Vaccine 37 (2019) 4963–4974

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The STING activator c-di-AMP exerts superior adjuvant properties than the formulation poly(I:C)/CpG after subcutaneous vaccination with soluble protein antigen or DEC-205-mediated antigen targeting to dendritic cells

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ARTICLE INFO

Article history:
Received 30 January 2019
Received in revised form 30 May 2019
Accepted 6 July 2019
Available online 15 July 2019

Keywords:
Adjuvant
c-di-AMP
Poly(I:C)/CpG
DEC-205
DC targeting
Subcutaneous route
Cell mediated immunity

ABSTRACT

Vaccination is the most efficient strategy to protect from infectious diseases and the induction of a protective immune response not only depends on the nature of the antigen, but is also influenced by the vaccination strategy and the co-administration of adjuvants. Therefore, the precise monitoring of adjuvant candidates and their immune modulatory properties is a crucial step in vaccine development. Here, one central aspect is the induction of appropriate humoral and cellular effector mechanisms.

In our study we performed a direct comparison of two promising candidates in adjuvant development, the STING activator bis-(3,5)-cyclic dimeric adenosine monophosphate (c-di-AMP) and the Toll-like receptor ligand formulation poly(I:C)/CpG. These were evaluated in C57BL/6 mice using the model antigen ovalbumin (OVA) in subcutaneous vaccination with soluble protein as well as in a dendritic cell (DC) targeting approach (αDEC-OVA). Strikingly, c-di-AMP as compared to poly(I:C)/CpG resulted in significantly higher antigen-specific IgG antibody levels when used in immunization with soluble OVA as well as in antigen targeting to DC. In vaccination with soluble OVA, c-di-AMP induced a significantly stronger CTL, Th1 and IFNγ-producing $CD8^+$ memory T cell response than poly(I:C)/CpG. The response was CTL and Th1 cell dominated, a profile shared by both adjuvants. In the context of targeting OVA to DC, c-di-AMP induced significantly increased Th1 and Th2 cell responses as compared to poly(I:C)/CpG. Interestingly, the Th1 response dominated the overall T cell response only when c-di-AMP was used, indicating a distinct modulatory property of c-di-AMP when the DC targeting immunization approach was exploited.

Taken together, we describe superior properties of c-di-AMP as compared to poly(I:C)/CpG in subcutaneous vaccination with soluble antigen as well as antigen targeting to DC. This indicates exceptionally effective adjuvant properties for c-di-AMP and provides compelling evidence of its potential for further adjuvant development, especially also when using DC targeting approaches.

Abbreviations: CDN, cyclic di-nucleotide; c-di-AMP, bis-(3′,5′)-cyclic dimeric adenosine monophosphate; c-di-GMP, bis-(3′,5′)-cyclic dimeric guanosine monophosphate; c-di-IMP, bis-(3′,5′)-cyclic dimeric inosine monophosphate; DDX41, DEAD (aspartate-glutamate-alanine-aspartate) box polypeptide 41; Poly(I:C), polyinosinic-polycytidylic acid; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1.

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https://doi.org/10.1016/j.vaccine.2019.07.019
0264-410X/

1. Introduction

Adjuvants are key components in vaccines that boost the potency and longevity of specific immune responses to antigens. They can also be exploited to modulate the immune responses elicited. Thus, the search for new and promising effective adjuvants plays a key role in vaccine development [1].

Toll-like receptors (TLR) play a crucial role in the activation of innate and adaptive immune responses and therefore represent effective targets for the design of vaccine adjuvants. Numerous adjuvant formulations are based on agonists for this class of pattern recognition receptors (PRR) and several TLR ligands are currently being evaluated in vaccine development [2]. Prominent examples are the TLR3 and TLR9 agonists polyinosinic-polycytidylic acid (poly(I:C)) and synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG), respectively. Both have been demonstrated to exhibit excellent adjuvant properties as indicated by the induction of strong antibody titers [3,4], a predominant Th1 T helper cell response [5–7] and cytotoxic T lymphocytes (CTL) [8,9]. Furthermore, poly(I:C) and CpG effectively trigger the activation of IL-12 and type I interferon production in dendritic cells (DC) [10,11] and their simultaneous administration was shown to have beneficial synergistic effects, resulting in superior adaptive immune responses [12–17]. Fairly recently, cyclic di-nucleotides (CDN) such as bis-(3,5)-cyclic dimeric guanosine, inosine or adenosine monophosphate (c-di-GMP, c-di-IMP and c-di-AMP, respectively) have moved to the focus of vaccine research. These molecules are ubiquitous signaling molecules of prokaryotes and have been described to exert strong adjuvant activities [18–24]. C-di-AMP was discovered as a secreted molecule of intracellular *Listeria monocytogenes* that triggers the host immune response [25]. It induces the production of type I interferons (IFN) through the activation of the PRR DDX41 (DEAD box polypeptide 41) and a cytosolic signaling axis composed of STING (stimulator of IFN genes), TBK1 (TANK-binding kinase 1) and IRF3 (IFN response factor 3) [26,27]; it also triggers expression of tumor necrosis factor (TNF) by a non-canonical induction pathway. As a mucosal adjuvant c-di-AMP promotes excellent humoral and cellular immune responses to model and disease related antigens and vaccines, such as Crucipain, in mice. Importantly, these responses are stronger than those induced by c-di-GMP or cholera toxin B adjuvants [19,21,24]. Mechanistically, DC and macrophages were identified as target cells of c-di-AMP, which induces surface expression of T cell co-stimulatory molecules and IFN-β production in these cells [28] and so far c-di-AMP adjuvant activity has been evaluated following mucosal or parenteral routes of administration [18,19,21,23,24] but not for subcutaneous (s.c.) applications.

Next to designing potent adjuvants, the choice of effective cellular targets is essential for successful vaccination. Here, DC as central players of the host immune response represent optimal target cells [29]. One of the most studied approaches involves *in vivo* targeting of specific surface receptors on DC by linking relevant antigens to receptor ligands or to receptor-specific antibodies. Over the last decades, more than 100 studies have evaluated a wide array of different receptors such as Fc receptors, CD40 or C-type lectin receptors (CLR) for their effectiveness in *in vivo* DC targeting [30]. In these, the CLR DEC-205 displays a prominent example. Antigen delivery to DC by means of DEC-205-directed (αDEC) antibody-antigen conjugates together with appropriate adjuvants (e.g. poly (I:C) alone or in combination with CpG) proved to be a powerful tool to elicit long-lived protective $CD4^+$ and $CD8^+$ T cell responses as well as antigen-specific antibody responses [7,31–34]. The effectiveness of DEC-205 targeting was confirmed for a variety of viral [35], bacterial [36], parasitic [37] and tumor antigens [33]. Importantly, we could recently show that targeting of DEC-205^+ DC displays an effective approach for the induction of anti-viral immunity in the liver [34], which is essential for designing effective vaccines protecting from viral hepatitis.

In our study we addressed the effectiveness of c-di-AMP as an adjuvant in s.c. vaccine administration in comparison to the formulation poly(I:C)/CpG frequently used in experimental models. Importantly, next to the administration of soluble antigen, we also evaluated its suitability for supporting *in vivo* DC targeting via DEC-205, which to our knowledge has not been investigated before. To this end, we s.c. vaccinated C57BL/6 mice with the model antigen ovalbumin (OVA) together with either c-di-AMP or poly(I:C)/CpG to directly compare the immunostimulatory properties of these adjuvant formulations. We show that c-di-AMP exhibits superior adjuvant activity as compared to poly(I:C)/CpG. Importantly, this also holds true for *in vivo* DC targeting through DEC-205. Our study thus identifies c-di-AMP as a promising vaccine adjuvant for subcutaneous antigen delivery and as well as for DEC-205-targeted antigen vaccination.

2. Materials and methods

2.1. Mice

Female, 6–8 week old C57BL/6 mice were purchased from Harlan Winkelmann (now Envigo; Borchen, Germany) and housed under specific pathogen-free conditions according to national and institutional guidelines. All experiments were approved by the local government agency (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit; file number 33.12-42502-04-10/0108).

2.2. Immunization protocols

Mice were immunized s.c. with 7 μg EndoGrade OVA (>98% purity) (Hyglos, Germany) together with 50 μg poly(I:C) (Invivogen, Germany; High Molecular Weight) and 50 μg CpG (5′ TCC ATG Acg TTC CTG Acg TT 3′, Eurofins MWG Operon, Germany) or together with 7.5 μg of c-di-AMP (BioLog, Bremen, Germany) in a total volume of 60 μl phosphate-buffered saline (PBS) on days 0, 14 and 28 (Fig. 1A). Purified rat DEC-205-specific IgG2a antibody (αDEC) was conjugated chemically to full-length OVA protein as previously described [34]. Mice were immunized with 30 μg αDEC-OVA or with 30 μg unconjugated αDEC antibody as a control together with 50 μg poly(I:C)/CpG or 7.5 μg c-di-AMP.

2.3. Sample collection

Blood samples were collected from the retro-orbital sinus on days −1, 13 and 27 and by cardiac puncture on day 42. Sera for the analysis of antigen-specific IgG were prepared as previously described [34]. For the isolation of splenocytes, organs were pooled for the experimental groups, transferred to IMDM (GlutaMAX-I) (Gibco) supplemented with 10% v/v FCS, 1% v/v penicillin/streptomycin and 0.25 mM 2-mercaptoethanol. Spleens were passed through a 100 μm cell strainer (BD Biosciences, USA), erythrocyte lysis was performed through osmotic shock (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2)) and splenocytes were resuspended in medium.

