Intranasal vaccination with an adjuvanted polyphosphazenes nanoparticle-based vaccine formulation stimulates protective immune responses in mice

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Abstract

The most promising strategy to sustainably prevent infectious diseases is vaccination. However, emerging as well as re-emerging diseases still constitute a considerable threat. Furthermore, lack of compliance and logistic constrains often result in the failure of vaccination campaigns. To overcome these hurdles, novel vaccination strategies need to be developed, which fulfil maximal safety requirements, show maximal efficiency and are easy to administer. Mucosal vaccines constitute promising non-invasive approaches able to match these demands. Here we demonstrate that nanoparticle (polyphosphazenes)-based vaccine formulations including c-di-AMP as adjuvant, cationic innate defense regulator peptides (IDR) and ovalbumin (OVA) as model antigen were able to stimulate strong humoral and cellular immune responses, which conferred protection against the OVA expressing influenza strain A/WSN/OVA$_I$ (H1N1). The presented results confirm the potency of nanoparticle-based vaccine formulations to deliver antigens across the mucosal barrier, but also demonstrate the necessity to include adjuvants to stimulate efficient antigen-specific immune responses.

Keywords: Nanoparticles; Polyphosphazenes; c-di-AMP; Mucosal; Influenza

Introduction

Despite the increasing knowledge about pathogens, their interaction with the host and the development of innovative prophylactic and therapeutic immune interventions, infectious

diseases still remain a major global health problem. Among different medical interventions, vaccination is the most valuable tool in order to not only prevent infectious diseases, but also contain their transmission in human and animal populations.[1, 2] However, for many pathogens vaccines are still not available. Another problem resides in the lack of acceptance by the public based on putative side effects and the missing awareness of the great threat posed even by the so-called childhood diseases. Currently, most vaccines are injected either intramuscularly, subcutaneously or intradermally. However, these conventional injection approaches show some disadvantages, which hamper broad acceptance and access to vaccination, particularly in developing countries. Among others, parenteral vaccine administration requires trained personnel and offers potential safety risks (*e.g*. needle-stick accidents, needle reuse, wound infection). In this context, easy-to-use and needle-free vaccination methods, such as mucosal vaccination strategies would be able to overcome these hurdles. Furthermore, mucosal immunizations also offer the possibility of self-administration, minimize the risk of cross-infections and show a better acceptance (*i.e*. better compliance) by the public, which makes them especial amenable for implementation in mass vaccination campaigns.[3] Moreover, in contrast to parenteral vaccination, immunization via mucosal routes can stimulate both systemic and mucosal immune responses. Mucosal immunity not only prevents disease but also infection thereby considerably reducing the risk of horizontal disease transmission from infected hosts to susceptible contacts.[4-6] However, targeting the mucosal inductive sites to induce immune responses at systemic and mucosal levels still represents a huge challenge. This holds especially true in the context of subunit vaccine candidates which show improved safety profiles but usually are only poorly immunogenic. In contrast, almost all mucosal vaccines currently approved are based on live attenuated pathogens showing strong immunogenicity but at the same time having the blemish of a potential reversion risk. On the other hand, mucosal subunit vaccines usually require increased antigen doses compared to parenteral vaccines in order to stimulate efficient immune responses, since part of the antigen will be lost when passing the mucosal barrier. Due to non-specific mechanisms, such as ciliary activity, mucosal enzymes and

