*^{tm1Cgn}/J), CD4^{-/-} (B6.129S2-*Cd4*^{tm1Mak}/J) and MyD88^{-/-} (B6.129P2-Myd88^{tm1.1Defr}/J) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were maintained in AAALAC approved barrier facilities at New York Blood Center (NYBC, New York, NY) or the University of Pennsylvania (Philadelphia, PA) and allowed to acclimatize for at least one week in the animal facility prior to use. All protocols involving mice were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at NYBC or University of Pennsylvania.

Ov-ASP-1 expression in *E. coli*:

In previous studies the recombinant Ov-ASP-1 (rOv-ASP-1) used for the adjuvanticity studies was expressed using the pTrcHis expression system (Invitrogen, Carlsbad, CA) and *E. coli* DH5 α (25). However, in this system, the expression levels were low due to the

vector's weak *trc* promoter. Therefore, using the same coding sequence a new construct was prepared using the T7-promoter driven pET28a expression vector (Novagen, Gibbstown, US). The sequence-confirmed recombinant plasmid DNA was transformed into *E. coli* BL21(DE3). The rOv-ASP-1 protein with a hexa His-tag at its N-terminus was expressed under induction of 1 mM IPTG for 5 hours. The resulting inclusion bodies were solubilized in 1% SDS and purified with IMAC as described (28); the protein remained soluble in PBS, pH 7.4 containing 0.1% SDS. The expression yield of rOv-ASP-1 was significantly increased in the pET28a/BL21 expression system compared to the previously employed pTrcHis/DH5 α system. After SDS-PAGE, the purified rOv-ASP-1 appeared as a major band of 25 kDa, as expected by coding sequence, plus two weak specific bands of approximately 18–20 kDa (Supplementary Fig. 1A and 1B). All bands were recognized by mouse anti-rOv-ASP-1 antisera, indicating they all constitute rOv-ASP-1-derived products (Supplementary Fig. 1C). The endotoxin level was less than 11.41 EU/mg, as measured by Endosafe cartridge (Charles River, Houston, US). To make sure that the residual LPS within the 25 μ g or 12.5 μ g rOv-ASP-1 (0.57 ng or 0.285 ng, respectively) used per mice for immunization does not influence the adjuvant specific outcomes of the *in vivo* experiments listed below, we pre-treated the protein with Polymyxin B sulfate (Sigma-Millipore) before use. Polymyxin B was shown to neutralize the effect of LPS present as a contaminant in recombinant proteins (29), and it is also used to remove LPS from the purified recombinant proteins using affinity columns (25). Briefly, the amount of rOv-ASP-1 needed for each experiment was incubated at room temperature for 60 minutes with 30 μ g/ml Polymyxin B. The mixture was then brought up to 100 μ l with PBS for injection into the mice; each inoculum did not contain more than 0.34 μ g Polymyxin B. For control, the same final amount of Polymyxin B was added to the PBS 0.1% SDS solution for 60 min before injection.

Immunization:

The trivalent inactivated influenza vaccine (IIV3) used in this study was Novartis 2013–14 and Novartis 2015–16. Each contains 15 μ g of hemagglutinin (HA) from each of the following 3 viruses in 0.5 ml volume: A/California/7/2009 NYMC X-181 (H1N1); A/Victoria/210/2009 NYMC X-187 (H3N2); and B/Brisbane/60/2008. Mice were immunized intramuscularly (i.m.) with different doses of IIV3 (10 μ l containing 0.9 μ g total HA or 0.3 μ g H1N1 HA, or 2.5 μ l containing 0.225 μ g total HA or 0.075 μ g H1N1 HA) or IIV3 mixed with a varied amount of purified rOv-ASP-1 pre-treated with Polymyxin B for 60 min. Control mice were injected with the PBS 0.1% SDS admixed with a similar final amount of Polymyxin B; the buffer solution of rOv-ASP-1 is referred to as control hereafter. Two weeks post immunization the mice were bled, and three weeks post immunization mice were challenged with the Influenza virus.

Protection against influenza virus:

Influenza A/California/7/2009 (H1N12009) virus strain was propagated in specific pathogen-free fertile chicken eggs (30). Groups of 3–5 mice were challenged intranasally (i.n.) after day 21 post primary immunization. Mice were anesthetized intraperitoneally with 2 mg ketamine and 0.15 mg xylazine, and inoculated i.n. with 30 μ l of 5000–20,000TCID₅₀ of H1N12009 influenza virus. The protective efficacy of immunization was measured by

following the weight of the mice and by their survival up to 15 days. Infected mice were weighed daily and mice that lost >25% of their original body weight were sacrificed according to IACUC guidelines. The weight represented in the figures thereafter is only from surviving mice in the various groups on the day of recording.

Anti-influenza specific IgG, IgG1 and IgG2c antibody responses:

The IIV3-specific antibody response was measured by ELISA as previously described (31). Briefly, 96-well ELISA plates were coated with IIV3 (0.09 µg of total HA). Antigen-bound serum antibodies were detected with HRP-labeled goat anti-mouse IgG, IgG1 or IgG2c antibodies (Molecular probes, Invitrogen). The titer of antibody was defined as the reciprocal of the highest dilution of sera giving an optical density greater than 2 times that of sera from control mice. Limit of detection (LOD) of IgG titer is indicated for each experiment in its respective figure. To determine antibody responses to individual viral proteins, 96-well ELISA plates were coated with 1µg/ml of recombinant Hemagglutinin (HA) or Neuraminidase (NA) proteins from influenza virus A/California/07/2009 (H1N1)pdm09 strain (BEI resources, NIAID, NIH). Mice sera were diluted 1 in 50 dilution and the antigen-bound serum antibodies were detected with HRP-labeled goat anti-mouse IgG (Molecular probes, Invitrogen). Absorbance was read at 450nm to determine O.D values. HAI antibodies were measured as previously described by Li et al., (32). Briefly, sera from immunized mice were serially diluted in U-bottom 96-well plates and 4 agglutinating doses of influenza virus with the A/California/07/2009 HA were added to each well in a total volume of 100 µl. These sera and virus mixtures were incubated at room temperature for 30 min. 12.5 µl of a 2% turkey red blood cell solution was then added and incubated at room temperature for 1 h, after which time the HAI antibody titer was determined as the reciprocal of the highest dilution at which hemagglutination was completely inhibited.

