1 A cell culture-derived whole virus influenza A
2 vaccine based on magnetic sulfated cellulose
3 particles confers protection in mice against lethal
4 influenza A virus infection

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16 **KEYWORDS**

influenza; vaccination; purification; magnetic particles; formulation; immunization;

ABREVIATIONS

TCID₅₀, 50% tissue culture infective dose; OD_{450nm}, absorbance at a wavelength of 450 nm; aMSCP, antigen-loaded MSCP; aq, aqueous; CB, coating buffer; CV, column volume; cPCA, crude positive control antigen; d, day; DVH, diafiltered virus harvest; DSP, downstream processing; HAU, hemagglutination units; HA, hemagglutinin/hemagglutination; HSPG, heparan sulfate proteoglycans; A/PR, influenza A/Puerto Rico/8/1934 virus; i.p., intraperitoneal; MDCK, Madin Darby canine kidney; MSCP, magnetic sulfated cellulose particles; MVA, Modified Vaccinia Ankara; NAFL, nonablative fractional laser; NP, nucleoprotein; PBS, phosphate buffer saline_(aq); PCA, positive control antigen; p.i., post viral infection; PFB, pre-formulation buffer; RSD, relative standard deviation; RT, room temperature; SRID, single radial immunodiffusion assay; SEM, standard error of the mean; SCMA, sulfated cellulose membrane adsorber; VB, virus broth; WB, washing buffer;

ABSTRACT

Downstream processing and formulation of viral vaccines employs a large number of different unit operations to achieve the desired product qualities. The complexity of individual process steps involved, the need for time consuming studies towards the optimization of virus yields, and very high requirements regarding potency and safety of vaccines results typically in long lead times for the establishment of new processes. To overcome such obstacles, to enable fast screening of potential vaccine candidates, and to explore options for production of low cost veterinary vaccines a new platform for whole virus particle purification and formulation based on magnetic particles has been established. Proof of concept was carried out with influenza A

virus particles produced in suspension Madin Darby canine kidney (MDCK) cells. The clarified, inactivated, concentrated, and diafiltered virus particles were bound to magnetic sulfated cellulose particles (MSCP), and directly injected into mice for immunization including positive and negative controls. We show here, that in contrast to the mock-immunized group, vaccination of mice with antigen-loaded MSCP (aMSCP) resulted in high anti-influenza A antibody responses and full protection against a lethal challenge with replication competent influenza A virus. Antiviral protection correlated with a 400-fold reduced number of influenza nucleoprotein gene copies in the lungs of aMSCP immunized mice compared to mock-treated animals, indicating the efficient induction of antiviral immunity by this novel approach. Thus, our data proved the use of MSCP for purification and formulation of the influenza vaccine to be fast and efficient, and to confer protection of mice against influenza A virus infection. Furthermore, the method proposed has the potential for fast purification of virus particles directly from bioreactor harvests with a minimum number of process steps towards formulation of low-cost veterinary vaccines, and for screening studies requiring fast purification protocols.

INTRODUCTION

Separation processes based on magnetic particles can be considered established technology for the purification of nucleic acids [1], peptides, proteins [2], viruses [3–5], organelles of animal cells or even entire cells [6]. The binding modes range from simple ionic interactions to exotic affinity ligands and depend on the species to be purified. In particular, the use of magnetic particle systems was investigated for medical applications like drug delivery, drug targeting, diagnosis, magnetic resonance imaging, or treatment with hyperthermia [7,8]. In addition, new applications for the formulation of vaccines are emerging. One example are nanoparticle vaccines which can be used as antigen carriers with specific immunostimulating features [9].

Furthermore, DNA vaccines based on magnetic particles showed promising results by employing magnetic fields for transfection [10].

The purification of particle-based vaccines, however, involves a traditional antigen purification process with subsequent particle formulation. Usually, the downstream processing (DSP) involves clarification, concentration, inactivation, purification, polishing, and sterile filtration steps and employs a wide range of different unit operations, like centrifugation, (dia-)filtration, and chromatography [11–13]. Therefore, the establishment of the DSP is often difficult to realize in research laboratories focusing on experimental validation of novel vaccine or gene therapy candidates as they often lack the expertise and the technical equipment. Moreover, there are only a few ready-to-use high-throughput DSP tools available for the purification of virus particles which would allow speeding up development of whole virus vaccine candidates or viral vectors for gene therapy [14].

