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Florian Krammer, Sabine Nakowitsch, Paul Messner, Dieter Palmberger,  
Boris Ferko, Reingard Grabherr

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**Swine-origin pandemic H1N1 influenza virus-like particles produced in insect cells induce hemagglutination inhibiting antibodies in BALB/c mice**

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1  
2  
3 Research Article  
4

5 **Swine-origin pandemic H1N1 influenza virus-like particles produced in insect**  
6 **cells induce hemagglutination inhibiting antibodies in BALB/c mice**  
7

8 Florian Krammer<sup>1</sup>, Sabine Nakowitsch<sup>2</sup>, Paul Messner<sup>3</sup>, Dieter Palmberger<sup>1</sup>, Boris  
9 Ferko<sup>2</sup> and Reingard Grabherr<sup>1</sup>  
10

11  
12  
13  
14  
15 <sup>1</sup>Department of Biotechnology, University of Natural Resources and Applied Life  
16 Sciences , Vienna, Austria  
17

18  
19  
20 <sup>2</sup>Avir Green Hills Biotechnology AG, Vienna, Austria  
21

22 <sup>3</sup>Department of Nanobiotechnology, University of Natural Resources and Applied  
23 Life Sciences , Vienna, Austria  
24

25  
26  
27 **Correspondence:** Dr. Reingard Grabherr, Vienna Institute of BioTechnology,  
28 University of Natural Resources and Applied Life Sciences, Muthgasse 11, 1190  
29 Vienna, Austria; check C.A. and title; block;

30  
31  
32  
33  
34 **E-mail:** Reingard.grabherr@boku.ac.at  
35

36  
37 **Keywords:** H1N1 / Influenza vaccine / Insect cells / Pandemic influenza / Virus-like  
38 particle  
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40  
41 **Abbreviations:** FCS, fetal calf serum; GFP, green fluorescent protein;  
42

43 **GMT**, geometric mean titer; **H1–H9**, HA subtypes 1–9; **HA**, hemagglutinin;  
44

45 **HAI**, HA inhibition; **HAU**, HA unit; **M1**, matrix protein; **NA**, neuraminidase;  
46

47 **RBC**, red blood cells; **rBV**, recombinant baculovirus; **VLP**, virus-like particle  
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50  
51 Recent outbreaks of influenza A highlight the importance of rapid and sufficient  
52 supply for pandemic and inter-pandemic vaccines. Classical manufacturing methods  
53 for influenza vaccines fail to satisfy this demand. Alternatively, cell culture-based  
54 production systems and virus-like particle (VLP)-based technologies have been  
55 established. We developed swine-origin pandemic H1N1 influenza VLPs consisting  
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3 of hemagglutinin (A/California/04/2009) and matrix protein. Hemagglutinin and  
4  
5 matrix protein were co-expressed in insect cells by the baculovirus expression  
6  
7 system. VLPs were harvested from infection supernatants, purified and used for  
8  
9 intraperitoneal immunization of BALB/c mice. Immunization induced high serum  
10  
11 antibody titers against A/California/04/2009 as well as hemagglutination inhibiting  
12  
13 antibodies. Additionally, we compared VLP production in two different insect cell  
14  
15 lines, Sf9 and BTI-TN5B1-4 (High Five<sup>TM</sup>). Taken together VLPs represent a  
16  
17 potential strategy for the fight against new pandemic influenza viruses.  
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## 25 **1 Introduction**

