A Trifunctional Dextran-Based Nanovaccine Targets and Activates Murine Dendritic Cells, and Induces Potent Cellular and Humoral Immune Responses In Vivo

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Abstract

Dendritic cells (DCs) constitute an attractive target for specific delivery of nanovaccines for immunotherapeutic applications. Here we tested nano-sized dextran (DEX) particles to serve as a DC-addressing nanocarrier platform. Non-functionalized DEX particles had no immunomodulatory effect on bone marrow (BM)-derived murine DCs in vitro. However, when adsorbed with ovalbumine (OVA), DEX particles were efficiently engulfed by BM-DCs in a mannose receptor-dependent manner. A DEX-based nanovaccine containing OVA and lipopolysaccharide (LPS) as a DC stimulus induced strong OVA peptide-specific CD4+ and CD8+ T cell proliferation both in vitro and upon systemic application in mice, as well as a robust OVA-specific humoral immune response (IgG1>lG2a) in vivo. Accordingly, this nanovaccine also raised both a more pronounced delayed-type hypersensitivity response and a stronger induction of cytotoxic CD8+ T cells than obtained upon administration of OVA and LPS in soluble form. Therefore, DEX-based nanoparticles constitute a potent, versatile and easy to prepare nanovaccine platform for immunotherapeutic approaches.

Introduction

Several classes of antigen-loaded particles have been demonstrated to passively target antigen presenting cells (APCs) including dendritic cells (DCs) as the most potent APC population by means of unspecific endocytotic internalization [1]. In the course of these studies, some types of particles have been demonstrated to exert immunostimulatory activity in DCs [2]. This property may be of advantage in order to evoke an antigen-specific immune response. However, a nanoparticle platform devoid of intrinsic immunomodulatory potential might be even more feasible as it allows to determine the polarization of the antigen-specific immune response solely by the quality of a particle-delivered adjuvant [3].

In this regard, we opted for dextran (DEX) nanoparticles, introduced almost thirty years ago as a carrier platform for protein antigens plus immunomodulatory compounds to elicit an antigen-specific humoral response after in vivo application [4]. In general, dextrans constitute dextrin-derives neutral biopolymers, which due to their excellent biocompatibility have been in widespread clinical use for decades, serving as blood volume expanders, and preventing thrombosis. Therefore, DEX particles may constitute an ideal platform for the development of functionalized nanocarriers. The DEX particles used in our study are based on commercially available dextran particles with an average Mw of 500 kDa. The model protein antigen ovalbumine (OVA), and lipopolysaccharide (LPS), a well established toll-like receptor (TLR)-4 ligand and TH1-promoting DC stimulus, were adsorbed to DEX particles by applying the protocol introduced by Schroder and co-workers [4,5].

It has been shown that uptake of OVA by pinocytosis in DCs resulted in the activation of OVA-specific CD4+ T cells, but evoked no CD8+ T cell response [6]. In contrast, endocytotic uptake of OVA, which is efficiently bound by the mannose receptor (MR) due to its mannosylation [7], resulted in strong activation of either T cell population. The MR belongs to a group of C-type lectin receptors which act as pattern recognition receptors and bind both endogenous as well as pathogen-derived structures [8]. Due to its rather restricted expression pattern, largely confined to macrophages and myeloid DC populations, the MR has become a well established target receptor for APC-specific vaccination [9].

In this study we analyzed the efficacy of a refined MR-targeting delivery system based on OVA, intended to serve both as a MR targeting molecule and as a source of antigen. We show that DEX-based nanoparticles as such are not internalized by DCs and lack unwanted immunomodulatory function. DEX particles adsorbed with OVA, however, were efficiently engulfed by murine DCs in vitro in a MR-dependent manner. Codelivery of OVA and LPS...
by DEX particles induced stronger and more sustained immune responses in vitro and in vivo than direct application of these compounds which confirms their usability for immunotherapeutic applications.

**Materials and Methods**

**Adsorption of Antigen and Adjuvant to Dextran (DEX) Particles**

DEX nanoparticles were mixed with OVA and LPS according to a general protocol described to result in efficient binding of distinct compounds to dextran-based nanospheres [5] with some modifications. Detailed information are obtainable from the Supporting Informations in Methods S1.

