Adenovirus-Based Vaccine with Epitopes Incorporated in Novel Fiber Sites to Induce Protective Immunity against Pseudomonas aeruginosa

Anurag Sharma1, Anja Krause1, Yaqin Xu2, Biin Sung1, Wendy Wu1, Stefan Worgall1,2*

1 Department of Genetic Medicine, Weill Cornell Medical College, New York, New York, United States of America, 2 Department of Pediatrics, Weill Cornell Medical College, New York, New York, United States of America

Abstract

Adenovirus (Ad) vector-based vaccines displaying pathogen-derived epitopes on Ad capsid proteins can elicit anti-pathogen immunity. This approach seems to be particularly efficient with epitopes incorporated into the Ad fiber protein. Here, we explore epitope insertion into various sites of the Ad fiber to elicit epitope-specific immunity. Ad vectors expressing the 14-mer Pseudomonas aeruginosa immune-dominant outer membrane protein F (OprF) epitope 8 (Epi8) in five distinct sites of the Ad5 fiber, loops CD (AdZ.F(CD)Epi8), DE (AdZ.F(DE)Epi8), FG (AdZ.F(FG)Epi8), HI (AdZ.F(HI)Epi8) and C terminus (AdZ.F(CT)Epi8), or the hexon HVRS loop (AdZ.HxEpi8) were compared in their capacity to elicit anti-P. aeruginosa immunity to AdOprF, an Ad expressing the entire OprF protein. Intramuscular immunization of BALB/c mice with AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 elicited higher anti-OprF humoral and cellular CD4 and CD8 responses as well as enhanced protection against respiratory infection with P. aeruginosa compared to immunization with AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(CT)Epi8 or AdZ.HxEpi8. Importantly, repeat administration of the fiber- and hexon-modified Ad vectors boosted the OprF-specific humoral immune response in contrast to immunization with AdOprF. Strikingly, following three doses of AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 anti-OprF immunity surpassed that induced by AdOprF. Furthermore, in the presence of anti-Ad5 immunity, immunization with AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8, but not with AdOprF, induced protective immunity against P. aeruginosa. This suggests that incorporation of epitopes into distinct sites of the Ad fiber is a promising vaccine strategy.

Introduction

Pseudomonas aeruginosa is one of the leading nosocomial bacterial pathogens worldwide and can cause serious infections of the respiratory tract. A vaccine against P. aeruginosa would be useful as treatment is often challenged by antibiotic resistance of the organism. No efficient and marketable vaccine is yet available [1,2]. P. aeruginosa outer membrane protein F (OprF) is one of the promising vaccine antigens. OprF is surface exposed, antigenically conserved in wild-type strains of P. aeruginosa and elicits cross-reactive, opsonizing and protective antibodies in various animal models and humans [1,3–7]. Various immunogenic peptides have been identified in the outer loops of OprF, including the 14-mer peptide Epi8 [8–10].

Adenovirus (Ad) vectors are attractive delivery vehicles for genetic vaccines due to their ability to act as immune system adjuvants and to rapidly evoke robust immune responses against the transgene product and viral capsid proteins [11–14]. Ad vectors could also serve as a vaccine platform against P. aeruginosa. Human Ad serotype 5 (Ad5) or non-human primate Ad serotype C7 (AdC7) expressing OprF induced robust protective immunity against pulmonary infections with P. aeruginosa in mice [15,16].

One of the limitations of Ad as vaccine carrier is that anti-Ad immunity elicited by the initial immunization usually prevents productive infection with subsequent immunizations, critical to achieve boosting of the anti-transgene immunity [13,17,18]. One of the prime-boost strategies for Ad-based vaccines is to incorporate vaccine epitopes into the Ad capsid [10,19–22]. Various Ad outer capsid proteins including hexon, fiber knob, penton base and protein IX have been targets for genetic modification [23]. Incorporation of influenza hemagglutinin (HA) epitopes into the fiber HI loop of the Ad5 fiber elicits stronger humoral and cellular immunity compared to incorporation of the same epitope into the more abundant hexon protein [20]. Here we explore different epitope-insertion sites within the Ad fiber protein to enhance the epitope-specific immune response of an Ad-based vaccine. We identify a novel site in the FG loop for epitope insertion to elicit robust epitope-specific immunity that can be boosted and is effective in Ad pre-immune animals.

