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Protective Anti-*Pseudomonas aeruginosa* Humoral and Cellular Mucosal Immunity by AdC7-mediated Expression of the *P. aeruginosa* Protein OprF

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Abstract

Replication-deficient adenoviral (Ad) vectors are an attractive platform for a vaccine against lung infections caused by *Pseudomonas aeruginosa*. Ad vectors based on non-human serotypes have been developed to circumvent the problem of pre-existing anti-Ad immunity in humans. The present study analyzes the anti-*P.aeruginosa* systemic and lung mucosal immunity elicited by a non-human primate-based AdC7 vector expressing the outer membrane protein F (AdC7OprF) of *P. aeruginosa*. Intramuscular immunization of mice with AdC7OprF induced similar levels of serum and mucosal anti-OprF IgG and increased levels of anti-OprF IgA in lung epithelial lining fluid (ELF) compared to immunization with a human serotype Ad5OprF vector ($p>0.05$). OprF-specific INF-γ in splenic T cells stimulated with OprF-pulsed syngeneic splenic dendritic cells (DC) was similar following immunization with AdC7OprF compared to Ad5OprF ($p>0.05$). In contrast, OprF-specific INF-γ responses in lung T cells stimulated with either spleen or lung DC were increased following immunization with AdC7OprF compared to Ad5OprF ($p<0.05$). Interestingly, direct administration of AdC7OprF to the respiratory tract resulted in an increase of OprF-specific IgG in serum, OprF-specific IgG and IgA in lung ELF, and OprF-specific INF-γ in lung T-cells compared to immunization with Ad5OprF, and survival following challenge with a lethal dose of *P. aeruginosa.* These data demonstrate that systemic or lung mucosal immunization with an AdC7-based vaccine vector induces superior pulmonary humoral and cellular anti-transgene immunity compared to immunization with an Ad5-based vector and favors AdC7-based vectors as vaccines to induce lung mucosal immunity.

Introduction

Pulmonary infections with *P. aeruginosa* are a frequent problem for patients with cystic fibrosis, immunodeficiency or bronchiectasis [1;2]. There is no current vaccine available against *P. aeruginosa.* The *P. aeruginosa* OprF protein, a major outer membrane protein that is surface exposed and antigenically conserved in various strains of *P. aeruginosa*, is a promising antigen for a vaccine [3]. Antibodies against OprF are associated with protection

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in animal models [4-7] and are present following immunization in humans [8-14]. Expression of OprF by a human serotype Ad5 vector induces anti-OprF humoral and cellular immunity and provides protection pulmonary infections with *P. aeruginosa* in mice [15].

Ad vectors are attractive platforms as vaccines due to their immunogenicity and their function as adjuvants [16-19]. The most commonly used human Ad serotypes 2 and 5 have the disadvantage of being less effective in the presence of the prevalent anti-human Ad 2 and 5 immunity among many populations [20-24]. Wild-type Ad5 is a ubiquitous pathogen and neutralizing titers are found in up to 50% of the adult US population impeding the efficacy of Ad5 based vaccines [20-24]. Less prevalent human or non-human Ad serotypes, including non-human primate-derived Ad vectors, have become attractive alternatives as Ad-based vaccines [21;25-27;27-36]. Non-human primate Ad serotypes do not circulate in the human population and should therefore not be affected by pre-existing immunity when used as vaccine carries in humans [29;37]. However this concept may not be completely viable for all populations, as neutralizing antibodies against chimpanzee Ad serotypes have been detected in a small percentage of people living in Sub-Saharan Africa [38;39]. Vaccine vectors based on the non-human serotype C3, C68, C6 and C7 can generate potent transgene-specific immune responses [28-30;33;40]. These immune responses can also be boosted by the sequential use of heterologous vectors [33;35;41-43].

The present study analyzes the systemic and airway mucosal immunogenic properties of an AdC7-based vector expressing OprF (AdC7OprF) to induce anti-*P. aeruginosa* protection compared to an Ad5-based vector. Interestingly, while weaker in inducing systemic and mucosal humoral anti-OprF IgG responses initially, immunization with AdC7OprF induced sustained higher levels of mucosal anti-OprF IgA and lung T-cell immunity and also resulted in protection against a lethal pulmonary challenge with *P. aeruginosa*. These data suggest that C7-based Ad vectors have a favorable profile to induce protective and mucosal immunity against a pulmonary pathogen such as *P. aeruginosa*.

Methods

Adenovirus Vectors

The recombinant Ad vectors used in this study are replication-defective E1-, E3- Ad vectors based on the human Ad5 or the chimpanzee AdC7 genome [35;44]. For construction of the AdC7OprF vector, the AdC7 plasmid pPan-GFP (kindly provided by JM Wilson, University of Pennsylvania) was digested with I-CeuI and PI-SceI. For both serotypes, an OprF expression cassette [15] was inserted into the E1 region, containing the human cytomegalovirus intermediate-early enhancer/promoter, the OprF cDNA, and a simian virus 40 poly(A) stop signal. Ad5Null, an Ad5 vector with no transgene [45] or AdC7Null (kindly provided by JM Wilson), an AdC7 vector with the green fluorescent protein cDNA under control of a prokaryotic promoter that does not lead to transgene expression in mammalian cells, were used as controls. The vectors were used on the basis of equal number of particle units (pu) and were propagated and purified as described previously [46;47].

