



This information is current as of July 3, 2013.

Immunization with Biodegradable Nanoparticles Efficiently Induces Cellular Immunity and Protects against Influenza Virus Infection

Torben Knuschke, Viktoriya Sokolova, Olga Rotan, Munisch Wadwa, Matthias Tenbusch, Wiebke Hansen, Peter Staeheli, Matthias Eppele, Jan Buer and Astrid M. Westendorf

J Immunol 2013; 190:6221-6229; Prepublished online 10 May 2013;
doi: 10.4049/jimmunol.1202654
<http://www.jimmunol.org/content/190/12/6221>

-
- | | |
|----------------------|--|
| References | This article cites 34 articles , 11 of which you can access for free at:
http://www.jimmunol.org/content/190/12/6221.full#ref-list-1 |
| Subscriptions | Information about subscribing to <i>The Journal of Immunology</i> is online at:
http://jimmunol.org/subscriptions |
| Permissions | Submit copyright permission requests at:
http://www.aai.org/ji/copyright.html |
| Email Alerts | Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/cgi/alerts/etoc |



Immunization with Biodegradable Nanoparticles Efficiently Induces Cellular Immunity and Protects against Influenza Virus Infection

Torben Knuschke,* Viktoriya Sokolova,[†] Olga Rotan,[†] Munisch Wadwa,* Matthias Tenbusch,[‡] Wiebke Hansen,* Peter Staeheli,[§] Matthias Eppe,[†] Jan Buer,^{*,1} and Astrid M. Westendorf^{*,1}

The ability of vaccines to induce T cell responses is crucial for preventing diseases caused by viruses or bacteria. Nanoparticles (NPs) are considered an efficient tool for inducing potent immune responses. In this study, we describe a novel vaccination approach with biodegradable calcium phosphate (CaP) NPs that serve as carrier of immunoactive TLR9 ligand (CpG) combined with a viral Ag from the influenza A virus hemagglutinin. Functionalized CaP NPs were efficiently taken up by dendritic cells in vivo and elicited a potent T cell-mediated immune response in immunized mice with high numbers of IFN- γ -producing CD4⁺ and CD8⁺ effector T cells. Most importantly, both i.p. and intranasal immunization with these NPs offered protection in a mouse model of influenza virus infection. This study demonstrates the great potential of CaP NPs as a novel vaccination tool that offers substantial flexibility for several infection models. *The Journal of Immunology*, 2013, 190: 6221–6229.

Traditional vaccination strategies with live attenuated viruses or inactivated pathogens have been widely used to treat and prevent diseases (1). Importantly, most of these strategies result in a humoral immune response but do not stimulate cellular immunity, which is required for a long-lasting and efficient response against infection with intracellular pathogens (2). Furthermore, an inactivated vaccine is insufficient protection against antigenic drift variants and does not provide protection against viruses from different subtypes. To this end, engineered live vaccine vectors, such as nonreplicating recombinant viruses expressing viral Ags, have been developed; these vectors induce the expansion of virus-specific T cells in murine models (3–5). Although these approaches have generated successful results, antivector immunity and safety concerns associated with live vectors complicate the development of live-vector vaccines (3). In contrast, nonliving synthetic vaccines delivering defined Ags can be designed to avoid antivector immunity (6). An ideal protein- or peptide-based vaccine should target professional APCs in vivo, protect the antigenic protein from degradation until it reaches the target cells, and, finally, induce APC maturation by interacting

with elements of the innate immune system, such as TLRs, to induce a protective T cell-mediated immune response.

The main entry site for many human pathogens is a mucosal surface such as the respiratory tract. Induction of mucosal immune responses for the local fight against the invading pathogen requires novel vaccine strategies (7, 8). Therefore, determining the optimal route of immunization is an important factor in the success of a vaccine. Systemic delivery of vaccines induces a high titer of neutralizing Abs that prevent the disease but are not sufficient to prevent the infection at the mucosal entry site. Delivery strategies that mimic the natural process of infection by stimulating the innate and adaptive immune system with TLRs and viral Ags are currently under investigation (9–11).

Nanoparticles (NPs) offer a new method for the design of vaccine systems. They are considered to be efficient Ag carriers and are being widely investigated for their biological potential (12–14). Recently, we generated biodegradable calcium phosphate (CaP) NPs that coencapsulate the TLR9 ligand CpG and a MHC class II-restricted influenza virus hemagglutinin (HA) peptide. We reported that treating dendritic cells (DCs) with these functionalized CaP NPs in vitro induces the activation and maturation of DCs with a strong Ag-specific CD4⁺ T cell response (15, 16).

In this study, we examined the in vivo efficiency of functionalized CaP NPs in activating APCs and inducing virus-specific CD4⁺ and CD8⁺ T cell responses. Functionalized CaP NPs were efficiently taken up by DCs in vivo; this uptake resulted in the upregulation of costimulatory molecules and elicited a potent T cell-mediated immune response in immunized mice with high numbers of IFN- γ -producing CD4⁺ and CD8⁺ effector T cells. Most importantly, i.p., intranasal and i.m. immunization with these NPs all offered strong protection in a mouse model of influenza virus infection.

Materials and Methods

Mice

The BALB/c mice used in all experiments were obtained from Harlan Laboratories (Harlan Winkelmann GmbH, Borcheln, Germany). TCR-HA

*Infection Immunology, Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen, 45122 Essen, Germany; [†]Institute of Inorganic Chemistry, University of Duisburg-Essen, Campus Essen, and Center for Nanointegration Duisburg-Essen, 45141 Essen, Germany; [‡]Department of Molecular and Medical Virology, Ruhr-Universität Bochum, 44801 Bochum, Germany; and [§]Department of Virology, University of Freiburg, 79106 Freiburg, Germany

¹J.B. and A.M.W. contributed equally to this work.

Received for publication September 21, 2012. Accepted for publication April 3, 2013.

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich/Transregio 60 to A.M.W., J.B., and M.E.).

Address correspondence and reprint requests to Prof. Astrid M. Westendorf, Infection Immunology, Institute of Medical Microbiology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany. E-mail address: astrid.westendorf@uk-essen.de

Abbreviations used in this article: CaP, calcium phosphate; DC, dendritic cell; MDCK, Madin-Darby canine kidney; NP, nanoparticle.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/\$16.00

transgenic mice expressing an α/β -TCR recognizing the MHC class II (H-2E^b:HA110–120)-restricted epitope of the HA protein have been described previously (17). Clone 4-TCR transgenic mice express an α/β -TCR that recognizes an epitope of the HA protein presented by the MHC class I (H-2K^d:HA512–520) complex (18). All mice used in the experiments were 8–10 wk old.

Preparation of functionalized NPs

The NPs were prepared by fast mixing of an aqueous solution of calcium nitrate (6.25 mM) with an aqueous solution of diammonium hydrogen phosphate (3.74 mM). The pH value of both solutions was adjusted beforehand to 9 with NaOH (0.1 M). Mixing was achieved by rapidly pumping both solutions into a glass vessel. Immediately after mixing, 1 ml of the CaP dispersion was mixed with 0.2 ml of an aqueous solution of CpG (63 μ M = 400 μ g/ml) in an Eppendorf tube.

For preparation of triple-shell NPs, 50 μ l of the HA protein (1 mg/ml), followed by 0.5 ml calcium nitrate solution (6.25 mM) and then 0.5 ml diammonium hydrogen phosphate solution (3.74 mM) were added to the dispersion of single-shell NPs. The final colloidal stabilization was then accomplished by adding 0.2 ml CpG solution (63 μ M). This leads to the final concentrations of 10.3 μ M CpG and 20.4 μ g/ml HA for triple-shell NPs (CaP/CpG/HA/CaP/CpG) (15, 16).

For uptake studies, green fluorescing CpG⁴⁸⁸ (labeled with Alexa488) was used for the preparation of triple-shell NPs (CaP/CpG⁴⁸⁸/CaP/CpG⁴⁸⁸) as described earlier without the addition of HA peptide.