**Electron Microscopy**

Shape and size distribution of DEX particles dispersed in PBS were studied using a Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR) at an accelerating voltage of 120 kV. Images were taken using a 1392 × 1042 SIS Megaview camera (Olympus, Münster, Germany).

**Dynamic Light Scattering**

The size of DEX particles resuspended in PBS and analysis of potential interaction with blood serum were determined by dynamic light scattering (DLS) analysis as described elsewhere [10]. Detailed descriptions are given in Methods S1.

**Ethics Statement**

All mouse strains were bred and maintained in the Central Animal Facilities of the University of Mainz under specific pathogen-free conditions according to the guidelines of the regional animal care committee. All animal experiments were performed in accordance with national and European (86/609/EEC) legislation, and in accordance with the Central Laboratory Animal Facility of the University of Mainz. The protocol was approved by the national investigation office of Rhineland-Palatinate (Permit Number: 31377-07/G00-1008).

For ethical reasons, blood samples were withdrawn under ketamine and xylazine anaesthesia and all efforts were made to minimize suffering.

**Mice**

All mouse strains were bred and maintained in the Central Animal Facilities of the Johannes Gutenberg-University of Mainz under specific pathogen-free conditions on a standard diet. The “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 1985) were followed. CD4+ T cells of OT-II (C57BL/6 background) and of DO11.10 (BALB/c) mice are transgenic for a δβTCR specific for OVA257–264 peptide in context of H-2 I-Ak and I-A^q; respectively, CD8^+ T cells of OT-I (C57BL/6) mice are transgenic for a δβTCR specific for OVA-derived SIINFEKL peptide (OVA257–264) in the context of H-2K^b. Both OT-I and OT-II strains (C57BL/6 background) were crossed to CD45.1^+ C57BL/6j congenic mice.

**Generation of Murine Bone Marrow-derived Dendritic Cells**

BM-DCs were generated as previously described [11] with some modifications. On day 6, non-adherent and loosely adherent BM-DCs were collected. Aliquots of BM-DCs were stimulated with DEX particle formulations at concentrations as indicated or with LPS (100 ng/ml) for 24 h.

**DC Viability**

To assess potential cytotoxic effects of DEX particle formulations, day 6 BM-DCs (2.5 × 10^6) were resuspended into wells of 96 well cell culture plates in a volume of 50 μl, and DEX particles were added at different concentrations as indicated. To assay cell viability, tetrazolium substrate was added which is reduced to a chromogenic formazan product by mitochondrial succinate dehydrogenase, and thereby correlates with the number of metabolically active cells. The reaction was stopped by addition of an organic solvent, and the concentration of solubilized formazan was detected spectrophotometrically in an ELISA reader according to the protocol provided by the manufacturer (Promega, Madison, WI).

**Cellular Uptake of Functionalized FITC-labeled DEX Particles**

BM-DCs (5 × 10^5 cells) or spleen cell suspensions (2 × 10^6 cells) derived from C57BL/6 mice were incubated with FITC-labeled DEX particle formulations (each 50 μl [BM-DCs] or 30 μl [spleen cells]) as indicated in a volume of 200 μl at 37°C in 96 wells of a cell culture plate for the indicated periods of time. To assess for MR-dependent endocytosis of OVA-adsorbed DEX particles by BM-DCs, cells (5 × 10^6 in 200 μl) were preincubated with mannan (200 μg/ml; Sigma-Aldrich, Deisenhofen, Germany) for 30 min at 37°C. After incubation, cells were harvested and stained for surface lineage marker expression as indicated for subsequent flow cytometry analysis (see below).

**Flow Cytometry**

Cells were washed in FACS buffer, and stained with PE-Cy7-conjugated anti-CD11c, PE-conjugated anti-CD80 or anti-CD3, FITC-conjugated anti-CD40, and e-fluor405-conjugated anti-MHCII or anti-F4/80 antibodies as indicated. For intracellular detection of IFN-γ, cells were stained with APC-Cy7-conjugated CD3, PE-conjugated anti-V^α2, and PE-Cy5-conjugated CD45.1, and fixed with 4% paraformaldehyde. Then, cells were permeabilized, and stained with APC-conjugated anti-IFN-γ. All antibodies were purchased from eBioscience (San Diego, CA). Expression intensities were assessed by flow cytometry (FACS LSR II, BD Biosciences, San Diego, CA).