Materials and Methods

Ethics statement

All animal studies were conducted in accordance to the protocols reviewed and approved by the Weill Cornell Institu-
Ad vectors

AdEasy™ adenoviral vector system (Agilent Technologies, Santa Clara, CA) was used to construct the replication-defective recombinant human Ad5 vectors. The vectors expressed either β-galactosidase, referred to as “Z” in the vector (AdZ), or no transgene (AdNull) [24]. The plasmid pAdEasy-1 (Agilent Technologies) was modified to insert gene encoding OprF 14-mer epitope Epi8 (NATAEGRAINRREVE) into loops CD (Gly450/Thr451), DE (Asn464/Gly465), FG (Gly509/Lys510), HI (Gly543/Asp544) or C terminus (CT) of the Ad5 fiber gene (Figure 1). The resultant plasmids and pAdEasy-1 were recombined with pShuttle-CMV-lacZ (Agilent Technologies) to obtain recombinant plasmids pAdZ.F(CD)Epi8, pAdZ.F(DE)Epi8, pAdZ.F(FG)Epi8, pAdZ.F(HI)Epi8, pAdZ.F(CT)Epi8 and pAdZ that were used for transfection to generate the fiber-modified Ad vectors AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8 and AdZ respectively. Fiber-modified Ad vectors were generated using a previously described strategy [25]. Because of potential inhibitory effects of the modified Ad fibers with the cellular Ad receptors, it is difficult to generate fiber-modified vectors in regular human embryonic kidney (HEK) 293 cells. Therefore, a HEK 293-derived cell line that constitutively expresses the Ad5 fiber protein (293F) was developed and used as packaging cell line. The 293F cell line was generated by transfection of a fiber-expressing plasmid (pcDNA3.1/Hyg-Fiber) and then screening single cell clones generated by transfection of a fiber-expressing plasmid developed and used as packaging cell line. The 293F cell line was confirmed by GelCode silver staining (Pierce, Rockford, IL). Western analysis

To evaluate the presence of the Epi8 epitope on the Ad fiber protein, purified Ad vectors (10¹⁰ virus particles) were denatured (95 °C for 5 min) in NuPAGE sample buffer (Invitrogen, Carlsbad, CA) and separated by 4 to 12% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; NuPAGE system; Invitrogen). Following transfer to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) equal loading was confirmed by GelCode silver staining (Pierce, Rockford, IL). The membrane was exposed to blocking solution (5% fat-free milk; Bio-Rad Laboratories) in PBS for 1 h and then incubated with either polyclonal anti-OprF serum, obtained from C57Bl6 mice immunized with recombinant OprF protein (1:500) [16], or anti-Ad fiber antibody (Abcam, Cambridge, MA) (1:500) for 1 h. Following addition of a peroxidase-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO) (1:10,000) for 1 h, a chemiluminescent peroxidase substrate (ECL reagent; Amersham Biosciences, Piscataway, NJ) was used for detection.

Infection with capsid-modified Ad vectors in vitro

To evaluate if incorporation of Epi8 in the different fiber sites interferes with the coxsackie-adenovirus receptor (CAR)-dependent or -independent infection in vitro, infection of A549 cells (high expression of CAR) or dendritic cells (DC; low expression of CAR) was analyzed. A549 cells (CCL185; American Type Culture Collection, Manassas, VA), maintained in complete Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin/mL, 100 μg of streptomycin/mL (all from Gibco BRL, Gaithersburg, MD) were infected with AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8, AdOpF, AdZ or AdNull at a dose of 10¹⁰ pu/animal diluted in PBS to the left thigh muscle using a 0.5 ml insulin syringe (Becton, Dickinson and Company, Franklin Lakes, NJ).

Mice

Female BALB/c mice were obtained from Taconic Farms. The animals were housed under specific pathogen–free conditions and were used at 6–8 weeks of age. Mice were inoculated by injection of 50 μl of the Ad vectors AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8, AdOpF, AdZ or AdNull at a dose of 10¹⁰ pu/animal diluted in PBS to the left thigh muscle using a 0.5 ml insulin syringe (Becton, Dickinson and Company, Franklin Lakes, NJ).
Anti-OprF, anti-Ad and anti-β-galactosidase humoral immune response

Mice were immunized intramuscularly with AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8, AdOprF or AdZ at a dose of 10¹⁰ pu/animal and boosted twice with the same virus after 2 and 5 wk. Serum was collected after 2, 5 and 8 wk of initial immunization. Lung bronchoalveolar lavage fluid was collected by intratracheal instillation and aspiration of 0.5 ml PBS, pH 7.4., which was centrifuged at 6000 rpm at 4°C for 10 min and the supernatant was stored at −80°C. Anti-OprF, anti-Ad and anti-β-galactosidase IgG were assessed by ELISA using flat bottomed 96-well EIA/RIA plates (Corning, New York, NY) coated with recombinant OprF (0.5 μg/well) [10], Ad5 (10⁹ pu/well) or β-galactosidase (0.1 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 25°C and two-fold serial serum dilutions were added to each well and incubated for 1 h at 25°C. Following three washes with PBS containing 0.05% Tween (PBS-Tween) a peroxidase-conjugated sheep anti-mouse IgG (Sigma-Aldrich), diluted 1:10,000 in PBS containing 0.05% Tween (PBS-Tween) a peroxidase-conjugated sheep anti-mouse IgG (Sigma-Aldrich), diluted 1:10,000 in PBS containing 0.05% Tween (PBS-Tween). 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.

