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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 r*Ov*-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 r*Ov*-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 r*Ov*-ASP-1 could effectively induce a mixed but Th1-skewed immune response in immunized mice. It appears that r*Ov*-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 r*Ov*-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)[4] 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, *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 (r*Ov*-ASP-1) is not only a protective Ag in vaccinated mice against *O. volvulus* third-stage larvae, but

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[4] Abbreviations used in this paper: PAMP, pathogen associated molecular pattern; ASP, activation-associated secreted protein; *Ov*-ASP-1, *Onchocerca volvulus* activation associated protein-1; SARS-CoV, severe acute respiratory syndrome coronavirus; S, spike protein; RBD, receptor-binding domain; DC, dendritic cell; MoDC, monocyte-derived DC; CBA, cytometric bead array; CAT, chloramphenicol acetyl transferase; NHD, normal healthy donor; wt, wild type; CHO, Chinese hamster ovary; HEK, human embryonic kidney cell; MPL, monophosphoryl lipid A; TDM, trehalose dicorynomycolate; HCV, hepatitis C virus.

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also a potent adjuvant for bystander proteins (2, 17–19). In OVA-immunized mice, r*Ov*-ASP-1 exceeded the efficacy of alum or monophosphoryl lipid A (MPL) plus trehalose dicorynomycolate (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 cell cytokines 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, *Ov*-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 r*Ov*-ASP-1 to induce both Th1- and Th2-associated Ab responses and, depending on the Ag, also 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 r*Ov*-ASP-1 and explored its mechanism of action. Consistently, r*Ov*-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 r*Ov*-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 (r*Ov*-ASP-1) was expressed as a histidine-tagged protein in *Escherichia coli* and purified as previously described (18). The purified r*Ov*-ASP-1 was tested negative in a *Limulus* amoebocyte lysate assay. A quantitative LPS testing by Cambrex Bio-Science showed that purified r*Ov*-ASP-1 contained <0.25 endotoxin units per milligram of the protein (25 pg endotoxin/mg). We considered it as an LPS-free stock and used it in all described experiments.

Immunization 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 AY278741) 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 20 μg of rS or rRBD resuspended in PBS plus 25 μg r*Ov*-ASP-1 or MLP plus TDM adjuvant as recommended (Sigma-Aldrich) and boosted with 10 μg of the same Ag with the corresponding adjuvant or PBS at 3-wk intervals. In a previous study (18), we found that 2.5 μg/mouse of r*Ov*-ASP-1 adjuvant was not significantly active for Ab responses, 10 μg/mouse gave adjuvanticity intermediate to the final dose that we have chosen to use (25 μg/mouse), and 50 μg/mice was not better than 25 μg/mouse. The source of r*Ov*-ASP-1 in this study is the same as that used in our previous studies and therefore we also used 25 μg/mice in this study. Preimmune sera were collected before immunization and antisera were collected 7 days after each boost. Sera were kept at 4°C before use.

ELISA

The reactivity of mouse antisera with rS protein was determined by ELISA. In brief, 1 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, biotinylated 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 10^4 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 1×10^6 cell/ml and 0.2×10^5 were plated to 96-well U-bottom plates for culture. The cells were stimulated with 1 μg/ml rRBD protein for 24 h. Cell culture 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 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 2×10^5 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

r*Ov*-ASP-1 and a similarly expressed control protein, chloramphenicol acetyl transferase (CAT), were labeled with biotin using the FluoReporter 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 r*Ov*-ASP-1 or CAT were then incubated with 1×10^6 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, r*Ov*-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).