extreme pH only reduced antigen quantities will get in contact with the antigen presenting cells (APCs) of the mucosa. These bottlenecks can be overcome, at least in part, by the use of adjuvants and nanoparticle-based carrier systems.[2, 7, 8] Such adjuvants can enhance the immunogenicity of antigens or protect them against degradation, thereby enhancing the potency of the vaccine and improving the quality and longevity of antigen-specific immune responses.[9] Nevertheless, despite the fact that different adjuvants are currently tested in clinical trials (e.g. the nanoemulsion W805EC (NanoBio Corporation, Michigan, USA), Flagellin (VaxInnate, New Jersey, USA)), only a few adjuvants are approved for human use, yet and except one, the orally delivered cholera toxin B subunit (cholera vaccine Dukoral®; Crucell, Leiden, Netherlands), all of them are administered via parenteral routes.[2, 10] For this reason, there is great interest in the development of new mucosal adjuvants and delivery systems. Therefore, the aim of the present study was to evaluate the potential of a new vaccine formulation based on the combination of three different adjuvants. Polyphosphazenes were used as nano carrier system for antigen delivery. They are high molecular weight polymers consisting of a long-chain backbone of alternating phosphorus and nitrogen atoms. Each of the phosphorus atoms has attached two organic side groups.[11] Because of this high amount of functional groups and the flexibility of the backbone chain, they can form water soluble polyplexes (nanoparticles (NPs)) with antigens under mild physiological conditions. Furthermore, polyphosphazenes allow a controlled release of the carried protein antigen due to their ability to degrade slowly in aqueous solutions.[11, 12] Thus, we demonstrated earlier that formation of polyphosphazenes with OVA resulted in spherical particles in the range of 0.7 to 3.0 µm in diameter with an incorporation efficacy of about 70%.[13] Such particles have been shown fostering the transport of antigens through the mucosa and facilitating the visibility for APCs.[11, 14, 15] Polyphosphazenes were shown to induce B cell and DC activation, caspase-1 dependent inflammatory cytokine production and recruitment of macrophages, DCs and lymphocytes to the place of infection (reviewed in [16, 17]). Furthermore, when combined with synthetic cationic IDR peptides, which are short derivatives of host defense peptides able to modulate the responses of DCs and other cells

of the adaptive immune system, antigen uptake and subsequent presentation to T cells was improved.[18-20] This leads to the stimulation of increased humoral and cellular responses when the adjuvants CpG and poly I:C, respectively, were included.[21, 22] However, neither CpG nor poly I:C have been licensed for human use yet (reviewed in [23, 24]). Consequently, we aimed in further improving the adjuvant system by including the strong mucosal adjuvant bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP). The STING agonist c-di-AMP promotes not only the stimulation of humoral responses, but also strong cellular immunity, encompassing mixed T helper (Th1/Th2/Th17) responses, and induction of multifunctional CD4 and CD8 T cells and cytotoxic T lymphocytes (CTL).[18, 25-27] Considering their specific molecular targets and the differences in their mode of action, this was expected to increase vaccine efficacy. To this end, mice were immunized by intranasal route using different polyphosphazene based vaccine formulations. Stimulated antigen-specific immune responses were characterized and vaccine efficacy was evaluated.

Methods

Mice

Female C57BL/6 (H-2b) mice 6–8 weeks of age were purchased from Harlan Germany and kept at the animal facility of the Helmholtz Centre for Infection Research (Germany) under specific pathogen-free conditions. All animal experiments in this study have been performed with ethical agreement by the local government of Lower Saxony (Germany) with the No. 33.11.42502-04-017/08.

Immunization protocol

Female C57BL/6 mice (n = 10) were immunized intranasally (i.n.) on day 0, 14, 28 and 62 using different vaccine formulations (Table 1, more details in supplementary materials).

Sample collection

Blood samples from immunized mice were taken from the retro-orbital complex on day −1, 13, 27, 61 and 81. Broncho-alveolar lavages (BAL), nasal washes and spleens were collected on day 81 (more details in supplementary materials).

Detection of antigen-specific antibodies by ELISA

OVA-specific antibodies in nasal washes, BAL and sera of individual animals were determined by ELISA as described previously (more details in supplementary materials). [18, 26]

Identification of multifunctional T cells and cytokine profiling by FACS staining

In order to evaluate the capacity of the different vaccine formulations to stimulate OVA-specific multifunctional T cells, splenocytes from immunized mice were taken and their capacity to produce different cytokines was evaluated by flow cytometry (more details in supplementary materials).

Determination of lymphocyte-mediated cytotoxicity *in vivo* (*In vivo* CTL)

In vivo CTL studies were performed in order to evaluate the capacity of the different vaccine formulations to stimulate antigen-specific CD8+ T cells (more details in supplementary materials).