Purification of Recombinant OprF Protein

A recombinant bacterial expression vector (pSUMO-OprF) with an N-terminal His tag was constructed and the recombinant OprF protein was purified as described previously[15]. Briefly, the PCR-amplified OprF gene was cloned into the expression vector pET SUMO (Invitrogen, Carlsbad, CA). The recombinant plasmid pSUMO-His-OprF was transformed into *Escherichia coli* BL21 (DE3), and the recombinant protein was purified by Ni-chelating affinity chromatography under native conditions [15].

Mice

Female C57BL/6 mice, obtained from Taconic Farms (Tarrytown, NY), were housed under specific pathogen-free conditions and used at 6 to 8 wk of age. The mice were immunized with the Ad vectors diluted in 50 μl PBS intramuscularly to the right thigh or intratracheally through a 24G angiocath inserted into the trachea.

Anti-OprF Humoral Responses

Mice were immunized with AdC7OprF, Ad5OprF, Ad5Null or AdC7Null at a doses from 10^9 - 10^{11} pu/animal via the intramuscular or intratracheal route. Serum and lung epithelial lining fluid (ELF), were collected after 4, 8 and 12 wk. Lung ELF was collected by three intratracheal instillations and aspirations of 1 ml PBS, pH 7.4, which was then centrifuged at 6000 rpm at 4° C for 10 min, and the supernatant was stored at −80° C. Anti-OprF total IgG and isotype antibody levels were assessed by ELISA using flat bottomed 96-well EIA/RIA plates (Corning, New York, NY) coated with recombinant OprF (0.5 μg/well in 0.05 M carbonate buffer, pH 7.4). The plates were blocked with 5% dry milk in PBS for 1 h at 23° C and serial serum dilutions were added to each well and incubated for 1 h at 23°C. Following three washes with PBS containing 0.05% Tween (PBS-Tween) a peroxidase-conjugated sheep anti-mouse IgG (Sigma), diluted 1:10,000 in PBS containing 1% dry milk, was added and incubated for 1 h at 23° C. Anti-OprF IgA and IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) were determined using an isotyping kit (Bio-Rad Laboratories, Hercules, CA). Absorbance at 415 nm was measured with a microplate reader (Bio-Rad Laboratories) and the antibody titers were calculated with a log(OD)-log(dilution) interpolation model and a cutoff value equal to 2-fold the absorbance of the background.

OprF-specific Cellular Responses

OprF-specific local and systemic cellular immune responses were assessed in T cells isolated from spleens 7 days following intramuscular and from lungs 8 wk following intratracheal administration of AdC7OprF, Ad5OprF, Ad5Null or AdC7Null (10^{10} pu per animal) using an interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. $CD3^+$T cells were purified from the spleen and lung using CD3 Microbeads (Miltenyi Biotec, Auburn, CA). Dendritic cells (DC) were purified from spleen and lung of naive syngeneic animals by positive selection using anti-CD11c microbeads (Miltenyi Biotec) and two consecutive purifications over MACS LS columns (Miltenyi Biotec) to serve as antigen-presenting cells. Purity for CD3 T cells was >95% and for DC was >90%, as determined by flow cytometry. DC (5×10^6/ml) were incubated for 2 hr with purified recombinant OprF protein (100 μg/ml) in RPMI medium supplemented with 2% fetal calf serum (FCS; HyClone, Logan, UT), 10 mM HEPES, pH 7.5 (Bio-Source International, Camarillo, CA) and 10^5 μM β-mercaptoethanol (Sigma-Aldrich). T cells (2×10^5) were incubated for 48 hr with splenic DC at a ratio of 4:1 with or without recombinant OprF protein on anti-IFN-γ-coated plates (R&D Systems), followed by incubation with the biotinylated anti-IFN-γ antibodies (R&D) for 14 hr at 4□C and with streptavidin-alkaline phosphatase conjugate (R&D) and the 3-amino-9-ethylcarbazole substrate (R&D). Spots were counted by computer-assisted ELISPOT image analysis (Zellnet Consulting, New York, NY).

Respiratory Tract Challenge with *P. aeruginosa*

To assess protective anti-*P. aeruginosa* immunity, mice that had received AdC7OprF, Ad5OprF, Ad5Null or AdC7Null (all at 10^{10} pu/mouse), were challenged after 8 wk with 50 μl of *P. aeruginosa* encapsulated in agar beads (5×10^6 CFU) via the intratracheal route. This model usually results in death of the mice 2-6 days following administration of the beads depending on the growth of the bacteria, which have to be prepared fresh, and the encapsulation efficiency into the agar beads [48;49].All mice were monitored daily for 14

days after the infection. Animals that appeared moribund were sacrificed, and this was recorded as the date of death.