To assess surface expression of Epi8 on the capsid-modified Ad vectors, ELISA plates were coated with intact or disrupted AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8 or AdZ (10¹⁰ pu/well). Disruption of Ad5 vector was carried out in 0.5% sodium dodecyl sulfate (56°C, 45 seconds) [29]. The plates were blocked with 5% dry milk in PBS for 1 h at 25°C and two-fold serial dilutions of anti-OprF or anti-Ad5 were added to each well and incubated for 2 h at 25°C. The plates were further processed and evaluated as described above.

Opf-specific cellular immune response

To evaluate Opf-specific cellular immune responses induced by the capsid-modified Ad vectors, BALB/c mice were immunized intramuscularly with 10¹⁰ pu of AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8 or AdZ (10¹⁰ pu/well). Disruption of Ad5 vector was carried out in 0.5% sodium dodecyl sulfate (56°C, 45 seconds) [29]. The frequency of Opf-specific CD4 and CD8 T lymphocytes was determined with a IL-4- and/or interferon-γ (IFN-γ)–specific enzyme-linked immunospot (ELISPOT) assay (R&D Systems) 7 days following the last immunization. Splenic CD4 or CD8 T cells were purified by positive selection with CD4 (L3T4) or CD8 (Ly-2) MACS microbeads (Miltenyi Biotec, Auburn, CA). The purity of the T cells was more than 95%. To serve as antigen-presenting cells, splenic DCs were purified from syngeneic naïve animals by positive selection with CD11c MACS beads (Miltenyi Biotec) and two consecutive purifications over MACS LS columns (Miltenyi Biotec). The purity of the DC was more than 90%. DC (5x10⁶/ml) were incubated for 2 h with purified recombinant Opf protein (100 μg/ml) in RPMI medium supplemented with 2% fetal bovine serum (HyClone, Logan, UT), 10 mM HEPES (pH 7.5; BioSource International, Camarillo, CA), and 10 μM β-mercaptoethanol (Sigma-Aldrich). CD4 or CD8 T cells (2x10⁵) were incubated with splenic DC with or without recombinant Opf protein at a ratio of 4:1 in IL-4 and/or IFN-γ plates (R&D Systems) for 48 h. Following washing, biotinylated anti-IFN-γ or anti-IL-4 (both from R&D Systems) antibodies were added and incubated overnight at 4°C. For final spot detection a streptavidin-alkaline phosphatase conjugate followed by 3-amino-9-ethylcarbazole substrate (both R&D Systems) was added. The spots were counted by computer-assisted ELISPOT image analysis (Zellnet Consulting, New York, NY).

Protection against pulmonary challenge with P. aeruginosa

The P. aeruginosa strain PAO1 was used to assess protective immunity. PAO1-containing agar beads were prepared based on the method of Starke et al. [30] and were used as described previously [31,32]. Briefly, a log-phase culture of PAO1 suspended in warm tryptic soy agar (52°C) was added to mineral oil with vigorous stirring and the mixture was cooled on ice. The PAO1-impregnated beads were washed extensively with PBS, and the density of viable bacteria enmeshed in agar beads was determined by plating of serial dilutions of homogenized beads. To evaluate if immunization with Epi8 capsid-modified Ad vectors resulted in protective immunity against a pulmonary challenge with P. aeruginosa, BALB/c mice were immunized intramuscularly with AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8, AdOprF or AdZ (all 10¹⁰ pu/mouse) followed by boost immunizations after 2 and 5 wk. Eight weeks following initial immunization, the mice were challenged intranasally with PAO1 (4x10⁶ cfu in 50 ul) encapsulated in agar beads. Mice were sacrificed 24 h post challenge and lung homogenates were plated on McConkey agar plates. The numbers of colonies were quantified after 48 h.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Two-Way ANOVA and statistical significance was determined at p<0.05.

Results

Generation and characterization of fiber-modified Ad vectors

The Ad vectors with Epi8 incorporated in the fiber loops CD (AdZ.F(CD)Epi8), DE (AdZ.F(DE)Epi8), FG (AdZ.F(FG)Epi8), HI (AdZ.F(HI)Epi8) or C terminal (AdZ.F(CT)Epi8) were generated in the HEK 293-derived cell line that constitutively expresses the Ad5 fiber protein (293F) and subsequently propagated in regular HEK 293 cells as described in material and methods section. The presence of Epi8 epitope in the intact fiber protein on each of the purified vectors was assessed by Western analysis with serum from mice that had been immunized with Opf (Figure 2A). Epi8 was detected in the fiber-modified Ad vectors at around 65 kDa, the size of the Ad5 fiber, and in AdZ.HxEpi8 at 120 kDa, the size of Ad5 hexon (Figure 2A). No signal was detected with the control AdNull. Detection of the fiber with an anti-fiber antibody showed a slightly increased size of the protein in the five fiber-modified vectors compared to AdNull and AdZ.HxEpi8 consistent with the presence of Epi8 (Figure 2B).

