Protein corona–mediated targeting of nanocarriers to B cells allows redirection of allergic immune responses

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Background: Nanoparticle (NP)–based vaccines are attractive immunotherapy tools because of their capability to codeliver antigen and adjuvant to antigen-presenting cells. Their cellular distribution and serum protein interaction ("protein corona") after systemic administration and their effect on the functional properties of NPs is poorly understood.

Objectives: We analyzed the relevance of the protein corona on cell type–selective uptake of dextran-coated NPs and determined the outcome of vaccination with NPs that codeliver antigen and adjuvant in disease models of allergy.

Methods: The role of protein corona constituents for cellular binding/uptake of dextran-coated ferrous nanoparticles (DEX-NPs) was analyzed both *in vitro* and *in vivo*. DEX-NPs conjugated with the model antigen ovalbumin (OVA) and immunostimulatory CpG-rich oligodeoxynucleotides were administered to monitor the induction of cellular and humoral immune responses. Therapeutic effects of this DEX-NP vaccine in mouse models of OVA-induced anaphylaxis and allergic asthma were assessed.

Results: DEX-NPs triggered lectin-induced complement activation, yielding deposition of activated complement factor 3 on the DEX-NP surface. In the spleen DEX-NPs targeted predominantly B cells through complement receptors 1 and 2. The DEX-NP vaccine elicited much stronger OVA-specific IgG_{2a} production than coadministered soluble OVA plus CpG oligodeoxynucleotides. B-cell binding of the DEX-NP vaccine was critical for IgG_{2a} production. Treatment of OVA-sensitized mice with the DEX-NP vaccine prevented induction of anaphylactic shock and allergic asthma accompanied by IgE inhibition.

Conclusions: Opsonization of lectin-coated NPs by activated complement components results in selective B-cell targeting. The intrinsic B-cell targeting property of lectin-coated NPs can be exploited for treatment of allergic immune responses. (J Allergy Clin Immunol 2018;142:1558-70.)

Key words: *Dendritic cells, B cells, complement, antibody, complement factor 3, lectin pathway, IgG_{2a}*

Allergen-specific immunotherapy (AIT) through repeated subcutaneous injections or sublingual application of increasing doses of allergen leading to allergen-specific tolerance is a well-established therapy of type 1 allergic diseases.[1] However, because of the long duration of this therapeutic approach and associated side effects, alternate vaccination strategies that aim to override T_H2 polarization have come into the focus of research.[2]

Conventional vaccines that include allergen plus adjuvant do not selectively address specific antigen-presenting cell (APC) types, and both components can act independently from each other. In this regard allergen can be recognized by IgE antibodies on the surfaces of basophils or mast cells and trigger allergic reactions, and the adjuvant can induce unwanted inflammatory side effects.[3] Indeed, it was shown that DNA that contained non-methylated CpG motifs and thereby activated B cells through Toll-like receptor (TLR) 9 induced production of anti-DNA antibodies.[4]

More recently, targeting of dendritic cells (DCs) with an allergen conjugated with a surface receptor–specific antibody[5] or a natural ligand[6] has been shown to promote T-cell tolerance induction. In these cases signaling of the targeted receptor induced a

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Abbreviations used
AIT: Allergen-specific immunotherapy
APC: Antigen-presenting cell
BCR: B-cell receptor
C3: Complement factor 3
CFSE: Carboxyfluorescein succinimidyl ester
CR: Complement receptor
DAPI: 4′-6-Diamidino-2-phenylindole dihydrochloride
DC: Dendritic cell
DEX-NP: Dextran-coated ferrous nanoparticle
FDC: Follicular dendritic cell
MBL: Mannose-binding lectin
NP: Nanoparticle
ODN: Oligodeoxynucleotide
OVA: Ovalbumin
TLR: Toll-like receptor

protolerogenic state in DCs. As an alternative, T_H2-dependent allergic reactions were inhibited by redirecting the immune response toward a T_H1 response through coapplication of adjuvants that imprint a T_H1-promoting capacity in DCs.[1] Therefore multifunctional vaccines hold great promise for AIT.[7,8] Because of advances in nanotechnology, complex nanoparticulate vaccines are now being explored that contain antigens, immunostimulants, and/or specific cellular targeting moieties.

However, the protein corona that covers the nanovaccine after serum contact can interfere with cell-specific targeting strategies through several mechanisms: (1) targeting moieties can be covered, preventing them from binding to their specific receptors on target cells[9]; (2) components of the serum protein corona of nanovaccines might mediate binding to cellular receptors, such as scavenger receptors[10]; and (3) the surface of the nanocarrier can be recognized as foreign by the complement system, triggering its activation and resulting in binding to complement receptors (CRs) highly expressed by APCs and other immune cell types.[11]

Nonetheless, the functional effect of the nanoparticle (NP) protein corona on its cell-binding properties *in vivo* has not been thoroughly assessed. Knowledge of functional consequences is essential for the design of the surfaces of nanocarriers to ensure their proper cellular targeting and functional efficacy.

In this study we investigated the suitability of dextran-coated ferrous nanoparticles (DEX-NPs), which are widely used in immunologic assays[12] and clinical trials,[13] to serve as nanovaccines for therapeutic treatment of systemic anaphylaxis and allergic asthma in mouse models. We show that lectin-coated surfaces of DEX-NPs activated the lectin complement pathway. Surface-deposited active complement factor 3 (C3) resulted in predominant targeting to CR1/2-expressing B cells. DEX-NPs coated with ovalbumin (OVA) as a model allergen plus immunostimulatory CpG oligodeoxynucleotides (ODNs) induced strong OVA-specific IgG_{2a} levels. Vaccination of sensitized mice with this model nanovaccine largely prevented allergic symptoms in anaphylaxis and asthma models associated with suppressed IgE levels and increased IgG_{2a} levels. We consider the complement-mediated B-cell targeting properties of lectin-coated NPs as an interesting approach for allergy treatment.

METHODS

Preparation and functionalization of dextran-coated colloidal NPs

Dextran-coated iron oxide NPs were synthesized and functionalized with endotoxin-depleted OVA (Sigma-Aldrich, Deisenhofen, Germany) and CpG ODN 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′; Metabion, Martinsried, Germany) by Miltenyi Biotec (Bergisch Gladbach, Germany), as previously described.[12] Details on DEX-NP generation are described in the Methods section in this article's Online Repository at www.jacionline.org. All DEX-NP formulations were labeled with Cy5.

Label-free quantitative proteomic analysis of corona components

NP protein complexes were isolated by means of centrifugation through a sucrose cushion, and the obtained pellets were washed to obtain the hard protein corona, as described previously.[14] Corona constituents were eluted from DEX-NP(OVA) by using 7 mol/L urea, 2 mol/L thiourea, and 2% CHAPS (all from Roth, Karlsruhe, Germany). Label-free quantitative proteomic analysis of protein corona components by using mass spectrometry with ion mobility–enhanced data-independent acquisition was performed, as described in detail previously.[15,16] The complete proteomic data set with all quantification results is shown in File E1 in this article's Online Repository at www.jacionline.org.

Mice

BALB/c, C57BL/6, and $C3^{-/-}$ (B6;129S4-$C3^{tm1Crr}$/J)[17]; mannose-binding lectin (MBL)$^{-/-}$ (B6.129S4-$Mbl^{1tm1Kata}$ $Mbl2^{tm1Kata}$/J)[18]; and OT-II[19] mice on the C57BL/6 background were bred and maintained in the Central Animal Facility of the Johannes Gutenberg-University Mainz under specific pathogen-free conditions on a standard diet according to the guidelines of the regional animal care committee. Female mice (BALB/c, C57BL/6, $C3^{-/-}$, and MBL$^{-/-}$ mice: 6 weeks; OT-II mice: 9-12 weeks) were used throughout all experiments. All animal experiments were performed in accordance with national and European (86/609/EEC) legislation. The "Principles of laboratory animal care" (National Institutes of Health publication no. 85-23, revised 1985) were followed.