2.4. Detection of antigen-specific total serum IgG

Antigen-specific total IgG as well as IgG1 and IgG2c subclasses were determined in serum samples by enzyme-linked immunosorbent assay (ELISA) as previously described [34]. Antibody binding to coated antigen was assessed using biotin-conjugated goat α-mouse IgG (Sigma, Germany), goat α-mouse IgG1 and goat

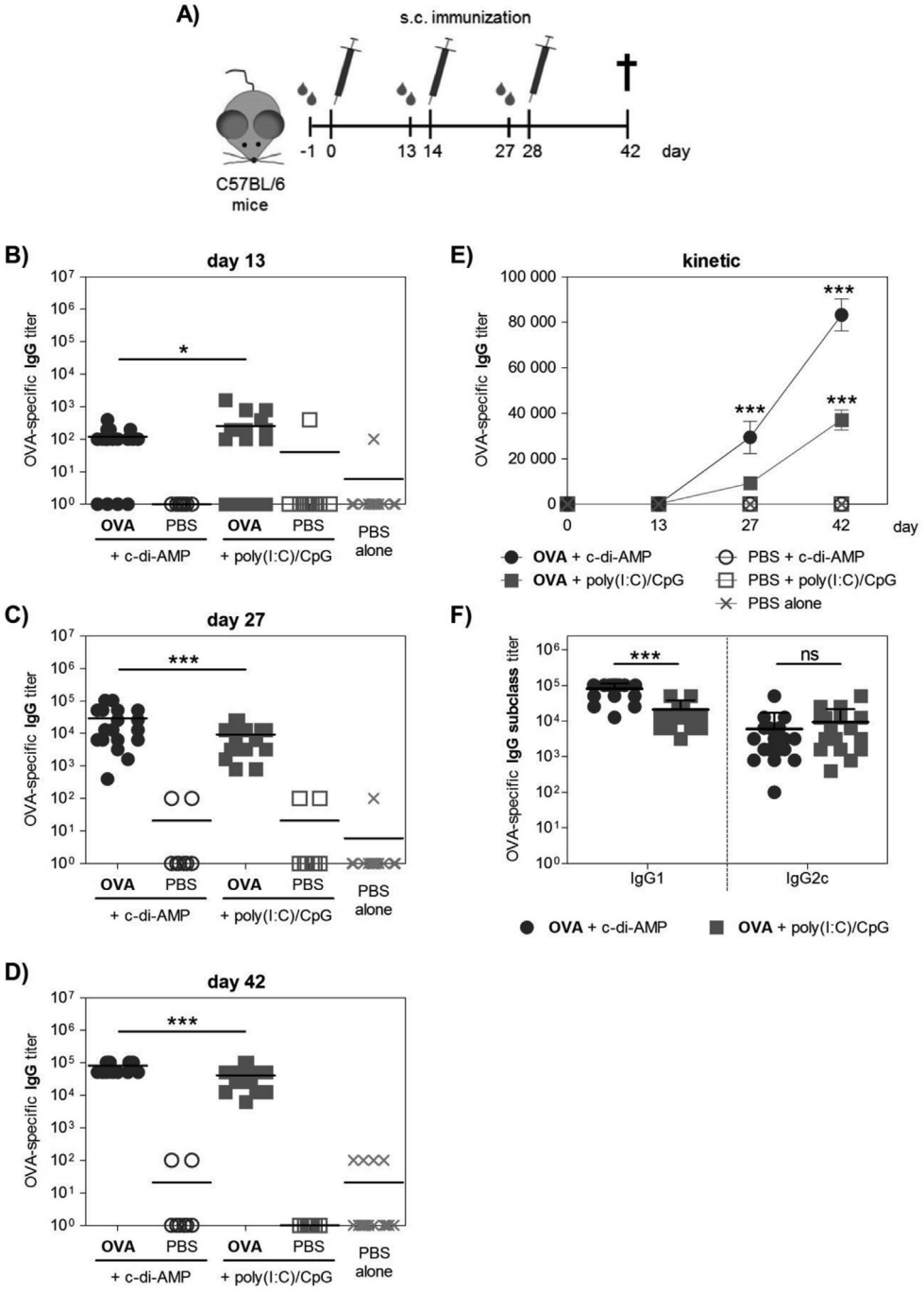

Fig. 1. Humoral immune responses following vaccination with soluble antigen and the adjuvants c-di-AMP or poly(I:C)/CpG. (**A**) Experimental plan. C57BL/6 mice (n = 5) were s.c. immunized with OVA + c-di-AMP or OVA + poly(I:C)/CpG or adjuvants alone on days 0, 14 and 28 (at least two independent experiments) and OVA-specific serum IgG titers were monitored. OVA-specific total IgG titers of individual mice as well as the mean are indicated for (**B**) day 13, (**C**) day 27 and (**D**) day 42. (**E**) shows the kinetic of OVA-specific total serum IgG titers (mean ± SEM). Statistical significance was determined using the one-way ANOVA followed by the Bonferroni test ($^{*}p < 0.05$; $^{***}p < 0.0001$). F) OVA-specific serum IgG1 and IgG2c subclass antibody titers of individual mice on day 42. Results (mean ± SD) were statistically analyzed using the two-way ANOVA ($^{***}p < 0.001$).

α-mouse IgG2c (Southern Biotech, USA) and Streptavidin-HRPO (BD Biosciences, Germany). Endpoint titers were expressed as the reciprocal value of the last serum dilution yielding an absorbance two-fold higher than that of negative controls.

2.5. Analysis of IFNγ- and IL-4-producing splenocytes by ELISPOT assay

Enzyme-linked immunosorbent spot (ELISPOT) kits for the detection of murine IFNγ (eBioscience, Germany) and IL-4 (BD Biosciences, Germany) were used according to the manufacturer's instructions. In brief, isolated splenocytes pooled for the experimental groups were cultured in the presence of either 5 μg/ml EndoGrade OVA protein (>98% purity) (Hyglos, Germany), 5 μg/ml of the immune dominant OVA peptides $CD4_{323-339}$ (ISQAVHAAHAEINEAGR) or $CD8_{257-264}$ (SIINFEKL) (synthesized in-house at the Helmholtz Centre for Infection Research, Germany) in triplicates (1×10^6 or 5×10^5 per well) for 18 h (IFNγ) or 36 h (IL-4). Positive spots were counted with an ELISPOT reader (C.T.L. Europe

GmbH, Germany) and analyzed using the ImmunoSpot image analyzer software v3.2 (C.T.L. Europe GmbH, Germany).

2.6. Analysis of IFNγ-producing CD44$^+$CD8$^+$ T cells by flow cytometry

Splenocytes were stimulated with a mixture of the OVA peptides $CD4_{323-339}$ and $CD8_{257-264}$ (5 μg/ml final concentration) or left unstimulated. After 2 h of incubation at 37 °C, 5 μg/ml Brefeldin A (Sigma, Germany) was added and cells were further incubated for 4 h. For flow cytometric analysis, splenocytes were stained for CD8 (clone 53–6.7) and CD44 (clone IM7) (BD Biosciences, Germany) following Fc-receptor blocking (anti-mouse CD16/CD36 antibody; BD Biosciences, Germany) and live/dead staining (LIVE/DEAD® Fixable Blue Dead Cell Stain kit; Life technologies GmbH, Germany). Cells were fixed (2% PFA/PBS), permeabilized using 0.1% Igepal® CA-630 in PBS (Sigma, Germany) and stained for intracellular IFNγ (clone XMG1.2) (eBioscience GmbH, Germany). Data were acquired on an LSR Fortessa instrument (BD Biosciences) and further analyzed using the FlowJo software 9.7.5 (Tree Star, USA).

2.7. Statistical analysis

Experimental results were statistically analyzed as indicated by one-way or two-way ANOVA followed by Bonferroni testing using the Graph Pad Prism 5 software (Graph Pad Software, La Jolla). A p-value below 0.05 was considered to indicate statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Upon s.c. protein immunization the adjuvant c-di-AMP induces stronger humoral immune responses than poly(I:C)/CpG

In order to directly compare the adjuvant properties of c-di-AMP to the well-established adjuvant formulation poly(I:C)/CpG, we analyzed their capacity to induce humoral immunity upon subcutaneous vaccination. To this end, C57BL/6 mice were immunized with either OVA + c-di-AMP or OVA + poly(I:C)/CpG or treated with the respective controls according to the vaccination regimen indicated in Fig. 1A. After we had experimentally proven that OVA protein without adjuvant does not induce specific IgG or T cell responses higher than that observed in PBS mock vaccinated animals (Supplementary Figs. 1 and 2), we decided to use PBS treated mice as the negative control indicating background responses. Regarding the adjuvants, a combination of 50 μg poly(I:C) and 50 μg CpG was chosen since these adjuvants are known to act synergistically with regard to immune activation and we and others have shown before that the selected dose induces robust humoral and cellular immunity in mouse vaccination approaches [12,14,16,17,33,34]. Finally, pilot experiments revealed 7.5 μg c-di-AMP to be sufficient to induce strong T and B cell responses in mice following s.c. vaccination (Supplementary Fig. 2) and was thus used for the study. Although the OVA-specific IgG titer was significantly higher in the OVA + poly(I:C)/CpG vaccinated group after the primary vaccination (day 13; Fig. 1B), the use of c-di-AMP as adjuvant resulted in a significantly higher OVA-specific IgG titer after the first (day 27; 3-fold increase, Fig. 1C) and second boost (day 42; 2-fold increase, Fig. 1D). Of note, every successive OVA + c-di-AMP vaccination resulted in a significant increase of the IgG titer as compared to the respective pre-vaccination serum antibody level (~3-fold increase from day 13 to day 27 and another ~3-fold increase from day 27 to day 42). In contrast, for the OVA + poly(I:C)/CpG formulation only the second boost vaccination on day 27 led to a significant further increase in the serum IgG titer (~4-fold increase from day 27 to day 42) (Fig. 1E). Both poly(I:C)/CpG and c-di-AMP strongly induced antigen-specific IgG1 and IgG2c subclass antibodies when co-administered with OVA (Fig. 1F). However, a significant (up to 4-fold) increase of OVA-specific IgG1 was observed in mice vaccinated with OVA + c-di-AMP as compared to OVA + poly(I:C)/CpG. At the same time, the IgG2c subtype titers were nearly unchanged between the vaccinated groups.