Challenge studies

Groups (n=4) of female C57BL/6 (H-2k) were challenged 41 days after the last immunization with a sub-lethal dose ($2x10^3$ FFU) of the influenza strain A/WSN/OVA_I (H1N1). This virus expresses the OVA CD8 epitope OVA257-264 (SIINFEKL) embedded in the neuraminidase protein (more details in supplementary materials).[28, 29]

Statistical analysis

Statistical significance of the observed differences was analyzed using the one-way ANOVA test of the Graph Pad Prism 5 software for Windows (Version 5.04). Differences were considered significant at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***), respectively.

Results

Characterization of immune responses induced after vaccination

Humoral immune responses

The levels of OVA-specific antibodies obtained following i.n. vaccination of mice with the different nanoparticle based formulations with or without co-administration of c-di-AMP as adjuvant are shown in Figure 1. Immunization of mice with OVA + PCPP + c-di-AMP was most efficient approach, which led to the stimulation of strong antibody production (factor 3.1 and 2.75 as compared to OVA + poly I:C and OVA + c-di-AMP, respectively) followed by OVA + PCEP + c-di-AMP and OVA + IDR + PCEP + c-di-AMP (Figure 1, A). Interestingly, incorporation of IDR does not seem to have a further impact on antibody production (Figure 1, A). Although not statistically significant, incorporation of c-di-AMP seems to be more efficient in stimulating humoral immune responses when compared to poly I:C (Figure 1). Different Th cell responses were stimulated according to the vaccine formulation, as indicated by the observed IgG1/IgG2c ratios (Figure 1, B). Combination of OVA with either of the polyphosphazenes stimulated a Th2 dominated response, as did the formulation encompassing OVA + IDR + PCEP + poly I:C. In contrast, all the other vaccine formulations which encompassed c-di-AMP stimulated a more balanced Th1/Th2 response (Figure 1, B).

When analyzing OVA-specific IgA titers in mucosal secretions of vaccinated animals the strongest OVA-specific antibody production was observed in BAL of mice receiving OVA in combination with PCPP and c-di-AMP (3.25- and 7.35-folds higher than in mice receiving

OVA + poly I:C and OVA + c-di-AMP, respectively) (Figure 2, A). No beneficial effect was observed when mice were immunized with OVA co-administered with any of the other PCEP based formulations with respect to those animals receiving OVA protein co-administered with either poly I:C or c-di-AMP (Figure 2, A). Interestingly, only marginal titers of OVA-specific IgA were detected in mice receiving OVA alone or in combination with either PCEP or PCPP. This indicates the necessity of including an adjuvant in the vaccine formulations in order to stimulate efficient local mucosal immune responses (Figure 2). Similar results have been observed in nasal washes of immunized mice (Figure 2, B). Nevertheless, titers detected in the upper respiratory tract were decreased as compared to those obtained for the lower respiratory tract.

Cellular immune responses

Cytokine profiles and multifunctional T cells

The cytokine profiles observed following immunization via the i.n. route are in accordance to the humoral immune responses. The increased IL-4/IFNγ ratios stimulated in mice using OVA with either of the polyphosphazenes or OVA in combination with PCEP, IDR and c-di-AMP also indicated a Th2 dominated response (Figure 3, A). As expected, all other formulations incorporating c-di-AMP as adjuvant stimulated a mixed Th1/Th2/Th17 response (Figure 3, A). In addition, combination of OVA with either of the polyphosphazenes and c-di-AMP stimulated similar IFNγ+ CD8+ T cell responses as compared to OVA + c-di-AMP alone, whereas formulations encompassing OVA + polyphosphazenes without c-di-AMP stimulated only marginal CD8+ T cell responses. However, incorporation of IDR seems to further boost the stimulation of OVA-specific CD8+ T cell responses, as observed by intracellular cytokine staining (Figure 3, B) and ELISPOT assay (data not shown).