Infectivity of Epi8 capsid-modified Ad vectors

To evaluate if incorporation of Epi8 into the different fiber sites affected CAR-dependent and CAR-independent infectivity, β-galactosidase (Z) transgene expression was assessed following infection of A549 cells or bone marrow-derived DC. In A549 cells, highest transgene expression was seen with AdZ and AdZ.HxEpi8 (p<0.05, both compared to all other vectors; Figure 3A). Of the fiber-modified vectors, AdZ.F(FG)Epi8 and AdZ.F(HI)Epi8 showed the highest expression levels (p<0.05 compared to AdZ.F(CD)Epi8, AdZ.F(DE)Epi8 and AdZ.F(CT)Epi8). Infectivity


of DC showed a similar pattern AdZ>Ad.Hx.Epi8>AdZ.F(G-FG)Epi8 and AdZ.F(HI)Epi8, the hexon-modified AdZ.HxEpi8 or AdNull as control (all 10^{10} particles) were separated on 4–12% polyacrylamide gradient SDS-PAGE, transferred to a polyvinylidene difluoride membrane and evaluated by Western blot analysis using A. anti-OprF serum; or B. anti-Ad fiber antibody.

doi:10.1371/journal.pone.0056996.g002

**Figure 2.** *P. aeruginosa* Epi8 epitope in various Ad vector fiber sites. The fiber-modified Ad vectors AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8 and AdZ.F(CT)Epi8, the hexon-modified AdZ.HxEpi8 or AdNull as control (all 10^{10} particles) were separated on 4–12% polyacrylamide gradient SDS-PAGE, transferred to a polyvinylidene difluoride membrane and evaluated by Western blot analysis using A. anti-OprF serum; or B. anti-Ad fiber antibody.

doi:10.1371/journal.pone.0056996.g002

**Figure 3.** Infectivity of fiber-modified Ad vectors in vitro. A549 cells (A) or murine bone-marrow-derived DC (B) were infected with the fiber-modified Ad vectors AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8 and AdZ.F(CT)Epi8, the hexon-modified AdZ.HxEpi8 or AdZ as control at 10^3 pu/cell (A549 cells) or 5×10^4 pu/cell (DC). ß-galactosidase activity was evaluated after 36 h by spectrophotometric assay and normalized to uninfected cells. Results represent mean ± SEM of three independent experiments. * denotes p<0.05, AdZ or AdZ.HxEpi8 compared to all others. § denotes p<0.05, AdZ.F(G-FG)Epi8 or AdZ.F(HI)Epi8 compared to AdZ.F(CD)Epi8, AdZ.F(DE)Epi8 or AdZ.F(CT)Epi8.

doi:10.1371/journal.pone.0056996.g003

**Figure 4.** Surface presentation of Epi8 epitope on capsid-modified Ad vectors. ELISA plates were coated with A. intact or B. disrupted AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8 or AdZ (10^{10} pu/well) and probed with anti-OprF serum. Data are shown as the mean ± SEM of 3 wells/vector.* denotes p<0.05, AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 compared to all others. § denotes p<0.05, AdZ.HxEpi8 compared to all others.

doi:10.1371/journal.pone.0056996.g004

Overall, these data suggest that incorporation of Epi8 into the fiber protein affects in vitro infectivity. Among the five fiber sites, insertion into the FG or HI loop has the least effect on Ad vector infectivity, whereas insertion into the loops CD, DE or CT strongly diminishes Ad infectivity.

**Surface presentation of Epi8 epitope on capsid-modified Ad vectors**

Epitopes inserted in capsid protein could be hidden by protein folding and so affect immune recognition. To investigate surface exposure and accessibility of the Epi8 epitope on the capsid-modified Ad vectors to antibody binding, intact or disrupted Ad vectors were probed with anti-OprF antibody in an ELISA plate. Intact AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 interacted strongly with anti-OprF compared to intact AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(CT)Epi8 and AdZ.F(HI)Epi8 (p<0.05 ) [Figure 3B]. Overall, these data suggest that incorporation of Epi8 into the fiber protein affects in vitro infectivity. Among the five fiber sites, insertion into the FG or HI loop has the least effect on Ad vector infectivity, whereas insertion into the loops CD, DE or CT strongly diminishes Ad infectivity.
Systemic humoral response to Epi8 capsid-modified Ad vectors

To assess the anti-OprF humoral immune response induced by immunization with the Epi8 capsid-modified Ad vectors mice were immunized intramuscularly with either AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8, AdOprF or AdZ (10^10 pu/mouse). At 2 weeks, anti-OprF titers were higher in animals immunized with AdOprF compared to animals immunized with capsid-modified vectors. Immunization with AdZ.F(FG)Epi8 and AdZ.F(HI)Epi8 elicited a systemic humoral immune response to AdZ.F(FG)Epi8 as P. aeruginosa Vaccine.