**T Cell Proliferation Assays**

Aliquots of day 6 BM-DCs (10^6 cells) were cocultured with DEX particle formulations (100 μl), LPS (100 ng/ml), and OVA protein (2 μg) as indicated for 24 h. For in vitro proliferation assays, (OT-II) T cells were purified from mouse spleens and lymph nodes by auto MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). BM-DCs were cocultured with OT-II T cells at the indicated cell numbers in triplicates for 48 h. Afterwards, cocultures were incubated with [3H]-thymidine for an additional
16 h. Genomic incorporation of \(^{3}\text{H}\)-thymidine was determined by liquid scintillation counting.

For in vivo analysis of T cell proliferation, splenocytes (OT-1xLy-5.1, OT-IIxLy5.1) were incubated with 0.5 \(\mu\text{M}\) carboxyfluorescein diacetate succinimidyl ester (CFSE, Life Technologies) for 10 min at room temperature. CFSE-labeled splenocytes (10\(^7\)) were transferred intravenously (i.v.) into C57BL/6 mice. After 48 h, 4 \(\mu\text{g}\) of OVA protein or the corresponding amount of DEX particle-bound OVA were injected i.v., either alone or combined with 100 ng of LPS as indicated. Four days later, spleens and peripheral lymph nodes (LNs) were removed and cell suspensions were analyzed for proliferation of CFSE-labeled OT-1xLy-5.1 or OT-IIxLy5.1 T cells by flow cytometry.

Foot Pad Swelling Assay to Assess Antigen-specific Delayed-type Hypersensitivity

DO11.10 CD4\(^+\) T cells (5 \(\times\) 10\(^5\) per mouse) were injected i.v. into BALB/c mice. One day later, OVA\(_{323-339}\) peptide (40 \(\mu\text{g/mouse}\)), LPS (100 ng/mouse), and DEX particle formulations (40 \(\mu\text{g/mouse}\)) were injected i.v. as indicated. Two weeks later, syngeneic day 7 BM-DCs, stimulated with LPS for 24 h, were pulsed with OVA\(_{323-339}\) peptide (0.1 \(\mu\text{g/ml}\)) for 4 h, and were injected subcutaneously (s.c.) into foot pads of pretreated mice (5 \(\times\) 10\(^3\) BM-DCs per foot pad). Starting on the day of injection, foot pad swelling was measured daily.

In Vivo Killing Assay

Spleen cells derived from OT-IxLy-5.1 mice were resuspended in PBS (5 \(\times\) 10\(^7\) /ml) and injected (200 \(\mu\text{l}\) i.v.) into mice via the tail vein. Two days later, groups of mice were immunized with OVA (4 \(\mu\text{g/mouse}\)), LPS (4 \(\mu\text{g/mouse}\)), and DEX particle-based nanovaccines (200 \(\mu\text{g per mouse}\)) as indicated. After 5 days, spleen cells were isolated from Ly-5.1 mice. One fraction was pulsed with 1 \(\mu\text{g/ml}\) OVA\(_{257-264}\) peptide (1 h, 37\(^\circ\)C) to serve as the target cell population. Target cells were labeled at a low concentration of CFSE (0.5 \(\mu\text{M}\), CFSE\(_{\text{low}}\) cells). The other fraction was left unpulsed and was labeled at higher CFSE concentration (0.05 \(\mu\text{M}\), CFSE\(_{\text{high}}\) cells) to serve as an internal control. Equal numbers of cells from both populations were mixed, and a total of 10\(^7\) cells in 200 \(\mu\text{I}\) of PBS was injected i.v. per mouse. Four h after injection, splenocytes were derived from treated mice, and the frequencies of CFSE\(_{\text{Ly-5.1}^+}\) cells were assessed by FACS analysis to determine the extent of in vivo killing. The level of specific cytotoxicity was calculated according to the following formula: 100\%\(-\text{CFSE}_{\text{low}}/\text{CFSE}_{\text{high}}\times100\%\).

