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Recombinant Ov-ASP-1, a Th1-Biased Protein Adjuvant Derived from the Helminth *Onchocerca volvulus*, Can Directly Bind and Activate Antigen-Presenting Cells

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We previously reported that *rOv*-ASP-1, a recombinant *Onchocerca volvulus* activation associated protein-1, was a potent adjuvant for recombinant protein or synthetic peptide-based Ags. In this study, we further evaluated the adjuvanticity of *rOv*-ASP-1 and explored its mechanism of action. Consistently, recombinant full-length spike protein of SARS-CoV or its receptor-binding domain in the presence of *rOv*-ASP-1 could effectively induce a mixed but Th1-skewed immune response in immunized mice. It appears that *rOv*-ASP-1 primarily bound to the APCs among human PBMCs and triggered Th1-biased proinflammatory cytokine production probably via the activation of monocyte-derived dendritic cells and the TLR, TLR2, and TLR4, thus suggesting that *rOv*-ASP-1 is a novel potent innate adjuvant. *The Journal of Immunology*, 2009, 182: 4005–4016.

Recently, recombinant proteins and synthetic peptides have been considered as alternative approaches for vaccine development, but their poor immunogenicity emphasizes a major unmet need for immunostimulatory adjuvants capable of safely boosting both humoral and cellular immune responses. Currently, alum remains the only adjuvant approved for human use in the majority of countries worldwide. Although alum is able to induce a good Ab (Th2) response, it has little capacity to stimulate strong cellular (Th1) immune responses which are so important for protection against many pathogens (1). In addition, alum has the potential to cause severe local and systemic side-effects that limit its wide application for various vaccines in humans.

Protozoan and helminth parasites have proven to be a source of molecules that have potent regulatory and, sometimes, stimulatory effects on the immune systems of their mammalian hosts (1–4). Some of these molecules were shown to contain pathogen associated molecular patterns (PAMP) that bind to endocytic-pattern recognition receptors on APCs. Several helminth products were also reported to act as adjuvants in experimental vaccine models, but they preferentially induced Th2-type immune responses (5–8). For example, proteins secreted by adult *Nippostrongylus brasiliensis* induced strong Th2 responses in mice immunized with hen egg lysozyme (5). Similarly, lacto-N-fucopentaose III, a carbohydrate found on the surface of the eggs of a human parasite, *Schistosoma mansoni*, acted as a Th2 adjuvant for human serum albumin when injected intranasally, s.c., or i.p. into mice (6, 7). In a recent report, a 19 aa synthetic peptide (GK-1) from *Taenia crassiceps* cysticerci was shown to also have a capacity of an adjuvant (8). When it was coadministered with the inactivated anti-influenza vaccine in both young and aged mice, it induced increased levels of anti-influenza Abs in aged mice before and after infection, reduced the local inflammation that accompanied influenza vaccination itself, and favored virus clearance after infection in both young and aged mice.

Activation-associated secreted proteins (ASP) of parasitic nematodes are highly immunogenic and have been extensively studied as potential vaccine components against their corresponding parasites, with the ASPs from hookworms being the major vaccine candidates tested in human trials (9–15). Like other filarial nematodes are highly immunogenic and have been extensively studied as potential vaccine components against their corresponding parasites, with the ASPs from hookworms being the major vaccine candidates tested in human trials (9–15). Like other filarial nematodes, *Onchocerca volvulus* worms secrete a large number of immunoregulatory molecules to subvert the immune responses and minimize severe pathology and thus enabling their ability to establish a chronic infection in humans that can last >15 years (16). The native ASP-1 protein of *O. volvulus* (*Ov*-ASP-1) is located in the secretory granules of the glandular esophagus of the infective third-stage larvae and is predicted to have multiple immunoregulatory functions (2, 17). Our previous studies demonstrated that recombinant *Ov*-ASP-1 (*rOv*-ASP-1) is not only a protective Ag in vaccinated mice against *O. volvulus* third-stage larvae, but also reported to act as adjuvants in experimental vaccine models, but they preferentially induced Th2-type immune responses (5–8). For example, proteins secreted by adult *Nippostrongylus brasiliensis* induced strong Th2 responses in mice immunized with hen egg lysozyme (5). Similarly, lacto-N-fucopentaose III, a carbohydrate found on the surface of the eggs of a human parasite, *Schistosoma mansoni*, acted as a Th2 adjuvant for human serum albumin when injected intranasally, s.c., or i.p. into mice (6, 7). In a recent report, a 19 aa synthetic peptide (GK-1) from *Taenia crassiceps* cysticerci was shown to also have a capacity of an adjuvant (8). When it was coadministered with the inactivated anti-influenza vaccine in both young and aged mice, it induced increased levels of anti-influenza Abs in aged mice before and after infection, reduced the local inflammation that accompanied influenza vaccination itself, and favored virus clearance after infection in both young and aged mice.

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also a potent adjuvant for bystander proteins (2, 17–19). In OVA-immunized mice, rOv-ASP-1 exceeded the efficacy of alum or monophosphoryl lipid A (MPL) plus trehalose dicymoniumcylate (TDM) adjuvants in terms of end-point total IgG or IgG1 and IgG2a Ab titers (18). Interestingly, IgG isotype responses to OVA were of a mixed Th1/Th2-associated Ab profile but with a Th1 dominance and the spleen cytokine profiles were exclusively of the Th1 type (18). We have also shown that although rOv-ASP-1 induced similar level of IgG1 responses as alum when formulated with HBsAg as the bystander vaccine protein, it clearly induced significantly higher levels of IgG2a and IFN-γ producing T cell responses against HBsAg than alum. Furthermore, rOv-ASP-1 improved both IgG1 and IgG2a responses to three commercial inactivated vaccines (hemorrhagic fever with renal syndrome, influenza, and rabies) when used separately or in combination (20). In comparison with the other helminth-derived adjuvants shown to be strong inducers of Th2 responses to bystander proteins in vaccines (5–7, 21), the property of rOv-ASP-1 to induce both Th1- and Th2-associated Ab responses and, depending on the Ag, a Th1-biased Ab and cellular responses highlights its potential utility as an effective adjuvant against many existing and emerging pathogens, for whom the protective responses are of the Th1 type.

