Published in final edited form as:

Vaccine. 2007 August 14; 25(33): 6211–6223.

Immunogenicity and Efficacy of Recombinant RSV-F Vaccine in a Mouse Model

Shree R. Singh^{a,*}, Vida A. Dennis^b, Christina L. Carter^a, Shreekumar R. Pillai^a, Ayanna Jefferson^b, Shivendra V. Sahi^c, and Eddie G. Moore^d

a Department of Mathematics and Science, Alabama State University, Montgomery, AL 36104, USA

b Division of Bacteriology and Parasitology, Tulane National Primate Research Center, Tulane University Health Science Center, Covington, LA 70433, USA

c Department of Biology, Western Kentucky University, Bowling Green, KY 42101, USA

d Department of Biological Sciences, Alabama State University, Montgomery, AL 36104, USA

Abstract

RSV vaccine development has constraints due to safety issues encountered by formalin inactivated FI-RSV vaccines. A desirable vaccine should induce Th₁ responses and a strong mucosal immunity to provide complete protection from RSV infection. In the present paper, we developed and evaluated a mucosal vaccine against RSV in a mouse model. The antigenic regions corresponding to residues 412-524 of RSV-F protein were amplified by RT-PCR and cloned into a vector containing the *ctxA₂B* gene of the cholera toxin. The recombinant protein was expressed in *E. coli* and properties of the recombinant protein were analyzed by SDS-PAGE, Western blot and G_{M1}-ELISA. The purified recombinant protein (rRF-412) was used to immunize BALB/c mice intranasally. The results from our studies show that the rRF-412 immunogen induced mucosal (IgA) and systemic antibody (IgG, IgG1, IgG2a, and IgG2b) responses which neutralized RSV. The IgG1/IgG2a ratios indicated a Th₁ biased antibody response. The Th₁ (TNF- α , IL-12p70, IFN- γ , IL-2) and Th₂ (IL-10, IL-4 and IL-5) cytokine profiles were analyzed after stimulation of spleen cells from mice immunized with purified RF-412 protein. Similar to the antibody response, we observed that the rRF-412 immunogen induced a mixed Th₁/Th₂ cytokine immune response with a Th₁ bias response. Serum antibodies were capable of neutralizing RSV and mice immunized with rRF-412 were significantly protected from live RSV challenge. Our data provides evidence that the rRF-412 immunogen may be a potential mucosal vaccine candidate against RSV.

Keywords

RSV; fusion protein; recombinant RSV-F protein; cholera toxin; cytokines; mucosal vaccine

1. Introduction

Respiratory syncytial virus (RSV) is a major cause of bronchiolitis and pneumonia in infants and young children infecting almost all infants less than two years of age. More than 90,000

* Corresponding Author. S. R. Singh, Alabama State University, 915, S. Jackson Street, Montgomery, AL 36101, USA, Tel.: +1-334-229-4168, Fax: +1-334-229-4955. E-mail address: ssingh@alasu.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

children in the US are hospitalized every year as a result of this viral infection [1]. RSV epidemics are an annual event occurring every winter, and reinfection with this virus is very common occurring throughout life [2]. RSV not only affects infants and young children, but also elderly and high-risk adults; the impact of RSV disease in the later groups is comparable to the influenza A disease [3]. Several approaches to develop vaccines against RSV have been employed which include formalin-inactivated RSV (FI-RSV), live attenuated RSV, inactivated RSV, subunit and DNA vaccines but none of them have been approved for use in humans [2].

Several approaches for RSV vaccine development have focused on the use of subunit proteins. The RSV genome is a negative sense single-stranded RNA which encodes ten genes and results into translation of eleven proteins: NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L [4]. Among these proteins, only the fusion (F) and attachment (G) glycoproteins have been studied in detail. The F glycoprotein is responsible for viral penetration, fusion of viral and cellular membranes and fusion of infected cells with surrounding cells (syncytium formation) [5]. The F protein induces high titers of neutralizing antibodies, RSV-specific cytotoxic and T-helper lymphocytes, and provides significant protection against RSV infection [6]. Additionally, the F protein is conserved among RSV strains, and antibodies to the F protein provide protection against both A and B strains of RSV. Immunization of mice with PFP leads primarily to a Th₁ immune response and based on encouraging results, the purified F protein (PFP) has been used in clinical trials [7,8]. In contrast, the RSV G protein [8] is associated with enhanced disease and induction of a Th₂ immune response. Thus, the RSV G protein may not be useful as a vaccine for humans.

RSV infects mucosal surfaces and in order to protect against RSV infection, induction of mucosal immunity in conjunction with a systemic immune response is important. Although most immunogens, including F protein, induce strong humoral and cellular immune responses, they do not provide complete protection from upper and lower respiratory tract infections [1]. Mucosal immunity is important in protecting the epithelium of the nasal cavity from RSV infections and the most important antibody isotype in mucosal secretions that functions to provide protective immunity at mucosal surfaces is IgA [9]. Salivary IgA (sIgA) found at mucosal surfaces provides protection from upper respiratory tract infections [10]. To induce mucosal antibodies several bacterial enterotoxin based adjuvants are used which include cholera toxin (CT). CT is a widely used and well understood adjuvant [11] which has been shown to induce mucosal antibody responses against several pathogens including RSV [6]. However, due to the toxic effects of the holotoxin, CT is not recommended for human applications.

The cholera toxin B (CTB) subunit lacking the toxic part has similar adjuvant activity [12] and also functions as an excellent carrier protein [13]. CTB can be either mixed with antigen or chemically coupled for immunizations, which improves antigen presentation [14]. However, chemical coupling is not consistent and coupling procedures can lead to poor immunogenicity [15]. A better approach is to genetically link antigens with CTB. In the *ctx* operon, the CTA subunit is expressed as A1-A2 subunits joined together by a disulfide bond. The CTA1 subunit is responsible for the typical cholera symptoms in humans. The CTB subunit is expressed from the *ctx* operon and forms a pentamer. One CTB pentamer associates with a CTA subunit resulting in a cholera holotoxin [16]. The A₂ subunit of the CTA associates with the CTB pentamer [17] and lack of A₁ subunit does not prevent holotoxin formation [18]. Following a genetic approach, in the *ctx* operon, the A1 subunit can be replaced with any antigen of interest without sacrificing the formation of a holotoxin-like chimera. This approach has been used by several investigators to link bacterial and viral proteins and the recombinant chimeras have been shown to induce protective immunity [18]. It has also been shown that the route of antigen administration to induce local immunity is also important. Intranasal immunization with RSV

antigens enhances the mucosal immune responses [19] and protect animals from upper and lower respiratory tract infections as opposed to other routes of immunizations [20].

Because the antigenic regions corresponding to residues 412-524 of the RSV F protein have been reported to contain several antigenic epitopes [21], we hypothesized that this RSV F protein fused with the CTA₂B subunit would induce systemic and mucosal immune responses in mice. To address this hypothesis, we first utilized recombinant DNA technology to express the RSV-F protein subunit, which is 112 amino acids long corresponding to residues 412-524 of the RSV F protein. This antigenic region was then cloned into a vector containing the modified cholera toxin. A chimeric protein (rRF-412) was expressed in *E. coli* and properties of the holotoxin-like recombinant RSV-F protein were analyzed. Finally, the recombinant protein was used to immunize BALB/c mice to assess its immunogenicity at the humoral and cellular levels. Here we present the results of this first report, which is aimed at developing a recombinant mucosal immunogen for RSV by genetically fusing antigenic regions of the RSV-F protein with a detoxified CTA₂B subunit of cholera toxin.

