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A new adjuvanted nanoparticle-based H1N1 influenza vaccine induced antigen-specific local mucosal and systemic immune responses after administration into the lung

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ABSTRACT

Annually influenza virus infections are responsible for hospitalization and mortality, especially in high risk groups. Constant antigenic changes in seasonal influenza viruses resulted from antigenic shifts and antigenic drifts, enable emerging of novel virus subtypes that may reduce current vaccine efficacy and impose the continuous revision of vaccine component. Currently available vaccines are usually limited by their production processes in terms of rapid adaptation to new circulating subtypes in high quantitites meeting the global demand. Thus, new approaches to rapidly manufacture high yields of influenza vaccines are required. New technologies to reach maximal protection with minimal vaccine doses also need to be developed.

In this study, we evaluated the systemic and local immunogenicity of a new double-adjuvanted influenza vaccine administered at the site of infection, the respiratory tract. This vaccine combines a plant-produced H1N1 influenza hemagglutinin antigen (HAC1), a silica nanoparticle-based (SiO2) drug delivery system and the mucosal adjuvant candidate bis-(3,5’)-cyclic dimeric guanosine monophosphate (c-di-GMP). Mice were vaccinated by intratracheal route with HAC1/SiO2 or HAC1[c-di-GMP (single-adjuvanted vaccine) or HAC1/SiO2/c-di-GMP (double-adjuvanted vaccine) and evaluated for target-specific immune responses, such as hemagglutination inhibition and hemagglutinin-specific IgG titers, as well as local antibody (IgG and IgA) titers in the bronchoalveolar lavage (BAL). Furthermore, the HAC1-specific T-cell re-stimulation potential was assessed using precision-cut lung slices (PCLS) of vaccinated mice.

The double-adjuvanted vaccine induced high systemic antibody responses comparable to the systemic vaccination control. In addition, it induced local IgG and IgA responses in the BAL. Furthermore, HAC1 induced a local T-cell response demonstrated by elevated IL-2 and IFN-γ levels in PCLS of c-di-GMP-vaccinated mice upon re-stimulation.

Overall, the present study showed the potential of the double-adjuvanted vaccine to induce systemic humoral immune responses in intratracheally vaccinated mice. Furthermore, it induced a strong mucosal immune response, with evidence of antigen-primed T-cells in the lung.

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1. Introduction

Annual influenza-associated cases of hospitalization and up to 500,000 deaths during frequent virus outbreaks and sporadic pandemics illustrate the serious health burden of influenza virus infections [1]. The high mutational rate of the virus and frequency of interspecies transmission and/or zoonosis leading to new
virus subtypes makes influenza infections highly unpredictable [2,3]. Therefore, there is a need of developing novel and effective influenza vaccines.

Traditionally, only systemic administration of inactivated influenza vaccines, mostly intramuscularly, has been used. In 2003 Flumist®, the first nasal influenza vaccine with live attenuated influenza viruses, has been approved in the US [4], which protects locally at the site of virus entry and infection. An advantage of delivering vaccines via the respiratory route is, besides the inductions of local immune responses at virus settlement, the non-invasive application which is likely to increase public compliance. However, it has been described that intranasal antigen administration induces poor immune responses when applied without an appropriate mucosal adjuvant [5]. Thus, many new effective mucosal adjuvants are in preclinical development (s. review [6]). In 2007, bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) was introduced as a mucosal adjuvant with promising activity [7]. Madhun et al. showed that c-di-GMP improved the immunogenicity of an intranasally delivered subunit influenza vaccine, compared to antigen only, by inducing strong mucosal and systemic immune responses [8]. Additionally, the authors showed that intranasal administration of the c-di-GMP adjuvanted antigen induced protective antibody titers and cellular immune responses that far exceeded the responses induced by intramuscular administration of the same vaccine [8]. Moreover, Svindland et al. tested vaccination with c-di-GMP combined with a second adjuvant, Chitosan, and showed that vaccination with the combination of these molecules can further improve the humoral and cellular immune responses against target antigens [9]. Besides its adjuvantive effects, Chitosan is used as an intranasal delivery system. Other drug delivery systems such as silica nanoparticle (NP) have also been previously shown to have adjuvant properties [10,11]. Recently, we have shown that a combination of a plant-produced recombinant hemagglutinin (HA) antigen from the H1N1 influenza virus A/California/04/09 (HAC1) with silica-NP (SiO$_2$) was able to recall a previously established immune response in human lung tissue [12]. Manufacturing of recombinant proteins in plants for influenza vaccine development evolved as an alternative to the conventional egg-based vaccine production to overcome the limitations in quantity and time consumption [13]. This bottleneck of egg-produced vaccines can have serious consequences during influenza pandemics, when the production of sufficient amounts of vaccine in an adequate time frame to serve the global market could be difficult.