In this study, we further evaluated the adjuvanticity of rOv-ASP-1 and explored its mechanism of action. Consistently, rOv-ASP-1 effectively boosted recombinant SARS-CoV spike protein (rS) or its receptor-binding domain (rRBD) to induce a mixed but Th1-skewed immune response in the immunized mice. It appears that rOv-ASP-1 bound primarily to the APCs among human PBMCs and triggered Th1 biased proinflammatory cytokines probably via the activation of monocyte-derived dendritic cells (MoDCs) and the TLR, TLR2, and TLR4, thus suggesting that rOv-ASP-1 is a novel potent innate adjuvant.

Materials and Methods
Preparation of recombinant Ov-ASP-1
The recombinant Ov-ASP-1 protein (rOv-ASP-1) was expressed as a histidine-tagged protein in Escherichia coli and purified as previously described (18). The purified rOv-ASP-1 was tested negative in a Limulus amoebocyte lysate assay. A quantitative LPS testing by Cambrex BioScience showed that purified rOv-ASP-1 im proved both IgG1 and IgG2a responses to three commercial inactivated vaccines (hemorrhagic fever with renal syndrome, influenza, and rabies) when used separately or in combination (20). In comparison with the other helminth-derived adjuvants shown to be strong inducers of Th2 responses to bystander proteins in vaccines (5–7, 21), the property of rOv-ASP-1 to induce both Th1- and Th2-associated Ab responses and, depending on the Ag, a Th1-biased Ab and cellular responses highlights its potential utility as an effective adjuvant against many existing and emerging pathogens, for whom the protective responses are of the Th1 type.

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Immunoization of mice
Mouse animal protocols were approved by the Institutional Animal Care and Use Committee at the New York Blood Center. The recombinant full-length rS and its rRBD of SARS-CoV Urbani (accession number YAY278741) were expressed by recombinant baculovirus system as previously described (22). Four BALB/c mice or five C57BL/6 (6 wk old) per group were s.c. immunized with 400 μg/ml rS protein was used to coat 96-well microtiter plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 2% nonfat milk, serially diluted mouse sera were added and incubated at 37°C for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Bound Abs were detected with goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 Abs (Sigma-Aldrich) as previously described (18). Then, biotylated rabbit anti-goat IgG and extravidin peroxidase conjugate (both from Sigma-Aldrich, 1/2000 dilution for 1 h at 37°C) were added sequentially. After washes, the reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine and the absorbance at 450 nm was measured by an ELISA plate reader (Tecan U.S.).

SARS pseudovirus and neutralization assay
A SARS-CoV pseudovirus system was developed in our laboratory as previously described (23, 24). In brief, human embryonic kidney cells (HEK) 293 T cells were cotransfected with a plasmid encoding the S protein corresponding to SARS-CoV Tor2 isolate and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) by using FuGENE 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudovirus were harvested 48 h post transfection and used for single-cycle infection of human or civet ACE2-transfected 293T (293T/ACE2) cells. In brief, 293T/ACE2 cells were plated in 96-well tissue culture plates at 104 cells/well and grown overnight. The supernatants containing SARS pseudovirus were preincubated with serially diluted antisera from vaccinated mice at 37°C for 1 h before adding to the 293T/ACE2 cells. The cultures were fed with fresh medium 24 h later and then incubated for an additional 48 h. The cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar), followed by addition of luciferase substrate (Promega). Relative light units were determined immediately in the Ultra 384 luminometer (Tecan U.S.). Values are presented as the Ab titers that provide 50% neutralization of rS binding to ACE2.

Stimulation of mouse splenocytes and measurement of cytokines
Spleens were harvested from the immunized mice and single-cell suspensions of splenocytes were obtained from the pooled spleens by mincing through cell strainers. Erythrocytes were lysed with ammonium chloride solution. Splenocytes were resuspended in complete RPMI 1640 medium to 1 × 107 cells/ml and 0.2 × 107 cells were plated to 96-well U-bottom plates for culture. The cells were stimulated with 1 μg/ml rRBD protein for 24 h. Cells were cultured supernatants were collected after stimulation and mouse cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and TNF-α) were quantified by cytometric bead array (CBA) kit (BD Biosciences) according to the manufacturer’s protocol.

IFN-γ ELISPOT assay
Specific IFN-γ-producing cells from the splenocytes of vaccinated mice were detected by BD ELISPOT Mouse IFN-γ ELISPOT Set (BD Biosciences) according to the manufacturer’s protocol (BD Biosciences). In brief, 96-well ELISPOT plates were coated with anti-IFN-γ mAb overnight at 4°C, and blocked by the blocking solution (RPMI 1640 containing 10% FBS). Single-cell suspensions prepared from the spleens of vaccinated mice were added to the wells at the concentration of 2 × 105 cells/well. Cells were incubated for 24 h in the presence or absence of the rRBD (1 μg/ml), followed by washes with PBS. The cells were sequentially incubated with biotinylated anti-mouse IFN-γ mAb for 2 h at room temperature, streptavidin-conjugated HRP for 1 h at room temperature and 3,3′,5,5′-tetramethylbenzidine substrate solutions for around 15 min, with extensive washes between incubations. The spots of IFN-γ-producing cells were counted by ELISPOT reader system.