2. Materials & methods

2.1. Animals and RSV stock preparation

BALB/c female mice (4–6 wk old) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in laminar flow racks under pathogen-free conditions with a cycle of 12 h of light and 12 h darkness. For all immunization studies, an approved protocol by Alabama State University Institutional Animal Care and Use Committee was followed.

Human RSV long strain was purchased from American Type Culture Collection (ATCC, Manassas, VA, ATCC # VR-26) and propagated in HEp-2 cells (ATCC # CCL-23). HEp-2 cells were grown in tissue culture flasks in MEM supplemented with 10% FBS and antibiotics. RSV with multiplicity of infection (m.o.i.) of 4:1 was added to the flask and virus adsorption was carried out for 1 hr at 37°C in a humidified atmosphere with 5% CO₂. MEM with 2% FBS was added to the flask and infection of cells was observed for an additional 3–4 days. RSV infected cells were centrifuged at 3,000 × g at 4°C to remove cellular debris, aliquoted, and were stored at –80°C until they were used.

2.2. Bacterial strains

E. coli Novablue and *E. coli* BL 21(DE3) cells were purchased from EMD Biosciences (San Diego, CA) and were used for cloning and expression experiments, respectively. The cells were grown at 37°C in Luria Bertani (LB) broth containing 100 µg/ml ampicillin. Clones used for protein expression were grown at 30°C.

2.3. RNA extraction, RT-PCR and construction of ctxA₂B based recombinant RSV-F vectors

Total RNA was isolated from frozen RSV stocks using RNeasy Mini kit (Qiagen Inc., Valencia, CA), which included DNase I digestion. RT-PCR was performed using superscript one step RT-PCR kit from Invitrogen (Carlsbad, CA) following the manufacturer's protocol. Reverse transcription was performed for 15 min at 55°C followed by PCR consisting of 35 cycles of denaturation at 94°C 1 min, annealing at 55°C for 30 s and extension at 72°C for 30 s. RSV-F specific forward (5'GTCGTTCCATGGCCATT GTGTCATGCTATGGC) and reverse (5' AAGTAAGTCGAC TTTGGTGGATTACCAGC) primers containing *Nco*I and *Sal*I restriction sites, respectively, were used in PCR reactions. The RT-PCR products which corresponded to residues 412-524 of RSV-F protein were purified from a low melt gel using a gel extraction kit (Qiagen). The purified DNA fragment was subjected to *Nco*I and *Sal*I restriction enzyme digestion followed by purification and subsequent cloning.

The modified cholera toxin *ctxA₂B* based vector, pUAB084, derived from the pET-32a(+) was used in this study. A similar vector, pCT^{ΔA1} has been described previously [22]. The RSV-F gene fragment was cloned into the pUAB084 vector at the 5' of the *ctxA₂B* gene (Fig. 1a). The resulting clone was designated as pASU-RSF and analyzed by restriction enzyme digestion and the junctional and RSV-F gene sequences were determined by DNA sequencing (Auburn University Genomics and Sequencing Laboratory, Auburn, AL). DNA sequences of the cloned RSV-F inserts and vector were compared and matched with the published sequences of the RSV-F gene [23] and the pUAB084 vector.

2.4. Expression, purification and in vitro association of recombinant protein

The purified pASU-RSF vector was used to transform *E. coli* BL21(DE3) expression host cells (containing inducible T7 RNA polymerase) and used for all protein expression experiments. The fusion protein consisting of the thioredoxin and RSV-F fragments, as well as the CTA₂ peptide was expressed by means of vector translation initiation codons, and a stop codon at the 3' end of the CTA₂ coding sequence. Translation of the CTB subunit occurred by independent signals promoted by its own ribosome-binding site, initially as monomers, and later assembled into pentamers, which then associated with the CTA₂ portion of the fusion protein (Fig. 1b). Following transformation, recombinant clones were selected and analyzed to ensure insert and vector content. The pASU-RSF vectors were grown at 30°C and when the cell density reached an OD₆₀₀ of 0.60, protein expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. The cells were harvested and cytoplasmic extracts (CE) were collected by suspending the cells in a wash buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA and 1% Triton X-100) and lysed using an Aminco French press cell (American Instrument Co., Urbana, IL) at 16,000 psi. The supernatant was saved as CE while the pellet containing the inclusion bodies (IB) was solubilized in a solubilization buffer (50 mM CAPS, pH 11.00, 0.5% N-lauroylsarcosine, and 0.1 mM DTT) for 30 min at 25°C. The IB solubilized fraction which contained a majority of the recombinant protein was also purified using His Bind Columns (EMD Biosciences) following the manufacturer's protocol with some modifications. Briefly, binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) was used to equilibrate the columns; protein was added to the column, and the column subsequently washed with wash buffer (binding buffer containing 20 mM imidazole and 6M Urea). The bound recombinant protein was eluted in elution buffer (wash buffer with 1 M imidazole) and further analyzed by SDS-PAGE and western blot. The purified protein fraction containing a single band was designated as RF-412, and the protein concentration was determined by the BCA assay (Pierce Biotechnology Inc., Rockford, IL).

Our analysis of the RF-412 protein revealed that CTB was not present in a proportion needed to form a holotoxin like chimeric protein. Therefore, RF-412 was reassociated with CTB (Sigma Chemical Company, St. Louis, MO) following the methods of Hardy et al. [24]. Unassociated CTB was removed from the protein mixture by dialysis (20 kDa m.w. cut off). The reassociated recombinant protein was named rRF-412 and its association with CTB was confirmed by G_{M1}-ELISA. The reassociated rRF-412 protein samples contained less than 0.25 endotoxin units per mg of protein, as assessed by *Limulus* amebocyte assay (Associates of Cape Cod Inc., Woods Hole, MA). The rRF-412 protein was used to immunize BALB/c mice as described below.

2.5. Protein analysis

The G_{M1}-ELISA assay was performed to determine the association of RF-412 and the CTB pentamer following procedures of Svennerholm and Holmgren [25]. Briefly, 96-well plates were coated with 0.01 μg/well of G_{M1} gangliosides (EMD Biosciences) followed by addition of 1 μg of reassociated rRF-412 protein to each well. The bound protein was detected using

RSV and CTB specific polyclonal antibodies. Since only cholera holotoxin like chimera are recognized by G_{M1}-ELISA, it confirmed the proper reassociation of the rRF-412 protein.