**Figure 5. Immunization with Epi8 fiber-modified Ad vectors induces anti-P. aeruginosa systemic humoral immunity.** BALB/c mice were immunized via the intramuscular route with the fiber-modified Ad vectors AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8 and AdZ.F(CT)Epi8, the hexon-modified AdZ.HxEpi8 or AdOprF (all 10^10 pu/mouse). Mice were boosted with the same vectors after 2 and 5 wk, respectively, and anti-OprF, anti-Ad and anti-β-galactosidase antibodies in serum were analyzed at 2, 5 and 8 wks by ELISA. A. Anti-OprF IgG. B. Anti-Ad IgG. C. Anti-β-galactosidase IgG. Data are shown as the mean ± SEM of 5 mice/group. Limit of detection is indicated by the dashed line. * denotes p<0.05. doi:10.1371/journal.pone.0056996.g005

**AdFiberEpi8 as P. aeruginosa Vaccine**

PLOS ONE | www.plosone.org 5 February 2013 | Volume 8 | Issue 2 | e56996
AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, and AdOprF. The hexon-modified AdZ.HxEpi8 induced higher OprF-specific IFN-γ titers than the capsid-modified AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, and AdOprF vectors in BALB/c mice. The data represent the mean of pooled cells from five mice per group from three separate experiments ± SEM. * denotes p < 0.05, AdOprF compared to all others. § denotes p < 0.05, AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 compared to AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdOprF, or AdZ.HxEpi8.

doi:10.1371/journal.pone.0056996.g007

Protection against pulmonary infection with P. aeruginosa

To evaluate if the most immunogenic Epi8 fiber-modified Ad vectors protect against pulmonary infection with P. aeruginosa, mice were immunized with AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdOprF or AdZ, boosted twice with the same vector and challenged by intranasal administration of agar-encapsulated PAO1 three weeks after the last vector administration. Mice immunized with AdZ.F(FG)Epi8, AdZ.F(HI)Epi8 and AdOprF showed reduction in the P. aeruginosa colony count compared to the AdZ group (p < 0.05; Figure 8). This suggests that the protective immunity generated by AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 is comparable to that induced by AdOprF.

doi:10.1371/journal.pone.0056996.g008

To evaluate the cellular immune responses, mice were immunized intramuscularly with AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8, AdZ.F(HI)Epi8, AdZ.F(CT)Epi8, AdZ.HxEpi8, AdOprF, or AdZ and boosted twice with the same vector. The frequencies of OprF-specific splenic CD4 and CD8 T cells stimulated by syngeneic DC pulsed with recombinant OprF protein were analyzed by ELISPOT. Immunization with AdOprF induced the highest OprF-specific IFN-γ CD4 (Figure 7A), IL-4 (Figure 7B) and IFN-γ CD8 T cell (Figure 7C) responses (p < 0.05) of the capsid-modified vectors, AdZ.F(FG)Epi8 and AdZ.F(HI)Epi8 induced higher OprF-specific IFN-γ CD4 (Figure 7A), IL-4 (Figure 7B) and IFN-γ CD8 T cell (Figure 7C) compared to AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(CT)Epi8 or AdZ.HxEpi8 (p < 0.05).

Figure 7. Immunization with Epi8 fiber-modified Ad vectors induces anti-P. aeruginosa cellular immunity. BALB/c mice were immunized via the intramuscular route with the fiber-modified Ad vectors AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(FG)Epi8 and AdZ.F(CT)Epi8, the hexon-modified AdZ.HxEpi8, AdOprF or AdZ (all 10¹⁰ pu/mouse) and boosted with the same vectors at 2 and 5 wk. Splenic CD4 and CD8 T cells were isolated 7 days following the last administration and incubated in vitro with syngeneic DC pulsed with recombinant OprF or DC alone. IL-4 and IFN-γ were determined by ELISPOT assay. A. CD4 IFN-γ; B. CD4 IL-4; C. CD8 IFN-γ. The data represent the mean of pooled cells from five mice per group from three separate experiments ± SEM. * denotes p < 0.05, AdOprF compared to all others. § denotes p < 0.05, AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 compared to AdZ.F(CD)Epi8, AdZ.F(DE)Epi8, AdZ.F(CT)Epi8 or AdZ.HxEpi8.
Efficacy of Epi8 fiber-modified Ad vectors in the presence of anti-Ad immunity