Taken together, these data indicated that the adjuvant capacity of c-di-AMP is superior to that of the combined TLR agonists poly(I:C) and CpG with respect to the induction of humoral immune responses following s.c. prime-boost vaccination.

3.2. Antigen-specific IFNγ-secreting effector CD4$^+$ and CD8$^+$ T cells are more efficiently induced through vaccination with c-di-AMP as adjuvant as compared to poly(I:C)/CpG

We next asked whether and how the nature of the induced antigen-specific cellular immune response would be affected by the different adjuvant formulations. Therefore, we quantified OVA-specific IFNγ- and IL-4-secreting T cells following *ex vivo* re-stimulation of splenocytes either with the full-length OVA protein or with the immune-dominant MHC class I (CD8) and II (CD4) OVA peptides two weeks after the third vaccination (day 42). As already observed for the antibody titers, vaccination with OVA + c-di-AMP showed a stronger potential to induce IFNγ-producing CD4$^+$ and CD8$^+$ effector T cells as compared to OVA + poly(I:C)/CpG (Fig. 2A–C). Of note, this observation was independent of the mode of splenocyte re-stimulation. While both adjuvant formulations also potently induced IL-4-secreting CD4$^+$ T cells (Fig. 2D, E), significantly more elevated numbers of IL-4$^+$CD4$^+$ T cells were observed following OVA + poly(I:C)/CpG as compared to OVA + c-di-AMP vaccination in response to *in vitro* re-stimulation with the OVA-CD4 peptide (Fig. 2E). In order to assess the adjuvants effects on the relative distribution of the induced CTL as well as T helper cell response, we compared the type of the T cell response detected in these analyses in more detail. Immunization using either adjuvant formulation predominantly induced CTL (IFNγ-producing splenocytes following MHC I (CD8) peptide stimulation) and Th1 (IFNγ-producing splenocytes following MHC II (CD4) peptide stimulation) responses as opposed to significantly lower levels of Th2 cells (IL-4-producing splenocytes following MHC II (CD4) peptide stimulation) (Fig. 2F).

In conclusion, our results demonstrate the effective induction of antigen-specific IFNγ- and IL-4-secreting T cells with an overall Th1- and CTL-dominated effector T cell profile for vaccination with both of the evaluated adjuvants. As compared to poly(I:C)/CpG, the adjuvant c-di-AMP proved to be superior in inducing IFNγ-producing Th1 cells and CTL, whereas poly(I:C)/CpG induced a stronger Th2 response following s.c. immunization.

3.3. Antigen-specific CD8$^+$IFNγ$^+$ memory T cells are more efficiently induced through vaccination with c-di-AMP as adjuvant as compared to poly(I:C)/CpG

Since both OVA + c-di-AMP and OVA + poly(I:C)/CpG immunization potently induced IFNγ-secreting T cells (Fig. 2A–C), we next evaluated the abundance of IFNγ$^+$CD44$^+$CD8$^+$ memory T cells induced following vaccination with either formulation. To this end, splenocytes isolated from immunized mice were antigen-specifically re-stimulated *in vitro* or left untreated and analyzed by flow cytometry. Independently of the applied adjuvant, OVA immunization resulted in a high abundance of antigen-specific IFNγ-producing CD44$^+$CD8$^+$ memory T cells that were entirely absent in the respective controls, i.e. solely adjuvant-treated mice and in unstimulated splenocytes (Fig. 3A). It is important to highlight that the frequency of IFNγ$^+$CD44$^+$CD8$^+$ memory T cells was

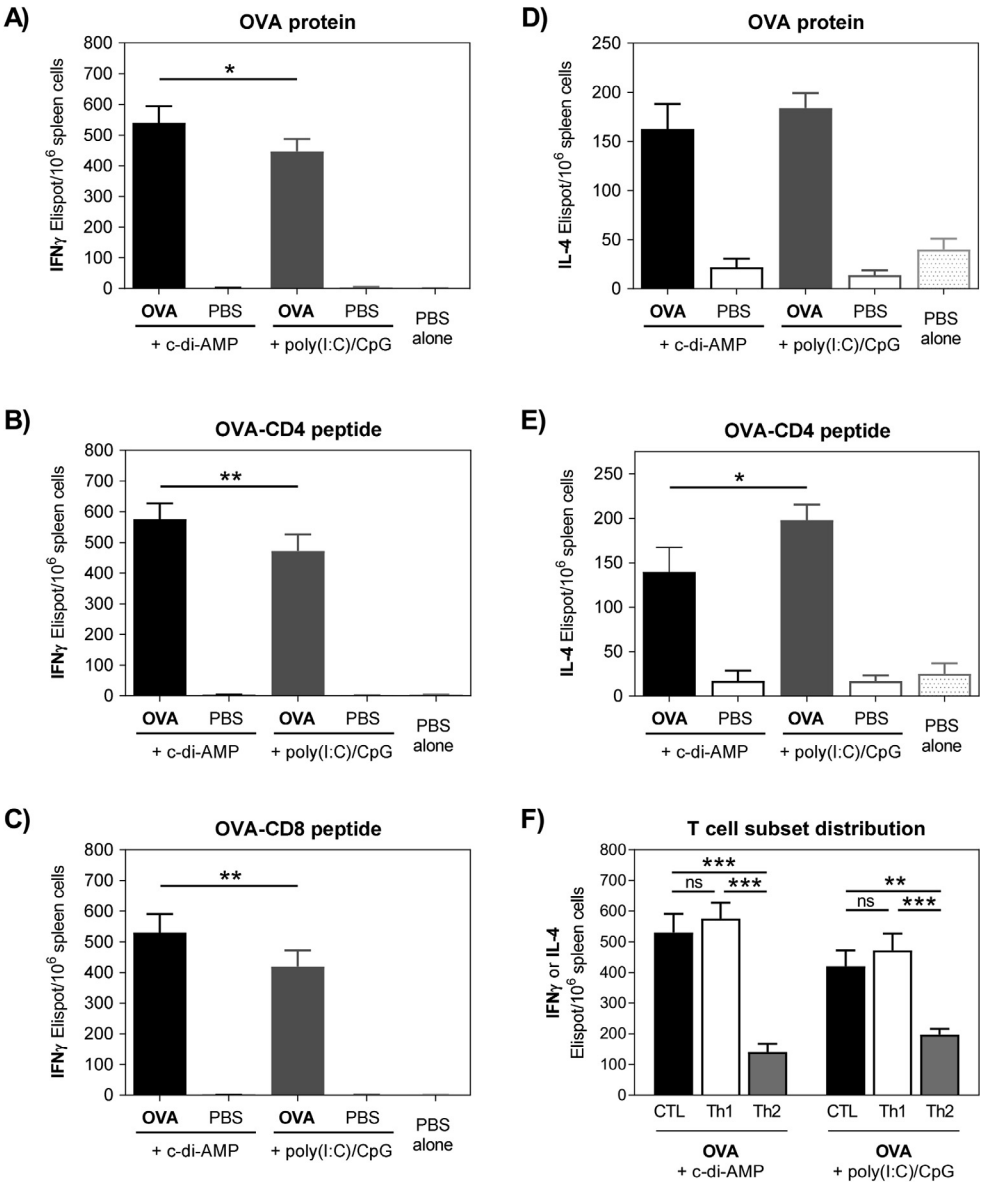

Fig. 2. Cellular immune responses following vaccination with soluble antigens and the adjuvants c-di-AMP or poly(I:C)/CpG. C57BL/6 mice (n = 5) were s.c. immunized as indicated on days 0, 14 and 28. On day 42, splenocytes were isolated, pooled for the experimental groups and re-stimulated with OVA protein **(A, D)**, OVA-CD4 peptide **(B, E)** or OVA-CD8 peptide **(C)** and ELISPOT assays for IFNγ **(A–C)** and IL-4 **(D, E)** were performed. Results are presented as spot forming units per 10^6 cells, from which the values obtained from non-stimulated samples were subtracted. The bars represent the mean ± SEM compiled from two independent experiments. **(F)** T cell subset distributions were evaluated from the results of the ELISPOT analyses. Black bars represent IFNγ-producing cells detected following re-stimulation with OVA-CD8 peptide (=CTL). White bars represent IFNγ-producing cells detected following re-stimulation with OVA-CD4 peptide (=Th1). Grey bars represent IL-4-producing cells detected following OVA-CD4 peptide stimulation (=Th2). Results were statistically analyzed using the one-way ANOVA followed by the Bonferroni test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$).

significantly higher in mice that were vaccinated with OVA + c-di-AMP as compared to those receiving OVA + poly(I:C)/CpG (Fig. 3B). This clearly indicated that as compared to poly(I:C)/CpG, the adjuvant c-di-AMP is superior at inducing IFNγ-producing memory $CD8^+$ T cells.

