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Post-Exposure Immunization by Capsid-Modified AdC7 Vector Expressing *Pseudomonas aeruginosa* OprF Clears *P. aeruginosa* Respiratory Infection

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Abstract

Respiratory infections with *Pseudomonas aeruginosa* are major health problems, particularly in patients with cystic fibrosis (CF). No vaccine against *P. aeruginosa* is yet available. A vaccine that controls colonization of the respiratory tract with *P. aeruginosa* could be useful to prevent chronic infection and exacerbations. Replication-deficient adenoviral (Ad) vectors based on non-human serotypes are attractive vaccine platforms as they can circumvent the problem of pre-existing anti-Ad immunity in humans. The primate-based AdC7 vector AdC7OprF.RGD that expresses the outer membrane protein F (OprF) of *P. aeruginosa* (AdC7OprF) and that displays an integrin-binding arginine–glycine–aspartic acid (RGD) sequence is a potent inducer of lung mucosal and protective immunity. Here, we investigated the efficacy of immunization with AdC7OprF.RGD to clear an already established *P. aeruginosa* respiratory infection in mice (wild-type and CF) and rats. Intratracheal administration of the clinical *P. aeruginosa* strain RP73 embedded in agar beads was used to establish persistent infection. Subsequent intranasal immunization with AdC7OprF.RGD induced robust *P. aeruginosa*-specific systemic and mucosal, humoral and cellular immune responses. Importantly, the AdC7OprF.RGD immunized mice effectively cleared *P. aeruginosa* from the lungs. Likewise, immunization with AdC7OprF.RGD to CF mice and Sprague Dawley rats with established *P. aeruginosa* respiratory infection showed enhanced anti-*Pseudomonas* immune responses and increased clearance of *P. aeruginosa* from the lungs. These data suggest that AdC7OprF.RGD can be effective as a post-exposure vaccine and may be useful in clinical settings in particular for patients with CF who frequently harbor the bacteria over prolonged periods.

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Introduction

Pseudomonas aeruginosa, a ubiquitous environmental gram-negative microorganism that is found in soil and aquatic environments, is one of the major opportunistic human pathogens. *P. aeruginosa* can cause chronic infections in the context of damaged or abnormal airway epithelium and compromised local pulmonary clearance mechanisms. Chronic respiratory infections with *P. aeruginosa* are major problems for patients with chronic pulmonary disorders, such as cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease (COPD) [1].

P. aeruginosa frequently acquires antibiotic resistance on top of an intrinsic resistance to various antimicrobial agents that often leads to treatment failures [2]. Therefore, alternate prophylactic and therapeutic approaches are needed. Despite some advances in the preclinical development of prophylactic vaccines against *P. aeruginosa*, a licensed product is not yet available [3]. Several studies have suggested that mucosal immunization can be an effective mode for the management of chronic *P. aeruginosa* respiratory infection [4–8]. Less effort has been focused on the development of a “therapeutic” vaccine that eliminates or reduces pre-existing *P. aeruginosa* burden in the respiratory tract [9].

Replication-deficient adenoviral (Ad) vectors are an attractive mucosal vaccine platform to protect against respiratory pathogens [10–13]. The outer membrane protein F (OprF) of *P. aeruginosa* is a sound vaccine antigen because it has been shown to be immunogenic in various models of acute and chronic lung infections and it is highly conserved [7, 14]. Our previous work identified a human Ad serotype 5 vector expressing OprF or displaying OprF epitopes on the viral capsid as a useful platform for *P. aeruginosa* vaccine [8, 15, 16]. We have also found that a non-human primate-based AdC7 vector expressing OprF was more potent in inducing lung mucosal and protective immunity compared to a human Ad5-based vector [6]. In addition, genetic modification of the AdC7 fiber to display an integrin-binding arginine–glycine–aspartic acid (RGD) sequence can further enhance mucosal protective immunogenicity of AdC7OprF [5]. Furthermore, the non-human origin of the Ad backbone can circumvent pre-existing anti-human Ad immunity which could limit the effectiveness of an Ad vaccine [17].

In this study we investigated the efficacy of AdC7OprF.RGD as a mucosal vaccine against established *P. aeruginosa* respiratory infections. We found that immunization with AdC7OprF.RGD induced robust humoral and cellular immunity against *P. aeruginosa* that resulted in the enhanced clearance of *P. aeruginosa* from the rodent lungs. These data suggest that the activation of OprF-specific mucosal immune response can be a useful therapeutic vaccine strategy for patients with chronic pulmonary disorders who are frequently colonized with *P. aeruginosa*.

Materials and methods

Ethics statement

All animal studies were conducted in accordance to the protocols reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee. All efforts were made to minimize suffering to the animals.

Animals

Female C57BL/6J mice (WT mice), obtained from Jackson Laboratory (Bar Harbor, ME), were housed under specific pathogen-free conditions and were used at 8 weeks of age. CFTR gene knockout mice (Cftr^{tm1UNC}, CF mice) were used at 7–10 weeks of age. Congenic C57BL/6J heterozygous breeding pairs (Cftr^{tm1UNC}) were maintained and continuously bred on regular mouse chow and polyethylene glycol water (GAVIS Pharmaceuticals, NJ) under specific pathogen-free conditions to prevent intestinal obstruction. To maintain congenic status and prevent genetic drift, each new generation of mice was bred to WT C57BL/6J mice and offspring were genotyped at 14 days of age by PCR analysis of tail-clip DNA. Sprague Dawley rats were obtained from Jackson Laboratory and housed under specific pathogen-free conditions and were used at 8 weeks of age.