To overcome these limitations and to explore options towards fast purification and formulation of veterinary vaccines, a novel approach based on magnetic beads was established in this study. Magnetic particles consisting of sulfated cellulose enabled the specific binding of the antigen of interest (influenza A virus particles), followed by direct formulation and injection into mice for immunization. This approach implements the vaccine formulation directly into the DSP after a clarification, concentration, and diafiltration step without the need of additional steps and the potential to reduce and simplify the whole process even further.

Proof of concept was carried out using inactivated influenza A/Puerto Rico/8/1934 (A/PR) virus particles produced in suspension MDCK cells and bound to magnetic sulfated cellulose particles (MSCP) described for analytical sample preparation in a previous study [3]. The antigen-loaded

MSCP (aMSCP) were directly used for the immunization of mice. The sulfated cellulose matrix of the MSCP acts as pseudo-affinity ligand potentially mimicking heparan sulfate proteoglycans (HSPG) and its use for virus purification was already shown for membrane based purification of influenza and Modified Vaccinia Ankara (MVA) virus particles [3,15–17]. HSPGs are involved in a wide range of natural pathogen-host interactions rendering the MSCP system suitable for the delivery of a wide range of different viral, bacterial, and parasitic antigens. Additionally, the used cellulose backbone has several further advantages: the raw material is abundantly available, cost-effective, shows low non-specific binding, and is known to be biocompatible [18–21]. The magnetic features of the MSCP are due to incorporated Fe_3O_4 particles that were also classified as biocompatible and nontoxic [10]. Therefore, the proposed MSCP vaccine system should not lead to any adverse effects *in vivo*. A further advantage regarding the use of micrometer-sized MSCP is that a fast and easy separation with cost-effective conventional magnetic separators can be achieved without the need of high-gradient magnetic field separation systems.

To assess the suitability of the MSCP influenza A/PR vaccine system we performed *in vivo* immunization studies in mice. Therefore, anti-A/PR antibody levels were monitored during the time course of the experiment. In addition, protection of immunized mice was investigated by challenging the animals with a lethal A/PR virus dose, followed by weight loss monitoring as an indicator for morbidity. Finally, the viral load in the lung tissue was determined to complement the picture.

MATERIALS AND METHODS

Materials. Aqueous (aq) buffers and cell culture-media were prepared from ultrapure water produced by a Milli-Q Advantage A10 water purification system (Merck KGaA, Darmstadt, Germany). All used chemicals had a purity of at least 99%.

Hemagglutination assay for virus quantification. The hemagglutination (HA) assay was carried out according to Kalbfuss et al. and is expressed in HAU units [22].

Single radial immunodiffusion assay for hemagglutinin antigen quantification. The amount of the major influenza surface protein hemagglutinin (HA) was quantified by a single radial immunodiffusion assay (SRID) assay based on Wood et al [23]. The SRID setup used a 7x7 diffusion matrix consisting of a 1% agarose gel with 64 μ g anti-A/PR serum/ml purchased from NIBSC (#03/242, Hertfordshire, England). An in-house sulfated cellulose membrane adsorber (SCMA) purified A/PR standard was used for HA quantification. For measuring the antigen amount of the starting and end solution of the MSCP antigen-loading process, sucrose was added to a final concentration of 10% (w/v), followed by freezing at -80°C and lyophilization overnight. Positive control antigen (PCA) samples could be directly used in the SRID assay. The relative standard deviation (RSD) for measuring the amount of MSCP-bound antigen was $\leq \pm 17\%$ (n = 17).

Virus antigen production. For producing influenza A/PR virus particles a MDCK suspension cell line was used [23,24]. MDCK cells were cultivated in a two-times concentrated chemically defined, protein- and peptide-free SMIF8_(aq) PGd medium purchased from Service Zellkultur Scharfenberg (Emden, Germany). The medium was supplemented with 5 g/l NaCl, 3.66 g/l D-(+)-glucose, 2 g/l NaHCO₃, 0.585 g/l L-glutamine, 0.352 g/l pyruvate, 0.242 g/l L-glutamic acid, 10 ml/l 10% Pluronic-F68, and 1 μ l/l 98% ethanolamine. The A/PR virus was obtained from the

Robert Koch Institute (Berlin, Germany) and was used to generate an in-house adherent MDCK-derived A/PR stock with a 50% tissue culture infective dose (TCID₅₀) of 10⁸ infectious virus particles/ml. Infection of MDCK cells was carried out with 10⁻⁵ trypsin units/cell purchased from Thermo Fisher Scientific (#27250-018, Massachusetts, USA) and the A/PR stock at a multiplicity of infection of 10⁻⁴ in a 5 L CT5-SK bioreactor (Sartorius-Stedim Biotech GmbH, Göttingen, Germany). Finally, the virus broth (VB) was harvested 72 hours post infection [24–26].