Statistical Analysis

The data are presented as mean ± standard error of the mean. Statistical analyses were performed using ANOVA. Survival evaluation was carried out using Kaplan-Meier analysis. Statistical significance was determined at $p<0.05$.

Results

Systemic Humoral and Protective Immunity Following Intramuscular Immunization

Serum anti-OprF IgG was detected 4, 8 and 12 wk following intramuscular administration of AdC7OprF and Ad5OprF (Supplemental Figure 1). The anti-OprF titers increased with increasing doses. At a dose of 10^{10} pu the titers following immunization with AdC7OprF were initially lower at 4 and 8 wk compared to Ad5OprF ($p<0.05$, both time points), but were similar at 12 wk ($p>0.1$). The dose of 10^{10} pu was used for all subsequent experiments. No anti-OprF antibodies were detected in the serum of mice that had received Ad5Null or AdC7Null at all time points. Analysis of the AdC7OprF-induced IgG isotypes at 8 wk showed that the anti-OprF IgG antibodies were predominantly of the IgG1 and IgG2b isotype, followed by IgG2a and IgG3 (Figure 1B), with lower IgG2a, IgG2b and IgG3 isotype titers compared to Ad5-immunized animals ($p<0.01$ for IgG2b, $p<0.05$ for IgG2a and IgG3).

To evaluate the protective effect of the immunizations with AdC7OprF and Ad5OprF against pulmonary infections with *P. aeruginosa*, mice were challenged with a lethal dose of agar-encapsulated *P. aeruginosa* 8 wk following vector administration. Dose escalation experiments showed dose-dependant protection, with 100% protection of the mice following immunization with Ad5OprF or AdC7OprF at a dose of 10^{11} pu (Supplemental Figure 1B). At an immunization dose of 10^{10} pu/mouse all mice that had received Ad5Null or AdC7Null died within the first 6 days after challenge (Figure 1C). In contrast, mice that had been administered with AdC7OprF or Ad5OprF showed prolonged survival ($p< 0.01$ AdC7OprF and Ad5OprF *vs* Ad5Null and AdC7Null, all comparisons; $p= 0.058$ Ad5OprF *vs* AdC7OprF). These data suggest, that systemic immunization with AdC7OprF induces protective immunity against *P. aeruginosa*, even in the presence of lower total systemic anti-OprF IgG titers compared to Ad5OprF.

AdC7OprF Induces Lung Mucosal Humoral Immunity

To evaluate if the protective anti-*P. aeruginosa* immunity was related to mucosal anti-OprF humoral immune responses, anti-OprF IgG and IgA levels were analyzed in lung ELF 4, 8 and 12 wk following administration of Ad5OprF, AdC7OprF, Ad5Null or AdC7Null. Mucosal anti-OprF IgG titers were higher 4 wk following administration of Ad5OprF compared to AdC7OprF (Figure 2A; $p<0.05$), but were similar at 8 and 12 wk. In contrast, mucosal anti-OprF IgA was higher following administration of AdC7OprF compared to Ad5OprF at all time points ($p<0.05$; Figure 2B). Similar results and statistical significance was observed in the repeat experiments (not shown). This suggests that transgene-specific lung mucosal humoral immunity is increased following immunization with the AdC7-based vector compared to Ad5.

AdC7OprF Induces Systemic and Lung Cellular Immunity after Intramuscular Administration

To evaluate the systemic and lung cellular responses following immunization with AdC7OprF and Ad5OprF vectors, OprF-specific IFN-γ responses in CD3 T cells from

spleen and lung of immunized mice were analyzed. Seven days following administration of Ad5OprF, AdC7OprF, Ad5Null or AdC7Null, lung $CD3^+$ T cells from immunized animals were stimulated with syngeneic pulmonary or splenic DC from naive mice, that were pulsed with recombinant OprF. Both Ad5OprF and AdC7OprF, induced an OprF-specific IFN-γ response in pulmonary $CD3^+$ T cells, which was higher in cells from AdC7OprF-immunized mice stimulated with pulmonary ($p<0.05$; Figure 3A) or splenic DC ($p<0.05$; Figure 3B). The systemic anti-OprF cellular response, determined in splenic $CD3^+$ T cells stimulated with OprF-pulsed splenic DC, showed no difference in the OprF-specific IFN-γ response between cells from Ad5OprF and AdC7OprF immunized mice ($p>0.05$, Figure 3C). IFN-γ levels in $CD3^+$ T cells derived from mice that had received Ad5Null or AdC7Null were low and not different to those in cells from naive control mice (Figure 3A,B,C). This suggests that intramuscular administration of AdC7OprF can induce a higher cellular anti-OprF response in the lung compared to Ad5OprF.