Flow cytometry-based binding assay
rOv-ASP-1 and a similarly expressed control protein, chloramphenicol acetyl transferase (CAT), were labeled with biotin using the FluorReporter Minibiotin-XX Protein Labeling Kit (Molecular Probes). Fresh healthy donor PBMCs (n = 4) were isolated by density gradient centrifugation over Ficoll (Sigma-Aldrich). Five μg of biotinylated rOv-ASP-1 or CAT were then incubated with 1 × 106 human PBMCs at 37°C for 30 min. After washing the cells at 4°C, the cells were incubated with streptavidin FITC (Amersham Pharmacia Biotech) for 20 min followed by 30 min incubation with PE or APC-labeled Abs (BD Pharmingen) directed against surface markers on PBMCs (T cells, monocytes, NK cells, and B cells). As controls for nonspecific binding, rOv-ASP-1-biotin labeled cells were incubated with appropriate APC or PE-labeled isotype and species-matched control Abs. After three washes the cells were resuspended in FACS buffer and analyzed using 2–3 color FACS analysis using a Becton Dickinson FACS CANTO flow cytometer and the DiVa software (NYBC FACS facility services).
For cytokine production, PBMCs (n = 14) were cultured in quadruplicate in round-bottom 96-well culture plates at 4 × 10^5/well in RPMI 1640 medium containing 10% FBS, 25 mM HEPES, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma-Aldrich). The cells were cultured for 5 days in the presence of rOv-ASP-1 (5 μg/ml) with or without polymyxin B at 37°C in a humidified 5% CO2 incubator. In preliminary experiments, 5 μg/ml LPS-free rOv-ASP-1 was established as an optimal dose for cytokine stimulation. For control purposes, we also treated PBMCs with recombinant CAT (5 μg/ml). In that CAT studies, we have established that CAT and other recombinant O. volvulus proteins expressed similarly in E. coli did not have any immunostimulatory activity. Cytokines (IFN-γ, TNF-α, IL-4, IL-5, and IL-10) were assayed in culture supernatants using a human Th1/Th2 CBA kit (BD Biosciences) according to the manufacturer’s protocol. To examine the potential involvement of the TLRs on the immunostimulatory activity of rOv-ASP-1 on PBMCs, we tested the inhibitory activity of anti-TLR Abs (TLR2 and TLR4) on the IFN-γ secretion from PBMCs stimulated by rOv-ASP-1. PBMCs were preincubated with 10 μg/ml goat anti-TLR2, -TLR4, or -IL-4 Abs (eBiosciences) at 37°C for 1 h before adding rOv-ASP-1 (5 μg/ml) to each treated well. Anti-IL-4 Abs served as unrelated control Ab and untreated wells served as the positive control for maximum IFN-γ secretion in the presence of rOv-ASP-1. The concentration of IFN-γ in the culture supernatants was tested by ELISA (25). Amounts of secreted cytokine are expressed in pg/ml per cultured wells.

**Stimulation of MoDCs with rOv-ASP-1**

Blood samples were collected from uninfected normal healthy donors (NHD) with no known risk factors for blood-borne virus infection (11 were male and 3 were female and the median age was 52). Ethical approval was obtained from Southampton and South-west Hampshire Joint Research Ethics Committee and all volunteers gave informed consent in writing before participating in the study. Fifty ml of freshly drawn blood was obtained from 14 NHD subjects. Blood was collected into K3-EDTA and separated immediately by centrifugation over Lymphoprep (Robbins Scientific). PBMCs were recovered and monocytes then isolated using the MACS CD14 isolation kit (Miltenyi Biotec), according to the manufacturer’s protocol. CD14+ cells were positively selected from PBMC using an AutoMACS machine (Miltenyi Biotec). Monocytes (10^6 cells/ml) were cultured in six-well plates (Greiner Bio-One) in 3 ml per well of complete RPMI medium (RPMI 1640 without phenol red (Invitrogen) with 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 10% heat-inactivated FCS (HyClone, Perbio Scientific)). Cultures were harvested and recovered after 24 h, then 25 ng/ml recombinant human granulocyte-macrophage CSF and 1000 IU/ml rhIL-4 (both from R&D Systems). After 2 and 5 days, 0.5 ml of culture medium was replaced with fresh cytokines. The day 5 immature DCs (2 × 10^6) were then cultured in complete medium (RPMI 1640 without phenol red (Invitrogen), with 2 mM t-glutamine, 100 U/ml LPS-free rOv-ASP-1) for 6 days. The end-point titers are the mean of four mice per group. As induction of high titer of IgG1 is considered indicative of a Th2-type immune response, high IgG2a, IgG2b, and IgG3 titers are typical of a Th1-type response. Apparently, rS alone induced a Th2 dominant response while rS/rOv-ASP-1 is a mixed but Th1 dominant.