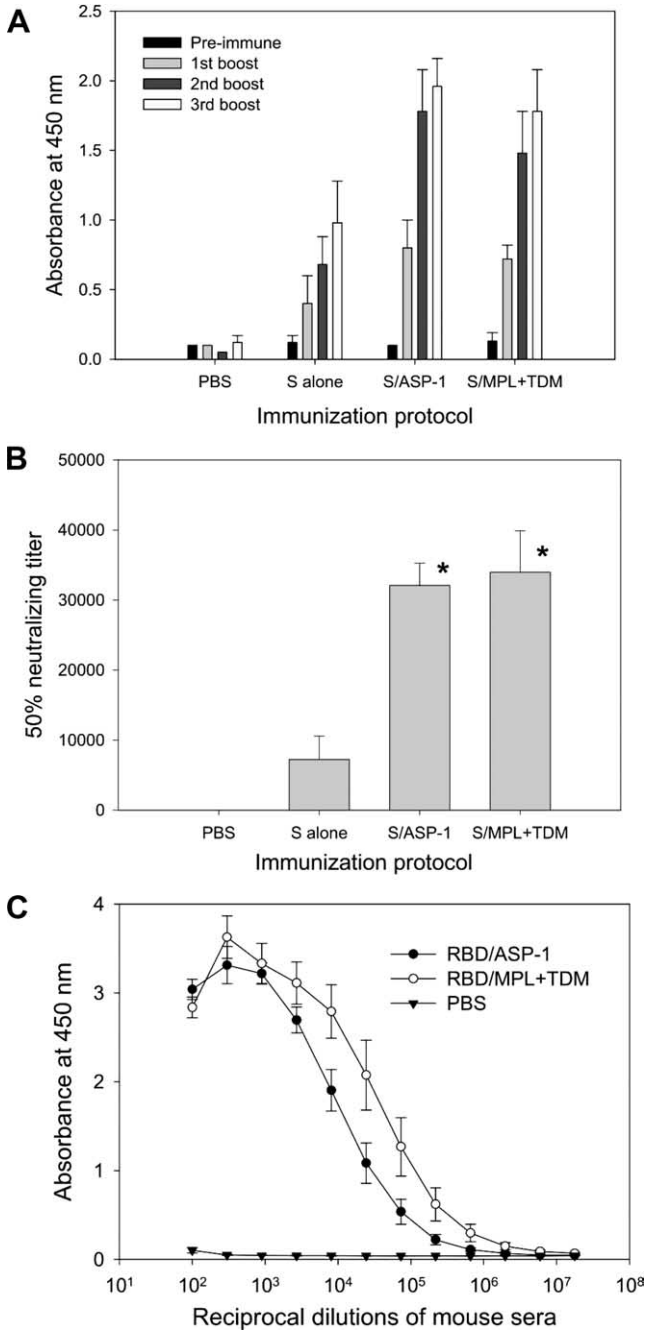

FIGURE 1. Ab responses of mice immunized with recombinant SARS-CoV S protein or its RBD in the absence or presence of the r*Ov*-ASP-1 or the MLP plus TDM adjuvant. *A*, Reactivity of mouse antisera with rS protein. The antisera were collected from mice before immunization and 7 days after each boost and tested by ELISA at 1/1000 dilution. *B*, Neutralization of SARS pseudovirus infection by mouse antisera from each rS immunization group. Infection of 293T/ACE2 cells by SARS pseudovirus was determined in the presence of antisera at a series of 2-fold dilutions, and 50% neutralization was calculated for each sample. *, $p < 0.01$ compared with the group of rS alone. *C*, Titers of anti-rS Abs from the rRBD-immunized mice. The antisera were tested by ELISA at a series of dilution.

*Stimulation of human naive PBMCs by r*Ov*-ASP-1*

Fresh human naive PBMCs were isolated by density gradient centrifugation over Ficoll (Sigma-Aldrich). 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 L-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 r*Ov*-ASP-1 (5 μg/ml) with or without polymyxin B at 37°C in a humidified 5% CO_2 incubator. In preliminary experiments, 5 μg/ml LPS-free r*Ov*-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 previous 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) in the cell culture supernatants were measured 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 r*Ov*-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 r*Ov*-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 r*Ov*-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 r*Ov*-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.

Table I. *Isotyping of antibodies against SARS rS protein after the second boost*[a]

Isotype	Immunization Protocol		
	rS alone	rS/r*Ov*-ASP-1	rS/MPL plus TDM
IgG1	72,900	656,100	656,100
IgG2a	2,700	656,100	218,700
IgG2b	2,700	218,700	72,900
IgG3	300	8,100	2,700

[a] 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/r*Ov*-ASP-1 is a mixed but Th1 dominant.

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 K_3-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 recommended 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 L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 10% heat-inactivated FCS (HyClone, Perbio Science)), containing 50 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 (2×10^5) were then cultured in complete medium (RPMI 1640 without phenol red (Invitrogen), with 2 mM L-glutamine, 100 U/ml

Table II. *Cytokine secretion from mouse splenocytes stimulated ex vivo by rRBD*

Cytokine (pg/20,000 cells)	Immunization Protocol		
	rRBD/PBS	rRBD/r*Ov*-ASP-1	rRBD/MPL plusTDM
IL-2	40.6	108.0	24.9
IL-4	0.0	5.3	6.3
IL-5	1.6	6.8	20.8
IFN-γ	75.5	950.9	267.6
TNF-α	16.8	66.9	24.3
IL-6	9.2	90.4	18.6
IL-10	7.6	41.5	51.3

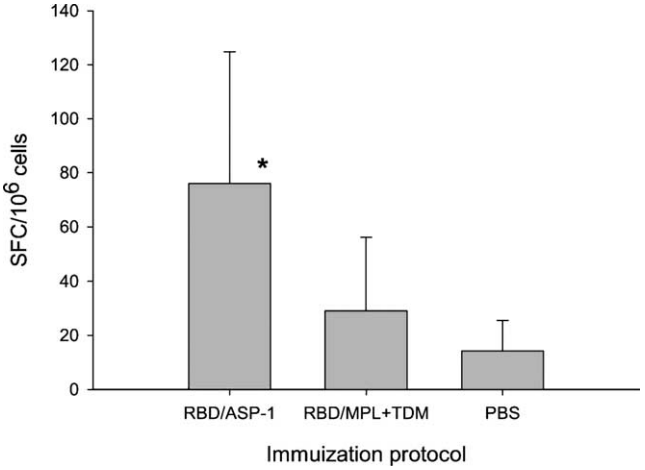

FIGURE 2. Detection of IFN-γ-producing cells by ELISPOT. Splenocytes from vaccinated mice were stimulated with SARS-CoV rRBD. Frequencies of IFN-γ-producing cells are expressed as mean ± SE of cytokine spot-forming cells (SFC)/10^6 cells of five independent experiments. *, $p < 0.05$ compared with RBD/MPL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

penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 10% heat-inactivated FCS (HyClone, Perbio Science)) alone or with 5 μg/ml r*Ov*-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 measure for cytokine secretion. For each condition, cells were resuspended and incubated on ice for 30 min in 50 μl wash buffer (PBS/0.05% NaN_3/0.5% 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; Milltenyi 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 also measured using a human Th1/Th2 CBA (BD Biosciences).