To evaluate the efficacy of fiber-modified Ad vectors in the presence of pre-existing anti-Ad immunity, Ad-immune mice, induced by repeat administration of AdNull, were immunized with AdZ.F(FG)Epi8 or AdOprF. In the presence of pre-existing anti-Ad immunity, AdOprF inoculated mice showed a marked reduction in anti-OprF titers that were close to basal levels (Figure 9A). In contrast, immunization with AdZ.F(FG)Epi8 elicited robust levels of anti-OprF IgG irrespective of pre-existing Ad immunity. Likewise, protection against P. aeruginosa, was similar when AdZ.F(FG)Epi8 was administered in the presence or absence of anti-Ad immunity (Figure 9B). This suggests that, in contrast to AdOprF, AdZ.F(FG)Epi8 can elicit protective anti-P. aeruginosa immunity even in the presence of anti-Ad immunity.

Discussion

A potent and effective vaccine against P. aeruginosa has long been sought after, but is so far not available. The present study demonstrates that fiber-modified Ad vectors expressing Epi8 induce anti-P. aeruginosa humoral and cellular protective immunity that can be boosted on repeated administration and is effective in presence of anti-Ad5 immunity.

Incorporation of epitopes into Ad vector capsid to induce epitope-specific immunity

One attractive feature of Ad-based vaccines is the feasibility to modify the Ad capsid to enhance immune responses or change the Ad tropism [23,33]. Incorporation of Epi8 into loop 1 of HVR5 of the Ad hexon protein has been shown to induce anti-epitope humoral and cellular immunity to protect against infections with P. aeruginosa in a murine model [16]. Incorporation of influenza HA or ovalbumin epitopes into various Ad capsid proteins demonstrated that incorporation into the fiber HI loop induces the strongest anti-epitope response [20,21]. The structure of the Ad5 fiber is known and there are multiple loops and sites that could theoretically be used as insertion sites for peptide sequences without disrupting the overall structure [34]. We developed and compared various Ad vectors that display Epi8 on the CD, DE, FG, HI loops or CT of Ad fiber knob. Immunization with fiber-modified Epi8 Ad vectors induced robust humoral and cellular responses. Following single administration anti-OprF immunity induced by the fiber-modified vectors was less compared to anti-OprF immunity induced by a vector expressing the entire OprF protein as transgene. Among the capsid-modified vectors, the strongest humoral response against the OprF protein was induced by AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8. The AdZ.HxEpi8, AdZ.F(CD)Epi8, AdZ.F(DE)Epi8 and AdZ.F(CT)Epi8 induced only low levels of anti-OprF humoral immunity. The stronger induction of Epi8-specific immune response by fiber-modified Ad vector compared to hexon-modified vector is consistent with our previous observations with HA epitope [20]. The low efficiency of CD, DE fiber loops CT or loop 1 of HVR5 of Ad hexon to generate epitope-specific immune responses can be explained by the interference of the inserted peptide with cellular infectivity, in particular of antigen-presenting cells or the impaired folding and exposure of the epitopes on the capsid. In contrast to our results, higher humoral immunity after single immunization was elicited against an ovalbumin epitope incorporated into the hexon protein compared to insertion of the epitope into the fiber HI loop [21]. It is likely that the nature of the two different
epitopes and the location of the epitope within the hexon (insertion within HVR5 in this study) versus replacement of HVR5 by Lanzi et al. influenced the epitope-specific immune responses. Consistent with our results, upon second administration a stronger humoral response was elicited when ovalbumin epitope was inserted into fiber protein compared to hexon [21].

Protective immunity against extracellular bacteria such as P. aeruginosa is mainly dependent on sufficient levels of humoral immunity induced by the vaccine. T cell-mediated immunity has received less attention in the development of a vaccine against P. aeruginosa but is a part of the response against natural infection with the organism [35,36]. Consistent with the humoral response, FG- and HI-modified vectors elicited strong OprF-specific Th1 and Th2 type cellular immunity, which was lower compared to AdOprF. The presence of multiple T-cell epitopes in the full length OprF protein used to pulse DCs, is likely responsible for higher T-cell activation by AdOprF. Importantly, the protective immunity generated by AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 was comparable to AdOprF. Although both humoral and cellular responses were induced after immunization with AdZ.F(FG)Epi8 or AdZ.F(HI)Epi8 in the present study, their individual contribution to the protection against P. aeruginosa challenge is not clear.

The HI loop in the fiber knob has been the usual choice for incorporation of antigenic epitopes or targeting moieties [20,21,35,36]. In the present study we identified the FG loop as a novel location in the fiber knob for peptide insertion. This site is comparable to the HI loop regarding in vitro infectivity and its capacity to elicit anti-peptide immunity. It would be interesting to assess infectivity and immunogenicity of a bispecific Ad vector with peptides at both FG and HI loops.