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29 In June 2009, the World Health Organization raised the pandemic level for swine-  
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31 origin H1N1 influenza A virus to phase 6. As demonstrated by this latest outbreak,  
32  
33 influenza A viruses represent a continuous pandemic threat [1–3]. Vaccination has  
34  
35 been the most effective way to prevent the disease resulting from influenza virus  
36  
37 infections. Classical egg-derived influenza vaccines can be produced in quantities  
38  
39 that are needed in an inter-pandemic seasonal influenza situation but, due to limited  
40  
41 egg supply, may be insufficient in case of a pandemic. Alternatively, cell culture-  
42  
43 based methods have been established for faster and higher yield production.  
44  
45 However, biosafety issues and difficulties with growth and yield of certain isolates  
46  
47 may cause constraints during production. Besides whole virus vaccines, subunit  
48  
49 vaccines comprising hemagglutinin (HA) and virus-like particles (VLPs) are  
50  
51 considered as alternative strategies and have been shown to successfully induce  
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53 neutralizing immune response [4–13].  
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3 Influenza A virus is a negative-sense single-stranded RNA virus belonging to the  
4  
5 *Orthomyxoviridae* family. The virion consists of a host cell-derived envelope, which  
6  
7 is lined on the inner side by the matrix protein (M1). On the surface there are  
8  
9 multiple copies of HA, which is responsible for attachment to the host cell surface  
10  
11 receptors, and neuraminidase (NA), which causes the release of new virus particles  
12  
13 from the host cell [14]. HA and NA are both glycoproteins and are the major  
14  
15 antigens of influenza A viruses. Successful prophylactic influenza vaccines have to  
16  
17 induce neutralizing antibodies that prevent HA from binding to cellular receptors.  
18  
19 Insect cell-derived VLPs have been produced previously and shown to be promising  
20  
21 candidates for vaccination [15]. VLPs are able to induce B cell-mediated immune  
22  
23 responses as well as cytotoxic T cell responses and CD4<sup>+</sup> proliferation [15]. The  
24  
25 market entry of GlaxoSmithKline's human papilloma virus vaccine (Cervarix<sup>TM</sup>)  
26  
27 finally demonstrated the potency of insect cell-derived VLP vaccines.  
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33  
34 Influenza VLPs have been expressed in various cell systems including mammalian  
35  
36 cells, plants and insect cells [4–13, 16]. Insect cell-derived influenza A VLPs have  
37  
38 been produced and studied by various investigators. Co-expression of HA, NA and  
39  
40 M1 or HA and M1, respectively, in insect cells by the baculoviral expression system  
41  
42 has been shown to lead to the formation of VLPs [7–13, 16, 17]. VLPs of HA  
43  
44 subtypes H1, H3, H5, H7 and H9 have been shown to elicit protective immune  
45  
46 responses against influenza A virus when administered intranasally, intraperitoneally  
47  
48 or intramuscularly [4, 6–12, 16].  
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53 In this study we generated VLPs as an alternative vaccine candidate for swine-origin  
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55 H1N1 pandemic influenza A virus. VLP production in two insect cell lines, Sf9 and  
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57 BTI-TN5B1-4 (High Five<sup>TM</sup>), was compared in terms of yield and purity.  
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3 Furthermore, mice were immunized with BTI-TN5B1-4-derived particles. Induction  
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5 of neutralizing antibodies and IgG levels were analyzed.  
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## 10 **2 Materials and methods**

### 11 **2.1 Cells**

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17 *Spodoptera frugiperda* Sf9 cells (ATCC CRL-1711) were maintained as adherent  
18 cultures in Roux flasks in modified insect cell medium IPL-41 [supplemented with  
19 lipid mixture (Sigma, St. Louis, USA) and yeast extract (Sigma)] and 3% fetal calf  
20 serum (FCS) at 27°C [18]. Sf9 cells dedicated to VLP production were cultivated in  
21 the same media in suspension in 500-mL shaker flasks at 100 rpm. *Trichoplusia ni*  
22 BTI-TN5B1-4 cells (ATCC CRL-10859) were maintained in shaker flasks in serum-  
23 free modified IPL-41 media at 27°C shaking at 100 rpm [18].  
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### 41 **2.2 Cloning and recombinant baculovirus generation**

42 The A/California/04/2009 HA gene (GenBank: GQ117044.1) was synthesized by  
43 Genart (Regensburg, Germany). The synthesis vector pGA was digested by the  
44 restriction endonucleases *EcoRV* and *NotI*. The hemagglutinin fragment was ligated  
45 into a *StuI* and *NotI*-digested pBacPAK8 (Clontech, CA, USA), resulting in  
46 pBacPAK8-SF.  
47  
48  
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52 The M1 gene from strain A/Udorn/307/1972 (H3N2) (GenBank: DQ508932.1) was  
53 synthesized by Sloning (Puchheim, Germany). The synthesis vector pPCR-M1 was  
54 digested by the restriction endonucleases *XhoI* and *NotI*. The M1 fragment was  
55 ligated into an *XhoI* and *NotI*-digested pBacPAK8, resulting in pBacPAK8-UM. The  
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3 green fluorescent protein (GFP) gene was ligated into an *Hind*III and *Xho*I-digested  
4  
5 pBac-5 (Merck, Darmstadt, Germany), resulting in pBac-5-GFP. Recombinant  
6  
7 baculoviruses (rBVs) were generated by cotransfection of pBacPAK8-SF,  
8  
9 pBacPAK8-UM, or pBac-5-GFP together with Baculogold *Autographa californica*  
10  
11 nuclear polyhedrosis virus (*AcNPV*) linearized DNA (PharMingen, San Diego, USA)  
12  
13 into Sf9 cells by Cellfectin (Invitrogen, Carlsbad, USA) according to the  
14  
15 manufacturer's instructions.  
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### 22 **2.3 Production, purification and characterization of VLPs**