Regarding the need of rapidly produced vaccines in times of pandemics and the time consuming limitation of the egg-based vac-

cines, the here presented study tested the recombinant antigen of a highly immunogenic H1N1 strain responsible for the 2009/2010 pandemic. Furthermore, the study extends the published work with HAC1 and SiO$_2$ and evaluates the immunogenicity of this vaccine formulation when combined with c-di-GMP and administered at the site of virus entry. Overall, it showed the potential of the c-di-

gMP/SiO$_2$, double-adjuvanted vaccine to induce systemic humoral and strong mucosal immune responses, with IgA in the Airways. Furthermore, it presented evidence of antigen-primed T-cells in the lung in intratraehal/ly vaccinated mice.

2. Materials and methods

2.1. Animals

Female wild-type BALB/c mice aged 6–8 weeks (Charles River, Sulzfeld, Germany) were kept at an animal facility under conventional housing conditions (22 °C, 55% humidity, 12-h day/night cycle) with food and tap water ad libitum. The randomized study was approved by a local agency (Application-No. 33.9-42502-04-11/0465) and conducted according to the German Animal Protection law.

2.2. Media

Reagents were, if not stated otherwise, purchased from Sigma–Aldrich (Munich, Germany). Phosphate buffered saline (PBS) without Ca$^{2+}$ and Mg$^{2+}$, pH 7.4, Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 HAM (DMEM) with g-lactamine, 15 mM HEPES and 7.5% w/v sodium bicarbonate without phenol red, pH 7.2–7.4, RPMI 1640 and Earle’s Balanced Salt Solution (EBSS) were obtained from Gibco (Darmstadt, Germany). Cell/tissue cul-

tivation medium was supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. Vaccine and adjuvants

HAC1 was produced as previously described [14]. Briefly, the HA nucleotide sequence, encompassing amino acids 18–530 of the A/California/04/09 influenza strain (H1N1, NCBI accession number ACQ76318.1) were optimized for expression in plants and synthesized. The optimized HA sequence contains a 6× His affinity purification tag and the ER retention signal KDEL at the C-terminus. This gene was inserted into the pGRD4 launch vector and transformed into Agrobacterium tumefaciens. The transformed bacterium was introduced into hydroponically grown Nicotiana benthamiana by vacuum infiltration and leaf tissues were harvested, homoge-

nized, extracted, filtered and chromatographically purified after a one-week growing period [14]. Aliquots of purified HAC1 were kept in PBS at −80 °C until usage. For silica-NP, DMEM and SiO$_2$ nanopowder (HDK-200, Wacker Chemie, Germany) were mixed and dispersed by ultrasonic sonotrode. The production of c-di-GMP has been described before [15,16]. Lyophilized c-di-GMP was stored at −20 °C. Immediately prior to immunization, HAC1 was admixed with the adjuvant and/or silica-NP and swirled ≥ 10 min on an overhead shaker to ensure complete mixing.