Cytokine analysis:

Quadriceps muscles from immunized mice were harvested after 4 h or 24 h of immunization, tendons were removed, and the muscles were homogenized in tissue protein extracting reagent (T-PER, Thermo-scientific) containing Complete Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics) at a proportion of 1 tablet/10 ml of T-PER. Homogenates were centrifuged at 10,000 x g for 10 min at 4°C and the total protein concentrations were determined in the supernatants using DC protein assay kit (BioRad). All muscle tissue homogenates were kept at 5 mg/ml concentration in T-PER buffer. Prior to analysis homogenates were diluted with assay buffer (Milliplex kit, Millipore) and 25 µl of the 1,500 µg/ml extract was taken in duplicates for cytokine/chemokine concentration measurement by magnetic-bead based assay (Milliplex kit, Millipore) according to manufacturer's instruction using the mouse 25-plex panel.

The profile of cells recruited into the injection site:

Quadriceps muscles from immunized mice were harvested after 18–24 h of the injection and cut into small pieces. A single cell suspension was prepared using skeletal muscle dissociation kit (Miltenyi Biotec) according to manufacturer's protocol. Briefly, small pieces of muscle were incubated in enzyme solution (enzyme D, enzyme P and enzyme A in DMEM) for 30 min at 37°C with gentle shaking, passed through 70 µm cell strainer and

wash with 10 ml DMEM. Centrifuge cell suspension at 300xg for 20 min. Cells were stained with a combination of the following fluorescently labeled antibodies: anti-CD45-AF700, anti-CD3-BV786, anti-CD19-BV605, anti-CD49b-PE, anti-CD11b-PE-Cy5, anti-CD11c-BV711, anti-F4/80-FITC, anti-Ly6C-PerCp, anti-Ly6G-PE/Cy7, anti-MHCII-PE/Dazzle594, anti-CD80-BV650, anti-CD40-APC, anti-CXCR3-BV510 and anti-CCR2-BV421 (all from BioLegend). Live/dead cells were analyzed using fixable viability dye efluor780 (eBioscience). The stained cells were acquired using BD LSRFortessa cell analyzer and data was analyzed using FlowJo software (Tree Star). Total immune cells (CD45⁺), myeloid cells (CD11b⁺), monocytes (CD11b⁺Ly6C⁺), macrophages (CD11b⁺F4/80⁺), neutrophils (CD11b⁺Ly6G⁺), dendritic cells (DCs; CD11c⁺MHCII⁺), natural killer cells (NK cells; CD49b⁺, CD3⁻), B cells (CD11b⁻CD11c⁻CD19⁺), T cells (CD11b⁻CD11c⁻CD3⁺) and NKT cells (CD49b⁺CD3⁺) were gated by flow cytometry.

Activation of monocyte-derived human dendritic cells with rOv-ASP-1:

Peripheral blood mononuclear cells (PBMCs) were isolated from leukopaks by Histopaque® –1077 (Sigma-Aldrich). CD14⁺ cells were then purified from the PBMCs by negative selection using Pan Monocyte isolation kit and MACS column from MACS Miltenyi Biotech as per manufacturer's protocol. Purified CD14⁺ cells were cultured in RPMI with 50 ng/ml of GM-CSF and 20 ng/ml of IL-4 (R&D Biosystems) in a 6-well culture plate for 5 days. On day 4, media was replenished with fresh media containing 50 ng/ml of GM-CSF and 20 ng/ml of IL-4. On Day 5, the loosely adherent dendritic cells (DCs) were harvested, counted and transferred to fresh plates at 3×10⁶ cells/ml and stimulated for 24 hrs with either 5 µg/ml of Polymyxin B pre-treated rOv-ASP-1 or with media alone (control) containing the same amount of PBS 0.1% SDS admixed with Polymyxin B that was used for the preparation of rOv-ASP-1.

RNA isolation and sequencing library preparations:

RNA was isolated from the rOv-ASP-1 stimulated or control DCs using Trizol extraction coupled with Qiagen RNEasy mini kit for better cleanup and purification. Poly-A enrichment was then done to deplete ribosomal RNA and other contaminants and sequencing libraries were prepared using NEB-ULTRA kit according to manufacturer protocol. Libraries were quantified using Qubit2.0 and size distribution assessed by TapeStation. Equimolar libraries were then pooled and sequencing was done using the Illumina Nextseq 500 platform with 2×75bp paired end reads. Samples were run using biological triplicates.

Differential expression analysis and pathway enrichment:

Alignment was done using Tophat mapper with UCSC hg38 human genome assembly as reference (<http://genome.ucsc.edu/cgi-bin/hgGateway?db=hg38>). The annotation used can be found at gencode.v23.chr_patch_hapl_scaff.annotation.gtf. CuffDiff was then used to perform differential expression analysis for pairwise comparisons calculating p-values and fold changes. The R package cummerbund (<http://bioconductor.org/packages/release/bioc/html/cummerbund.html>) was used to visualize cufflinks outputs, perform clustering and report pairwise differentially expressed gene counts. A total of 5309 genes were differentially expressed. Data was then filtered to select those with a minimum *p*-value of

0.001 and a minimum of 1.8 fold change. A total of 2564 genes were selected post-filtering and those significantly up or down-regulated genes were then identified. Ingenuity Pathways Analysis (IPA) was then used to identify enriched pathways. Enriched pathways were then inspected and expression data overlaid to generate integrated pathway maps. Pheatmap R package was used to visualize expression profiles for relevant genes and pathways.

Statistical analysis:

The unpaired, two-tailed Student's t-test was used to determine if the difference in immune responses between groups of mice was significant. Multiple t-test comparisons were done using the Holm-Sidak method. Datasets involving comparisons of more than two groups were analyzed by two-way ANOVA followed by Tukey' post-hoc test. Survival rates were analyzed by the log-rank (Mantel-Cox) test. Antibody titers were expressed as mean $\pm$ SEM. $p < 0.05$ was considered statistically significant.

