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Routing dependent immune responses after experimental R848-adjuvated vaccination

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ABSTRACT

Most traditional vaccines are administered via the intramuscular route. Other routes of administration however, can induce equal or improved protective memory responses and might provide practical advantages such as needle-free immunization, dose sparing and induction of tissue-specific (mucosal) immunity. Here we explored the differences in immunological outcome after immunization with model antigens via two promising immunization routes (intradermal and intranasal) with or without the experimental adjuvant and TLR7/8-agonist R848. Because the adaptive immune response is largely determined by the local innate cells at the site of immunization, the effect of R848-adjuvation on local cellular recruitment, antigenic uptake by antigen-presenting cells and the initiation of the adaptive response were analyzed for the two routes of administration. We show a general immune-stimulating effect of R848 irrespective of the route of administration. This includes influx of neutrophils, macrophages and dendritic cells to the respective draining lymph nodes and an increase in antigen-positive antigen-presenting cells which leads for both intradermal and intranasal immunization to a mainly T_H1 response. Furthermore, both intranasal and intradermal R848-adjuvated immunization induces a local shift in DC subsets; frequencies of $CD11b^+$DC increase whereas $CD103^+$DC decrease in relative abundance in the draining lymph node. In spite of these similarities, the outcome of immune responses differs for the respective immunization routes in both magnitude and cytokine profile. Via the intradermal route, the induced T-cell response is higher compared to that after intranasal immunization, which corresponds with the local higher uptake of antigen by antigen-presenting cells after intradermal immunization. Furthermore, R848-adjuvation enhances *ex vivo* IL-10 and IL-17 production after intranasal, but not intradermal, T-cell activation. Quite the opposite, intradermal immunization leads to a decrease in IL-10 production by the vaccine induced T-cells. This knowledge may lead to a more rational development of novel adjuvanted vaccines administered via non-traditional routes.

1. Introduction

Vaccines play an essential role in prevention of many life-threatening infectious diseases [1]. Nowadays, most vaccines are administered via the intramuscular route, but other routes of administration show equal or improved immunological effectiveness [2–5] and provide practical advantages e.g. needle-free administration and dose-sparing [6,7]. Local antigen-presenting cells (APC), most importantly dendritic cells (DC), take up antigen, mature and transport antigen to local draining lymph nodes where they prime naïve T-cells [8], thus determining the type and magnitude of the adaptive response [9,10]. Therefore, vaccination strategies may improve by choosing a distinct route of administration.

With its high numbers of APC, efficient drainage and easy access, the skin is an attractive location for immunization [11]. Cutaneous APC, the epidermal-based Langerhans cells (LC), dermal DC ($CD11b^+$ or $CD103^+$ migrating DC), macrophages and migrating LC [11,12] are well equipped to initiate a protective immune response. Animal experiments [13,14] and e.g. rabies- and Fluzone Intradermal®-vaccinations [15,16] show successful vaccine-responses after intradermal immunization. Another promising route of administration is intranasal immunization for its ease in administration and enrichment of classical DC in the nasopharynx [17]. Phenotype and function of nasal APC were recently described

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[18]. A minor CD103$^+$DC- and a major CD11b$^+$DC-population were found in the nose-associated lymphoid tissue (NALT), next to macrophages and B-cells. In addition to one FDA-approved intranasal vaccine (FluMist®, a live attenuated influenza vaccine that conveys protection in healthy adults [19]), animal experiments also demonstrate the potential of intranasal immunization [14,20].

Skin and mucosal immune cells are exposed to environmental antigens and have well-developed tolerance mechanisms. To induce an immune response to vaccine-antigens and overcome tolerance, adjuvants are often required. Synthetic mimics of pathogen-associated molecular patterns, a novel class of adjuvants [8,21], efficiently stimulate pattern recognition receptors on innate cells, most importantly Toll-like receptors (TLR; reviewed in [22]). TLR-agonists preferentially induce advantageous T_H1 responses, supporting cellular immunity, important in protective vaccine-responses [23,24]. Resiquimod (R848) is an imidazoquinoline and a TLR7/8-agonist. Its less potent equivalent Imiquimod is already FDA-approved for topical administration in anti-viral cream and TLR7/8-agonists are being studied in clinical trials for their adjuvanticity [25]. R848 was shown to activate local innate cells, to induce pro-inflammatory cytokines [26,27] and to affect APC-maturation [27–29] which, when co-delivered with vaccine-antigens, predominantly leads to T_H1-responses after both intranasal and intradermal immunization in animal models [14,30].

With increasing interest in new routes of administration, it becomes evident that immunization with identical antigenic components via different routes influences responses and even the effect of adjuvant may depend on the route [2,14]. The exact mechanisms of these differences are, however, not (completely) known. Here we explored differences in immunological outcome after immunization with model antigens, with or without R848, via two promising immunization routes (intranasal and intradermal). Effects on APC-recruitment and antigen-uptake as well as initiation of adaptive responses were studied. A similar local cellular influx after R848-adjuvation was found for both routes of administration, consisting of neutrophils, macrophages, and also DC. Furthermore, R848-adjuvation induced *in vivo* DC maturation, expansion of CD11b$^+$ DC and a decline of CD103$^+$ DC-subset after both intradermal and intranasal immunization. However, intradermal immunization resulted in more antigen-uptake by local APC compared to intranasal immunization, resulting in heightened T-cell response. R848 enhanced T-cell proliferation for both routes, but (T-cell-) cytokine production differed; most importantly IL-10, which was, compared to non-adjuvated vaccinations, upregulated after intranasal, but downregulated after intradermal immunization. In conclusion, we show a general immune-stimulating effect for R848 but different outcomes of immune responses for intradermal and intranasal immunization when presented with the same model-vaccine.

2. Material and methods

2.1. Mice

BALB/c wildtype mice (8–10 weeks) were obtained from Charles River Laboratories and human proteoglycan specific Thy1.1$^+$TCR-5/4E8-transgenic mice [31,32] were bred at the Central Animal Laboratory of Utrecht University, The Netherlands. Mice were kept under standard conditions and received water and food *ad libitum*. Experiments were approved by the Utrecht University Animal Experiments Committee.