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

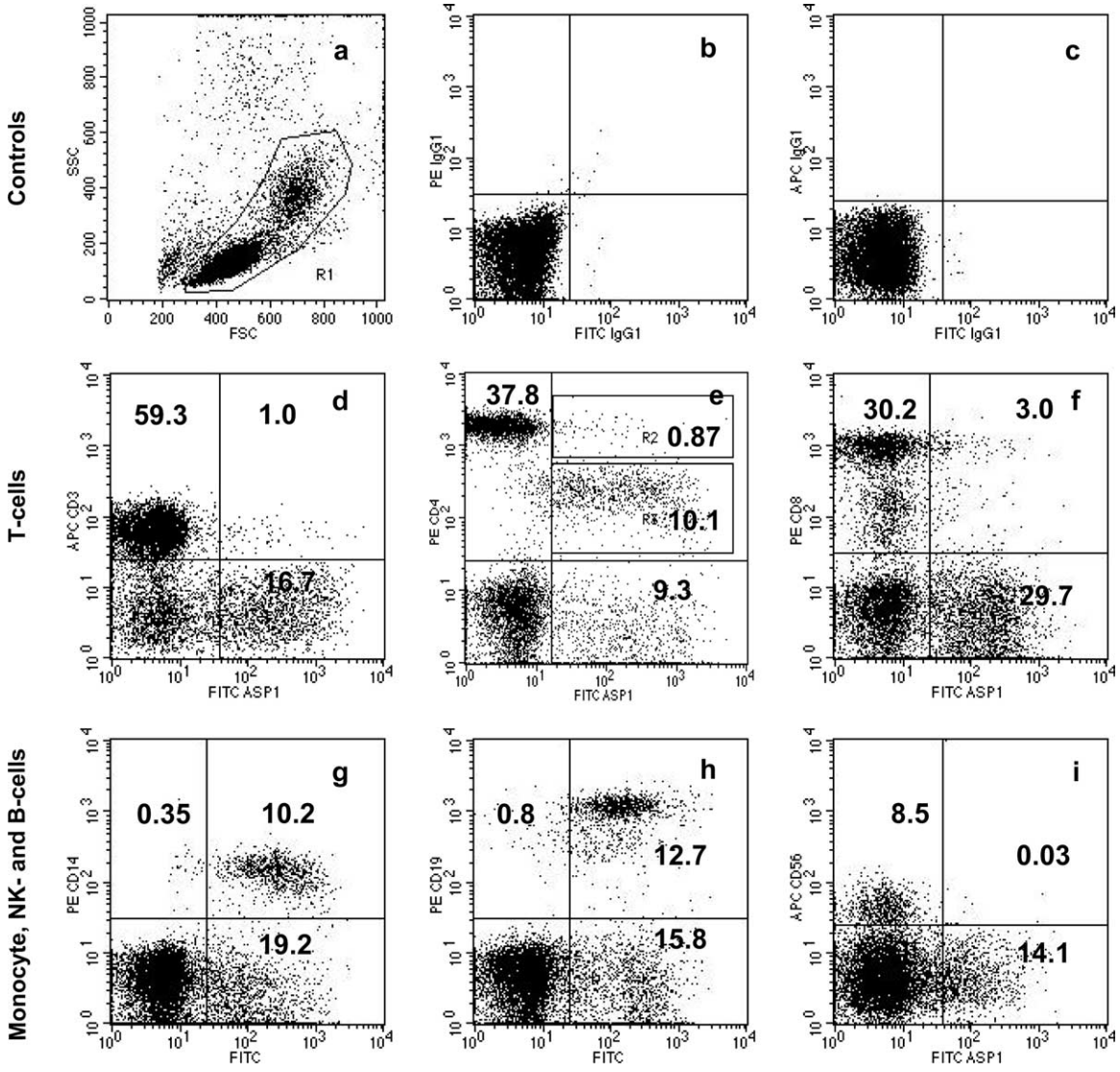

FIGURE 3. Binding of biotinylated r*Ov*-ASP-1 to human PBMC as determined by flow cytometry. Cells were gated on lymphocytes (R1) (*a*). The isotype-specific Abs to IgG-PE or IgG-APC were used as control (*b* and *c*). Cells were stained with rOv-ASP-1 biotin-streptavidin FITC and for T cells with CD3/CD4/CD8 (*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.

