Naked DNA immunization with full-length attachment gene of human respiratory syncytial virus induces safe and protective immune response

M. E. HAMAD¹, H. M. AMER¹,², M. A. FARRAG¹, A. H. OSMAN³, F. N. ALMAJHDI¹*

¹Department of Botany and Microbiology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia; ²Department of Virology, ³Department of Pathology, Faculty of Veterinary Medicine, Cairo University, 11221 Giza, Egypt

Received January 30, 2017; revised September 13, 2017; accepted March 28, 2018

Summary. – Development of potent vaccine for human respiratory syncytial virus (HRSV) that confers better protection than natural infection remains a global challenge. Vaccination with naked DNA is considered successful approach for the control of many viral diseases. In this study, the potential of DNA vaccination using full-length attachment gene of HRSV type A Saudi strain cloned in pcDNA3.1+ vector (pcDNA/GA) was evaluated in BALB/c mice. The expression efficiency of pcDNA/GA was first confirmed in HEp-2 cells on RNA and protein levels. Mice immunization with either pcDNA/GA or the positive control formalin-inactivated vaccine (FI-RSV) has generated significant serum antibody concentration in ELISA (7.31±0.418 and 9.76±0.006 µg/ml, respectively) with superior neutralizing activity. Similarly, both immunogens evoked robust HRSV-specific CD8+ T-cell response in ELISPOT assay compared to mice immunized with pcDNA3.1+ vector or saline (negative controls). Challenge of the immunized mice with the wild-type HRSV did not provoke clinical symptoms or mortality in any mice group. On the 7th day post-challenge, mice were euthanized and lungs were extirpated for evaluation of viral load, histopathological changes and cytokine profile. A significant diminish in the viral load and histology score were concluded in lungs of pcDNA/GA immunized mice compared to those immunized with FI-RSV and negative controls. The pulmonary cytokine profile of pcDNA/GA immunized mice displayed notable upregulation of Th1-associated cytokines while that of FI-RSV immunized mice exhibited high levels of Th2-associated cytokines. In conclusion, the DNA vaccine candidate pcDNA/GA has proven prominent efficacy and safety in mouse model, which encourages further evaluation in clinical trials.

Keywords: attachment glycoprotein; challenge; DNA vaccination; human respiratory syncytial virus; mice immunization; T-helper biased response

Introduction

Human respiratory syncytial virus (HRSV) is a common cause of acute lower respiratory tract infection (ALRTI) manifested by bronchiolitis, pneumonia and chronic obstructive pulmonary disease (COPD) in infants and young children (Polack, 2015). It has been estimated that 64 million children are suffering from HRSV-associated ARTIs annually worldwide (Tregoning and Schwarze, 2010). More severe lower respiratory tract infection that requires hospitalization is developed in approximately 3.4 million children with an estimated mortality of 66,000–199,000 each year (Nair et al., 2010). HRSV natural infection does not induce long-lasting immunity, which causes children to undergo multiple infections throughout their lives. HRSV has been identified by WHO as a global disease and is currently on the ‘watching list’ of WHO’s initiative vaccine research group (WHO, 2005).

Vaccination is considered the most effective and economic measure for prevention of a multitude of infectious diseases worldwide (WHO, 2005). To date, no effective HRSV vaccines are commercially available. The development of HRSV
DNA vaccines have emerged during the last two decades as a novel way of vaccination that provides targeted immunogenicity and higher safety. DNA vaccines are easy to construct and stable upon long-term storage. They permit prolonged expression of folded and glycosylated antigens, induce effective antibodies and CD8 T-cells, and generate significant immunological memory (Li et al., 2012; Liu et al., 2006). The ability of DNA vaccines to induce Th1 biased immune response and to overcome the inhibitory effects of maternal antibodies highlights the promise of DNA vaccine in developing effective HRSV vaccine candidates (Wu et al., 2009). Several studies are ongoing and animal/clinical studies are still under investigation (Stab et al., 2013; Wu et al., 2009; Xu et al., 2014).