2.2. Immunizations

Wildtype mice were immunized intradermally (pinnae of the ear; [33]) or via the intranasal route [34] (see also the Supplementary M&M). Intradermal and intranasal treatment consisted of antigen in PBS ± R848 (Invivogen, 25 μg) (in 10 μl or 20 μl respectively). The contralateral ear was left untreated and used as control. Antigens were human proteoglycan peptide, the cognate antigen of Thy1.1$^+$-TCR-5/4E8-CD4$^+$ T-cells, (hPGp; 100 μg, ^{70}ATEGRVRVNSAYQDK^{84}; GenScript), Ovalbumin-peptide$_{323-339}$ (pOVA; 250 μg; GenScript,) or DQ-Ovalbumin (DQ-OVA; 50 μg; Thermo Fisher).

2.3. Single cell suspensions

Twenty-four hours after intradermal immunization, immune cells from both ears were isolated as described [35]. Twenty-four hours after intranasal immunization, NALT was isolated as described [36]. Also, spleens and draining lymph nodes were collected. Draining lymph nodes were the auricular lymph node, cervical lymph node and deep cervical lymph node (intranasal immunization) or the auricular lymph node (intradermal immunization). Single cell suspensions from spleens, lymph node and NALT were prepared as described [32].

2.4. Co-cultures

2.4.1. Co-culture with TCR-5/4E8-Tg CD4+ T-cells

Single cell suspensions from spleens of Thy1.1$^+$TCR-5/4E8-Tg donor mice were CD4$^+$ T-cell enriched and CD25-depleted (PC61) and labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (protocol adapted from [37]). The purity of the CD4$^+$ T cells used in co-cultures was between 83 and 85%. After CD25 depletion, 0.4% (intranasal experiment) and 0.7% (intradermal experiment) of the CD4$^+$ T cells still expressed CD25. Immune cells from ear and lymph node harvested 24 h post immunization (hpi) with hPGp were labeled with CellTrace Violet (Invitrogen) with 5 μM CellTrace Violet in PBS according to manufacturer's instructions.

CFSE-labeled TCR-5/4E8-Tg CD4$^+$ T-cells were co-cultured with CellTrace-labeled cells ('APC') isolated from ear, auricular or cervical lymph node at a 1:2 ratio (T-cell:APC) with or without hPGp (10 μg/ml) for 72 h at 37 °C, 5% CO_2 in IMDM containing FBS (Lonza, 5%), β-mercaptoethanol (Gibco; 5×10^{-5} M), penicillin (Gibco; 100 units/ml) and streptomycin (Gibco; 100 μg/ml). Cells from the deep cervical lymph node or NALT were co-cultured in a 2:1 ratio, since cell yield in these organs was not sufficient for the 1:2 ratio. Cells were cultured. Subsequently, supernatants were collected for cytokine assays.

2.4.2. Co-culture with hybridoma DO11.10-GFP CD4± T-cell

DO11.10-GFP hybridoma cells [38] were grown under geneticin selection (0.5 mg/ml; Invivogen) and, to distinguish from other cells, stained with CellTrace Violet as described above. Immune cells from ear, NALT and lymph node ('APC') harvested 24 h after pOVA immunization, were co-cultured in Opti-MEM (Gibco) containing FBS (10%), β-mercaptoethanol (5×10^{-5} M), penicillin (100 units/ml) and streptomycin (100 μg/ml) for 18 h at 37 °C, 5% CO_2 with the CellTrace$^+$DO11.10 cells in a 5:1 (APC: T-cell) ratio with or without pOVA (0.2 μg/ml).

2.5. In vivo transfer studies

One day before hPGp immunization, acceptor wild type mice received 3×10^6 CFSE-labeled CD4$^+$-enriched T-cells from Thy1.1$^+$-TCR-5/4E8-Tg donor mice intravenously in 200 μl PBS. The purity of the transferred CD4$^+$ T cells was 68% and 70% for the intranasal and intradermal experiment respectively. Four days post immunization (dpi), ears, NALT, draining lymph node and spleen were harvested. Cells were directly used for flow cytometric analysis or stimulated (2×10^5 cells/well; 200 μl) with hPGp (10 μg/ml) for 72 h at 37 °C, 5% CO_2 for supernatants for cytokine assays.

2.6. Standardization of experiments

Paired intradermal and intranasal experiments were performed within the same timeslot (<1–4 days) and experimental conditions were kept as stable as possible: (1) immunized mice were from the same cohort and randomly divided in 4 groups before paired experiments were performed (intranasal PBS, intranasal R848, intradermal PBS or intradermal R848), (2) Immunizations were performed by the same researcher, (3) both the FACS antibody mix and the $CD4^+$ T cell enrichment antibody mix for individual experiments was prepared as one mix and kept cool and in the dark to prevent reduction in quality, (4) FACS and Magpix settings were kept the same and (5) the $CD4^+$ T cell donor mice were from the same cohort and randomly divided in 2 groups before experiments (intranasal or intradermal).

2.7. Cytokine analysis

Supernatants were used for multiplex cytokine analysis of IFN-γ, IL-2, IL-6, IL-10 and IL-17, using the Magpix (Luminex XMAP) system according the manufacturer's instructions and as described [39]. The antibody pairs (coat:detect) were AN-18:XMG1.2, JES6-1A12:JES6-5H4, MP5-20F3:MP5-32C11, JES5-2A5:SCC-1 and TC11-18H10:TC11-8H4.1 for IFN-γ, IL-2, IL-6, IL-10 and IL-17 respectively. The concentrations of cytokines in the tested samples were calculated using standard curves and the MFI data was analyzed using a 5-parameter logistic method (xPONENT software, Luminex, Austin, USA).

2.8. Flow cytometry analysis

Cells were stained in PBS supplemented with 2% FBS with the monoclonal antibodies F4/80-FITC/Pe-Cy7 (BM8), CD45-APC-H7 (2D1), Ly6C-PE (HK1.4), Ly6G-BV510 (1A8) (Biolegend), I-A/I-E-HorizonV450/FITC (M5/114.15.2), CD11b-PerCPCy5.5 (M1/70), CD11c-APC (N418), CD3-eFluor450 (17A2), CD69-APC (H1.2F3), Thy1.1-PerCPCy5.5 (HIS51) (eBioscience) and/or CD4-APC/eFLuorV510 (RM4-5), CD24-V450/Pe-Cy7 (M1/69), CD86-FITC (GL1), CD40-PE (3/23), CD103-BV510 (M290), CD19-APC-H7 (1D3) (BDBiosciences).