Immunohistochemistry

Mice were injected intravenously with DEX-NP formulations (each with 2×10^{12} particles), as indicated. In blocking experiments mice were pretreated with 50 μg of rat anti-mouse CD21/CD35 antibody (clone 7G6; BD Biosciences, San Jose, Calif) or corresponding isotype control antibody (clone RTK4530; BioLegend, San Diego, Calif) 1 hour before application of DEX-NP formulations. Four hours after DEX-NP injection, inner organs were retrieved, and derived cryosections were incubated with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; Life Technologies, Carlsbad, Calif) and Alexa Fluor 488–labeled antibodies (BioLegend) to detect immature (GR-1, clone RB6-8C5) and differentiated (CD11b, M1/70) myeloid cells, macrophages (CD68, FA-11), DCs (CD11c, N418), B cells (CD19, 6D5), and follicular dendritic cells (FDCs; FDC-M1). BX61 and IX81 fluorescence microscopes (Olympus, Tokyo, Japan) were equipped with cellF software (Soft Imaging System, Münster, Germany).

Confocal microscopy

Immunosorted (Miltenyi Biotec) and untouched splenic B cells were incubated with Cy5-labeled DEX-NP(OVA-CpG), as indicated; washed with FACS buffer (PBS, 1% FCS, and 0.5 mmol/L EDTA); and transferred onto chamber slides (IBIDI, Martinsried, Germany). Cells were incubated with anti-CD19 antibody (green) and DAPI (blue). The LSM510-UV confocal microscope (Zeiss, Jena, Germany) was used.

NP binding studies

Immunosorted splenic $CD19^+$ B cells were seeded into wells of 96-well cell culture plates (5×10^5/200 μL). Aliquots of nonfunctionalized DEX-NPs (DEX-NP[−]; 5×10^{11} per sample) were preincubated (for 1 hour at 37°C) with albumin (5 mg/mL; Sigma-Aldrich), mouse total IgG (100 μg/mL; BioLegend), or mouse serum (10 μL per sample) and left untreated or heat inactivated (for 30 minutes at 56°C) in a volume of 20 μL. Aliquots of dextran- and starch-coated NPs (each 10 μg) were preincubated with mouse serum, as indicated. In some experiments seeded mouse B cells were preincubated (for 30 minutes at 37°C) with rat anti-mouse CD21/CD35 antibody (5 μg/mL). After 4 hours, samples were incubated with Alexa Fluor–labeled anti-mouse CD19 (clone 6D5) antibodies or corresponding isotype control antibodies.

For *in vivo* analysis, DEX-NP formulations were administered intravenously through the tail vein, and spleens were retrieved after 4 or 24 hours. Spleen cell suspensions were incubated with cell type–specific antibodies (see above). Frequencies of DEX-NP(−) binding immune cells were assessed by using flow cytometry (LSR II; BD Biosciences) and analyzed with FlowJo software (FlowJo, Ashland, Ore).

In vivo T-cell proliferation

To assess OVA-specific $CD4^+$ T-cell proliferation *in vivo*, we used OT-II mice, which have $CD4^+$ T cells that express a transgenic T-cell receptor that recognizes $OVA_{323\text{-}339}$ (ISQAVHAAHAEINEAGR) in the context of $I\text{-}A^b$. To this end, splenocytes derived from OT-IIxCD45.1 mice were labeled with 0.5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) for 10 minutes. CFSE-labeled splenocytes (10^7 in 200 μL of PBS) were transferred intravenously into C57BL/6 mice. After 48 hours, DEX-NP formulations at equivalent doses of OVA (4 μg per mouse) and CpG ODN 1826 (0.4 μg/mouse) were injected intravenously. Four days later, spleens and peripheral lymph nodes were removed, and cell suspensions were analyzed for proliferation of CFSE-labeled T cells by using flow cytometry.

Antibody detection

Mice were bled 14 days after immunization from the retro-orbital plexus, and sera were collected. OVA-specific IgG_1, IgG_{2a}, and IgE levels were quantitated by means of ELISA. For this, plates were coated with OVA (25 mg/mL) and incubated with diluted sera (for IgG_1 detection, $1{:}10^5$; for IgG_{2a} detection, 1:500; for IgE detection, 1:10). Afterward, IgG class-specific rat anti-mouse antibodies conjugated with horseradish peroxidase were applied (Dianova, Hamburg, Germany).

Cytokine quantification

Cytokine contents (TNF-α and IL-6) in supernatants of spleen cells were detected by using the CBA assay (BD Biosciences), a previously described.[20] Samples were analyzed in the FL3 channel of an LSR-II flow cytometer. Results were analyzed by using CBA Analysis software (BD Biosciences).

Anaphylaxis model

In a therapeutic model of systemic anaphylaxis, BALB/c mice were sensitized subcutaneously 3 times (days 0, 7, and 14) with 10 μg of OVA in PBS (10 μg per mouse). One week after the last sensitization, mice were vaccinated intravenously 3 times every other day with different DEX-NP formulations at equivalent doses of OVA (4 μg) or corresponding numbers of DEX-NP(−) in 200 μL of PBS or with PBS as a solvent control alone. At days 14 (before the first vaccination) and 28 (after the third vaccination), blood samples were collected for antibody measurements. One week after the last vaccination, untreated (naive) and differentially pretreated mice were challenged with OVA (25 μg per mouse) by means of intravenous injection, and body temperature was monitored rectally every 15 minutes.

Asthma model

In a therapeutic model of asthma, BALB/c mice were sensitized with 10 μg of OVA emulsified in 1.5 mg of Alum (Pierce, Rockford, Ill) administered intraperitoneally 2 times (days 0 and 7). Mice were vaccinated with DEX-NP formulations (days 10 and 12) at equivalent doses of OVA (4 μg) or with corresponding numbers of DEX-NP(−) in 200 μL of PBS or with PBS alone. Untreated (naive) and differentially treated mice were challenged daily with nebulized OVA (1% in PBS for 20 minutes) 3 times, starting on day 14. Two days after the last challenge, differentially pretreated mice were administered serially increasing doses of methacholine, and airway hyperresponsiveness was measured, as previously described.[21] Afterward, bronchoalveolar lavage was performed to yield lung-infiltrating immune cells. Bronchoalveolar lavage fluid–derived cells were cytospun (5×10^5 cells per slide) onto microscope slides, stained with Diff-Quick (Dade Behring, Marburg, Germany), and photographed for morphologic analysis. Sections of lung tissue were stained with hematoxylin and eosin to identify inflammatory cells. Lung inflammation was scored (no change, score = 0; mild, score = 1; moderate, score = 2; and severe, score = 3), as previously described.[22]

Bioinformatics and statistical analysis

Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, Calif). Results were expressed as means ± SEMs. Differences among groups were tested by using ANOVA. Data were normally distributed, and variance between groups was not significantly different. Differences between 2 groups were tested by using the paired Student *t* test, assuming significance at a *P* value of less than .05.

RESULTS

Systemically applied DEX-NPs target B cells

Generation of DEX-NP formulations and their physicochemical characterization are described in the Methods section and in Table E1 in this article's Online Repository at www.jacionline.org. Atomic force microscopy showed a spherical shape of DEX-NPs and confirmed limited size variability (see Fig E1 in this article's Online Repository at www.jacionline.org). To assess the suitability of DEX-NPs as vaccines, we first monitored their organ biodistribution and cell type–specific accumulation after intravenous injection into mice. DEX-NP(−) accumulated preferentially in the liver (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org), where they colocalized with the $CD11b^+$ macrophage population (see Fig E2, *B*). However, a considerable fraction of DEX-NP(−) also accumulated to the spleen. Within the spleen, DEX-NP(−) scarcely colocalized with phagocytic myeloid cells, namely $GR\text{-}1^+$ immature myeloid cells/neutrophils and $CD68^+$ macrophages, but with $CD11c^+$ DCs (Fig 1, *A*). Surprisingly, the vast majority of DEX-NP(−) accumulated within the B-cell areas of the spleen (Fig 1, *B*, left panel) and colocalized with $CD19^+$ B cells (Fig 1, *B*, right panel). FDCs displayed some engagement of DEX-NP(−) (Fig 1, *A*).