To confirm the identity of the recombinant protein, electrophoresed proteins were transferred onto polyvinylidene difluoride membranes (PVDF), probed with anti-RSV polyclonal antibodies (Chemicon International, Inc. Temecula, CA) for 1 h at RT and then by appropriate HRP-conjugated secondary antibodies (Southern Biotech Associates Inc.; SBA, Birmingham, AL). Immunoreactive protein bands were detected using ECL fluorescent detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.6. Immunization and RSV challenge studies

The immunogenicity and protective efficacy of the rRF-412 antigen were studied in female BALB/c mice. Three groups of 4–6 wk old mice were immunized intranasally (i.n.) with 50 µg of rRF-412, PBS or 10⁵ PFU of live RSV. At least ten mice per group were immunized at days 1, 15 and 29 with 50 µg of rRF-412 antigen (50 µl volume in PBS) by slow application to the nasal nares. Live RSV was used for immunization only once on day 1 to simulate natural infection. Blood samples were collected from the tail vein of all groups of mice on days 0, 14, 28, and 49. Saliva samples were collected at the same time intervals using a pipette fitted with plastic tips after i.p. injection of carbachol (0.5 µg) to stimulate salivation. Serum and saliva samples were stored at –20°C until they were analyzed. On day 49, spleens were harvested from 3 mice and used for cytokine analysis. On day 50, anesthetized mice were challenged with 10⁵ PFU of live RSV intranasally.

2.7. Analysis of antibody response

Antibody titer and isotypes of all sera and saliva samples were determined following published protocols [26]. ELISA plates were either coated with 100 µl of purified RF-412 antigen (1 µg/ml) or RSV; blocked and then ten-fold serial dilutions of serum and saliva samples were added to designated wells overnight. The plates were incubated for 1 h at room temperature, washed, followed by addition of HRP-conjugated goat anti-mouse Ig (100 µl, 1:2,000) (SBA). The enzymatic reaction was developed by adding 100 µl TMB substrate (Kirkegard and Perry Laboratories, KPL, Gaithersburg, MD) and the absorbance was read at 450 nm using a Tecan ELISA plate reader (Tecan, Research Triangle Park, NC). Isotypes determination kits (SBA) were used for antibody isotyping (IgA, IgG, IgG1, IgG2a, and IgG2b) as described previously [27].

2.8. Cell isolation and stimulation

Spleen cells from each group of mice (3 per group) were pooled and cells were isolated using 40-mesh stainless steel screens (Sigma). Spleen cells (3×10⁶/ml) were cultured *in vitro* in 24-well plates (Costar, Cambridge, MA) in the presence of RF-412 purified antigen at 1, 0.5 and 0.25 µg/ml concentrations. The concentration of antigen eliciting the maximal response was used for data analysis. Some wells received 5 µg/ml of Concanavalin A (Con A; Sigma) as a nonspecific control. The control supernatant was generated by culturing cells in medium alone. Culture medium consisted of 45% RPMI 1640, 45% Iscove's modified Dulbecco's medium (Gibco-Invitrogen), 10% heat-inactivated FBS (JRH Biosciences, Inc., Lenexa, KS), 1 mM Hepes (Gibco), 0.5 mM sodium pyruvate (Sigma), 2 mM L-glutamine, 0.05 mM mercaptoethanol (Sigma), and 1 µg/ml gentamicin sulfate (Gibco-Invitrogen) (complete medium). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Cell free-supernatants were harvested at 24, 48 and 72 hr and stored at –70°C until they were used.

2.9. Measurement of cytokine concentrations

Cytokine ELISAs were performed as previously described.[28] Concentrations of Th₁ (TNF- α , IL-12p70, IFN- γ , IL-2) and Th₂ (IL-10, IL-4 and IL-5) cytokines were quantified in cell free-supernatants of spleen cultures using Opti-EIA sets (BD-PharMingen, San Diego, CA) according to the manufacturer's instructions.

2.10. Virus neutralization assay

The virus neutralization assay was performed following protocols of Sastre et al. (2004) with modifications [29]. Five-fold serially diluted (in a total of 100 μ l PBS) sera were heat inactivated (56°C for 30 min) and approximately 1,000 PFU of RSV were mixed with the sera and incubated for 2 hr at 4°C. Approximately, 15,000 HEp-2 cells in 100 μ l MEM-10 were added to each well of a 96-well microtiter plate. The RSV-serum mixture was added to the appropriate wells and incubated for 3 days in a 5% CO₂ incubator at 37°C. The plates were washed with PBS and fixed with 80% acetone followed by blocking with 3% blocking buffer. Goat anti-RSV antibody was added to the appropriate wells and incubated for 60 min at RT. After three washings, rabbit anti-goat HRP-conjugated antibody was added and the enzymatic reaction was developed using TMB substrate (KPL) and ODs were read at 450 nm. The neutralization titer was calculated from the average OD of the wells by extrapolating the inverse of the serum dilution resulting in 50% reduction of RSV activity.

2.11. Lung RSV titer determination

Mice were sacrificed at day 55 (5 days after challenge) and the lungs were collected and frozen at -70°C. Individual lungs were thawed out, homogenized and mixed in 1 ml of MEM containing 2% FBS followed by centrifugation at 3,000 $\times$ g for 10 min. Supernatants containing the virus were serially diluted ten-fold in PBS and used for RSV titer determination. BD Falcon 8-chambered tissue culture slides (BD Biosciences, San Jose, CA) were used to grow HEp-2 cell monolayers to 60–70% confluency in minimal essential media with 10% fetal bovine serum. The media from slides were removed; 5 μ l of lung samples containing RSV were added to the chambers; adsorption was carried out for 30 min at 37°C in a 5% CO₂ incubator followed by addition of 1 ml of MEM with 2% FBS to each chamber. RSV infection was carried out for 48 hr in a 5% CO₂ incubator at 37°C. Cells were fixed with 10% TCA followed by successive washes with 70% and 90% alcohol. After 30 min blocking in 3% dry milk powder, anti-RSV F monoclonal (200 μ l of 1:100) antibody was added to each chamber for 1 hr at RT. The chambers were washed three times followed by incubation for 1 hr at RT with FITC-conjugated goat anti-mouse secondary antibody (200 μ l of 1:1000). After two washings, slides were viewed at 200X magnification under an Olympus IX51 fluorescent microscope and the number of cells in each chamber with fluorescence was counted towards the total number of PFU. Total number of PFU/ml was determined by averaging the number of PFU from at least four chambers for each sample and then adjusting for the dilution factor.

2.12. Statistical analysis

Comparisons of antibody concentrations and isotypes between various groups of mice at different time intervals were performed by the two-tailed unpaired Student's *t*-test for samples with unequal variances. Cytokine data were analyzed using the two-tailed unpaired Student's *t*-test. *P* < 0.05 was considered significant.

3. Results

3.1. Recombinant vectors express functional RSV-F protein associated with cholera toxin B subunit

The recombinant clone, pASU-RSF, was expressed in *E. coli* BL21 (DE3) cells and the soluble and insoluble fractions of the protein were analyzed on SDS gels. The solubilized protein fractions contained a protein band of ~36 kDa that corresponded to the expected size of the RF-412 (Fig. 2a, lane 4). To confirm the presence of recombinant RSV-F protein, proteins were transferred onto a PVDF membrane and probed with an anti-RSV polyclonal antibody. Presence of the purified RSV-F protein was confirmed following analysis of western blot results (Fig. 2b, lane 2).