Subsequently, cells were measured on a FACSCanto II Flow cytometer (BDBiosciences). Analysis was performed with FlowJo v10 (Tree Star). Representative dot plots, including gating strategies, can be found in the Supplemental Figs. 1–3.

2.9. Statistical analysis

Unpaired, two-tailed t-tests with Welch correction were performed using Prism v6.05 (GraphPad). Differences were considered significant at $p < 0.05$ and indicated as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. R848-adjuvation changes local cellular environment and antigen-uptake after intradermal and intranasal immunization

Initiation of vaccine-responses starts with uptake of vaccine-antigen by local innate cells. Therefore, we determined the cellular composition at local sites 24 h after intranasal or intradermal immunization with model-antigen hPGp, adjuvated or not with R848. Furthermore, antigen-uptake was determined by immunization with DQ-OVA that fluoresces after intracellular processing.

Twenty-four hours post intranasal immunization with hPGp + R848, frequencies of neutrophils, macrophages, and DC but not inflammatory monocytes were increased in cervical lymph node, compared to hPGp only (Fig. 1a). R848-adjuvation led to higher expression of CD40 (Fig. 1b) and CD86 (56% vs 91%; not shown) on DC and relatively more $CD11b^+$DC, but less $CD103^+$DC were observed (Fig. 1c). In auricular lymph node and deep cervical lymph node, R848-adjuvation induced similar changes in cell composition and DC phenotypes as in the cervical lymph node (not shown). In NALT a potential increase in $\%MHCII^+$-cells could be seen, whereas frequencies of macrophages and inflammatory monocytes appeared to decrease (not shown). After intranasal immunization, DQ-OVA was taken up and processed by only a small number of cells and this tended to be increased by R848-adjuvation only in DC and B-cells in the cervical lymph node (Fig. 1d).

R848-adjuvation of intradermal immunization also affected cellular composition and DQ-OVA uptake in skin. Frequencies of macrophages and inflammatory monocytes went down with hPGp + R848 compared to hPGp only, but had no obvious effect on neutrophil-, DC- or LC-frequency (Fig. 2a). Skin-DC expressed

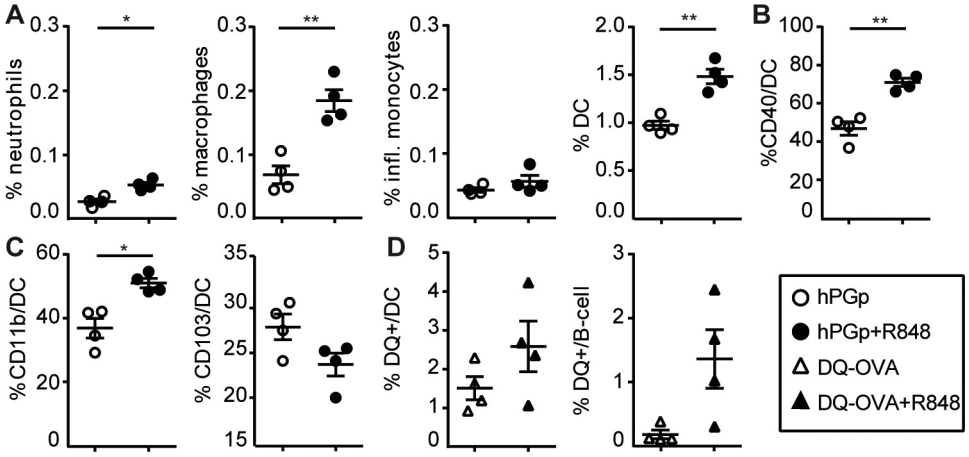

Fig. 1. Addition of adjuvant R848 to intranasal immunization changes local cellular composition and antigenic uptake. Mice were intranasally immunized with hPGp ± R848 (A–C) or DQ-OVA ± R848 (D) and 24hpi cellular composition and uptake of DQ-OVA in the cervical lymph node was analyzed. Flow cytometric analysis of (A) neutrophils ($CD11b^+Ly\text{-}6G^+$), macrophages ($CD11b^+F4/80^+Ly\text{-}6G^-$), inflammatory monocytes ($CD11b^+Ly\text{-}6C^+F4/80^-Ly\text{-}6G^-$) and DC ($CD11c^+MCHII^+$) and (B) the expression of markers CD40 and (C) CD11b and CD103 on DC. Cell populations are indicated as % cells in the live single cell gate. In (D) the % B-cells ($CD19^+MHCII^+$) and DC that have taken up and processed DQ-OVA is shown. Each symbol represents an individual animal and mean ± SEM are indicated. N = 4/group.