B-cell targeting by DEX-NPs depends on activation of the lectin complement pathway and deposition of activated C3 on the particle surface

Next, we investigated the mechanism leading to specific B-cell targeting by DEX-NP(−). In contrast to the *in vivo* situation, DEX-NP(−) bound poorly to immunomagnetically sorted $CD19^+$ splenic B cells *in vitro* (Fig 2, *A*), which suggested a critical role for mouse serum components that might form a

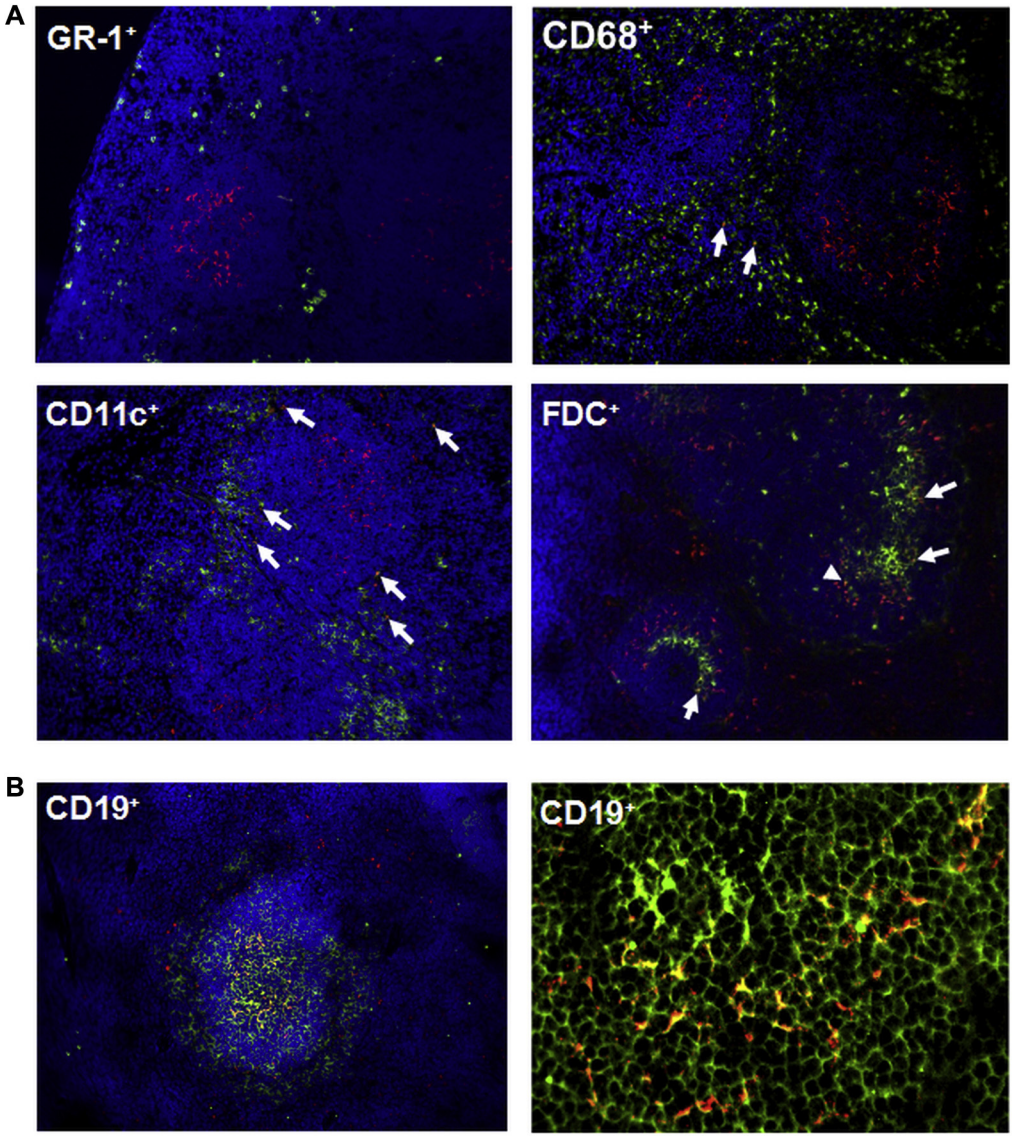

FIG 1. DEX-NPs colocalize with CD19$^+$ B cells. Cy5-labeled DEX-NPs (DEX-NP[−]) were administered intravenously into mice. After 4 hours, retrieved spleen sections were incubated with antibodies (green fluorescence) and DAPI (blue) to identify immature myeloid cells/neutrophils (GR-1$^+$), macrophages (CD68$^+$), DC (CD11c$^+$), and FDCs (FDC$^+$; *arrows* denote cells colocalizing with DEX-NP[−]; **A**) and B cells (CD19$^+$; **B**). Results are representative of 4 experiments. Fig 1, *A* and *B*, *left panel*, ×20 magnification; Fig 1, *B*, *right panel*, ×40 magnification.

protein corona around DEX-NP(−) *in vivo*. However, *in vitro* pretreatment of DEX-NP(−) with known major constituents of protein coronae[12] had no effect on subsequent B-cell binding (Fig 2, *A*). In contrast, pretreatment of DEX-NP(−) with native mouse serum yielded strong binding and uptake of DEX-NPs by B cells, as confirmed by using confocal microscopy (Fig 2, *B*). Heat treatment (at 30 minutes for 56°C) of mouse serum before incubation with DEX-NP(−) prevented B-cell binding (Fig 2, *A*), which suggested a decisive role of complement factors.[23] Indeed, quantitative proteomic analysis of the protein corona of DEX-NP(−) pretreated with native mouse serum identified components of the lectin-activated complement cascade, including MBL-associated proteases 1 and 2 (Table I). Both proteins are recruited and activated by MBL, which recognizes polysaccharides on cell surfaces of pathogens.[24] MBL-associated proteases 1 and 2 can activate C3 convertase, which results in deposition of activated C3b on lectin-coated surfaces. In line with this, C3 was identified as a major constituent of the DEX-NP(−) protein corona (Table I and Fig 2, *C*). All CRs preferentially bind C3 products.[25] Therefore activated C3 constituted a likely candidate for binding of serum-opsonized DEX-NPs to B cells.

Preincubation of DEX-NP(−) with recombinant C3 protein yielded no enhanced B-cell binding (Fig 2, *D*), which demonstrates that C3 activation on the NP surface is required for subsequent B-cell binding (Fig 2, *E*). In accordance with this, DEX-NP(−) opsonized with serum derived from C3$^{-/-}$ and MBL$^{-/-}$ mice showed strongly reduced B-cell binding. Serum-pretreated DEX-NP(−) had no effect on overall viability of spleen cells (see Fig E3, *A*, in this article's Online Repository