### Table II. Isotyping of antibodies against SARS rS protein after the second boost

<table>
<thead>
<tr>
<th>Isotype</th>
<th>rS alone</th>
<th>rS/rOv-ASP-1</th>
<th>rS/MPL plus TDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>72,900</td>
<td>656,100</td>
<td>656,100</td>
</tr>
<tr>
<td>IgG2a</td>
<td>2,700</td>
<td>656,100</td>
<td>218,700</td>
</tr>
<tr>
<td>IgG2b</td>
<td>2,700</td>
<td>218,700</td>
<td>72,900</td>
</tr>
<tr>
<td>IgG3</td>
<td>300</td>
<td>8,100</td>
<td>2,700</td>
</tr>
</tbody>
</table>

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penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 10% heat-inactivated FCS (HyClone, Perbio Science) alone or with 5 μg/ml rOv-ASP or 100 ng/ml LPS in 48-well cell culture plates. After 24 h of stimulation, DCs were stained for two-color flow cytometry (FACSCalibur dual laser flow cytometer, BD Biosciences) and analyzed using CellQuest software (BD Biosciences) and the supernatants were measured for cytokine secretion. For each condition, cells were resuspended and incubated on ice for 30 min in 50 μl wash buffer (PBS/0.05% NaNO3/0.5% BSA) containing 100 μg/ml human Fcγ fragments (Jackson ImmunoResearch Laboratories) (“blocking buffer”). Cells were then stained with 50 μl of APC-labeled anti-human CD83, CD86, CD40 (BD Biosciences) or CD303-APC (BDCA2; Miltenyi Biotec) or FITC-labeled anti-human HLA-DR (BD Biosciences), all diluted to their optimal concentrations in blocking buffer or APC- or FITC-labeled isotype controls (murine IgG1 and IgG2b; BD Biosciences) at equivalent dilutions. After staining, cells were washed, then fixed in 1% formaldehyde and stored on ice in the dark pending analysis. Using two-color analysis, the presence of the surface markers, HLA-DR, CD83, CD86, and CD40 on immature and mature MoDCs was determined by collecting ~40,000 events in the total cell population. All positive staining was compared with appropriate isotype controls. Multiple cytokines in the 24 h culture supernatants from the stimulated MoDCs were determined by collecting ~40,000 events in the total cell population. All positive staining was compared with appropriate isotype controls. Multiple cytokines in the 24 h culture supernatants from the stimulated MoDCs were determined by collecting ~40,000 events in the total cell population. All positive staining was compared with appropriate isotype controls. Multiple cytokines in the 24 h culture supernatants from the stimulated MoDCs were determined by collecting ~40,000 events in the total cell population. All positive staining was compared with appropriate isotype controls.

To measure cytokine in response to CD40 ligation, 2 × 10^5 washed immature or matured MoDCs were added to 2 × 10^5 human

FIGURE 2. Detection of IFN-γ-producing cells by ELISPOT. Splenocytes from vaccinated mice were stimulated with SARS-CoV rRBD. Frequencies of IFN-γ-producing cells are expressed as mean ± SE of cytokine spot-forming cells (SFC)/10^6 cells of five independent experiments. *, p < 0.05 compared with RBD/MPL plus TDM or PBS group.

FIGURE 3. Binding of biotinylated rOv-ASP-1 to human PBMC as determined by flow cytometry. Cells were gated on lymphocytes (R1) (a). The isotype-specific Abs to IgG-PE or IgG-APC were used as control (b and c). Cells were stained with rOv-ASP-1 biotin-streptavidin FITC and for T cells with CD3/CD4/CD8 (d–f), for monocytes with CD14 (g), for B-cells with CD19 (h), and for NK cells with CD56 (i). Representative of the binding profile of PBMCs from one normal individual.
CD40L-transfected Chinese hamster ovary (CHO) cells (hCD40L-CHO) or wild-type (wt) cells (wt-CHO) (both gifts from Professor M. Glennie, Tenovus Laboratories, Southampton, U.K.) adhering to the wells of 48-well cell culture plates in a final culture volume of 0.5 ml complete RPMI 1640 medium with or without 50 ng/ml rOv-ASP or 100 ng/ml LPS. Further wells contained MoDCs, hCD40L-CHO, or wt-CHO cells alone. After 24 h, supernatants were harvested and centrifuged for 10 min at 2500 g, then frozen pending further analysis using a human Th1/Th2 CBA as described above.

**MLR assay**

To establish whether rOv-ASP was able to enhance MoDC Ag-presenting capacity, a MLR assay was performed and proliferation was measured by tritiated thymidine incorporation. Day 5 MoDCs isolated from five NHDs were cultured (1 × 10^5) in complete medium supplemented with IL-4 and GM-CSF with or without rOv-ASP (5 μg/ml) for 24–48 h. DCs were then thoroughly washed before adding to naive CD4^+ T cells to ensure all rOv-ASP was washed off. As a result, the T cell response was detected if presented by the stimulated DCs. As responder cells in the MLR, naive CD4^+ T cells were isolated by negative selection from five allogeneic normal healthy donors using a CD4^+ T cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer’s recommended protocol. Purified CD4^+ T cells (2 × 10^5) were cocultured in triplicates with MoDCs at various DC:T cell ratios (1:20–1:80) in U-bottom 96-well plates in complete RPMI 1640 for 5 days at 37°C, 5% CO2. During the last 18 h of incubation, the cultures were pulsed with 1 μCi/ml [3H]thymidine. Cells were then harvested and the incorporated tritium thymidine was measured on a beta plate reader (Topcount, Packard) to determine induction of the allogeneic naive CD4 T cell proliferation.

**TLR2, TLR4, and TLR9 activation**

Stable lines of HEK293 expressing surface TLR constructs (TLR2, TLR4, or TLR9) were obtained under Material Transfer Agreement from Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA). These HEK cells naturally lack TLR2, TLR4, or TLR9 expression and genetic complementation with TLR constructs renders the cells responsive to the respective TLR ligands (23, 24). The cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin (PAA), 0.1 g/ml streptomycin (PAA), and 200 mM L-glutamine (Invitrogen) in a 5% saturated CO2 atmosphere at 37°C. Cells were plated at 5 × 10^4 cells per well in flat-bottom 96-well tissue-culture plates and incubated for 3 h to allow adhesion. Cells were then stimulated with rOv-ASP-1 or control TLR ligands (Pam3CysK4; a bacterial lipoprotein analog for TLR2; LPS for TLR4; or the synthetic DNA analog CpG-ODN2216 for TLR9) at 37°C for 18 h. The culture supernatants were collected for quantitation of IL-8 production using commercial ELISA kit (R&D systems). Values are expressed in pg/ml per well.