We further analyzed the quality of the stimulated antigen-specific T cell responses by evaluating their capacity to produce more than one cytokine, as it was shown that

multifunctional T cells are associated with enhanced protection against certain infections.[30-33] Immunization via the i.n. route using OVA + c-di-AMP alone or in combination with either of the polyphosphazenes efficiently stimulated multifunctional CD4+ T cells (Figure 4, A). The same is true when mice received OVA + c-di-AMP + PCEP + IDR. Thus, mice receiving OVA + c-di-AMP + PCEP and OVA + c-di-AMP + PCPP, respectively, showed significantly increased levels of bifunctional (IFNγ+/TNFα+, IFNγ+/IL-2+; with $P<0.001$ for PCEP and $P<0.01$ for PCPP), as well as trifunctional (IFNγ+/TNFα+/IL-2+; with $P<0.01$ for PCEP and $P<0.05$ for PCPP) antigen-specific CD4+ T cells (Figure. 4, A). In contrast, besides low levels of IFNγ producing cells, incorporation of poly I:C as adjuvant was ineffective in terms of stimulating bi- and trifunctional CD4+ T cells. Interestingly, although statistically not significant the vaccine formulations OVA + c-di-AMP + PCPP (48% bi- and 4% trifunctional CD4+ T cells) and OVA + c-di-AMP + PCEP + IDR (56% bi- and 5% trifunctional CD4+ T cells) seem to be slightly superior in terms of stimulating multifunctional CD4+ T cells, as compared to OVA + c-di-AMP alone (30% bi- and 3% trifunctional CD4+ T cells) and OVA + c-di-AMP + PCEP (36% bi- and 2% trifunctional CD4+ T cells), respectively (Figure 4, A). Similar results have been observed in terms of multifunctional antigen-specific CD8+ T cells. OVA + c-di-AMP in combination with either of the polyphosphazenes, and OVA + c-di-AMP + PCEP + IDR were the most efficient formulations in terms of stimulating multifunctional CD8+ T cells as indicated by increased levels of bi- (19%, 19% and 18%, respectively) and trifunctional (1% for both OVA + c-di-AMP + PCPP and OVA + c-di-AMP + PCEP + IDR) CD8+ T cells (Figure 4, B). However, only combination of OVA + c-di-AMP + PCPP and OVA + c-di-AMP + PCEP + IDR resulted in significantly increased numbers of bifunctional CD8+ T cells ($P<0.05$ and $P<0.01$, respectively) compared to all other groups, whereby only vaccination with OVA + c-di-AMP + PCEP + IDR stimulated also significantly increased numbers of bifunctioinal CD8+ T cells with respect to those obtained using OVA + c-di-AMP. Moreover, significantly increased levels of trifunctional CD8+ T cells were stimulated only in mice receiving OVA + c-di-AMP + PCPP ($P>0.05$) (Figure 4, B) as compared to all groups but OVA + c-di-AMP + PCEP + IDR. Thus, in contrast to what was observed for multifunctional

CD4+ T cells, these formulations performed not only better than the formulations incorporating poly I:C but also better than OVA + c-di-AMP alone (7% bi- and 0% trifunctional; $P<0.05$) (Figure 4, B). Therefore, incorporation of polyphosphazenes seems to be needed in order to stimulate improved quality of CD8+ T cell responses.

In vivo CTL

In order to further the immune response profiles stimulated by the different vaccine formulations, we performed an *in vivo* CTL study. In line with the results obtained for humoral and cellular responses, immunization of mice by i.n. route using OVA + c-di-AMP + PCPP or OVA + c-di-AMP + PCEP + IDR seem to be most efficient approaches for stimulating CTL responses (Figure 5). Thus, although differences were not significant compared to those observed in mice receiving OVA + c-di-AMP + PCEP and OVA with either of the adjuvants alone, stimulation of protective immune responses seem to be more robust as indicated by the smaller standard deviations (0.55 and 2.14 vs 6.31, 5.55 and 3.66). This assumption is further supported by the fact, that the differences observed with respect to mice immunized with OVA + poly I:C + PCEP + IDR were only statistically significant when compared with the results obtained following vaccination with OVA + c-di-AMP + PCPP and OVA + c-di-AMP + PCEP + IDR, respectively (Figure 5).