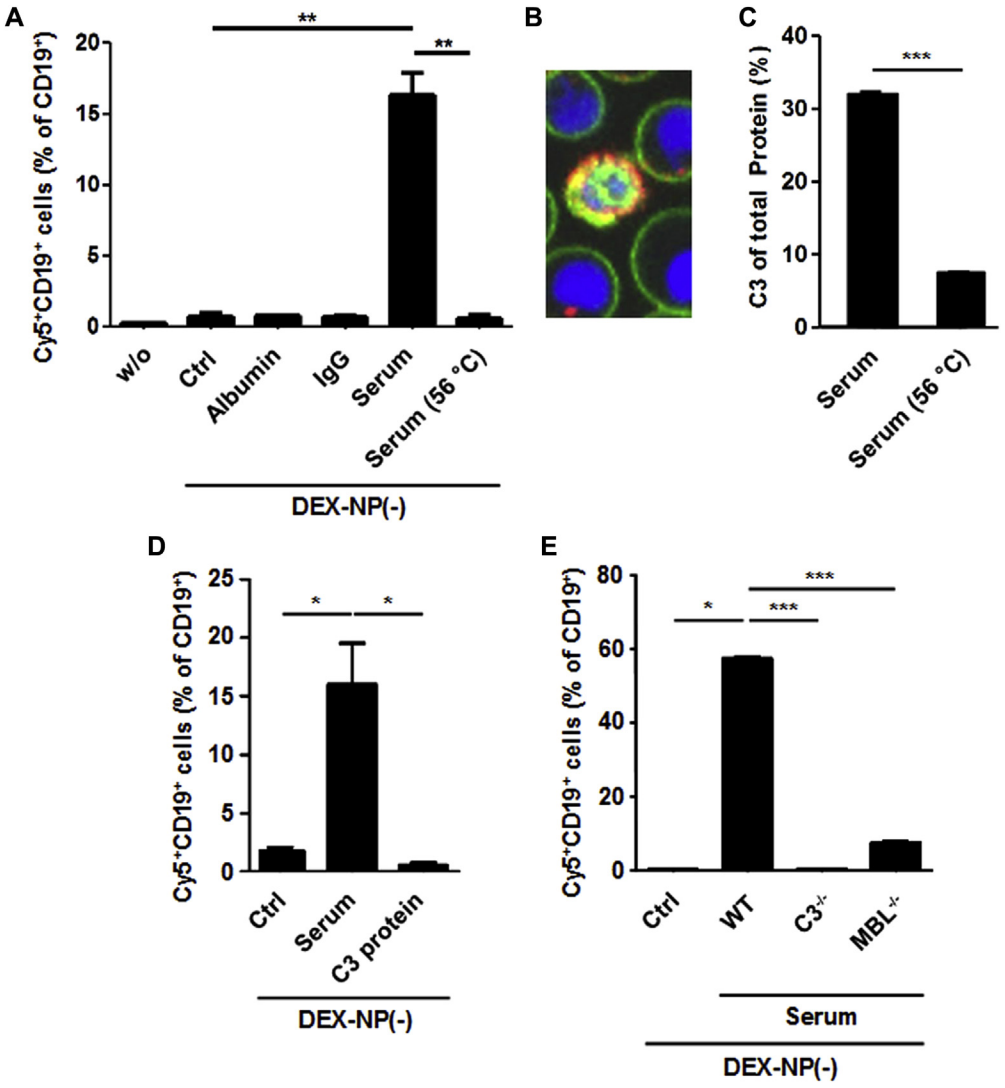

FIG 2. Complement opsonization of DEX-NPs mediates B-cell binding. **A** and **C-E,** Frequencies of B cells *(in vitro)* binding differentially pretreated DEX-NP(−) derived from wild-type (*WT*; Fig 2, *A* and *D*) or knockout (Fig 2, *E*) mice after 4 hours of coincubation (n = 3). **B,** Internalization of DEX-NP(OVA-CpG) by B cells *(in vitro)* after 4 hours. Fig 2, *D,* Complement C3 abundance in the DEX-NP(−) protein corona (n = 3). Fig 2, *A* and *C-E*: **P* < .05, ***P* < .01, and ****P* < .001.

at www.jacionline.org) and also did not activate splenic leukocytes, as shown by unaltered cytokine profiles (see Fig E3, *B*), which suggests that complement activation is confined to the DEX-NP(−) surface.

In accordance with our findings for mouse B cells, pretreatment of DEX-NP(−) with human plasma resulted in a strongly enhanced binding to $CD19^+$ human B cells, which was abolished by heat inactivation of complement as well (see Fig E4 in this article's Online Repository at www.jacionline.org). Thus our results obtained in mice might be of translational relevance for clinical application in human subjects.

C3-opsonized DEX-NPs bind B cells through CR1/2

Antibody-mediated blockade of CR1/2 (CD21/CD35) on B cells completely prevented binding of serum-pretreated DEX-NP(−) *in vitro* (Fig 3, *A*) and reduced colocalization of DEX-NP(−) with splenic B cells *in vivo* (Fig 3, *B* and *C*). Other solid-core NP formulations that were coated with starch (Fig 3, *D* and *E*) or dextran (Fig 3, *F*) engaged B cells in a complement-dependent manner as well. In contrast, standard silica NPs, which bound to splenic B cells to a high extent in the absence of serum, displayed strongly reduced B-cell binding after serum preincubation (Fig 3, *G*). This suggests that only lectin-coated NPs bind B cells in a complement-mediated fashion by triggering the lectin pathway of complement activation.

DEX-NPs that codeliver antigen and adjuvant induce APC activation and an antigen-specific $CD4^+$ T-cell response

Next, we evaluated the suitability of DEX-NP–derived formulations for B cell–focused vaccinations. DEX-NPs coupled with the model antigen OVA (DEX-NP[OVA]) and with the T_H1-promoting immunostimulatory CpG ODN 1826 (DEX-NP

TABLE I. Top 20 most abundant corona proteins detected after incubation of DEX-NP(OVA) with untreated and heat-inactivated mouse serum*

UniProt accession no.	Protein	Isoelectric point	Molecular weight (kDa)	Reported peptides	Total protein (%)	
					Native serum	Heat-inactivated serum
P01027	**Complement C3**	6.27	188	125	7.7	3.6
P11276	Fibronectin	5.25	276	111	3.2	3.4
P07724	Serum albumin	5.68	71	51	3.1	4.5
O08677	**Kininogen-1**	6.05	74	29	3.1	3.1
P06909	Complement factor H	6.60	144	73	3.0	1.9
P35441	Thrombospondin-1	4.54	134	72	2.9	2.4
Q61838	α_2-Macroglobulin	6.23	167	70	2.7	4.2
P01029	**Complement C4-B**	7.25	195	82	2.3	2.0
P26262	Plasma kallikrein	7.89	73	41	2.2	0.9
P98064	**MBL-associated lectin serine protease 1 (MASP-1)**	5.21	82	35	2.2	1.5
Q9ESB3	**Histidine-rich glycoprotein**	7.33	60	23	2.1	2.2
P19221	**Prothrombin**	6.00	72	41	2.1	0.6
P28665	Murinoglobulin-1	5.96	167	72	1.9	2.4
Q921I1	Serotransferrin	6.85	79	44	1.8	2.2
Q91WP0	**MBL-associated serine protease 2 (MASP-2)**	5.63	77	34	1.8	0.5
O88783	Coagulation factor V	5.61	248	55	1.7	2.2
Q8VDD5	Myosin-9	5.38	228	26	1.7	2.5
P06684	**Complement C5**	6.37	191	75	1.6	0.9
P01872	Ig mu chain C region secreted form	6.58	51	24	1.5	1.5
Q61702	Inter-α-trypsin inhibitor heavy chain H1	6.51	102	33	1.4	2.2

*Proteins associated with the lectin pathway of complement activation are shown in boldface. The complete data set is shown in File E1.

[OVA-CpG]) in addition retained their B-cell targeting properties *in vivo* (see Fig E5 in this article's Online Repository at www.jacionline.org). Within the spleen, CpG ODN–coupled DEX-NPs mediated upregulation of CD86 expression by B cells (Fig 4, *A* and *B*), engaged no $CD86^+$ macrophages but did engage $CD11c^+$ DCs (Fig 4, *C*) and activated these (Fig 4, *D*). Furthermore, application of DEX-NP(OVA) yielded moderate proliferation of adoptively transferred OT-II $CD4^+$ T cells, which were enhanced strongly on coinjection of CpG ODN 1826 (Fig 4, *E* and *F*).

Induction of a T_H1-biased antibody response by antigen/adjuvant-codelivering DEX-NPs depends on B-cell targeting

As expected, only vaccination with DEX-NP(OVA-CpG) mounted very high levels of T_H1-associated OVA-specific IgG_{2a} antibodies but only slightly increased levels of T_H2-associated OVA-specific IgG_1 and IgE (Fig 5, *A*). Moreover, DEX-NP–mediated induction of OVA-specific IgG_{2a} was much stronger than application of equimolar amounts of soluble OVA protein plus soluble CpG in parallel experiments (Fig 5, *B*).

In accordance with the critical role of activated C3 to mediate B-cell targeting of DEX-NPs, immunization of $C3^{-/-}$ and $MBL^{-/-}$ mice with DEX-NP(OVA-CpG) yielded no or strongly attenuated OVA-specific IgG_{2a} production. Congruently, antibody-mediated blockade of CR1/2 strongly reduced the frequency of splenic $CD19^+$ B cells that colocalized with DEX-NP(OVA-CpG) (Fig 5, *C*) and inhibited OVA-specific IgG_{2a} antibody production (Fig 5, *D*).