3.4. αDEC-OVA administered with c-di-AMP is superior at inducing humoral immunity as compared to αDEC-OVA administered with poly (I:C)/CpG

In vivo targeting of antigen to DEC-205^+ DC has been demonstrated to result in the potent induction of antigen-specific $CD4^+$ and $CD8^+$ T cells as well as humoral immune responses [33,34]. Based on our results obtained from s.c. vaccination with soluble OVA, we next sought to evaluate the adjuvant potential of c-di-AMP in an αDEC-205-based *in vivo* targeting approach in comparison to the previously used and well-established poly(I:C)/CpG formulation [35,36]. After successful chemical conjugation of the αDEC-205 (αDEC) antibody to the OVA antigen (αDEC-OVA) following a previously published protocol [34], mice were vaccinated s.c. with either αDEC-OVA + c-di-AMP or αDEC-OVA + poly(I:C)/CpG or treated with uncoupled αDEC in combination with the respective adjuvants as controls. Evaluation of the OVA-specific total IgG titer revealed only slightly increased antibody levels after the first vaccination (day 13, Fig. 4A), but strongly increased levels two weeks after the second (day 27, Fig. 4B) and third (day 42, Fig. 4C) vaccination in mice that received αDEC-OVA + c-di-AMP or αDEC-OVA + poly(I:C)/CpG. Interestingly, OVA-specific IgG levels were significantly increased following the first (Fig. 4B) and second boost (Fig. 4C) in mice vaccinated with αDEC-OVA

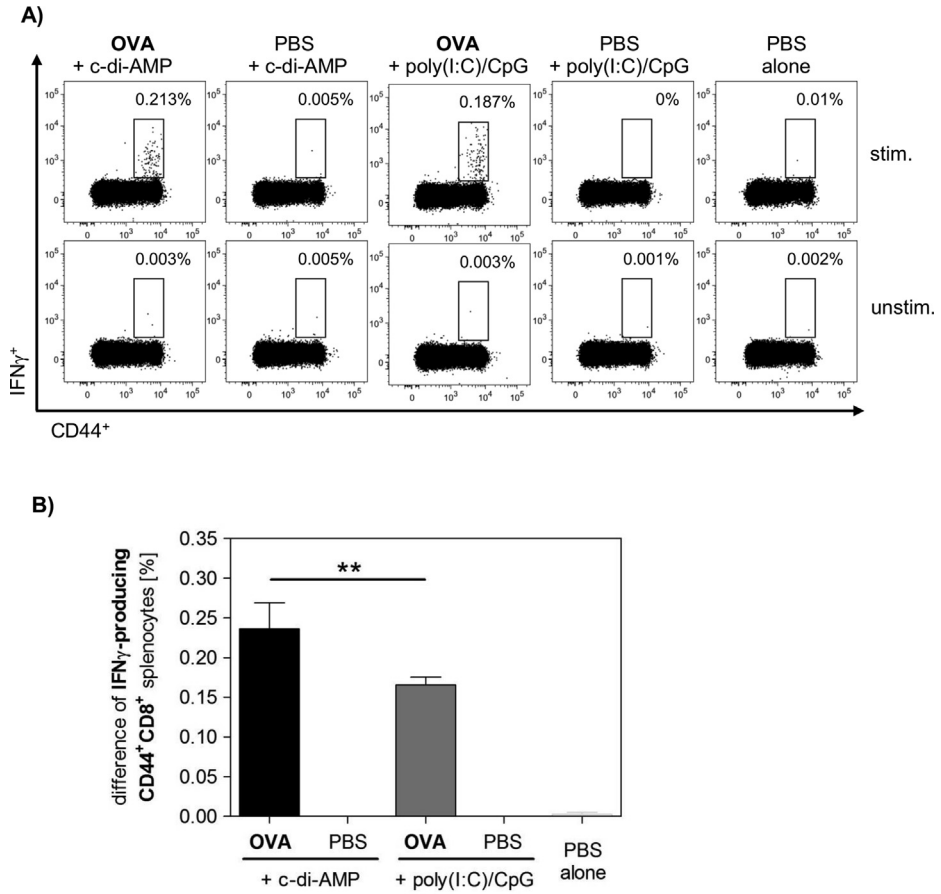

Fig. 3. Evaluation of IFNγ-producing $CD8^+$ memory T cells in mice vaccinated with soluble antigen using c-di-AMP or poly(I:C)/CpG as adjuvants. For flow cytometric analyses, splenocytes of s.c. immunized (as indicated; on days 0, 14 and 28) C57BL/6 mice (n = 5) were isolated on day 42, pooled for each experimental group and re-stimulated with a mixture of OVA-CD4 and OVA-CD8 peptides or left unstimulated. (**A)** Representative dot plots of $IFN\gamma^+CD44^+$ cells (gated on live, single $CD8^+$ splenocytes). (**B)** Bars (mean ± SEM, three independent experiments) represent the frequencies of $IFN\gamma^+CD44^+CD8^+$ cells following stimulation (background values obtained from non-stimulated cells were subtracted). Statistical significance was determined using the one-way ANOVA followed by the Bonferroni test ($^{**}p < 0.01$).

+ c-di-AMP as compared to the established adjuvant formulation poly(I:C)/CpG. In addition, only in mice that were vaccinated with αDEC-OVA + c-di-AMP a significant increase of total OVA-specific serum IgG was observed after the first (day 13 to day 27) and second (day 27 to day 42) boost vaccination (Fig. 4D). Analyses of the IgG subclasses IgG1 and IgG2c revealed significantly higher IgG1 titers following αDEC-OVA + c-di-AMP compared to αDEC-OVA + poly(I:C)/CpG vaccination (~3.5 fold increase), but comparable levels of serum IgG2c in both experimental groups (Fig. 4E). Of note, this observation was similar to the results from the previous vaccination experiments with soluble OVA (Fig. 1F).

Taken together, as was already observed for OVA protein alone, the adjuvant capacity of c-di-AMP was significantly increased with respect to the induction of antigen-specific IgG and IgG1 subclass responses as compared to poly(I:C)/CpG in *in vivo* DC targeting via DEC-205.

3.5. αDEC-OVA administered with c-di-AMP is more effective with respect to the induction of $IFN\gamma^+$Th1 and IL-4^+Th2 cells than αDEC-OVA administered with poly(I:C)/CpG

Next to inducing a humoral immune response, *in vivo* targeting of antigens to DEC-205^+ DC in combination with adjuvants that induce DC maturation and co-stimulatory capacity efficiently induces cellular immune responses [31,32,34,35]. Thus, we next asked whether as compared to poly(I:C)/CpG, αDEC-OVA administered with c-di-AMP would also induce stronger CTL, Th1 and Th2 cell responses following s.c. prime-boost-vaccination. ELISPOT assays revealed the efficient induction of IFNγ-secreting T cells in both αDEC-OVA + c-di-AMP as well as αDEC-OVA + poly(I:C)/CpG immunized mice (Fig. 5A–C). Remarkably, more elevated numbers of IFNγ-secreting T cells were detected in the spleens of mice immunized with αDEC-OVA + c-di-AMP as compared to αDEC-OVA + poly(I:C)/CpG (Fig. 5A–C). This observed increase was strongest and statistically significant when splenocytes were re-stimulated with full-length OVA protein or the MHC II (CD4) peptide, i.e. for Th1 cells (Fig. 5A, B). Strikingly and in contrast to vaccination with soluble OVA (Fig. 2D, E), αDEC-OVA + c-di-AMP but not αDEC-OVA + poly(I:C)/CpG vaccination resulted in a pronounced induction of OVA-specific IL-4-secreting Th2 cells (Fig. 5D, E). The evaluation of the T cell subset distribution induced using either c-di-AMP or poly(I:C)/CpG revealed that c-di-AMP led to the induction of a clearly Th1 dominated profile in combination with less prominent but balanced CTL and Th2 immune responses (Fig. 5F). In contrast, the numbers of CTL and Th1 cells were equally high following αDEC-OVA + poly(I:C)/CpG vaccination, whereas negligible numbers of Th2 cells were induced.

In conclusion, c-di-AMP proved to be highly effective in stimulating T cell responses following αDEC-OVA vaccination as mirrored by the superior induction of antigen-specific IFNγ-producing Th1 cells as well as the induction of CTL and IL-4-producing Th2 cells. Furthermore, there was a distinct shift in the relative distribution towards Th1 cells in vaccination with αDEC-OVA + c-di-AMP.