Our project focus was to express a recombinant RSV-F protein associated with the CTB pentamer to form a CT-like holotoxin chimera. In the *E. coli* BL21 expression host, recombinant protein is expressed as a fusion protein consisting of Thioredoxin-RSV-F-CTA₂ protein (Fig. 1b). The CTB fraction is expressed as monomers and forms a pentamer which associates with the CTA₂ portion of the fusion protein (Fig. 1b). The G_{M1}-ELISA was employed to test the reassociation of the recombinant protein since this test detects only holotoxin-like chimera. In order to confirm the association of RSV-F protein with CTB, we used anti-RSV antibodies in G_{M1}-ELISA. The G_{M1}-ELISA data revealed that a low amount of CTB was present in our purified RF-412 protein resulting in poor association of purified protein with CTB. Therefore, we denatured CTB and added it to the purified recombinant protein. Following this experiment, we were able to achieve the folding and reassociation of the recombinant protein confirmed by G_{M1}-ELISA (Table 1).

3.2. Recombinant antigen induces systemic and mucosal antibody responses

To address our hypothesis that rRF-412 would induce systemic and mucosal immune responses, 3 groups of mice were immunized i.n. with rRF-412, PBS or live RSV. Mice were immunized at least thrice with PBS and RF412, but only once with RSV to simulate natural infection. Serum and saliva samples were collected at two-week intervals following each immunization. These samples were analyzed by ELISA using RF-412 protein as antigen to detect RSV-F specific antibodies. The rRF-412 chimeric protein when administered i.n. resulted in significantly higher ($P<0.05$) serum IgG antibody response as compared with the PBS and RSV groups (Fig. 3a). Initial responses were first detected at day 14 (after the first immunization) and these responses continued to increase after the second immunization. Since a Th₁ immune response has been shown to induce protective immunity against RSV infection, our interest was to analyze Th₁ (IgG2a) and Th₂ (IgG1, IgG2b) specific antibody isotypes. Serum from mice immunized with rRF-412 had significantly higher levels of IgG1, IgG2a and IgG2b responses ($P<0.05$) as compared to the PBS and RSV groups of mice (Fig. 3). Overall, these serum specific antibody isotype responses were observed after the first immunization (Figs. 3b–d) and their levels increased after the second immunization. Serum IgG2a antibody responses were more prominent than the IgG1 isotype (Figs. 3 b and c). Although significantly higher ($P<0.05$) serum IgG2b antibody levels were observed (Fig. 3d), again it was comparatively lower than the IgG2a response (Fig. 3c). Following studies of Durbin et al. [30], we also calculated the ratios of IgG1/IgG2a and IgG2b/IgG2a isotype responses (Table 2) which are indicators of Th₁ vs. Th₂ response. All serum and salivary antibody isotype ratios between Th₂-Th₁ were consistently lower than 1, thus indicating a prominent Th₁-driven immune response to the rRF-412 immunogen in mice. Mice immunized with RSV had IgG, IgG1, IgG2a, and IgG2b isotype responses but at low levels compared to RF-412 immunized mice (Figs. 3 a–d). This was not surprising since only RSV-F specific antibodies were being detected in our antibody analysis.

The mucosal antibody response (IgA) in serum was also analyzed. The rRF-412 immunized mice had significantly higher ($P < 0.05$) serum IgA responses after the first immunization, which continued to increase after the second immunization (Fig. 3e), although this increase was not statistically significant. Overall the IgA responses in sera were 2–3 fold lower than other antibody isotypes analyzed. Mice immunized with RSV had significantly higher ($P < 0.05$) serum IgA responses as compared to the PBS control mice (Fig. 3e), but this response was lower than the RF-412 immunized group.

Mucosal antibody immune responses were also assessed in saliva samples obtained from groups of mice. In general, the mucosal antibody response in saliva was 2–3 fold lower than that of serum antibody response (Fig. 4). Similar to the serum antibody responses, mice immunized with rRF-412 had a significantly higher salivary antibody response as compared with the PBS and RSV groups. Salivary IgG antibody response increased in rRF-412 immunized mice after the first immunization (Fig. 4a) and remained high following subsequent immunizations. The RSV group also had a significantly higher salivary IgG response in comparison with the PBS group (Fig. 4a). The salivary IgG1 antibody response after vaccination of mice with rRF-412 was significantly higher ($P < 0.05$) after the first immunization, which remained high after the second and third immunizations (Fig. 4b). The salivary IgG1 response from the RSV group was significantly higher than the PBS group (Fig. 4b); although at a lower level in comparison with the rRF-412 group. When we analyzed the IgG2a and IgG2b antibody response in saliva, again the rRF-412 immunogen successfully induced significantly higher amounts of IgG2a and IgG2b antibodies (Figs. 4c and d). The salivary IgG2a isotype response was higher than the IgG1 and IgG2b isotype responses. The salivary Th₁-Th₂ antibody isotype indicator ratios of IgG1/IgG2a and IgG2b/IgG2a ranged between 0.352–0.665 (Table 2), further indicating that the rRF-412 immunogen induces a mixed Th₁/Th₂ immune response with a prominent Th₁ driven response.

RSV infection is mainly localized to the epithelial tissues in the mucosa and in order to provide effective protection, the local induction of IgA antibodies is necessary prerequisite. Therefore we evaluated the ability of the rRF-412 immunogen to induce a local IgA response after mice were immunized i.n. with rRF-412. Surprisingly, IgA antibody isotype response in saliva was comparatively lower than IgG1, IgG2a and IgG2b isotypes. The salivary IgA response was detected after the first immunization ($P < 0.05$) in mice immunized i.n. with rRF 412, which remained statistically unchanged after the second and third immunizations (Fig. 4e). The rRF-412 immunogen induced higher amounts of mucosal IgA response compared to the RSV group (Fig. 4e). Overall, the antibody results indicate that the rRF-412 immunogen induces both systemic and mucosal antibody responses, which could potentially provide protection against RSV infection.

3.3. rRF-412 immunogen is capable of inducing Th₁/Th₂ mixed immune response

Induction of either a Th₁ or Th₂-mediated immune response plays a very important role in immunity against RSV, and selective induction of one of these immune responses can determine the success or failure of an immunogen. Cytokine ELISA was conducted to determine the production of Th₁ (IFN- γ , IL-2, IL-12p70 and TNF- α) and Th₂ (IL-4, IL-5, and IL-10) cytokines in media- RF-412- and Con A-stimulated spleen cell cultures of mice immunized with rRF-412, RSV and PBS. IFN- γ was produced in RF-412-stimulated cultures of rRF-412 and RSV immunized mice; however, the level produced by cells of rRF-412 mice was significantly more ($P < 0.02$) than the level produced by cells of RSV mice (Fig. 5a). Cells from rRF-412 immunized animals when stimulated with RF-412 antigen produced significantly more IL-2 ($P < 0.001$) than cells from RSV mice did (Fig. 5b). Of interest was the observation that IL-12p70 was spontaneously produced in media-stimulated cultures of only rRF-412 immunized mice. Stimulation of rRF-412 cells with RF-412 antigen caused a

further significant increase in IL-12p70 level ($P < 0.05$); this level of IL-12p70 was equivalent to the significant amount ($P < 0.001$) of this cytokine that was produced by cells from RSV mice (Fig. 5c). Spontaneous production of TNF- α was observed in media only cultures of RSV animals and with significantly more production ($P < 0.04$) in RF-412-stimulated cultures (Fig. 5d). rRF-412 immunized animals did not produce TNF- α . With the exception of IL-12p70, spleen cells from PBS immunized mice did not produce any other cytokine in response to stimulation with RF-412 antigen. Cells from all animals, albeit at different levels produced all Th₁ cytokines (IFN- γ , IL-2 and IL-12p70) in response to Con A (Figs. 5a–c). There was marginal production of TNF- α in Con A-stimulated cultures (Fig. 5d). These results indicate a robust Th₁ cytokine response induced in spleen cells by the rRF-412 antigen.