In a therapeutic setting vaccination of mice with antigen/adjuvant-codelivering DEX-NPs prevents IgE-mediated anaphylactic shock

To assess the therapeutic potential of DEX-NPs, we used a model of systemic anaphylaxis (Fig 6, *A*). Only vaccination of sensitized mice with the antigen/adjuvant-codelivering NP formulation (DEX-NP[OVA-CpG]) prevented a decrease in body temperature after challenge (Fig 6, *B*), which was associated with an inhibition of OVA-specific IgE (Fig 6, *C*) but a strong induction of OVA-specific IgG_{2a} antibody production (Fig 6, *D* and *E*). Levels of OVA-specific IgG_1 remained largely unaltered in all groups of mice (see Fig E6 in this article's Online Repository at www.jacionline.org).

Vaccination of mice with antigen/adjuvant-codelivering DEX-NPs prevents asthma in a therapeutic setting

Similarly, in a therapeutic model of allergic asthma (Fig 7, *A*), only sensitized mice vaccinated with DEX-NP(OVA-CpG) before challenge with nebulized OVA showed no strong airway hyperresponsiveness in response to pretreatment with higher doses of methacholine (Fig 7, *B*), lacked lung-infiltrating eosinophils (Fig 7, *C*), and had no increased lung inflammation score (Fig 7, *D*, and see Fig E7 in this article's Online Repository

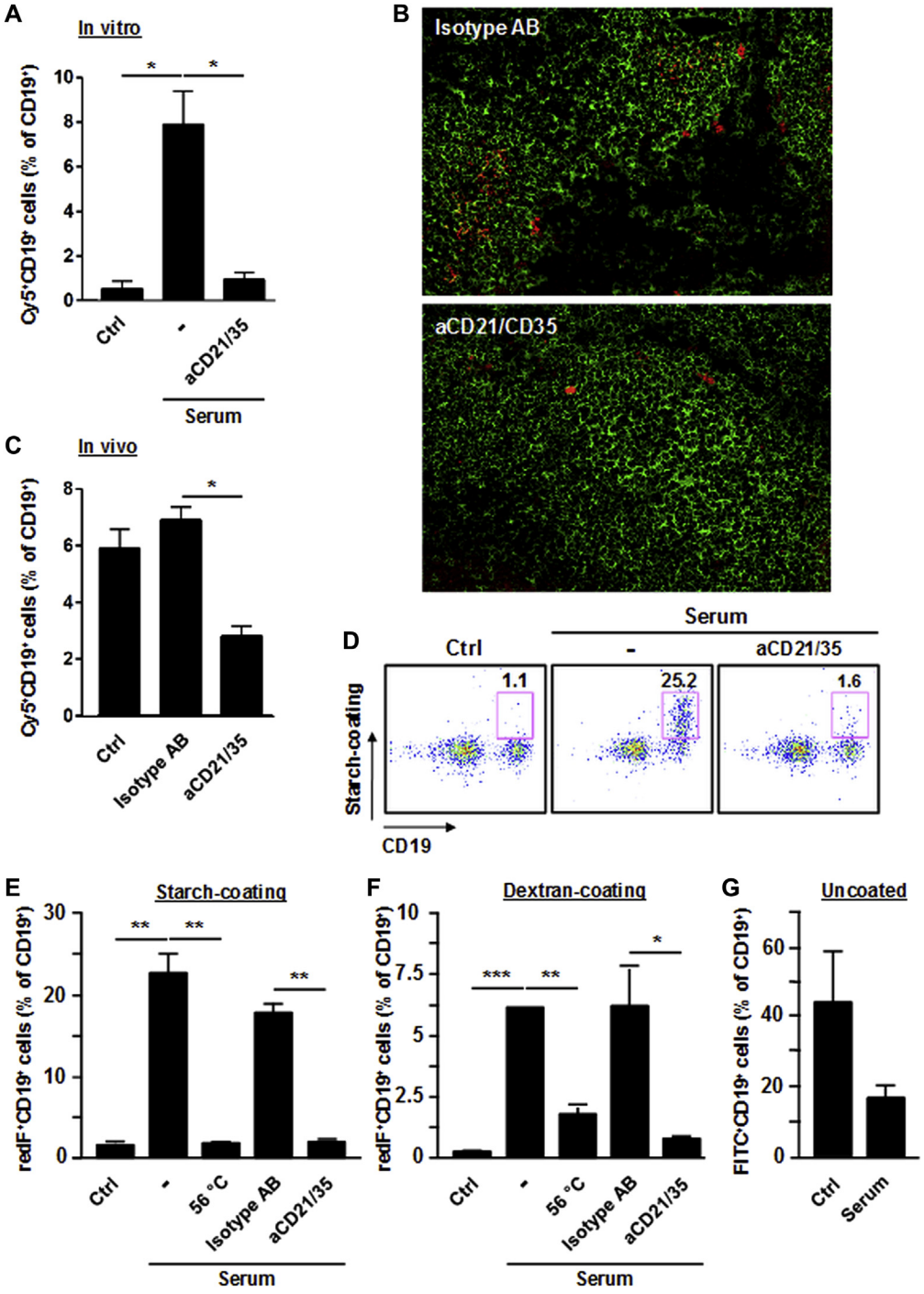

FIG 3. Serum-opsonized NPs bind B cells through CD21/CD35. **A,** Frequencies of B cells *(in vitro)* binding DEX-NP(−) in the presence of neutralizing anti-CD21/CD35 antibody (n = 3). **B,** DEX-NPs in spleens of mice pretreated with isotype control or anti-CD21/CD35 antibody assessed 4 hours after injection (3 mice per group, n = 2; magnification ×10). **C,** Quantification of NP-binding B cells (300 cells counted per experiment, n = 2). **D,** B cells *(in vitro)* binding starch-coated NPs (gated, n = 3). **E-G,** Frequencies of B cells binding starch-coated (Fig 3, *E*) and dextran-coated (Fig 3, *F*) NPs (n = 3) and carboxylated silica NPs (Fig 3, *G*; n = 4). Fig 3, *A, C,* and *E-G*: **P* < .05, ***P* < .01, and ****P* < .001.

at www.jacionline.org). In line with this, only these mice showed no induction of OVA-specific IgE (Fig 7, *E*, upper panel) but strongly upregulated OVA-specific IgG_{2a} (Fig 7, *E*, lower panel). In general, levels of OVA-specific IgG_1 remained largely unaffected (see Fig E8 in this article's Online Repository at www.jacionline.org).