Adenovirus

The recombinant Ad vectors used in this study are replication-defective E1-, E3- deficient Ad vectors based on the chimpanzee AdC7 genome (kindly provided by JM Wilson, University of Pennsylvania) [5]. AdC7OprF contains the human cytomegalovirus intermediate-early enhancer/promoter, the OprF cDNA, and a simian virus 40 poly(A) stop signal expression cassette in the E1 region as previously described [8]. The RGD peptide was incorporated at the C-terminal of the fiber gene [8]. AdC7Null (kindly provided by JM Wilson), an AdC7 vector that does not lead to transgene expression in mammalian cells, was used as control. The vectors were used on the basis of equal number of particle units (pu) and were propagated, purified and quantified as described previously [18].

Pseudomonas aeruginosa strains

The clinical CF *P. aeruginosa* strain RP73 (CCBJ/EMBL/GenBank accession number CP006245.1), that can be used to establish persistent respiratory infections in mice [19], was kindly provided by Dr. Bragonzi (San Raffaele Scientific Institute, Italy). *P. aeruginosa* embedded in agar beads were used to develop murine models of persistent *P. aeruginosa* lung infection. The embedding of *P. aeruginosa* within agar beads retains the bacteria within the airways and has been developed to model a CF-like environment of microaeriosis and bacterial biofilms. Retention of the bacteria presumably avoids physical elimination and leads to a persistent stimulation of host defenses without accounting for initial bacterial colonization [19]. Therefore, the establishment of infection is almost instant, but also wanes over a few weeks. Agar beads embedded with *P. aeruginosa* were prepared using a modified version of the method described previously [19]. Bacteria from glycerol stocks were streaked for isolation on a tryptic soy agar (TSA) plate and incubated at 37°C overnight. A single colony was used to inoculate 5 ml of tryptic soy broth (TSB). A small aliquot of this

overnight culture was used to inoculate 50 ml of TSB. Bacteria were grown to late log phase in a shaking incubator at 37°C, centrifuged at 2,700g for 15 min, and suspended in 1ml of TSB. The bacterial suspension was embedded into agar beads by mixing with 1.5% (for mice) or 2% (for rats) w/v agar (DIFCO™ AGAR granulated, Beckton & Dickenson) in TSB and was then spun into warmed heavy mineral oil. The mineral oil was rapidly stirred for 6 min at room temperature, then cooled for 4°C for 35 min and on ice for 20 min. The beads were centrifuged at 2,700 g for 15 min and then washed six times with phosphate buffered saline (PBS). Only beads sized between 100µm and 200µm were selected using cell strainers (PluriSelect, San Diego, CA) and stored at 4°C. *P. aeruginosa* embedded agar beads were prepared the day before inoculation. *P. aeruginosa* concentration was measured in an aliquot of homogenized bead slurry by plating on TSA and calculated as colony forming unit (cfu)/ml. The slurry was diluted with PBS to obtain the desired bacterial concentration before inoculation.

Administration of *P. aeruginosa* embedded in agar beads to mice and rats

Wild-type mice were intratracheally injected with 1×10^6 cfu of *P. aeruginosa* embedded in agar beads in 100µl PBS under isoflurane anesthesia. CF mice and rats were injected with 5×10^5 or 5×10^6 cfu of *P. aeruginosa* embedded in agar beads respectively. Control mice received non-bacterial agar beads prepared similarly with sterile PBS. Aliquots of beads preparations were stained with alcian blue, the numbers of the beads were counted under microscope, and approximately same numbers of beads were used for injection. Body weight was recorded daily.

Post-exposure immunization of mice with adenovirus vector

Mice were immunized by intranasal inoculation of AdC7OprF.RGD (10^{10} pu diluted in 50µl PBS) 4 days following administration of *P. aeruginosa*. Control mice received AdC7Null or just PBS intranasally. Rats were immunized with AdC7OprF.RGD or AdC7Null (both at 5×10^{10} pu) 1 day after the administration of *P. aeruginosa* embedded in agar beads. The timing of vaccine administration was chosen based on the recovery of mice or rats from the stress of intratracheal administration (minor surgical procedure).

Anti-OprF humoral immune responses

Serum or lung homogenates were collected to measure anti-OprF humoral immune responses. Whole lungs homogenized in 700µl of PBS were centrifuged at 1000 rpm at 4°C for 10 min, and the supernatants were collected to measure anti-OprF IgA titers in lungs. Anti-OprF total IgG, IgG isotypes, IgM and IgA antibody titers 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) as described [8]. The plates were blocked with 5% dry milk in PBS for 1 h at RT. Serial dilutions of serum or lung homogenate supernatants were added to each well and incubated for 1 h at RT. Following three washes with PBS containing 0.05% Tween20 (PBS-T) 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 RT. Anti-OprF IgG isotypes (IgG1, IgG2a, IgG2b and IgG3), IgM and IgA 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. Serum from the non-infected and non-immunized mice was used to determine the non-specific reaction levels for the assay. For rats, peroxidase-conjugated sheep anti-rat IgG (Sigma; 1:10,000 dilution) was used as secondary antibody to detect anti-OprF IgG in serum by ELISA.