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27 BTI-TN5B1-4 or Sf9 cells were co-infected with rBV expressing HA and M1 at a  
28  
29 multiplicity of infection of 10 and a cell density of  $1 \times 10^6$  cells/mL in 500-mL  
30  
31 shaker flasks. Cells were harvested 72 h post infection and separated from  
32  
33 supernatant by low-speed centrifugation for 10 min at  $2000 \times g$  at room temperature.  
34  
35 The supernatant was tested for VLP content by hemagglutination assay and for  
36  
37 baculovirus titer by plaque assay on Sf9 cells [19]. VLPs were pelleted by  
38  
39 ultracentrifugation at  $136,000 \times g$  at  $20^\circ\text{C}$  for 90 min. Pellets were resuspended in  
40  
41 PBS for 1 h at room temperature. VLPs were further purified by discontinuous  
42  
43 sucrose density gradient centrifugation (20, 30, 40, 50, 60%) at  $190\,000 \times g$  at  $4^\circ\text{C}$   
44  
45 for 16 h. Gradients were split into fractions, which were analyzed by SDS-PAGE,  
46  
47 Western blot and protein quantification with Bradford reagent (Bio-Rad, Hercules,  
48  
49 USA). The two fractions containing most of the VLP material were diluted with PBS  
50  
51 and were pelleted by ultracentrifugation at  $4^\circ\text{C}$  at  $136\,000 \times g$  for 90 min. The pellets  
52  
53 were resuspended in SPGN buffer (6% sucrose, 3.8 mM  $\text{KH}_2\text{PO}_4$ , 7.2 mM  $\text{K}_2\text{HPO}_4$ ,  
54  
55 4.9 mM L-glutamic acid, 75 mM NaCl, pH 7.5) and tested for VLP content by  
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3 hemagglutination assay and for baculovirus titer by plaque assay on Sf9 cells. An  
4  
5 rBV expressing GFP as negative control for animal testing was produced by three-  
6  
7 step ultracentrifugation from similar gradient fractions as for VLPs.  
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#### 10 11 12 **2.4 SDS-PAGE and blots**

13  
14  
15  
16  
17 Sucrose gradient fractions, infection supernatants and vaccination preparation were  
18  
19 examined by SDS-PAGE and Coomassie staining [20]. Western Blot analysis was  
20  
21 performed using anti-M1 (Institute of Virology, Bratislava) and anti-H1 antibodies  
22  
23 (Institute of Virology, Bratislava) and a secondary anti-mouse IgG alkaline  
24  
25 phosphatase-labeled goat antibody (Sigma).  
26  
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#### 31 32 **2.5 Transmission electron microscopy**

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35  
36 Sucrose gradient fractions were used for electron microscopy. VLPs were adsorbed  
37  
38 for 1 min on copper grids directly from sucrose gradient fraction 7 and washed with  
39  
40 PBS containing 1% BSA. Negative staining was performed using uranyl acetate  
41  
42 (pH 4.5) [21]. Samples were examined on a transmission electron microscope at  
43  
44 various magnifications.  
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#### 50 51 **2.6 Hemagglutination assay**

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54  
55 VLPs were serially twofold diluted in PBS and incubated at room temperature for 2 h  
56  
57 with 50  $\mu$ L 1.25% human red blood cells (RBC, blood group 0). The  
58  
59 hemagglutination extent was estimated visually. The hemagglutination titer  
60



1  
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3 designated as hemagglutination units (HAU) was determined by the reciprocal of the  
4  
5 highest dilution without complete sedimentation of RBC.  
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## 10 **2.7 Animals and vaccination**

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13  
14  
15 BTI-TN5B1-4-derived influenza VLPs were pooled from sucrose gradient fractions,  
16  
17 diluted in PBS and pelleted by ultracentrifugation at  $136\,000 \times g$  at  $4^{\circ}\text{C}$ . Pellets were  
18  
19 resuspended in SPGN buffer (6% sucrose, 3.8 mM  $\text{KH}_2\text{PO}_4$ , 7.2 mM  $\text{K}_2\text{HPO}_4$ ,  
20  
21 4.9 mM L-glutamic acid, 75 mM NaCl, pH 7.5).  
22  
23

24  
25 For immunization, 6-week-old female BALB/c mice were used. Mice (three to four  
26  
27 mice per group) were vaccinated with purified VLPs (0.25  $\mu\text{g}$  HA), with GFP-  
28  
29 expressing rBV ( $\sim 100$  ng, the same amount as the baculovirus background found in  
30  
31 the VLP preparation) and with SPGN, respectively, via intraperitoneal injection at  
32  
33 week 0. Mice were boosted on day 25 with VLPs carrying total 3  $\mu\text{g}$  HA, whereas  
34  
35 the negative control animals received baculovirus preparation or SPGN. On days 0,  
36  
37 25 and 47, blood was collected from anesthetized mice via the orbit and transferred  
38  
39 to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at  $-$   
40  
41  $20^{\circ}\text{C}$ . Immunological tests in mice were approved by the Austrian Federal Ministry  
42  
43 of Science and Research according to Austrian law (ZI. 200910116).  
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## 50 **2.8 Evaluation of humoral immune response**

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55 A/California/04/2009-specific serum antibodies were determined by ELISA. The 96-  
56  
57 well microtiter plates (Nunc-Immuno Plate Maxisorp) were coated with 2  $\mu\text{g}/\text{mL}$   
58  
59 A/California/07/2009 standard (NIBSC, London, UK) in coating buffer for 2 h at  
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1  
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3 room temperature. Plates were blocked with PBS containing 3% BSA and 0.005%  
4  
5 Tween 20 (room temperature, 2 h) and incubated with serial twofold dilutions of  
6  
7 each 1:3200 pre-diluted serum samples (room temperature, 2 h). Afterwards, plates  
8  
9 were washed with PBS containing 0.005% Tween 20 and incubated with peroxidase-  
10  
11 conjugated anti-mouse IgG antibody (Sigma, A3673) in PBS containing 1% BSA  
12  
13 and 0.005% Tween 20 (room temperature, 1 h). Unbound antibody was removed and  
14  
15 plates were washed. Samples were incubated with substrate (*o*-phenylenediamine  
16  
17 and H<sub>2</sub>O<sub>2</sub>), reaction was stopped using H<sub>2</sub>SO<sub>4</sub> and the colorimetric change was  
18  
19 measured at 492 nm.  
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## 27 **2.9 Hemagglutination inhibition assay**