Protection against challenge with influenza strain A/WSN/OVA$_I$

We next investigated the protective capacity of the stimulated immune responses by infecting mice 41 days after the last immunization with a sub-lethal dose of the influenza strain A/WSN/OVA$_I$ (H1N1). In almost all groups the maximal weight loss was reached on day 7 after infection (Figure 6). Mice immunized with OVA + PCPP + c-di-AMP showed the highest level of immunity losing only 1% of weight and recovering within one day (Figure 6B). Similar results have been observed for mice receiving OVA + c-di-AMP alone (3.9%; Figure 6A), OVA + PCEP + c-di-AMP (3.9%) and OVA + c-di-AMP + PCEP + IDR (4.9%; Figure 6C). The

convalescence of these animals took longer (3-6 days) as compared to those immunized with OVA + PCPP + c-di-AMP (Figure 6B). Control mice receiving only PBS (7.5% weight loss) and animals vaccinated with OVA alone (11% weight loss), OVA with either of the polyphosphazenes alone (13.6% and 8% weight loss for PCEP and PCPP, respectively) or in combination with IDR, PCEP and poly I:C (11.6% weight loss) were least protected against the challenge with influenza (Figure 6, Table 2).

Discussion

Emerging immunization approaches, such as subunit and mucosal vaccines make essential the use of adjuvants. In this regards, purified antigens usually are very poorly immunogenic when given by mucosal routes. Furthermore, mucosal vaccination often requires high antigen doses, since only part of the antigen comes across the mucosal barrier. Thus, adjuvants able to improve the strength of antigen-specific immune responses and carrier systems enabling efficient delivery of the antigens to the APCs can overcome these hurdles. Micro- and nanoparticle-based delivery systems such as liposomes, proteasomes, solid lipid NPs, outer membrane vesicles or virus-like particles (VLPs) emerged as promising tools offering versatile possibilities to generate safe and efficient vaccines (reviewed in [34, 35]). The majority of the polymeric NPs currently used in vaccine applications are based on polylactic-co-glycolic acid (PLGA) and polylactic acid (PLA).[36, 37] Although these NPs were shown to improve vaccine performance, they still have some limitations such as limited protein release efficacy due to protein instability. Also, chemical modifications of PLGA aiming in more targeted delivery weren't as successful as expected (reviewed in [38]). In contrast, polyphosphazenes like PCPP and PCEP can be easily conformed into NPs using ionic complexation processes with benign agents in aqueous solutions. This in turn makes them superior to the hydrophobic PLGA NPs. Likewise, aqueous solutions are highly protein compatible, whereas formation of hydrophobic NPs requires the use of organic solvents or complex manufacturing equipment (reviewed in [16]). Many studies of polyphosphazenes clearly demonstrated their potential as adjuvants to enhance the magnitude and the quality of

antigen-specific immune responses, especially of the humoral arm of the immune system.[15, 39-43] Moreover, vaccine formulations containing PCPP have been reported to be safe and immunogenic in humans (reviewed in [14, 16, 44]). However, although PCPP is able to enhance both Th1 and Th2 immune responses, it favors the stimulation of a Th2 dominated immune responses.[43] In contrast, PCEP is known to stimulate a more balanced Th1/Th2 response as indicated by increased production of IgG2a antibodies, which is in line with the results obtained in the present study.[39, 45] However, their capacity to promote CTL responses is rather limited. Furthermore, the biological activity of polyphosphazenes seems to depend on different physico-chemical properties, such as conformation-activity and molecular weight-activity relationships as well as ionic sensitivity (reviewed in [14, 16]). Therefore, in order to achieve optimal results compatibility with a specific antigen and/or other adjuvants need to be investigated. For example, polyphosphazenes were already shown to be compatible with well-established adjuvants, such as CpG and poly I:C, resulting in improved immune responses.[21, 22, 46] Although it has been shown in clinical trials that CpG and poly I:C can improve antigen-specific immune responses, none of them has been licensed for human use yet (reviewed in [23, 24]). Therefore, in the present work we investigated the potential of a new adjuvant combination based on polyphosphazenes and the mucosal adjuvant c-di-AMP.