Virus broth clarification and inactivation. The VB was sequentially filtered through a 5 µm and 0.65 µm depth filter (#CFAP0508YY and #CFAP9608YY, GE Water & Process Technologies, Trevose, USA). Subsequently, chemical inactivation of the virus particles was carried out by adding β-propiolactone (Serva Electrophoresis GmbH, Heidelberg, Germany) to a final concentration of 6 mM and incubation for 24 h at 37°C. Finally, the inactivated virus particle solution was filtered through a 0.45 µm depth filter (#CMMP9408YY, GE Water & Process Technologies, Trevose, USA) to collect the inactivated and clarified virus harvest. Inactivation was verified before the subsequent steps were performed as published previously [27].

Concentration and diafiltration. The inactivated and clarified virus harvest was 20x volumetrically concentrated using a cellulose Sartoon Slice 200 Hydrosart cassette (Sartorius-Stedim Biotech GmbH, Göttingen, Germany) with a molecular weight cut-off of 750 kDa. In a next step a 10x volume buffer exchange by diafiltration was carried out to a pre-formulation buffer (PFB; 50 mM NaCl, 10 mM Tris-HCl_(aq), pH 7.4) to facilitate binding of the virus particles to the MSCP and SCMA pseudo-affinity matrices in subsequent steps. The obtained diafiltered virus harvest is referred to as DVH in the following. For concentration and

diafiltration a Äkta crossflow tangential flow filtration device (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used.

Magnetic sulfated cellulose particles preparation. MSCP are based on MG 200 magnetic macroporous cellulose particles with a diameter of 100 to 250 μm purchased from Iontosorb (Ústí nad Labem, Czech Republic) which were sulfated as described previously [3,28]. Briefly, 15 g of magnetic cellulose particles were dispersed in a 1:20 chlorsulfonic acid-pyridine reaction mixture and incubated for 15 min at 42°C. Finally, resulting MSCP were washed with ultrapure water to remove remaining chlorsulfonic acid-pyridine solution and resuspended in 70% ethanol_(aq) for storage at room temperature (RT) until further usage.

Antigen loading and formulation of magnetic sulfated cellulose particles. 500 μl MSCP in 70% ethanol_(aq) were washed 5x with PFB to condition the MSCP for virus particle binding. 100 μl of the DVH solution were used to measure the antigen amount in the starting solution by SRID assay. 400 μl DVH, including 6.8 μg HA, were added to the conditioned MSCP and incubated for 10 min at RT to enable virus particle binding to the MSCP. After binding, the residual HA amount in the supernatant of the end solution was determined again by SRID, and the amount of HA bound to aMSCP estimated from the difference between the starting and the end solution. Afterwards, aMSCP were washed 5x with 500 μl PFB to remove residual DVH solution. Finally, aMSCP were suspended in 700 μl formulation buffer (FB) consisting of PFB with 250 μg polyinosinic:polycytidylic acid (poly I:C)/ml (Invivogen, Toulouse, France) and 250 μg CpG/ml (Eurofins Genomics, Ebersberg, Germany) as adjuvant [29]. The aMSCP system was optimized to contain a minimum antigen content of 1.0 μg HA per immunization. The antigen loss after aMSCP loading was investigated by measuring HA activity of washing and formulation supernatants. In total, a loss of approximately 11% HAU was observed and therefore

considered negligible (data not shown). For vaccination, 200 μ l of the aMSCP formulation were used per mouse and injection with a $\varnothing$ 0,45 x 12 mm needle (#466 5457, B. Braun Melsungen AG, Melsungen, Germany) and a 1 mL syringe (#9161406V, B. Braun Melsungen AG, Melsungen, Germany). Two batches of aMSCP were used for immunization with 2.1 μ g HA for the first immunization and 1.2 μ g HA for the booster injection, respectively (immunization protocol, see below).