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31 Sera of immunized mice were treated with receptor-destroying enzyme (Szabo  
32  
33 Scandic, Vienna, Austria) overnight at 37°C and heat-inactivated at 56°C for 30 min  
34  
35 [22]. Serial twofold dilutions were prepared in a 96-well microtiter plate and 25 µL  
36  
37 containing 4 HAU antigen A/Texas/5/2009 re-assortant (IDCDC-RG15 CDC)- or  
38  
39 A/California/04/2009-presenting VLPs, respectively, was added. After 30 min of  
40  
41 incubation 50 µL 0.5% chicken erythrocytes solution (derived from adult chicken  
42  
43 blood) was added. The assay was incubated for 2 h at +4°C and hemagglutination  
44  
45 extent was estimated visually. The hemagglutination inhibition (HAI) titration end  
46  
47 point was determined by the highest dilution of antiserum that still inhibits complete  
48  
49 hemagglutination of chicken erythrocytes. The presented value is the reciprocal of  
50  
51 the last serum dilution inhibiting virus hemagglutination. For a better comparison of  
52  
53 the two antigens (re-assortant virus and VLPs) geometric mean titers (GMT) of all  
54  
55 analyzed groups were calculated.  
56  
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### 3 Results

#### 3.1 Production and characterization of influenza A/California/04/2009 VLPs in two different insect cell lines

VLPs were generated by co-infection of Sf9 or BTI-TN5B1-4 cells with HA- (A/California/04/2009) and M1- (A/Udorn/307/1972) expressing baculoviruses. VLPs were detected and quantified in the supernatant by hemagglutination assay, while the concentration of baculoviruses was measured by plaque assay. Baculovirus titers were  $\sim 1 \times 10^8$  PFU/mL in Sf9 supernatants and  $\sim 1 \times 10^6$  PFU/mL in BTI-TN5B1-4 supernatants (Table 1). Sf9 and BTI-TN5B1-4 supernatants showed a biological activity of 16 HAU/50  $\mu$ L, although BTI-TN5B1-4 supernatants contained a sevenfold higher HA concentration when analyzed by Western blotting (Table 1). Thus, a total higher content of HA protein was detected in BTI-TN5B1-4-derived VLPs, in spite of the same hemagglutination titer as for Sf9-derived VLPs. ((Table 1))

For investigating the size and shape of the virions, VLPs produced in both cell systems were purified and compared with respect to their density in a sucrose gradient. VLPs were harvested from supernatants by ultracentrifugation, resuspended pellets were further purified by sucrose density gradient centrifugation and different fractions were analyzed by Western blot analysis. As shown in Fig. 1, the HA protein was detected as an approximately 70-kDa protein and the M1 protein was detected as a 29-kDa protein. In the VLP preparation derived from the BTI-TN5B1-4 cell culture supernatant (Fig. 1), M1 was found in fractions 4–11 and in the pellet. The highest concentrations of HA and therefore of VLPs were found in fraction 7 and 8 (~40–

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3 45% sucrose) in the BTI-TN5B1-4 preparation and in fraction 6 and 7 (~35–40%  
4  
5  
6 sucrose) in the Sf9 preparation (data not shown). To confirm the structure of insect  
7  
8 cell-derived VLPs, electron microscopy was performed. Particles from fraction 7 of  
9  
10 both purifications were stained with uranyl acetate (pH 4.5) as described and used for  
11  
12 transmission electron microscopy (Fig. 2) [21]. Both cell lines produced spheric and  
13  
14 partially polymorphic VLPs of 80–120-nm diameter, which is consistent with the  
15  
16 morphology of wild-type H1 influenza virions. VLPs containing HAs from various  
17  
18 strains were produced in Sf9 cells, and all fractions of the sucrose gradient showed a  
19  
20 similar picture (80–120 nm in diameter) when analyzed by electron microscopy (data  
21  
22 not shown).  
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27 ((Figs. 1, 2))  
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### 31 **3.2 Analysis of immune response**