Systemic Humoral Immune Responses Following Lung Mucosal Immunization

Based on the above results that intramuscular administration of AdC7OprF resulted in better lung mucosal immunity against *P. aeruginosa* compared to administration of Ad5OprF, anti-OprF humoral and anti-*P. aeruginosa* protective immunity following direct administration of the vectors to the respiratory tract by intratracheal administration was evaluated. Anti-OprF serum IgG levels were similar 4 wk following administration of AdC7OprF and Ad5OprF ($p>0.05$; Figure 4A). Interestingly, anti-OprF IgG level was higher in the AdC7OprF group after 8 and 12 wk ($p<0.01$ and $p<0.05$, respectively). Anti-OprF titers increased until 8 wk and were sustained up to 12 wk, whereas in the Ad5OprF group titers decreased at 8 wk and reached background levels at 12 wk following administration. No anti-OprF IgG was detected in mice that had received AdC7Null or AdNull at all time points. Similar results and statistical significance was observed in the repeat experiments (not shown). The serum anti-OprF IgG isotype response in the AdC7OprF group at 8 wk was predominantly IgG2b followed by IgG2a, IgG3 and IgG1 (Figure 4B). All IgG isotypes were higher with AdC7OprF compared to Ad5OprF ($p<0.001$ for IgG2a and IgG2b; $p<0.01$ for IgG1 and IgG3).

Mucosal Humoral and Protective Immune Responses Following Lung Mucosal Immunization

Lung mucosal anti-OprF antibodies were determined in the lung ELF 4, 8 and 12 wk following intratracheal administration of AdC7OprF, Ad5OprF, Ad5Null and AdC7Null. Lung mucosal anti-OprF IgG titers were similar 4 wk following intratracheal administration of AdC7OprF and Ad5OprF ($p>0.1$, Figure 5A). Mice immunized with AdC7OprF showed a sustained anti-OprF IgG response up to 12 wk, whereas no anti-OprF IgG titer was detected 8 and 12 wk following administration of Ad5OprF ($p<0.001$, both time points; Figure 5A). Anti-OprF IgA titers were higher following administration of AdC7OprF compared to Ad5OprF at all time points ($p<0.01$ at 4 and 12 wk, $p<0.001$ at 8 wk; Figure 5B), suggesting that, similar to intramuscular administration, intratracheal administration of the AdC7-based vector induces higher mucosal humoral immunity compared to an Ad5-based vector.

To evaluate the protective effect of lung mucosal immunization against a pulmonary challenge with *P. aeruginosa*, AdC7OprF, Ad5OprF, Ad5Null and AdC7Null were administered intratracheally and the mice were challenged with a lethal dose of agar-encapsulated PAO1 after 8 wk. All mice that had received Ad5Null or AdC7Null died within 7 days (Figure 5C). In contrast, mice that had received AdC7OprF or Ad5OprF showed similar prolonged survival ($p<0.01$ AdC7OprF and Ad5OprF *vs* Ad5Null and

AdC7Null, all comparisons; p= 0.023 Ad5OprF *vs* AdC7OprF), although they differed in systemic and mucosal anti-OprF immunity.

AdC7OprF Induces Lung Cellular Immunity Following Lung Mucosal Administration

To address the systemic and lung cellular responses following immunization with AdC7OprF and Ad5OprF vectors, OprF-specific IFN-γ responses in CD3 T cells from lung of immunized mice were analyzed. 8 wk following administration of Ad5OprF, AdC7OprF, Ad5Null or AdC7Null, lung $CD3^+$ T cells from immunized animals were stimulated with syngeneic pulmonary or splenic DC from naive mice, that were pulsed with recombinant OprF. Both Ad5OprF and AdC7OprF, induced an OprF-specific IFN-γ response in pulmonary $CD3^+$ T cells, but the response was higher in cells from AdC7OprF-immunized mice stimulated with pulmonary ($p<0.001$; Figure 6A) or splenic DC ($p<0.001$; Figure 6B). The overall systemic anti-OprF cellular response, determined in splenic $CD3^+$ T cells stimulated with OprF-pulsed splenic DC, was under the detectable level (data not shown). IFN-γ levels in $CD3^+$ T cells derived from mice that had received Ad5Null or AdC7Null were low and not different to those in cells from naive control mice (Figure 6 A and B). This shows that intratracheal administration of AdC7OprF can induce persistent cellular anti-OprF response in the lung. Overall, these data suggest that, as seen for systemic immunization, administration of AdC7OprF to the respiratory tract results in humoral, cellular and protective anti-*P. aeruginosa* immunity and this protective effect may be based on different immune parameters compared to Ad5OprF.