The attachment (G) glycoprotein is the major transmembrane spike of HRSV that mediates virus adsorption to host cell (Ghildyal et al., 2005). Recombinant G glycoprotein produced in Escherichia coli either alone (BBG2Na) or in combination with fusion (F) and matrix (M2) proteins (rRFM2G) was immunogenic and protected mice and cotton rats from HRSV infection (Goestch et al., 2000; Power et al., 1997; Subbarayan et al., 2010). Recombinant influenza, Sendai and Newcastle disease viruses that express chimeric HRSV G protein on their surfaces were able to elicit high levels of neutralizing antibodies and significantly decreased the lung viral load in immunized animals (Jones et al., 2012; McGinness et al., 2011; Quan et al., 2011). Albeit immunogenic in BALB/c mice, utilization of G protein in the form of adjuvanted subunit or viral vectored vaccines was not recommended due to several safety considerations (Bembridge et al., 1998; Sperer et al., 1998).

In the current report, the potential of DNA vaccination using full-length HRSV G gene for disease control in infants and young children was investigated in a mouse model. The generated vaccine candidate has elicited potent antibody and cytotoxic T lymphocytes (CTL) responses and confirmed pulmonary Th1 biased immune response post-challenge. These preliminary data suggested the compatibility of the DNA vaccine candidate for further testing in non-human primates and in clinical trials.

**Materials and Methods**

*Virus and cells.** HRSV-A strain Riyadh 38/2008 was isolated and characterized from a clinical specimen obtained from a 6-month-old baby hospitalized in a primary care center in Riyadh (Almajhdi et al., 2014). Human epidermoid carcinoma (HeP-2) cells were maintained in Dulbecco Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Thermo Scientific, USA) at 37°C and 5% CO₂. The virus was propagated in HeP-2 cells for 3 successive passages and a high-titer stock of 10⁶ TCID₅₀/ml was aliquoted and stored at -80°C until use.

*Construction of recombinant immunization vector.** Viral RNA was extracted using QIAamp viral RNA mini kit and cDNA was synthesized with Sensiscript reverse transcription kit (Qiagen, Germany) according to the manufacturer’s instructions. Full-length G gene sequence (Genbank Acc. No. JX131638) was amplified using GeneAmp High-Fidelity PCR kit (Applied Biosystems, USA) and the primers: HRSV-GAF-HindIII (5’-CC CAAGCTTATGGTCGAGCTCAAA ACAAAGGAC-3’) and HRSV-GAR-EcoRI (5’-CCG GATATTCTATTTGTTGTGTTGGA TG-3’). The amplified PCR product was agarose gel-purified using QIAquick gel extraction kit (Qiagen) and was cloned into the immunization vector pcDNA3.1+ (Thermo Scientific) via 5’ HindIII and 3’ EcoRI. The recombinant plasmid clone (designated as pcDNA/GA) was verified by restriction endonuclease digestion, PCR, and DNA sequencing. A high concentration of endotoxin-free plasmid stock was prepared using PureYield™ plasmid maxiprep system (Promega, USA).

*Preparation of formalin-inactivated HRSV vaccine (FI-RSV).** FI-RSV vaccine was prepared for use as a positive control in animal experiments as described before (Kim et al., 1969). Briefly, semi-confluent monolayers of HeP-2 cells were infected with HRSV strain Riyadh 38/2008 at MOI of 0.1. Virus adsorption was allowed for 1 h before fresh DMEM with 1% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B were added. After complete development of CPE (3–4 days), cells were subjected to 3 cycles of freezing and thawing, and medium was clarified by centrifugation at 3,000 x g for 10 min (4°C). Formalin was added to the clarified supernatant (1:4,000) and incubated at 37°C for 72 h. Inactivated virus particles were precipitated by ultra-centrifugation at 50,000 x g for 30 min (4°C). The pellet was resuspended in serum free medium (1:25 of the original volume), and aluminum hydroxide (4 mg/ml) was added and incubated overnight for virus adsorption. The mixture was precipitated at 1000 x g for 10 min (4°C) and the pellet was resuspended in serum free medium (1:100 of the original volume) (Waris et al., 1996).
Transfection and expression analysis. In vitro expression of pcDNA/GA was confirmed in HEp-2 cells on both RNA and protein levels. Cells were plated in two separate 6-well plates at a density of 2 x 10^4 cells/ml one day before transfection. Transfection was done with 1 µg/well of the plasmid preparation with the aid of FuGene 6 transfection reagent (Promega). Empty pcDNA3.1+ transfected and non-transfected cells were included as negative controls. Three days later, RNA was extracted from the transfected cells of one plate using RNeasy mini kit (Qiagen) and genomic DNA was eliminated using RNase-free DNase set (Qiagen) according to the manufacturer’s protocol. Amplification of G gene sequence was performed using OneStep RT-PCR kit (Qiagen) and the primers: HRSV-GA-F (5’-TCATT TTGTGTTTG TTGGATGAAGATGG-3’) and HRSV GA-R (5’-TCATT TTGGTTTG TTGGATGAAGATGG-3’). Cells of the second plate were fixed 72 h post-transfection using chilled 80% acetone and were stained with HRSV-specific FITC-conjugated goat polyclonal antibodies (Thermo Scientific). The plate was examined, and images were captured by IN Cell analyzer 2000 (GE Healthcare, UK).