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36 Immunogenicity of VLPs was examined in BALB/c mice (6 weeks) by  
37  
38 intraperitoneal injection, at days 0 and 25, of purified antigen (VLPs carrying total  
39  
40 0.25 and 3  $\mu$ g HA, four animals), of purified GFP-expressing rBV (~100 ng, four  
41  
42 animals) and of SPGN (sucrose-phosphate-glutamine-NaCl buffer; three animals).  
43  
44 Pre-immune sera and post-vaccination antisera 25 days after the first vaccination and  
45  
46 22 days after the boost were evaluated for the induction of specific immune response  
47  
48 by ELISA and by HAI assay. Serum antibodies with the ability to prevent virus-  
49  
50 induced agglutination of chicken RBCs are a main indicator for vaccine-induced  
51  
52 immune response and consequently for the efficiency of vaccines. Four out of four  
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54 mice sero-converted after the first immunization and responses significantly  
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56 increased after boosting. As can be seen in Fig. 3, sera from pre-immunized mice and  
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3 from the negative controls showed no immune response, whereas sera from mice  
4  
5 single immunized with VLPs (0.25  $\mu$ g HA) showed a GMT of 181 (titers between  
6  
7 128 and 256 HAI units) with A/Texas/5/2009 re-assortant as antigen, and a GMT of  
8  
9 45 (titers between 32 and 64 HAI units) with A/California/04/2009 VLPs used as  
10  
11 antigen (Fig. 3). After the second immunization with VLPs carrying a total of 3  $\mu$ g  
12  
13 HA, the GMT titers with both antigens increased to 2048 HAI units (Fig. 3). IgG  
14  
15 levels against A/California/04/2009 were tested by IgG ELISA. ELISA titers of  
16  
17 1:3200 pre-diluted sera (absorbance at 492 nm) ranged from 0.027 to 0.098 in  
18  
19 immunized mice, whereas sera from animals from both negative controls showed  
20  
21 only background reactivity ( $-0.004$  to  $0.001$ ). After boosting, a significant rise of  
22  
23 titers from immunized mice could be observed. Titers reached values between 0.215  
24  
25 and 0.439; both negative controls showed again only background reactivity ( $-0.004$   
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27 to  $0.002$ ) (Fig. 4).

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33 ((Figs. 3, 4))

#### 34 35 36 37 38 39 **4 Discussion**

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43 This report describes the production and the immunogenicity of swine-origin  
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45 pandemic H1N1 A/California/04/2009 HA-containing influenza A VLPs. VLPs  
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47 produced in the two cell lines Sf9 and in BTI-TN5B1-4 (High Five<sup>TM</sup>) were  
48  
49 compared. Supernatants from both cell lines showed a similar and constant biological  
50  
51 activity of 16 HAU/50  $\mu$ L, but BTI-TN5B1-4 supernatants contained a sevenfold  
52  
53 higher HA concentration when analyzed by Western blot analysis (Table 1).  
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57 Additional analysis by electron microscopy and by gradient centrifugation showed  
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59 that VLPs from both cell lines had a similar size and shape; however, the main  
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3 portion of BTI-TN5B1-4-derived VLPs accumulated at a higher density during  
4 centrifugation. We assume that two preparations of similar VLPs accumulating at  
5  
6 different densities have different numbers of HA molecules integrated in their  
7  
8 membranes. This fact could explain the higher amount of HA protein found in the  
9  
10 BTI-TN5B1-4 cell culture supernatant.  
11  
12