Antibody Detection

Mice were immunized with DEX particle formulations equivalent to 4 \(\mu\text{g}\) of OVA protein (DEX[OVA], DEX[OVA+LPS]) or 200 \(\mu\text{g per mouse}\) of DEX particles (DEX[\(-\)], DEX[LPS]) as indicated. One and two weeks after immunization mice were bled from the retro-orbital plexus. OVA-specific IgG1 and IgG2a levels were determined in defined sera by ELISA. The antibody titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis. IgG contents were standardized by testing reference sera in parallel.

Results

Characterization of Functionalized DEX Particle Formulations

DEX-based nanoparticles (DEX[\(-\)]) were of spherical shape and rather uniform in size as assessed by electron microscopy (Figure 1A). DEX particle formulations containing OVA and LPS either alone or in combination were comparable in terms of appearance and size (data not shown). Actual sizes of dextran T500 and the different derived types of DEX-based particles were assessed by DLS analysis. The angular dependency of the hydrodynamic radii of the different types of dextran particles is shown in Figure 1B. Extrapolation to zero scattering angle (scattering vector \(q = 0\)) resulted in the z-average values of the hydrodynamic radii of the untreated Dextran T500 polyacrylate (<\(R_d\),\(^2\>) = 17 nm), which were somewhat larger in case of the DEX particle formulations (DEX[\(-\)]: 23 \(\pm\) 3 nm, DEX[OVA]: 20 \(\pm\) 3 nm, DEX[LPS]: 19 \(\pm\) 3 nm, and DEX[OVA+LPS]: 20 \(\pm\) 3 nm). Due to the largely comparable sizes and scattering intensities of Dextran T500 and derived DEX particles, the latter mostly consist of single dextran molecules interacting with OVA and LPS, respectively, in a yet unknown manner, not elucidated in the present work. DLS analysis of different DEX particle types (DEX[OVA], DEX[LPS]) preincubated with human serum showed no significant alterations of particle-associated parameters (Figure S1), which excludes considerable interaction of these DEX particles with serum components.

OVA-containing DEX Nanoparticles are Engulfed by BM-DCs in a Mannose Receptor-dependent Manner

Since DEX-based particles exerted no detrimental effect on BM-DC viability (Figure 1C), next we assessed the intracellular uptake of FITC-labeled DEX particles by unstimulated BM-DCs. In a time kinetics assay DEX particle types devoid of OVA protein (DEX[\(-\)], DEX[LPS]) showed no binding to BM-DCs over 24 h of coincubation (Figure 2A). In contrast, incubation with OVA-containing DEX particle formulations (DEX[OVA], DEX[OVA+LPS]) resulted in steadily increasing frequencies of FITC\(^{+}\) BM-DCs. Confocal microscopy confirmed pronounced cellular uptake of OVA-containing versus non-containing DEX particles by BM-DCs (Figure 2B) as assessed 4 h (left panel) and 24 h (right panel) after the onset of coincubation. In light of the OVA-dependent binding and uptake of DEX particles by BM-DCs, we asked for involvement of the MR receptor. In competition experiments, preincubation of BM-DCs with the prototypic MR ligand mannan at high concentration significantly reduced cellular binding of subsequently applied DEX[OVA] (Figure 2C).

Based on the finding of MR-dependent binding of DEX particles to BM-DCs, we evaluated their suitability to specifically target primary APCs as well, an important prerequisite for their intended in vivo application. For this, isolated spleen cells were coincubated with FITC-labeled DEX particles. As shown in Figure 2D, only CD11c\(^{+}\) DCs and F4/80\(^{+}\) macrophages efficiently bound FITC\(^{+}\) DEX[OVA+LPS], but not control DEX particles. In contrast, CD19\(^{+}\) B cells and CD3\(^{+}\) T cells, known to lack MR expression, showed no efficient binding of either type of DEX particles.

LPS-loaded DEX Formulations Efficiently Stimulate BM-DCs, and DEX Particles Codelivering OVA and LPS Evoke Strong CD4\(^{+}\) T Cell Proliferation

In previous studies, polymers functionalized with mannos to target APCs via binding to the MR mediated efficient internalization, but at the same time induced DC activation [12]. Therefore, we analyzed the different types of DEX particles for their DC-activating capacity.