For intranasal immunization, triple-shell CaP NPs (CaP/CpG/HA/CaP/CpG) were synthesized as described earlier. After the final step, the triple-shell NPs were removed from the supernatant by ultracentrifugation for 30 min at 66,000 $\times$ g. Afterward, the centrifuged NPs were redispersed in water (in one quarter of the initial volume) with an ultrasonic processor (cycle 0.8, amplitude 60%; UP50H, Hielscher; Ultrasound Technology) for 10 s. The final concentrations of CpG and HA peptide were 20.6 μ M and 40.8 μ g/ml, respectively.

All inorganic salts were of pro analysi quality. Ultrapure water (Purelab ultra instrument from ELGA) was used for all preparations. All formulations were prepared and analyzed at room temperature. The particles were characterized by scanning electron microscopy (ESEM Quanta 400) with palladium-sputtered samples. Dynamic light scattering was performed with a Zetasizer nanoseries instrument (Malvern Nano-ZS, λ = 532 nm). The particle size data refer to scattering intensity distributions (z-average).

Peptides and TLR ligand

Conserved influenza A/PR/8/34 (H1N1) HA peptide sequences containing MHC class II and MHC class I epitopes were synthesized (Intavis AG, Cologne, Germany) with the following sequences: HA_{110–120}, SVSSFERFERFEIFPKES; HA_{512–520}, YQILAIYSTVASSLVLL. The phosphorothioate-modified B-type CpG 1826 was purchased from Eurofins MWG Operon (Ebersberg, Germany).

Single-cell suspension

Splenocytes. Spleens were rinsed with an erythrocyte lysis buffer and washed with PBS containing 2% FCS and 2 mM EDTA.

Dendritic cells. DCs were isolated by cutting spleen tissue into small pieces and treating it with 1 mg/ml collagenase type D (Roche Diagnostics GmbH, Mannheim, Germany) and 10 μ g/ml DNase I type II (Sigma-Aldrich Chemie GmbH, St. Louis, MO) diluted in PBS with 2% FCS and 2 mM EDTA. CD11c⁺ cells were then positively selected on MACS columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany).

T cells. CD4⁺ and CD8⁺ T cells were isolated from the spleens of TCR-HA or clone 4-TCR transgenic mice by using the MACS CD4 or CD8 T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). For proliferation assays, T cells were labeled with eFluor670 (eBioscience, Frankfurt, Germany).

Abs and flow cytometry

The mAbs anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11c (HL3), anti-CD80 (16-10A1), and anti-CD86 (GL1) were obtained from BD Biosciences Pharmingen (Heidelberg, Germany). An allophycocyanin-conjugated recombinant MHC class I pentamer (Proimmune, Oxford, U.K.) was used to stain influenza virus-specific CD8⁺ T cells. The H-2K^d pentamer contains the IYSTVASSL sequence of HA A/PR/8/34 from influenza virus A. Flow cytometry was performed with an LSR II instrument using DIVA software (BD Biosciences).

Uptake of functionalized CaP NPs in vitro

Splenocytes (1×10^6) were incubated for 24 h in Iscove's modified Dulbecco's medium-complemented medium containing 10% FCS, 25 μ mol 2-ME, and 100 μ g/ml penicillin/streptomycin with NPs functionalized with Alexa488-modified CpG (CaP/CpG⁴⁸⁸/CaP/CpG⁴⁸⁸). In total, 40 μ l of the particle dispersion was added to 0.4 ml cell medium with splenocytes. After 24 h, the cells were washed with PBS to remove all dispersed CaP NPs, stained for CD11c, and analyzed by flow cytometry.

For the specific in vitro stimulation of DCs, 5×10^5 DCs/well were incubated with soluble CpG and the respective HA peptide, with triple-shell CaP NPs containing CpG and the indicated HA peptide (CaP/CpG/HA/CaP/CpG) or with triple-shell CaP NPs containing nonimmunogenic oligonucleotide sequences and the HA_{110–120} peptide in 400 μ l medium in a 48-well dish at 37°C. After 24 h, cells were washed for the removal of any CaP NPs that were not taken up from the supernatant. Finally, activated DCs were used for T cell proliferation assays.

T cell proliferation

DCs were isolated and stimulated with functionalized CaP NPs (CaP/CpG/HA/CaP/CpG) for 24 h as described earlier. DCs were harvested, washed, and cocultured for 48–72 h with eFluor670-labeled HA-specific CD4⁺ or CD8⁺ T cells at a 1:10 ratio. Cells were stained for the expression of CD4 or CD8. Flow cytometry with gating on CD4⁺ or CD8⁺ T cells was used to measure proliferation by the loss of eFluor670 dye.

Immunization of mice

Mice were immunized i.p. with 300 μ l PBS, soluble CpG/HA_{110–120};512–520, and CaP NPs functionalized with CpG/HA_{110–120};512–520 (10.3 μ M CpG and 20.4 μ g/ml HA peptide; CaP/CpG/HA/CaP/CpG) on days 0, 14, and 28. For nasal application, mice were anesthetized with ketamine/xylazine and immunized with 50 μ l PBS, soluble CpG/HA_{110–120};512–520, and CaP NPs functionalized with CpG/HA_{110–120};512–520 (20.6 μ M CpG and 40.8 μ g/ml HA peptide; CaP/CpG/HA/CaP/CpG) on days 0, 14, and 28. For i.m. immunization, mice were injected with 50 μ l PBS, soluble CpG/HA_{110–120};512–520, and CaP NPs functionalized with CpG/HA_{110–120};512–520 (20.6 μ M CpG and 40.8 μ g/ml HA peptide; CaP/CpG/HA/CaP/CpG) on days 0, 14, and 28 in the right caudal thigh muscle.

Influenza virus challenge

On day 42 after initial immunization, mice were anesthetized by ketamine/xylazine and challenged with 1000 PFUs influenza virus A/PR/8/34 (50 μ l); where indicated, conscious mice were infected with 80,000 PFUs influenza virus A/PR/8/34. Seven days later, lung viral titers or viral copy numbers were determined.

Determination of lung viral titers by plaque assay

Lungs were collected on day 7 after virus challenge; they were homogenized in PBS containing 0.3% BSA and centrifuged for 8 min. Supernatants were serially diluted in OptiMem Medium containing 0.3% BSA in 24-well plates. Samples were then transferred to 12-well plates containing a Madin-Darby canine kidney (MDCK) cell monolayer. Lung supernatants and MDCK cells were incubated for 1 h at room temperature. After incubation, the medium containing diluted lung supernatants was removed, and 2 ml DMEM containing trypsin (0.2 μ g/ml) and Avicel (3%) was added to MDCK cells. On day 4, cells were fixed with 4% paraformaldehyde, and plaques in the MDCK cell layer were stained with crystal violet (Roth, Karlsruhe, Germany).

Determination of lung viral copy numbers by quantitative PCR

Lungs were collected on day 7 after virus challenge; they were homogenized in PBS containing 0.3% BSA and centrifuged for 8 min. Viral RNA was isolated from 140 μ l lung homogenates using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of viral RNA was determined by real-time RT-PCR (RT-quantitative PCR) using the QuantiTect Probe RT-PCR Kit (Qiagen) with SYBR-Green. A conserved sequence in the matrix gene of influenza A/TCTTAACCGAGGTCGAAACG-3' + 5'-AGGGCATTTTGGACAAAG/TCGTCTA-3') (19).

IFN- γ ELISPOT assay

IFN- γ -producing cells were evaluated by ELISPOT with a mouse ELISPOT^{PLUS} kit (Mabtech AB, Nacka Strand, Sweden). Cells collected from the spleen or the lung lymph nodes of immunized mice were restimulated

with HA_{110–120} or HA_{512–520} peptide in a 96-well ELISPOT plate for 24 h. IFN- γ -producing cells were stained according to the manufacturer's instructions. After the plate was dried, the number of spots was counted by an AID ELISPOT reader using ELISPOT 6.0 software (Mabtech).