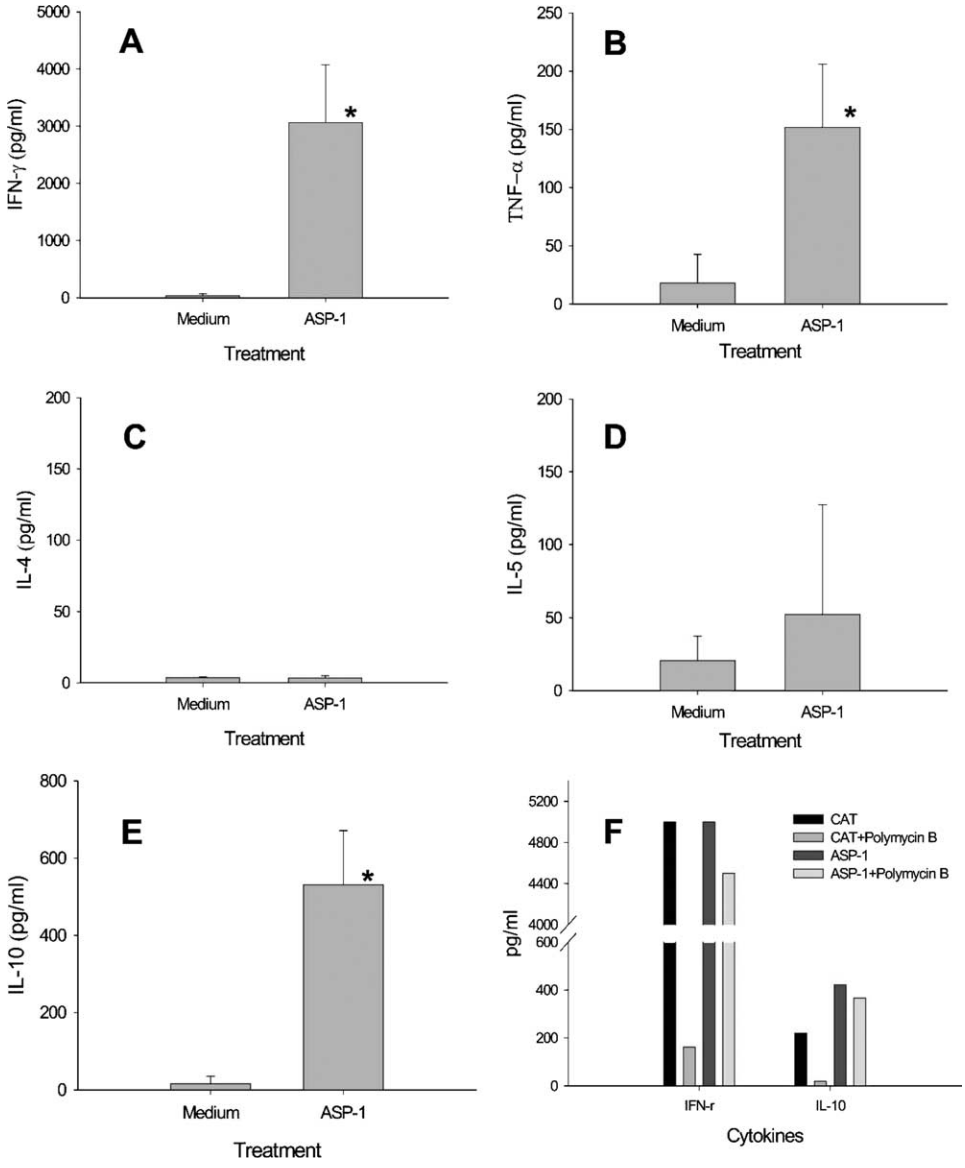

FIGURE 4. Cytokine secretion of human PBMCs stimulated by r*Ov*-ASP-1. Human PBMCs were collected from normal healthy donors ($n = 14$), and treated with or without r*Ov*-ASP-1 (5 μg/ml) for 5 days. The culture supernatants were tested by human CBA kits for cytokines IFN-γ (*A*); TNF-α (*B*); IL-4 (*C*); IL-5 (*D*); IL-10 (*E*). *, $p < 0.01$ compared with untreated control wells. *F*, IFN-γ and IL-10 secretion of human PBMCs triggered by r*Ov*-ASP-1 or recombinant CAT in the presence or absence of polymycin B (20 μg/ml). The r*Ov*-ASP-1 induced cytokine secretion was not inhibited by polymycin B.

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 5 μg/ml r*Ov*-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 $\times$ *g*, then frozen pending further analysis using a human Th1/Th2 CBA as described above.

MLR assay

To establish whether r*Ov*-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 r*Ov*-ASP (5 μg/ml) for 24–48 h. DCs were then thoroughly washed before adding to naive $CD4^+$ T cells to ensure all r*Ov*-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 RMPI 1640 for 5 days at 37°C, 5% CO_2. During the last 18 h of incubation, the cultures were pulsed with 1 μCi/ml [^{3}H]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, Worchester, 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 μg/ml streptomycin (PAA), and 200 mM L-glutamine (Invitrogen) in a 5% saturated CO_2 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 r*Ov*-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-κB-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

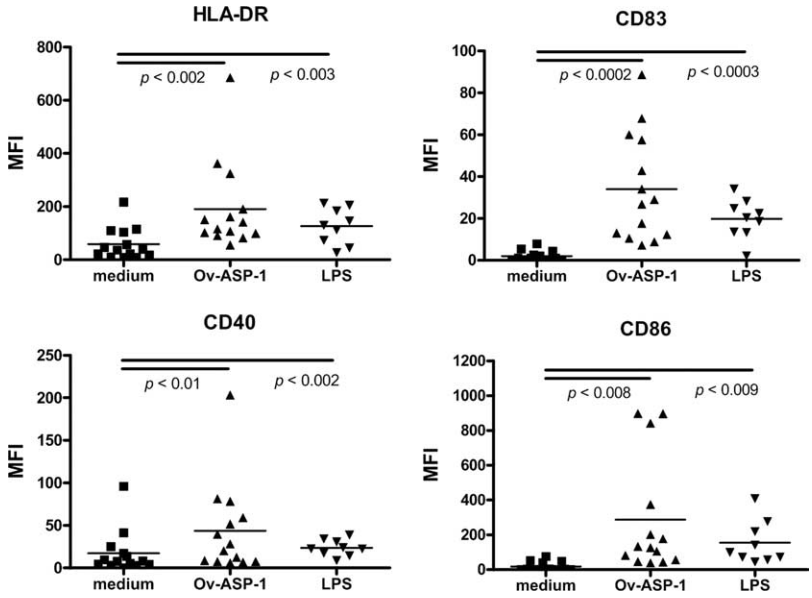

FIGURE 5. Phenotypic maturation of MoDCs treated by r*Ov*-ASP-1. MoDCs ($n = 14$) were cultured with or without 5 μg/ml r*Ov*-ASP for 24 h. Using FACS analysis, the expression of maturation markers CD40, CD83, CD86, and HLA-DR were measured. All four of the maturation markers were up-regulated after treatment with r*Ov*-ASP-1. LPS was used as a positive control.

(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 1×10^5 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 r*Ov*-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 20 h of stimulation, luciferase activity was measured in cell extracts with the Luciferase 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 $\pm$ SD of triplicate experiments.