13  
14 Another main difference between both production platforms was the concentration of  
15  
16 baculoviruses in the production supernatants. Sf9 supernatants contained  
17  
18 approximately  $\sim 1 \times 10^8$  PFU baculovirus/mL, whereas BTI-TN5B1-4 supernatants  
19  
20 contained  $\sim 1 \times 10^6$  PFU/mL. The presence of baculoviruses and hence DNA  
21  
22 contamination is considered a major problem for insect cell-derived influenza VLP  
23  
24 vaccine production [11]. Baculovirus has been shown to transduce a variety of  
25  
26 mammalian cells very efficiently and *in vivo* provokes expression of various kinds of  
27  
28 cytokines, which may lead to an un-intended adjuvant effect as well as to unwanted  
29  
30 inflammatory reactions [23, 24]. Additionally, a theoretical risk of integration of  
31  
32 baculovirus DNA into the cellular genome of the vaccinee and consequential DNA  
33  
34 damage cannot be ruled out [23]. So far, generation of insect cell-derived influenza  
35  
36 VLP vaccines have usually been carried out in Sf9 cells. In terms of downstream  
37  
38 processing, it has to be noted that Sf9 cells were cultivated in the presence of FCS,  
39  
40 while BTI-TN5B1-4 cells are easily cultivated in serum-free media. We consider the  
41  
42 establishment of BTI-TN5B1-4 cells an advantageous production system, as it  
43  
44 decreases the protein background (FCS) as well as the baculovirus background and,  
45  
46 hence, DNA contamination. Additionally, Hi-5 Rix4446 cells, which are derived  
47  
48 from a BTI-TN5B1-4 cell subclone, are used as production cell line for the  
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50 production of a human papilloma virus vaccine (Cervarix<sup>TM</sup>). Cervarix<sup>TM</sup> was  
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3 approved by the European Medicines Agency (EMA). Consequently, our further  
4 immunological studies were performed with BTI-TN5B1-4 cell-derived VLPs.  
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6 Fractions of homogeneous consistency were achieved using purification of the  
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8 influenza VLPs by sucrose gradient centrifugation (Fig. 1). The calculated mass of  
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10 A/California/04/2009 HA was 63 kDa. Nevertheless, the HA band in the blot appears  
11  
12 slightly above 70 kDa, which leads to the conclusion that the HA is glycosylated.  
13  
14 Insect cells perform complex glycosylation but in a slightly different manner than  
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16 mammalian cells. Insect cells are not able to add sialic acid or galactose to their  
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18 terminal *N*-glycan structures; generally they show a paucimannose type of  
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20 glycosylation [25]. In the case of BTI-TN5B1-4 cells some galactosylation activity  
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22 has been shown previously [26]. Nevertheless, a negative effect of the different  
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24 glycosylation pattern found in insect cells, as compared to mammalian cells, on the  
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26 immunogenicity of the recombinant HA in the *in vivo* assay has not yet been  
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28 reported.  
29  
30 Neutralizing antibodies against the influenza HA are the main indicator for protective  
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32 immunity against infection. We demonstrated that vaccination with  
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34 A/California/04/2009 VLPs lead to seroconversion of four out of four tested mice.  
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36 HAI assays showed high titers of neutralizing antibodies in all four vaccinated mice.  
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38 Both negative control groups showed no or just background antibody activity.  
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40 Hemagglutination inhibition assay was carried out with two different antigens, an  
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42 A/Texas/5/2009 H1N1 re-assortant and A/California/04/2009 HA presenting VLPs.  
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44 At lower titers the hemagglutination inhibition assay seems to have a higher  
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46 sensitivity with the A/Texas/5/2009 re-assortant as antigen. On the other hand the use  
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48 of VLPs as antigen in the hemagglutination assay has advantages from the safety  
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50 point of view. In general, ELISA data on anti-HA IgG reflects the results from HAI  
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3 assay (Fig. 4). Influenza A VLPs have been produced in insect cells (Sf9) displaying  
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5 various subtypes of HA before [7, 9, 11, 27]. Although, different experimental  
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7 settings were applied, in all cases IgG levels in mice increased after boosting. The  
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9 HAI titers shown in our study compare to reports by others. For example, Pushko *et*  
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11 *al.* [11] showed HAI titers in the range 320–640 after a second boosting, while Ross  
12  
13 *et al.* [9] were able to induce HAI titers up to 2048. Virus neutralization assay and  
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15 challenge studies will give additional and definite information about protective  
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17 immunity induced by A/California/04/2009 VLPs in mice; however, for the scope of  
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19 this first study we considered fast production and proof of principle as the main  
20  
21 goals. As a first promising step towards an alternative pandemic influenza vaccine  
22  
23 strategy, we demonstrated that A/California/04/2009 VLPs were able to induce high  
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25 levels of hemagglutination inhibiting antibodies and a high anti-HA serum IgG level  
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27 in absence of adjuvants.  
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32 Not only efficacy, but also time is a very critical factor in vaccine production,  
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34 especially in a pandemic situation. It took us 51 days from beginning of the cloning  
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36 work to the production of the first VLP batch for mouse tests. Taken into account  
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38 that gene synthesis of a 1700-bp gene will take approximately 2 weeks, the vaccine  
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40 production for a new influenza subtype could be started within less than 10 weeks  
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42 after first isolation of the RNA sequence of a new strain. VLP vaccines circumvent  
43  
44 problems like slow growth of isolates and therefore unpredictable yields, mutation of  
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46 HA through host adaption and the need for biosafety level (BSL)-3 facilities, which  
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48 are obligatory in cell culture-based production of inactivated pandemic influenza  
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50 vaccines. VLP vaccines could be produced in any standard-equipped good  
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52 manufacturing practice BSL-1 laboratory with an additional ultracentrifuge, a  
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54 laminar flow hood and a shaker or a wave bag device. Insect cell-derived influenza  
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VLPs not only represent a very promising candidate for inter-pandemic annual influenza vaccine, they are also a very fast, safe and effective alternative vaccine approach especially for newly emerging pandemic influenza strains like swine-origin H1N1 or avian H5N1.

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27 **Figure 1.** Western blot of BTI-TN5B1-4 cell culture-derived A/California/04/2009  
28 VLPs in a discontinuous sucrose density gradient (20–60% sucrose). The blot was  
29 developed using anti-M1 and anti-H1 mAbs and an alkaline phosphatase-conjugated  
30 anti-mouse IgG mAb, respectively. Lane 1 is the top fractions with the lowest  
31 sucrose concentration, lane 11 harbors the highest sucrose concentration, P indicates  
32 the pellet. Positions of HA and M1 are indicated.  
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41 **Figure 2.** Transmission electron micrographs of negatively stained  
42 A/California/04/2009 VLPs derived from BTI-TN5B1-4 cells (A) and Sf9 cells (B).  
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45 **Figure 3.** HAI titers. Day 0, 25 (post-immunized) and 47 (22 days post-boost) serum  
46 HAI antibody responses were assessed against an H1N1 A/Texas/5/2009 re-assortant  
47 (A) and H1N1 A/California/04/2009-presenting VLPs (B). Bars indicate GMT.  
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51 Negative controls (neg. contr.) and baculovirus controls (BV) showed only  
52 background levels at all time points, whereas A/California/04/2009 VLPs (VLP)  
53 raised serum antibodies able to prevent virus-induced agglutination of chicken RBCs.  
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60 Antibody levels increased to 2048 HAI units after boosting.