2.4. Vaccination and sample collection

Mice were immunized on days 0 and 21 with either 5 μg antigen (HAC1), single- or double-adjuvanted vaccine (5 μg HAC1/10 μg SiO$_2$; 5 μg HAC1/7.5 μg c-di-GMP; 5 μg HAC1/10 μg SiO$_2$/7.5 μg c-di-GMP) by intratracheal route (50 μl). For intratracheal immu-

nization mice were tilted (~45°) and the vaccine administered into the deep lung with subsequent insufflation with an air bolus. A sys-

temic control group to ensure the effectiveness of the vaccination protocol, received 1 μg HAC1 adsorbed on aluminum hydroxide (Alum) intraperitoneally (200 μl). Blood was obtained by retro-

bulbar sampling and sera were collected on days 0, 21, 35, and 49 to determine HA-specific antibody response by hemagglutina-

tion inhibition (HAI) and enzyme-linked immunosorbent (ELISA) assays. On day 49, mice were sacrificed with an intraperitoneal overdosing of pentobarbital-Na (Merial, Hallbergmoos, Germany) and cutting the Vena cava inferior. BAL fluids, agarose-filled lungs, and spleens were sampled and used for immunoglobulin (Ig) mea-

surements and re-stimulation assays.

2.5. HAI assay

Collected sera were treated with a receptor-destroying enzyme (Denka Seiken, Japan). HAI assay was performed using 0.75% turkey erythrocytes and A/California/07/09 × 179A virus (CDC #2009713114) with an initial serum dilution of 1:20 as described previously [14]. HAI endpoint titers were determined as reciprocal
of the highest serum dilution causing complete HAI. Seroconversion in vaccinated animals was defined as an HAI antibody titer at a serum dilution of ≥1:40 [17,18].

2.6. Antibody measurement

HA-specific serum IgG antibodies and nasal IgG and IgA were assessed using ELISA assay, as previously described [19]. Briefly, ELISA plates were coated with inactivated A/California/07/09 virus and samples of BAL or serum were tested in series of two- or four-fold dilutions. Antigen-specific IgG and IgA were detected using horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibody (Jackson Immunoresearch Laboratories Inc., PA, USA), respectively. Endpoint titers were determined as reciprocal serum dilutions that gave mean optical density (OD) values three times greater than those from pre-immune sera at a 1:100 or 1:50 dilution for IgG and IgA, respectively.

2.7. Splenocyte proliferation assay

Isolated splenocytes (1 × 10^5 cells) of vaccinated mice were incubated in 96-well round bottom plates with RPMI supplemented with 5% fetal bovine serum, containing 10 μg/ml HAC1 or PBS (negative control) at 37 °C for 72 h. Proliferation was measured as described before [20]. Cells were pulsed with 5 μCi/ml ^3H-thymidine (Amersham Buchler, Braunschweig, Germany) for 18 h and harvested on filter mats (Canberra-Packard, Dreieich, Germany). A Topcount Microplate Scintillation Counter (Canberra-Packard, Dreieich, Germany) measured ^3H-thymidine-positive cells as counts per minute.

2.8. Antigen stimulation of precision-cut lung slices (PCLS)

Murine PCLS were prepared as described before [21,22]. Two PCLS (approx. 300 μm thick) per well were treated with 10 μg/ml HAC1 or medium (non-stimulated) and cultured under cell culture conditions (37 °C, 5% CO2 and 95% air humidity) for 24 h. Supernatant was collected and stored at −80 °C until use.

2.9. Cytokine measurement

Cytokines interleukin (IL)-2, interferon-gamma (IFN-γ), IL-5, and IL-10 in the supernatant of re-stimulated PCLS were measured using the murine Th1/Th2 tissue culture kit from Mesoscale Discovery (MSD) Assays (Gaithersburg, MD, USA). The assay was performed and results were analyzed according to manufacturer’s specifications using MSD plates, MSD Sector Imager 2400, and Discovery workbench software. Total protein concentrations were measured in PCLS lysates using the BCA Protein Assay kit (Pierce, Rockford, IL, USA) [12]. Cytokines were correlated to total protein (ng/mg) and compared to the non-stimulated cytokine baseline level as fold induction.