**NF-kB-dependent reporter gene assay**

HEK293 cells, either with or without stable transfection with hCD14, were maintained in medium including sodium pyruvate and supplemented with 10% FCS (Biochrom), 100 U/ml penicillin (PAA), 0.1 μg/ml streptomycin.
(PAA), and 200 mM l-glutamine (Invitrogen). Transient transfection of HEK293 cells was performed using FuGENE 6 transfection reagent (Roche Diagnostics) following the manufacturer’s instruction. Cells were plated in 12-well plates with 1 × 10⁴ cells per well. When cultures reached 50–80% confluence, they were incubated overnight with plasmids containing NF-κB reporter luciferase (120 ng) and, to control for differences in transfection efficiency, with RSV-β-galactosidase (40 ng). Cells were co-transfected either with hTLR4 (2 ng) and hMD2 (40 ng), or with hTLR2 (40 ng). After 24 h, cells were stimulated with various amounts of rOv-ASP-1 or control ligands in DMEM with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 200 mM l-glutamine. The control ligands for TLR2 were: Triacylated lipopeptides (Pam3CSK4; LP3) and diacylated lipopeptides (Pam2CSK4; LP2) (EMC Microcollections) as previously described (26). LPS (Sigma-Aldrich) was used as the ligand for TLR4. After 24 h of stimulation, luciferase activity was measured in cell extracts with the Lucif-Gal Reporter Gene Assay kit (Roche Diagnostics) according to the manufacturer’s instruction. Luciferase and β-galactosidase activity were estimated with chemiluminescence assays (luciferase and β-Gal Reporter Gene Assay (Roche Diagnostics)) and results were expressed as the ratio of luciferase to β-galactosidase and graphed as the mean ± SD of triplicate experiments.

Statistical analysis
Pairwise t tests and, where appropriate, Mann-Whitney tests were used to compare means. Analyses were performed using Prism version 4.0 for Windows (GraphPad Software).

Results

rOv-ASP-1 potentiates both Th1- and Th2-associated IgG2a and IgG1 responses

The adjuvanticity of rOv-ASP-1 was firstly evaluated by using recombinant SARS-CoV rS as a model Ag. Four BALB/c mice were immunized four times with the rS in the absence or presence of rOv-ASP-1 or with a control adjuvant, MLP plus TDM. As shown in Fig. 1A, both adjuvants, rOv-ASP-1 and MLP plus TDM, augmented the IgG Ab responses to rS protein vs rS alone in terms of total IgG titers. Further comparison between the rOv-ASP-1 and MLP plus TDM-induced responses by IgG isotypes (Table I) shows: 1) similar IgG1 (1:656,100); and 2) three-fold higher IgG2a (656,100 vs 218,700) and IgG3 (218,700 vs 72,900) end point titers in the presence of rOv-ASP-1. Each adjuvant/Ag model performed differently depending on the adjuvant, a skewed Th2 response with MLP plus TDM (IgG1/IgG2a = 3), but a mixed Th1/Th2 (IgG2a/IgG1 = 1) response with rOv-ASP-1 with a dominance of Th1 Abs (IgG2a and IgG2b). These results indicate that rOv-ASP-1 induced a more Th1 skewing Ab response than MLP plus TDM. Moreover, when the neutralizing activities of the Abs were analyzed (Fig. 1B), it appeared that the antisera from the mice immunized with rS in the presence of rOv-ASP-1 contained similar levels of the neutralizing Abs against infection by SARS pseudovirus (1/29,096) as those from the mice administered with rS plus MLP plus TDM adjuvant (1/33,960). Therefore, rOv-ASP-1 is a potent adjuvant for SARS-CoV rS protein and can induce functional antiviral Abs.

rOv-ASP-1 effectively induced both humoral and Th1-dominated cellular immune responses against the rRBD of SARS-CoV S protein

We have previously shown that the RBD of SARS-CoV is a major target of neutralizing Abs (22, 27–29) and that recombinant RBD-based vaccines can induce protective immunity in the immunized animals (23, 30–33). In this study, the recombinant RBD was further used to test the adjuvanticity of rOv-ASP-1 in comparison with the MLP plus TDM adjuvant in C57BL/6 mice. As shown in Fig. 1C, both immunization protocols elicited similar high titers of Abs against the rS protein after three vaccinations. The pooled splenocytes from each group of immunized mice were stimulated with the rRBD (1 μg/ml) as a recall Ag and the production of cytokines were measured by CBA kit (Table II). In previous vaccines studies using rOv-ASP-1 as the adjuvant and rS or rRBD as the bystander Ag (32, 34), we found that the variation between individual mice was very low and therefore we are confident that the results we obtained using the pooled spleens are a good representation of what would have been the outcome if we had used individual mice. Notably, rOv-ASP-1 exceeded the efficacy of MPL plus TDM adjuvant in boosting the production of Type I proinflammatory cytokines (IL-2, IFN-γ, TNF-α, and IL-6). Secretion of the Th2/regulatory cytokine IL-10 was elevated but less than that induced by MPL plus TDM. There was no significant recall induction of the IL-4 or IL-5 cytokines by rRBD. The highly dominant IFN-γ recall response was further supported by enumerating the number of the IFN-γ-producing cells in the splenocytes from the vaccinated mice cultured for 24 h post the presence of rRBD. As shown in Fig. 2, RBD/ASP-1 vaccination induced a significantly higher frequency of the IFN-γ-producing cells than those of RBD/MPL plus TDM and PBS groups (p < 0.01). These results suggested that rOv-ASP-1 is a potent adjuvant inducing not only strong...
humoral response but also an RBD specific Th1-biased cellular response.