DNA immunization and mice challenge. Six-to-eight weeks old female BALB/c mice were obtained and maintained in SPF conditions with standard feeding at the animal facilities of College of Pharmacy, King Saud University, Riyadh. Mice were divided into 5 groups (12 mice each) and were immunized twice (days 0 and 28). Mice groups were injected intramuscularly (i.m.) in musculus tibialis anterior with 100 µl of pcDNA/GA (50 µg in saline), pcDNA3.1+ (50 µg in saline), FI-RSV (3 x 10^7 TCID_50 equivalent in saline) or saline-alone (negative and mock-infected control groups). Fourteen days after the second immunization, mice of each group were separated into two clusters; one cluster (n = 3) was euthanized for serum and spleen collection, while the other cluster (n = 9) was anesthetized and challenged by intranasal administration of HRSV strain Riyadh 38/2008 (10^6 TCID_50/mouse). Mock-infected control group only received DMEM by the intra-nasal route. Post-challenge, physiological parameters, clinical signs and mortality were daily recorded. All animal experiments were approved by the Research Ethics Committee, King Saud University (code 4/67/352665).

Evaluation of antibody and T cell responses. Mice sera, collected after the second immunization, were screened for HRSV specific antibodies using mouse respiratory syncytial virus IgG ELISA kit (MyBioSource, USA) according to the manufacturer's instructions. The antibody level was measured at wavelength 405 nm by EL x 800 microplate reader (BioTek, USA) and the concentration of antibodies was calculated according to the standard curve generated by six standards included in the kit. Neutralizing antibodies were determined by micro-neutralization assay as described earlier (Graham et al., 1988). The neutralization titer was expressed as the reciprocal of the highest serum dilution that blocked virus infectivity (neutralization units (NU)/ml).

Specific T cell response was assay on mice splenocytes using interferon gamma (IFN-γ) ELISPOT kit (U-CyTech biosciences, Netherlands) according to the manufacturer’s protocol (De Rose et al., 2005). Briefly, splenocytes (2 x 10^6 cells) of each mouse group were seeded in RPMI-1640 medium supplemented with 10% FBS and antibiotics at 48 well plates. One row was left untreated as negative control and a second received concanavalin A (2 µg/well) as positive control. Cells in the other wells were pre-stimulated by a peptide mixture of HRSV-G derived immunodominant CDB+ T cell epitopes (0.5 µg of each peptide/well; purity >98%). The peptides include SNNPTCW AICKRIPNKKP (aa: 177–194), WAICKRIPNKKPG (aa: 183–195), ESNPPSPQVYTTYSEH (aa: 183–197), EGNPPSQVSTTSEY (aa: 271–285) (Thinkpeptide, UK) (Lukens et al., 2006; McDermott et al., 2014; Varga et al., 2000). After 24 hours of incubation at 37°C, cells were transferred to 96-well ELISPOT plate (coated with anti-IFN-γ antibodies) in 3-fold serial dilutions from 1 x 10^3 to 3 x 10^4/well (in triplicates). Incubation with the appropriate stimulus was extended for further 16 h to allow spot formation using biotinylated detection antibodies and GABA solution. Spots were developed using activator solution and examined under an inverted microscope (Eclipse TS100, Nikon, Japan). Spots of negative control were subtracted from the spots in test wells.