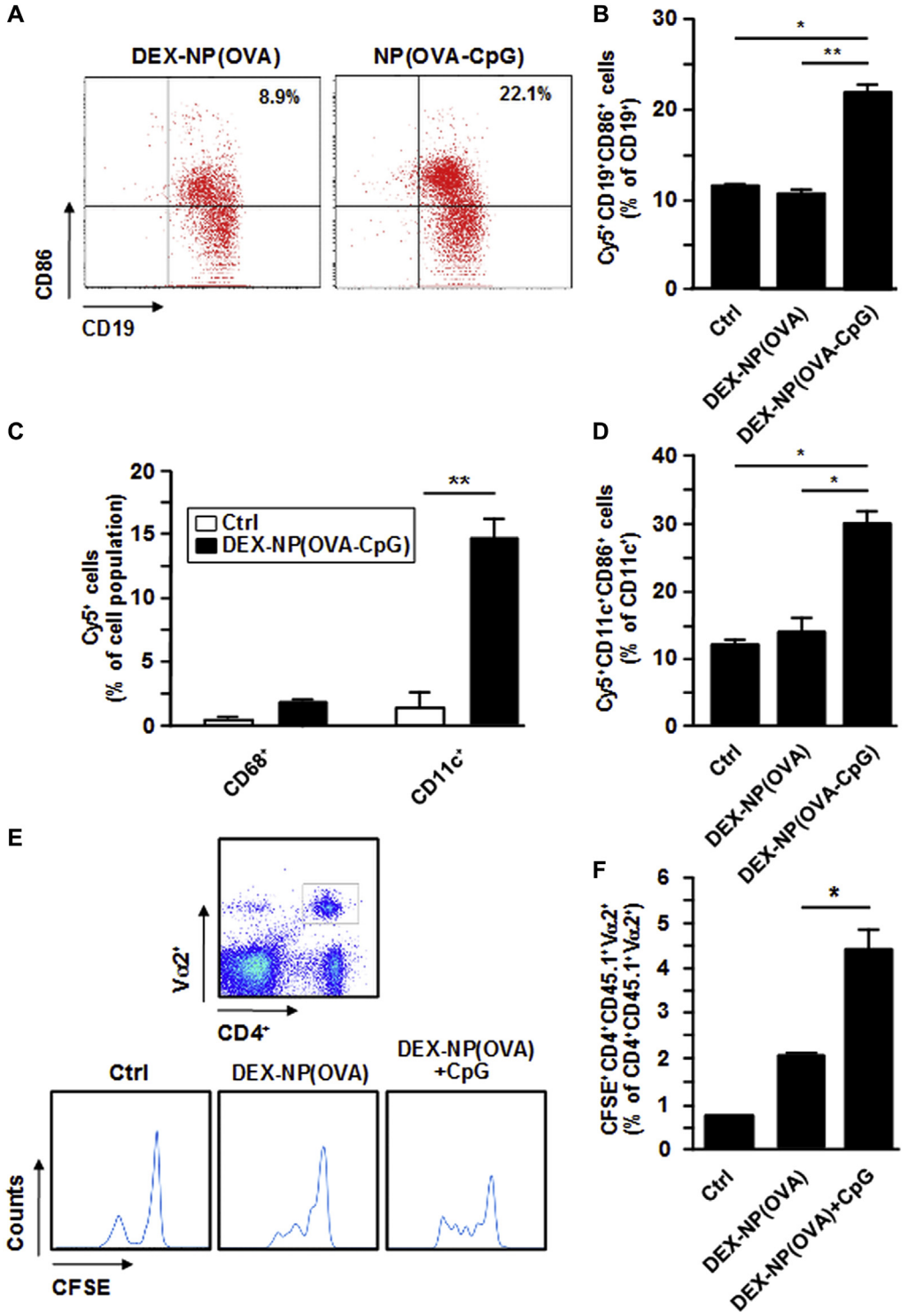

FIG 4. Functionalized DEX-NPs activate B cells and DCs *in vivo* and evoke $CD4^+$ T-cell proliferation. **A-D,** Frequencies of activated B cells (24 hours; Fig 4, *A* and *B*), DCs and macrophages (4 hours; Fig 4, *C*), and activated DCs (24 hours; Fig 4, *D*) after intravenous application of DEX-NP formulations (3 mice per group, compiled from n = 2 [total of 6 mice per group]). **E** and **F,** Proliferation of $CD4^+$ OT-II T cells assessed 3 days after vaccination with DEX-NP(OVA) and soluble CpG (4 mice per group; representative of 3 experiments; Fig 4, *E*) and according quantification (n = 3; a total of 12 mice per group; Fig 4, *F*). Fig 4, *B-D* and *F*: $*P < .05$, $**P < .01$, and $***P < .001$.

Altogether, these results confirm that vaccination of sensitized mice with DEX-NP(OVA-CpG) prevents induction of a T_H2-associated humoral immune response, which in turn drives allergies by promoting T_H1-associated IgG_{2a} antibody switching. Therefore we conclude that lectin-coated NP vaccines, which intrinsically target B cells and codeliver antigen and a

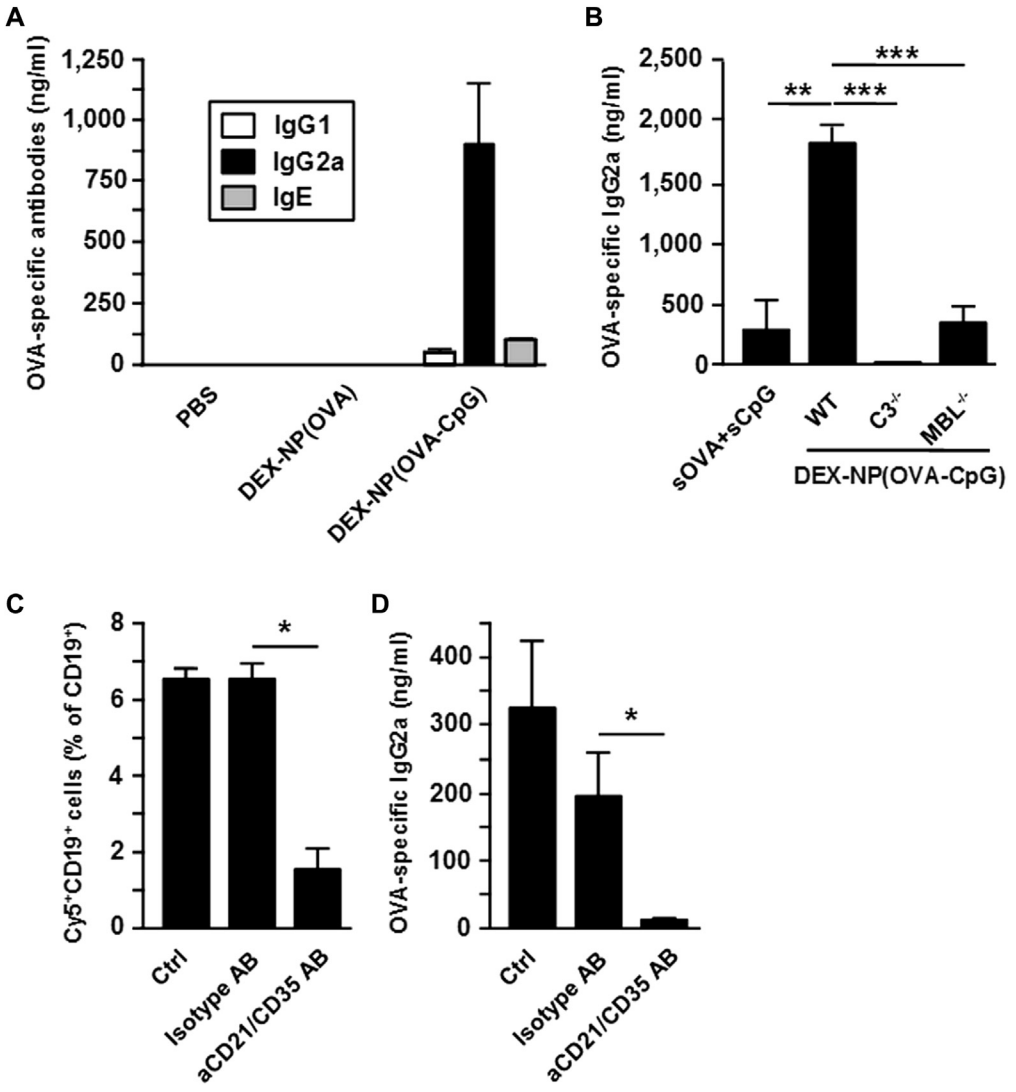

FIG 5. Functionalized DEX-NPs induce profound antibody production dependent on complement activity and binding to CD21/CD35. **A, B,** and **D,** OVA-specific IgG_1, IgG_{2a}, and IgE in sera of mice obtained 10 (Fig 5, *B* and *D*) or 14 (Fig 5, *A*) days after vaccination with OVA and soluble CpG or DEX-NP formulations or PBS alone (control group) and in the presence of anti-CD21/CD35 or isotype control antibody (Fig 5, *D*). **C,** Frequencies of splenic B cells that colocalized with DEX-NPs 4 hours after application. Fig 5, *A* and *C*, Five mice per group compiled from n = 2 (a total of 10 mice per group); Fig 5, *B* and *D*, 3 mice per group compiled from n = 3 (a total of 9 mice per group). Fig 5, *A-D*: $*P < .05$, $**P < .01$, and $***P < .001$.

T_H1-promoting adjuvant, such as CpG ODN, are suitable candidates for therapeutic treatment of allergies.

DISCUSSION

Here we show that DEX-NPs trigger activation of the lectin pathway of complement activation[26] and demonstrate the essential role of active C3 deposited on DEX-NPs to mediate their binding to $CD19^+$ B cells through CR1/2. To the best of our knowledge, our study is the first to demonstrate specific B-cell binding of an NP not functionalized with a B-cell targeting antibody but rather because of its intrinsic complement-activating potential. These key findings are summarized in Fig E9 in this article's Online Repository at www.jacionline.org.