Preparation of the positive control antigen. To obtain the crude positive control antigen (cPCA), DVH was purified by sulfated cellulose membrane adsorber (SCMA) chromatography [30]. The used SCMA prototype had a column volume (CV) of 3 ml and was kindly provided by Sartorius-Stedim Biotech GmbH (Göttingen, Germany). For the chromatographic purification, the SCMA was equilibrated with 20x CV of PFB, loaded with 65 ml DVH, washed with 20x CV PFB, and eluted with 10x CV of a buffer containing 600 mM NaCl 10 mM Tris-HCl_(aq) pH 7.4. Finally, the elution peak was pooled according to the light scattering signal indicating the presence of purified virus particles to obtain the cPCA. The cPCA was dialyzed overnight to 150 mM NaCl 50 mM Tris-HCl_(aq) pH 7.4 followed by sucrose addition to a final concentration of 10% (w/v) and frozen at -80°C. Finally, the antigen sample was lyophilized overnight and stored at -80°C to obtain the positive control antigen (PCA). PCA samples were diluted in FB to the desired HA amount per 200 μ l before being injected into mice with a $\varnothing$ 0,45 x 12 mm needle (#466 5457, B. Braun Melsungen AG, Melsungen, Germany) and a 1 mL syringe (#9161406V, B. Braun Melsungen AG, Melsungen, Germany).

SDS-PAGE with silver staining. To compare the purity of the cPCA and the aMSCP samples a discontinuous SDS-PAGE with subsequent silver-staining was carried out. Therefore, pure acetone was added at five times the sample volume followed by precipitation overnight at -20°C.

The cPCA was used directly for the precipitation. The antigen loaded onto the aMSCP was first eluted with 600 mM NaCl 10 mM Tris-HCl_(aq) pH 7.4 (the same buffer used for the elution of the cPCA from the SCMA). Then, the samples were centrifuged at 16000 g for 30 min, the supernatant discarded, and the pellet used for all subsequent steps. The SDS-PAGE consisted of a 4% stacking gel combined with a 12% acrylamide separating gel according to Laemmli et al. [31]. SDS-PAGE were run with a Mini-Protean® Tetra System (Bio-Rad Laboratories, Hercules, USA) at a constant current of 10 mA until the running front reached the separating gel boarder followed by increasing the current to 20 mA. To estimate the protein size a PageRuler™ Prestained Protein Ladder (#26616, 10 to 180 kDa, Thermo Fisher Scientific, Massachusetts, USA) was used. Silver staining was carried according to Blum et al. [32]. The SDS-PAGE pictures were obtained with ViewPix 900 Scanner (biostep GmbH, Burkhardtsdorf, Germany) at a resolution of 300 dpi.

Mice. Eight week old female C57Bl/6 mice were purchased from Harlan Laboratories (AB Venray, The Netherlands). Mice were kept under specific pathogen-free conditions according to the guidelines of the regional animal care committee.

Collection of blood and preparation of sera. Sera were prepared from blood samples collected one day prior to the immunizations and at the end of the immunization experiments by retrobulbar bleeding of mice. For this purpose mice were anesthetized by inhalation of isoflurane (Baxter AG, Volketswil, Switzerland). Blood samples were incubated for 20 min at room temperature and for further 20 min at 4°C. Afterwards, blood samples were spun down for

215 10 min with 14000 rpm at 4°C. Supernatants were collected and stored at -20°C until further
216 analysis.

217 **Detection of anti-A/PR antibodies by ELISA.** A sandwich ELISA was used for the detection
218 of anti-A/PR antibodies in sera of immunized mice. For immobilizing the capturing antibodies
219 100 µl of an anti-A/PR serum purchased from NIBSC (#03/242, Hertfordshire, England) diluted
220 1:1000 in 50 mM Na₂CO_{3(aq)} pH 9.6 coating buffer (CB) was incubated at 4°C overnight in Nunc
221 MaxiSorp 96-well ELISA plates obtained from Thermo Fisher Scientific (Massachusetts, USA).
222 Subsequently, plates were incubated with 200 µl of 10% fetal calf serum purchased from Sigma-
223 Aldrich Co. LLC. (St. Louis, USA) for 1 h at RT for blocking, followed by washing 5x with
224 washing buffer (WB) consisting of 0.05% Tween-20 in phosphate buffer saline_(aq) (PBS). Next,
225 plates were incubated with 100 µl inactivated and clarified virus harvest for 2 h at RT, followed
226 by a 5x wash with WB. Then, 100 µl of the diluted mouse sera were added to the plates followed
227 by incubation for 2 h at RT and subsequent 7x washing with WB. As positive control 100 µl of
228 PBS containing 0.5 µg/ml monoclonal anti-influenza virus type A hemagglutinin antibody C102
229 purchased from HyTest Ltd. (Turku, Finland) was used; as negative control PBS was used. Next,
230 100 µl of the 1:5000 in PBS diluted anti-mouse IgG (whole molecule)-peroxidase antibody
231 purchased from Sigma-Aldrich Co. LLC (St. Louis, USA) was added and incubated for 1.5 h at
232 RT and subsequent 7x washing with WB. Finally, 100 µl of 3,3', 5,5' -tetramethylbenzidine
233 liquid substrate purchased from Sigma-Aldrich Co. LLC. (St. Louis, USA) was added. The
234 enzymatic reaction was stopped after 15 min with 50 µl of 2 M H₂SO₄ purchased from Merck
235 KGaA (Darmstadt, Germany) to measure the absorbance at a wavelength of 450 nm
236 (OD450nm) indicating antibody levels. The RSD for the ELISA was ≤ ±15% (n = 3).