Immunization of mice by i.n. route with vaccine formulations based on polyphosphazenes in combination with c-di-AMP strongly enhanced antigen-specific mucosal immune responses, as indicated by the increased IgA titers detected in lung and nasal washes of immunized mice. Similar IgA titers have been observed in mice vaccinated by i.n. route with antigens in combination with PCPP and or PCEP and IDR.[21, 43] In agreement with what observed at mucosal level, at systemic level humoral and cellular immune responses were also improved following immunization with either of the polyphosphazenes combined with c-di-AMP as compared to vaccination with antigen and NPs alone. The strongest antibody production was observed in mice receiving the model antigen OVA in combination with PCPP and c-di-AMP. Interestingly, while PCPP favors the stimulation of Th2 dominated immune responses, this effect is compensated by the inclusion of c-di-AMP in the formulation, leading to a clear

stimulation of a strong Th1/Th2/Th17 mixed response.[18, 27]. Similar results have been obtained when PCEP was combined with c-di-AMP, suggesting that c-di-AMP rather than the polyphosphazenes is triggering the modulation of the antigen-specific Th2 dominated response towards a more balanced Th1/Th2 response. This is further supported by the observation that combination of PCEP with poly I:C and IDR did not result in the stimulation of increased IgG2c titers. These findings are in line with previously published data showing that combination of a RSV antigen with poly I:C, PCEP and IDR strengthen the magnitude of the antigen-specific immune responses but did not alter their quality.[21] In contrast, incorporation of c-di-AMP in vaccine formulations resulted in redirection with improved Th1 and CTL responses.[18, 25-27] Nevertheless, the modulation of the immune response towards a more balanced Th1/Th2 response pattern might also depend on other factors, among others the route of administration, the PCEP dosage and the intrinsic properties of the included antigen. Thus, when mice were immunized subcutaneously or intranasally using 50 µg of PCEP in combination with the influenza antigen X:31, PCEP stimulated enhanced production of IgG2a.[47] The same is true when mice received pertussis toxoid in combination with CpG, IDR and 10 µg of PCEP by subcutaneous route.[15]

However, only little is known about the quality of the stimulated cellular immune responses following mucosal or parenteral vaccination using polyphosphazenes as adjuvant carrier system. Therefore, we investigated the T cell responses stimulated after i.n. immunization with the different vaccine formulations. Interestingly, polyphosphazenes alone did not improve the quality of the antigen-specific T cell responses. Only PCPP seem to be able to stimulate minor numbers of bi-functional CD8+ T cells. Nevertheless, combination of polyphosphazenes with c-di-AMP seems to have a beneficial effect, leading to the stimulation of significantly increased levels of bi- ($P<0.001$ for PCEP and $P<0.01$ for PCPP) and even trifunctional ($P<0.01$ for PCEP and $P<0.05$ for PCPP) CD4+ and CD8+ T cells compared to the corresponding formulations without c-di-AMP. Incorporation of IDR also seems to further boost the stimulation of multifunctional T cells when formulated with PCEP and c-di-AMP. The obtained results are in line with previous observations showing that combination of