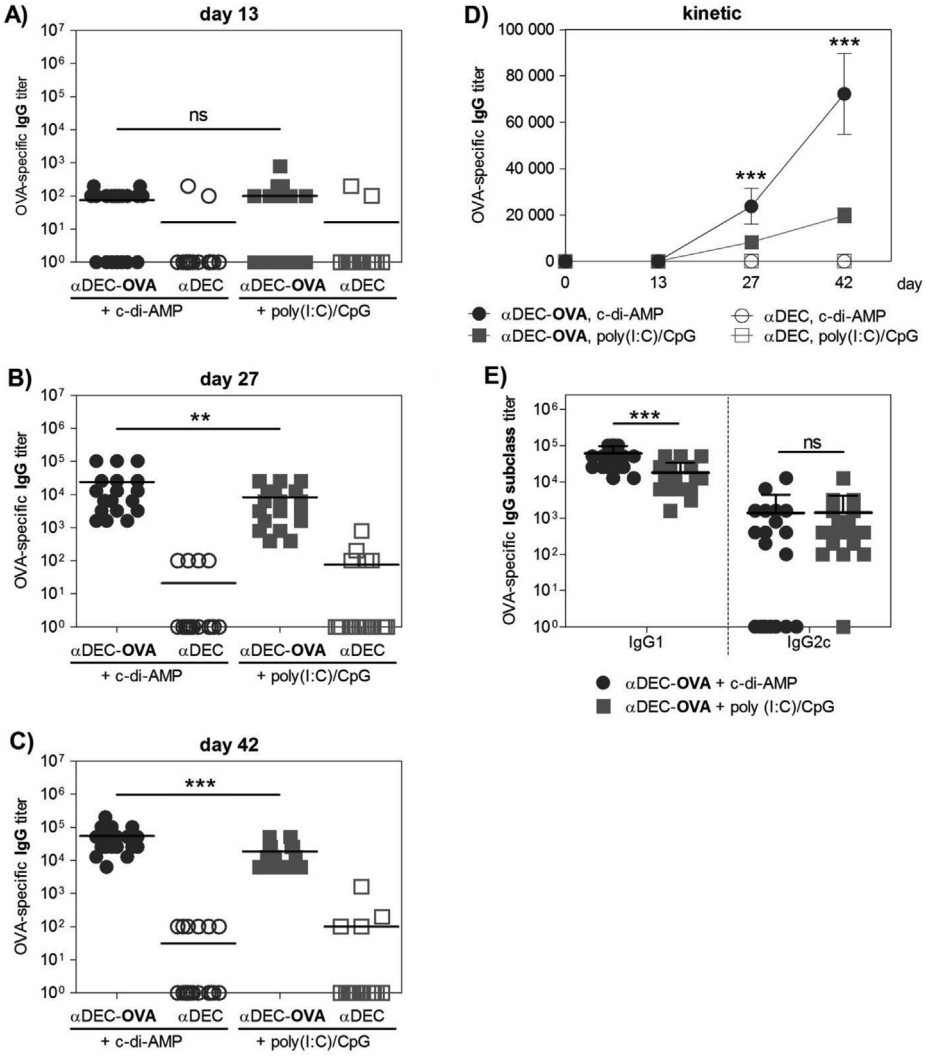

Fig. 4. Humoral immune responses following antigen targeting to DEC-205 using c-di-AMP or poly(I:C)/CpG as adjuvants. C57BL/6 mice (n = 5) were s.c. immunized with αDEC-OVA + c-di-AMP or αDEC-OVA + poly(I:C)/CpG or treated with uncoupled αDEC + adjuvants on days 0, 14 and 28 (four independent experiments) and OVA-specific serum IgG titers were monitored. OVA-specific total IgG titers of individual mice as well as the mean are indicated for (**A)** day 13, (**B)** day 27 and (**C)** day 42. (**D)** shows the kinetic of OVA-specific total serum IgG titers (mean ± SEM). Statistical significance was determined using the one-way ANOVA followed by the Bonferroni test (**$p < 0.01$; ***$p < 0.0001$). **(E)** OVA-specific serum IgG1 and IgG2c subclass antibody titers of individual mice as well as the mean ± SD on day 42. Results were statistically analyzed using the two-way ANOVA (***$p < 0.001$).

3.6. c-di-AMP and poly(I:C)/CpG are equally potent in inducing IFNγ⁺CD8⁺ memory T cells following vaccination with αDEC-OVA

We next compared the efficiency of memory T cell induction upon αDEC-OVA vaccination with either c-di-AMP or poly(I:C)/CpG. As expected, flow cytometric analyses revealed IFNγ⁺CD44⁺CD8⁺ memory T cells to be detectable exclusively following antigen-specific re-stimulation of splenocytes obtained from αDEC-OVA immunized mice, but not of splenocytes isolated from the control groups vaccinated with uncoupled αDEC or in untreated splenocytes (Fig. 6A). However, in terms of potential differences in the abundance of memory IFNγ-producing CD8⁺ T cells induced following prime-boost vaccination, αDEC-OVA administered with poly(I:C)/CpG induced slightly more elevated numbers of IFNγ-producing memory CD8⁺ T cells as compared to mice vaccinated with αDEC-OVA + c-di-AMP (Fig. 6B). This difference did however not reach statistical significance. Thus, we conclude that upon *in vivo* antigen targeting to DEC-205⁺ DC, c-di-AMP and poly(I:C)/CpG induce IFNγ-secreting memory CD8⁺ T cells nearly to the same extent.

4. Discussion

In our study we compared the efficacy of the recently discovered vaccine adjuvant c-di-AMP to the well-established TLR agonist formulation poly(I:C)/CpG in two different experimental vaccination approaches: in mice, we applied both adjuvants for s.c. vaccination using either the soluble model antigen OVA or αDEC-OVA for *in vivo* DC targeting. In the latter, OVA is chemically conjugated to an antibody specific for the CLR DEC-205. DEC-205 was first described to be expressed on DC and thymic epithelial cells and later weak DEC-205 expression was also demonstrated for murine B cells, T cells and granulocytes. Notably, DEC-205 is particularly highly expressed on certain DC subsets including CD8⁺ resident DC from lymphoid tissue (reviewed in [38,39]). Interestingly, a unique CD8$^{neg/low}$ DEC-205⁺ DC population has been identified in murine lymph nodes [40]. Further studies revealed that DEC-205 expression can be induced on CD8⁻ DC, which in their immature stage are negative for this marker, during their induced maturation [41,42]. To our knowledge no publication exists to date demonstrating DEC-205 expression on murine