2.10. Statistical analysis

Statistical analyses were performed by either the Kruskal–Wallis test with Dunn’s multiple comparison post hoc tests or by the Mann–Whitney test using GraphPad 4.03 (GraphPad, San Diego, CA, USA). Data were expressed as mean ± standard error of the mean (SEM) or median ± quartiles. Differences between treatment groups and controls were considered statistically significant at p < 0.05. The number of mice is indicated in the figure legends.

3. Results

3.1. Intratracheal administration of double-adjuvanted HAC1 with SiO2 and c-di-GMP induced robust systemic antibody responses

As main readout parameters for a systemic antibody response HAI and HAC1-specific IgG titers were analyzed in the blood of vaccinated mice. The non-adjuvanted group vaccinated with HAC1 only did not develop detectable HAI or antigen-specific IgG antibodies in the serum (Fig. 1). On the contrary, administration of HAC1 intraperitoneally with Alum served as a positive control and induced very robust HAI (4096 ± 627.1; Fig. 1A) and IgG (286,720 ± 75,248; Fig. 1B) antibody titers after the second vaccination (day 35). Mice vaccinated with either
HAC1/SiO2 or HAC1/c-di-GMP developed low titers of HAI antibodies after the second vaccination (43 ± 30 and 12 ± 7; Fig. 1A), as well as modest serum IgG titers following the booster dose (205 ± 81 and 2980 ± 1419; Fig. 1B). The group receiving the double-adjuvanted vaccine, HAC1/SiO2/c-di-GMP, developed high HAI titers (770 ± 470; Fig. 1A) and antigen-specific IgG titers (43,840 ± 23,923; Fig. 1B).

### 3.2. Addition of c-di-GMP adjuvant was necessary to induce antigen-specific splenocytes

To further evaluate the systemic immune response following intratracheal vaccination, the proliferation index of splenocytes upon antigenic re-stimulation was assessed (Fig. 2). Splenocytes isolated from immunized mice were re-stimulated in vitro with HAC1 followed by 3H-thymidine labeling. The cell proliferation level was compared to non-stimulated splenocytes from the same animal. Results showed no detectable antigen-induced proliferation of splenocytes in the non-adjuvanted, positive control, or the HAC1/SiO2 groups (HAC1: 1.2 ± 0.1; HAC1-Alum: 1.5 ± 0.2; HAC1/SiO2: 1.2 ± 0.2).

In contrast, in the single-adjuvanted group (HAC1/c-di-GMP) the level of proliferation was two-fold compared to non-stimulated splenocytes (2.2 ± 0.4) and the double-adjuvanted vaccine induced the highest level of splenocyte proliferation (4.4 ± 1.7) upon HAC1 re-stimulation.

### 3.3. The double-adjuvanted vaccine induced local IgG and IgA antibody responses in the BAL

Local immune responses in the lung were assessed by measuring HA-specific IgG or IgA titers in BAL samples (Fig. 3A and B). The non-adjuvanted group vaccinated with HAC1 only did not develop detectable IgG or IgA in the BAL (baseline IgG/IgA level 25; Fig. 3A and B). In contrast, the positive control group (HAC1-Alum) showed antigen-specific IgG titers in the BAL (115 ± 37) comparable to the double-adjuvanted group, while IgA levels were undetectable. HAC1/SiO2 or HAC1/c-di-GMP did not induce detectable IgG or IgA in the BAL of immunized mice. However, addition of c-di-GMP to HAC1/SiO2 did induce detectable levels of IgG in 2/5 mice (115 ± 73; Fig. 3A) and in one mouse detectable levels of IgA (Fig. 3B).