OprF-specific cellular immune responses

OprF-specific cellular immune responses were assessed 2 weeks after the immunization with AdC7OprF.RGD or AdC7Null. Mice splenocytes were isolated as a single-cell suspension and cultured in RPMI medium supplemented with 2% fetal bovine serum (HyClone, Logan, UT), 10 mM HEPES (pH 7.5; Biosource International, Camarillo, CA), and 10 μ M β -mercaptoethanol (Sigma-Aldrich) in 96-well (Millipore) plates. The splenocytes were stimulated with 10 μ g/ml OprF for 48 h and culture supernatants were collected. The mouse Interferon- γ (IFN- γ) and IL-4 were measured in the culture supernatant with specific ELISA kits (eBioscience).

Bacterial loads in lungs

Both the lungs from the sacrificed mice and rats were homogenized in 700 μ l and 3ml of PBS respectively, serially diluted and plated on TSA plates. The concentration of *P. aeruginosa* were determined after 48 h and bacterial load in lung were calculated as cfu per gram lung.

Statistical analysis

The data are presented as mean \pm standard error of the mean (SEM). Statistical analyses were performed using ANOVA followed by Tukey's multiple comparisons or unpaired t test. Statistical significance was determined at $p < 0.05$.

Results

Systemic and mucosal humoral immune responses induced by AdC7OprF.RGD in mice with established *P. aeruginosa* respiratory infection

Mice were infected with *P. aeruginosa* embedded in agar beads to mimic the disease state of chronic lung infection with *P. aeruginosa*. The animals were then immunized with AdC7OprF.RGD, AdC7Null or PBS 4 days after the administration of *P. aeruginosa*. All *P. aeruginosa*-administrated mice lost about 15% of their body weight by day 3 and regained their starting weight by day 10 (Figure S1). Serum anti-OprF IgG was detected in all groups at 1 and 2 weeks after the immunization. Anti-OprF IgG only increased in the AdC7OprF.RGD-immunized mice at 2 weeks after the immunization, but not in the non-immunized and AdC7Null-immunized mice ($p < 0.001$ all comparisons; Figure 1A). All IgG isotypes were higher in AdC7OprF.RGD immunized mice compared to the mice that had received AdC7Null ($p < 0.05$ all comparisons; Figure 1B).

Serum anti-OprF IgM was also detected at 1 week after the immunization in all groups but was higher in the mice that had received AdC7OprF.RGD compared to AdC7Null ($p < 0.001$; Figure 1C). Mucosal anti-OprF IgA titers in lungs 2 weeks following immunization were also higher in the mice that had received AdC7OprF.RGD compared to AdC7Null ($p < 0.05$;

Figure 1D). This suggests that mucosal administration of AdC7OprF.RGD to the mice with established *P. aeruginosa* infection enhances the systemic and mucosal humoral immune responses induced by the initial *P. aeruginosa* infection.

Systemic cellular immune responses induced by AdC7OprF.RGD in mice with established *P. aeruginosa* respiratory infection

In addition to humoral immune responses, cellular immune responses are known to play an important role to clear the *P. aeruginosa* infections [20]. Both OprF-specific IFN- γ and IL-4 in splenocytes from the mice with established *P. aeruginosa* respiratory infection that were immunized with AdC7OprF.RGD were higher compared to AdC7Null or PBS controls ($p < 0.05$, all comparisons; Figure 2). This suggests that both Th1 and Th2 type of cellular immune responses were augmented by immunization with AdC7OprF.RGD in the mice with established *P. aeruginosa* respiratory infection. Additionally, immunization with AdC7OprF.RGD enhanced the expression of IFN- γ and IL-17A in the lung tissue compared to AdC7Null or PBS controls ($p < 0.05$ all comparisons; Figure S3A–B). No significant change in IL-4 mRNA levels was observed in lungs (Figure S3C).

Enhanced clearance of *P. aeruginosa* from the lungs after the post-exposure immunization with AdC7OprF.RGD

To evaluate the efficacy of post-exposure immunization with AdC7OprF.RGD to clear the established *P. aeruginosa* respiratory infection, lung *P. aeruginosa* bacterial loads were quantified at 1 and 2 weeks after the immunization. Compared to the mice that had received AdC7Null or that were not immunized, the mice that had received AdC7OprF.RGD showed decreased bacterial loads at both time points ($p < 0.05$, both comparisons; Figure 3).

Efficacy of post-exposure immunization with AdC7OprF.RGD in CF mice with established *P. aeruginosa* respiratory infection

We investigated the efficacy of post-exposure immunization with AdC7OprF.RGD in CF mice with established *P. aeruginosa* respiratory infection. Although it is not an equivalent model for human CF lung disease, CF mice are more susceptible to *P. aeruginosa* compared to WT mice (Figure S2B). Immunization with AdC7OprF.RGD elicits comparable anti-OprF IgG titers in CF and WT mice (Figure S2A) and protects against a *P. aeruginosa* challenge in both mice (Figure S2B).