**rOv-ASP-1 binds primarily to APCs among human PBMCs**

Our data have clearly demonstrated that rOv-ASP-1 is a potent vaccine adjuvant that induces a Th1-dominant cellular response and, depending on the bystander Ag, we also found that it can elicit a dominant Th1-associated Ab response (18, 20). To explore the possible mechanism of rOv-ASP-1 adjuvanticity, we analyzed first the binding profile of biotinylated rOv-ASP-1 with human PBMCs (n = 4) by flow cytometry (Fig. 3). We found that biotin-labeled rOv-ASP-1 bound mostly to monocytes (93–98% of CD14+ or 87–90% of CD3-CD4+low) and to B cells (41–94% of CD19+). A small fraction of other cells also bound rOv-ASP-1: 1–3% CD4+ (CD3-CD4+high), 3.9–8% of CD8+ T cells, and 0.5–9% of NK cells. As a control, biotinylated-CAT did not bind to >2% of any leukocyte population (data not shown). These results demonstrated that rOv-ASP-1 primarily binds to the APCs, monocytes, and B cells, among human PBMCs, suggesting that rOv-ASP-1 may interact with the specific receptors presented on the surface of the APCs to activate the immune cells.

**rOv-ASP-1-stimulated human PBMCs to produce proinflammatory cytokines**

We then determined whether rOv-ASP-1 can trigger cytokine production of human PBMCs from normal healthy donors. As shown in Fig. 4, rOv-ASP-1 protein stimulated significant production of Th1-type cytokines, IFN-γ, and TNF-α, and the immunoregulatory T cell cytokine, IL-10. No significant Th2 cytokines (IL-4 or IL-5) were detected in the PBMC culture supernatants, although the IL-5 response was somewhat increased. Inhibition of LPS activity using polymyxin B (20 μg/ml) had no effect on the bioactivity of rOv-ASP-1 on human PBMCs (Fig. 4F). In contrast, it appeared that the activity of the recombinant control protein, CAT, was due to contamination by LPS, as its cytokine-inducing activity could be abolished by polymyxin B. Therefore, the rOv-ASP-1 protein appears to be a potent inducer of proinflammatory (IFN-γ and...
TNF-α and immunoregulatory (IL-10) cytokine secretion from the normal human PBMCs.

**rOv-ASP-1 induces phenotypic maturation of MoDCs and stimulates the secretion of proinflammatory and anti-inflammatory cytokines**

It is well known that TLRs activate signal transduction cascades leading to expression of costimulatory molecules on DCs and the secretion of IL-12 following recognition of their respective ligands (35). To determine whether rOv-ASP-1 induced DC maturation, MoDCs from NHDs (n = 14) were cultured with or without rOv-ASP-1 at 5 μg/ml for 24 h. Using FACS analysis, we measured expression of surface molecules that are characteristic of DCs after stimulation. As shown in Fig. 5, all four phenotypic markers that were increased after treatment with rOv-ASP-1. Notably, rOv-ASP-1 induced secretion of IL-12 was CD40-dependent; significant enhancement of IL-12 secretion was observed only after providing the MoDCs with costimulation through the CD40-CD40L pathway (i.e., by including CD40L-expressing CHO cells). Costimulation through the CD40-CD40L pathway also enhanced the rOv-ASP-1-induced secretion of IL-10 and TNF-α (Fig. 6; immature plus CD40L vs rOv-ASP-1 plus CD40L). These results provide evidence that rOv-ASP-1 may initiate DC-induced adaptive immune responses by multiple distinct mechanisms.

To assess whether rOv-ASP-1 treatment of MoDCs was able to enhance their accessory/Ag presentation function, we used the MLR assay with allogeneic Ags. Fig. 7A shows the results of the MLR experiments indicating that MoDCs treated with 5 μg/ml rOv-ASP-1-enhanced their ability to stimulate proliferation of allogeneic CD4+ T cells. Moreover, rOv-ASP-1 primed DCs were able to induce IFN-γ secretion from naive PBMCs (Fig. 7B). The ability of rOv-ASP-1 to stimulate human DCs further substantiates the prospect of its use as an innate vaccine adjuvant in humans that can boost the activation of an adaptive immune response.

**The rOv-ASP-1-induced IFN-γ secretion is TLR2 and TLR4 dependent**

Previous studies have indicated that signaling from TLR on APCs after ligand binding can induce subsequently the production of proinflammatory cytokines from T and NK cells and provide help to B cells, and thus this mechanism is thereby thought to contribute considerably to the enhanced Ab and cellular-enhancing effects of innate vaccine adjuvants (36, 37). The secretion of IFN-γ by T and NK cells is mostly induced by IL-12, which is primarily produced by DCs and macrophages (38–40). Therefore, we investigated whether rOv-ASP-1 can activate TLR2 and/or TLR4 presented on the surface of APCs and thereby result in proinflammatory cytokine production. To test this hypothesis, PBMCs from three healthy donors were treated with rOv-ASP-1 in the absence or presence of antibodies against TLR2, TLR4, or IL-4 Abs (10 μg/ml) at 37°C for 1 h before adding 5 μg/ml rOv-ASP-1 for stimulation over a 5-day period. The concentration of IFN-γ in the culture supernatants was tested by ELISA. *p < 0.05 when compared with the rOv-ASP-1 untreated control.
presence of anti-TLR2, anti-TLR4, or anti-IL-4 Abs, and the secretion of IFN-γ was then measured by ELISA. As shown in Fig. 8, the IFN-γ secretion was significantly inhibited with both anti-TLR2 and anti-TLR4 Abs and not with anti-IL-4 Abs, suggesting that the rOv-ASP-1-induced IFN-γ secretion from human PBMCs is TLR2 and TLR4 dependent.