Histopathology, virus titration and cytokine profiling of lungs after challenge. Seven days post-challenge, mice were euthanized, and lungs of each group were harvested and divided into three sets (3 mice each); one set was formalin-fixed and prepared in thin sections for staining with hematoxylin and eosin (Murawski et al., 2010). Histopathological examination of lung tissues was conducted in a blind-fashion and images were documented using Eclipse E-800 microscope equipped with DXM1200C digital camera (Nikon, Japan). Scoring of the histopathological changes followed the system developed by Hwang et al. (2014) that ranged from 0 – 3 according to the degree of inflammatory cell infiltration in the peri-bronchiolar space. The second set of lungs was individually homogenized, clarified by centrifugation at 3,000 rpm for 30 min (4°C), and filtered using 0.22 µm syringe filters. The lung virus titers were determined by infectivity assay and were expressed as TCID_50 per gram of lung tissue. The third set of lungs was homogenized in liquid nitrogen and total RNA was isolated from the tissue homogenate using RNeasy mini kit (Qiagen). Pulmonary gene expression of Th1 and Th2 cytokines was evaluated using RT-2 PCR Profiler arrays (Qiagen) on ABI Prism 7500 Sequence detection system (Applied Biosystems). Cycle-threshold (C) values were obtained using a constant baseline for all PCR runs. Five endogenous expression controls provided by the array (Actb, B2m, Gapdh, Gush, and Hsp90ab1) were used to calculate the arithmetic mean which was then set as the C value for normalization. RT-PCR array results were analyzed and heatmaps were generated by Scatter-plots using online RT-2 PCR Profiler data PCR array analysis software (http://www.qiagen.com/sa/shop/genes-and-pathways/data-analysis-center-overview-page). Experimental gene expression was defined as ∆∆Ct and calculations were normalized to the housekeeping genes.

Statistical analysis. The data are presented as means and standard deviations. Difference of means between test and control groups was analyzed using unpaired Student’s t-test and was considered statistically significant when p value is less than 0.05.
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Results

**pcDNA/GA vector expresses HRSV G protein in HEp-2 cells**

The potential of pcDNA/GA vector to express HRSV G protein was first verified in HEp-2 cells before being utilized in immunization studies. Cells transfected with pcDNA/GA, pcDNA3.1+ or DNA-free medium were incubated for 3 days and total RNA was extracted. DNA elimination was further conducted to avoid amplification signals developed from the remnants of transfected plasmid constructs. Distinct RT-PCR amplicons with the expected size of full-length G gene (894 bp) were generated from HEp-2 cells transfected with pcDNA/GA but not from those transfected by pcDNA3.1+ or DNA-free medium (data not shown). On the other hand, the protein expression was assayed by direct immunofluorescence. Only cells transfected with pcDNA/GA have shown diffuse intracellular staining similar to that developed in HRSV infected cells (Fig. 1).

**Immunization with pcDNA/GA elicits high level of antibodies in mice**

Induction of HRSV-specific antibody response in BALB/c mice immunized i.m twice with pcDNA/GA (4-week interval) was measured in sera samples collected 14 days after the second immunization by ELISA. Significant antibody levels were developed in mice immunized with both FI-RSV and pcDNA/GA with a mean antibody concentration of 9.76 µg/ml (SD = 0.006, CV = 0.46%) and 7.31 µg/ml (SD = 0.418, CV = 4.28%), respectively; p value 0.001. No distinct antibodies were recognized in the sera of pcDNA3.1+ immunized and negative control mice (Fig. 2). The neutralizing activity of the raised antibodies was further evaluated by micro-neutralization assay in HEp-2 cells. HRSV-specific antibodies in the sera of mice immunized with FI-RSV and pcDNA/GA were capable to inhibit the virus activity with a mean antibody titer of $3.7 \pm 1.3 \times 10^3$ and $4.8 \pm 1.7 \times 10^3$ NU/ml, respectively. Sera of control mice failed to develop signs of virus neutralization.

**pcDNA/GA evokes robust HRSV-specific CD8+ T cell response**

The immunogenicity of pcDNA/GA vector was further confirmed by measuring IFN-γ secretion of splenocytes *ex vivo* using ELISPOT assay. The assay results were calculated by subtracting the mean number of background
spots developed in the negative control wells (Fig. 3a) from the mean number of spots developed in the test wells (Fig. 3b) and were expressed as the mean spot forming count of T cells (SFC). Concanavalin A was used as a positive control for test validation. pcDNA/GA was able to induce a robust IFN-γ production in response to HRSV-A G-derived peptide with a mean SFC range of 187±6.4 to 25±3.5 at cell densities of 1 x 10^5 and 3 x 10^3 cells/well, respectively. FI-RSV showed potent IFN-γ induction with a mean SFC that ranged from 283±20 to 46±5.2 at cell densities of 1 x 10^5 and 3 x 10^3 cells/well, respectively, while pcDNA3.1+ immunized and negative control mice did not show any results that would differ from the negative control (Fig. 3c).