Statistical analysis

Paired *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

r*Ov-ASP-1 potentiates both Th1- and Th2-associated IgG2a and IgG1 responses*

The adjuvanticity of r*Ov*-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 r*Ov*-ASP-1 or with a control adjuvant, MLP plus TDM. As shown in Fig. 1*A*, both adjuvants, r*Ov*-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 r*Ov*-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 three-fold higher IgG2b (218,700 vs 72,900) end point titers in the presence of r*Ov*-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 r*Ov*-ASP-1 with a dominance of Th1 Abs (IgG2a and IgG2b). These results indicate that r*Ov*-ASP-1 induced a more Th1 skewing Ab response than MLP plus TDM. Moreover, when the neutralizing activities of the Abs were analyzed (Fig. 1*B*), it appeared that the antisera from the mice immunized with rS in the presence of r*Ov*-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, r*Ov*-ASP-1 is a potent adjuvant for SARS-CoV rS protein and can induce functional antiviral Abs.

r*Ov-ASP-1 effectively induced both humoral and Th1-dominant 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 r*Ov*-ASP-1 in comparison with the MLP plus TDM adjuvant in C57BL/6 mice. As shown in Fig. 1*C*, 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 r*Ov*-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 have had used individual mice. Notably, r*Ov*-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 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 r*Ov*-ASP-1 is a potent adjuvant inducing not only strong

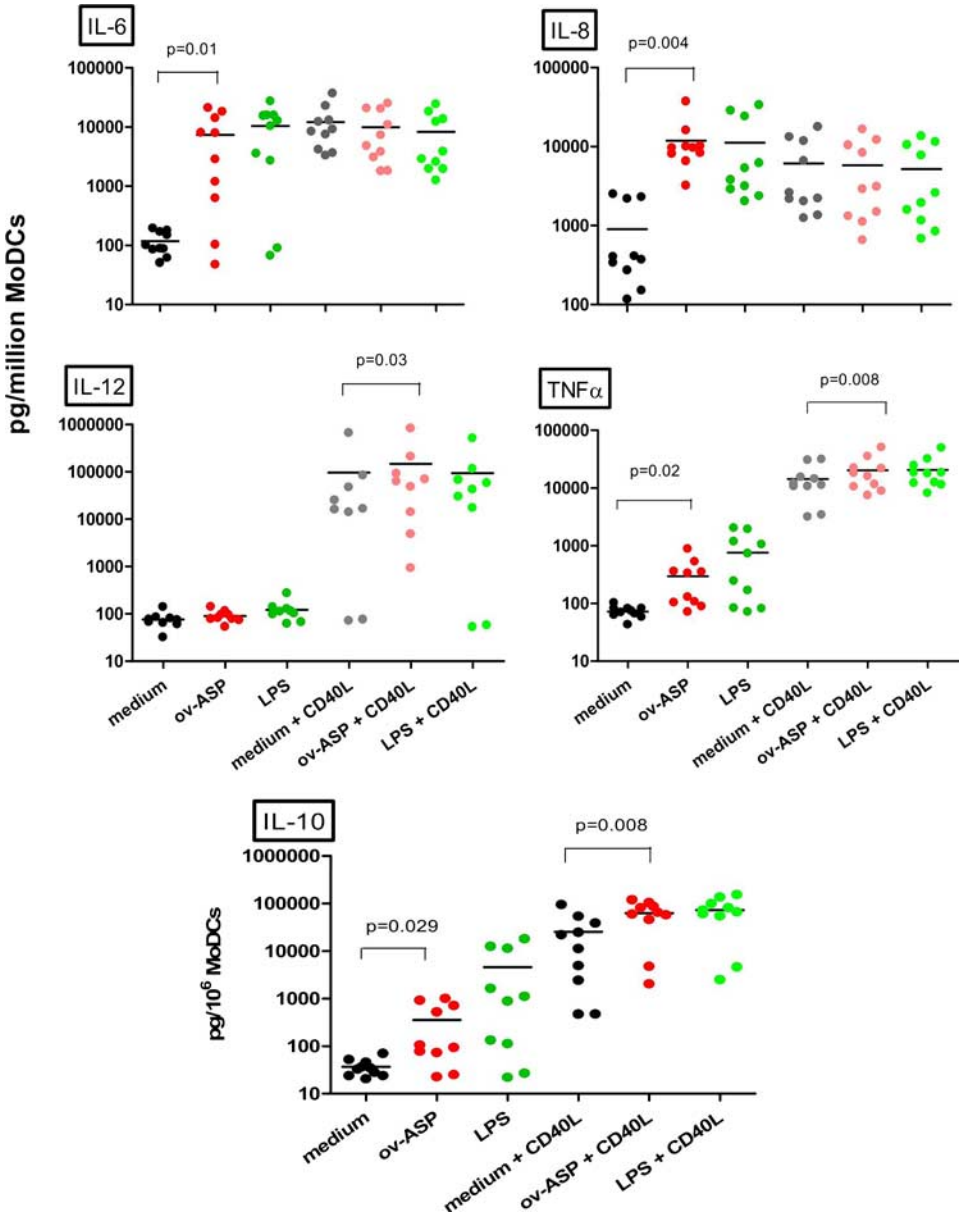

FIGURE 6. Cytokine production from r*Ov*-ASP-1 treated MoDCs with and without CD40L. MoDCs (n = 9–10) were cultured for 24 h with medium alone or in a 1:1 ratio with a hCD40L-CHO with or without 5 μg/ml r*Ov*-ASP. LPS was used as a positive control. r*Ov*-ASP alone stimulated significant amounts of proinflammatory cytokines, IL-6, IL-8, IL-2, and TNF-α but not IL-12. However, when MoDCs were costimulated with CD40L, significant enhancement of IL-12 secretion was seen. MoDCs stimulated with r*Ov*-ASP significantly stimulated the production of the anti-inflammatory cytokine, IL-10 and this was further enhanced when MoDCs were costimulated with CD40L.

humoral response but also an RBD specific Th1-biased cellular response.