***In vivo* titration of the vaccine antigen dose.** Three different doses of SCMA purified HA antigen, i.e. 1.0 μg , 7.5 μg and 15.0 μg HA PCA, were administered intraperitoneally (i.p.) into mice ($n = 3/\text{antigen dose}$) in a final volume of 100 μl PBS containing 50 μg poly I:C and 50 μg CpG as adjuvants. The first immunization (d1) was followed by booster injections after two (d14) and four weeks (d28), respectively. Sera were collected at day 13 (d13), 27 (d27), and 42 (d42) to measure the anti-A/PR antibody levels by ELISA.

Immunization with antigen-loaded MSCP. The aMSCP and the respective controls were administered i.p into the mice in a final volume of 200 μl FB containing 50 μg poly I:C and 50 μg CpG as adjuvants. The experimental setup consisted of four mice groups ($n = 5$): G1: aMSCP in FB; G2: 1 μg HA PCA in FB as positive control; G3: 1 μg HA PCA antigen in FB and empty MSCP in FB injected at two different sites of the abdomen as antigen-particle interaction control; and G4: FB only as negative control. Two weeks after the first immunization a booster injection was applied. Sera were collected at day 13 (d13) and 27 (d27) for measuring the anti-A/PR antibody levels by ELISA.

Challenge of mice with a lethal virus dose. 28 days after the first immunization and 14 days after the second immunization, respectively, the mice were infected with an MDCK cell-derived influenza A/PR virus obtained as described by Stegemann et al.[33] For this, a final volume of 25 μl A/PR diluted in PBS was administered onto the nostrils of mice. A lethal dose was examined by preceding titration studies where a loss of 25% body weight was considered as lethal [33]. Prior intranasal infection, mice were anesthetized by i.p. injection of 2% xylazin (6.8mg/kg body weight, Ceva Tiergesundheit GmbH, Düsseldorf, Germany) and 10% ketamine (90-120 mg/kg body weight, CP-Pharma, Burgdorf, Germany) in 0.9% $\text{NaCl}_{(\text{aq})}$. Mice were

weighed daily from the day of challenge until the end of the infection experiment and were sacrificed at day 6 post viral infection (p.i.) by CO₂ euthanasia.

Determination of viral load by quantitative real-time PCR. For virus quantification in the lung tissue of immunized and challenged animals, mice were sacrificed at day 6 p.i. by CO₂ inhalation followed by perfusion of the lungs with ice-cold PBS. Subsequently, lungs were carefully excised and stored until further analysis in RNAlater purchased from Qiagen (Hilden, Germany) at -20°C. RNA was isolated from whole lung homogenates using the RNeasy Mini Kit purchased from Qiagen (Hilden, Germany). DNA was digested by using RNase-Free DNase set (Qiagen, Hilden, Germany) and the RNA was eluted in 50 µl nuclease-free ultrapure water. RNA content was determined with a NanoDrop ND-1000 spectrophotometer purchased from Thermo Fisher Scientific (Massachusetts, USA). Equal amounts of RNA (1 µg) were used for cDNA synthesis in a reverse transcription reaction using a mixture of 0.25 µg oligo-dT and 1.5 µg random primers in nuclease-free ultrapure water in a total volume of 12 µl. Samples were incubated for 10 min at 70°C and placed on ice for 10 min. Afterwards, reverse transcription reaction mix was added containing 4 µl 5x first-strand buffer, 2 µl 0.1 M dithiothreitol, 1 µl 10 mM dNTP mix, and 1 µl SuperScript II Reverse Transcriptase all obtained from Invitrogen (California, USA). For the cDNA synthesis the RNA samples were incubated for 60 min at 42°C in a thermocycler (Peglab, Erlangen, Germany) and the obtained cDNA concentration was measured with the spectrophotometer. Afterwards, real-time PCR was performed in triplicate with a LightCycler 480 SYBR Green I Master reaction mix with a LightCycler 480 system both obtained from Roche Diagnostics GmbH (Mannheim, Germany) using 25 ng of cDNA/reaction. Nucleoprotein (NP) gene primers were used at a final concentration of 500 nM and an external NP gene copy standard was used for generating a standard curve. Sense primer:

5'GAGGGGTGAGAATGGACGAAAAAC-3'; anti-sense primer: 5'-
CAGGCAGGCAGGCAGGACTT-3'. The RSD for the qPCR was $\leq \pm 37\%$ (n = 3).

Statistical analysis. Results from the immunization experiments are displayed as mean $\pm$ standard error of the mean (SEM). Data were analyzed for significance with an unpaired, two-tailed student *t*-test and two-way ANOVA with Bonferroni post-test. Data evaluation was performed with GraphPad Prism v 5.04 from GraphPad Software Inc. (La Jolla, CA, USA).

RESULTS AND DISCUSSION

Establishment of the antigen dose needed to induce antiviral immunity in mice. Before probing the efficacy of the aMSCP vaccination approach we first determined the antigen dose needed for the efficient induction of antiviral immunity using a standard vaccination protocol based on the use of soluble antigen admixed to adjuvants. Immunogenicity of the HA antigen was tested by application of three different antigen concentrations: 1.0, 7.5, and 15.0 μ g HA. PCA mixed with Poly I:C and CpG were administered i.p. into mice followed by two booster injections in a two week interval, i.e. on day 14 and day 28 following primary vaccination (Figure 1A). As depicted in Figure 1B, immunization with all three antigen concentrations induced anti-A/PR antibody responses already by day 13 after application of a single antigen dose, with the highest anti-A/PR antibody response observed in mice immunized with 7.5 μ g and 15.0 μ g HA PCA. At day 42, i.e. after three immunizations, virtually no differences were observed in the level of A/PR-specific serum antibodies between the groups indicating that all three antigen doses were able to induce a potent immune response in mice (Figure 1B).

To further demonstrate that low-dose immunization would confer protection of mice against influenza A virus infection, mice immunized on d1, d14 and d28 with 1.0 μ g HA PCA were

304 infected intranasally with a lethal A/PR virus dose. Then the body weight was monitored for
305 6 days followed by subsequent estimation of the viral load in the lung tissue by measuring viral
306 NP gene copies. Body weight loss and the number of NP gene copies in lungs of immunized
307 mice were compared to those of the negative control group which was mock-immunized with
308 PBS. Strongly correlating with the presence of a robust anti-A/PR antibody response, mice
309 immunized with 1.0 μ g HA PCA showed no body weight loss ($98.65 \pm 1.06\%$) or any other
310 signs of disease after infection with a lethal A/PR virus dose indicating efficient antiviral
311 protection. In direct contrast, mock-immunized mice exhibited pronounced morbidity following
312 viral challenge with a significant decrease in body weight ($88.0 \pm 3.07\%$, $p < 0.0001$, Figure 1C)
313 already at day 4 p.i. Here, strong viral replication became evident in the lungs which displayed a
314 392-fold increase in NP gene copies with 142067 ± 93981 NP gene copies/25 ng cDNA for the
315 negative control group, compared to 363 ± 101 NP gene copies/25 ng cDNA in the PCA
316 immunized group ($p = 0.0007$, Figure 1D). These results clearly indicate the efficacy of low-dose
317 PCA vaccination and thus the dosage of 1.0 μ g HA antigen was used in the subsequent aMSCP
318 immunizations.

Figure 1. (A) Experimental scheme for the determination of the antigen concentration needed to induce an antiviral immunity in mice. (B) Anti-A/PR antibody responses determined by ELISA of 1:1000 diluted mice sera before and after immunization with 1.0, 7.5 and 15.0 µg HA (n = 3 mice/group, horizontal line expresses the mean). (C) Mice immunized three times with 1.0 µg HA or with PBS as negative control were infected with a lethal dose of influenza A/PR virus at day 42 post the first immunization. Body weight loss was monitored until day 6 post infection (p.i., n= 3-4 mice/ group, mean ± SEM, **** indicates significant differences compared to the negative control group, p < 0.0001). (D) Viral load in the lung tissue was measured at day 6 p.i. by determining viral nucleoprotein gene copies using real-time PCR (horizontal line expresses the mean, *** indicates significant differences, p = 0.0007).

