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Evaluation of adenovirus 19a as a novel vector for mucosal vaccination against influenza A viruses

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ABSTRACT

Since preexisting immunity and enhanced infection rates in a clinical trial of an HIV vaccine have raised some concerns on adenovirus (Ad) serotype 5-based vaccines, we evaluated the subgroup D adenovirus serotype Ad19a for its suitability as novel viral vector vaccine against mucosal infections. In BALB/c mice, we compared the immunogenicity and efficacy of E1/E3-deleted Ad19a vectors encoding the influenza A virus (IAV)-derived antigens hemagglutinin (HA) and nucleoprotein (NP) to the most commonly used Ad5 vectors. The adenoviral vectors were applied intranasally and induced detectable antigen-specific T cell responses in the lung and in the spleen as well as robust antibody responses. A prior DNA immunization significantly improved the immunogenicity of both vectors and resulted in full protection against a lethal infection with a heterologous H3N2 virus. Nevertheless, the Ad5-based vectors were slightly superior in reducing viral replication in the lung which corresponded to higher NP-specific T cell responses measured in the lungs.

1. Introduction

Human adenoviruses belong to the genus *Mastadenovirus* and are classified in seven species (A-G) currently including 84 serotypes [1]. These viruses are mostly associated with mild diseases like keratoconjunctivitis, gastrointestinal and respiratory tract infections [2]. Beside their role in these epidemic outbreaks, human adenoviruses have attracted great attention in vaccinology because adenovirus-based viral vectors combine several excellent vaccine characteristics. Replication-deficient adenoviral vectors can be easily produced and are suitable for mass production since they can be grown to high titers in cell lines able to complement for the viral gene products deleted from the vector backbone, like E1 [3]. The viral DNA genome tolerates manipulation and does not integrate into the host cell genome, minimizing the risk for insertion mutagenesis. Several human serotypes and adenoviruses from animals like chimpanzees are currently under investigation as viral vector vaccines [4–6] with the human subgroup C serotype 5 being the best characterized one. It has been extensively studied in animal models [7–10] and in numerous clinical trials, including vaccinations against IAV, HIV, Ebola, Tuberculosis and Malaria [11–15]. In most of these studies, Ad5 vectors were safe and well tolerated and proved strong immunogenicity with an efficient induction of both antibody and T cell responses.

Unfortunately, the global seroprevalence against Ad5 is estimated to 60–90% depending on the age and region [16]. The influence of this preexisting immunity on the Ad5 vaccine efficacy is a matter of debate. Several studies suggest a decreased vaccine efficacy if volunteers are seropositive for Ad5 [17–20]. Interestingly, the largest Ad5 vaccine trial, the HIV STEP study, demonstrated a relatively low impact of the preexisting immunity on the immunogenicity. However, a dramatic twist of this study was that the prevalence of high titers of anti-Ad5 antibodies apparently led to higher infection rates after the vaccination [12].

Thus, in light of the high seroprevalence and yet unclear consequences of Ad5 anti-vector immunity, the exploitation of new adenoviral vector systems based on rare serotypes is of particular interest. In this report, we evaluate the immunogenicity and efficacy of a replication-deficient adenoviral vector based on the human species D serotype 19a [21,22] in comparison to a prototypical Ad5 vector. A frequency of neutralizing antibodies to Ad19a of 16–19% was reported for Ad19a [23,24], which was also recently renamed to Ad64 [25]. Since mucosal immune responses are

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essential for robust protection against IAV infections [26–28], the vectors encoding HA and NP were delivered intranasally and evaluated as single-shot vaccination or in a DNA prime viral vector boost regimen.

2. Materials and methods

2.1. Adenoviral vectors and DNA vaccines

Replication-deficient, E1/E3-deleted vectors of serotype 5 or 19a encoding the codon-optimized gene sequences of hemagglutinin derived from pH1N1 A/Hamburg/4/2009 and nucleoprotein derived from H1N1 A/Puerto Rico/8/1934 were produced by Sirion Biotech GmbH (Martinsried, Germany). A CMV promotor controls transgene expression in these constructs. For the analysis of antigen expression, A549 cells were transduced with a multiplicity of infection (MOI) of 10 of each recombinant vector. Forty-eight hours later, the expression levels of HA and NP were determined by Western Blot analysis with post-IAV infection sera of mice as detection antibodies. For DNA priming, pVax1-based plasmid DNA encoding the same antigens was prepared using the NucleoBond®Xtra Maxi EF Kit (Macherey-Nagel, Düren, Germany).

2.2. Animals, immunizations, and infections

6–8 weeks-old female BALB/cJRj mice were purchased from Janvier (Le Genest-ST-Isle, France) and housed in individually ventilated cages in accordance with the national law and institutional guidelines. The study was approved by an external ethics committee authorized by the North Rhine-Westphalia State Office for Consumer Protection and Food Safety and performed under the project license AZ 84-02.04.2013-A371. In case of DNA priming, animals received a mixture of the plasmids pV-HA and pV-NP (10 μg each) intramuscularly followed by electroporation as described before [29]. The adenoviral vectors encoding HA and NP were mixed with 2×10^6 infectious units (IU) of each vector, referred to as Ad5 or Ad19a, and were applied intranasally in a single dose of 50 μl PBS under anesthesia (100 mg/kg ketamine and 15 mg/kg xylazine). Two weeks after the adenoviral vector immunization, four animals of each group were sacrificed and bronchoalveolar lavage fluid (BALF) was collected before spleen and lungs were prepared for T cell analyses, as described elsewhere [30]. Serum samples from 13 animals of each group were collected four weeks after the adenoviral vector immunization. Seven animals per group were challenged five weeks after the adenoviral vector immunization with 10^4 plaque-forming units (10 LD_{50}) of the heterologous, mouse-adapted H3N2 A/Hong Kong/1/1968 (kindly provided by Prof. Georg Kochs, University Hospital Freiburg, Germany) by intranasal instillation in a volume of 50 μl. Disease progression and detection of viral RNA in BALF were monitored as described before [31]. As indirect measure of tissue damage and disruption of barrier function, total protein content in the cell-free BALF was measured using bicinchoninic acid assay (Pierce).