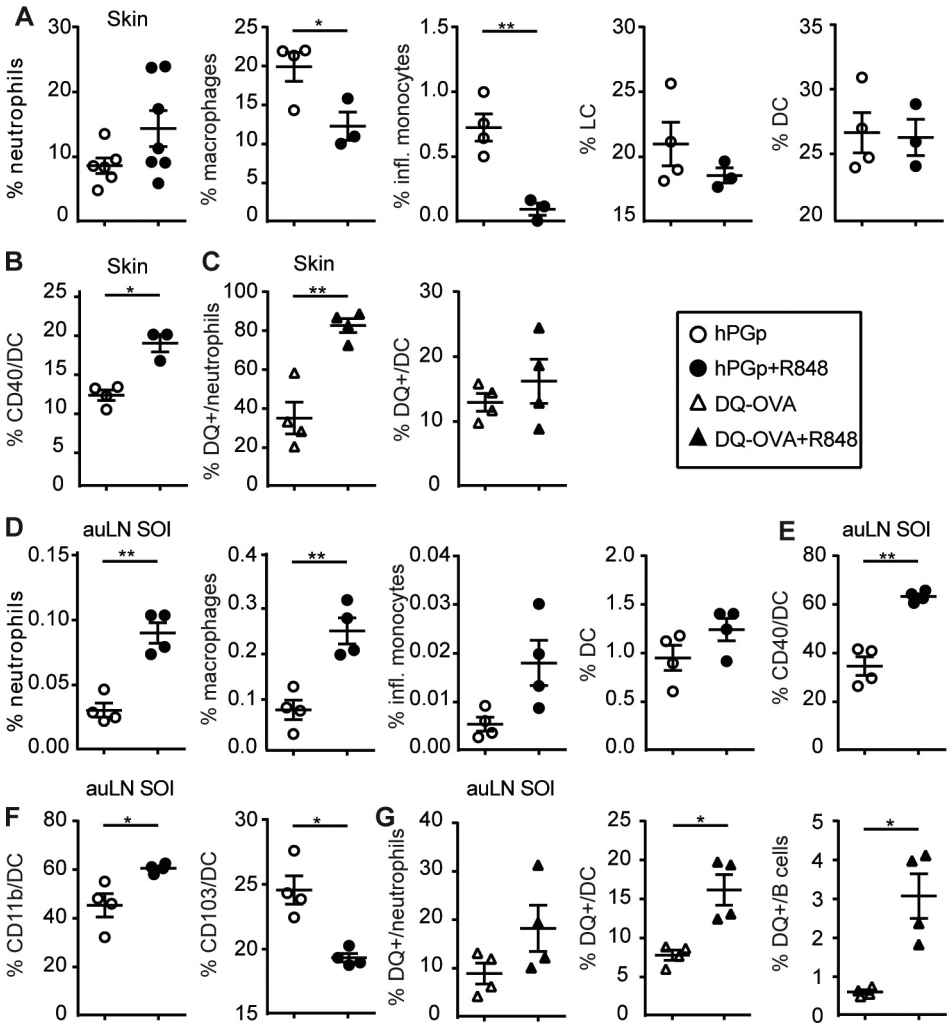

Fig. 2. Addition of adjuvant R848 to intradermal immunization changes local cellular composition and antigenic uptake. Mice were intradermally immunized with hPGp ± R848 (A,B, D–F) or DQ-OVA ± R848 (C,G) and 24hpi cellular composition of the skin (A,B) and auricular lymph nodes (D,E) and uptake of DQ-OVA in the ear skin (C) and auricular lymph nodes (G) at the site of injection were analyzed. Flow cytometric analysis of (A) neutrophils ($CD11b^+Ly\text{-}6G^+$), macrophages ($CD11b^+F4/80^+Ly\text{-}6G^-CD11c^-$), inflammatory monocytes ($CD11b^+Ly\text{-}6C^+F4/80^-Ly\text{-}6G^-CD11c^-$), LC ($MHCII^+CD24^+CD11b^+$) and DC ($CD11c^+MHCII^+$) and (B) the expression of marker CD40 on DC in skin. In skin, cell numbers are indicated as % cells in the live, $CD45^+$, single cell gate. In (D) neutrophils, macrophages, inflammatory monocytes and DC are shown in the auricular lymph node. In (E) the expression of markers CD40 and (F) CD11b and CD103 on DC in auricular lymph node are shown. In the auricular lymph node, cell populations were gated as described in Fig. 1 legend. In (C,G) the % neutrophils and DC in skin (C) and neutrophils, B-cells and DC in the auricular lymph node (G) that have taken up and processed DQ-OVA are shown. Graphical representation as described for Fig. 1. N = 4–7/group. Abbreviations: auLN: auricular lymph node, SOI: site of injection.

more CD40 (Fig. 2b) and took up DQ-OVA. However, DQ-OVA was mainly processed by skin-neutrophils in the presence of R848 (Fig. 2c); over 90% of neutrophils contained processed DQ-OVA. In auricular lymph node, frequencies of mostly neutrophils, macrophages, inflammatory monocytes and DC increased by R848-adjuvation (Fig. 2d). Similar to intranasal immunization, intradermal hPGp + R848 immunization led to higher expression of CD40 (Fig. 2e) and CD86 (56% vs 91%; not shown) on DC, and increasing $CD11b^+$DC- but decreasing $CD103^+$DC-frequencies in draining lymph nodes (Fig. 2f). Twenty-four hours after intradermal immunization, neutrophils and DC, and to a smaller extent B-cells, had taken up and processed DQ-OVA and R848-adjuvation increased this significantly (Fig. 2g).

3.2. Intradermal immunization with R848, but not intranasal immunization, enhances ex vivo antigen-presenting capacities of local APC

To assess effects of routing and R848-adjuvation on antigen-presenting capacities of APC, we measured the initiation of adaptive responses. Cells from skin and/or draining lymph nodes from mice injected 24 h prior with hPGp ± R848 either via the intradermal or intranasal route, were co-cultured with hPGp-specific $CD4^+$ T-cells.

APC from auricular lymph node, cervical lymph node or deep cervical lymph node of intranasal immunized mice did not induce proliferation of, or CD69-upregulation on the hPGp-specific $CD4^+$ T-cells (Fig. 3a,b). Supplementation of hPGp in culture, however, did result in proliferation and enhanced CD69-expression (not shown) irrespective of adjuvation, indicating that T-cells could proliferate. Although no effect of R848 on proliferation was observed, hPGp-stimulated T-cells showed higher IFN-γ, IL-6, IL-17 and IL-10 production when the APC were derived after R848-adjuvation (Fig. 3c–e), suggesting a stimulating effect of R848.

Co-cultures containing skin or auricular lymph node cells derived from hPGp-intradermally immunized mice led to proliferation of the hPGp-specific $CD4^+$ T-cells, and R848-adjuvation further enhanced this, in particular for auricular lymph node cells (Fig. 4a). Co-culture with skin and auricular lymph node cells also increased CD69-expression on $CD4^+$ T-cells when immunizations