Results

The rOv-ASP-1 protein is an effective adjuvant for the influenza vaccine and allows a 40-times antigen sparing:

The pTrcHis/DH5 α expressed rOv-ASP-1 has been previously reported to enhance anti-influenza antibody responses in both adult and aged immunized mice (18, 24). The previously employed *E. coli* pTrcHis/DH5 α expression system resulted in low levels of protein expression. To increase the expression yield of the rOv-ASP-1 adjuvant we have re-cloned its coding sequence in the pET28a expression vector. To confirm that the newly pET28a/BL21 expressed rOv-ASP-1 protein lot is active as an adjuvant, mice were immunized with 25 μ g and 12.5 μ g rOv-ASP-1-adjuvanted IIV3 (0.9 μ g total HA or 0.3 μ g H1N1 HA). It was observed that when rOv-ASP-1 is used in half amount than those used in previous studies induced similar titers of anti-IIV3 antibodies and the mice were protected; the immunized mice did not lose weight after an influenza viral challenge as compared to IIV3 immunized mice (Fig. 1A and 1B). To test whether the amount of the IIV3 within the adjuvanted-IIV3 vaccine can be further reduced, we compared the antibody responses between 0.9 μ g total HA or 0.3 μ g H1N1 HA (10X antigen sparing) vs. 0.225 μ g total HA or 0.075 μ g H1N1 HA (40X antigen sparing) IIV3 adjuvanted with 12.5 μ g rOv-ASP-1. The rOv-ASP-1-adjuvanted IIV3-spared vaccine had significantly increased anti-IIV3 antibody titers and the mice did not lose weight after challenge with the virus (Fig. 1C and 1D). These results suggest that rOv-ASP-1 increased influenza vaccine potency even when used in a much reduced (0.225 μ g total HA or 0.075 μ g H1N1 HA) antigen dose.

To evaluate the adjuvanticity of rOv-ASP-1 with various batches of seasonal IIV3 vaccine, we compared its adjuvanticity using two different annual batches of the Novartis IIV3 vaccine; 2013–14 and 2015–16. Notably, rOv-ASP-1 enhanced anti-IIV3 specific antibody titers and decreased disease severity (no weight loss) after H1N12009 virus challenge regardless of the batch of the vaccine used (Supplementary Fig. 2).

In preparation for product development of rOv-ASP-1 for human testing, it became apparent that the purified rOv-ASP-1 needed to be kept in 0.1% SDS to stay soluble and effective. To

identify other detergents that can be used to support the solubility of rOv-ASP-1, the recombinant protein in PBS containing 0.1% SDS, 0.4% polysorbate-80 or 0.3% tergitol was tested for their adjuvanticity *in vivo* with the IIV3 vaccine. As expected, rOv-ASP-1 in PBS containing 0.1% SDS enhanced the efficacy of the IIV3 vaccine, while rOv-ASP-1 in PBS containing polysorbate-80 or tergitol did not show any adjuvant effect in terms of stimulating antibody responses and/or protecting mice from virus challenge (Supplementary Fig. 3). Other expression systems for the production and storage of this protein adjuvant may need to be evaluated once it is moved forward for pre-clinical development.

Both Th1 and/or Th2 associated antibody responses are important for rOv-ASP-1 adjuvanticity:

rOv-ASP-1 has been shown to induce a balanced Th1/Th2-associated antibody response. To test which of the antibody responses, Th1 (IgG2c) and/or Th2 (IgG1) associated, are important for IIV3+rOv-ASP-1 mediated protection against the influenza virus, we immunized wildtype (WT), IFN $\gamma^{-/-}$ (Th1 cytokine *i.e.*, mice will be IgG2c deficient) and IL-4 $^{-/-}$ (Th2 cytokine *i.e.*, mice will be IgG1 deficient) mice with IIV3 or IIV3+rOv-ASP-1. Notably, similar levels of anti-IIV3 IgG antibodies were observed in IIV3 immunized WT, IFN $\gamma^{-/-}$ and IL-4 $^{-/-}$ mice. However, the titer of the anti-IIV3 antibodies after immunization with IIV3+rOv-ASP-1 was significantly lower in IL-4 $^{-/-}$ mice than in WT mice, while it was similarly elevated in the IFN $\gamma^{-/-}$ mice and WT mice (Fig. 2A). As expected, immunization with IIV3+rOv-ASP-1 induced anti-IIV3 antibodies of both IgG1 and IgG2c isotypes in the WT mice, while immunization with IIV3 alone induced only IgG1 antibodies. The anti-IIV3 Th2-associated IgG1 antibody response was much reduced in IL-4 $^{-/-}$ mice than in the WT mice immunized with IIV3+rOv-ASP-1, while anti-IIV3 IgG1 antibodies were induced in the IIV3+rOv-ASP-1 immunized IFN $\gamma^{-/-}$ mice (Fig. 2B). No anti-IIV3 Th1-associated IgG2c antibody response was induced in the IFN $\gamma^{-/-}$ mice after immunization with IIV3+rOv-ASP-1, while significantly elevated anti-IIV3 IgG2c antibody responses were observed in the IL-4 $^{-/-}$ mice after IIV3+rOv-ASP-1 immunization (Fig. 2C).

To dissect out the antibody responses to individual protein components of the influenza virus, we further tested IgG antibody levels against the HA and NA proteins from the H1N1 influenza virus pandemic strain in WT, IFN $\gamma^{-/-}$ and IL-4 $^{-/-}$ mice immunized with IIV3 or IIV3+rOv-ASP-1. We observed that IIV3+rOv-ASP-1 immunized WT, IFN $\gamma^{-/-}$ and IL-4 $^{-/-}$ mice had significantly higher anti-HA IgG antibody response than mice that received IIV3 alone. No difference was observed in anti-HA antibody responses after immunization with IIV3+rOv-ASP-1 between IFN $\gamma^{-/-}$ mice and WT mice, while the anti-HA antibody responses were significantly lower in the IL-4 $^{-/-}$ mice than in WT mice (Fig. 2D). No anti-NA antibody responses were observed in IIV3 or IIV3+rOv-ASP-1 immunized WT, IFN $\gamma^{-/-}$ and IL-4 $^{-/-}$ mice (data not shown).

Although there were differences in the titers of anti-IIV3 IgG1 and/or IgG2c antibody responses in WT, IFN $\gamma^{-/-}$ and IL-4 $^{-/-}$ IIV3+rOv-ASP-1 immunized mice, none of the mice had body weight loss after virus infection, and 100% of the mice survived infection. This suggests that the presence or absence of either Th1- or Th2- associated antibody response after immunization with the rOv-ASP-1-adjuvanted IIV3 vaccine does not affect the

outcome of protection from virus challenge. The IIV3 immunized WT, IFN $\gamma^{-/-}$ and IL-4 $^{-/-}$ mice had on the other hand a similar weight loss profile and a comparable percentage of survival (28.5%, 14.3%, and 14.3%, respectively) (Fig. 2E and 2F).