**Immunization with pcDNA/GA protects challenged mice from disease progression**

The capacity of pcDNA/GA to protect vaccinated subjects was primarily discerned by challenging immunized mice and recording signs of disease progression in terms of mortality rate, weight loss, clinical symptoms, viral load in lungs and pulmonary histopathology. No mortality or significant weight loss was recognized in any of the immunized and control mice groups until the 7th day post-challenge when mice were sacrificed. Similarly, no obvious clinical symptoms were observed.

Lungs of sacrificed mice were extirpated after euthanasia and prepared for virus titration. There was no indication of HRSV replication in lungs of pcDNA/GA immunized and mock-infected mice while the virus titer was calculated as 10^{1.40±0.10}, 10^{3.70±0.13}, 10^{3.55±0.09} TCID_{50} per gram of lung tissue in FI-RSV immunized, pcDNA3.1+ immunized and negative control groups, respectively. Histopathological examination of formalin-fixed lung tissues collected from FI-RSV immunized mice showed massive peribronchiolar leukocytic aggregation; mainly lymphocytes and plasma cells, with focal necrosis of bronchiolar epithel (Score-3). Mice immunized with pcDNA 3.1+ vector showed perivascular focal aggregation of mononuclear cells and focal damage of alveolar septa (Score-2). Few peri-bronchiolar lymphocytic aggregations with papillary epithelial cell proliferation in the bronchiolar lumen were observed in lung tissues of pcDNA/GA immunized mice (Score-1). Thickening of the alveolar wall and infiltration by foamy macrophages was also noticed. No signs of inflammation were observed in mock-infected group (Score-0) (Fig. 4).
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Fig. 4

Pulmonary histopathology of immunized mice post-challenge

(a) Lung section of mock-infected group showing no infiltration of inflammatory cells. (b) Focal aggregation of mononuclear cells and damage of alveolar septa in lung section of pcDNA 3.1+ immunized group. (c) Few lymphocytic infiltrations in the peri-bronchial spaces with polypoids in the bronchiolar epithel and thickening of the alveolar walls in lung tissue of pcDNA/GA immunized mice. (d) Extensive infiltration of inflammatory cells in the peri-bronchial space and focal necrosis of the epithelial lining in lungs of FI-RSV immunized group. Sections were stained with hematoxylin and eosin. Magnification 200x.

Cytokine analysis predicts effective and safe response in pcDNA/GA immunized mice

The ability of pcDNA/GA to induce safe immune reaction that does not involve Th2 biased response in lungs after challenge was evaluated using RT²-PCR Profiler array. The array determines the gene expression profile of 84 mouse-specific antiviral receptors, mediators and signaling components. Fold-regulation changes derived by ΔΔCt were calculated in relevance to the positive control group (FI-RSV immunized mice). Genes with at least two-fold change were only considered for analysis. Comparing data of both challenged mice groups showed that pcDNA/GA immunized mice displayed upregulation in interleukin 2 (IL-2), granulocyte macrophage colony stimulating factor (GM-CSF), toll-like receptor 6 (TLR6), B-cell leukemia/lymphoma 6 (BCL6), CCAAT/enhancer binding protein beta (CEBPB), interleukin 4 receptor alpha (IL-4Ra), Janus kinase 3 (JAK3), Jun-B oncogene (JUNB), nuclear factor of activated T-cells 1 (NFATC1), protein tyrosine phosphatase receptor type C (PTPRC), suppressor of cytokine signaling 3 (SOCS3), signal transducer and activator of transcription 6 (STAT6), tumor necrosis factor receptor superfamily member 4 (TNFRSF4) and tumor necrosis factor ligand superfamily member 4 (TNFSF4) (Fig. 5a,c). In particular, the characteristic markers for Th1 immune response such as IL2, GM-CSF, and TLR6 were over-expressed by 22-, 3- and 2.4-folds, respectively. Conversely, FI-RSV immunized mice exhibited remarkable upregulation of the inflammatory mediators and cytokines: IL-4, IL-5, IL-6, IL-10, IL-13, interleukin 18 receptor 1
(IL-18R1), IL-25, IL-27, IL-27Ra, GATA binding protein 3 (GATA3), chemokine C-X-C motif receptor 3 (CXCR3), chemokine C-C motif receptor 4 (CCR4), chemokine C-C motif ligand 5 (CCL5), CCL7, CCL11, and SOCS5 (Fig. 5b,d). Th2 biased response was obvious in FI-RSV immunized mice group and was manifested by over-expression of IL-5, IL-6, IL-13, IL-25, IL-27, CCR4 cytokines with a fold increase ranging from 16 (IL-27) to 550 (IL-5).