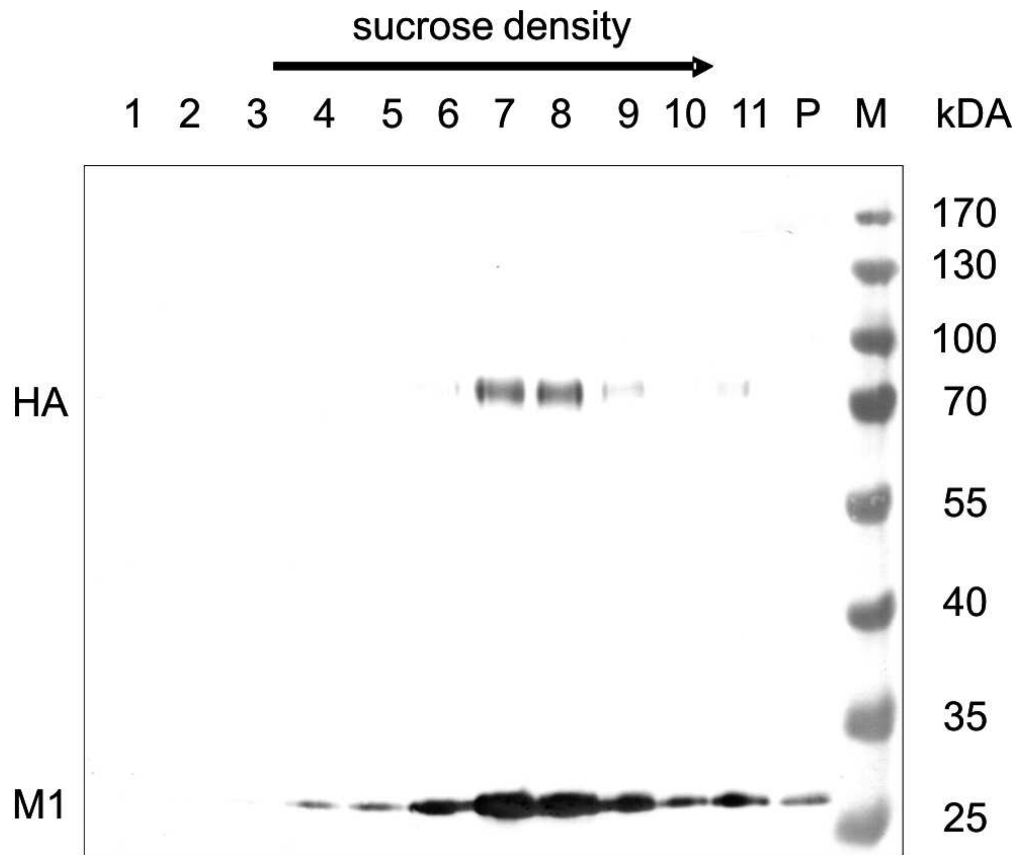
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3 **Figure 4.** Influenza HA-specific total serum IgG responses. Day 0, 25 (post-  
4 immunized) and 47 (22 days post-boost) serum IgG antibody responses were  
5 assessed by ELISA against H1N1 A/California/07/2009 standard antigen (NIBSC).  
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7 Sera were 1:3200 pre-diluted. Negative controls (neg. contr.) and baculovirus  
8 controls (BV) showed only background levels of specific anti-HA IgG at all time  
9 points, whereas A/California/04/2009 VLPs (VLP) raised high levels of specific anti-  
10 HA antibodies. Absorbances were read at 492 nm and results are expressed as  
11 arithmetic mean titer.  
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**Table 1.** Comparison between Sf9 and BTI-TN5B1-4 cells as production platforms for influenza A VLPs

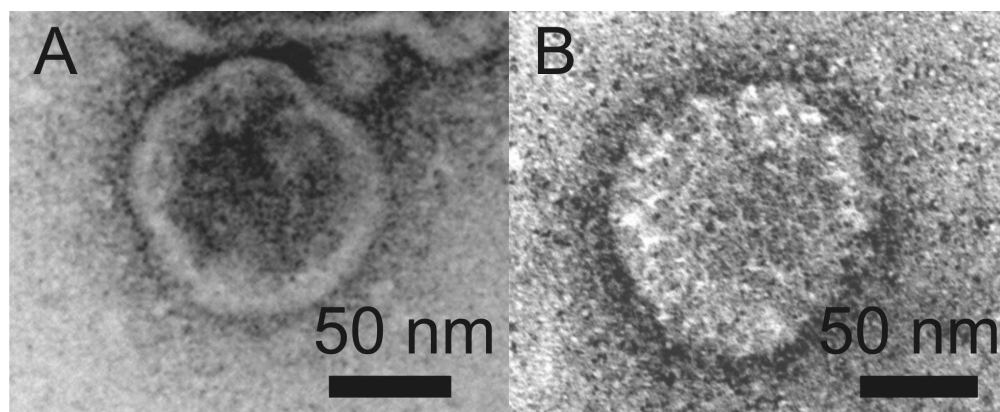
	<b>Sf9</b>	<b>BTI-TN5B1-4</b>
<b>HAU/mL</b>	320	320
<b>HA protein yield in <math>\mu\text{g/mL}</math></b>	0.4	3
<b>Baculovirus titer PFU/mL</b>	$\sim 10^8$	$\sim 10^6$