![Fig. 2. Proliferation index of splenocytes upon re-stimulation with the recombinant HAC1 antigen. Spleen cells of vaccinated mice were isolated on day 49 and re-stimulated with 10 μg/mL HAC1 followed by labeling with 3H-thymidine. The proliferation indexes upon antigen stimulation and in the medium alone were compared. Data are presented as mean ± SEM. *p < 0.05 compared to the proliferation index of non-stimulated splenocytes from the same animal; Mann-Whitney test (n = 5). HAC1: plant-derived recombinant hemagglutinin protein, SiO2 = silica nanoparticles, c-di-GMP: bis-(3′,5′)-cyclic dimeric guanosine monophosphate, Alum: Aluminum hydroxide, i.t.: intratracheally, i.p.: intraperitoneally.](image1)

![Fig. 3. Titers of antigen-specific IgG and IgA in the bronchoalveolar lavage (BAL) fluid of vaccinated mice. BAL fluid from mice vaccinated with 5 μg HAC1 or 10 μg HAC1 was collected on day 49 and analyzed for HAC1-specific IgG and IgA (A and B). Data are presented as Boxplot and whiskers median ± quartiles (n = 5) and compared using the Kruskal–Wallis test and Dunn’s multiple comparison post-hoc test. *p < 0.05 and **p < 0.01, comparison to vaccination with antigen only. HAC1: plant-derived recombinant hemagglutinin protein, SiO2 = silica nanoparticles, c-di-GMP: bis-(3′,5′)-cyclic dimeric guanosine monophosphate, Alum: Aluminum hydroxide, i.t.: intratracheally, i.p.: intraperitoneally.](image2)

In order to ensure that the induction of mucosal IgA in the single positive mouse was a result of vaccination, mice were immunized with a higher antigen concentration (10 μg HAC1) and the BAL was examined for the presence of HAC1-specific IgG and IgA (Fig. 3A and B). The non-adjuvanted group (10 μg HAC1) showed no increased local IgG or IgA titers (Fig. 3A and B). One mouse given HAC1/SiO2 developed mucosal IgG titers above baseline (30 ± 5 vs. 25) while two mice developed detectable IgA (titre 45 ± 15 vs. 25). HAC1/c-di-GMP induced elevated titers of mucosal IgG (135 ± 68) and IgA (385 ± 172) with positive titers in 80% of the vaccinated mice. Mice receiving HAC1/SiO2/c-di-GMP developed enhanced levels of mucosal IgG (540 ± 271) and IgA (490 ± 283) in 100% of vaccinated mice. Additionally, doubling the antigen dose increased IgG by 4.3-fold (Fig. 3A).

### 3.4. Animals immunized with HAC1/SiO2/c-di-GMP showed a T-cell immune response upon antigenic re-stimulation

To determine the local antigen-specific T-cell-mediated immune response at the cytokine level, PCLS from vaccinated
mice were re-stimulated with HAC1. Cytokine secretion upon antigen stimulation was compared to the non-stimulated cytokine baseline level and expressed as fold induction. The non-adjuvanted group (HAC1 only) showed no altered IL-2 or IFN-γ expression upon antigen-stimulation compared to non-stimulated PCLS (fold induction ≤ 2; Fig. 4A and B). The positive control mice, however, secreted low levels of IL-2 compared to non-stimulated samples (fold induction 37 ± 35) but showed no increase in IFN-γ production (27 ± 27). Results also showed that in contrast to HAC1/SiO2 re-stimulation with HAC1/c-di-GMP did induce antigen-specific cells producing IL-2 and IFN-γ (155 ± 60 and 244 ± 118, respectively). Additionally, re-stimulation of PCLS from HAC1/SiO2/c-di-GMP vaccinated mice also induced IL-2 and IFN-γ (262 ± 132-fold and 275 ± 138-fold).