Based on the intrinsic B-cell targeting properties of DEX-NPs and their concurrent binding to a fraction of splenic $CD11c^+$ DCs required to induce primary $CD4^+$ T-cell responses,[5] we evaluated the suitability of DEX-NPs to serve as a nanovaccine for the induction and therapeutic modulation of humoral immune responses. We used the model antigen OVA, which is frequently used in mouse allergy models.[27] In addition, we used CpG ODN 1826, which binds to TLR9 expressed by splenic DCs and B cells,[28] as a T_H1-promoting adjuvant to alter allergy-inducing T_H2 responses, as already reported by others.[29] Activation of splenic DCs by CpG ODN–conjugated DEX-NPs *in vivo* is in line with previous reports on endocytotic uptake of CpG ODN–coupled NPs by DCs and hence accessibility of endosomal/lysosomal TLR9.[30] Our finding of concurrent B-cell activation indicates that targeted B cells must have internalized at least a fraction of C3-opsonized DEX-NP(OVA-CpG) to trigger TLR9 as well.[28,31]

The induction of pronounced OVA-specific IgG_{2a} but largely unaltered IgG_1 antibody levels on vaccination with

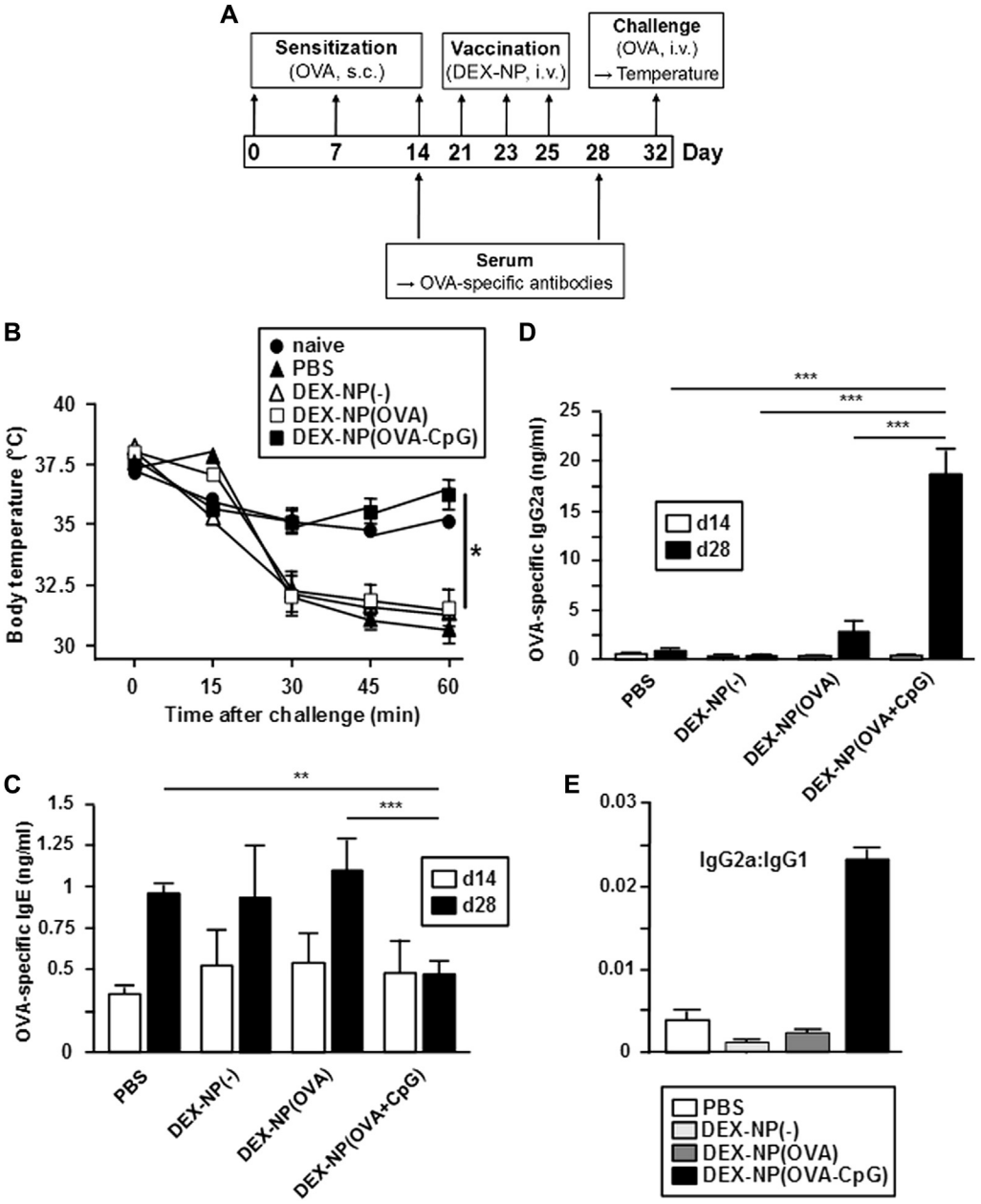

FIG 6. Vaccination of OVA-sensitized mice with OVA CpG−codelivering DEX-NPs prevents IgE induction and inhibits anaphylactic shock. **A,** Experimental design. *i.v.*, Intravenous; *s.c.*, subcutaneous. **B,** Alterations of body temperature after challenge (15 mice per group, compiled from n = 3). **C** and **D,** OVA-specific IgE (Fig 6, *C*) and IgG_{2a} (Fig 6, *D*) in serum obtained before the first (day 14) and after the last (day 28) vaccination with indicated DEX-NP formulations or PBS alone (control group). **E,** IgG_{2a}/IgG_1 ratio. Fig 6, *C-E*, Five mice per group; 1 experiment representative of 5. Fig 6, *B-E*, $*P < .05$, $**P < .01$, and $***P < .001$.

DEX-NP(OVA-CpG) is in accordance with the T_H1-skewing properties of the type of CpG ODN used.[29] Application of DEX-NP(OVA-CpG) to $C3^{-/-}$ mice yielded no OVA-specific antibody response, which is in agreement with our finding of C3-dependent B-cell targeting of DEX-NP formulations. $C3^{-/-}$ mice in general are characterized by diminished antibody production after protein immunization.[32,33] However, $MBL^{-/-}$ mice show no such defect[15] but also mounted poor antibody responses after vaccination with DEX-NP(OVA-CpG). These findings underscore the strict requirement for lectin-induced complement C3 activation on the DEX-NP surface for subsequent B-cell targeting, which in turn is indispensable for induction of antigen-specific antibody responses.

Indeed, it was shown previously that complement opsonization of pathogens and proteins resulted in their binding to CR1/2 on B cells.[33,34] In case of an antigen-specific B-cell receptor (BCR), concomitant engagement of CR1/2 and the BCR strongly increased uptake of complement-opsonized material and, consequently, antibody production. Nucleic acids applied in soluble form were reported to activate autoreactive B cells.[4] Therefore soluble CpG,