Discussion

A vaccine against pulmonary infections with *P. aeruginosa* is needed for patients at risk, in particular individuals with CF before their lungs become colonized with this pathogen. The present study demonstrates that an AdC7-based vector expressing OprF induces longterm anti-*P. aeruginosa* systemic, mucosal and protective immunity following systemic and airway mucosal immunization. Furthermore, mucosal immunization with AdC7OprF led to sustained high levels of mucosal anti-OprF IgG, IgA and lung T-cell immunity. In comparison, Ad5OprF induces anti-*P. aeruginosa* protective immunity with lower anti-OprF IgA levels after systemic administration or short term mucosal anti-OprF IgG and IgA response after mucosal administration, suggesting that C7-based Ad vectors may use a different immune profile to induce protective and mucosal immunity against *P. aeruginosa*.

Vaccine-induced Protective Immunity Against *P. aeruginosa*

Several *P. aeruginosa* vaccine candidates have been assessed in experimental animals and humans, including sub-cellular fractions, capsule components, purified proteins and recombinant proteins [3;50]. The utility of most of these vaccines has been demonstrated in rodent models of *P. aeruginosa* infection, including the agar-encapsulated bacteria challenge model used in the present study. Humoral immunity seems to be paramount in evoking protection against *P. aeruginosa* in this model [51]. Since *P. aeruginosa* is an extracellular pathogen, humoral immunity induced by a vaccine should prevent infection. OprF is one of the promising *P. aeruginosa* antigen candidates for a vaccine [3] as humoral systemic immunity against OprF is associated with protection in animal models [4-7] and serum antibodies are present following immunization in humans [8-14]. An Ad5 vector expressing the OprF cDNA as transgene and containing an OprF-epitope in the Ad capsid has been shown to provide protective immunity in mice [15]. Both, Ad5OprF and AdC7OprF induced systemic humoral anti-OprF immunity, with Ad5OprF more superior after 4 and 8 wk following intramuscular administration and AdC7OprF more superior after 8 and 12 wk following intratracheal administration at equal doses of 10^{10} particles. This dose was chosen for based on the similar levels at 12 wk following intramuscular immunization and the

antibody response for both viruses at this dose being increased compared to the next lower dose in the dose escalation studies. Although, T cell-mediated anti-*P. aeruginosa* immunity has received less attention in the vaccine development, human T cell proliferation in response to *P. aeruginosa* [51;52]and protective CD4 and CD8 T cell-dependent immunity was achieved in several models [52-54]. Comparing CF patients with and without chronic lung infection suggested that a Th2 type response correlated with infection, implying that a Th1 type response may be more protective [55;56] and that the Th1 cytokine IFN-γ seems to play a important role in the clearance of *P. aeruginosa* infection in the lung [55;56]. OprF-specific IFN-γ responses were detected in spleen and lung T cells 7 days and 8 wk following immunization with both OprF-expressing vectors, with a higher mucosal anti-OprF specific IFN-γ response induced by AdC7OprF.

AdC7-based Vectors as Vaccine Platforms

AdC7 and other primate-derived Ad vectors are closely related to human adenoviruses and share many of their structural and biological characteristics including the capacity to act as a vaccine carrier [29;42]. Humoral responses against transgenes following intramuscular or subcutaneous administration have been lower compared Ad5-based vectors after administration in rodents [28;40;42]. In contrast, following intranasal administration both, simian Ad vector and Ad5 vectors, induce comparable levels of serum and mucosal humoral immunity up to 2 wk [28], as observed in the present study after intratracheal administration up to 4 wk. However, after12 wk both vectors induced equal levels of systemic and mucosal OprF-specific IgG following intramuscular administration. In contrast, a sustained systemic and mucosal OprF-specific IgG level was only present following lung mucosal administration of AdC7OprF and not with Ad5OprF indicating a longterm predominance of mucosal immune induction by a primate-derived Ad vector. Different parameters could play a role including infectivity and stimulation of different target cells or differences in the adjuvant effect of the Ad vectors itself [57;58]. Particularly in the respiratory tract, infectivity and stimulation of various cell types as well as interaction with innate immune molecules such as Toll-like receptors by primate-derived Ad vector has so far not been evaluated and could be at last partially responsible for the prolonged mucosal immune response.

Mucosal Immunity Induced by AdC7

Mucosal surfaces are sites of entry for numerous pathogens and mucosal immune responses, including secretory IgA and cytotoxic T cells play crucial roles in host protection against these pathogens [59-64]. In general, induction of mucosal immunity, especially for the respiratory tract, requires efficient antigen delivery and adjuvant systems, that can target the mucosal immune-inductive sites and appropriately stimulate the innate immune system to generate effective adaptive immunity [64]. Several vaccine strategies, including Ad-based vaccines have been studied as mucosal vaccines [65;66]. Ad-based vaccines, administered by the intramuscular route, have been shown to also induce mucosal immune responses to some extent [67-71]. As adenovirus can invade and replicate in mucosal surfaces of the respiratory and gastrointestinal tract, mucosal immunization may even elicit a more potent immune response. Intranasal administration of Ad-based vaccines generated mucosal immune responses and protective immunity [67;68;72-75]. Transgene-specific humoral and T-cell responses at mucosal sites were detected following intramuscular administration, however at much lower levels compared to the systemic responses [67-71]. In accordance with these studies, systemic administration of AdC7Oprf and Ad5OprF resulted in similar but time dependant systemic humoral and cellular immune responses, but interestingly, mucosal anti-OprF IgA levels were higher following immunization with AdC7OprF. Immunization with AdC7OprF also resulted in increased mucosal T cell-mediated anti-OprF immune response compared to Ad5OprF. This effect was even more pronounced following