Analysis of Th₂ related cytokines induced in spleen cells by rRF-412 antigen revealed that cells from mice immunized with rRF-412 did not produce IL-4 and IL-5 (Fig. 6a and b). On the contrary, cells from RSV immunized animals produced significant levels of IL-4 ($P < 0.04$) and IL-5 ($P < 0.05$) (Fig. 6a and b). IL-10 was significantly induced in cell cultures of rRF-412 ($P < 0.05$) and RSV ($P < 0.04$) immunized mice (Fig. 6c). Albeit the level of IL-10 was higher in RSV than rRF-412 immunized mice, this difference was not statistically significant. PBS mice did not produce any detectable levels of the Th₂ cytokines investigated. Cells from all mice produced Th₂ cytokines in response to Con A (Figs. 6a–c). Overall, the rRF-412 immunogen induced a predominant Th₁ than Th₂ response in rRF-412 immunized animals, and more of a mixed Th₁/Th₂ response in RSV immunized animals (Fig. 6d). Therefore, in conjunction with the antibody response data, it can be concluded that the rRF-412 antigen induces a mixed Th₁/Th₂ immune response with a prominent Th₁ response.

3.4. rRF-412 immunogen induces neutralizing antibodies and provides protection

We assessed the capability of serum antibodies to neutralize RSV using the RSV neutralization assay. Our data indicate that the rRF-412 immunogen induced neutralizing serum titer of log₁₀ 2.649 in comparison to PBS group (log₁₀ 0.849) which did not induce significant neutralizing antibodies (Table 3). RSV immunization provided better neutralizing serum antibody titer (log₁₀ 2.709) compared to mice immunized with the rRF-412 immunogen (Table 3). We also measured the RSV titer in lungs of immunized animals that were subsequently challenged with live RSV. Mice immunized with rRF-412 immunogen were significantly protected from a challenged RSV infection, as indicated by the reduced number of PFU compared to the PBS control group of mice. RSV immunized mice were better protected from RSV challenge compared to the rRF-412 immunogen (Table 3). However, our data shows that the rRF-412 immunogen can induce RSV neutralizing antibodies and protect from a RSV challenge infection.

4. Discussion

The development of a RSV vaccine has been a formidable task due to stringent requirements that the candidate vaccine must be able to induce strong systemic, cellular and mucosal immune responses to protect from upper and lower respiratory tract infections. Even more importantly, the candidate immunogen must be able to induce Th₁ immunity since a prominent Th₂ immune response leads to exacerbated disease after RSV exposure [31,32]. Therefore, vaccine development against RSV is focused more on a Th₁ type immune response, which can provide protective immunity and induce a local antibody response. In the present study, we developed a recombinant RSV-F protein in combination with modified CT using a genetic fusion approach. The results from our study clearly demonstrated that this chimeric immunogen induced both Th₁ and Th₂ immune responses, with a prominent Th₁ response in immunized mice.

The genetic fusion approach used in our study avoids the use of adding adjuvants separately to formulate a vaccine; therefore one antigenic molecule is presented to the immune system. This approach is particularly suited for pathogens causing upper respiratory diseases such as RSV where i.n. immunizations can induce local antibody responses. The genetic manipulation is fairly simple and this approach has been utilized for design and development of mucosal vaccines against various pathogens [27]. Despite these benefits, one of the limitations in our expression system was that the fusion protein (RF-412-CTA₂) was expressed as inclusion bodies (insoluble fraction) while CTB subunits were present in the soluble fractions. This resulted in suboptimal levels of required CTB ratio (5CTB:1CTA₂) to form a holotoxin [24]. Therefore, it became necessary to add CTB to achieve the formation of holotoxin. The same problem was encountered in our previous study; it was similarly resolved and resulted in a chimeric immunogen that was effective in inducing protective immune responses [27].

Analysis of serum and salivary antibody responses from rRF-412 immunization of mice revealed that the rRF-412 immunogen is capable of inducing a robust systemic and mucosal immune response. In general, antibody responses in serum were higher compared to the salivary antibody responses. This is consistent with similar studies which also demonstrated that serum antibody responses are higher than other mucosal antibody responses [20,26]. In our studies, mice immunized with RSV showed lower levels of all antibody isotypes; this may be due to our experimental design, which was targeted to test for only RF-412 specific antibody responses rather than total antibody responses to all RSV antigens. In addition, mice were immunized only once with 10⁵ PFU of live RSV to simulate natural infection.

The RF-412-specific IgA response in serum and saliva was significantly high but the salivary IgA response was about one-half of the serum IgA response. This finding is consistent with other reports where mucosal IgA responses in comparison to serum IgA responses were lower when RSV-F in combination with modified CT was administered mucosally [26]. The low mucosal IgA levels observed in our present study was surprising based on the genetic approach used to develop the rRF-412 immunogen. We had expected a high mucosal IgA response based on our previously reported MOMP protein (developed in a similar fashion) that induced comparable amounts of serum and salivary IgA responses [27]. Thus, the ability of the RSV-F protein to induce a local IgA antibody response may be a limiting factor of this immunogen. Several studies [26] and our studies support the notion that the RSV-F can induce significant mucosal IgA response only when used in combination with adjuvants. Interestingly most of these studies employed either CTB or CT as adjuvant. In our present study, we selected modified CTA₂B as a fusion partner and the i.n. vaccination route, because modified CT is safer and stimulates a strong mucosal immune response [22]. Our results clearly demonstrate that the genetically linked RSV-F gene with CTA₂B can be successfully expressed in a bacterial system and when the chimeric antigen is used to immunize mice via i.n. route it can induce strong mucosal as well as systemic immune responses.

The effectiveness of rRF-412 to induce Th₁ and Th₂ immune responses was studied by analyzing indirect evidence of Th₁ (IgG2a) and Th₂ (IgG1, IgG2b) specific antibody isotypes. We found a strong IgG2a response in both serum and saliva samples from mice immunized with rRF-412 antigen. However, we also found significant IgG1 antibody isotype response. The ratios of Th₂/Th₁ antibody isotypes were consistently lower than 1 and in case of salivary antibody responses they ranged from 0.352 to 0.665. Thus, the Th₁-Th₂ antibody response results indicate that recombinant rRF-412 immunogen elicits both Th₁ and Th₂ antibody responses with a prominent Th₁ response. The Th₁/Th₂ mixed immune response may be due to the presence of more than one antigenic site in the RSV-F immunogen. The ability of a single epitope to induce Th₁ as well Th₂ immune response has been reported by others [33]. Induction of a mixed Th₁/Th₂ with a Th₁-biased response to rRF-412 may not be solely linked to the RSV fusion protein. The role of modified CT (CTA₂B), which was part of the recombinant

antigen is a strong possibility. CT and other enterotoxin based adjuvants have been shown to induce Th₁/Th₂ mix immune responses in combination with RSV antigens [34,35].