For this, unstimulated BM-DCs were incubated in parallel assays with OVA protein, LPS, or the different types of DEX particles for 24 h, and the expression of DC activation markers
(CD40, CD80, CD86) was analyzed. Stimulation of BM-DCs with LPS resulted in marked upregulation of either activation marker, while OVA and OVA-containing DEX particle formulations (DEX[OVA]) were devoid of DC-stimulatory activity (Figure 3A). Only DEX particle types containing LPS (DEX[LPS], DEX[OVA + LPS]) facilitated robust DC activation as reflected by upregulation of CD40, CD80 and CD86, to similar extent as mediated by LPS in case of the two latter.

Due to efficient engulfment of OVA-adsorbed DEX particles and the DC-stimulatory capacity of particulate LPS, we tested the efficacy of DEX particles to mediate an antigen-specific T cell response. While BM-DCs preincubated with soluble OVA protein alone induced no marked proliferation of subsequently cocultured OVA peptide-specific OT-II CD4\(^+\) T cells, pretreatment of BM-DCs with DEX[OVA] facilitated robust T cell activation (Figure 3B). In accordance, pretreatment of BM-DCs with DEX particles codelivering OVA and LPS (DEX[OVA+LPS]) facilitated strongly enhanced T cell activation, which was significantly higher as induced by BM-DCs pretreated with OVA plus LPS.

Codelivery of OVA and LPS by a DEX-based Nanovaccine Results in a Potent and Sustained Immune Response in vivo

Due to the strong bioactivity of OVA and LPS when applied as particulate formulations to mediate DC-dependent activation of antigen-specific CD4\(^+\) T cells in vitro, we assessed the suitability of these DEX particles to mount an OVA-specific CD4\(^+\) T cell response, when applied directly in vivo, which requires targeting of MR-expressing APCs. To this end, proliferation of CFSE-labeled splenocytes derived from OT-II mice and injected i.v. into C57BL/6 mice was analyzed after treatment of recipient mice with OVA and LPS as soluble or particulate formulations. As shown in Figure 4A, in all groups of mice which had received OVA plus LPS, strong proliferation of OT-II T cells was detected. Based on this result, the suitability of DEX-based nanovaccines to elicit a robust CD4\(^+\) T cell-dependent immune response was evaluated in a model of antigen-specific foot pad swelling. For this, syngeneic OVA peptide-specific CD4\(^+\) T cells were transferred into recipient mice, sensitized with OVA plus LPS, DEX(O-VA+LPS) or DEX(-) as a control, and challenged by injection of syngeneic, OVA peptide-pulsed stimulated BM-DCs into the hind foot pads. While sensitization with soluble OVA plus soluble LPS resulted in a transient foot pad swelling only, that returned to background levels already at day 2 after challenge, application of particle-bound OVA plus LPS (DEX[OVA+LPS]) induced a marked and prolonged delayed-type hypersensitivity response that persisted for days (Figure 4B).

**Figure 1.** DEX particles are of spherical appearance and uniform in size. (A) Shape and size distribution of DEX(–) dispersed in PBS was studied by electron microscopy. (B) Hydrodynamic radii of Dextran T500 and derived DEX particle formulations as function of q\(^2\) in DPBS buffer (0.33 mg/ml) were determined by DLS (see Methods). Graphs denote the angular dependency of the apparent diffusion coefficient of the different dextran solutions in buffer solution. (C) To assess cytotoxic effects of DEX particles on BM-DC viability, cells (2.5 \( \times \) 10\(^5\)) were seeded into wells of 96 well cell culture plates in a volume of 50 µl in triplicates, and DEX(–) particles (20 mg/ml) were added at different amounts as indicated. One day later, viability of BM-DCs was assessed as described (see Methods). The viability of untreated BM-DCs was set to 100% (dashed line).

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DEX-based Nanovaccines Induce Strong CD8\(^+\) T Cell Activation in vivo

To assess the suitability of DEX-based nanovaccines to induce robust CD8\(^+\) T cell responses, the proliferation of OVA peptide-specific CD8\(^+\) T cells derived from OT-1 mice and transferred into syngeneic C57BL/6 mice was assessed after treatment of recipient mice with OVA and LPS in different formulations. In comparison, coapplication of soluble OVA and LPS mounted low CD8\(^+\) T cell proliferation only, which was significantly higher in case of prior immunization with particulate OVA (DEX[OVA]), coadministered with soluble LPS (Figure 5A). However, DEX particles

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adsorbed with OVA plus LPS (DEX[OVA+LPS]) evoked the strongest OVA-specific CD8\(^+\) T cell proliferation of all groups compared. Similarly, the frequency of OT-I T cells producing the Th1 cytokine IFN-\(\gamma\) was lowest in mice treated with OVA plus LPS, intermediate when DEX(OVA) plus LPS had been coapplied, and highest in the group immunized with DEX(O-VA+LPS) (Figure 5B).