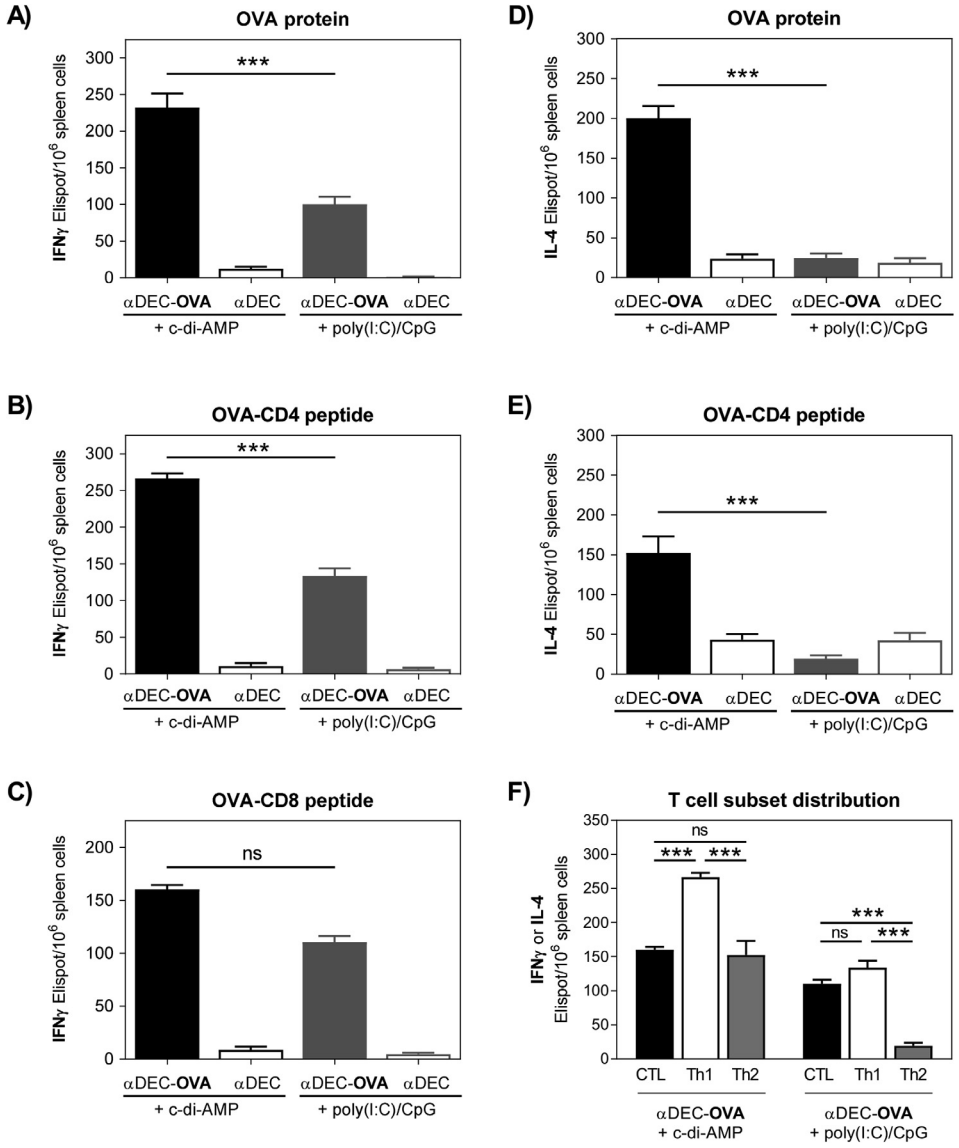

Fig. 5. Cellular immune responses following antigen targeting to DEC-205 using c-di-AMP or poly(I:C)/CpG as adjuvants. C57BL/6 mice (n = 5) were s.c. immunized as indicated on days 0, 14 and 28. On day 42, splenocytes were isolated and re-stimulated with OVA protein **(A, D)**, OVA-CD4 peptide **(B, E)** or OVA-CD8 peptide **(C)** (in triplicates) and ELISPOT assays for IFNγ **(A–C)** and IL-4 **(D, E)** were performed. Results are presented as spot forming units per 10^6 cells, from which the values obtained from non-stimulated samples were subtracted. The bars represent the mean ± SEM compiled from two independent experiments. **(F)** T cell subset distributions were evaluated from the results of the ELISPOT analyses. Black bars represent IFNγ-producing cells detected following re-stimulation with OVA-CD8 peptide (=CTL). White bars represent IFNγ-producing cells detected following re-stimulation with OVA-CD4 peptide (=Th1). Grey bars represent IL-4-producing cells detected following OVA-CD4 peptide stimulation (=Th2). Results were statistically analyzed using the one-way ANOVA followed by the Bonferroni test ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$).

plasmacytoid (p)DC and therefore murine pDC are considered to be DEC-205$^-$. Thus, while we cannot exclude that the observed effects following αDEC-OVA vaccination in our study are at least in part due to cells other than CD8$^+$ conventional DC, these cells are generally accepted to be the major targets in *in vivo* αDEC-205-mediated vaccination approaches.

Our study revealed that as compared to poly(I:C)/CpG, c-di-AMP co-administered with soluble antigen is of superior efficacy in terms of inducing both humoral and cellular immune responses. We detected high and with every boost steadily and significantly rising OVA-specific IgG titers in the sera of OVA + c-di-AMP immunized mice (Fig. 1C, D, E), which is in line with previous reports by other groups [19,24]. While poly(I:C) and CpG are well-known for their potential to induce antigen-specific IFNγ$^+$ effector T cells following vaccination [5–9], our data uncovered a superior efficiency of c-di-AMP in this respect (Fig. 2A–C). Even though higher numbers of IL-4-producing Th2 cells were observed in the poly(I:C)/CpG-adjuvanted group (Fig. 2D, E), both adjuvant formulations overall induced an IFNγ- (CTL, Th1) rather than IL-4- (Th2) dominated cytokine profile in T cells (Fig. 2F). While this is well in line with the described adjuvant effects of poly(I:C)/CpG [5–9], inconsistent data have been published regarding the nature of effector T cells induced by c-di-AMP adjuvanted vaccine formulations. A balanced Th1/Th2 response was observed upon administration of c-di-AMP as a mucosal adjuvant after intranasal vaccination using ß-galactosidase as model antigen [19], whereas a Th1 (IFNγ, IL-2) dominated cytokine profile was stimulated using the nucleoprotein of the influenza virus as antigen by the same administration route [24]. Moreover, a non-invasive transcutaneous c-di-AMP adjuvanted vaccination approach for OVA resulted in a Th2 cytokine dominated response, which was shifted to a balanced Th1/Th2 pattern when the antigen was packed in nanoparticles [23].

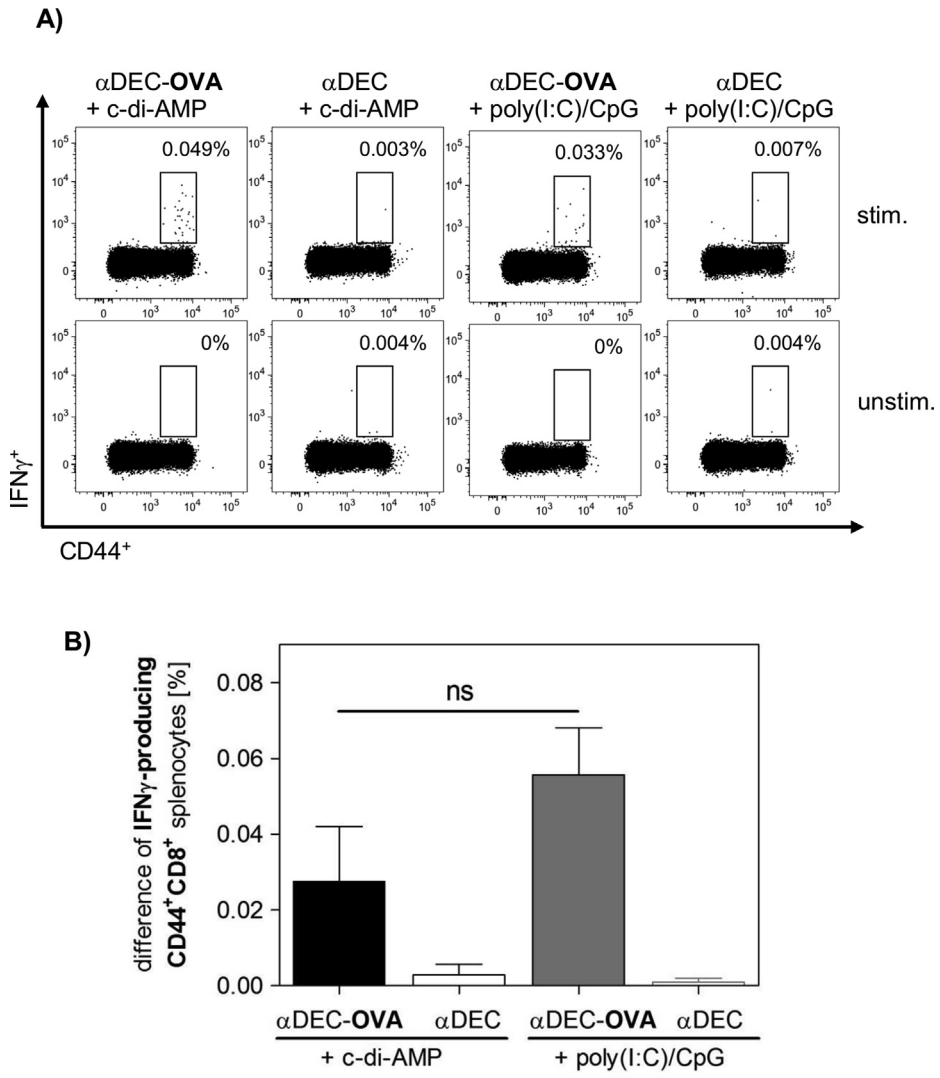

Fig. 6. Evaluation of IFNγ-producing $CD8^+$ memory T cells following antigen targeting to DEC-205 using c-di-AMP or poly(I:C)/CpG as adjuvants. For flow cytometric analyses, splenocytes of s.c. immunized (as indicated; days 0, 14 and 28) C57BL/6 mice (n = 5) were isolated on day 42, pooled for each experimental group and re-stimulated with a mixture of OVA-CD4 and OVA-CD8 peptides or left unstimulated. (**A**) Representative dot plots of $IFN\gamma^+CD44^+$ cells (gated on live, single $CD8^+$ splenocytes). (**B**) Bars (mean ± SEM, three independent experiments) represent the frequencies of $IFN\gamma^+CD44^+CD8^+$ cells following stimulation (background values obtained from non-stimulated cells were subtracted). Data were statistically analyzed using the one-way ANOVA followed by the Bonferroni test.

Next to experimental variations such as antigen and adjuvant concentrations or the nature of the antigen and delivery system, the route of administration certainly plays a critical role with respect to the quality and quantity of the induced immune responses [43]. We recently observed that in contrast to intramuscular immunization, three intranasal administrations of antigen adjuvanted with c-di-AMP were not sufficient to induce neutralizing antibodies [21]. In a c-di-GMP vaccination study, sublingual vaccine application was beneficial in inducing local as well systemic humoral and cellular immune responses as compared to the intranasal and intramuscular route, respectively [44]. To our knowledge this is the first study to show that the excellent adjuvant properties of c-di-AMP also hold true for the s.c. route of administration and that these effects are superior as compared to poly(I:C)/CpG. Most of the c-di-AMP adjuvant studies primarily focused on its high potential as a mucosal adjuvant [18,19,21,24]. Certainly, mucosal vaccination holds the advantage of improved general compliance, ease of administration and stimulation of local immune responses at the pathogen portal of entry [45]. Nevertheless, i.n. application has been associated for certain adjuvants with neurological side effects [46] and to date the application in clinical practice has been limited due to technical and logistic challenges [45]. Thus, it is important to emphasize the excellent adjuvant activity of c-di-AMP for the most commonly used s.c. route of vaccine administration.