administration of AdC7OprF to the respiratory tract, with longterm sustained systemic and mucosal anti-OprF IgG and mucosal anti-OprF IgA compared to Ad5OprF. Previous studies have shown that chimpanzee-based Ad induce similar levels of transgene-specific humoral mucosal immunity compared to Ad5 [28], but these studies were only extended to 2 wk post administration.

Simian Ad vectors stimulate transgene-specific Th1 T cell responses in mice that are similar or slightly superior to those achieved with Ad5 [30;41] based on their increased induction of type I interferons in DC [58]. An even more striking effect was seen when we analyzed the OprF-specific T cell mediated response in the lung. AdC7OprF induced more OprF-specific INF-γ producing lung T cells after intramuscular administration compared to Ad5OprF and similar results were obtained after administration to the respiratory tract (not shown). Although the mucosal anti-OprF immunity, particular the anti-OprF IgA responses, induced by AdC7OprF was higher compared to Ad5OprF, the protective immunity induced by AdC7OprF against a lethal challenge with *P. aeruginosa* was comparable. The protective values of IgA has been demonstrated for a variety of pathogens [76], but has not been studied in detail for Ad-based anti-*P. aeruginosa* vaccines. Further studies are needed to investigate if the prevalent mucosal immune induction by AdC7 vectors may lead to a higher protective outcome in other mucosal pathogen models.

Overall, the favorable immunological mucosal response in mice following immunization with the AdC7 vaccine vector favors the further development of non-human primate based Ad vectors as vaccines to induce protective pulmonary mucosal immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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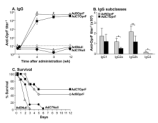

Figure 1.

Systemic humoral and protective anti-OprF immunity following intramuscular immunization with AdC7OprF and Ad5OprF. C57BL/6 mice were immunized with AdC7OprF, Ad5OprF, Ad5Null or AdC7Null at a dose of 10^{10} pu/mouse. **A.** Serum anti-OprF IgG antibodies 0, 4, 8 and 12 wk following administration analyzed by ELISA using recombinant OprF as antigen. **B.** Serum anti-OprF IgG1, IgG2a, IgG2b and IgG3 antibodies 8 wk following administration. Data are shown as mean ± SEM, n=4/group, of one of three representative experiments. The dashed line represents the limit of detection. **C.** Protection against pulmonary challenge with *P. aeruginosa*. Eight wk following immunization mice (n=7/group) were challenged with a lethal intratracheal dose of agar-encapsulated *P. aeruginosa* (10^6 cfu), and survival was monitored for 12 days. * and ** denote significance of $p<0.05$ and ** $p<0.01$, respectively, between AdC7OprF and Ad5OprF.

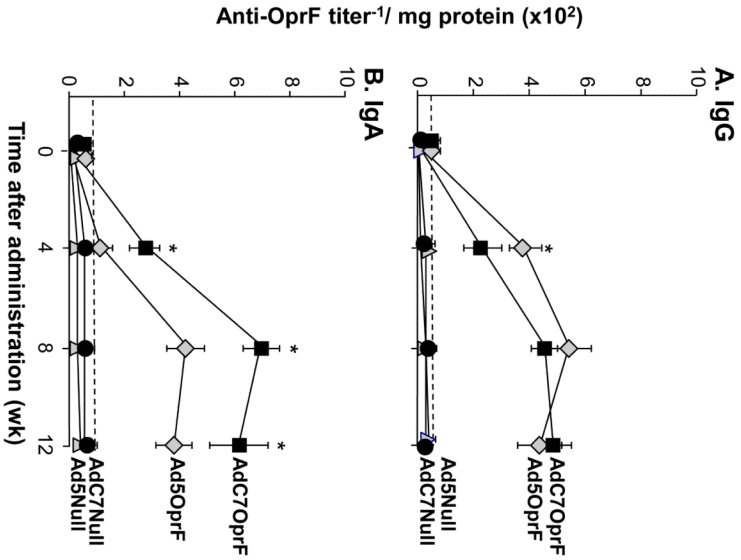

Figure 2.