In order to achieve an effective RSV vaccine, the targeted antigen must drive CD4⁺ or CD8⁺ cells towards induction of prominent Th₁ cytokines. Based on our results, the rRF-412 antigen used in this study elicited a prominent Th₁ response in rRF-412 immunized mice. We observed strong induction of Th₁ cytokines (IFN- γ , IL-2 and IL-12p70) in rRF-412 immunized mice when spleen cells were stimulated with the RF-412 antigen. In fact, spleen cells from rRF-412 immunized mice produced the highest concentration of IFN- γ , a cytokine associated with protective immunity in RSV infection. However, a comparatively low level of the anti-inflammatory cytokine, IL-10, a Th₂ type response was also observed IL-10, as produced by cells from rRF-412 immunized mice, may function to balance the proinflammatory milieu in these mice which is a well-known function ascribed to this cytokine [36]. The inability of spleen cells from rRF-412 immunized mice to produce IL-4, IL-5 or TNF- α cytokines, which are typically associated with immunopathology in RSV [37,38] strongly suggest the attractiveness of rRF-412 as a RSV vaccine candidate.

Our results demonstrated that the rRF-412 immunogen induced a mixed Th₁/Th₂ response in RSV immunized mice. Although several studies have indicated that live RSV immunization leads to a Th₁ response [39] there are a few studies that have indicated that there is a Th₁/Th₂ mixed response from live RSV infection [40] with a favored Th₁ response. This may correlate with the fact that natural RSV infection does not provide complete protection, and this may account for reinfection occurring throughout life. Studies by Joshi et al. [41] indicate that lower level of IFN- γ is produced in babies following natural RSV infection suggesting suppression of Th₁ response.

Although induction of CD4⁺ T cells prominently expressing Th₂ cytokines are responsible for enhanced eosinophilia and exacerbated disease in mice immunized with RSV antigens [37], a mixed Th₁/Th₂ response to antigens as observed in our study, may not lead to exacerbated disease following RSV exposure. One RSV G protein immunogen inducing a prominent Th₂ response was demonstrated not to induce a Th₂ response following RSV challenge [42]. Also, the same antigen administered with DDA adjuvant induced a balanced Th₁/Th₂ immune response and provided protection when tested in mice and cotton rats [43]. The IgA antibody isotype which is necessary to protect the upper respiratory tract from RSV infection is a Th₂ associated antibody since switching to IgA isotype is dependent on the IL-10 cytokine response [44]. Thus IL-10 induction with a prominent Th₁ response should provide several advantages of a desirable vaccine. In our report, induction of a Th₁ prominent cytokine response along with IgA antibody response may be due to the combined effect of the RSV-F subunit linked to the CTA₂B forming a holotoxin like chimera. Bacterial and viral antigens linked to CTA₂B have been shown to enhance IgA production in animals [22]. Other factors such as route of immunization (i.n.) and the importance of selected RSV-F antigenic regions cannot be ignored as the mediators of the enhanced mucosal response observed in our study. In fact, studies by others show that purified F protein along with CT or CTB [6] is effective in inducing protective immune response when administered intranasally. Our studies are comparable to those since rRF-412 antigen provides significant protection and induces serum neutralizing antibodies.

To our knowledge this is the very first report demonstrating that a genetically linked RSV-F gene with CTA₂B (rRF-412) can be successfully expressed in a bacterial system. When the chimeric antigen is used to immunize mice via i.n. route it can induce a strong mucosal as well as systemic immune response and protect animals from RSV challenge. Analysis of indirect Th₁ (IgG2a) and Th₂ (IgG1/IgG2b) antibody responses in serum and saliva in conjunction with analysis of Th₁-Th₂ cytokine profiles (IFN- γ , IL-2, IL-12p70, IL-4, IL-5, IL-10) generated

from stimulated spleen cells indicate a Th₁/Th₂ mixed immune response with a Th₁ favored response. The RSV lung titer and serum antibody neutralization data support this conclusion. We therefore conclude that rRF-412 immunogen is a potential vaccine candidate against RSV.

Acknowledgements

The authors would like to thank Dr. Susan Hollingshead for her kind gift of vector pUAB084. We thank Dr. N. K. Singh for critically reading the manuscript. This work was supported by National Institutes of Health Grants 2S06 GM008219-200012 (to S.R.S.) and 1P20 MD000547 (to S.R.S.).

References

- Domachowske JB, Rosenberg HF. Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment. *Clin Microbiol Rev* 1999;12(2):298–309. [PubMed: 10194461]
- Dudas RA, Karron RA. Respiratory syncytial virus vaccines. *Clin Microbiol Rev* 1998;11(3):430–9. [PubMed: 9665976]
- Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *New Engl J Med* 2005;352(17):1749–57. [PubMed: 15858184]
- Tripp RA, Oshansky C, Alvarez R. Cytokines and respiratory syncytial virus infection. *Proc Am Thorac Soc* 2005;2:147–49. [PubMed: 16113483]
- Lamb, RA.; Kolakofsky, D. Paramyxoviridae: the viruses and their replication. In: Fields, BN.; Knipe, DM.; Howley, PM., editors. *Fundamental Virology*. Philadelphia: Lippincott-Raven; 1996. p. 577–604.
- Walsh EE. Humoral, mucosal and cellular immune response to topical immunization with a subunit respiratory syncytial virus vaccine. *J Infect Dis* 1994;170:345–50. [PubMed: 8035021]
- Munoz FM, Piedra PA, Glenzen WP. Safety and immunogenicity of respiratory syncytial virus purified fusion protein-2 vaccine in pregnant women. *Vaccine* 2003;24:3465–67. [PubMed: 12850361]
- Culley FJ, Pennycook AMJ, Tregoning JS, Hussell T, Openshaw PJM. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. *J Immunol* 2006;80(9):4521–27.
- Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. *J Infect Dis* 2004;190:373–78. [PubMed: 15216475]
- Graham BS, Bunton LA, Wright PF, Karzon DT. Reinfection of mice with respiratory syncytial virus. *J Med Virol* 1991;34:7–13. [PubMed: 1885946]
- Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. *Clin Microbiol Rev* 2001;14:430–45. [PubMed: 11292646]
- Tamura, S-i; Ishihara, K.; Miyata, K.; Aizawa, C.; Kurata, T. Mechanism of enhancement of the immune responses to influenza vaccine with cholera toxin B subunit and a trace amount of holotoxin. *Vaccine* 1995;13(4):339–41. [PubMed: 7793127]
- Elson, CO. Cholera toxin as a mucosal adjuvant. Kiyono, H.; Ogra, PL.; McGhee, JR., editors. *Mucosal Vaccines*: Academic Press; 1996. p. 59-72.
- Tamura S, Samegai Y, Kurata H, Nagamine T, Aizawa C, Kurata T. Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. *Vaccine* 1988;6:409–13. [PubMed: 2848377]
- Verhuel AFM, Versteeg A, DeReuver MJ, Jansze M, Snippe H. Modulation of the immune response to pneumococcal type 14 capsular polysaccharide-protein conjugates by the adjuvant quil A depends on the properties of the conjugates. *Infect Immun* 1989;57:1078–83. [PubMed: 2925240]
- Holmgren J. Actions of cholera toxin and the prevention and treatment of cholera. *Nature* 1981;292:413–17. [PubMed: 7019725]
- Sixma TK, Pronk SE, Kalk KH, Wartna ES, Zanten BAMv, Witholt B, et al. Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E.coli*. *Nature* 1991;351:371–7. [PubMed: 2034287]
- Jobling MG, Holmes RK. Fusion proteins containing the A2 domain of cholera toxin assemble with B polypeptides of cholera toxin to form immunoreactive and functional holotoxin-like chimeras. *Infect Immun* 1992;60(11):4915–24. [PubMed: 1399002]