No induction of IL-5 secretion was detected in the non-adjuvanted, positive control or HAC1/SiO2 vaccinated groups upon antigenic re-stimulation (fold induction ≤ 2; Fig. 4C). However, the c-di-GMP-adjuvanted HAC1 antigen induced cells to secret slightly elevated levels of IL-5 upon HAC1 re-stimulation (2.2 ± 0.1 and 2.4 ± 0.1 for single- and double-adjuvanted, respectively) compared to non-stimulated PCLS.

The release of the anti-inflammatory cytokine IL-10 was at baseline levels in PCLS from the non-adjuvanted and positive control groups (fold induction ≤ 2; Fig. 4D) as well as HAC1/SiO2 immunized mice. In contrast, IL-10 levels were enhanced in PCLS samples from HAC1/c-di-GMP as well as HAC1/SiO2/c-di-GMP vaccinated mice, when re-stimulated with HAC1 (12 ± 4 and 7 ± 2, respectively).

4. Discussion

The present study evaluated the systemic and local immunogenicity of a double-adjuvanted influenza vaccine (HAC1/SiO2/c-di-GMP) delivered via the respiratory tract. The vaccine is intended to be used as an inhalable needle-free vaccine targeting the upper and lower respiratory tract. However, for the work described here, we administered the vaccine intratracheally as a practical alternative to evaluate effects of the vaccine in the deeper lung before conducting an inhalation study prior to the challenge experiments. Minne and colleagues described the impact of vaccine delivery site on the immune responses and concluded that targeting the lower lungs for an inhaled influenza vaccination can induce systemic and local immune responses most efficiently [23].
Recent results with the NP-admixed antigen in a human lung tissue model showed that HAC1/siO₂ was able to re-activate formerly primed T-cells [12]. Even though HAC1/siO₂ had a re-activating potential in human PCLS, vaccination of mice intratracheally was barely able to induce seroprotection (HAI titer >1:40). Moreover, it did not induce any local immune response, such as antigen-specific Ig secretion or T-cell induction upon re-stimulation, when administered at a lower antigen dose (5 μg HAC1). However, addition of the mucosal adjuvant c-di-GMP to HAC1/siO₂ induced HA and IgG antibodies and T-cells that are considered potential markers for systemic and local protective immune responses against influenza importation. In no adverse side effects or clinical signs of decreased well-being of the study animals were observed after intratracheal administration of the double-adjuvanted vaccine. These increased antigen-specific immune responses demonstrated the synergistic effect of the combination of nontoxic concentrations of siO₂ and c-di-GMP and were in line with the work of Svindland et al. [9]. Although mucosal IgG and IgA were induced by the single-adjuvanted vaccine HAC1/c-di-GMP, a higher antigen dose was required. With regard to the shortage of vaccine supply during influenza pandemics and the focus on new vaccine strategies on antigen dose-sparing formulations, the double-adjuvanted vaccine showed promising results as it provided a dose-sparing effect. However, 10 μg of antigen were required to induce local IgG and IgA in 100% of the vaccinated mice. At a first view, systemic vaccination seemed to be more effective than local vaccination of the antigen dose required to induce systemic HA and IgG titers. On the contrary, 1 μg HAC1 given systemically was not sufficient to induce local IgA titers. In fact, this study was not designed to compare dose-sparing effects of local versus systemic applications, but rather to evaluate an additive effect of combined adjuvants. The systemic administration was only used as a control for the vaccination protocol as well as antigen stability and not meant as a comparative group to evaluate superior efficacy of the respiratory vaccination to the systemic vaccination.

The importance of mucosal IgA during influenza infection and its ability to neutralize virus in infected epithelial cells has previously been shown [24,25]. Also the role of IgA in cross-protection against drifted virus strains has been shown to contribute to protection, albeit it is not essential [26,27]. New insights into immune protection have altered second generation influenza vaccines from being designed to induce systemic IgG toward the induction of broader cross-protective responses against the virus, including other antibody isotypes, such as IgA. This new protection strategy combines the induction of systemic and local as well as humoral and cellular immune responses [25]. In this study, the double-adjuvanted vaccine demonstrated the ability to induce systemic functional antibody responses as well as local cellular immune responses suggesting the advantage of combining proper adjuvants and the relevance of immunizing at the site of infection.