Cytokine profile of *ex vivo* restimulated splenocytes

Spleens of intranasally immunized mice were washed as described earlier and 5×10^5 splenocytes/well were restimulated with 10 μ g/ml HA_{110–120} or HA_{512–520} peptide for 48 h. Supernatants were taken and the quantification of cytokines was performed with a Procarta Cytokine assay kit (Panomics, Fremont, CA) according to the manufacturer's recommendations. The assay was run with a Luminex 200 instrument using the Luminex IS software (Luminex Corporation, Austin, TX).

Determination of total IgG levels in serum

Blood samples were taken before or 7 d after challenge infection. Sera were prepared and stored at -20°C until usage. IgG levels were determined by using the ELISA for mouse IgG kit (MABTECH AB, Nacka Strand, Sweden) according to the manufacturer's instructions. Serum samples were serially diluted and IgG concentrations were calculated from OD values at 405 nm.

Statistics

One-way ANOVA followed by Bonferroni's multiple-comparisons test or Student *t* test was used to compare groups; *p* values <0.05 were considered significant.

Results

CaP NPs designed as a potential immunization vehicle

Recently, we introduced CaP NPs as nontoxic carriers of immunoinactive TLR ligands and a virus-derived peptide with the ability to mature DCs and induce Ag-specific CD4⁺ T cell responses *in vitro* (15, 16). In this study, we characterized these CaP NPs with regard to their potential use as versatile vaccination vehicle. Therefore, we prepared multishell CaP NPs by using a multistep process of subsequent precipitation and functionalization. The CaP cores contained immunogenic peptides of the influenza virus A/PR/8/34 HA: HA_{512–520} (MHC class I) or HA_{110–120} (MHC class II). These peptides were protected from degradation by an additional CaP shell. Finally, the NPs were colloiddally stabilized by the addition of the TLR9 ligand CpG (Fig. 1). On average, the diameter of these triple-shell NPs was ~ 250 nm.

Uptake of CaP NPs by DCs

DCs are the most effective APCs and play an important role in initiating T cell-mediated immune responses (20). DCs can control a substantial part of the adaptive immune response by internalizing and processing Ags through the MHC class I and II pathways and, finally, by presenting antigenic peptides to CD4⁺ and CD8⁺ T cells (21, 22). Therefore, targeting DCs with a maturation trigger combined with Ag delivery is one of the important goals in the development of new vaccines. To determine whether DCs take up functionalized CaP NPs *in vitro*, we stimulated whole splenocytes with Alexa488-labeled CpG-functionalized CaP NPs. After 24 h, splenocytes were harvested, and CD11c⁺ DCs were analyzed for the uptake of Alexa488-labeled NPs. Importantly, CD11c⁺ DCs showed a high uptake efficiency; $\sim 37\%$ of all DCs was positive for Alexa488 (Fig. 2A). In addition, unlike untreated splenic DCs, Alexa488-labeled DCs exhibited a specific upregulation of the costimulatory molecules CD80 and CD86 (Fig. 2B). To analyze whether CaP NPs alone possess an adjuvant activity, we compared CpG-functionalized CaP NPs and CaP NPs functionalized with nonimmunogenic control oligonucleotides for DC activation. Importantly, only CpG-functionalized CaP NPs induced the activation of CD11c⁺ DCs with specific upregulation of the costimulatory molecules CD80, CD86, and CD40, and elevated secretion of IL-2, IL-12, and TNF- α ($p < 0.01$, $p < 0.001$; Fig. 2C).

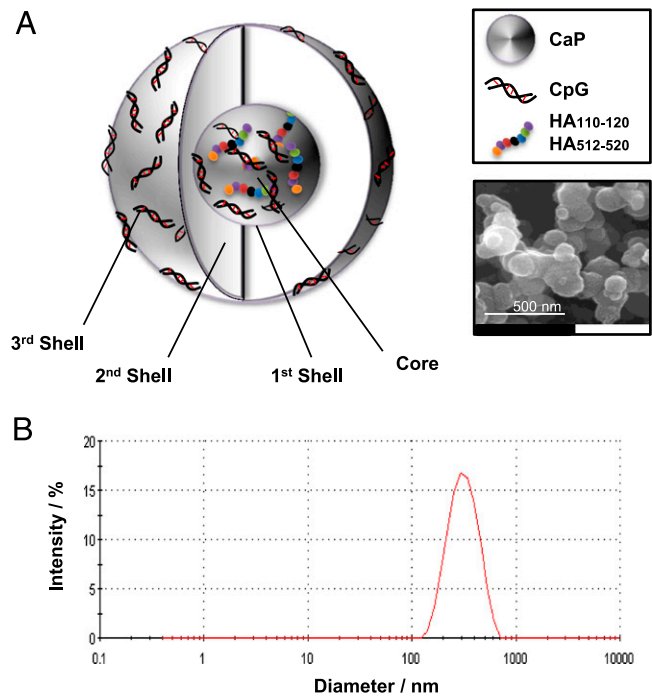


FIGURE 1. Schematic illustration of functionalized CaP NPs. **(A)** CaP NPs are prepared in multiple steps to a final composition of three layers of CaP and TLR ligand CpG (CaP/CpG/CaP/CpG). The core contains the HA peptide HA_{110–120} or HA_{512–520}. The triple-shell NPs were characterized by scanning electron microscopy. **(B)** Results of dynamic light scattering of triple-shell CaP NPs, functionalized with CpG and HA peptides. The average particle diameter is illustrated.

In previous studies, we demonstrated that CaP NPs loaded with an MHC class II-restricted HA peptide of the influenza virus strongly induced an influenza-specific CD4⁺ T cell response *in vitro* (16). For a potent vaccine candidate, the induction of both virus-specific CD4⁺ and CD8⁺ T cell immunity is essential. Therefore, we determined whether DCs present encapsulated HA Ag on MHC class II molecules and cross-present this Ag on MHC class I molecules. CD11c⁺ DCs were isolated from the spleen and stimulated with functionalized CaP NPs or with soluble CpG and HA_{512–520} or HA_{110–120} peptides. Next, stimulated DCs were cocultured with HA-specific CD4⁺ or CD8⁺ T cells, and the proliferation of T cells was measured. As depicted in Fig. 2D, functionalized CaP NPs did not negatively influence the presentation of HA peptides and induced strong HA-specific CD4⁺ and CD8⁺ T cell responses *in vitro* ($p < 0.001$), a finding suggesting the great potential of functionalized CaP NPs for Ag delivery *in vivo*.

Functionalized CaP NPs induce potent CD4⁺ and CD8⁺ effector T cells

We tested the *in vivo* efficiency of CpG/HA_{512–520} or CpG/HA_{110–120} functionalized CaP NPs as a vaccination tool (Fig. 3A). Naive BALB/c mice were immunized *i.p.* three times at intervals of 14 d with PBS, soluble CpG, and either HA_{512–520}/HA_{110–120} peptides or CpG/HA_{512–520}/HA_{110–120} functionalized CaP NPs. Two weeks after the last immunization, splenocytes were analyzed for the presence of HA-specific CD8⁺ T cells and for IFN- γ production. The percentage of HA-specific CD8⁺ T cells detected in mice immunized with functionalized CaP NPs was significantly higher than that detected in mice immunized with PBS and, more important, was also significantly higher than that detected in mice immunized with soluble CpG and HA peptides ($p < 0.05$; Fig. 3B).