To evaluate the efficacy of immunization on the established *P. aeruginosa* respiratory infection in CF mice, AdC7OprF.RGD or AdC7Null were intranasally inoculated to CF mice that had been administrated *P. aeruginosa* embedded in agar beads 4 days prior to the immunization. Ten days after the immunization, the mice that had received AdC7OprF.RGD showed higher levels of serum anti-OprF IgG (Figure 4A) and lower *P. aeruginosa* bacterial loads in lungs (Figure 4B) compared to the mice that had received AdC7Null ($p < 0.0001$, both comparisons). This suggests that post-exposure immunization with AdC7OprF.RGD is effective to enhance clearance of *P. aeruginosa* from the lungs in CF mice.

Efficacy of immunization with AdC7OprF.RGD in rats with established *P. aeruginosa* respiratory infection

To evaluate the efficacy of AdC7OprF.RGD in another rodent species, we established persistent *P. aeruginosa* infections in rat lungs and immunized with AdC7OprF.RGD or AdC7Null 1 day after the administration of *P. aeruginosa* embedded in agar beads. Two weeks after the immunization, the rats that had received AdC7OprF.RGD had higher levels of serum anti-OprF IgG ($p < 0.01$; Figure 5A) and lower *P. aeruginosa* bacterial loads in lungs ($p < 0.05$; Figure 5B) compared to the rats that had received AdC7Null. This suggests that post-exposure immunization with AdC7OprF.RGD is also effective to enhance pulmonary clearance of *P. aeruginosa* in rats.

Discussion

This study introduces a novel application for an Ad-based vaccine platform against established *P. aeruginosa* respiratory infections. Ad-based vaccines against *P. aeruginosa* such as AdC7OprF.RGD have been mostly developed to function in a classic active immunization approach to prevent infections. With the growing realization of the commonality of bacterial colonization in respiratory tracts that has been a particular problem for patients with chronic pulmonary disorders, a successful vaccine strategy for these conditions is also needed to be able to control pre-existing colonization and to prevent bacterial overgrowth leading to clinical exacerbations. Here we tested, for the first time, such a strategy against respiratory infections with *P. aeruginosa* and demonstrated that immunization with AdC7OprF.RGD in rodents with already established *P. aeruginosa* respiratory infection can lead to faster elimination or reduction of the bacteria in the lungs. This can be useful strategy for individuals with CF who are frequently colonized with *P. aeruginosa* at an early age before their lung function declines.

In CF affected individuals, *P. aeruginosa* colonizes in respiratory tract early in life with median age at first infection of 5.5 years, and most adults harbor *P. aeruginosa* in their respiratory tracts [21]. Despite some success of widespread implementation of antibiotic therapy to eradicate initial *P. aeruginosa* acquisition, the occurrence of multi-drug resistant *P. aeruginosa* continues to rise [2, 22, 23]. Therefore, a therapeutic vaccine to clear *P. aeruginosa* colonization would be an attractive strategy. Most therapeutic vaccines have been developed against viruses such as rabies [24], ebola [25, 26], varicella [27], human immunodeficiency virus, hepatitis B and hepatitis C [28]. Bacterial targets have been mainly *Mycobacterium spp*s [29].

We evaluated in the efficacy of post-exposure immunization with AdC7OprF.RGD in wild-type C57BL/6J mice, Cfr^{tm1UNC} mice and Sprague Dawley rats. The exaggerated inflammatory response and increased susceptibility for *P. aeruginosa* that is characteristic for human CF lung disease is partially mimicked in Cfr^{tm1UNC} mice that are backcrossed on a C57BL/6 background [30–32]. Rats were tested to confirm our findings in another rodent model that is commonly used to study pulmonary *P. aeruginosa* infections [33, 34]. Based on this and prior data by us and others we assume that humoral, mucosal and systemic opsonizing immunity are effective to prevent and clear colonization against this extracellular pathogen [20]. T-cell responses have also been shown to mediate protective immunity in