The above results suggest that the rOv-ASP-1-induced proinflammatory cytokine production from human PBMCs might be dependent on TLR2 and TLR4 activation. We then used the stable lines of HEK293 cells expressing TLR2, TLR4, or TLR9 to test its potential to activate TLR directly. Each of the cell lines was respectively incubated with rOv-ASP-1 at the indicated amounts and the TLR activation was expressed by human IL-8 production. As shown in Fig. 9, the specific control ligands for TLR2, TLR4, or TLR9 stimulated the cells expressing the appropriate receptors, while rOv-ASP-1 activated both TLR2- and TLR4-expressing cells at dose-dependent manners, but not TLR9. The wt HEK293 cells, which do not express TLR2 and TLR4, were unresponsive to the rOv-ASP-1 (data not shown) in the IL-8 release assay.

rOv-ASP-1 triggered cellular activation via TLR2 and TLR4

The above results suggest that the rOv-ASP-1-induced proinflammatory cytokine production from human PBMCs might be dependent on TLR2 and TLR4 activation. We then used the stable lines of HEK293 cells expressing TLR2, TLR4, or TLR9 to test its potential to activate TLR directly. Each of the cell lines was respectively incubated with rOv-ASP-1 at the indicated amounts and the TLR activation was expressed by human IL-8 production. As shown in Fig. 9, the specific control ligands for TLR2, TLR4, or TLR9 stimulated the cells expressing the appropriate receptors, while rOv-ASP-1 activated both TLR2- and TLR4-expressing cells at dose-dependent manners, but not TLR9. The wt HEK293 cells, which do not express TLR2 and TLR4, were unresponsive to the rOv-ASP-1 (data not shown) in the IL-8 release assay.

The rOv-ASP-1 cellular activation via TLR2 and TLR4 was further determined by a more sensitive NF-κB-dependent reporter gene assay. Consistently, rOv-ASP-1 strongly induced luciferase activity in the TLR2- or TLR4-transfected HEK293 cells in a dose-dependent manner (Fig. 10). These data strongly support the view
that both TLR2 and TLR4 are involved in transducing rOv-ASP-1 stimulation.

**Discussion**

The identification of new adjuvants that stimulate both Ab and cellular responses is an important challenge for vaccine development. In particular, there is a demand for safe and nontoxic adjuvants able to stimulate cellular (Th1) immunity for fighting against the existing and emerging pathogens. We have previously shown that rOv-ASP-1 has potent adjuvant effects for several vaccine Ags including recombinant proteins, synthetic peptides, and commercial inactivated vaccines (18, 20). Importantly, rOv-ASP-1 could induce a mixed Th1/Th2 response with a Th1-biased Ab profile against some of the bystander Ags in the immunized mice. In the present work, the adjuvanticity of rOv-ASP-1 was further evaluated in comparison with MPL plus TDM adjuvant using the recombinant SARS-CoV S and RBD proteins, which have been shown to induce in the presence of the MPL plus TDM adjuvant potent neutralizing Abs and have thereby been considered as an ideal SARS vaccine candidates (22, 30). Our present study has shown that rOv-ASP-1 also was highly effective in eliciting functional Ab responses in immunized mice. Its adjuvanticity to the SARS-CoV S protein exceeded the MPL plus TDM adjuvant, especially in the induction of Th1-associated IgG2a and IgG2b Ab isotype responses. Furthermore, we have demonstrated that Ov-ASP-1 is a potent adjuvant also for the induction of Th1-biased cellular response. Impressively, immunization with the rRBD protein and rOv-ASP-1 significantly promoted the IFN-γ and TNF-α production in the immunized mice. These results further confirmed that rOv-ASP-1 can be used as a potent vaccine adjuvant for the recombinant protein-based vaccine Ags which are generally far less immunogenic than old fashion vaccines consisting of live or killed whole organisms.

The molecular mechanism underlying adjuvant activity has been poorly understood for a long time, but this field has been rapidly evolving since the discovery of the TLR family of proteins and their corresponding innate ligands. TLRs are prominent pattern recognition receptors of the innate immune system recognizing various invading pathogens through conserved motif named PAMPs (36). Interaction of PAMPs with TLR on the surface of APCs initiates a signaling cascade resulting in the recruitment of adaptor proteins such as the MyD88 and the downstream activation of NF-κB and MAPK (35). Consequently, the TLR signaling stimulates the activation and maturation of APCs including the regulated presentation of Ags, up-regulation of costimulatory molecules, and secretion of proinflammatory chemokines and cytokines. These events mediate not only innate but ultimately also adaptive immunity (37, 41). Vaccinations with adjuvants that mimic TLR ligands are advantageous as they are capable of eliciting positive effects across the entire spectrum of innate and adaptive immunity. Therefore, innate immune signals mediated by TLRs have been thought to contribute significantly to the Ab-enhancing effects of vaccine adjuvants as well as their ability to elicit cellular protective immunity. Previous studies have demonstrated that human parasite-derived products can also stimulate the immune responses via the TLR pathways. For example, a schistosome-specific phosphatidylserine was shown to activate TLR2 and induce a mixed Th1/Th2 response. Impressively, immunization with the parasite protein rOv-ASP-1 through an unknown as yet uncharacterized mechanism was able to stimulate human DCs to produce Th2-type IL-4 and IL-5 cytokines, which are important for parasite-induced IL-12 production and optimal resistance to infection in vivo (1). Furthermore, the adjuvant candidate lacto-N-fucopentaose III from *S. mansoni* acted as an innate Th2 adjuvant via TLR4 signaling (43, 44).