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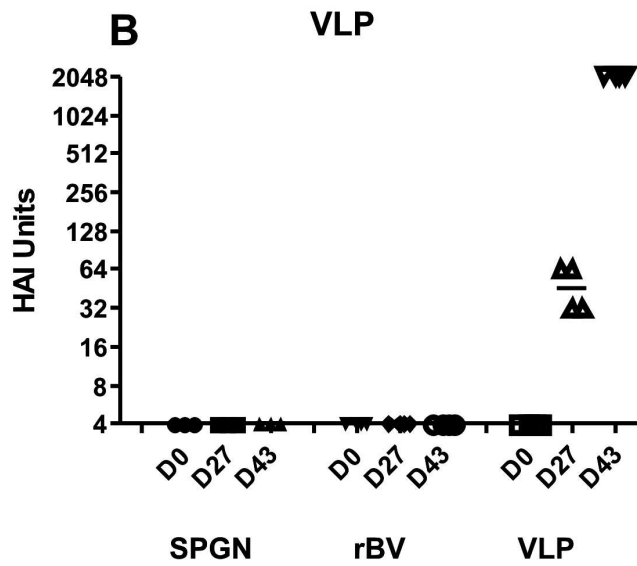
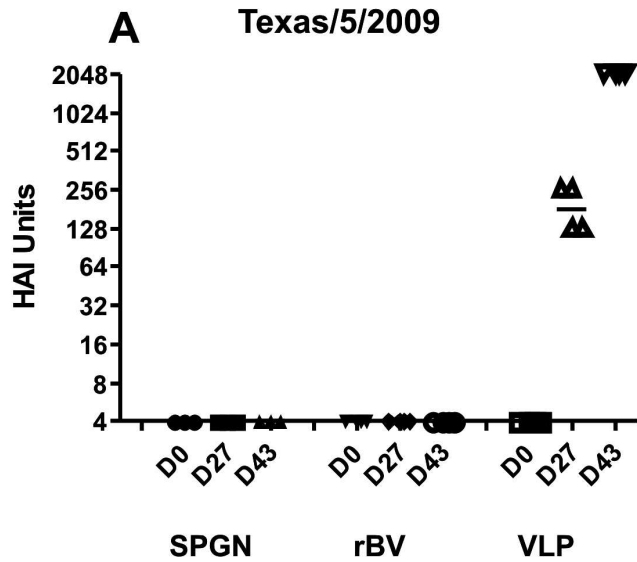


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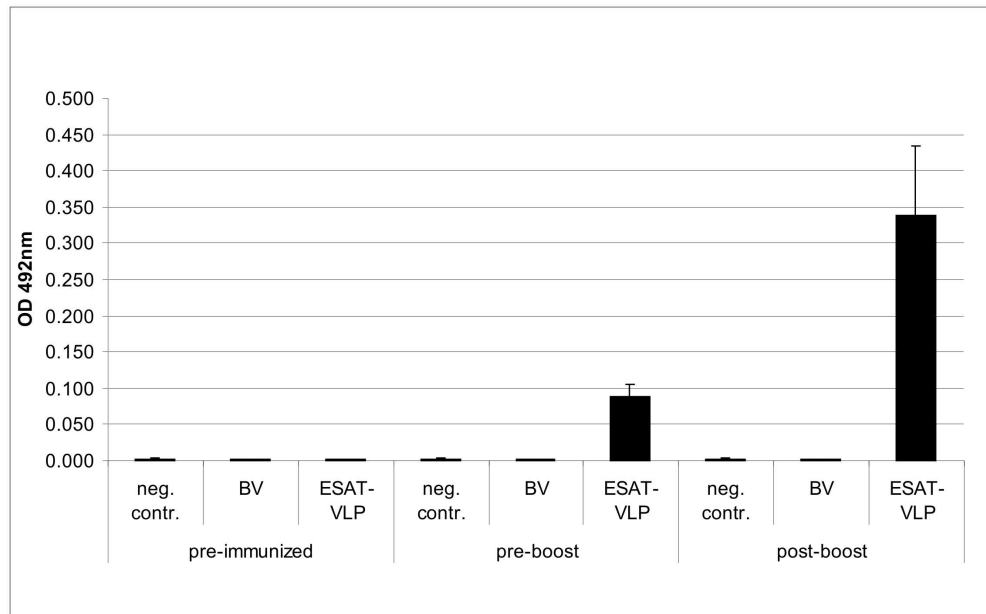
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