individuals with *P. aeruginosa* infections [35–37]. We chose a potent capsid-modified AdC7 vector, which has robust respiratory mucosal immune stimulatory properties, as a vaccine platform to induce strong humoral and cellular immunity to clear established *P. aeruginosa* respiratory infections. Mucosal immunization with AdC7OprF.RGD induced increased levels of anti-*P. aeruginosa* humoral and cellular immune responses above those that were induced by the pre-existing *P. aeruginosa* respiratory infection in mice. The induction of all 4 subclasses of IgG indicates broad activation of transgene-specific humoral responses: IgG2a and IgG1 suggest induction of both Th1 and Th2 type of immune responses; as IgG1, IgG2, and IgG3 play important roles in opsonization, their induction by the vaccine is important as OprF is a major target for opsonizing antibodies to clear the infection [38, 39]. Increased levels of anti-*P. aeruginosa* humoral immune responses were also observed in CF mice that are characterized by an increased susceptibility to *P. aeruginosa* [30, 40, 41]. However, CF mice do not mimic the severity and other critical mucus characteristics of human CF lung disease. Other CF animal models such as CF pigs or ferrets could be useful to further evaluate the preclinical feasibility of AdC7OprF.RGD as a therapeutic vaccine [40]. While it would have been interesting to evaluate the bacterial lung titers beyond 2 weeks post-immunization, we anticipate that, based on our prior studies with AdC7OprF.RGD [5, 6], to observe a similar or even higher levels of anti-OprF immunity, the waning *P. aeruginosa* load over time would have worked against detection of this stronger effect. The choice of this vaccine vector is supported by the promising potential of adenovirus-based vaccines to induce robust anti-transgene immunity as evident in numerous preclinical and clinical studies against a vast variety of infectious agents [42–44]. AdC7, in addition to circumventing the pre-existing immunity against common human Ad serotypes, induces long-term anti-transgene systemic, mucosal and protective immunity in the respiratory tract, particularly when administered via the mucosal route to the respiratory tract [5, 6, 8]. Although only a few mucosal vaccines have been developed for humans, the importance of mucosal immunization against mucosal pathogens is well recognized [45–49]. Addition of RGD to AdC7 vector further enhances the mucosal humoral immunity and protective efficacy of the vaccine [5]. The strategy of RGD incorporation to the fiber of Ad vectors have been previously applied to improve the vaccine and immunomodulatory cancer therapies [50–52]. The RGD peptide facilitates the infections to the cells expressing high levels of $\alpha v \beta 3$ or $\alpha v \beta 5$ integrins, in particular dendritic cells, which promotes the antigen presentation through both class I and class II pathways. Furthermore, adenovirus-based vaccines are known to favor Th1-biased transgene-specific immune responses [53, 54], which is further enhanced by the addition of the RGD peptide [51]. This could be beneficial in CF where immune responses are often skewed to Th2 [55–57]. Innate immune responses are known to be abnormal in CF [58]. This has also been partially seen in CF mice, which may affect the strength of adaptive immunity induced by vaccines [32, 59]. Interestingly, immunization with AdC7OprF.RGD induced comparable adaptive anti-OprF immunity in the CF mice compared to the WT controls (Figure S2A). Adjuvant properties of Ad vector and the RGD-mediated targeting of dendritic cells might have played a role in induction of these strong adaptive immune responses in the CF mice. In addition to IFN- γ and IL-4, immunization with AdC7OprF.RGD also augmented IL-17A expression, which is a critical component of vaccine-induced protection against pulmonary infection and to control chronic

lung infections with *P. aeruginosa* [60–62]. A more detailed analysis of lung immune cells would be informative and could be subject of future studies.

Overall, our study suggests that AdC7OprF.RGD could be a promising vaccine for patients with chronic pulmonary disorders, especially with colonized *P. aeruginosa* in their lungs or with chronic *P. aeruginosa* respiratory infection with frequent clinical exacerbations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. A vaccine against established *P. aeruginosa* respiratory infections would be helpful.
2. Capsid-modified AdC7 vectors are potent inducers of respiratory mucosal immunity.
3. Immunization with AdC7OprF.RGD against established *P. aeruginosa* lung infections was evaluated.
4. AdC7OprF.RGD immunization induced robust humoral and cellular immunity against *P. aeruginosa*.
5. The induced immunity enhanced clearance of *P. aeruginosa* from rodent lungs.