*r*Ov-*ASP-1 binds primarily to APCs among human PBMCs*

Our data have clearly demonstrated that r*Ov*-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 r*Ov*-ASP-1 adjuvanticity, we analyzed first the binding profile of biotinylated r*Ov*-ASP-1 with human PBMCs (n = 4) by flow cytometry (Fig. 3). We found that biotin-labeled r*Ov*-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 r*Ov*-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 r*Ov*-ASP-1 primarily binds to the APCs, monocytes, and B cells, among human PBMCs, suggesting that r*Ov*-ASP-1 may interact with the specific receptors presented on the surface of the APCs to activate the immune cells.

*r*Ov-*ASP-1-stimulated human PBMCs to produce proinflammatory cytokines*

We then determined whether r*Ov*-ASP-1 can trigger cytokine production of human PBMCs from normal healthy donors. As shown in Fig. 4, r*Ov*-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 r*Ov*-ASP-1 on human PBMCs (Fig. 4*F*). 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 r*Ov*-ASP-1 protein appears to be a potent inducer of proinflammatory (IFN-γ and

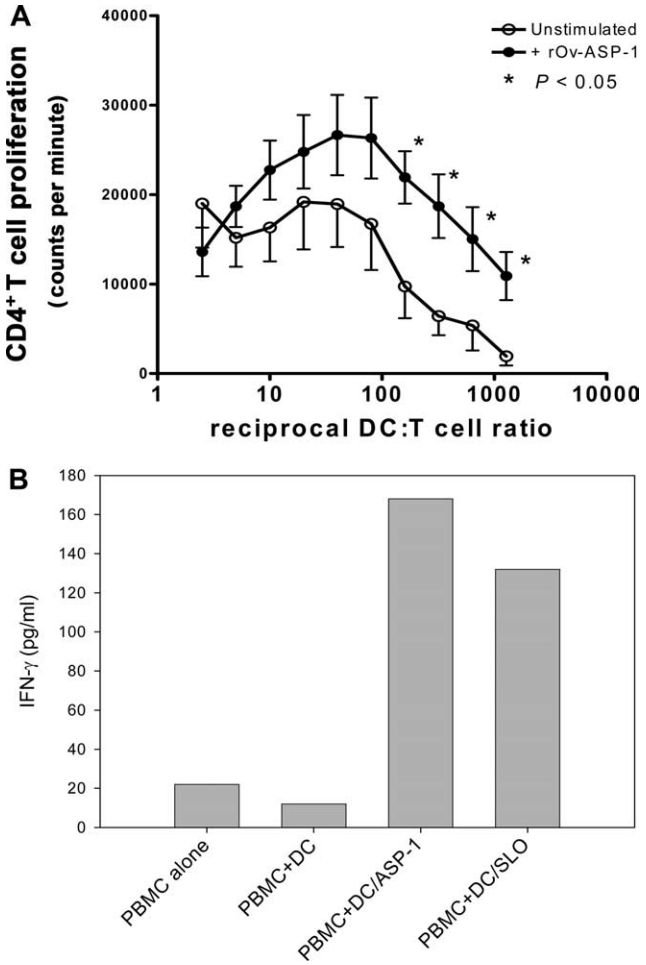

FIGURE 7. r*Ov*-ASP-1 treated MoDCs have enhanced allo-stimulatory function. *A*, MoDCs treated with 5 μg/ml r*Ov*-ASP-1 showed enhanced ability to stimulate the proliferation of allogenic CD4$^+$ T cells. The CD4$^+$ T cell proliferation (cpm) in response to r*Ov*-ASP-1 is shown. Each point represents the mean (+SD) of results from five individuals. *, $p < 0.05$. *B*, r*Ov*-ASP-1-primed human DCs induced IFN-γ secretion from naive PBMCs. MoDCs were treated with 2 μg/ml r*Ov*-ASP-1 and then washed and cocultured (1×10^4/well) with autologous normal PBMCs (2×10^5/well) in round-bottom 96-well plate for 5 days. The level of IFN-γ secretion was determined by ELISA. Streptolysin O (SLO) served as a positive immunopotentiator.

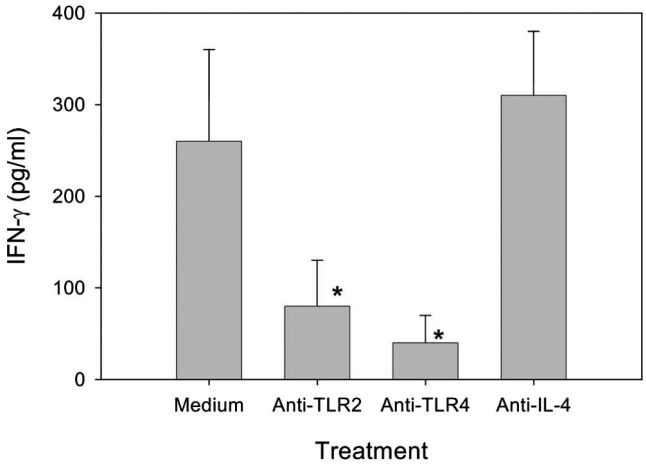

FIGURE 8. Inhibition of r*Ov*-ASP-1-induced IFN-γ secretion from human PBMC ($n = 4$) by anti-TLR Abs. The PBMCs were preincubated with anti-TLR2, anti-TLR4, or IL-4 Abs (10 μg/ml) at 37°C for 1 h before adding 5 μg/ml r*Ov*-ASP-1 for stimulation over a 5-day period. The concentration of IFN-γ in the culture supernatants was tested by ELISA. *, $p < 0.05$ when compared with the r*Ov*-ASP-1 untreated cells.