Even though a challenge study would be necessary to prove that the local and systemic immune responses observed here can provide protection against influenza virus infection, there is convincing evidence in the literature that the measured immune responses discussed above have been linked to protective efficacy [28–30]. For example, Liu et al. compared different routes of immunization and their effect on local and systemic immune responses and combined this with lung protection against an influenza infection [29]. Their results regarding the induction of mucosal IgA, serum IgG and systemic HA titers after vaccine administration into the lower airways of the lung were in line with the results presented above. They detected only in the primed intrapulmonary immunization mucosal sIgA in the lung, but not the intramuscular administration. Furthermore, they observed the highest nasal and lung IgG titers in mice primed (and boosted) via the mucosal route [29]. Of note, the challenge study performed by Liu et al. demonstrated that the immune responses induced by the adjuvanted intrapulmonary vaccination were sufficient to protect against influenza virus infection and reduce the virus titers in the lung post infection to levels below detection [29].

It has been shown previously that intranasal administration of c-di-GMP as an adjuvant for influenza vaccines can induce multifunctional influenza-specific CD4⁺ Th1 cells in the spleen of immunized mice [8,9]. Furthermore, multifunctional Th1 cells have also been shown to be present in the blood of vaccinated human volunteers and in the non-inflamed normal human lung tissue, as determined by their potential to produce IL-2, IFN-γ and/or TNF-α upon re-activation [31,32]. Consistent with the cytokine profile of influenza-specific multifunctional Th1 cells, our study showed increased IL-2 and IFN-γ levels in antigen re-stimulated PCLS of mice vaccinated with HAC1/c-di-GMP. The induction of Th1 cytokines in re-stimulated PCLS indicates that the antigen was recognized by HAC1-specific memory T-cells. These results are in line with the hypothesis by Jul-Larsen and colleagues who discussed that addition of an adjuvant improves the efficacy of HAC1 toward the induction of a robust T-cell response [32].

Additionally, our results aligned with previous studies on intranasally administered c-di-GMP showing an induction of a Th1-biased cytokine profile in re-stimulated splenocytes against target antigen [8,9,33]. Yet, our study also showed a mild induction of the Th2 cytokine IL-5 and the anti-inflammatory cytokine IL-10 in re-stimulated PCLS of intratracheally c-di-GMP-vaccinated mice. The fold induction of the Th1 cytokines for the double-adjuvanted vaccinated mice, however, far exceeded the level of Th2 cytokines that were induced (IFN-γ/IL-5; 119-fold; IFN-γ/IL-10; about 39-fold). Nevertheless, the double-adjuvanted vaccine, as well as the c-di-GMP admixed antigen, induced IL-10 secretion in PCLS upon antigenic re-stimulation which exceeded the non-stimulated IL-10 baseline level. Among other cytokines, IL-10 can be released by influenza-specific CD4⁺ memory T-cells and has been described as having a putatively crucial role in regulating inflammation during acute influenza infection [34]. The fact that the double-adjuvanted vaccine induced IL-10-competent cells might also contribute to a reduced level of inflammation in the lungs with repeated exposure to the virus post vaccination.

Overall, the data presented in the current study demonstrate that the double-adjuvanted HAC1 vaccine is immunogenic in the mouse model when administered intratracheally. Even though the protective efficacy of the double-adjuvanted HAC1 vaccine needs to be evaluated in a relevant animal model, the present study demonstrates that the double-adjuvanted HAC1 induces systemic functional antibody response as well as local humoral and cellular immune responses when administered via the respiratory tract, indicating potential for future needle-free vaccine applications.

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