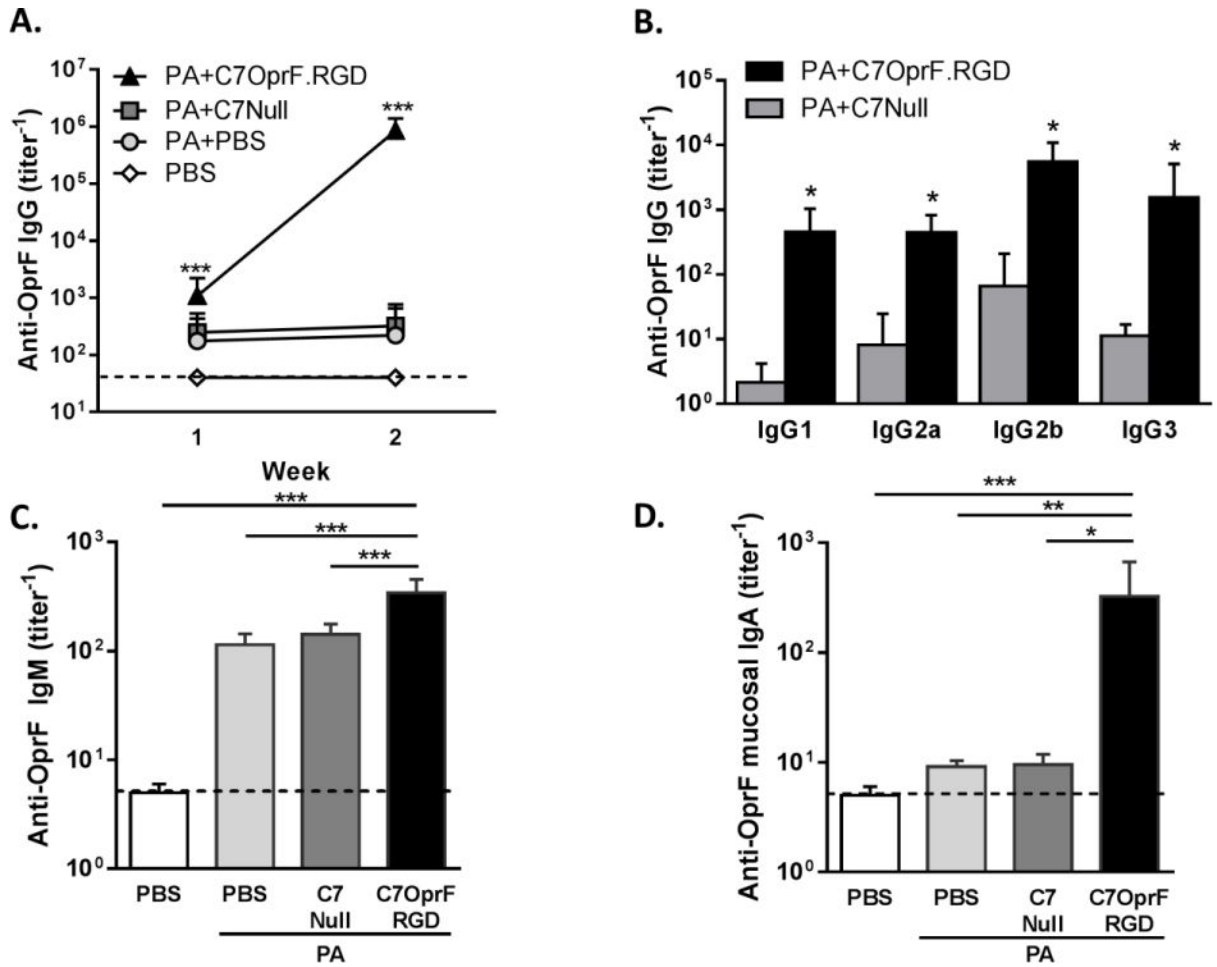


Figure 1. Anti-*P. aeruginosa* humoral immune responses after the immunization with AdC7OprF.RGD in mice with established *P. aeruginosa* respiratory infection

C57BL/6 mice were intratracheally administrated with 1×10^6 cfu of *P. aeruginosa* embedded in agar beads and immunized intranasally with either 1×10^{10} particle units of AdC7OprF.RGD (PA+AdC7OprF.RGD), AdC7Null (PA+AdC7Null) or PBS (PA+PBS) 4 days after the administration of *P. aeruginosa*. Non-infected and non-immunized mice were used as controls (PBS). Anti-OprF antibody titers were determined in serum at 1 and 2 weeks after the immunization and in lung homogenate supernatants at 2 weeks after the immunization by ELISA. **A.** Serum anti-OprF IgG (1 wk and 2 wk). **B.** Serum anti-OprF IgG isotypes (2 wk). **C.** Serum anti-OprF IgM (1 wk). **D.** Anti-OprF IgA in lung homogenate supernatants (2wk). Data are presented as mean \pm SEM of $n=7-8$ mice per group. *, ** and *** denote significance of $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

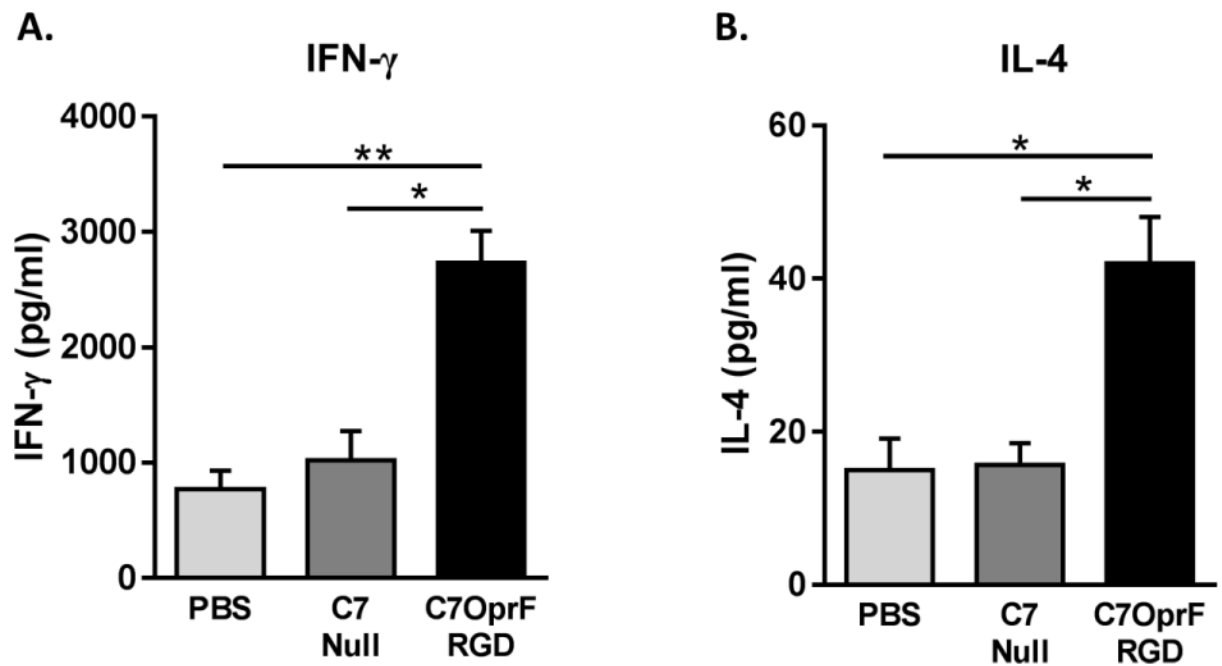


Figure 2. Cellular immune responses after the immunization with AdC7OprF.RGD in mice with established *P. aeruginosa* respiratory infection