different adjuvants, such as CpG, poly I:C, c-di-nucleotides, IDR and polyphosphazenes resulted in immune responses not only strengthened in magnitude but also in quality.[20, 48-51] These synergistic effects most likely are based on the stimulation of different immune cells and signaling pathways by the different adjuvants. While CpG and poly I:C constitute TLR9 and TLR3 agonists, respectively, c-di-AMP act via STING, IDR interact with surface as well as intracellular receptors of APCs, and polyphosphazenes seem to induce the NLRP3 inflammasome. In this context, CpG, poly I:C, c-di-AMP constitute pathogen-associated molecular patterns (PAMPs) that provide a danger signal to APCs by interacting with their corresponding pathogen recognition receptors (PRRs). Polyphosphazenes on the other hand activate the inflammasome leading to DC activation and maturation, as assessed by upregulation of co-stimulatory molecules and cytokines and enhanced antigen presentation in the context of MHC molecules.[16, 52, 53] Importantly, without a co-delivered danger signal NPs cannot induce DC maturation, which in turn explains the week responses observed when mice were immunized with the model antigen OVA and either of the polyphosphazenes alone. Thus, danger signal plus antigen presentation (signal 1) plus expression of co-stimulatory molecules and cytokines (signal 2) efficiently activate adaptive immune responses such as CD4+ and CD8+ T cell responses. IDR on the other hand are recognized by G protein–coupled receptors resulting in the recruitment of APCs to the site of vaccine application and the activation of these cells (reviewed in [20]). Poly I:C signaling is primarily dependent on TLR3 and strongly drives both cell-mediated immunity and a potent type I interferon response.[53] Furthermore, when poly I:C was combined with an adjuvant formulation (cationic formulation; CAF05) containing a cationic liposome as delivery system and a synthetic mycobacterial cord factor as adjuvant, it was shown to efficiently stimulate also antigen-specific CTL responses in mice following intraperitoneal immunization, similarly to what we have observed after i.n. vaccination of mice.[54] However, in the present work combination of poly I:C with PCEP and IDR was less efficient in stimulating OVA-specific multifunctional CD4+ and CD8+ T cells, as compared to c-di-AMP. Moreover, this vaccine formulation was also less efficient in stimulating OVA-specific cytotoxic CD8+ T cells.

Consistent with these observations mice receiving the poly I:C encompassing formulation showed only minor levels of protection against a sub-lethal challenge with the influenza strain A/WSN/OVA_I (H1N1), whereas mice immunized with OVA in combination with either of the polyphosphazenes and c-di-AMP were protected most efficiently. This is further supported by the fact that the convalescence in the c-di-AMP groups was also considerable shortened as compared to that of mice receiving OVA alone, OVA in combination with NPs only or the formulation encompassing OVA together with PCEP, IDR and poly I:C. Interestingly, although combination of PCEP with c-di-AMP and IDR seem to be more efficient in improving the quality of the stimulated antigen-specific T cell responses, this did not provide an advantage in terms of protection. This might not be surprising as the correlate for protection against influenza infection currently is the hemagglutinin-specific antibody titer whereas influenza-specific T cell responses are only known to play a role in relieving the severity and duration of an influenza infection.[55] However, the ability of IDR to improve T cell response quality when incorporated in a polyphosphazene based vaccine formulation still makes them a promising adjuvant component for many infectious agents for which multifunctional T cells have been shown to play an important role in the control of the disease.[56-58]

Taken together, the results presented in this work have demonstrated that polyphosphazenes constitute a promising system for efficient antigen delivery across the mucosal barrier. The use of polyphosphazenes in combination with c-di-AMP and IDR not only improves the strength of the stimulated antigen-specific immune responses, but also their quality in terms of promoting a more balanced Th1/Th2 response pattern, as well as stimulation of multifunctional Th cells and CTL. Thus, the co-formulation of these three adjuvants overcome many of the limitations of most currently approved vaccines, making this approach especially suitable for development vaccines against resilient intracellular persistent pathogens. Similar effects have been obtained co-administering three, different TLR agonists MALP-2 (TLR2), poly I:C (TLR3), and CpG (TLR9) included in an HIV vaccine, resulting in increased protection of mouse against viral challenge.[59, 60] The results reported in the present work confirmed this hypothesis, demonstrating that more efficient adaptive immune responses are

induced, which conferred protective immunity against a recombinant influenza virus challenge.