Mucosal humoral anti-OprF response following intramuscular immunization. C57BL/6 mice were immunized with AdC7OprF, AdOprF, Ad5Null or AdC7Null at a dose of 10^{10} pu/mouse. **A.** Anti-OprF IgG antibodies in the lung epithelial lining fluid 0, 4, 8 and 12 wk following vector administration. **B.** Anti-OprF IgGA antibodies in lung epithelial lining fluid 0, 4, 8 and 12 wk following administration. Data are shown as mean ± SEM, n=4 /group, of one of three representative experiments. The dashed line represents the limit of detection. * denotes significance of $p<0.05$, respectively, between AdC7OprF and Ad5OprF.

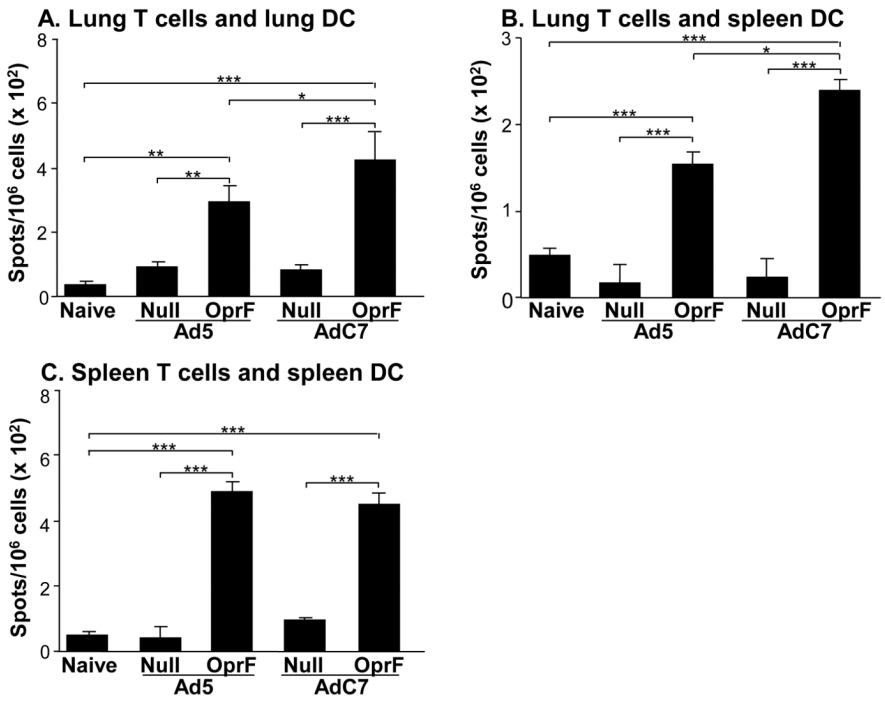

Figure 3.

Systemic and mucosal cellular IFN-γ response to OprF following intramuscular immunization. AdC7OprF, AdOprF, Ad5Null or AdC7Null were administered intramuscularly at a dose of 10^{10} pu/mouse. Seven days following administration, $CD3^+$ T cells were isolated from spleens and lungs and incubated *in vitro* with splenic or lung DC alone or pulsed with recombinant OprF and OprF-specific IFN-γ responses were determined in an ELISPOT assay. **A.** INF-γ in lung $CD3^+$ T cells stimulated with pulsed syngenic pulmonary DC. **B.** INF-γ in lung $CD3^+$ T cells stimulated with pulsed syngenic splenic DC. **C.** INF-γ in splenic CD3 T cells stimulated with pulsed syngenic splenic DC. The data represent the mean of pooled cells from five mice per group (n =5). Values are presented after subtraction of background IFN-γ secretion by corresponding $CD3^+$ T cells induced by DC alone. *, ** and *** denote significance of $p<0.05$, $p<0.01$ and $p<0.001$, respectively

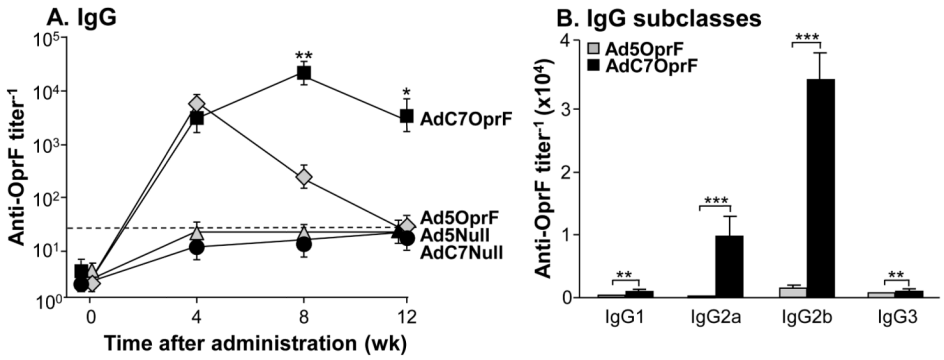

Figure 4.