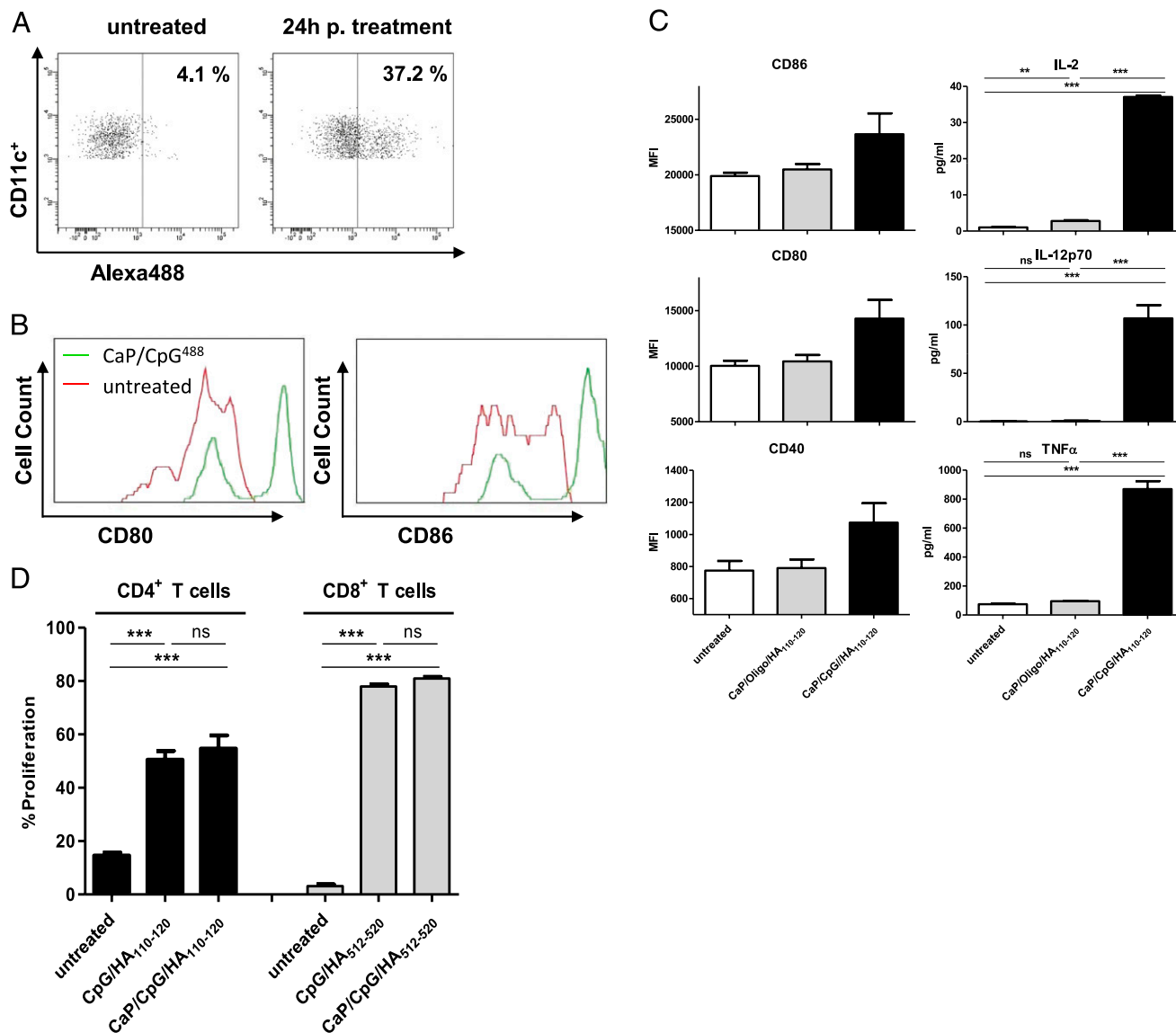


FIGURE 2. Uptake of CaP NPs and presentation of virus-specific Ags in vitro. **(A)** Spleen cells were incubated in vitro with Alexa488-labeled CaP NPs. After 24 h, cells were stained for CD11c and analyzed for CD11c⁺Alexa488⁺ cells. **(B)** Alexa488⁺CD11c⁺ DCs were analyzed for the expression of CD80 and CD86. Results are demonstrated in histograms. **(C)** CD11c⁺ splenic cells were incubated with CpG functionalized CaP NPs or CaP NPs functionalized with nonimmunogenic oligonucleotides. Expression of CD80, CD86, and CD40 and cytokine levels of IL-2, IL-12, and TNF- α in cell culture supernatants were analyzed 24 h later. **(D)** CD11c⁺ splenic cells were incubated with functionalized CaP NPs or with soluble CpG and HA₅₁₂₋₅₂₀ or HA₁₁₀₋₁₂₀ peptides. After 24 h, DCs were washed and cocultured with eFluor670-labeled HA-specific CD4⁺ or CD8⁺ T cells for 48–72 h. Proliferation was measured by loss of eFluor670 dye and is indicated as a percentage of proliferating cells. The figure comprises the results of three independent experiments. Bars represent mean $\pm$ SEM. ** p < 0.01, *** p < 0.001. ns, Not significant.

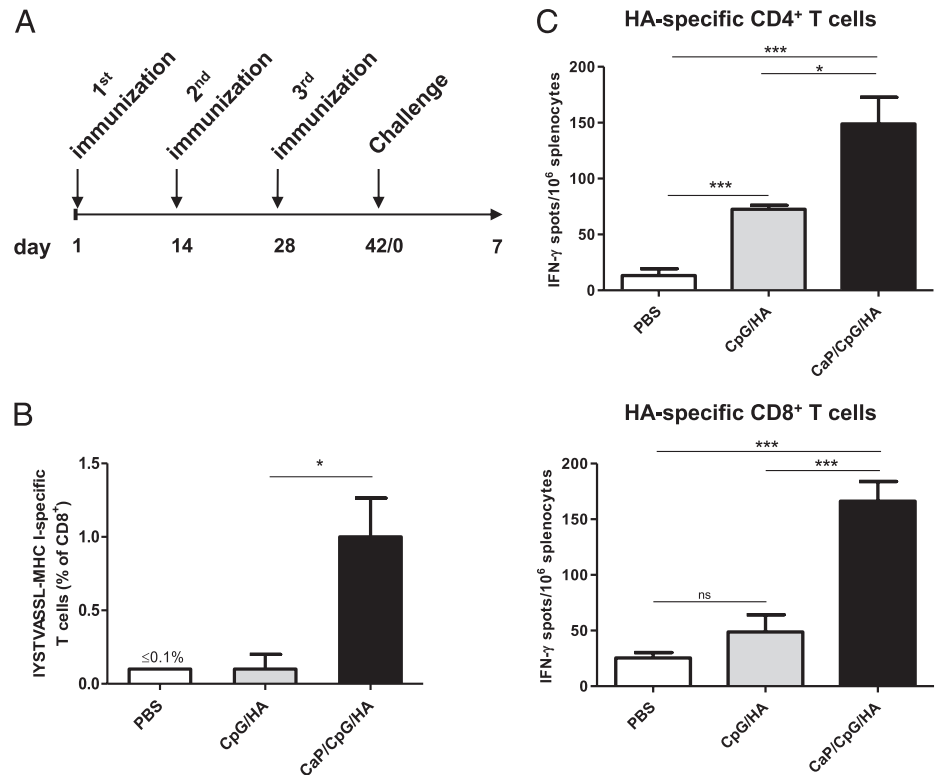
Splenocytes from mice immunized with CaP NPs were restimulated with HA₅₁₂₋₅₂₀ or HA₁₁₀₋₁₂₀ peptide; this restimulation demonstrated the induction of IFN- γ -producing CD4⁺ and CD8⁺ T cells (Fig. 3C). In contrast, restimulation of splenocytes from mice immunized with soluble CpG and HA peptides induced a significantly lower IFN- γ response (p < 0.05 and p < 0.001). Of interest, no humoral IgG Ab response against HA peptides was detected in any of the immunized mice (data not shown). These results indicate that i.p. immunization with functionalized CaP NPs primes cellular immunity much more efficiently than immunization with soluble factors.