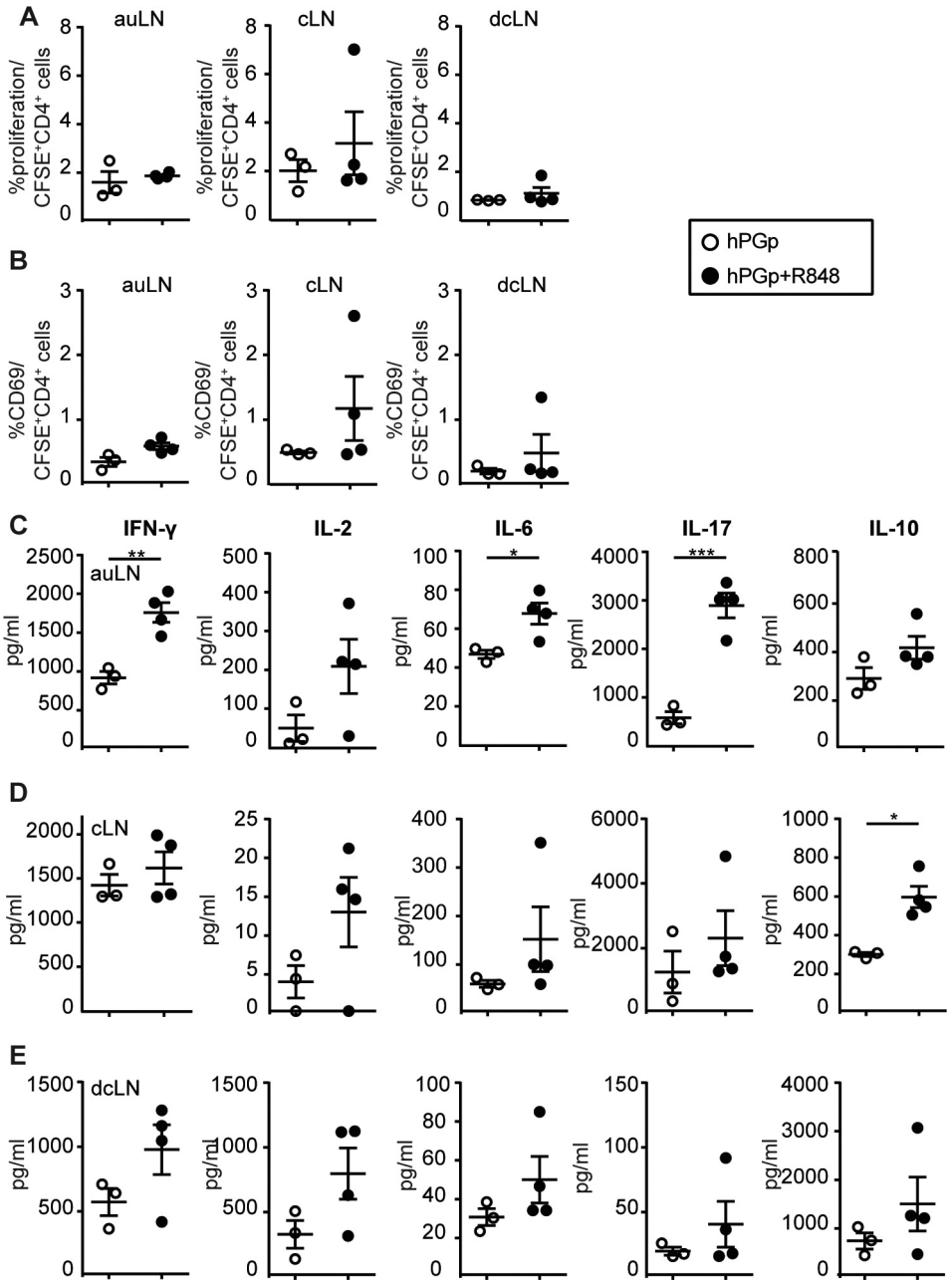

Fig. 3. Addition of adjuvant R848 to intranasal immunization has no effect on *ex vivo* proliferation or activation of cognate $CD4^+$ T-cells, but does change their cytokine production. Mice were intranasally immunized with hPGp ± R848 and after 24 h auricular lymph nodes, cervical lymph nodes and deep cervical lymph nodes were harvested and co-cultured with CFSE-labeled hPGp-specific $CD4^+$ T-cells for 3 days. Proliferation of (A) and CD69 expression on (B) CFSE-labeled hPGp-specific $CD4^+$ T-cells. Proliferation is defined as %divided $CFSE^+CD4^+$ cells. (C-E) Net cytokine concentrations (pg/ml) for IFN-γ, IL-2, IL-6, IL-17 and IL-10 in supernatant of co-cultures with (C) auricular lymph nodes, (D) cervical lymph nodes and (E) deep cervical lymph nodes are shown. Net cytokine concentration: cytokine $production_{hPGp\ restimulated}$ - cytokine $production_{medium}$. Graphical representation as described for Fig. 1. N = 3–4/group. Abbreviations: auLN: auricular lymph node, cLN: cervical lymph node, dcLN: deep cervical lymph node.

were R848-adjuvated (Fig. 4b). Stimulation of the cultures with *in vitro* added hPGp, led to substantial cytokine production, but differences between groups were minor. Though not significant, in skin-T-cell co-cultures IFN-γ, IL-6 and IL-10 production appeared higher when skin cells were derived from hPGp + R848-immunized mice, compared to hPGp (Fig. 4c). This was not the case in auricular lymph node-T-cell co-cultures. There, only IL-17 cytokine production was significantly higher when the immunization contained R848 (Fig. 4d), albeit much lower than after intranasal hPGp + R848-immunization.

To confirm the difference in *ex vivo* antigen-presentation after intranasal and intradermal immunization, we made use of pOVA-specific hybridoma cells. These MHC-II-restricted cells become GFP-fluorescent when activated and were co-cultured with cells from mice intranasally or intradermally immunized with pOVA. In accordance with what we observed in the hPGp-co-cultures, no obvious T-cell activation was observed after co-culture with cells derived from intranasally pOVA-immunized mice (Table S1a). A similar experiment with cells from intradermally pOVA-immunized mice did lead to T-cell activation, which was

further increased by R848 with skin and auricular lymph node derived cells (Table S1b).

3.3. R848-supplementation enhances in vivo proliferation of transferred cognate CD4⁺ T-cells, and changes their ex vivo cytokine production

To compare the immunization routes in an *in vivo* setup we transferred hPGp-specific T-cells 1d prior to intranasal or intradermal immunization with hPGp ± R848. Four days after intranasal immunization, T-cells had proliferated in the auricular lymph node, cervical lymph node, and spleen, and this was significantly increased with R848 (Fig. 5a). In the cervical lymph node, transferred T-cells upregulated CD69 after R848-adjuvation (Fig. 5b; trend) concurring with high proliferation. *Ex vivo* hPGp-restimulation resulted in IFN-γ and IL-2 production by auricular lymph node and cervical lymph node cells, which was increased with R848-adjuvation (Fig. 5c,d). Furthermore, hPGp + R848-injected mice showed IL-6, IL-17 and IL-10 production in the cervical lymph node. This in contrast to hPGp-injection only, where production was mostly below detection limit (Fig. 5d).