HAI (Hemagglutinin inhibition) titers are currently regarded as an immunological correlate of protection induced by vaccines against influenza infection (33); they reflect the presence of antibodies that block receptor binding of influenza viruses. Notably, in our studies one immunization of mice with IIV3 (0.9 μ g of total HA or 0.3 μ g H1N1 HA, or with 0.225 μ g of total HA or 0.075 μ g H1N1 HA) or with rOv-ASP-1 (25 μ g or 12.5 μ g) adjuvanted IIV3 (0.9 μ g of total HA or 0.3 μ g H1N1 HA, or with 0.225 μ g of total HA or 0.075 μ g H1N1 HA) did not elicit HAI titers (<1:20) albeit, the mice immunized with rOv-ASP-1-adjuvanted IIV3 survived H1N1 viral challenge (Figs. 1 and 2). HAI titers >1:40 were elicited only after a boost immunization with the adjuvanted IIV3 vaccine (data not shown). Notably, several novel influenza virus vaccines that are currently under development do not target the hemagglutinin head domain, but rather more conserved sites, including the hemagglutinin stalk. Importantly, antibodies against the hemagglutinin stalk do not show activity in hemagglutination inhibition assays and will require different methods for quantification (34–37). These studies and our results suggest that the protective immunity induced *in vivo* by the rOv-ASP-1-adjuvanted IIV3 vaccine might be independent of the presence of HAI antibodies.

CD4⁺ T cell derived immune responses are indispensable for IIV3 and rOv-ASP-1-adjuvanted IIV3 vaccine:

It is known that adjuvants utilize CD4⁺ Th help cells for antibody generation by B cells (39, 40). However, a recent report have shown that even in the absence of CD4⁺ T cell responses the MF59-adjuvanted IIV3 vaccine was able to generate IgG antibody responses with memory (41). Our studies have shown that the rOv-ASP-1 mediated antibody response was CD4⁺ T cell dependent; the CD4 $^{-/-}$ mice did not produce any anti-IIV3 or anti-HA IgG antibodies after immunization with IIV3 or IIV3+rOv-ASP-1 (Fig. 3A and 3B). Consequently, all the CD4 $^{-/-}$ mice in the IIV3 and IIV3+rOv-ASP-1 immunized groups had severe weight loss and succumbed to infection within 7 days of viral challenge (Fig. 3C and 3D). These results indicate that CD4⁺ T cell help is necessary in mediating protective antibody response against the influenza virus, and rOv-ASP-1 adjuvant is also dependent on CD4⁺ T cells to mediate this protective immunity.

Immunization with rOv-ASP-1 or rOv-ASP-1-adjuvanted IIV3 induces an increased level of chemokines into the muscle:

Most adjuvants work by activating the functions of the innate immune system which includes induction of chemokine and cytokine, chemotaxis of immune cells, antigen processing and presentation (20). Hence, we also studied the immune microenvironment at the site of injection *i.e.*, muscles. Muscles from control, IIV3 or IIV3+rOv-ASP-1 immunized B6 mice were isolated after 4 h and 24 h after immunization and the presence of a total of 25 chemokines and cytokines in their lysates was analyzed. Significantly increased levels of several chemokines and cytokines, including KC, G-CSF, IP-10, MCP-1, MIP-1 β , MIP-2, and IL-6 were observed as early as 4 h after immunization with IIV3+rOv-ASP-1

when compared to muscles of mice after immunization with IIV3 alone (Fig. 4A). After 24 h, the amount of measured chemokines and cytokines was much reduced except for IP-10 and MCP-1 that maintained their higher levels also after 24 h (Fig. 4B). IL-6 after 24 h was much decreased. IIV3 immunization did not induce a significant increase in the levels of any chemokine or cytokine more than injection in control mice (Fig. 4A and 4B). To examine the contribution of the rOv-ASP-1 adjuvant in driving the increased level of chemokines and cytokines into the muscle, rOv-ASP-1 without IIV3 was injected. Apparently, the Polymyxin B treated rOv-ASP-1 alone also significantly stimulated the increased level of the same immune factors that were induced by immunization with IIV3+rOv-ASP-1; similar levels and at both time points 4 and 24 h (Supplementary Fig. 4). Other chemokines/cytokines measured including MIP-1 α , RANTES, GM-CSF, IL-1 α , IL-1 β and T-cell derived cytokines like TNF- α , IL-2, IFN- γ , IL-4, IL-5, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15 and IL-17 were either not induced by the immunization or did not show any difference in the levels among the various immunized groups of mice (Supplementary Table 1).

Myeloid immune cells are recruited to the site of injection *i.e.*, muscle in response to immunization with rOv-ASP-1:

rOv-ASP-1 induced release of chemokines which are known to recruit immune cells from circulation and secondary lymphoid organs to their site (42, 43). To profile the various types of immune cells recruited after immunization, muscles from mice after 18–24 h of immunization were analyzed. Significantly higher numbers of leukocytes (CD45⁺) were observed in the muscles of IIV3+rOv-ASP-1 immunized mice when compared to muscles from IIV3 immunized mice; most of these recruited leukocytes were CD11b⁺ myeloid cells (Fig. 5B and 5C). When the recruited cells were further characterized into different cell types based on differential cell surface marker expression, it was observed that immunization with IIV3+rOv-ASP-1 significantly increased the numbers of monocytes (CD11b⁺Ly6C⁺), macrophages (CD11b⁺F4/80⁺) and neutrophils (CD11b⁺Ly6G⁺) as compared to control or IIV3. Immunization with IIV3 recruited significantly more NK cells than control group. No increase in the numbers of dendritic cells (CD11c⁺MHCII⁺), NK cells (CD49b⁺) and/or adaptive immune cells like B (CD19⁺), T (CD3⁺) and NKT (CD3⁺CD49b⁺) cells was observed in any of the immunized mice (Fig. 5D). Immunization with rOv-ASP-1 alone recruited significantly higher numbers of myeloid cells including monocytes, macrophages, and neutrophils than control group (Supplementary Fig. 5), consistent with the chemokine profiles induced by injecting rOv-ASP-1 only into the muscle.

Cells recruited to the muscle after immunization were activated and expressed receptors specific to chemokines present in the muscle:

The activation of recruited cells in the muscle was analyzed by determining the expression of MHCII and CD80 on the surface of monocytes and macrophages. It was observed that the number of monocytes and macrophages that expressed MHCII was significantly higher in IIV3+rOv-ASP-1 than IIV3 immunized mice. The number of monocytes and macrophages expressing CD80, however, was higher in IIV3+rOv-ASP-1 than IIV3 immunized mice, although not significantly (Fig. 6A). As MCP-1 and IP-10 chemokines were highly present in the muscles, the expression of their receptors CCR2 and CXCR3, respectively, was also

examined. Recruitment of significantly higher number of CCR2⁺ monocytes was found in response to immunization with IIV3+rOv-ASP-1 than IIV3 immunized mice. Although the number of CXCR3⁺ monocytes in the IIV3+rOv-ASP-1 immunized mice was also increased, the increase was not statistically significant (Fig. 6B). The number of macrophages expressing either CCR2 or CXCR3 was not found to be significantly different among the groups of immunized mice (data not shown). The cell recruitment into the muscle after immunization with the Polymyxin B pre-treated rOv-ASP-1 alone was also analyzed; increased numbers of MHCII⁺ monocytes and macrophages were observed in the muscles after immunization of rOv-ASP-1 without IIV3 (Supplementary Fig. 6A). Additionally, immunization with rOv-ASP-1 alone also recruited monocytes which expressed CCR2 and CXCR3 on their surface as observed similarly with IIV3+rOv-ASP-1 immunization (Supplementary Fig. 6B). These results suggest that immune modulation at the site of injection is induced predominantly by rOv-ASP-1, while the IIV3 vaccine antigens have a minimal role at least with the tested attributes.