To explore the mechanism of rOv-ASP-1 adjuvanticity, we initially analyzed the binding profile of rOv-ASP-1 with normal human PBMCs by a flow cytometry-based assay. It was found that rOv-ASP-1 binds primarily to the human monocytes and B cells, the major APCs in the peripheral blood. Significantly, rOv-ASP-1 stimulated human PBMCs to produce Th1-type proinflammatory cytokines (IFN-γ and TNF-α) and the immunoregulatory IL-10 cytokine. It has been known that IFN-γ is mainly released by activated Th1 and NK cells while TNF-α is mainly produced by macrophages but also by a broad variety of other cell types including Th1 cells. Both cytokines are involved in the regulation of the immune and inflammatory responses. Recently, we have shown that rOv-ASP-1 induced IFN-γ secretion from purified CD56− cells but not from CD4+ or CD8+ cells (25). Moreover, this immunostimulatory effect was dependent on contact between CD56− and CD56+ fractions of PBMC, most likely between APCs, including DCs, in the CD56− fraction.

Interestingly, rOv-ASP-1 also induced significant IL-10 secretion by naive human PBMCs. This cytokine is mainly expressed by monocytes and Th2 cells and is capable of inhibiting synthesis of proinflammatory cytokines like IFN-γ, IL-2, TNF-α, and GM-CSF made by cells such as macrophages and the Th1 cells. As rOv-ASP-1 could not stimulate human PBMCs to produce Th2-type IL-4 and IL-5 cytokines, it is possible that a monocytes subpopulation is responsible for the secretion of IL-10. Initial studies of PBMCs vs monocytes have indicated that monocytes secrete IL-10 in the presence of rOv-ASP-1 through an unknown as yet mechanism. Based on the profile of cytokines stimulated by rOv-ASP-1 we hypothesize that the Ov-ASP-1 protein (≈23 kDa) may contain a few putative Th1 PAMPs that bind to different receptors on APCs that activates differently the secretion of Th1 cytokines and IL-10 through distinct pathways. Innate recognition of PAMP signals APCs, typically human monocyte-derived DCs, to express costimulatory molecules and secrete cytokines, which drive the polarization of naive CD4+ Th type 1 and type 2 cells toward the Th1 or Th2 phenotype (45). Th1 cells express IFN-γ and TNF-α that instruct B cells to produce Ag-specific IgG2a, whereas Th2 cells produce IL-4, IL-5, and IL-13 to promote IgG1 and IgE class switching.

Human monocyte-derived DCs treated with the parasite protein rOv-ASP-1 have shown evidence of phenotypic maturation, enhanced cytokine production and enhanced allo-stimulatory function. These findings confirm the immunostimulatory properties of this protein are mediated by its ability to activate APC population. The ability of rOv-ASP-1 to stimulate human DCs further substantiates the prospect of its use as an innate vaccine adjuvant in humans that can boost the activation of an adaptive immune response. The ability of rOv-ASP-1 to stimulate human DCs may enable it to be used as a human therapeutic. In the case of chronic hepatitis C virus (HCV) infection, where DC function has been reported to be impaired by the virus, rOv-ASP-1 could enhance DC-driven T cell anti-HCV responses that would shift the virus-host equilibrium in favor of viral clearance. In support of this, recent in vitro evidence showed that rOv-ASP-1 was able to enhance and/or induce anti-HCV Core IFN-γ responses in patients with chronic HCV infection (25).

The adjuvant activity of rOv-ASP-1 appears to be mediated by TLR signaling presented on the APCs. In agreement with this hypothesis, the IFN-γ secretion from human PBMCs could be significantly inhibited by both anti-TLR2 and anti-TLR4 Abs. Consistently, rOv-ASP-1 also triggered the activation of HEK293 cells transfected with TLR2 and TLR4 but not with TLR9 in dose-dependent manners. Previous studies indicate that each TLR mediates the response to specific PAMPs shared by a set of molecular interactions.
structures (41). Among the TLRs, TLR2 mediates the response to the most diverse set of molecular structures including lipopolysaccharide, peptidoglycan, and lipoteichoic acids, while it is generally accepted that TLR4 mainly responds to LPS from Gram-negative bacteria. But, they share a common activation pathway mediated through their Toll-IL-1R signaling domain resulting in activation of NF-κB and MAPK. Previous results have also demonstrated that a number of pathogen-derived PAMP-containing ligands could simultaneously activate TLR2 and TLR4 (46–48), such as the major surface protein of Wolbachia endosymbionts in filarial nematodes (43) and the glycosylphosphatidylinositols derived from T. gondii (46). Structural homology analyses of Ov-ASP-1 with the crystallized closely related hookworm-secreted protein Na-ASP-2 suggest that Ov-ASP-1 has three distinct functional domains (49). It is possible that such distinct subdomains of Ov-ASP-1 may contain distinct bioactive sites. If this is the case, we may be able to express a subunit that stimulates IFN-γ without concurrent IL-10 secretion or specially activates TLR2 or TLR4 pathways. To further corroborate the involvement of TLR2 and concurrent IL-10 secretion or specially activates TLR2 or TLR4-ASP-2 suggest that Na-ASP-2 may be able to express a subunit that stimulates IFN-γ without concurrent IL-10 secretion or specially activates TLR2 or TLR4.

In conclusion, rOv-ASP-1 has potential to be developed as a novel innate vaccine adjuvant since it can bind to APCs, matures and activates DCs, and subsequently induces proinflammatory cytokines leading to a Th1-dominant immune response, potentially via TLR2 and TLR4 activation.

Disclosures

The authors have no financial conflict of interest.

References