Functionalized CaP NPs accelerate clearance of influenza virus from the lung

To determine whether HA-specific CD4⁺ and CD8⁺ T cells induced by i.p. immunization with CaP NPs exhibited protective

activity, we challenged immunized mice intranasally with a high dose of influenza virus A/PR/8/34 2 wk after the last of three immunizations. Lung viral titers were determined 7 d after challenge. Importantly, all mice immunized with CaP NPs exhibited significantly lower viral titers in the lung than the PBS group ($\sim 10^{3.0}$ versus $\sim 10^{3.7}$ PFUs; p < 0.001). More interestingly, 35% of the mice had completely cleared the virus by this time point. In contrast, immunization with soluble CpG and HA peptides did not clear the virus; it merely resulted in slightly lower viral titers compared with the PBS control group (Fig. 4A). This accelerated clearance of virus from the lungs of mice immunized with CaP NPs was strongly accompanied by a specific increase in the number of HA-specific CD8⁺ T cells in the spleens of challenged mice (p < 0.001; Fig. 4B). However, total IgG serum concentrations showed no significant differences between the different groups, neither before nor on day 7 postinfection, indicating

FIGURE 3. Immunization protocol and induction of influenza-specific T cell responses. **(A)** BALB/c mice were immunized three times i.p. with 300 μ l functionalized CaP NPs containing CpG and HA₅₁₂₋₅₂₀ or HA₁₁₀₋₁₂₀ peptides (10.3 μ M CpG and 20.4 μ g/ml HA peptide), soluble CpG plus the HA peptides (10.3 μ M CpG and 20.4 μ g/ml HA peptide). Two weeks after the final immunization, BALB/c mice were sacrificed for cellular analysis. **(B)** Total spleen cells from immunized mice were stained for the presence of HA-specific CD8⁺H-2K^d:YSTVASSL⁺ T cells (three mice per group). Bars represent mean $\pm$ SEM. **(C)** Splenocytes from immunized BALB/c mice (three mice per group) were restimulated in vitro with 10 μ g/ml HA₅₁₂₋₅₂₀ or HA₁₁₀₋₁₂₀ peptide. After 24 h, the numbers of IFN- γ -producing CD4⁺ and CD8⁺ T cells were determined by ELISPOT in triplicate. Spots were counted and expressed as spots per splenocyte. The figure illustrates results from one of three independent experiments. Bars represent mean $\pm$ SEM. * p < 0.05, *** p < 0.001. ns, Not significant.



a minor effect of virus-specific T cells on IgG induction in this setting (Fig. 4C).

Intranasal immunization with functionalized CaP NPs induces effective antiviral mucosal immunity

The lung is the local microenvironment in which primary immune protection against respiratory pathogens such as influenza virus takes place. Therefore, it is believed that efficient protection against

influenza virus requires the induction or recruitment of virus-specific T cells into the lung. For this reason, we determined whether intranasal administration of functionalized CaP NPs was sufficient to induce mucosal immunity and protection against influenza virus infection. First, we measured the specific uptake of NPs by lung lymph node DCs. Alexa488-labeled CaP NPs were administered intranasally to BALB/c mice. After 4 h, lung lymph node cells were stained with anti-CD11c and examined for an

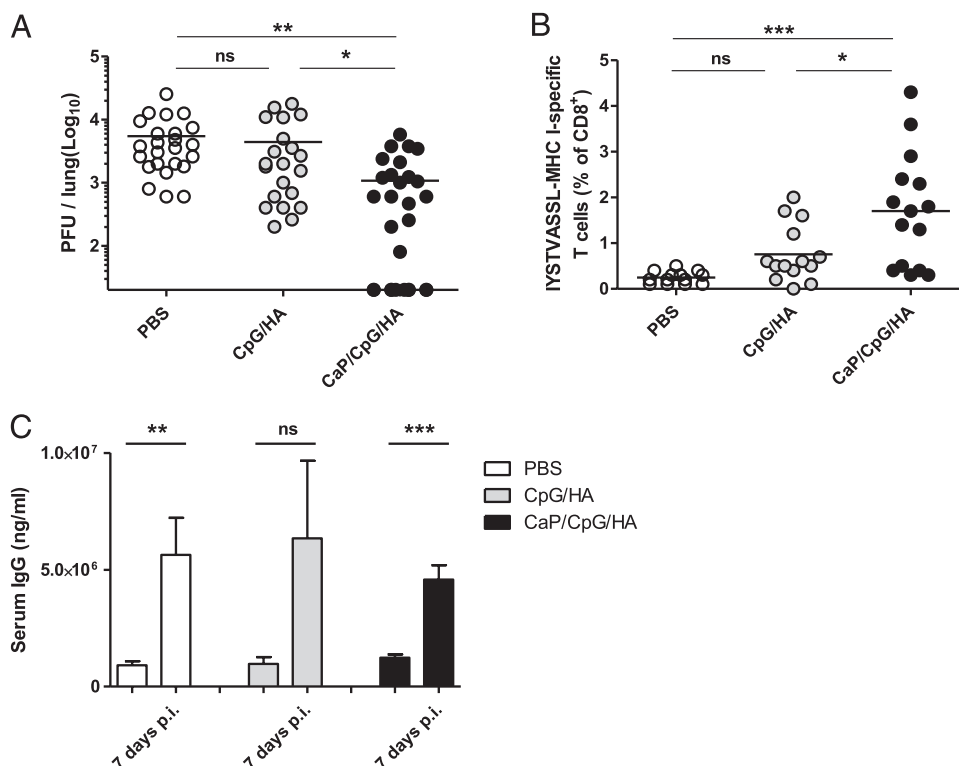


FIGURE 4. Protection of immunized mice against influenza virus replication. **(A)** Fourteen days after the last of three immunizations, immunized mice were challenged intranasally with 1000 PFUs influenza virus A/PR/8/34. After 7 d, mice were sacrificed and lung virus titers were measured by plaque assay. y-Axis starts at the influenza virus detection limit. The figure illustrates results from four independent experiments. Each data point represents one animal. **(B)** Total spleen cells from influenza-challenged mice (7 d after challenge) were stained for the presence of HA-specific CD8⁺H-2K^d:YSTVASSL⁺ T cells. Each data point represents one animal. **(C)** IgG concentrations in sera of immunized mice were determined by ELISA 2 wk after the final immunization and at 7 d postinfection. The figure illustrates the results of two independent experiments (four mice per group). * p < 0.05, ** p < 0.01, *** p < 0.001. ns, Not significant.

Alexa488⁺ population. Approximately 29% of the CD11c⁺ DCs were Alexa488⁺ (Fig. 5A). In the next step, naive BALB/c mice were immunized intranasally according to the same immunization protocol as that used before for PBS, soluble CpG, and HA₁₁₀₋₁₂₀/HA₅₁₂₋₅₂₀ peptides or CpG/HA₅₁₂₋₅₂₀/HA₁₁₀₋₁₂₀ functionalized CaP NPs. Two weeks after the last of three immunizations, splenocytes were isolated, restimulated with the corresponding HA peptides, and analyzed for IFN- γ , TNF- α , and IL-2 production. As demonstrated in Fig. 5B and 5C, a higher number of IFN- γ , IL-2-, and TNF- α -producing CD4⁺ and CD8⁺ T cells were detected in mice immunized with CaP NPs than in other mice ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Furthermore, 7 d after influenza virus challenge, viral replication in the lungs of mice immunized with CaP NPs was significantly lower ($\sim 10^{3.2}$ PFUs) than in mice treated with PBS ($\sim 10^{4.2}$ PFUs; $p < 0.01$; Fig. 6A), and this decrease was accompanied by a higher percentage of HA-specific CD8⁺ T cells in the lung lymph nodes and the spleen ($p < 0.05$ and $p < 0.001$; Fig. 6B).

Although mice represent a good animal model for influenza infection, they show some differences in viral manifestation in comparison with humans. Viral replication in mice, for example, is mainly confined to the lower respiratory tract (23, 24). Human influenza infection is mainly limited to the upper respiratory tract. As a consequence, influenza infection in mice is more severe than in humans. To simulate the situation of upper respiratory tract infection, conscious rather than narcotized mice were challenged

with influenza virus. In general, mice infected without anesthesia showed reduced morbidity 7 d after influenza virus infection, but CpG/HA₅₁₂₋₅₂₀/HA₁₁₀₋₁₂₀ functionalized CaP NP-immunized mice exhibited the highest reduction of the viral load in lungs ($p < 0.05$ and $p < 0.001$; Fig. 6C). Furthermore, elevated numbers of HA-specific CD8⁺ T cells indicate an activation of pre-existing virus-specific T cells after intranasal immunization with functionalized CaP NPs (Fig. 6D).