Protection induced by the rOv-ASP-1 adjuvanted IIV3 vaccine is observed in the absence of MyD88:

We have previously reported that rOv-ASP-1 appears to primarily bind to human monocytes and B cells, and that it also has the ability to mature and activate DCs to induce a Th-1 biased immune response in a TLR2/4-dependent signaling pathway (27). To further evaluate whether rOv-ASP-1 is a novel potent innate adjuvant that could activate TLR2/4 signaling pathways in mice, MyD88^{-/-} mice were either immunized with IIV3 or IIV3+rOv-ASP-1. Similar to WT mice, MyD88^{-/-} mice after immunization with IIV3+rOv-ASP-1 had significantly higher levels of anti-IIV3 and anti-HA IgG antibody responses than mice immunized with IIV3 only. However, the anti-HA antibody responses were significantly lower in the MyD88^{-/-} mice than in WT mice after immunization with IIV3+rOv-ASP-1 (Fig. 7A and 7B). Notably, 71.4% MyD88^{-/-} mice vaccinated with IIV3+rOv-ASP-1 survived H1N1 viral challenge while none of the MyD88^{-/-} mice immunized with IIV3 survived the viral challenge (Fig. 7C and 7D). These data suggests that rOv-ASP-1-adjuvanted IIV3 vaccine mediate protection to influenza viral challenge also in the absence of MyD88 pathway.

Stimulation of human naïve DCs with rOv-ASP1 up-regulates anti-viral immunity related genes:

To further understand rOv-ASP-1 immunostimulatory properties on innate cells, we performed RNAseq of rOv-ASP-1-activated monocyte-derived human DCs. The transcriptome data further corroborate our previous finding; stimulation of DCs with rOv-ASP-1 elicited the up-regulation of several signaling pathways including the TLR-signaling, IFN signaling and the maturation of DCs (Fig. 8A). It is interesting to note the presence of the antiviral immunity related, IRF-activation pathway, within the top overrepresented pathways (Fig. 8A). A closer look at the regulation of TLR pathway related genes in response to rOv-ASP-1 stimulation (Fig.8B) suggests that a specific TLR4 pathway was up-regulated (Fig. 8C). However, the up-regulation of TICAM1 and TICAM2 as adaptor proteins and the lack of TIRAP overexpression favor a pathway that might be operating also in the absence of MyD88-dependent TLR4 signaling pathway (44). It has been previously

shown that TLR4 MyD88-independent signaling through TICAM1 preferentially induces IFN β overexpression (44). This cascade activates anti-viral immunity genes, which appear to be also regulated in response to rOv-ASP-1 stimulation (Fig. 8D). It is known that the activation of type I IFN and IFN-responsive genes requires the activation of IRFs (45), a pathway that also appears to be overrepresented in our transcriptome data (Fig. 8E). We also observed from our transcriptome data the up-regulation of interferon signaling (Fig. 8F), which is known to occur after TLR4 activation (44), and more specifically, type I interferon-signaling pathway (Fig. 8G).

Discussion

Annual vaccination against infectious influenza virus can prevent deaths due to epidemic and pandemic disease (46). Development of new adjuvants for influenza vaccine is an active area of research. Recently, MF59 has been approved by FDA to be used in influenza vaccine formulation for elderly people, >65 years of age in USA. Our group has identified an *O. volvulus*-derived protein named rOv-ASP-1, which enhances immune responses when co-administered with several bystander vaccine antigens (25, 47). The *E. coli* expressed recombinant rOv-ASP-1 protein has to be dissolved in PBS with 0.1% SDS in order to stay soluble. As SDS will pose a problem in the future when the adjuvant is developed for human testing, we tested other detergents that are considered generally recognized as safe (GRAS) in humans; polysorbate 80 and tergitol. Unfortunately, formulation of rOv-ASP-1 with these two detergents have affected its efficacy as an adjuvant; other expression systems for the production of the recombinant protein such as the yeast expression system may need to be evaluated in the future once we move forward for human vaccine development.

Reports from our group have shown that this protein effectively enhances anti-influenza IgG response and protects mice following lethal influenza virus challenge. Moreover, the rOv-ASP-1-adjuvanted IIV3 vaccine induced cross-protection of aged mice from influenza virus (18, 24). The present study further validates the potent adjuvanticity of rOv-ASP-1 with the seasonal influenza vaccine; it supported antigen sparing of the IIV3 vaccine up to 40 times of the protective un-adjuvanted dose in mice. In addition, when the amount of rOv-ASP-1 was reduced by half (from our earlier published data) also showed similar efficacy with IIV3 in the present study. Our present results demonstrate the adjuvanticity of rOv-ASP-1 in its ability to enhance anti-influenza protective responses with antigen sparing, and that are not necessarily dependent on the elicitation of significant titers of HAI antibodies. Notably, this adjuvant can be potentially used in a simple aqueous formulation with a much reduced seasonal IIV3 vaccine dose, and may thus help overcome the operational challenges of producing sufficient quantities of the influenza virus antigens for the vaccine formulation at times of epidemic or pandemic outbreaks. Presently, people are vaccinated with 500 μ l IIV3 that contains 45 μ g of total HA; 15 μ g HA from each of the three viruses within IIV3. We were able to elicit protection in mice using rOv-ASP-1-adjuvanted IIV3 using only 0.225 μ g total HA or 0.075 μ g H1N1 HA versus the full protecting dose of the un-adjuvanted IIV3; 9 μ g total HA or 3 μ g H1N1 HA. However, whether such considerable antigen sparing effect will also be effective for human vaccines will demand clinical trials for validation.