Discussion

Protection of infants and young children from the repeated HRSV infections is still a worldwide concern. Failure of the early vaccination trials using FI-RSV in children at the 1960s has hampered the progress toward developing alternative vaccine candidates for decades. As a rule of thumb, based on several animal experiments and clinical trials
(reviewed by Anderson, (2013), an effective HRSV vaccine should have the following key features: 1) capable to induce protective levels of neutralizing antibodies and CD8+ T cells; 2) stimulate a pattern of CD4+ T cell response similar to that of wild-type virus; 3) induce a balanced Th1/Th2 immune response; 4) would not provoke signs of pulmonary involvement and disease enhancement.

In contrast to FI-RSV, purified peptides and recombinant vaccinia vectors that showed enhancement of pulmonary inflammation and eosinophilia in lungs after virus challenge, live HRSV and naked DNA vaccines induced balanced Th1/Th2 responses with minimal safety constraints (Andersson et al., 2000; Bembridge et al., 2000; Li et al., 2000; Miller et al., 2002). The genetic stability and prolonged expression of the viral immunogens in DNA vaccines make it a promising approach for the development of an effective HRSV vaccine candidate. Previous studies demonstrated that DNA vectors expressing HRSV G or F proteins can provide protection in other animal models like cotton rats and non-human primates essential to verify the extent and duration of immunity in repeated-exposure to challenge virus. Similar studies are also required to recognize the differential response of immunized live virus challenge (Kim et al., 2003; Waris et al., 1996). To evaluate the safety of pcDNA/GA in mice, the cytokine profile of Th1 and Th2 immune responses was evaluated in lungs post-challenge. It was obvious that Th1 – but not Th2 – associated cytokines exhibited significant over-expression as compared to FI-RSV immunized control mice. In particular, the key Th1 associated cytokine IL-2 showed 22-fold increased expression in the lungs of pcDNA/GA immunized mice. On the contrary, a substantial increase (16–550-folds) in the level of Th2 associated cytokines (e.g. IL-4, IL-5, IL-6, IL-10 and IL-13) in lung tissue augments eosinophil recruitment and development of hypersensitivity response following HRSV challenge (Kim et al., 2003; Waris et al., 1996). As outlined earlier, induction of Th1/Th2 balanced immune response and absence of lung immunopathology are two critical identifiers for successful HRSV vaccination. Prior studies have indicated that the over-expression of Th2 associated cytokines (particularly IL-4, IL-5, IL-6, IL-10 and IL-13) in lung tissue augments eosinophil recruitment and development of hypersensitivity response following HRSV challenge (Kim et al., 2003; Waris et al., 1996). To evaluate the safety of pcDNA/GA in mice, the cytokine profile of Th1 and Th2 immune responses was evaluated in lungs post-challenge. It was obvious that Th1 – but not Th2 – associated cytokines exhibited significant over-expression as compared to FI-RSV immunized control mice. In particular, the key Th1 associated cytokine IL-2 showed 22-fold increased expression in the lungs of pcDNA/GA immunized mice. On the contrary, a substantial increase (16–550-folds) in the level of Th2 associated cytokines (e.g. IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25) was noticeable in lungs of control but not in pcDNA/GA immunized mice. Absence of replicating virus and inflammatory signs in lung tissues of pcDNA/GA immunized mice further confirmed the potential of this construct to prevent disease progression and to avoid lung immunopathology.

Collectively, these results support that immunization with pcDNA/GA does not predispose for enhanced disease in the mouse model. Yet it induces robust humoral and cellular immune responses and provides sufficient protection against live virus challenge in vivo. Additional experiments may be required to recognize the differential response of immunized animals to virus challenge at different time frames and to repeated-exposure to challenge virus. Similar studies are also essential to verify the extent and duration of immunity in other animal models like cotton rats and non-human primates before proceeding to clinical trials in human subjects.

Acknowledgment. This study was supported by the research grant (RGP-VPP), for the Deanship of Scientific Research, King Saud University. The authors would like to thank the members of animal facility unit at College of Pharmacy, King Saud University for their help during animal experiments.

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