TNF-α) and immunoregulatory (IL-10) cytokine secretion from the normal human PBMCs.

*r*Ov-*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 r*Ov*-ASP-1 induced DC maturation, MoDCs from NHD ($n = 14$) were cultured with or without r*Ov*-ASP-1 at 5 μg/ml for 24 h. Using FACS analysis, we measured expression of surface molecules that are characteristically up-regulated in DCs after stimulation. As shown in Fig. 5, all four phenotypic markers that were tested increased after treatment with r*Ov*-ASP-1. MoDCs from NHDs ($n = 9$–10) were then cultured with or without r*Ov*-ASP-1 (5 μg/ml) for 24 h and cytokine production was determined. Notably, r*Ov*-ASP-1-stimulated significant amounts of the proinflammatory cytokines, IL-6, IL-8, and TNF-α but not IL-12 (Fig. 6). There was no significant induction of other cytokines tested IL-2, IL-4, IL-5, or IL-1β but the MoDCs cultured with r*Ov*-ASP-1 significantly stimulated also the production of the anti-inflammatory cytokine, IL-10. Notably, the r*Ov*-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 r*Ov*-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 r*Ov*-ASP-1 may initiate DC-induced adaptive immune responses by multiple distinct mechanisms.

To assess whether r*Ov*-ASP-1 treatment of MoDCs was also able to enhance their accessory/Ag presentation function, we used the MLR assay with allogeneic Ags. Fig. 7*A* shows the results of the MLR experiments indicating that MoDCs treated with 5 μg/ml r*Ov*-ASP-1-enhanced their ability to stimulate proliferation of allogeneic CD4$^+$ T cells. Moreover, r*Ov*-ASP-1 primed DCs were able to induce IFN-γ secretion from naive PBMCs (Fig. 7*B*). The ability of r*Ov*-ASP 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 r*Ov-*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 r*Ov*-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 r*Ov*-ASP-1 in the absence or

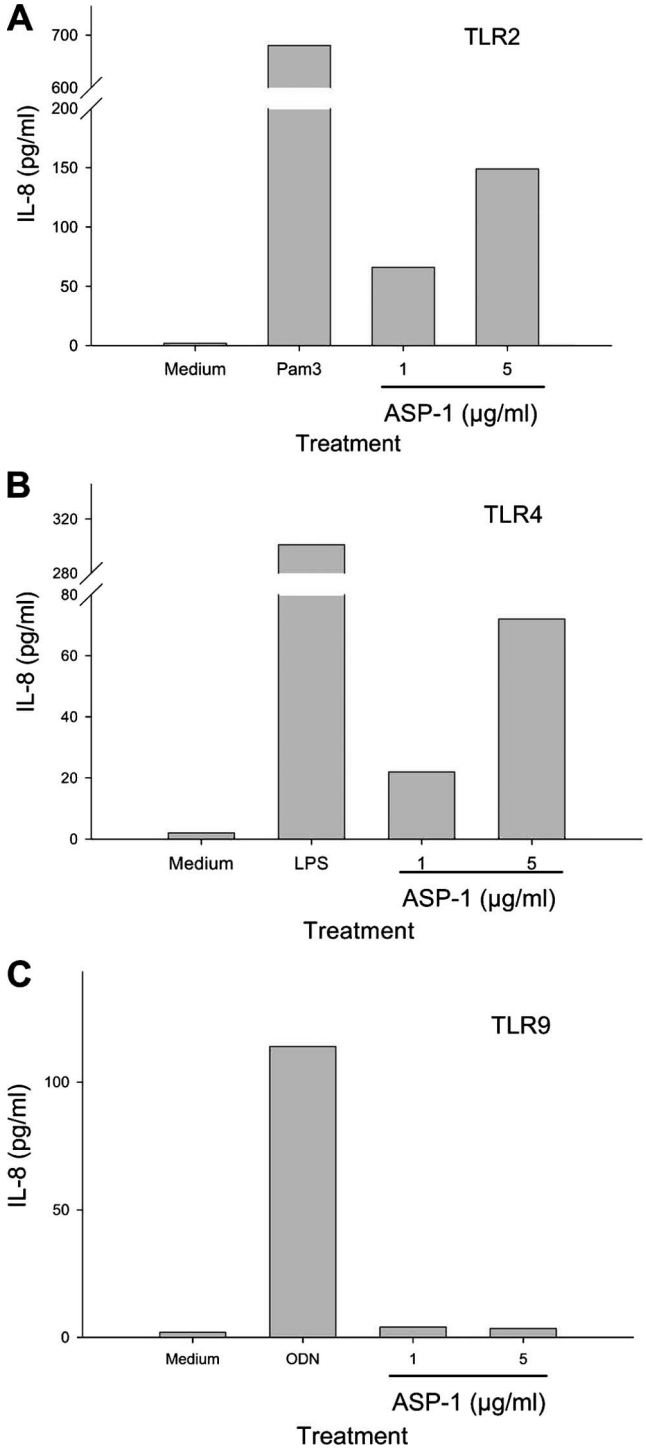

FIGURE 9. Activation of TLR2 and TLR4 by r*Ov*-ASP-1 in a dose-dependent manner. Human HEK293 cells expressing TLR2 (*A*), TLR4 (*B*), or TLR9 (*C*) were stimulated with 1 μg or 5 μg/ml rOv-ASP-1 for 18 h and the activation was measured by human IL-8 secretion. TLR control ligands (Pam3CSK4 for TLR2, LPS for TLR4, and ODN2216 for TLR9) activated the secretion of IL-8 from the appropriate cell lines as expected.