Immunogenicity of antigen-loaded MSCP. Next we immunized mice with aMSCP and respective controls in a two-week interval (d1 and d14). Since we had observed before a robust anti-A/PR antibody response after two vaccinations with 1.0 μ g HA antigen (Fig. 1B) we refrained from performing a third booster immunization. One day prior injection and viral infection (d0, d13, d27), respectively, mice were bled retrobulbar and sera were analyzed by ELISA for the presence of anti-A/PR antibodies. As summarized in Figure 2B high anti-A/PR antibody concentrations were detectable in mice immunized with aMSCP (G1), the 1.0 μ g HA positive control (G2) as well as mice immunized with 1.0 μ g HA in combination with empty MSCP injected at two different sites of the abdomen as antigen-particle interaction control (G3). According to our expectation, no A/PR-specific antibodies were detectable in mock-immunized control animals (G4) (Figure 2B). The comparable strength of the antibody responses induced with the MSCP bound (aMSCP, G1) and free antigen (G2, G3) might be the consequence of desorption of bound virus particles from the aMSCP after injection. To evaluate purity differences between the aMSCP and the cPCA samples, a SDS-PAGE with subsequent silver-staining was carried out. Both samples contained the viral proteins. The cPCA sample contained high impurity levels in the high molecular weight range whereas the aMSCP sample contained higher impurity levels in the low molecular weight range (Figure 3).

Infection of mice with a lethal influenza virus dose. To correlate antibody levels with antiviral protection by aMSCP, immunized mice (G1) and respective control groups (G2-G4) were intranasally infected with a lethal dose of active A/PR virus. Health status and body weight loss were monitored over a period of six days. Already at day 3 p.i., the mock-immunized group (G4) showed a significant decrease in body weight ($93.5 \pm 0.9\%$) in comparison to the aMSCP group and all other antigen-containing control groups (G1: $101.3 \pm 1.3\%$, G2: $101.7 \pm 1.2\%$, G3:

101.4 $\pm$ 1.5%, Figure 2C). In mock-immunized mice, the body weight further declined until day 6 p.i. (G4: 76.8 $\pm$ 1.9%) and was accompanied with signs of anorexia, lethargy and ruffed fur confirming a severe influenza A/PR infection-associated disease. At the same time, all mice that were immunized with aMSCP or the respective antigen-containing controls maintained their initial body weight (G1: 100.6 $\pm$ 1.7%, G2: 103.3 $\pm$ 1.2%, G3: 103.0 $\pm$ 2.5%). In addition, there were no signs of influenza disease during the entire observation period of 6 days following the lethal challenge which is indicative for an efficient antiviral protection in these groups (Figure 2C). At day 6 p.i. all mice were sacrificed and the viral load in the lung tissue was determined by quantification of viral NP gene copies. Strikingly and well in line with the antibody levels and the overall disease course in the different experimental groups, a 408-fold higher NP gene copy number was observed in the lung tissue of the mock-immunized mice (G4: $3.8 \times 10^6 \pm 1.2 \times 10^6$ NP gene copies) compared to aMSCP-immunized mice (G1: $0.9 \times 10^4 \pm 0.2 \times 10^4$ NP gene copies; Figure 2D). Administration of 1.0 μ g HA PCA antigen (G2) and 1.0 μ g HA PCA with empty MSCP (interaction control, G3) resulted in a 138-fold (G2: $2.7 \times 10^4 \pm 1.1 \times 10^4$ NP gene copies) and 316-fold (G3: $1.2 \times 10^4 \pm 0.5 \times 10^4$ NP gene copies) decrease, respectively, when compared to the negative control group (G4, Figure 2D). Together, our results revealed that aMSCP vaccinated animals were fully protected against a lethal influenza A/PR infection. Importantly, protective antibody-mediated immunity of the aMSCP group (G1) was comparable to the positive controls (G2, G3), corroborating that the tested MSCP vaccine system represents a time saving and cost effective alternative to conventional vaccine purification and formulation processes.