The finding of robust CD8\(^+\) T cell proliferation and IFN-\(\gamma\) production as induced by DEX-based nanovaccines in \textit{vivo} prompted us to assess the functional activity of OT-I T cells in terms of cytotoxic activity. In an \textit{in vivo} killing assay, lysis of OVA peptide-presenting target cells occurred only in groups of mice cotreated with OVA and LPS (Figure 5C, upper panel). Lysis of CFSE\(^{\text{low}}\) target cells was lowest after coadministration of soluble OVA and LPS, somewhat elevated in the group which had received DEX(OVA) plus LPS, and strongest in mice injected with DEX(OVA+LPS) (Figure 5C, lower panel).

**DEX Particles that Codeliver OVA and LPS Induce a Th2-biased Humoral Response**

In light of the essential role of humoral immune responses for pathogen clearance and their contribution to anti-tumor responses, we asked for the potential of DEX-based nanovaccines to mount the production of OVA-specific antibodies. For this, naive mice were injected \textit{i.v.} with the different types of DEX particles,
and sera derived one and two weeks later were assayed for OVA-specific IgG titers. At either time point, OVA-specific IgG1 and IgG2a were detectable only in sera obtained from mice immunized with OVA-containing DEX-based nanovaccines, thereby confirming antigen-dependency of antibody production (Figure 6). As expected, OVA-specific antibody titers were higher

Figure 3. BM-DCs are strongly activated by LPS-containing DEX particle formulations, and codelivery of OVA results in robust antigen-specific CD4⁺ T cell activation. (A) Aliquots of unstimulated day 6 BM-DCs (10⁶ cells; C57BL/6) were left untreated (−), stimulated with LPS (100 ng/ml), or were coincubated with DEX particles (100 μl) as indicated for 24 h. Afterwards, expression of CD40, CD80, and CD86 was assessed by flow cytometry. Graphs denote mean fluorescence intensities (MFI) ± SEM of three experiments. Statistical significant differences between groups are indicated (**p<0.01, ***p<0.001). (B) Unstimulated day 6 BM-DCs (10⁶ cells; C57BL/6) were treated with soluble OVA (2 μg) or as described in A for 24 h. Titrated numbers of BM-DCs were cocultured with sorted CD4⁺ OT-II T cells in triplicates for 3 days at the ratios indicated. T cell proliferation was assessed as incorporation of ³H-thymidine added for the last 16–18 h. Data represent mean ± SEM of triplicates and are representative of three independent experiments. Statistical significant differences between DEX(OVA-LPS) versus OVA plus LPS and DEX(OVA) versus OVA for each T cell/BM-DC ratio are indicated (*p<0.05, **p<0.01).

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when OVA plus LPS were coredelivered (DEX[OVA+LPS]) than mounted in response to OVA alone. At either time point assessed, more IgG1 than IgG2a was detected, reminiscent of a Th2-skewed IgG pattern. Taken together, these findings show that DEX-based nanovaccines are capable to induce both a cellular and a humoral immune response in vivo in an antigen-specific manner.

**Discussion**

DCs constitute an attractive target for immunotherapeutic strategies based on their versatile functional properties, namely to maintain peripheral tolerance under steady state conditions [13], but to induce potent immune responses when activated by pathogen-associated danger signals [14]. In conventional vaccination strategies protein antigen(s) and APC-activating adjuvant(s) are coinjected [15]. However, it is well established by now, that antigen in combination with adjuvant induces a stronger immune response when codelivered as a particulate formulation [16].