2.3. FACS-based antibody analysis

The flow cytometric antibody analysis was conducted as described elsewhere [29]. Briefly, HEK 293 T cells were transfected with plasmid DNA encoding the antigen of interest together with a plasmid encoding a blue fluorescent protein (BFP). 48 h post-transfection, the cells were incubated with sera or BALF diluted in FACS-PBS (PBS with 0.5% BSA and 1 mM sodium azide) to bind to HA on the surface, or diluted in permeabilization buffer (0.5% saponin in FACS-PBS) to bind to intracellular NP. Afterwards, specifically bound antibodies were detected with polyclonal anti-mouse Ig-FITC or anti-mouse IgA-FITC (clone C10-3, all BD Biosciences). The median FITC fluorescence intensity of BFP^+ cells was measured on a BD FACSCanto™ II and analyzed using FlowJo™ software (Tree Star Inc.).

2.4. Influenza microneutralization assay

To determine influenza-specific neutralizing antibody titers in sera or BALF, a microneutralization assay on MDCK II cells was performed as previously described [31].

2.5. Intracellular cytokine staining and pentamer staining

Animals were sacrificed two weeks after the adenoviral immunization in order to isolate lymphocytes from lung and spleen tissue. Lung tissue was cut into small pieces and treated for 45 min at 37 °C with 500 units Collagenase D and 160 units DNase I in 2 ml R10 medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-Glutamine, 10 mM HEPES, 50 μM β-Mercaptoethanol and 1% penicillin/streptomycin). Digested lung tissues and spleens were mashed through a 70 μm cell strainer before the suspensions were subjected to an ammonium-chloride-potassium lysis. 10^6 splenocytes or one fifth of the total lung cell suspension were plated per well in a 96-well round-bottom plate and incubated for six hours in 200 μl R10 medium containing monensin (2 μM), anti-CD28 (1 μg/ml, eBioscience), anti-CD107a-FITC (clone eBio1D4B, eBioscience) and 5 μg/ml of the peptides $HA_{110\text{-}120}$ (SFERFEIFPKE), $HA_{518\text{-}526}$ (IYSTVASSL), $NP_{55\text{-}69}$ (RLIQNSLTIERMVL), or $NP_{147\text{-}155}$ (TYQRTRALV), respectively. Non-stimulated samples were used for subtraction of background cytokine production (negative values were set as zero). After the stimulation, cells were stained with anti-CD8a-Pacific blue (clone 53–6.7, BD Biosciences), anti-CD4-PerCP (clone RM4-5, eBioscience) and Fixable Viability Dye eFluor® 780 (eBioscience). After fixation and permeabilization, cells were stained intracellularly with anti-IL-2-APC (clone JES6-5H4, BD Biosciences), anti-TNFα-PECy7 (clone MPG-XT22, BD Biosciences) and anti-IFNy-PE (clone XMG1.2, eBioscience).

For the pentamer staining, the same amounts of lymphocytes were incubated with APC-labeled H-$2K^D$ $NP_{147\text{-}155}$ pentamer (ProImmune) for 20 min at 4 °C followed by a second staining step with anti-CD127-FITC (clone A7R34, eBioscience), anti-CD103-PE (clone 2E7, eBioscience), anti-CD69-PerCP (clone H1.2F3, BD Biosciences), anti-KLRG1-PE-Cy7 (clone 2F1, eBioscience), anti-CD8a-Pacific-Blue (clone 53-6.7, BD Biosciences) and anti-CD45.2-APC-Cy7 (clone 104, Biolegend). After fixation, data were acquired on a BD FACSCanto™ II and analyzed using FlowJo™ software (Tree Star Inc.).

2.6. Flow cytometric analyses of cellular infiltration

One quarter of the cellular BALF fraction was stained with anti-Gr1-FITC (clone RB6-8C5, eBioscience), anti-CD49b-PE (clone DX5, eBioscience), anti-CD45-PerCP (clone 30-F11, BD Biosciences), anti-CD19-PE-Cy7 (clone 1D3, BD Biosciences), anti-F4/80-APC (clone BM8, eBioscience), anti-CD11b-APC-Cy7 (clone M1/70, BD Biosciences), anti-CD11c-Pacific-Blue (clone HL3, BD Biosciences) and anti-CD3e-BV510 (clone 145-2C11, BD Biosciences).

2.7. Statistical analyses

Results are shown as mean ± standard error of the mean (SEM) or in log-scale figures as median ± interquartile range. One-way ANOVA with Tukey's or Kruskal-Wallis post-test was performed with Prism 5.0 (GraphPad Software, Inc.). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Statistical comparisons to naïve mice are not shown for more clarity of the figures.

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