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 r*Ov*-ASP-1-induced IFN-γ secretion from human PBMCs is TLR2 and TLR4 dependent.

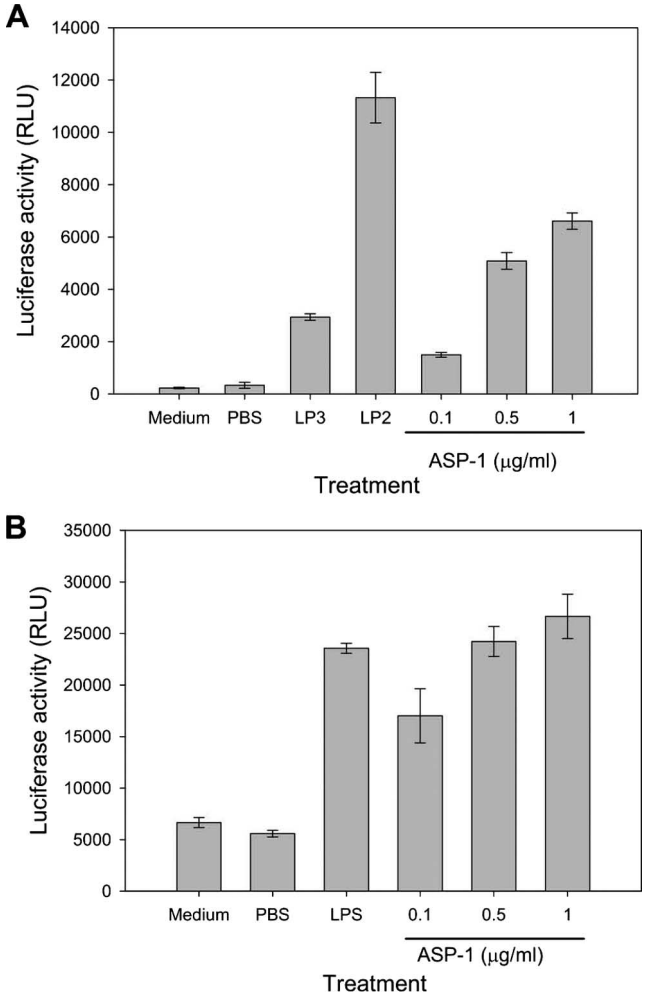

FIGURE 10. The r*Ov*-ASP-1-induced cellular activation as determined by NF-κB-dependent reporter gene assay. HEK293 cells were cotransfected with an NF-κB-dependent luciferase reporter construct and the expression plasmids for TLR2 (*A*) or TLR4/MD2 (*B*). The cells were challenged with increasing amounts of r*Ov*-ASP-1. The control ligands for TLR2 were Pam3CSK4 (LP3) and Pam2CSK4 (LP2); LPS was used as a ligand for TLR4. The luciferase activity was quantified after 20 h of stimulation.

r*Ov-ASP-1 triggered cellular activation via TLR2 and TLR4*

The above results suggest that the r*Ov*-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 r*Ov*-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 r*Ov*-ASP-1 activated both TLR2- and TLR4-expressing cells at dose-dependent manners, but not TLR9. The wt HEK293 cells, which do not expression TLR2 and TLR4, were unresponsive to the r*Ov*-ASP-1 (data not shown) in the IL-8 release assay.

The r*Ov*-ASP-1 cellular activation via TLR2 and TLR4 was further determined by a more sensitive NF-κB-dependent reporter gene assay. Consistently, r*Ov*-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 r*Ov*-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 r*Ov*-ASP-1 has potent adjuvant effects for several vaccine Ags including recombinant proteins, synthetic peptides, and commercial inactivated vaccines (18, 20). Importantly, r*Ov*-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 r*Ov*-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 MLP 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 r*Ov*-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 r*Ov*-ASP-1 significantly promoted the IFN-γ and TNF-α production in the immunized mice. These results further confirmed that r*Ov*-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 affect DCs such that mature DCs gained the ability to induce the development of IL-10-producing regulatory T cells (42). The *Toxoplasma gondii* profilin activate DCs through TLR11, which was required 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 r*Ov*-ASP-1 adjuvanticity, we initially analyzed the binding profile of r*Ov*-ASP-1 with normal human PBMCs by a flow cytometry-based assay. It was found that r*Ov*-ASP-1 binds primarily to the human monocytes and B cells, the major APCs in the peripheral blood. Significantly, r*Ov*-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 r*Ov*-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, r*Ov*-ASP-1 also induced significant IL-10 secretion from 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 r*Ov*-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 r*Ov*-ASP-1 through an unknown as yet mechanism. Based on the profile of cytokines stimulated by r*Ov*-ASP-1 we hypothesize that the *Ov*-ASP-1 protein ($\sim$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 r*Ov*-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 r*Ov*-ASP 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 r*Ov*-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, r*Ov*-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 r*Ov*-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 r*Ov*-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, r*Ov*-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

structures (41). Among the TLRs, TLR2 mediates the response to the most diverse set of molecular structures including lipoproteins, 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 in 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 TLR4 in r*Ov*-ASP-1 bioactivities and thus back our indirect studies of anti-TLR2 and anti-TLR4 Abs, additional studies using TLR2 and TLR4 knockout mice are needed. These would include testing whether the adjuvanticity of r*Ov*-ASP-1 is lost in these mice and if innate cytokines production and DC maturation is reduced.

In conclusion, r*Ov*-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.

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