Intramuscular immunization with functionalized CaP NPs enhances viral clearance

Although intranasal immunization represents an effective application route for inducing potent mucosal immune responses, i.m. injection is the predominant route for many viral vaccines including influenza. To test the efficiency of functionalized CaP NPs for i.m. immunization, we immunized mice according to the standard immunization protocol, but by i.m. injection. Two weeks after the last immunization, conscious mice were challenged with influenza virus. Importantly, only in CaP/CpG/HA NP-immunized mice, viral RNA in lungs was below detection limit at day 7 postinfection. This accelerated clearance of virus from the lungs of mice immunized with CaP NPs was strongly accompanied by a specific increase in the number of HA-specific CD8⁺ T cells in the lung lymph node of challenged mice (Fig. 7B). In contrast, i.m. immunization with soluble HA/CpG did not affect viral clearance in this experimental setting (Fig. 7A, 7B).

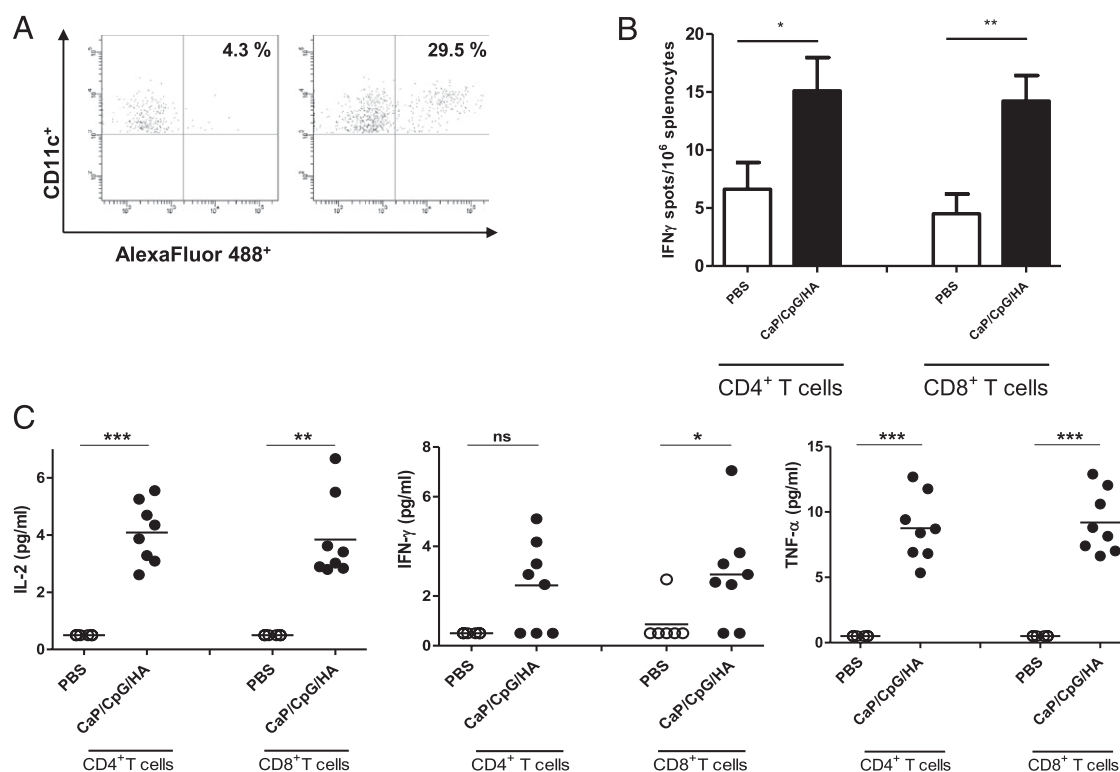


FIGURE 5. Intranasal immunization with functionalized CaP NPs prevents influenza virus replication. **(A)** Alexa488-labeled CaP NPs were administered intranasally to BALB/c mice. After 4 h, lung lymph node cells were isolated and stained for CD11c. The percentage of Alexa488⁺CD11c⁺ DCs is shown. **(B)** BALB/c mice were immunized intranasally with 50 μ l CpG/HA₁₁₀₋₁₂₀;512-520 functionalized CaP NPs (20.6 μ M CpG and 40.8 μ g/ml HA peptide), soluble CpG plus HA₁₁₀₋₁₂₀;512-520 (20.6 μ M CpG and 40.8 μ g/ml HA peptide) on days 1, 14, and 28. Two weeks after the last of three immunizations, BALB/c mice were either sacrificed for cellular analysis or challenged intranasally with 1000 PFUs influenza virus strain A/PR/8/34. Splenocytes from CaP NP immunized mice were restimulated in vitro with 10 μ g/ml of the HA₅₁₂₋₅₂₀ or HA₁₁₀₋₁₂₀ peptide. After 24 h, the numbers of IFN- γ -producing CD4⁺ and CD8⁺ T cells were determined by ELISPOT in triplicate. Spots were counted and expressed as spots per lung lymph node cells. The figure illustrates the findings of two independent experiments (five mice per group). Bars represent mean $\pm$ SEM. **(C)** Splenocytes of immunized mice were cultured in the presence of 10 μ g/ml of the HA₅₁₂₋₅₂₀ or HA₁₁₀₋₁₂₀ peptide. After 48 h, supernatants were analyzed for indicated cytokines by Luminex technology. The figure illustrates the results of two independent experiments. Each data point represents one sample. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, Not significant.