Mechanism of the adjuvanticity of rOv-ASP-1 in mice has not been fully understood. It is known that rOv-ASP-1 induces a balanced Th1/Th2 antibody response; however, whether any of these subtypes are important for protection against influenza is not known. IFN γ and IL-4 play important roles in antibody class-switching (48), where IFN γ is known to favor Th1 associated isotypes of antibody isotypes (IgG2a, IgG2b and IgG2c) while IL-4 induces Th2 (IgG1) class of antibodies (49, 50). The rOv-ASP-1 adjuvanted IIV3 vaccine stimulated high anti-IIV3 and anti-HA IgG antibody responses in mice deficient for IFN γ and IL-4. IgG1 and IgG2c production was compromised in IL-4 and IFN γ knockout mice respectively. Regardless, the IgG, IgG1 and IgG2c antibody responses against the virus antigens or against the HA protein appeared to be protecting mice against virus challenge even in the absence of HAI antibodies, thus suggesting the presence of unidentified specificities of protective antibodies regardless of the antibody isotype.

CD4⁺ Th cells play multifaceted role in immune response like secretion of cytokines, activation of B cells, isotype switching of antibodies, and regulation of immune response (51, 52). CD4⁺ Th cells are very important for eliciting immune response with split vaccine (53–55). As expected, IIV3 vaccine responses are completely dependent on help provided by the CD4⁺ Th cells. Conversely, MF59 adjuvant has recently been shown to induce effective antibody responses against influenza virus in absence of CD4⁺ T cell responses (41). In our studies, although the isotype of antibodies induced by the rOv-ASP-1 adjuvanted influenza vaccine were not important for protection, the CD4⁺ T cell responses were critically important. CD4⁺ Th cells are important for enhancing B cell mediated immunity activation of antibody secretion from B cells, which are capable of neutralizing pathogens (56–59). Our data suggests that rOv-ASP-1 adjuvanted IIV3 are dependent on CD4⁺ Th cells to mediate antibody response and protect mice against influenza. As the precise role of T-mediated immunity in influenza infection has not been fully characterized, additional studies focusing on the importance of secondary lymphoid organs for the education of adaptive immune cells will have to be undertaken to further our understanding of the protective mechanism(s) elicited by our adjuvanted IIV3 vaccine.

The HAI (Hemagglutinin inhibition) assay is commonly used to measure protective antibodies that bind at or near the HA receptor binding site (RBS) and therefore inhibit virus-induced hemagglutination (35). From our studies, we found that single rOv-ASP-1 adjuvanted IIV3 immunization conferred protection in mice to the H1N1 viral challenge even without inducing HAI titers (<1:20). This could be due to the fact that low amounts of influenza vaccine (0.9 μ g or 0.225 μ g of total HA or 0.3 μ g or 0.075 μ g of H1N1 HA) used for immunization with or without rOv-ASP-1 adjuvant were not sufficient to induce detectable titers of HAI antibodies. When we boosted the mice we were able to detect HAI titers >1:40 two weeks post one boosting immunization (data not shown), however, such a regimen of immunization is not easily translatable to human studies, and therefore was not continued. Other recent studies (34–37) suggest a significant role of protective immunity *in vivo* by antibodies other than HAI antibodies. Future experiments will have to be done to determine the characteristics of the antibodies our vaccine has induced, and that the protection we have conferred by one immunization with the rOv-ASP-1 adjuvanted IIV3 is mediated by the induction of other non-HAI antibodies using passive transfer of sera experiments. Interestingly, a recent study has shown that influenza virus hemagglutinin

stalk-specific antibodies in human serum can also be a surrogate marker for *in vivo* protection in a serum transfer mouse challenge model (38). Preliminary studies with AID^{-/-} mice confirmed that antibodies are essential for the ability of the ASP-1-adjuvanted IIV3 vaccine to confer protection to mice against challenge with the influenza virus (data not shown).

Most of the adjuvants are conventionally thought to activate innate immune system, enhance antigen presentation by antigen presenting cells, which subsequently activate variety of T helper cells and cytotoxic T cells followed by antibody secretion by B cells (20, 22, 60–64). Most human vaccines are administered intramuscularly, where the local immune environment is modulated by the vaccine adjuvants by affecting the innate immune responses such as recruitment of antigen presenting cells and neutrophils (65–67). The Polymyxin B pre-treated rOv-ASP-1 adjuvant, with or without the influenza vaccine, elicited an increased level of variety of chemokines such like KC, G-CSF, IP-10, MCP-1, MIP-2 and IL-6 into the site of injection soon after immunization. KC is known to chemoattract neutrophils (68, 69); G-CSF is a growth factor important for survival, proliferation and differentiation of neutrophils (70); IP-10 is a chemoattractant for monocytes/macrophages, dendritic cells, NK cells and T cells (71); MCP-1 mainly recruits monocytes (72); and MIP-2 is chemotactic for polymorphonuclear leukocytes (73). IL-6 acts as a pro-inflammatory chemokine as well as anti-inflammatory myokine (74). It might have secreted by myocytes because of injection injury or after activation of immune cells by rOv-ASP-1. Apparently, rOv-ASP-1 induced formation of a microenvironment at the site of injection which is conducive for the recruitment, survival and proliferation of variety of immune cells which can process and present influenza antigen, and can subsequently activate antigen-specific T and B cells.

Injection of mice with Polymyxin B pre-treated rOv-ASP-1 also elicited the recruitment of high numbers of myeloid immune cells to the site of injection which are mainly monocytes, macrophages and neutrophils. This observation corroborates with the environment of chemokines present in the muscle after rOv-ASP-1 immunization. Monocytes and macrophages recruited by rOv-ASP-1 were activated as observed by MHCII expression on their surface which may be involved in antigen processing and presentation. These recruited monocytes may differentiate to macrophages and dendritic cells which are potent antigen presenting cells and activate T cell immunity. Chemokine-chemokine receptor signaling plays very important role in mounting immune responses (43, 75). IP-10 and MCP-1 were two chemokines which were present in high concentration in muscles after 24 h of immunization and expression of their receptors CXCR3 and CCR2 was observed on the recruited monocytes. CCR2 expressing monocytes are inflammatory in nature and they help in generating heightened immune response against immunized antigens and activate T cells (76–78). These observations suggest that rOv-ASP-1 elicits secretion of chemokines and recruitment of immune cells at the site of injection which is favorable for antigen processing and presentation which subsequently help for the activation of adaptive long lasting immunity.

