Vaccine 36 (2018) 1405–1413

Contents lists available at ScienceDirect

Vaccine

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

Routing dependent immune responses after experimental R848-adjuvated vaccination

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ARTICLE INFO

Article history:
Received 2 October 2017
Received in revised form 15 December 2017
Accepted 29 January 2018

Keywords:
R848
Adjuvant
Route of administration
Intranasal
Intradermal
Vaccine

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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https://doi.org/10.1016/j.vaccine.2018.01.077
0264-410X/

[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.