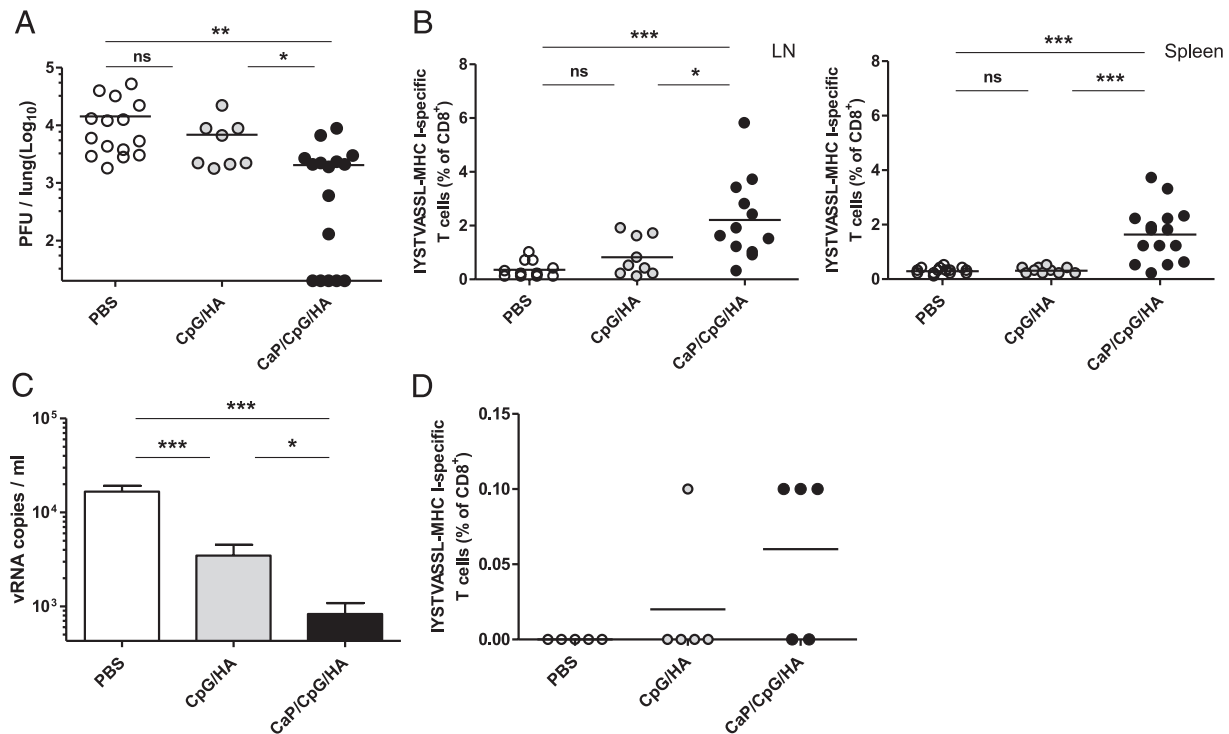


FIGURE 6. Intranasal immunization suppresses virus replication. **(A)** Fourteen days after the last of three immunizations, mice were challenged intranasally with 1000 PFUs influenza virus A/PR/8/34. After 7 d, mice were sacrificed, and lung virus titers were measured by plaque assay. y-axis starts at the influenza virus detection limit. The figure illustrates the results from three independent experiments. **(B)** Lung lymph node cells and total spleen cells from influenza-challenged mice (7 d after challenge) were stained for the presence of HA-specific CD8⁺H-2K^d:IYSTVASSL⁺ T cells. The figure illustrates the results from three independent experiments. **(C)** Fourteen days after the last of three immunizations, conscious mice were challenged intranasally with a 80,000 PFUs influenza virus A/PR/8/34. Viral RNA was isolated on day 7 postinfection from homogenized lungs. Viral copy numbers were determined by qPCR (five mice per group). The figure comprises the results of two independent experiments. Bars represent mean $\pm$ SEM. **(D)** Lung lymph node cells from influenza-challenged mice (7 d after challenge) were stained for the presence of HA-specific CD8⁺H-2K^d:IYSTVASSL⁺ T cells. Each data point represents one animal. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, Not significant.

In summary, these findings suggest that functionalized CaP NPs are sufficient to induce protective antiviral immunity when administered by various immunization routes.

Discussion

In this study, we found that CaP NPs can deliver Ag to DCs and can drive sufficient maturation and signal transduction in DCs to facilitate Ag processing and presentation, including cross-presentation. i.p. and, even more important, intranasal and i.m. administration of MHC class I/II-restricted HA peptides and TLR ligand 9 CpG, coencap-

sulated in CaP NPs, were effective in inducing protective cellular immunity against influenza virus in mice.

Synthetic or natural biomaterials have been used as drug-delivery carriers and as vaccine adjuvant agents (25–27). However, the advanced formulation of these materials for specific targeting and administration through various biological routes is still under investigation. In the case of infection with respiratory viruses such as influenza, particular attention should be paid to the lung as a target for mucosal immunization because the lung is an entrance point for respiratory viruses (28). Virus-specific CD4⁺ and CD8⁺ T cells in

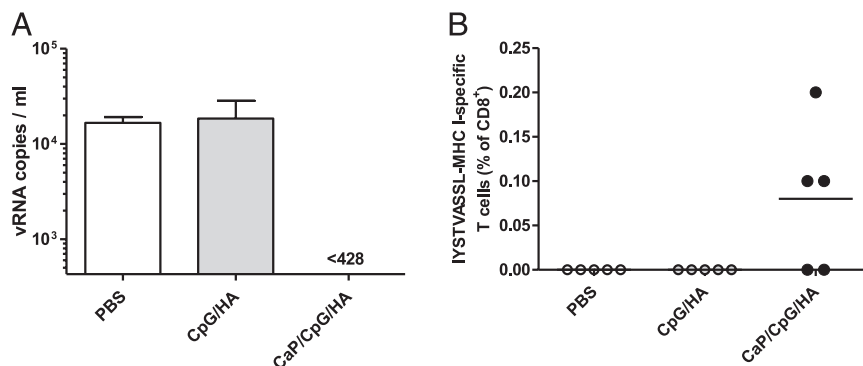


FIGURE 7. Intramuscular immunization with functionalized CaP NPs prevents influenza virus replication. **(A)** Fourteen days after the last of three i.m. immunizations, conscious mice were challenged intranasally with 80,000 PFUs influenza virus A/PR/8/34. Viral RNA was isolated on day 7 postinfection from homogenized lungs. Viral copy numbers were determined by qPCR (five mice per group). The figure comprises the results of two independent experiments. Bars represent mean $\pm$ SEM. **(B)** Lung lymph node cells from influenza-challenged mice (7 d after challenge) were stained for the presence of HA-specific CD8⁺H-2K^d:IYSTVASSL⁺ T cells. Each data point represents one animal.

the lung are considered to confer protection against respiratory viruses (28). However, the requirements for efficient induction of potent virus-specific T cells in the lung are still unclear. In general, Ags introduced via the mucosal route are often only poorly immunogenic because of the immunosuppressive environment, and they induce a state of tolerance (29). Therefore, it seems important for mucosal vaccination with peptides to use effective delivery systems that provide both carrier and adjuvant functions. In this study, intranasal immunization was found to be quite effective for the clearance of influenza virus. The CaP-based nanoparticulate carrier used in this study can be easily prepared and functionalized as a multishell system. After being taken up by the cells, the calcium ions are rapidly pumped out of the cell; therefore, the intracellular calcium level does not exceed a harmless level (30). In addition, adjuvants can be controlled, added, and released while nanoparticulated CaP alone is not sufficient to act as an adjuvant on DCs.

Unmethylated CpG was used as an adjuvant in our study. It binds to TLR9 and is a well-established, safe molecule with clinical translational potential, as demonstrated by the findings of three completed clinical trials of CpG as a vaccine adjuvant (31). CpG can evoke a range of immunostimulatory effects; it can enhance the activity of lymphocytes and APCs, trigger DC maturation, and drive the immune system toward the Th1 immune response against specific Ags (32). This finding is in accordance with our results: we demonstrated the maturation of DCs and the induction of IFN- γ -producing CD4⁺ and CD8⁺ effector cells after mice were immunized with CaP NPs functionalized with CpG/HA peptide. Although the control of influenza virus infection is generally assumed to be primarily mediated by neutralizing Abs after immunization with inactivated influenza virus, cellular immunity may play a role as well, because CD8⁺ and CD4⁺ T cells participate in the immune response against influenza infection (33). Notably, in this study, we demonstrate that, after immunization with functionalized CaP NPs, Th1-based T cells alone are sufficient to provide protective immunity and prevent replication of influenza virus in mice. In future experiments, it would be of interest to incorporate a panel of different epitopes and to combine humoral and cellular immune responses for protective immunity, to induce a broader immune response against different influenza virus strains.

The route of vaccination is important in influencing immune responses at the initial site of pathogen invasion. Systemic immunization is adequate for successful vaccines against some mucosal pathogens, where high titers of neutralizing Abs are capable of clearing the virus and preventing disease (34). However, some mucosal pathogens may require strategies that activate multiple arms of the innate and adaptive immune system for a strong and long-lasting response (35). CaP NPs seem to represent an excellent delivery system for mucosal vaccines as the intranasal immunization with HA/CpG-functionalized CaP NPs was more efficient in inducing protection at mucosal sites than i.p. immunization.

Biomimicry by NPs is a powerful method for modulating the immune response for active immunization. Biodegradable CaP NPs loaded with influenza Ag and with a surface modified by bacterial component CpG can prime a vigorous immune response against the encapsulated Ag and can provide protective immunity. Our findings demonstrate the high potential of CaP NPs as a novel vaccination tool that allows the flexible dosing of Ags and adjuvant, and therefore provides control over many of the variables that are important in optimizing an effective vaccine delivery system.