Many studies have indicated that innate immune responses are important in enhancing adaptive immune responses. The TLR signaling in antigen-presenting cells and other cell

types is known to induce the production of various cytokines to augment subsequent adaptive immune responses (79, 80). We have previously reported that rOv-ASP-1 trigger Th1-biased proinflammatory cytokine production probably via the activation of monocyte-derived dendritic cells, and TLR2 and TLR4 (27). Most of the TLR signaling is through the MyD88 adaptor molecule with the exception of TLR3 (which is through the TRIF adaptor molecule). It is noted that TLR4 can also signal through a MyD88-independent pathway. Our current data show that MyD88^{-/-} mice that received rOv-ASP-1 adjuvanted IIV3 vaccine were able to induced anti-IIV3 and anti-HA IgG antibody response, and protect mice from H1N1 viral challenge. This data suggests that the rOv-ASP-1 adjuvanted IIV3 vaccine can induce protective immunity also in the absence of a MyD88 pathway.

As such, all MyD88-dependant TLR pathways seem to be irrelevant leaving cytoplasmic TLR4 and potentially TRAF3 mediated TLR3 signaling as the initial pathways induced in the innate cells. Transcriptome data from human DCs treated with polymyxin pre-treated rOv-ASP-1 provides further evidence in favor of a cytoplasmic TLR4 activation. The differential expression of TLR related adaptor proteins, and more precisely TICAM1, 2, coupled with a lack of expression of TIRAP further suggests a MyD88-independent TLR4 signaling. Identifying which pathway is essential still requires further testing and validation. Yet, it has been previously reported that TLR4-TRAF6 mediated signaling plays a crucial role in mounting anti-viral immunity responses (81, 82). The treatment with rOv-ASP-1 also seems to activate anti-viral immunity through type I interferon signaling and potentially IRF activation. The overexpression of TBK1 and IKKε has been shown to upregulate phosphorylation of IRF3 and IRF7, both playing a pivotal role in response to viral antigens (45). It has also been reported that both TBK1 and IKKε, feedback into type I IFN expression and upregulate IFNβ by modulating its promoter activity. This activation further enhances type I IFN signaling and antiviral immunity related genes. Type I IFN signaling is known to trigger the transcription of Interferon-stimulated genes (ISGs) such as, IRF1, IRF9, ISG20, ISG15, MX1, OAS1, IFIT2, IFIT3, IFITM1 and IFITM3. All of the aforementioned genes have been reported to play major roles in antiviral immunity (83) and our data shows a corresponding overexpression of these genes. This finding is of extreme relevance to the adjuvanticity of this novel protein adjuvant as it triggers anti-viral immunity pathways on its own and without the presence of co-administered viral components. This translates into higher immune responses when challenged with live virus and may potentially explain the mode of action of rOv-ASP-1.

In conclusion, rOv-ASP-1 is a highly potent adjuvant which allows antigen sparing for the seasonal influenza vaccine to a large extent (40X), which can be mixed with the vaccine easily to make formulation available to big population during needed times. The rOv-ASP-1 adjuvant activates humoral immunity in a T cell dependent manner resulting in an enhanced protective effect against influenza, and interestingly it is independent of the type of antibody response generated and/or the presence of HAI antibodies, which could help certain human population with cytokine gene polymorphism. The rOv-ASP-1 adjuvant activates the formation of immune niche at the site of immunization which may help antigen processing and presentation, T cell immune response, antibody generation and potentially also long-lasting memory responses. These findings indicate that rOv-ASP-1 could be used as a potential vaccine adjunct to improve human health.

Our initial transcriptomic studies of rOv-ASP-1 activated human DCs points to the possibility that the protection induced by the rOv-ASP-1-adjuvanted IIV3 antigen sparing immunization is mediated via novel pathways that are independent of the presence of HAI antibodies. Alternative possibilities could be the role of other neutralizing antibodies that are non-HAI such as those directed against the hemagglutinin stalk (34–37), the activation of the complement system or the interaction with accessory cells, similar to what was observed with the H7N1 vaccine (35), or the induction of antibody-dependent cellular cytotoxicity (38). Moreover, it appears that protection could be elicited also via signaling pathways that are operational in the absence of MyD88; through type I interferon signaling and potentially IRF activation. Future studies will need to be performed to validate these hypotheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

HA	Hemagglutinin
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
rOv-ASP-1	Recombinant <i>Onchocerca volvulus</i> activation associated protein-1
Th cells	T helper cells
T-PER	Tissue protein extraction reagent
IIV3	Trivalent inactivated influenza vaccine

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Highlights

- The rOv-ASP-1 adjuvant with 40-times less IIV3 can induce protection against the influenza virus
- The rOv-ASP-1 adjuvanted vaccine is independent of the elicited IIV3-specific antibody isotype response, and independent of the presence of HAI antibodies
- Adjuvanticity of rOv-ASP-1 for IIV3 is CD4⁺ T cell dependent, and is induced in the absence of the MyD88 pathway
- The rOv-ASP-1 adjuvant induces increased level of chemokines and innate cells into the injection site that may augment protective responses against influenza infection

Fig. 1.

The rOv-ASP-1 adjuvant enhances the efficacy of the influenza IIV3 vaccine using 40-times lower antigen dose. B6 mice were immunized with 0.9 or 0.225 µg IIV3 alone or in combination with 12.5 and 25 µg of rOv-ASP-1. (A and C) After two weeks sera was collected and influenza-specific IgG antibodies were measured by ELISA using IIV3 as an antigen. LOD is limit of detection for the assay. Three weeks post immunization mice were intranasally challenged with a 5000 TCID₅₀ of influenza A virus H1N12009. (B and D) Body weight and survival was recorded up to 12 days post challenge. Numbers in red represent the number of survived mice versus total number of mice per group. Mice that lost 25% weight during infection were euthanized and recorded as dead. Data is representing the results from one experiment that was repeated twice independently, and resulted with similar outcomes. Titers are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ (unpaired, two-tailed Student's t-test).

Fig. 2.

Both Th1/Th2 associated cytokines and antibody responses are not necessary for rOv-ASP-1 adjuvanticity of the influenza vaccine. WT, $IFN\gamma^{-/-}$ and $IL-4^{-/-}$ mice were immunized with 0.225 μ g IIV3 (0.075 μ g H1N1 HA) alone or adjuvanted with 12.5 μ g ASP-1. (A, B and C) Anti-IIV3 IgG antibody titers and the Th1- or Th2- associated IgG1 and IgG2c isotype antibody responses and (D) anti-HA IgG antibody response, respectively, were measured in sera collected 2 weeks after immunization by ELISA. LOD = Limit of detection for the assay. (E and F) Percent body weight and survival were recorded for 15 days post challenge with 7500 TCID₅₀ of influenza A (H1N12009 virus) 3 weeks after immunization of the WT, $IFN\gamma^{-/-}$ and $IL-4^{-/-}$ mice. Death was defined as $\geq 25\%$ weight loss. Titers of antibodies are expressed as mean $\pm$ SEM. The pooled experimental data are from 6–7 mice in each experimental group and from two separate experiments. Significance was calculated using unpaired, two-tailed Student's t-test or two-way ANOVA followed by Tukey's post-hoc test.

for A, B, C and D datasets; unpaired, two-tailed Student's t-test and log-rank (Mantel-Cox) test were used for data in Fig. E and F, respectively. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 3.