After intradermal immunization transferred T-cells had proliferated in both auricular lymph node (site of injection and contralateral) and spleen, and R848 significantly increased proliferation and CD69-expression (Fig. 6a,b). In ear skin, no (transferred) T-cells could be detected. hPGp-restimulation induced (limited) cytokine production in auricular lymph node at the site of injection (Fig. 6c) and spleen (Fig. 6d), but hardly any in the contralateral (not shown). IFN-γ production was enhanced when R848 was included in both auricular lymph node and spleen as was IL-6 in auricular lymph node. In auricular lymph node, IL-10 production was significantly lower after intradermal hPGp + R848-immunization compared to hPGp only, in contrast with responses after intranasal immunization.

4. Discussion

Improved initiation of vaccine-responses is a potential means to strengthen vaccine-efficacy and properly combined with adjuvant, might steer adaptive responses suited for protective immunity. Here we explored the potential differential effects of R848-adjuvant upon different routes of administration; intranasal and intradermal immunization. We show that, when using an identical model-vaccine, intranasal and intradermal immunization lead to differences in magnitude and type of adaptive responses and that R848-adjuvant enhances local and systemic responses via both routes to a different extent.

First, we compared local cellular compositions shortly after immunization (Figs. 1 and 2). Surprisingly, in skin and NALT, the effects of R848-adjuvation were similar in that macrophages and inflammatory monocytes went down in frequency (skin: Fig. 2a and NALT-data not shown), possibly indicating an efflux to draining lymph nodes since macrophage, inflammatory monocytes and DC frequencies went up in draining lymph nodes after both routes of administration (Figs. 1a 2d). Furthermore, R848-supplemented intradermal immunization slightly increased neutrophil frequency in skin (Fig. 2a). Neutrophil-influx was also observed after laser-assisted intradermal immunization [40] and intradermal immunization in pig-skin [13] already 6hpi, corresponding with neutrophil's function as first responders [41]. In the relevant draining lymph node, neutrophil frequencies were significantly increased for both routes of administration (Figs. 1a, 2d). Additionally, after intradermal immunization, in particular in skin (Fig. 2c) and to a lesser extent in draining lymph node (Fig. 2g),

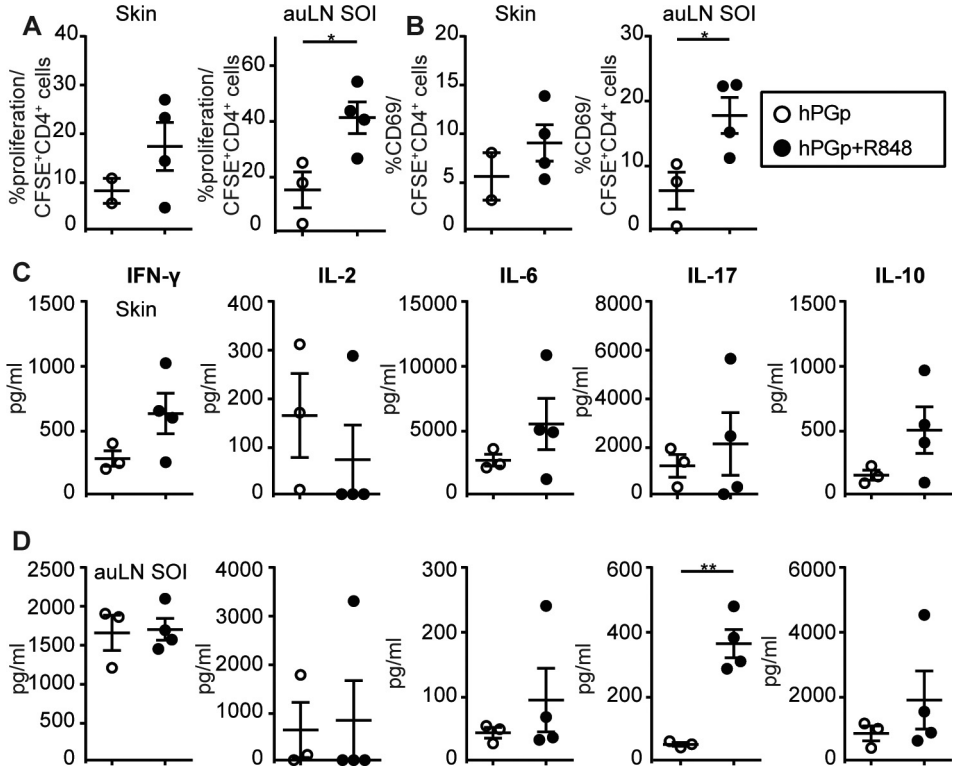

Fig. 4. Addition of adjuvant R848 to intradermal immunization enhances *ex vivo* proliferation and activation of cognate CD4⁺ T-cells, and changes their cytokine production. Mice were intradermally immunized with hPGp ± R848 and after 24 h, skin and auricular lymph nodes from the site of injection were harvested and co-cultured with CFSE-labeled hPGp-specific CD4⁺ T-cells for 3 days. (A) Proliferation of and (B) CD69 expression on CFSE-labeled hPGp-specific CD4⁺ T-cells. (C–E) Net cytokine concentrations (pg/ml) for IFN-γ, IL-2, IL-6, IL-17 and IL-10 in supernatant for co-cultures with (C) skin or (D) auricular lymph node cells. Proliferation and cytokine production was determined as in Fig. 3. Graphical representation as described for Fig. 1. N = 3–4/group. Abbreviations: auLN: auricular lymph node, SOI: site of injection.