Systemic anti-OprF humoral response following mucosal immunization. C57BL/6 mice were immunized with AdC7OprF, AdOprF, Ad5Null or AdC7Null at a dose of 10^{10} pu/mouse. **A.** Serum anti-OprF IgG antibodies 0, 4, 8 and 12 wk after administration analyzed by ELISA using recombinant OprF as antigen. **B.** Serum anti-OprF IgG1, IgG2a, IgG2b and IgG3 antibodies 8 wk following administration. Data are shown as mean ± SEM, n=4/group, of one of three representative experiments. The dashed line represents the limit of detection. *, ** and *** denote significance of $p<0.05$, $p<0.01$ and $p<0.001$, respectively, between AdC7OprF and Ad5OprF.

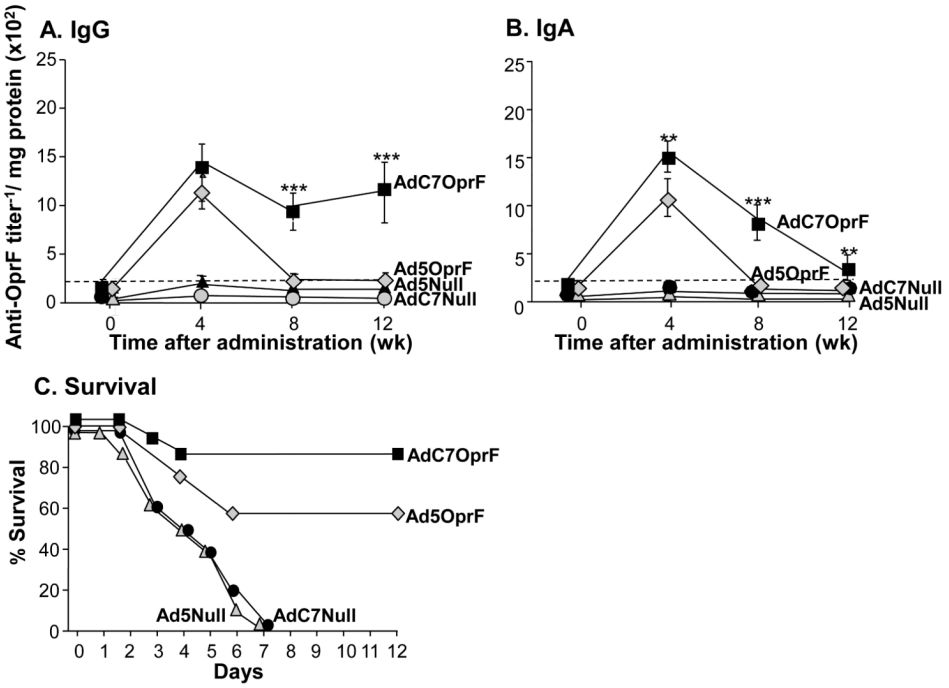

Figure 5.

Mucosal humoral and protective anti-OprF immunity following mucosal immunization. C57BL/6 mice were immunized with AdC7OprF, AdOprF, Ad5Null or AdC7Null at a dose of 10^{10} pu/mouse. **A.** Anti-OprF IgG antibodies in lung epithelial lining fluid 0, 4, 8 and 12 wk following vector administration. **B.** Anti-OprF IgA antibodies in the lung epithelial lining fluid 0, 4, 8 and 12 wk following administration. Data are shown as mean ± SEM, n=4/group, of one of three representative experiments. The dashed line represents the limit of detection. **C.** Protection against pulmonary challenge with *P. aeruginosa* following intratracheal immunization. AdC7OprF, AdOprF, Ad5Null or AdC7Null were administered intratracheally at a dose of 10^{10} pu/mouse. Mice (n=10/group) were challenged 8 wk later with a lethal intratracheal dose of agar-encapsulated *P. aeruginosa* (10^6 cfu), and survival was monitored for 12 days. Shown is a representative of 1 out of 2 experiments. ** and *** denote significance of $p<0.01$ and $p<0.001$, respectively, between AdC7OprF and Ad5OprF.

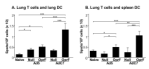

Figure 6.

Mucosal cellular IFN-γ response to OprF following mucosal immunization. AdC7OprF, AdOprF, Ad5Null or AdC7Null were administered intratracheally at a dose of 10^{10} pu/mouse. Eight wk following administration, $CD3^+$ T cells were isolated from lungs and incubated *in vitro* with splenic or lung DC alone or pulsed with recombinant OprF and OprF-specific IFN-γ responses were determined in an ELISPOT assay. **A.** INF-γ in lung $CD3^+$ T cells stimulated with pulsed syngenic pulmonary DC. **B.** INF-γ in lung $CD3^+$ T cells stimulated with pulsed syngenic splenic DC. The data represent the mean of pooled cells from five mice per group (n =5). Values are presented after subtraction of background IFN-γ secretion by corresponding $CD3^+$ T cells induced by DC alone. *, ** and *** denote significance of $p<0.05$, $p<0.01$ and $p<0.001$, respectively.