CD4 T cells play an important role in rOv-ASP-1 mediated adjuvanticity with IIV3 vaccine. 0.225 μ g IIV3 (0.075 μ g H1N1 HA) with or without 12.5 μ g ASP-1 was administered in WT and CD4^{-/-} mice. (A) anti-IIV3 and (B) anti-HA IgG antibody responses 2 weeks after primary immunization were detected using ELISA with IIV3 antigen or recombinant HA protein. LOD = Limit of detection for ELISA. (B and C) body weight and survival in CD4^{-/-} mice post challenge with influenza A H1N12009 (7500 TCID₅₀) virus was recorded for 15 days. Titers of antibodies or O.D values are represented as mean $\pm$ SEM and are pooled experimental data of two experiments with 3–4 mice per group. Significance was calculated using unpaired, two-way ANOVA followed by Tukey' post-hoc test for antibody titers and unpaired, two-tailed Student's t-test and log-rank (Mantel-Cox) test were used to calculate significances between groups for weight loss and survival. ** p < 0.01 and *** p < 0.001.

Fig. 4.

The rOv-ASP-1 adjuvanted IIV3 vaccine induces increased level of chemokines into the site of injection *i.e.*, muscle. B6 mice were injected intramuscularly with 0.9 μ g IIV3 with or without 25 μ g rOv-ASP-1; PBS with 0.1% SDS injected intramuscularly was used as the control. (A and B) Concentration of various chemokines and cytokines present at the site of injection of the immunized mice were measured after 4 h and 24 h using milliplex kit. The pooled experimental data present the mean $\pm$ SEM from two independent experiments with a total of 6 mice in each experimental group. * p < 0.05, unpaired, two-tailed Student's *t*-test between the IIV3 and the IIV3+ASP mice groups.

Fig. 5.

The rOv-ASP-1 adjuvanted IIV3 vaccine elicits the recruitment of myeloid cells to the site of injection. 0.9 μ g IIV3 with or without 25 μ g rOv-ASP-1 was injected into muscle of B6 mice and single cell suspensions were prepared after 18–24 h. Cells were stained with fluorescently labeled antibodies for different cell surface markers and analyzed by flow cytometry. (A) Plots represent gating strategy adopted to segregate flow acquired cells into different cell types based on differential expression of surface markers. (B) Representative FACS data to show myeloid cells (CD11b⁺) recruitment in the muscles of control, IIV3 or IIV3+ASP immunized mice. (C and D) Numbers of recruited total immune cells, myeloid cells and different immune cells per muscle of the immunized mice were calculated and presented using a logarithmic scale as mean $\pm$ SEM pooled from two independent experiments with total of 6 mice in each experimental group. Immune cells were segregated into different types based on their surface expression of markers. First, total immune cells

were defined as CD45⁺ which were then differentiated into myeloid cells (CD11b⁺), monocytes (CD11b⁺Ly6C⁺), macrophages (CD11b⁺F4/80⁺), neutrophils (CD11b⁺Ly6G⁺), dendritic cells (DCs; CD11c⁺MHCII⁺), natural killer cells (NK cells; CD49b⁺), B cells (CD11b⁻CD11c⁻CD19⁺), T cells (CD11b⁻CD11c⁻CD3⁺) and NKT cells (CD49b⁺CD3⁺). **p* < 0.05 (unpaired, two-tailed Student's t-test and corrected for multiple comparisons using the Holm-Sidak method).

Fig. 6.

Activation markers and chemokine receptors are expressed on the surface of myeloid cells recruited to the muscles in response to IIV3+r-Ov-ASP-1 immunization. B6 mice were injected in muscles with 0.9 μ g IIV3 with or without 25 μ g rOv-ASP-1. (A) Numbers of MHCII and CD80 expressing monocytes and macrophages were measured in muscles of mice immunized with control, IIV3 or IIV3+rOv-ASP-1 after 18–24 h of injection. (B) Number of monocytes expressing CCR2 and CXCR3. Data represents mean $\pm$ SEM from two independent experiments with total of 6 mice each group. * $p < 0.05$ (unpaired, two-tailed Student's t-test and corrected for multiple comparisons using the Holm-Sidak method).

Fig. 7.

Protection induced by rOv-ASP-1 adjuvanted IIV3 vaccine in the absence of the MyD88 pathway. 0.225 μ g IIV3 (0.075 μ g H1N1 HA) with or without 12.5 μ g ASP-1 was administered in WT and MyD88^{-/-} mice. (A) anti-IIV3 and (B) anti-HA IgG antibody responses 2 weeks after primary immunization were detected using ELISA with IIV3 antigen or recombinant HA protein. LOD = Limit of detection for ELISA. (C and D) body weight and survival in MyD88^{-/-} mice post challenge with H1N12009 (7500 TCID₅₀) was recorded for 15 days. Titers of antibodies or O.D values are represented as mean $\pm$ SEM and are representative of two experiments with 3–4 mice per group. Significance was calculated using two-way ANOVA followed by Tukey' post-hoc test for antibody titers and unpaired, two-tailed Student's t-test and log-rank (Mantel-Cox) test were used to calculate significances between groups for weight loss and survival. ** p < 0.01 and *** p < 0.001.

Fig. 8. Transcriptome sequencing of human monocyte-derived dendritic cells (DCs) treated with rOv-ASP-1.

(A) Stacked bar chart representing the top 15 overrepresented pathways with the corresponding p-values shown. The total number of genes in each pathway is reported. (B) Differential expression profile of genes related to Toll-Like Receptor (TLR) signaling pathways. (C) TLR signaling pathway with up-regulated genes highlighted in pink. Red arrows correspond to the TLR4 MyD88-independent activation pathway. (D) Expression profile of Interferon signaling related genes. (E) Type I interferon pathway with up-regulated genes highlighted in pink (Increasing intensities correspond to higher expression values). (F and G) Differential expression of genes involved in anti-viral immunity responses. Expression data shown in B, D and F corresponds to log10 of FPKM values.