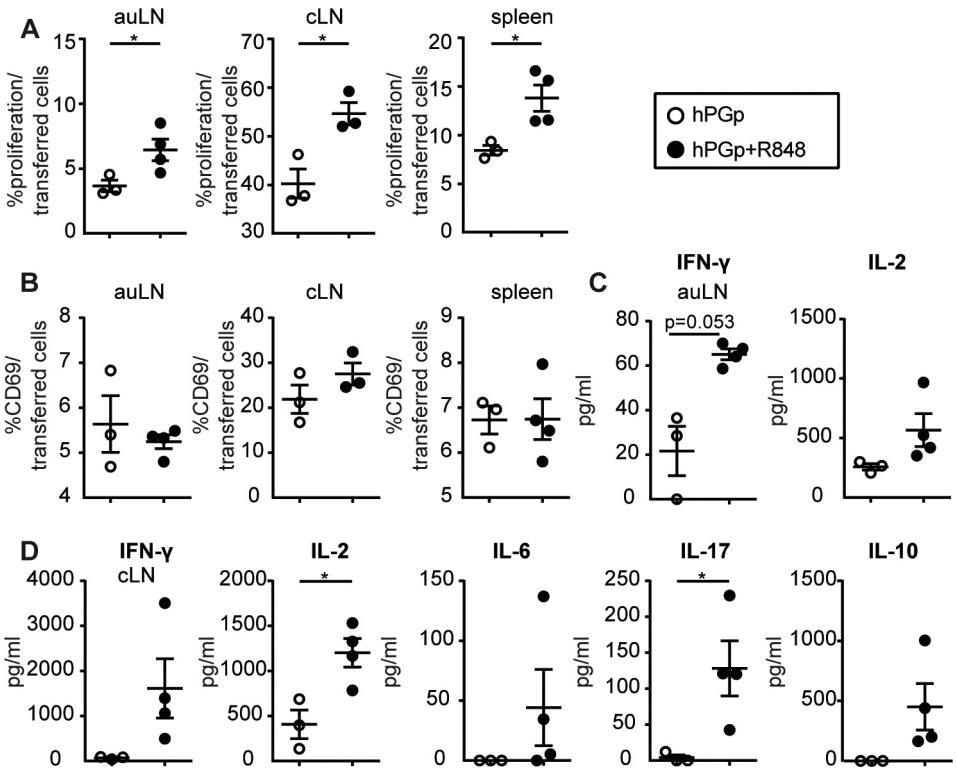

Fig. 5. Addition of adjuvant R848 to intranasal immunization enhances local and systemic *in vivo* proliferation of transferred cognate CD4$^+$ T-cells, and changes their *ex vivo* cytokine production. Mice received an intravenous transfer of CFSE-labeled hPGp specific CD4$^+$ T-cells 1d prior to intranasal immunization with hPGp ± R848. (A) Proliferation of and (B) CD69 expression on transferred CFSE-labeled hPGp-specific CD4$^+$ T-cells 4dpi in auricular lymph node, cervical lymph node and spleen. (C-D) Net cytokine concentrations (pg/ml) for IFN-γ, IL-2, IL-6, IL-17 and IL-10 in supernatant after 72 h of stimulation with hPGp (10 μg/ml) in (C) auricular lymph nodes (IFN-γ, IL-2 only) and (D) cervical lymph nodes. Proliferation and cytokine production was determined as in Fig. 3. Graphical representation as described for Fig. 1. N = 3–4/group. Abbreviations: auLN: auricular lymph node, cLN: cervical lymph node.

mainly neutrophils contained processed antigen similar to other studies [18,42]. After intranasal immunization only limited numbers of neutrophils processed antigen (not shown) in contrast with other reports [18,43]. Besides neutrophils, DC and B-cells were the main antigen$^+$cells in draining lymph nodes for both routes of administration, although also in these cells at this particular time point, intradermal immunization ensured higher antigen-uptake than intranasal immunization (Figs. 1d, 2c,g). Irrespective of route of administration, addition of R848 induced more mature DC (Figs. 1b, 2b,e), corresponding with other observations [27–29].

To assess effects of routing and R848-adjuvation on antigen-presenting capacities of APC, we measured initiation of adaptive responses by means of *ex vivo* co-cultures. In line with the higher antigen-uptake after intradermal immunization compared to intranasal immunization, APC in skin and draining lymph nodes of intradermally immunized animals induced *ex vivo* activation and proliferation of naïve CD4$^+$ T-cells (Fig. 4A,B), confirming that APC *in vivo* took up antigen and received maturation signals. This in contrast with APC from intranasally immunized animals, that did not induce significant proliferation in co-culture (Fig. 3A,B). A possible explanation for this might be the existing disagreement of the actual draining lymph node after intranasal immunization. Both mediastinal lymph node [43–45] and cervical lymph node [18,46] have been described as draining lymph node after intranasal immunization. The draining lymph node of mice intranasally immunized under anesthesia is the mediastinal lymph node [47]. However, we have immunized superficially breathing conscious mice and thus the nasal cavity is the main priming site which is drained by the cervical lymph node [18]. Of course this does not exclude other draining lymph nodes involved during the course of an intranasal immune response as is also demonstrated by the observed effects in the auricular lymph node after intranasal immunization (Fig. 5A,C). However, in [18] it was shown that 24 h after intranasal immunization antigen$^+$DC are significantly increased in cervical lymph node indicating that we analyzed the correct draining lymph node and at the right time for this route of administration. Nonetheless, the time point might explain differences between the success of the two routes since routes may differ in time at which antigen-carrying, mature APC arrive in the respective draining lymph nodes. This was demonstrated by [2] where the peak of antigen$^+$APC in the draining lymph node arrived at different times after intraperitoneally and intramuscular immunization and even DC-subsets arrive differently in the draining lymph node for the routes of administration tested [2].