19. Wright, PF. The rationale for a mucosal approach to the prevention of respiratory syncytial virus-associated pulmonary disease. Kiyono, H.; Ogra, PL.; McGhee, JR., editors. *Mucosal Vaccines*: Academic Press; 1996. p. 295-301.
20. Walsh EE. Mucosal immunization with a subunit respiratory syncytial virus vaccine in mice. *Vaccine* 1993;11:1135–8. [PubMed: 8249433]
21. Lopez JA, Bustos R, Orvell C, Berois M, Arbiza J, Garcia-Barreno B, et al. Antigenic structure of human respiratory syncytial virus fusion glycoprotein. *J Virol* 1998;72(8):6922–8. [PubMed: 9658147]
22. Hajishengallis G, Hollingshead SK, Koga T, Russell MW. Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. *J Immunol* 1995;154:4322–32. [PubMed: 7722290]
23. Collins PL, Huang YT, Wertz GW. Nucleotide sequence of the gene encoding the fusion (F) glycoprotein of human respiratory syncytial virus. *Proc Natl Acad Sci USA* 1984;81:7683–7. [PubMed: 6096849]
24. Hardy SJS, Holmgren J, Johansson S, Sanchez J. Coordinated assembly of multisubunit proteins: Oligomerization of bacterial enterotoxins *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* 1988;85:7109–13. [PubMed: 3050987]
25. Svennerholm AM, Holmgren J. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM₁-ELISA) procedure. *Curr Microbiol* 1978;1:19.
26. Tebbey PW, Scheuer CA, Peek JA, Zhu D, LaPierre NA, Green BA, et al. Effective mucosal immunization against respiratory syncytial virus using purified F protein and a genetically detoxified cholera holotoxin, CT-E29H. *Vaccine* 2000;18:2723–34. [PubMed: 10781860]
27. Singh SR, Hulett K, Pillai SR, Dennis VA, Oh MK, Scissum-Gunn K. Mucosal immunization with recombinant MOMP genetically linked with modified cholera toxin confers protection against *Chlamydia trachomatis* infection. *Vaccine* 2006;24:1213–24. [PubMed: 16194585]
28. Ganapamo F, Dennis VA, Philipp MT. Differential acquired immune responsiveness to bacterial lipoproteins in Lyme disease-resistant and -susceptible mouse strains. *Eur J Immunol* 2003;33:1934–40. [PubMed: 12884859]
29. Sastre P, Melero JA, Garcia-Barreno B, Palomo C. Comparison of antibodies directed against human respiratory syncytial virus antigens present in two commercial preparations of human immunoglobulins with different neutralizing activities. *Vaccine* 2004;23:435–43. [PubMed: 15530691]
30. Durbin JE, Johnson TR, Durbin RK, Mertz SE, Morotti RA, Peebles RS, et al. The role of IFN in respiratory syncytial virus pathogenesis. *J Immunol* 2002;168:2944–52. [PubMed: 11884466]
31. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *J Virol* 1996;70:2852–60. [PubMed: 8627759]
32. Graham BS, Henderson GS, Tang Y-W, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. *J Immunol* 1993;151:2032–40. [PubMed: 8345194]
33. Varga SM, Wissinger EL, Braciale TJ. The attachment (G) glycoprotein of respiratory syncytial virus contains a single immunodominant epitope that elicits both Th1 and Th2 CD4+ T cell responses. *J Immunol* 2000;165:6487–95. [PubMed: 11086089]
34. Lycke N. The mechanism of cholera toxin adjuvanticity. *Res Immunol* 1997;148:504–20. [PubMed: 9588829]
35. Simmons C, Hussell T, Sparer T, Walzl G, Openshaw P, Dougan G. Mucosal delivery of a respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits protective, immunopathogenic and immunoregulatory antiviral CD8+ T cell responses. *J Immunol* 2001;166(2):1106–13. [PubMed: 11145691]
36. Donnelly RP, Dickensheets H, Finbloom DS. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J Interferon Cytokine Res* 1999;19:563–73. [PubMed: 10433356]

37. Hussell T, Baldwin CJ, O'Garra A, Openshaw P. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. *Eur J Immunol* 1997;27:3341–49. [PubMed: 9464822]
38. Walzl G, Matthews S, Kendall S, Gutierrez-Ramos JC, Coyle AJ, Openshaw PJ, et al. Inhibition of T1/ST2 during respiratory syncytial virus infection prevents T helper cell type 2 (Th2)- but not Th1-driven immunopathology. *J Exp Med* 2001;193:785–92. [PubMed: 11283151]
39. Openshaw PJM. Immunity and immunopathology to respiratory syncytial virus. *Am J Respir Crit Care Med* 1995;152:559–62.
40. Tripp RA, Moore D, Barskey A, Jones L, Moscattiello C, Keyserling H, et al. Peripheral blood mononuclear cells from infants hospitalized because of respiratory syncytial virus infection express T helper-1 and T helper-2 cytokines and CC chemokine messenger RNA. *J Infect Dis* 2002;185:1388–94. [PubMed: 11992272]
41. Joshi P, Shaw A, Kakakios A, Isaacs D. Interferon-gamma levels in nasopharyngeal secretions of infants with respiratory syncytial virus and other respiratory viral infections. *Clin Exp Immunol* 2003;131:143–47. [PubMed: 12519398]
42. Corvaia N, Tournier P, Nguyen TN, Haeuw JF, Power UF, Binz H, et al. Challenge of BALB/c mice with respiratory syncytial virus does not enhance the Th2 pathway induced after immunization with a recombinant G fusion protein, BBG2NA, in aluminum hydroxide. *J Infect Dis* 1997;176:560–69. [PubMed: 9291300]
43. Klinguer-Hamour C, Libon C, Plotnicky-Gilquin H, Bussat M-C, Revy L, Ngueyen T, et al. DDA adjuvant induces a mixed Th1/Th2 immune response when associated with BBG2NA, a respiratory syncytial virus potential vaccine. *Vaccine* 2002;20:2743–51. [PubMed: 12034101]
44. Defrance T, Vanbervliet B, Briere F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* 1992;175:671–82. [PubMed: 1371300]

a **b** **Fig 1.**

Genetic construct of pASU-RSF vector and expression of fusion genes. A. An RSV F gene fragment corresponding to regions 412–524 residues of the F protein were amplified by RT-PCR and ligated into pUAB084 vector at 5' of the *ctxA₂B* gene between *NcoI* and *SalI* restriction sites and the pASU-RSF vector was generated. B. Diagram showing expression of the RSVF-CTA₂B fusion protein and association of CTB pentamer with fusion protein to form a holotoxin-like chimera.