Boosting of anti-OprF immune response by repeat administration of fiber-modified Ad vectors

Anti-Ad immune responses impair efficacy of Ad vectors of the same serotype, as pre-existing neutralizing antibodies against Ad prevent the cellular uptake of Ad and expression of the transgene in a previously immunized host [37,38]. To circumvent this issue Ad vaccine vectors derived from rare human or nonhuman Ad serotypes that evade anti-Ad5 immunity have been developed [39–41]. However, all of these Ad vectors generate potent anti-vector immunity that diminishes the utility of vector re-administration/boosting. Anti-Ad immunity can be boosted by repeated infection with wild-type Ad or Ad vectors, leading to an increase in anti-Ad humoral responses with subsequent infections [37,38,42]. Consequently, immune responses against viral epitope placed on an Ad capsid protein can be boosted by repeated vector administration. Immunization with the capsid-modified Ad vectors in this study enabled repeated administration of the same vector resulting in boosting of the anti-epitope and not the anti-transgene humoral response. Strikingly, anti-OprF IgG was higher following three doses of FG- or HI-modified vector compared to AdOprF, thus highlighting the utility of fiber-modified Ad vectors for vaccine delivery. One of the mechanisms that explains the boosting of humoral response is the Fcγ receptor-mediated uptake of Ad vector-antibody immune complexes by antigen-presenting cells and subsequent increased stimulation of specific immune cells [43].

The prevalence of pre-existing vector immunity in humans may limit the utility of human Ad serotype vaccines. Notably, in sharp contrast to immunization with AdOprF, AdZ.F(FG)Epi8 induced protective anti-P. aeruginosa immunity even in the presence of high levels of pre-existing anti-Ad immunity. This suggests that pre-existing Ad vector immunity can be effectively circumvented by the incorporation of antigenic epitopes into the fiber protein.

Taken together, incorporation of antigenic peptides on the FG or HI loop of Ad fiber knob is an efficient strategy to generate protective immunity that can be boosted by repeated administration and is effective in the presence of pre-existing anti-Ad immunity. The display of antigenic epitopes on the Ad fiber should also be valuable in the development of Ad-based vaccines against other pathogens. The robust protective immunity induced by AdZ.F(FG)Epi8 and AdZ.F(HI)Epi8 make both of these sites attractive for the insertion of epitopes as a general vaccine strategy.

Author Contributions

Conceived and designed the experiments: SW AS AK. Performed the experiments: AS BS WW YX. Analyzed the data: AS AK SW. Contributed reagents/materials/analysis tools: AK. Wrote the paper: AS SW.