Ongoing studies aim on the development of reliable targeting strategies which enable APC-focussed delivery of nanovaccines [17], but major restrictions arise from the laborious synthesis and functionalization, limited biodegradability, cytotoxicity, and intrinsic immunomodulatory properties of such formulations [18]. In light of these limitations, we sought to develop a nanovaccine largely devoid of the aforementioned hindrances. For this, we evaluated the suitability of DEX particles which have been introduced almost thirty years ago as a biodegradable, and non-cytotoxic carrier system for proteins and other compounds, reported to elicit antigen-specific humoral responses in vivo after s.c. immunization of mice, at much higher extent than direct immunization with antigen [4].

In accordance with the general properties of dextran biopolymers, in our study DEX particles were devoid of cytotoxic or direct immunomodulatory effects. In this regard, it is noteworthy that other types of nanoparticles, like zinc oxide [19] or amorphous silica nanoparticles [20], which are contained in a variety of consumer products, have been shown to induce cytotoxic effects in isolated immune cells.

In earlier approaches, particulate carriers have been optimized in terms of structural composition and size, aimed to result in enhanced uptake by myeloid APCs by means of endocytosis and phagocytosis [21]. Based on these studies, particles of 0.1–1 μm in diameter have been demonstrated to passively target APCs [22,23]. However, cell-type unspecific uptake of such types of particles may limit their usability in terms of APC-specific vaccination [24]. In contrast, here we show that DEX particles as such, despite their minor size, were not engulfed by immune cells to a great extent, which underlines their potential to serve as inert carriers for macromolecules that facilitate cell type-specific targeting.

Accordingly, we sought to exploit the intrinsic myeloid APC targeting property of OVA, which by itself constitutes an important model antigen frequently employed to study adaptive immune responses. In agreement with the well established MR-targeting properties of OVA [6,7], here we demonstrated that DEX particles containing OVA were efficiently engulfed by murine BM-DCs in an MR-dependent manner, as suggested by efficient inhibition in the presence of mannan at high concentration. Moreover, OVA-containing DEX particles efficiently bound primary DCs and macrophages, shown to express the MR [25], while B cells and T cells as MR-deficient lymphoid immune cells were not targeted. These results suggest that mannosylation of a given protein antigen may suffice to mediate binding and cellular uptake of a conjugated nanovaccine by MR-expressing APCs. Accordingly, as exploited in our study, a candidate protein at the same time may serve both as a source of antigen, and as an APC-targeting molecule. In confirmation of the latter, the uptake of...
protein antigens by APCs was strongly elevated when these proteins were mannosylated due to expression in engineered yeast cells [26] or in vitro prior to application [27].

Several studies have suggested that uptake of MR-targeting nano-carriers, like polyanhydride [12] and PLGA (D, L-lactide-co-glycolic acid) [28] nanoparticles, resulted in DC activation, which may be explained in part by signaling pathways activated in response to MR-mediated protein uptake [29]. In contrast, in our study BM-DCs incubated with DEX(OVA) remained unstimulated, which indicates that MR engagement as such is not

Figure 5. The nanovaccine DEX(OVA+LPS) induces profound activation of antigen-specific CD8+ T cells in vivo. C57BL/6 mice received CFSE-labeled, OVA-specific OT-I T cells (10^7) i.v. Two days later, groups of mice (each five animals) were either left untreated or were immunized with OVA (4 µg per mouse), LPS (4 µg), and DEX particle formulations (each 200 µl) as indicated. (A) On day 5, the frequencies of proliferating CD8+CD45.1+Vα2 OT-I T cells were assessed by flow cytometry. (B) In the same experiments, the frequencies of IFN-γ-producing CD8+ OT-I T cells in each group were significantly higher than in the non-immunized control group. Other statistical significant differences between groups are indicated (*p<0.05, **p<0.01, ***p<0.001). (C) On day 4 after immunization, mice were injected with CFSElow target cells (loaded with OVA257–264) and CFSEhigh control cells (each 10^7 cells) derived from syngeneic Ly-5.1+ mice. 4 h later, splenocytes were isolated and frequencies of CFSE-labeled cell populations were assessed by flow cytometry. Upper panel: Data represent mean±SEM of two independent experiments. Statistical significant differences between groups are indicated (*p<0.05, **p<0.01). Lower panel: Frequencies of Ly-5.1+ target cells (CFSElow) and control cells (CFSEhigh) in spleen cell suspensions derived from one mouse of either group are shown as histograms. Graphs are representative of two independent experiments.