C57BL/6 mice were immunized intranasally with either 1×10^{10} particle units of AdC7OprF.RGD, AdC7Null or PBS 4 days after the administration of *P. aeruginosa* embedded in agar beads. Two weeks after the immunization, the splenocytes were collected, cultured and stimulated with OprF protein for 48 h. **A.** IFN- γ and **B.** IL-4 levels were measured in the culture supernatants by ELISA. Data are presented as mean \pm SEM of n=8 mice per group. * and ** denote significance of $p < 0.05$ and $p < 0.01$ respectively.

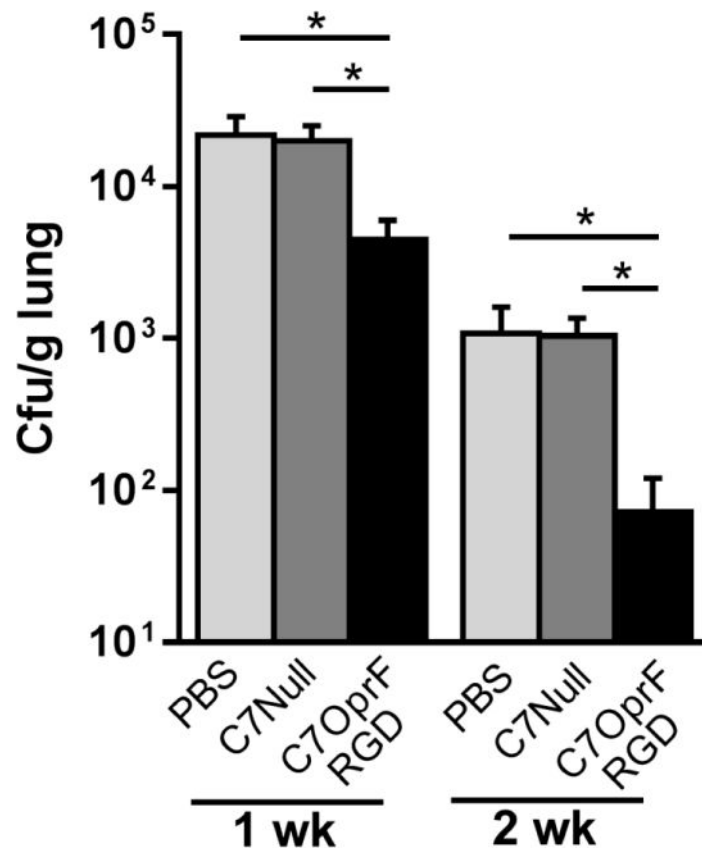


Figure 3. Clearance of *P. aeruginosa* from lungs after the immunization with AdC7OprF.RGD C57BL/6 mice were immunized intranasally with either 1×10^{10} particle units of AdC7OprF.RGD, AdC7Null or PBS 4 days after the administration of *P. aeruginosa* embedded in agar beads. *P. aeruginosa* bacterial loads were determined in lung homogenates 1 and 2 weeks after the immunization. Shown are cfu per gram lung tissue. Data are presented as mean \pm SEM of $n=7-8$ mice per group. * denotes significance of $p < 0.05$.