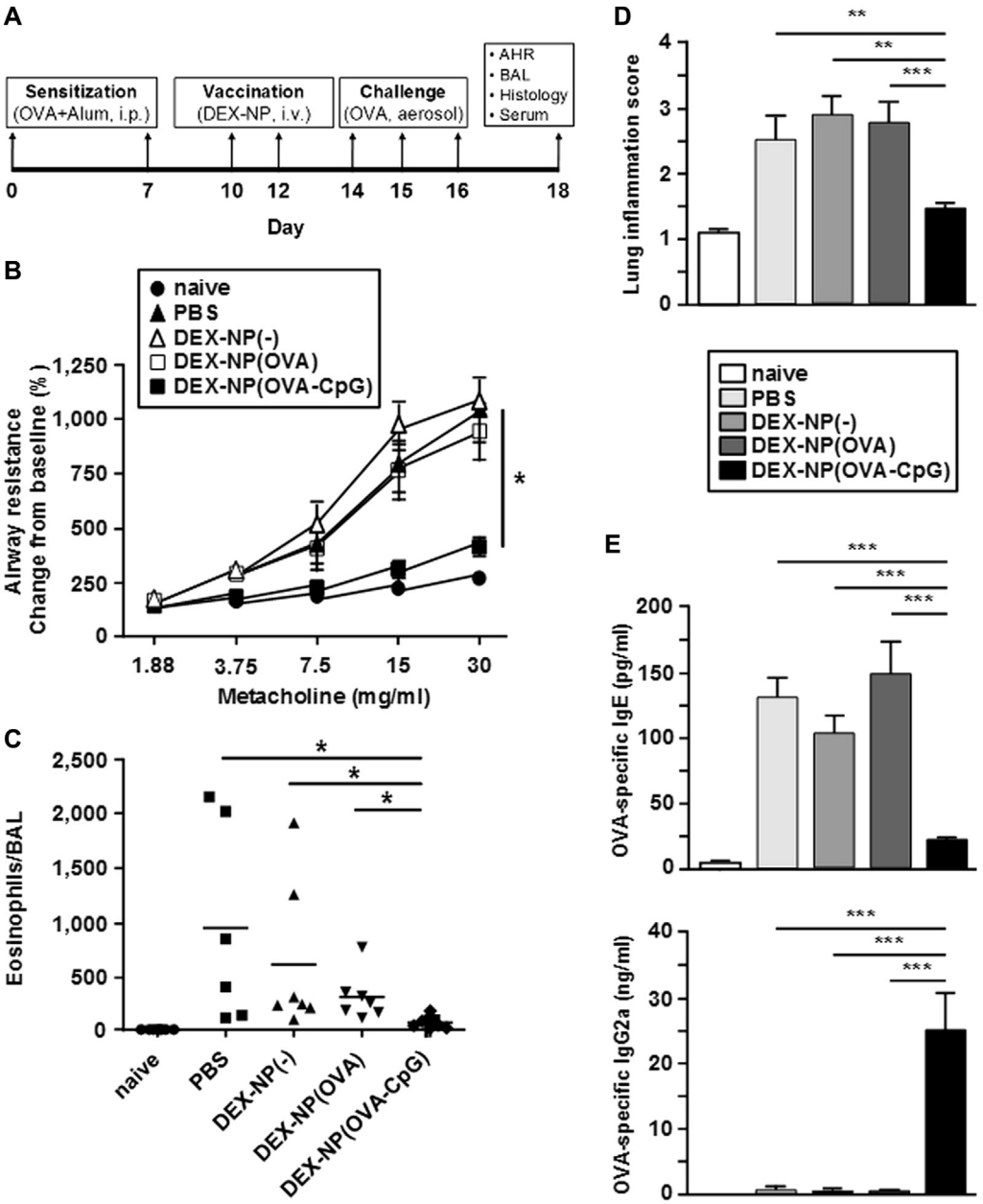

FIG 7. Treatment of OVA-sensitized mice with OVA/CpG-codelivering DEX-NPs prevents asthma. **A,** Experimental design. *i.p.*, Intraperitoneal; *i.v.*, intravenous. **B,** Airway resistance measurements of mice vaccinated with the indicated DEX-NP formulations or PBS alone after challenge and administration of methacholine (6 mice per group, representative of n = 2). **C,** Eosinophil numbers in bronchoalveolar lavage fluid (*BAL*; 300 cells counted per sample; 7 samples per group; compiled from n = 2). **D,** Lung inflammation scores (40 sections scored per group; 10-12 mice per group; n = 2). **E,** OVA-specific antibodies in sera (10-12 mice per group; compiled from n = 2 [a total of 20-24 mice per group]). Fig 7, *B-E*: $*P < .05$, $**P < .01$, and $***P < .001$.

when used as an adjuvant in conventional vaccination, might contribute to the risk of inducing autoimmune diseases. The strategy to use a CpG ODN–carrying nanocarrier with internalization that is not mediated by macropinocytosis, as in case of soluble CpG ODN, but depends on interaction of codelivered allergen with the BCR and of opsonizing C3 with CR1/2 might limit in part unwanted stimulation of autoreactive B cells.

Our finding of low antibody levels in $C3^{-/-}$ and $MBL^{-/-}$ mice immunized with DEX-NP(OVA-CpG) suggests that binding only through OVA to a specific BCR is insufficient for effective NP uptake by B cells. Our finding of superior antibody induction by vaccination with particulate antigen/adjuvant combinations is in line with previous studies.[35] Thus far, this effect was largely attributed to stronger activation and antigen transfer to DCs. However, our results support that B-cell targeting might play a major role because it was previously shown that stimulated B cells that had internalized protein antigen through the BCR exerted potent $CD4^+$ T-cell stimulatory activity.[36] Therefore we cannot

rule out that in our setting DEX-NP–targeted B cells constitute the dominant APC population and strongly promote T_H1 polarization.

In models of systemic anaphylaxis and asthma, we show that vaccination of sensitized mice with our effective nanovaccine DEX-NP(OVA-CpG) completely inhibited allergic reactions. In both allergy models we observed an inhibition of OVA-specific T_H2-associated IgG_1 and IgE induction at the expense of strongly upregulated T_H1-asociated IgG_{2a}. After NP therapy, in the anaphylaxis model body temperatures remained unaffected after challenge, and in the asthma model no lung inflammation was observed, probably because of the lack of IgE. In similar approaches poly(lactic-co-glycolic acid)–based NPs loaded with a relevant allergen plus CpG ODN were efficient in the treatment of house dust mite–induced asthma in a preventive setting[37] and for therapeutic application in a peanut allergy model.[38] Therefore the strategy to prevent or override T_H2 polarization and hence IgE production in favor of T_H1 for allergy treatment is well substantiated.

In these vaccination studies the authors proposed that their nanovaccines acted predominantly on the level of DC activation and antigen transfer to result in T_H1-biased antibody responses. In this context the importance of B cells as the actual inducers of humoral immune responses was not assessed. Somewhat surprisingly, specific B-cell targeting has thus far been addressed only with regard to the delivery of chemotherapeutics to kill B lymphoma cells.[39] Toward this purpose, different types of nanocarriers have been conjugated with antibodies that recognize B cell–specific surface receptors.[40]

In contrast to ongoing vaccination approaches that focus on DCs as critical inducers and regulators of T_H cell responses,[35] our work shows that the intrinsic B-cell targeting properties of lectin-coated NPs can be successfully exploited for improvement of AIT and that direct B-cell targeting is actually critical to mount antibody responses. Moreover, DEX-NPs *in vivo* also bound FDCs, which express CR1/2 as well, and serve to retain native antigen for long periods of time.[34] It remains to be shown whether binding of C3-opsonized NPs to FDCs yields according long-term effects of vaccination, including B-cell memory.

We show that other types of NPs coated with dextran or starch bound B cells in a complement-dependent manner. We propose that serum complement adsorption might be a general feature of lectin-coated NPs, which suggests that functionalization of nanovaccines with a lectin coating is sufficient to trigger recognition by the complement system and C3-mediated B-cell targeting. We observed complement-dependent targeting of DEX-NPs for human B cells, which suggests clinical relevance of this approach. In addition to lectin-dependent complement activation, other types of NPs can induce distinct pathways of innate immune recognition. Previously, we have shown that various types of polystyrene and silica NPs were opsonized by IgG and the complement component C1q after incubation with human plasma,[12] which is indicative of an activation of the classical complement pathway.[41]

We are confident that the strategy to design nanovaccines that mimic pathogens, such as by applying a lectin coat, will allow B cell–focused vaccinations to induce and shape humoral immune responses. In addition, the inherent property of nanocarriers to (co)deliver different kinds of biologicals and drugs can also be exploited to address the whole B-cell compartment (B cells and FDCs) through binding of C3-opsonized nanocarriers to CR1/2. Further studies are planned to elucidate the potential of C3-mediated targeting of this compartment to yield long-lasting therapeutic effects through induction of B-cell memory, which also requires FDC-dependent long-term storage of native allergen.[42]

Subsequent studies need to address the therapeutic potential of the B-cell compartment targeting nanovaccines that deliver complex allergens, as required for successful AIT.[43] For this, a nanocarrier can be coated with different allergens. Alternatively, nanocarriers are conjugated with one type of recombinant allergen out of a panel and are pooled before administration. In any case, the inherent B-cell targeting property of according nanovaccines might minimize unwanted binding of particulate allergens to IgE receptors, as observed for soluble allergens.

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Clinical implications: Lectin-coated NPs trigger complement activation, which results in deposition of activated C3 on the NP surface. C3-coated NPs target B cells through CR1/2. This mechanism can be exploited for allergy treatment by using NPs that codeliver an allergen and a T_H1-promoting adjuvant, as shown in mouse models of anaphylaxis and asthma.

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