References

1. Sharma A, Krause A, Worgall S (2011) Recent developments for Pseudomonas vaccines. Hum Vaccin 7: 999–1011.
2. Doring G, Pier GB (2008) Vaccines and immunotherapy against Pseudomonas aeruginosa. Vaccine 26: 1011–1024.
3. Mutharia LM, Hancock RE (1983) Surface localization of Pseudomonas aeruginosa outer membrane protein F by using monoclonal antibodies. Infect Immun 42: 1027–1033.
4. Mutharia LM, Nica TI, Hancock RE (1982) Outer membrane proteins of Pseudomonas aeruginosa serotype strains. J Infect Dis 146: 770–779.
5. Gilleland HE, Jr, Gilleland LB, Matthews-Greer JH (1988) Outer membrane protein F preparation of Pseudomonas aeruginosa as a vaccine against chronic pulmonary infection with heterogeneous immunity strain in a rat model. Infect Immun 56: 1017–1022.
6. Price BM, Galloway DR, Baker NR, Gilleland LB, Szczek J, et al. (2001) Protection against Pseudomonas aeruginosa chronic lung infection in mice by genetic immunization against outer membrane protein F (OprF) of P. aeruginosa. Infect Immun 69: 3510–3515.
7. Sorichter S, Baumann U, Baumgart A, Walterspacher S, von Specht BU, et al. (2005) Synthetic peptides representing two epitopes and the location of the epitope within the hexon (insertion within HVR5 in this study) versus replacement of HVR5 by Lanzi et al. influenced the epitope-specific immune responses. Consistent with our results, upon second administration a stronger humoral response was elicited when ovalbumin epitope was inserted into fiber protein compared to hexon [21].
8. Gilleland LB, Gilleland HE, Jr (1995) Synthetic peptides representing two protective, linear B-cell epitopes of outer membrane protein F of Pseudomonas aeruginosa elicit whole-cell-reactive antibodies that are functionally pseudomonal specific. Infect Immun 63: 2347–2351.
9. Hughes EE, Gilleland LB, Gilleland HE, Jr (1992) Synthetic peptides representing epitopes of outer membrane protein F of Pseudomonas aeruginosa that elicit antibodies reactive with whole cells of heterologous immunotype strains of P. aeruginosa. Infect Immun 60: 3547–3563.
10. Worgall S, Krause A, Riva M, Herr KK, Vintayen EV, et al. (2005) Protection against P. aeruginosa with an adenosine vector containing an OprF epitope in the capsid. J Clin Invest 115: 1281–1289.
11. Wilson JM (1996) Adenoviruses as gene-delivery vehicles. N Engl J Med 334: 1105–1107.
12. Lasaro MO, Enil HC (2009) New insights on adenovirus as vaccine vectors. Mol Ther 17: 1333–1339.
13. Zais AK, Machado HB, Herschman HR (2009) The influence of innate and pre-existing immunity on adenovirus therapy. J Cell Biochem 108: 778–790.
14. Sharma A, Taedson M, Bangari DS, Mital SK (2009) Adenoviral vector-based strategies for cancer therapy. Curr Drug Deliv 4: 117–130.
15. Krause A, Wha WZ, Xu Y, Jho J, Crystal RG, et al. (2011) Protective anti-P. aeruginosa aeruginosa humoral and cellular mucosal immunity by AdG7-mediated expression of the P. aeruginosa protein OprF. Vaccine 29: 2131–2139.
16. Worgall S, Krause A, Qin J, Jho J, Hackett NR, et al. (2007) Protective immunity to pseudomonas aerugionas induced with a capsid-modified adenovirus expressing P. aeruginosa OprF. J Virol 81: 13801–13809.
17. Hashimoto M, Boyer JL, Hackett NR, Wilson JM, Crystal RG (2005) Induction of protective immunity to anthrax lethal toxin with a nonhuman primate adenovirus-based vaccine in the presence of preexisting anti-human adenovirus immunity. Infect Immun 73: 6805–6809.
18. Ahi YS, Bangari DS, Mital SK (2011) Adenoviral vector immunity: its implications and circumvention strategies. Curr Gene Ther 11: 307–320.
19. Crompton J, Toogood CJ, Wallis N, Hay RT (1994) Expression of a foreign epitope on the surface of the adenovirus hexon. J Gen Virol 75 (Pt 1): 133–139.
20. Krause A, Jho JH, Hackett NR, Roelvink PV, Bruder JT, et al. (2006) Epitopes expressed in different adenovirus capsid proteins induce different levels of epitope-specific immunity. J Virol 80: 7525–7530.
21. Lanzi A, Ben Youssef G, Perricaudet M, Benihoud K (2011) Anti-adenovirus humoral responses influence on the efficacy of vaccines based on epitope display on adenovirus capsid. Vaccine 29: 1463–1471.

22. Matthews QL, Fatima A, Tang Y, Perry BA, Tsuruta Y, et al. (2010) HIV antigen incorporation within adenovirus hexon hypervariable 2 for a novel HIV vaccine approach. PLoS One 5: e11815.

23. Matthews QL (2011) Capsid-incorporation of antigens into adenovirus capsid proteins for a vaccine approach. Mol Pharm 8: 3–11.

24. Hezhi J, Crystal RG, Bewig R (1993) Modulation of gene expression after replication-deficient, recombinant adenovirus-mediated gene transfer by the product of a second adenovirus vector. Gene Ther 2: 124–131.

25. Koizumi N, Misuguichi H, Sakarazi F, Yamaguchi T, Watanabe Y, et al. (2003) Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. J Virol 77: 13062–13072.

26. Mitteler N, March KL, Trapnell BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 70: 7498–7509.

27. Rosenfeld MA, Siegfried W, Yoshimura K, Yoneyama K, Fukayama M, et al. (1991) Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo. Science 252: 431–434.

28. Song W, Kong HL, Carpenter H, Torii H, Granstein R, et al. (1997) Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. J Exp Med 186: 1247–1256.

29. Hackett NR, Kaminsky SM, Sondhi D, Crystal RG (2000) Antivector and antitransgene host responses in gene therapy. Curr Opin Mol Ther 2: 376–382.

30. Yang Y, Li Q, Ertl HC, Wilson JM (1995) Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J Virol 69: 2004–2015.

31. Bangari DS, Mittal SK (2006) Development of nonhuman adenoviruses as vaccine vectors. Vaccine 24: 849–862.

32. Stone D, Lieber A (2006) New serotypes of adenoviral vectors. Curr Opin Mol Ther 8: 423–431.

33. Sharma A, Bangari DS, Tandon M, Pandey A, HogenEsch H, et al. (2009) Comparative analysis of vector biodistribution, persistence and gene expression following intravenous delivery of bovine, porcine and human adenoviral vectors in a mouse model. Virology 396: 44–54.

34. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, et al. (2001) Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther 3: 697–707.

35. Abdell-Motal U, Wang S, Lu S, Wigglesworth K, Galili U (2006) Increased immunogenicity of human immunodeficiency virus gp120 engineered to express Galalpha1-3Galbeta1-4GlcNAc-R epitopes. J Virol 80: 6943–6951.