To compare routes of administration and effects of R848-adjuvation in an *in vivo* setup, we performed CD4$^+$ T-cell-transfers prior to immunization. Both intradermal and, surprisingly also intranasal immunization, despite its associated low antigen-uptake (Fig. 1d) and lack of *ex vivo* induction of T-cell proliferation in co-culture (Fig. 3a, Table S1a), led to *in vivo* proliferation of naïve T-cells (Figs. 5a, 6a). For both routes of administration, the effect was systemic and R848-adjuvation enhanced responses significantly both in draining lymph nodes and in spleen. However, responses were much higher after intradermal immunization, in line with high antigen-uptake by APC (Fig. 2c,g) and co-cultures (Fig. 4, Table S1b). IFN-γ production by vaccine-specific CD4$^+$ T-cells was observed after both routes of administration without R848, albeit higher after intradermal immunization, and was increased by R848-adjuvation (Figs. 5d, 6c), in line with R848's T_H1-profile [29]. Furthermore, although IL-17 production was generally below detection limits in the non-adjuvated situations, R848-adjuvation induces significant IL-17 production by the intra-

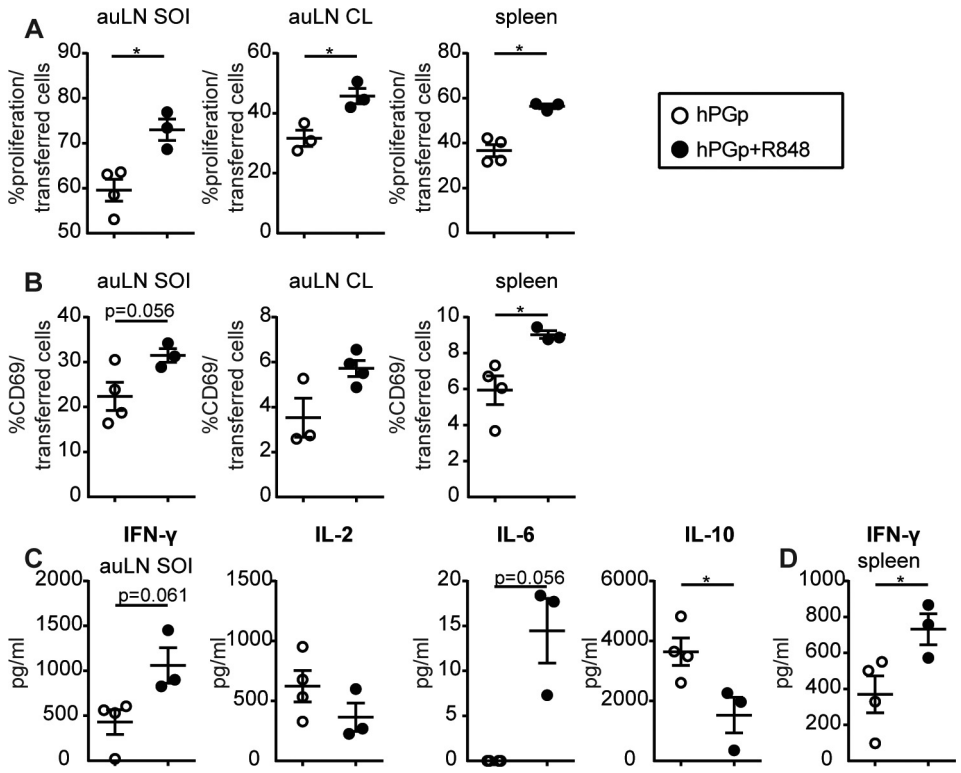

Fig. 6. Addition of adjuvant R848 to intradermal immunization enhances local and systemic *in vivo* proliferation of transferred cognate CD4$^+$ T-cells, and changes their *ex vivo* cytokine production. Mice received an i.v. transfer of CFSE-labeled hPGp specific CD4$^+$ T-cells 1d prior to intradermal immunization with hPGp ± R848. (A) Proliferation of and (B) CD69 expression on transferred CFSE-labeled hPGp-specific CD4$^+$ T-cells 4dpi in auricular lymph nodes at the site of injection and contralateral and spleen. (C-D) Net cytokine concentrations (pg/ml) for IFN-γ, IL-2, IL-6 and IL-10 in supernatant after 72 h of stimulation with hPGp (10 μg/ml) in (C) auricular lymph node at the site of injection and (D) spleen (IFN-γ only). Proliferation and cytokine production was determined as in Fig. 3. Graphical representation as described for Fig. 1. N = 3–4/group. Abbreviations: auLN: auricular lymph node, SOI: site of injection, CL: contralateral.

nasal, but not intradermal route. In addition, IL-10 production is also route-dependent. Non-adjuvated, intradermal, but not intranasal, immunization induces IL-10, which is decreased when R848 is supplemented, as expected with R848's pro-inflammatory profile [29]. However, IL-10 increases after intranasal immunization if R848-supplemented. In our setup it is unclear if R848 directly acts on T-cells, as was found *in vitro* for human effector memory CD4$^+$ T-cells [48], or on surrounding cells. The increase in IL-10 after intranasal R848-adjuvation might result from higher proliferation and represent the contracting-phase of immune responses. Similarly, IL-10 is high after intradermal immunization, where significant proliferation is observed and restoration of homeostasis via IL-10 might be required.

Differences in magnitude and cytokine-profiles after different routes of administration likely follow from differences in priming, in particular the DC-subsets involved [10,12]. We distinguished two subsets of DC, CD11b$^+$DC, likely a mixture of DC and macrophages, and good primers of CD4$^+$ T-cells [17] and the subset CD103$^+$DC, known for their migratory and cross-presenting capacities and potential in tolerance induction [17,49]. However, after intranasal and intradermal immunization, relative frequencies of CD11b$^+$DC and CD103$^+$DC are similar in the respective draining lymph node, but R848-adjuvation ensures an increase in CD11b$^+$-DC parallel to a decrease in CD103$^+$DC irrespective of routes of administration (Figs. 1c, 2f). TLR-7/8-agonists in mice are topic of debate since in mice TLR-8 might not be functional and R848 thus signals via TLR-7 [26] which is not expressed on all APC, but mainly on CD8$^+$DC or pDC and macrophages/monocytes [26]. To pinpoint the cause of the differential responses for the respective routes of administration and R848-adjuvation, a more thorough DC-subset analysis is required, in particular now it becomes clear that multiple distinct DC-subsets are important for coordinated T_H1 responses [50]. This was, however, outside the scope of our research.

In both routes of administration, R848-adjuvation results in enhanced immune responses as was demonstrated by an increase in cell influx, proliferation and more pronounced T_H1 responses. However, the final R848-induced response differs for the intradermal and intranasal route of administration. Our results demonstrate the necessity for more research on different route of administration, which can aid in the development of more effective vaccines.

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Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.01.077.

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