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sufficient to mediate DC activation. In general, structurally distinct nanoparticle formulations were characterized by an intrinsic DC-activating immunomodulatory function, i.e. γ-PGA (poly[γ-glutamic acid]) [30], poly(propylene)sulfide [31], or LDH (layered double hydroxide) [32]. Yet other types of nanoparticles have been reported to exert inhibitory activity on DCs, like PVA-SPIONS (poly[vinylalcohol]-coated super-paramagnetic iron oxide nanoparticles) [33]. Altogether, these reports demonstrate that nanoparticles often exert immunomodulatory activity, which may modulate the character of an intentionally induced immune response in an unwanted manner, e.g. in terms of T-cell polarization [34]. Therefore, the lack of immunomodulatory activity of DEX particles on DCs clearly broadens their range of application, because it may allow to shape a nanovaccine-induced immune response solely according to the properties of codelivered adjuvants [35]. Here we employed the TLR4 ligand LPS as an adjuvant, well known to activate myeloid DCs, which in turn favor Th1-biased immune responses [36]. LPS-containing DEX particles readily activated BM-DCs to similar extent as LPS applied directly. Interestingly, DEX particles engineered to contain only LPS were not internalized by BM-DCs. In contrast, Demento and co-workers [37] reported that PLGA-based nanoparticles were engulfed by murine DCs at higher efficiency when decorated with LPS than at non-functionalized state. This observation suggests that TLR4 engagement per se may be sufficient for subsequent internalization of TLR4 ligand-coated nanovaccines. However, the discrepancy between the findings of Demento and co-workers and our results may be explained by differences in particle-surface LPS densities.

The DEX-based nanovaccine which codelivered OVA and LPS (DEX(OVA+LPS)) was most effective in inducing pronounced T-cell responses both in vitro when incubated with BM-DCs, as well as in vivo after direct application. Whereas both soluble and DEX-bound OVA were able to stimulate the proliferation of CD4⁺ T cells, only the delivery of DEX(OVA+LPS) exerted a sustained immune response as demonstrated in a foot pad swelling assay, a well established model for T-cell mediated delayed-type hypersensitivity. DEX-based nanovaccines also proved substantially more efficient to mediate cross presentation of OVA peptides by DCs in vivo than soluble OVA [5] as evidenced by the induction of a Th1-biased activation of OVA-specific CD8⁺ OT-I T cells. Taken together, these data demonstrate that the immunomodulatory effect of antigen (OVA) and adjuvant (LPS) on a particulate carrier (DEX) that targets DCs induces superior T-cell-mediated immune responses in vivo when compared to immunization with soluble antigen and adjuvant.

Besides mounting potent T-cell responses, in line with the results obtained by Schroeder et al. [4], DEX-based nanovaccines containing OVA also induced a Th2-biased OVA-specific IgG isotype pattern (IgG1>IgG2a). Thus, in context with DEX particle-derived nanovaccines other adjuvants than LPS may be required to induce a pronounced Th1-skewed pattern of antibody production.

Taken together, the modular character of the DEX-based nanovaccine platform evaluated in this study may enable the generation of vaccine formulations that are able to specifically target glycosylated protein antigens to DCs in a MR-mediated fashion in vivo. In addition, these DEX-based nanocarriers are also able to specifically administer adjuvants or other immunomodulatory agents to DCs in situ in order to shape immune responses as required for immunotherapeutic applications.

Supporting Information

Figure S1 DEX particles functionalized with OVA or LPS display no interaction with serum. DLS analysis of DEX particle formulations preincubated with human serum was performed as described in the Materials and Methods section. Graphs denote correlation functions (scattering angle 30°) of DEX(OVA) (upper panel) and DEX(LPS) (lower panel) in human serum. Force fit (eq. 3 and residuum (bottom line) are shown. (TIF)

Methods S1 Detailed information on the generation of DEX particles, and of DLS analysis are given in Methods S1. (DOC)

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of cellular uptake of DEX particle formulations by BM-DCs. This work was performed in partial fulfillment of the requirements of the doctoral thesis of Limei Shen.

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Author Contributions

Conceived and designed the experiments: LS MB SG HS. Performed the experiments: LS TH EM KR IT NV SP. Analyzed the data: LS TH MK MS MB. Contributed reagents/materials/analysis tools: LS. Wrote the paper: LS SG MB.