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Figure caption

Figure 1. Systemic humoral immune responses stimulated in C57BL/6 mice after four immunizations with different OVA containing formulations via the intranasal (i.n.) route. (A) OVA-specific IgG titers in sera of mice 20 days after the last immunization. Differences between the group receiving OVA + PCPP + c-di-AMP and groups receiving OVA alone and PBS, respectively, were considered significant at $p<0.05$ (*). (B) OVA-specific IgG1 subclass in sera of immunized mice. Differences between IgG1 titers of the group receiving OVA + PCPP + c-di-AMP and groups receiving PBS, OVA alone and OVA in combination with either of the polyphosphazenes were considered significant at $p<0.05$ (*). Differences between

IgG1 titers of the group receiving OVA + PCEP + c-di-AMP and groups receiving PBS and OVA alone, respectively, were considered significant at $p<0.05$ (*). (C) OVA-specific IgG2c subclass in sera of immunized mice. Differences between IgG2c titers of the group receiving OVA + PCPP + c-di-AMP and groups receiving PBS, OVA alone, OVA in combination with either of the polyphosphazenes and the formulations encompassing IDR, respectively, were considered significant at $p<0.05$ (*). Results are expressed as average of the last dilution (end point dilution, e.p.d.) giving the double value (OD450 nm) of the background value (negative control). Bullet points indicate titers for individual animals of each experimental group. Standard error of mean (SEM) is indicated by vertical lines.

Figure 2. Mucosal immune responses stimulated in C57BL/6 mice after four immunizations with different OVA containing formulations via the i.n. route. OVA-specific IgA titers in BAL (A) and nasal washes (NW) (B) of mice measured 20 days after the last immunization. Results are expressed as average of the last dilution (end point dilution, e.p.d.) giving the double value (OD450 nm) of the background value (negative control). To compensate for variations in the efficiency of recovery of secretory antibodies among animals, the results were normalized and expressed as end point titers of antigen-specific IgA per µg of total IgA present in the sample. Standard error of mean (SEM) is indicated by vertical lines.

Figure 3. Cellular responses stimulated in C57BL/6 mice after i.n. immunization with different OVA containing formulations. Splenocytes from vaccinated animals were restimulated with 10µg/ml of OVA protein (A) and 10 µM OVA CD8+ peptide (SIINFEKL) (B) for 20 h. Cytokine profiles of antigen-specific CD4+ (A) and CD8+ T cells (B) stimulated by the different vaccine formulations were evaluated by intracellular cytokine staining. Results are expressed as average percentage (n=3) of all CD4+ and CD8+ T cells, respectively. Standard error of mean (SEM) is indicated by vertical lines.

Figure 4. Quality of the T cell responses stimulated following i.n. vaccination. Results are expressed as average percentage (n=3) of all IFNγ+ CD4+ and CD8+ T cells, respectively, acquired per sample (approx. 130,000 CD4+ cells). This subpopulation was further divided into mono-functional (IFNγ positive) (light gray), bi-functional (IFNγ / IL-2 or IFNγ / TNFα positive) (dark gray) and tri-functional cells (IFNγ, IL-2 and TNFα positive) (black). Differences are statistically significant with $P<0.05$ and $P<0.01$, respectively, with respect to, OVA + PCEP and OVA + PCPP (∗ indicating differences between values of bifunctional T cells and o indicating differences between values of trifunctional T cells. Standard error of mean (SEM) is indicated by vertical lines.

Figure 5. Lymphocyte-mediated cytotoxicity stimulated *in vivo* following i.n. vaccination of C57BL/6 mice. Results are expressed as percentage of specific killing. Differences are statistically significant with $P<0.0001$ with respect to OVA alone (∗), OVA + PCEP (+), OVA + PCPP (o) and with $P<0.05$ with respect to OVA + peptide + PCEP + Poly I:C (#).

Figure 6. Protection of vaccinated mice against influenza A virus infection. C57BL/6 mice (n=4) received four immunizations with different OVA encompassing formulations by i.n. route, whereas control animals received PBS. Mice were challenged with a sub-lethal dose ($2x10^3$ FFU) of the influenza strain A/WSN/OVA_I (H1N1) given by i.n. application 41 days after the last immunization. For a period of three weeks animal body weight was monitored. Results are expressed as average percentage of weight loss. (A) control groups, (B) PCPP encompassing formulations, (C) PCEP encompassing formulations.