Fig 2.

Detection of recombinant protein expressed from vector pASU-RSF. A. The pASU-RSF clone was expressed and cytoplasmic extracts (CE), and purified protein (RF-412) were analyzed on a 10–20% linear gradient after staining with Coomassie blue (1%). Lane 1, MW; lane 2, pUAB084 vector CE; lane 3, RF-412 CE; lane 4, purified RF-412. B. Purified RF-412 was transferred onto a PVDF membrane and probed with anti-RSV polyclonal antibody. Lane 1, MagicMark XP Western Protein Standard; lane 2, RF-412.

Fig 3.

Systemic antibody response to recombinant protein, rRF-412. Serum samples were collected at two weeks interval after mice were immunized with rRF 412, PBS and live RSV via the i.n. route. RSV F specific antibody isotypes IgG (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgA (E) were determined by ELISA. Results are presented as an average of data from three groups in triplicate; error bars represent standard deviation. *, significantly different ($P < 0.05$) from PBS group; **, significantly different ($P < 0.05$) from PBS and RSV groups. P values were calculated by use of Student's t -test.

Fig 4.

Mucosal antibody response to recombinant protein, rRF-412. Saliva samples were collected at two weeks interval after mice were immunized with rRF 412, PBS and live RSV via the i.n. route. RSV F specific antibody isotypes IgG (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgA (E) were determined by ELISA. Results are presented as an average of data from three groups in triplicate; error bars represent standard deviation. *, significantly different ($P < 0.05$) from PBS group; **, significantly different ($P < 0.05$) from PBS and RSV groups. P values were calculated by use of Student's t -test.

Fig 5.

RF-412-induced Th₁ cytokine production in spleen cells from immunized mice. Spleen cells (3×10^6) from PBS, rRF-412 and RSV immunized mice were stimulated with RF-412 or with Con A for 24 hr (TNF- α) or 48 hr (IFN- γ , IL-2, IL-12p70). All cultures were set up in duplicate. A sandwich ELISA was performed in duplicate for cytokine detection. The lower limit of detection of the ELISA was respectively, 30 pg/ml, 3 pg/ml, 15 pg/ml and 20 pg/ml for IFN- γ (A), IL-2 (B), IL-12p70 (C) and TNF- α (D). Each bar, which is the mean $\pm$ standard error of duplicate determinations, represents the concentration of cytokine in the culture supernatant of spleen cells from three mice per group. *, significantly different from values of cells incubated in media alone ($P < 0.05$ to 0.0007); **, significantly different from values of cells incubated in media alone, and the PBS and RSV groups ($P < 0.1$ to 0.003). P values were calculated by use of Student's t -test. Cytokine determinations were repeated at least twice for each duplicate culture.

Fig 6.

RF-412-induced Th₂ cytokine production in spleen cells from immunized mice. Spleen cells (3×10^6) from PBS, rRF-412 and RSV immunized mice were stimulated with RF-412 or with Con A for 48 hr (IL-4, IL-5, IL-10). All cultures were set up and analyzed as described for Th₁ cytokine production in figure 5. The lower limit of detection of the ELISA was respectively, 7.8 pg/ml, 15 pg/ml, 30 pg/ml for IL-4 (A), IL-5 (B) and IL-10 (C). *, significantly different from values of cells incubated in media alone ($P < 0.05$ to 0.02); **, significantly different from values of cells incubated in media alone, and the PBS and rRF-412 groups ($P < 0.05$ to 0.04). ***, significantly different from values of cells incubated in media alone, and the PBS group ($P < 0.02$). P values were calculated by use of Student's t -test. (D), comparison of Th₁ (IFN- γ , IL-2, IL-12, X axis) versus Th₂ cytokine (IL-4, IL-5, IL-10, Y axis) production in immunized mice. Cytokine determinations were repeated at least twice for each duplicate culture.

Table 1G_{M1}-ELISA absorbance values^a

[[Antigen]]	[[PBS]]	[[CTB]]	[[reassociated rRF-412]]	[[Unassociated RF-412]]
[[[]]]				
[[Antibodies]]	anti-CTB anti-RSV	[[anti-CTB]]	[[anti-RSV]]	[[anti-RSV]]
[[[]]]				
[[Absorbance]] ^b	[[0.0805±0.044]]	[[2.796±0.083]]	[[3.76±0.08]]	[[0.061±0.001]]

^a 96-well plates were coated with G_{M1} gangliosides and PBS, CTB, reassociated and unassociated RF-412 samples were added to wells and indicated primary antibodies were used to detect the bound antigens as described in the methods.

^b Absorbance was read at 450 nm. Absorbance values represent four replicates with Standard Error.

Table 2IgG1/IgG2a and IgG2b/IgG2a ratios of serum and saliva antibody response^a

[[[]]]	Serum		Saliva	
	[[IgG1/IgG2a]]	[[IgG2b/IgG2a]]	[[IgG1/IgG2a]]	[[IgG2b/IgG2a]]
[[Day 14]]	[[0.854±0.046]]	[[0.783±0.096]]	[[0.665±0.073]]	[[0.536±0.040]]
[[Day 28]]	[[0.864± 0.119]]	[[0.839±0.292]]	[[0.486 ±0.104]]	[[0.352±0.069]]
[[Day 49]]	[[0.912± 0.149]]	[[0.777±0.186]]	[[0.478± 0.056]]	[[0.491±0.092]]

^aSera and saliva samples from mice immunized with rRF-412 were used to determine the antibody isotype and their titers as described in the materials and methods section. Mean values from IgG1, IgG2a and IgG2b were used to calculate the ratios as indicated. Values represent at least 6 replicates with Standard Error.

Table 3
Serum neutralization titer^a and lung RSV titer^b (log₁₀)

[[Antigen]]	[[Serum Neutralization titer]]	[[Lung titer]]
[[rRF-412]]	[[2.649± 0.092]]	[[3.823±0.072]]
[[RSV]]	[[2.709± 0.095]]	[[3.585± 0.073]]
[[PBS]]	[[0.849± 0.086]]	[[4.836± 0.001]]

^aSera from all immunized mice at day 49 were used to determine the RSV neutralization titer as described in the methods.

^bLung samples from mice immunized with indicated antigens and subsequently challenged with RSV (10⁵ PFU) were collected and used to collect RSV. RSV titer was determined as described in the methods. Data are presented from at least 6 replicates with Standard Error.