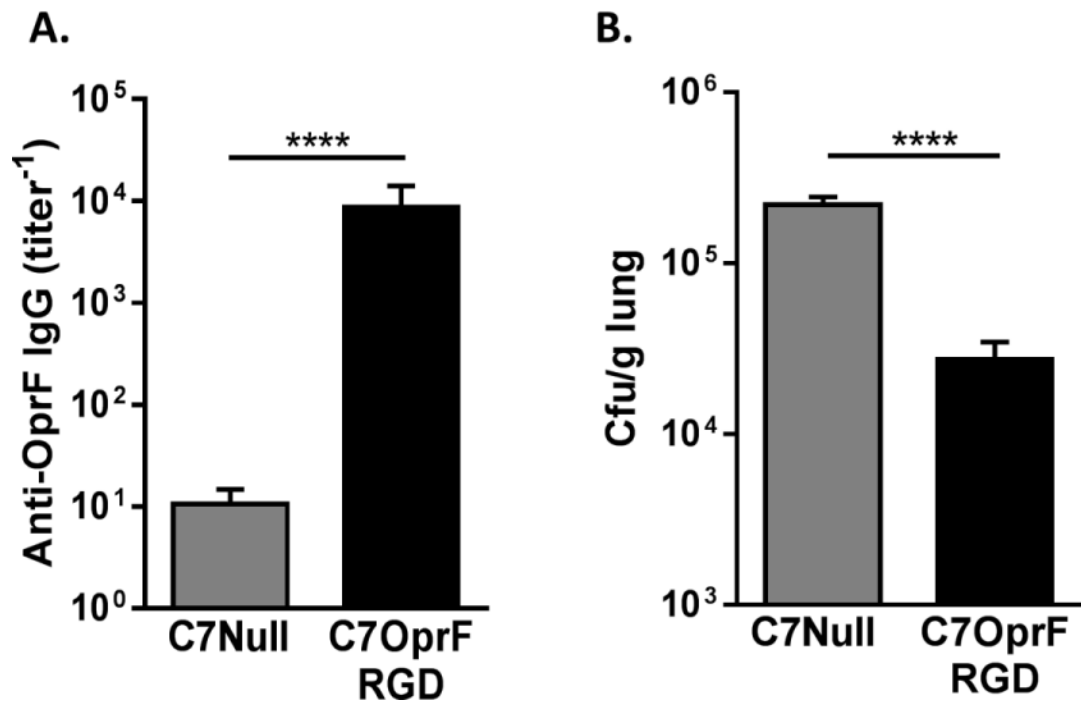


Figure 4. Anti-*P. aeruginosa* humoral immune responses and enhanced *P. aeruginosa* clearance from lungs after the immunization with AdC7OprF.RGD in CF mice with established *P. aeruginosa* respiratory infection

CF mice (*Cfr*^{tm1UNC}) were immunized intranasally with either 1×10^{10} particle units of AdC7OprF.RGD or AdC7Null 4 days after the administration of *P. aeruginosa* embedded in agar beads. Ten days after the immunization, anti-OprF IgG antibody titers were determined in serum and *P. aeruginosa* bacterial loads were determined in lung homogenates. **A.** Serum anti-OprF IgG. **B.** *P. aeruginosa* bacterial loads in lung homogenates. Data are presented as mean \pm SEM of $n=8$ mice per group. **** denotes significance of $p < 0.0001$.

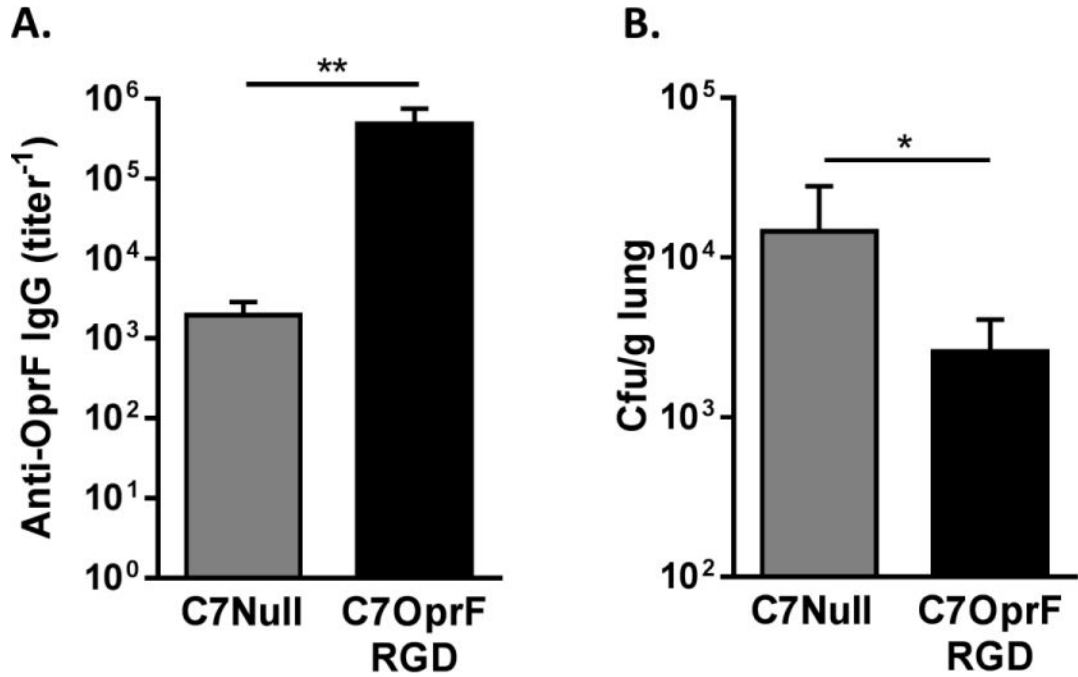


Figure 5. Anti- *P. aeruginosa* humoral immune responses and enhanced *P. aeruginosa* clearance from lungs after the immunization with AdC7OprF.RGD in rats with established *P. aeruginosa* respiratory infection

Rats (Sprague Dawley) were immunized intranasally with either 5×10^{10} particle units of AdC7OprF.RGD or AdC7Null 1 day after the administration of *P. aeruginosa* embedded in agar beads. Two weeks after the immunization, anti-OprF IgG antibody titers were determined in serum and *P. aeruginosa* bacterial loads were determined in lung homogenates. **A.** Serum anti-OprF IgG. **B.** *P. aeruginosa* bacterial loads in lung homogenates. Data are presented as mean \pm SEM of $n=5$ mice per group. * and ** denote significance of $p < 0.05$ and $p < 0.01$ respectively.