Figure 2. (A) Experimental scheme for the immunization with the MSCP vaccine system. Immunization with antigen-loaded MSCP (aMSCP) conferred robust protection against a lethal A/PR infection. (B) Anti-A/PR antibody responses after immunization with aMSCP in formulation buffer (FB, G1) and respective control groups (G2: 1.0 μg positive control antigen (PCA) in FB as positive control; G3: 1.0 μg PCA in FB and empty MSCP in FB injected at two different sites of the abdomen as antigen-particle interaction control; G4: FB mock-immunized mice as negative control). Mice were bled retrobulbar at indicated time points and sera were diluted 1:1000 followed by quantification of anti-A/PR antibody levels by ELISA (n = 5 mice/group, mean ± SEM). (C) Relative body weight changes in mice after a lethal A/PR challenge 28 days post the first and 14 days post the secondary immunization (n = 4-5 mice/

group, mean $\pm$ SEM; indicated significant differences compared to the negative group: **, $p < 0.001$, ****, $p < 0.0001$). (D) Estimation of viral load at day 6 post infection by quantification of nucleoprotein gene copies in whole lung homogenates using real-time PCR (n = 4-5 mice/ group, horizontal line expresses the mean; indicated significant differences compared to the negative control group: **, $p \leq 0.002$; ***, $p \leq 0.0009$).

Figure 3. SDS-PAGE with subsequent silver staining of the cPCA and the antigen eluted from the aMSCP. The cPCA sample contained high impurity levels in the high molecular weight range, and the aMSCP sample contained higher impurity levels in the low molecular weight range. For optimal comparison of the samples, the scanned SDS-PAGE image was set to greyscale, brightness reduced by -100%, and contrast increased by +50%.

CONCLUSION

The production of an influenza A/Puerto Rico/8/34 (A/PR, H1N1) whole virus vaccine by pseudo-affinity purification and formulation with magnetic sulfated cellulose particles (MSCP) was successfully carried out. We performed the first proof-of-concept-study conclusively

400 demonstrating that the resulting antigen-loaded MSCP (aMSCP) can be directly used for
401 immunization. The immunized mice showed an efficient induction of anti-A/PR antibodies that
402 conferred full protection against a lethal A/PR virus challenge.

403 In our experimental setup a conventional DSP process was used for clarification, inactivation,
404 concentration, and diafiltration of virus harvests. Only the SCMA chromatography step, used for
405 further virus purification, was replaced by MSCP. However, optimized upstream processing and
406 harvest pretreatment could reduce significantly the overall process step number. This and the
407 easy handling of magnetic particle systems without the need of elaborate equipment and
408 intensive training could enable the usage for purifying and formulating new virus vaccine
409 candidates, in particular for veterinary applications where relatively high contamination levels
410 are accepted. In addition, the use of aMSCP could support the purification and formulation of
411 gene therapy vectors and viral vectors for disease treatment. Finally, commercially available
412 high-throughput magnetic particle handling systems could be used to allow for high-throughput
413 screening applications to speed up the development of viral vaccines and gene therapies even
414 further.

415 The MSCP consisting of cellulose with incorporated Fe_3O_4 particles were not degraded during
416 the time course of the *in vivo* experiments. Furthermore, we did not observe any adverse effects
417 in the mice that received MSCP formulations. These results are in agreement with literature on
418 the biocompatibility and nontoxicity of cellulose and Fe_3O_4 particles [10,18–21]. Nevertheless,
419 potential effects of the MSCP on hosts have to be carefully evaluated in future studies. A way to
420 potentially degrade the cellulose matrix could be a co-injection of an cellulose-disintegrating
421 enzyme mixture as shown previously for dextran microparticles [34]. Another approach would
422 be to avoid the injection of magnetic particles into the host at all. This could be implemented by

topical application of the aMSCP, i.e., on nonablative fractional laser (NAFL) pretreated skin [35], or intradermal applications, i.e., topical application of aMSCP with microneedles for transferring the antigen through the skin barrier [36,37].

In summary, the very effective induction of a protective immunity against a viral pathogen in combination with the ease of applicability, scalability and the option to bind a wide range of pathogens and antigens to the MSCP renders the outlined system an interesting tool for a variety of possible applications ranging from basic research to the development of vaccines or viral vectors. Follow up studies to optimize the system regarding the MSCP size, the amount of adjuvant added, the immunization schedule, and the route of application are currently performed.

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Author Contributions

‡ MP and SF contributed equally to this work. MP and SF carried out the experiments and wrote the manuscript; MP, SF, DB and MW conceived the study and planned the experiments; MP, SF, DB, MW and UR revised the manuscript.

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Notes

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