Side-by-side comparisons of the c-di-IMP, c-di-GMP and c-di-AMP with gold standard adjuvants, such as the B subunit of the cholera toxin, LPS or alum suggested that CDN exert stronger adjuvant properties than many of the standard compounds [19,20,22,47]. Moreover, the adjuvant effects of c-di-AMP were equal or even stronger as compared to c-di-GMP (e.g. regarding the proliferative and cytotoxic capacity of T cells) [19]. In our study, we also detected superior adjuvant qualities of c-di-AMP compared to the frequently used TLR agonist formulation poly(I:C)/CpG. Even though both adjuvant formulations trigger the innate immune system, they act through different signaling pathways. Poly(I:C) and CpG are detected by endosomal TLR3 and TLR9, respectively, which triggers the induction of type I IFNs through the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent and myeloid differentiation primary response protein

88 (MyD88)-dependent signaling pathways, respectively [48]. Moreover, activation of multiple downstream pathways via poly (I:C) and CpG causes a synergistic effect in terms of activating DC and T cell mediated immune responses [12,14,16,17]. C-di-AMP acts through the PRR DDX41 and a cytosolic signaling axis composed of STING/TBK1/IRF3, thus utilizing an alternative signaling cascade that nevertheless also results in the activation of type I IFNs [26,27]. Thus, both pathways result in type I IFN production, which exert direct effects on $CD8^+$ T cells [49]. Moreover, type I IFNs inhibit Th2 cytokine production (IL-4 and IL-5), whereas at the same time stimulating IFNγ-production in $CD4^+$ T cells [50,51]. Moreover, c-di-AMP promotes a non-canonical activation of TNFα, which in turn is required for antibody responses [52]. Blaauboer et al. showed that c-di-AMP activates STING-dependent, IFN-I-independent TNFα production for cellular responses [52]. In contrast, Lirussi et al. showed that not TNFα but the strong STING-dependent stimulation of type I IFN represents a key feature, which is essential for the stimulation of CTL responses by cross priming as well as for optimal Th cell responses [53]. Of note, CDN promote a "self-limited" immune activation by targeting STING for degradation, which is in turn of high interest for the development of adjuvanted vaccines with regard to safety [54].

Taken together, this is generally well in line with our data (Fig. 2F). Both, c-di-AMP and poly(I:C)/CpG have been shown to exert immunostimulatory effects on DC [10,11,28]. The excellent performance of c-di-AMP as compared to poly(I:C)/CpG in vaccination with soluble OVA encouraged us to extend our studies to a vaccination trial focusing on *in vivo* antigen targeting to the DEC-205 endocytosis receptor on DC. As for the soluble antigen, c-di-AMP turned out to exhibit superior adjuvant capacities as compared to poly(I:C)/CpG, which was demonstrated by the more efficient induction of antigen-specific IgG (Fig. 4) as well as Th1 and Th2 cells (Fig. 5). This was in as much surprising as the adjuvant poly(I:C) has been most frequently used in DEC-205 vaccination studies, either alone [7,36] or in combination with αCD40 [32,35,37,55]. Importantly, in combination with DEC-205- targeted antigens these adjuvant formulations always resulted in a highly effective induction of antibody and T cell responses [7,32,33,35,36]. Moreover, Longhi et al. compared a number of different TLR ligands, including poly(I:C) (TLR3), MALP-2 (TLR2/6), Pam3cys (TLR1/2), LPS (TLR4), R-848 (TLR7/8) and CpG (TLR9) in an αDEC-205-based DC targeting approach and identified poly(I:C) as the most potent adjuvant [15]. In our study, we now identified c-di-AMP to be even superior to poly(I:C)/CpG with respect to the magnitude of B and T cell responses induced in an αDEC-205-based *in vivo* DC targeting regimen. A quantitatively stronger DC activation property displays one conceivable reason for the stronger adjuvant effects observed for c-di-AMP, although both c-di-AMP and poly(I:C) are capable to stimulate the induction of T cell co-stimulatory molecules (MHC II, CD80, CD86) on murine and human DC *in vitro* and *in vivo* [10,11,19,28], as well as to trigger their type I IFN production [26,27,48].

Previous studies reported αDEC-antigen vaccination to induce an overall Th1 cell-dominated immune response [7,15,35,37] in addition to CTL [31,34,35], regardless of the chosen adjuvant formulations, antigen or route of immunization, with OVA being described to induce an exceptionally strong $CD8^+$ T cell response in addition to $CD4^+$ T cells [31] (reviewed [38,56]). This is well in line with the results of our study in the case of αDEC-OVA + poly(I:C)/CpG immunized mice (Fig. 5F). In contrast, αDEC-OVA + c-di-AMP vaccination did not only induce Th1 cells and CTL, but also reasonable numbers of Th2 cells. This can be at least in part due to the capacity of c-di-AMP to promote also TNF-α induction. As such, αDEC-OVA + c-di-AMP was found to be superior to αDEC-OVA + poly(I:C)/CpG vaccination in terms of inducing balanced $CD4^+$ T cell responses. IL-4 has been shown to counteract CTL development [57], which is one possible explanation for the lower relative CTL frequency as compared to the Th1 subset in αDEC-OVA + c-di-AMP vaccination (Fig. 5F and 2F). Importantly, the nature of the induced T cell response strongly depended on whether c-di-AMP was used as an adjuvant in combination with a soluble antigen or with an antigen targeted to DEC-205^+ DC *in vivo*. The DEC-205 antibody used in this study particularly targets the cross-presenting $CD8^+$ DC subset that is known for its capacity to induce CTL and Th1 immunity [58]. Idoyaga et al. demonstrated that this effect is not only primarily dependent on the targeted DC subset but also influenced by the adjuvant, though independent from the targeted uptake receptor (e.g. DEC-205, Langerin or Clec9A) [55]. Further studies are needed in order to elucidate the mechanisms underlying the observed impact of c-di-AMP on the bias in the T cell responses induced by soluble as compared to DC-targeted antigen.

Yet another interesting aspect to be followed in the future is the suitability of the αDEC-antigen + c-di-AMP formulation for mucosal vaccination. Since c-di-AMP displays an excellent mucosal adjuvant [18,19,21,24] and the αDEC-205 targeting strategy also effectively induces mucosal immunity [59], the nature of immune responses induced following administration of αDEC-205-targeted antigens adjuvanted with c-di-AMP by the mucosal route is a promising field for future investigations.

While soluble OVA protein adjuvanted with c-di-AMP induced a significantly stronger memory T cell response than OVA + poly(I:C)/CpG (Fig. 3B), this was not the case when OVA was targeted to DEC-205^+ DC (Fig. 6B). Bonifaz et al. demonstrated that αDEC-205-mediated antigen targeting to DC in combination with αCD40 treatment led to a sustained memory $CD8^+$ T cell formation [31], which was later confirmed by Trumpfheller et al. [35]. Since IL-4 is known for its CTL counteracting potential [57], we may speculate that the higher proportion of IL-4-producing Th2 cells in the αDEC-OVA + c-di-AMP vaccinated group (Fig. 5E) is one possible reason for the similar size of memory $CD8^+$ T cell pool induced by the DEC-205 targeting approach but not following vaccination with soluble antigen. Here, OVA + poly(I:C)/CpG induced higher frequencies of Th2 cells (Fig. 2E), which indeed correlated with significantly reduced memory $CD8^+$ T cell formation (Fig. 3B). Certainly, further investigations are needed in order to elucidate the impact of c-di-AMP on the nature of effector and memory T cell responses in the context of αDEC-205-based vaccination in more detail.

In conclusion, we show for the first time that the CDN c-di-AMP is generally well suitable for s.c. vaccination and moreover exhibits superior adjuvanticity during s.c. vaccination as compared to the frequently used combined TLR ligands poly(I:C) and CpG. This holds true for its use both in combination with soluble protein antigen as well as with antigen conjugated to a DEC-205 antibody targeting DC *in vivo*. Thus, we suggest c-di-AMP to be considered as an exceptionally effective alternative to established adjuvant formulations for s.c. vaccination approaches. Furthermore, we clearly show that especially for applications where in addition to a strong antibody and CTL response not only Th1 but also Th2 cell responses are desired, DEC-205-targeted antibody-antigen conjugates adjuvanted with c-di-AMP need to be taken into account.

Declaration of Competing Interest

CAG and TE are named as inventors in a patent covering the use of c-di-AMP as adjuvant (PCT/EP 2006010693).

Acknowledgements

We thank Tatjana Hirsch, Silvia Prettin and Elena Reinhard for expert technical assistance. This work was supported by a grant

of the Helmholtz Association of German Research Centers (HGF) which was provided in frame of the Helmholtz Alliance "Immunotherapy of Cancers" (HCC_WP2b). Moreover, D.B. was supported by the President's Initiative and Networking Fund of the HGF under contract number W2/W3-029. The work was supported in part by the European Commission under the project "Univax" (contract no. 601738) and "TRANSVAC" (contract no. 228403).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.019.