Acknowledgments

We thank Patrick Juszczak, Mechthild Hemmler-Roloff, and Klaus Sure for excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

- Sallusto, F., A. Lanzavecchia, K. Araki, and R. Ahmed. 2010. From vaccines to memory and back. *Immunity* 33: 451–463.
- Zinkernagel, R. M. 2002. Immunity, immunopathology and vaccines against HIV? *Vaccine* 20: 1913–1917.
- Bråve, A., K. Ljungberg, B. Wahren, and M. A. Liu. 2007. Vaccine delivery methods using viral vectors. *Mol. Pharm.* 4: 18–32.
- Flatz, L., A. N. Hegazy, A. Berghaler, A. Verschoor, C. Claus, M. Fernandez, L. Gattinoni, S. Johnson, F. Kreppel, S. Kochanek, et al. 2010. Development of replication-defective lymphocytic choriomeningitis virus vectors for the induction of potent CD8⁺ T cell immunity. *Nat. Med.* 16: 339–345.
- Haglund, K., I. Leiner, K. Kerksiek, L. Buonocore, E. Pamer, and J. K. Rose. 2002. Robust recall and long-term memory T-cell responses induced by prime-boost regimens with heterologous live viral vectors expressing human immunodeficiency virus type 1 Gag and Env proteins. *J. Virol.* 76: 7506–7517.
- Hubbell, J. A. 2010. Drug development: Longer-lived proteins. *Nature* 467: 1051–1052.
- Chen, K., and A. Cerutti. 2010. Vaccination strategies to promote mucosal antibody responses. *Immunity* 33: 479–491.
- Nembrini, C., A. Stano, K. Y. Dane, M. Ballester, A. J. van der Vlies, B. J. Marsland, M. A. Swartz, and J. A. Hubbell. 2011. Nanoparticle conjugation of antigen enhances cytotoxic T-cell responses in pulmonary vaccination. *Proc. Natl. Acad. Sci. U. S. A.* 108: E989–E997.
- Ichinohe, T., A. Iwasaki, and H. Hasegawa. 2008. Innate sensors of influenza virus: clues to developing better intranasal vaccines. *Expert Rev. Vaccines* 7: 1435–1445.
- Perrone, L. A., A. Ahmad, V. Vaguilla, X. Lu, G. Smith, J. M. Katz, P. Pushko, and T. M. Tumpey. 2009. Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. *J. Virol.* 83: 5726–5734.
- Wee, J. L., J. P. Scheerlinck, K. J. Snibson, S. Edwards, M. Pearce, C. Quinn, and P. Sutton. 2008. Pulmonary delivery of ISCOMATRIX influenza vaccine induces both systemic and mucosal immunity with antigen dose sparing. *Mucosal Immunol.* 1: 489–496.
- Bachelder, E. M., T. T. Beaudette, K. E. Broaders, J. M. Fréchet, M. T. Albrecht, A. J. Mateczun, K. M. Ainslie, J. T. Pesce, and A. M. Keane-Myers. 2010. In vitro analysis of acetalated dextran microparticles as a potent delivery platform for vaccine adjuvants. *Mol. Pharm.* 7: 826–835.
- Tai, W., L. Roberts, A. Seryshev, J. M. Gubatan, C. S. Bland, R. Zabriskie, S. Kulkarni, L. Soong, I. Mbawuike, B. Gilbert, et al. 2011. Multistrain influenza protection induced by a nanoparticulate mucosal immunotherapeutic. *Mucosal Immunol.* 4: 197–207.
- Demento, S. L., N. Bonafé, W. Cui, S. M. Kaeck, M. J. Caplan, E. Fikrig, M. Ledizet, and T. M. Fahmy. 2010. TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. *J. Immunol.* 185: 2989–2997.
- Sokolova, V., T. Knuschke, J. Buer, A. M. Westendorf, and M. Eppler. 2011. Quantitative determination of the composition of multi-shell calcium phosphate-oligonucleotide nanoparticles and their application for the activation of dendritic cells. *Acta Biomater.* 7: 4029–4036.
- Sokolova, V., T. Knuschke, A. Kovtun, J. Buer, M. Eppler, and A. M. Westendorf. 2010. The use of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin to induce dendritic cell maturation and T cell activation. *Biomaterials* 31: 5627–5633.
- Kirberg, J., A. Baron, S. Jakob, A. Rolink, K. Karjalainen, and H. von Boehmer. 1994. Thymic selection of CD8⁺ single positive cells with a class II major histocompatibility complex-restricted receptor. *J. Exp. Med.* 180: 25–34.
- Morgan, D. J., R. Liblau, B. Scott, S. Fleck, H. O. McDevitt, N. Sarvetnick, D. Lo, and L. A. Sherman. 1996. CD8⁺ T cell-mediated spontaneous diabetes in neonatal mice. *J. Immunol.* 157: 978–983.
- Fouchier, R. A., T. M. Bestebroer, S. Herfst, L. Van Der Kemp, G. F. Rimmelzwaan, and A. D. Osterhaus. 2000. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *J. Clin. Microbiol.* 38: 4096–4101.
- Hart, D. N. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 90: 3245–3287.
- Choy, J. H., S. Y. Kwak, Y. J. Jeong, and J. S. Park. 2000. Inorganic Layered Double Hydroxides as Nonviral Vectors This work was in part supported by the Korean Ministry of Science and Technology through the NRL project and by the Korean Ministry of Education (BSRI-99-3413). S.Y.K. expresses her thanks to the BK21 fellowship. *Angew. Chem. Int. Ed. Engl.* 39: 4041–4045.
- Macatonia, S. E., N. A. Hosken, M. Litton, P. Vieira, C. S. Hsieh, J. A. Culpepper, M. Wysocka, G. Trinchieri, K. M. Murphy, and A. O'Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4⁺ T cells. *J. Immunol.* 154: 5071–5079.
- Belser, J. A., X. Lu, T. R. Maines, C. Smith, Y. Li, R. O. Donis, J. M. Katz, and T. M. Tumpey. 2007. Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. *J. Virol.* 81: 11139–11147.
- Belser, J. A., D. A. Wadford, C. Pappas, K. M. Gustin, T. R. Maines, M. B. Pearce, H. Zeng, D. E. Swayne, M. Pantin-Jackwood, J. M. Katz, and

- T. M. Tumpey. 2010. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice. *J. Virol.* 84: 4194–4203.
25. Fahmy, T. M., S. L. Demento, M. J. Caplan, I. Mellman, and W. M. Saltzman. 2008. Design opportunities for actively targeted nanoparticle vaccines. *Nano-medicine (Lond)* 3: 343–355.
26. Liu, J., Z. Jiang, S. Zhang, C. Liu, R. A. Gross, T. R. Kyriakides, and W. M. Saltzman. 2011. Biodegradation, biocompatibility, and drug delivery in poly (ω -pentadecalactone-co-p-dioxanone) copolyesters. *Biomaterials* 32: 6646–6654.
27. Lo, C. T., P. R. Van Tassel, and W. M. Saltzman. 2010. Poly(lactide-co-glycolide) nanoparticle assembly for highly efficient delivery of potent therapeutic agents from medical devices. *Biomaterials* 31: 3631–3642.
28. Kohlmeier, J. E., and D. L. Woodland. 2009. Immunity to respiratory viruses. *Annu. Rev. Immunol.* 27: 61–82.
29. Lambrecht, B. N., and H. Hammad. 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity* 31: 412–424.
30. Neumann, S., A. Kovtun, I. D. Dietzel, M. Eppe, and R. Heumann. 2009. The use of size-defined DNA-functionalized calcium phosphate nanoparticles to minimise intracellular calcium disturbance during transfection. *Biomaterials* 30: 6794–6802.
31. Klinman, D. M., S. Klaschik, T. Sato, and D. Tross. 2009. CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. *Adv. Drug Deliv. Rev.* 61: 248–255.
32. Krishnamachari, Y., and A. K. Salem. 2009. Innovative strategies for co-delivering antigens and CpG oligonucleotides. *Adv. Drug Deliv. Rev.* 61: 205–217.
33. Sun, J., R. Madan, C. L. Karp, and T. J. Braciale. 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat. Med.* 15: 277–284.
34. Herremans, T. M., J. H. Reimerink, A. M. Buisman, T. G. Kimman, and M. P. Koopmans. 1999. Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus. *J. Immunol.* 162: 5011–5018.
35. Neutra, M. R., and P. A. Kozlowski. 2006. Mucosal vaccines: the promise and the challenge. *Nat. Rev. Immunol.* 6: 148–158.