References

[1] Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. Trends Immunol 2009;30:23–32.
[2] Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. Vaccine 2011;29:3341–55.
[3] Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.
[4] Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, et al. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005;79:2910–9.
[5] Stahl-Hennig C, Eisenblatter M, Jasny E, Rzehak T, Tenner-Racz K, Trumpfheller C, et al. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS Pathog 2009;5:e1000373.
[6] Stern BV, Boehm BO, Tary-Lehmann M. Vaccination with tumor peptide in CpG adjuvant protects via IFN-gamma-dependent CD4 cell immunity. J Immunol 2002;168:6099–105.
[7] Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, et al. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. Proc Natl Acad Sci USA 2008;105:2574–9.
[8] Fujimoto C, Nakagawa Y, Ohara K, Takahashi H. Polyriboinosinic polyribocytidylic acid [poly(I:C)]/TLR3 signaling allows class I processing of exogenous protein and induction of HIV-specific CD8+ cytotoxic T lymphocytes. Int Immunol 2004;16:55–63.
[9] Schwarz K, Storni T, Manolova V, Didierlaurent A, Sirard JC, Rothlisberger P, et al. Role of Toll-like receptors in costimulating cytotoxic T cell responses. Eur J Immunol 2003;33:1465–70.
[10] Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med 1999;189:821–9.
[11] Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998;28:2045–54.
[12] Grossmann C, Tenbusch M, Nchinda G, Temchura V, Nabi G, Stone GW, et al. Enhancement of the priming efficacy of DNA vaccines encoding dendritic cell-targeted antigens by synergistic toll-like receptor ligands. BMC Immunol 2009;10:43.
[13] Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK, et al. TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol 2011;186:2422–9.
[14] Liu C, Chu X, Sun P, Feng X, Huang W, Liu H, et al. Synergy effects of Polyinosinic-polycytidylic acid, CpG oligodeoxynucleotide, and cationic peptides to adjuvant HPV E7 epitope vaccine through preventive and therapeutic immunization in a TC-1 grafted mouse model. Hum Vaccin Immunother 2018;14:931–40.
[15] Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med 2009;206:1589–602.
[16] Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005;6:769–76.
[17] Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, Klinman DM, et al. Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. Proc Natl Acad Sci USA 2008;105:16260–5.
[18] Ebensen T, Debarry J, Pedersen GK, Blazejewska P, Weissmann S, Schulze K, et al. Mucosal administration of cycle-Di-nucleotide-adjuvanted virosomes efficiently induces protection against influenza H5N1 in mice. Front Immunol 2017;8:1223.
[19] Ebensen T, Libanova R, Schulze K, Yevsa T, Morr M, Guzman CA. Bis-(3',5')-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. Vaccine 2011;29:5210–20.
[20] Ebensen T, Schulze K, Riese P, Morr M, Guzman CA. The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant. Clin Vaccine Immunol 2007;14:952–8.
[21] Landi A, Law J, Hockman D, Logan M, Crawford K, Chen C, et al. Superior immunogenicity of HCV envelope glycoproteins when adjuvanted with cyclic-di-AMP, a STING activator or archaeosomes. Vaccine 2017;35:6949–56.
[22] Libanova R, Ebensen T, Schulze K, Bruhn D, Norder M, Yevsa T, et al. The member of the cyclic di-nucleotide family bis-(3′, 5′)-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. Vaccine 2010;28:2249–58.
[23] Mittal A, Schulze K, Ebensen T, Weissmann S, Hansen S, Lehr CM, et al. Efficient nanoparticle-mediated needle-free transcutaneous vaccination via hair follicles requires adjuvantation. Nanomedicine 2015;11:147–54.
[24] Sanchez MV, Ebensen T, Schulze K, Cargnelutti D, Blazejewska P, Scodeller EA, et al. Intranasal delivery of influenza rNP adjuvanted with c-di-AMP induces strong humoral and cellular immune responses and provides protection against virus challenge. PLoS One 2014;9:e104824.
[25] Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science 2010;328:1703–5.
[26] Parvatiyar K, Zhang Z, Teles RM, Ouyang S, Jiang Y, Iyer SS, et al. The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. Nat Immunol 2012;13:1155–61.
[27] Sauer JD, Sotelo-Troha K, von Moltke J, Monroe KM, Rae CS, Brubaker SW, et al. The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infect Immun 2011;79:688–94.
[28] Skrnjug I, Rueckert C, Libanova R, Lienenklaus S, Weiss S, Guzman CA. The mucosal adjuvant cyclic di-AMP exerts immune stimulatory effects on dendritic cells and macrophages. PLoS One 2014;9:e95728.
[29] Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767–811.
[30] Kastenmuller W, Kastenmuller K, Kurts C, Seder RA. Dendritic cell-targeted vaccines–hope or hype? Nat Rev Immunol 2014;14:705–11.
[31] Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 2004;199:815–24.
[32] Boscardin SB, Hafalla JC, Masilamani RF, Kamphorst AO, Zebroski HA, Rai U, et al. Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. J Exp Med 2006;203:599–606.
[33] Johnson TS, Mahnke K, Storn V, Schonfeld K, Ring S, Nettelbeck DM, et al. Inhibition of melanoma growth by targeting of antigen to dendritic cells via an anti-DEC-205 single-chain fragment variable molecule. Clin Cancer Res 2008;14:8169–77.
[34] Volckmar J, Gereke M, Ebensen T, Riese P, Philipsen L, Lienenklaus S, et al. Targeted antigen delivery to dendritic cells elicits robust antiviral T cell-mediated immunity in the liver. Sci Rep 2017;7:43985.
[35] Trumpfheller C, Finke JS, Lopez CB, Moran TM, Moltedo B, Soares H, et al. Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. J Exp Med 2006;203:607–17.
[36] Do Y, Didierlaurent AM, Ryu S, Koh H, Park CG, Park S, et al. Induction of pulmonary mucosal immune responses with a protein vaccine targeted to the DEC-205/CD205 receptor. Vaccine 2012;30:6359–67.
[37] Soares H, Waechter H, Glaichenhaus N, Mougneau E, Yagita H, Mizenina O, et al. A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. J Exp Med 2007;204:1095–106.
[38] Macri C, Dumont C, Johnston AP, Mintern JD. Targeting dendritic cells: a promising strategy to improve vaccine effectiveness. Clin Transl Immunol 2016;5:e66.
[39] Lehmann CH, Heger L, Heidkamp GF, Baranska A, Luhr JJ, Hoffmann A, et al. Direct delivery of antigens to dendritic cells via antibodies specific for endocytic receptors as a promising strategy for future therapies. Vaccines (Basel) 2016;4.
[40] Vremec D, Shortman K. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. J Immunol 1997;159:565–73.
[41] Moffat JM, Segura E, Khoury G, Caminschi I, Cameron PU, Lewin SR, et al. Targeting antigen to bone marrow stromal cell-2 expressed by conventional and plasmacytoid dendritic cells elicits efficient antigen presentation. Eur J Immunol 2013;43:595–605.
[42] Reuter A, Panozza SE, Macri C, Dumont C, Li J, Liu H, et al. Criteria for dendritic cell receptor selection for efficient antibody-targeted vaccination. J Immunol 2015;194:2696–705.
[43] Mohanan D, Slutter B, Henriksen-Lacey M, Jiskoot W, Bouwstra JA, Perrie Y, et al. Administration routes affect the quality of immune responses: A cross-sectional evaluation of particulate antigen-delivery systems. J Control Release 2010;147:342–9.
[44] Pedersen GK, Ebensen T, Gjeraker IH, Svindland S, Bredholt G, Guzman CA, et al. Evaluation of the sublingual route for administration of influenza H5N1 virosomes in combination with the bacterial second messenger c-di-GMP. PLoS One 2011;6:e26973.

[45] Lycke N. Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 2012;12:592–605.
[46] Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004;350:896–903.
[47] Gray PM, Forrest G, Wisniewski T, Porter G, Freed DC, DeMartino JA, et al. Evidence for cyclic diguanylate as a vaccine adjuvant with novel immunostimulatory activities. Cell Immunol 2012;278:113–9.
[48] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;11:373–84.
[49] Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med 2005;202:637–50.
[50] Brinkmann V, Geiger T, Alkan S, Heusser CH. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. J Exp Med 1993;178:1655–63.
[51] Wenner CA, Guler ML, Macatonia SE, O'Garra A, Murphy KM. Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. J Immunol 1996;156:1442–7.
[52] Blaauboer SM, Gabrielle VD, Jin L. MPYS/STING-mediated TNF-alpha, not type I IFN, is essential for the mucosal adjuvant activity of (3'-5')-cyclic-di-guanosine-monophosphate in vivo. J Immunol 2014;192:492–502.
[53] Lirussi D, Ebensen T, Schulze K, Trittel S, Duran V, Liebich I, et al. Type I IFN and not TNF, is essential for cyclic Di-nucleotide-elicited CTL by a cytosolic cross-presentation pathway. EBioMedicine 2017;22:100–11.
[54] Rueckert C, Rand U, Roy U, Kasmapour B, Strowig T, Guzman CA. Cyclic dinucleotides modulate induced type I IFN responses in innate immune cells by degradation of STING. FASEB J. 2017;31:3107–15.
[55] Idoyaga J, Lubkin A, Fiorese C, Lahoud MH, Caminschi I, Huang Y, et al. Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. Proc Natl Acad Sci USA 2011;108:2384–9.
[56] Trumpfheller C, Longhi MP, Caskey M, Idoyaga J, Bozzacco L, Keler T, et al. Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. J Intern Med 2012;271:183–92.
[57] Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787–93.
[58] Henri S, Vremec D, Kamath A, Waithman J, Williams S, Benoist C, et al. The dendritic cell populations of mouse lymph nodes. J Immunol 2001;167:741–8.
[59] Lakhrif Z, Moreau A, Herault B, Di-Tommaso A, Juste M, Moire N, et al. Targeted delivery of toxoplasma gondii antigens to dendritic cells promote immunogenicity and protective efficiency against toxoplasmosis. Front Immunol 2018;9:317.