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# Subunit vaccines based on recombinant yeast protect against influenza A virus in a one-shot vaccination scheme

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ABSTRACT

Here we report on new subunit vaccines based on recombinant yeast of the type *Kluyveromyces lactis* (*K. lactis*), which protect mice from a lethal influenza A virus infection. Applying a genetic system that enables the rapid generation of transgenic yeast, we have developed *K. lactis* strains that express the influenza A virus hemagglutinin, HA, either individually or in combination with the viral M1 matrix protein. Subcutaneous application of the inactivated, but otherwise non-processed yeast material shows a complete protection of BALB/c mice in prime/boost and even one-shot/single dose vaccination schemes against a subsequent, lethal challenge with the cognate influenza virus. The yeast vaccines induce titers of neutralizing antibodies that are readily comparable to those induced by an inactivated virus vaccine. These data suggest that HA and M1 are produced with a high antigenicity in the yeast cells. Based on these findings, multivalent, DIVA-capable, yeast-based subunit vaccines may be developed as promising alternatives to conventional virus-based anti-flu vaccines for veterinary applications.

## 1. Introduction

Influenza A viruses (IAV) are among the most important respiratory pathogens worldwide, infecting a wide range of host species and posing a major threat to human and animal health [1]. In humans, the highly contagious virus leads to annual seasonal epidemics, which may result in up to 600,000 deaths worldwide [2]. In poultry, periodic outbreaks of avian influenza cause serious damage every year, with an estimated economic loss of around US $20 billion [1]. In swine, influenza is one of the most frequently reported infectious diseases and also causes severe economic losses [3,4].

IAV belong to the *Orthomyxoviridae* family of enveloped viruses containing a single-stranded, eight-segmented RNA genome of negative polarity [5]. Segmentation favors the generation of reassortant viruses with novel genetic and biological characteristics that may trigger pandemic spread [6–8]. Due to the rapid change of circulating viruses through antigenic drift, caused primarily by error-prone replication, or antigenic shift, caused by re-assortment, the development of effective vaccines is a considerable challenge [9].

Conventional influenza vaccines, such as those for domestic poultry and swine, contain inactivated representative influenza viruses [10,11] that are mostly amplified in fertilized chicken eggs or vertebrate cell lines [12]. These vaccines have several limitations including an elaborate, time-consuming production, which can lead to unsatisfactory efficacy due to a suboptimal match between selected vaccine strains and the circulating viruses. Other disadvantages involve the dependence on fertilized chicken eggs [13] and the risks associated with cell culture production [14,15]. This situation indicates that there is a crucial need for alternative flu vaccines, which should be safe and efficacious, but also rapid and inexpensive to manufacture and to apply. Other requirements include rapid adaptation to emerging virus strains and, specifically for veterinary vaccines, the potential to differentiate between vaccinated and non-vaccinated animals (DIVA).

Here, we describe a novel type of subunit vaccine that can be generated rapidly. The vaccine is based on a transgenic *Kluyveromyces lactis* (*K. lactis*) yeast that is safe, efficacious, and inexpensive to manufacture.

## 2. Results

### 2.1. Generation and characterization of a K. lactis strain expressing hemagglutinin

We applied genetically modified strains of the yeast *Kluyveromyces lactis* (*K. lactis*) as a vaccination platform, which, in rapid

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one-step procedures, enable stable integration of one or more foreign genes at defined loci in the yeast genome (SFig. 1). Effective gene expression is mediated by the strong *LAC4* promoter and can be induced by the addition of lactose to the growth medium [16]. Previous approaches have shown that subcutaneous vaccination with unprocessed, inactivated *K. lactis*, containing a heterologously expressed viral subunit, successfully protects target animals against infections by the cognate viral pathogen [17].

Initially, we applied the *K. lactis* strain VAK367-D4, which permits the defined insertion of a gene of interest (*GOI*) into the *LAC4* locus (SFig. 1). To generate a *K. lactis*-based influenza vaccine, we employed the highly immunogenic antigen hemagglutinin (HA), which has been already successfully applied in multiple subunit vaccination approaches [18–24]. The HA envelope protein, which forms a trimeric complex on the viral membrane, interacts with the cellular receptor sialic acid and is the major driver of the IAV fusion entry process. HA is synthesized as HA0 precursor and later on processed into HA1 and HA2 [5]. Specifically, we applied the *HA* gene of the human IAV A/PR/8/34 (designated here as $HA_{PR8}$). A/PR/8/34 is a well characterized example of the influenza virus H1N1 type that is widespread in birds, swine and humans. It was chosen as a model because it causes lethal respiratory disease in laboratory mice but shows no virulence in humans [25,26].

Following the procedure shown in SFig. 1, the $HA_{PR8}$ gene was recombined into VAK367-D4. Recombinant strains were selected and, after confirmation of genomic gene integration, expression of the $\mathrm{HA}_{\mathrm{PR8}}$ protein [27] was evaluated after lactose induction by western-blot. Several resulting *K. lactis* strains, including one, VAK952, which was further investigated, expressed high amounts of an 80 kDa protein that was undetectable in the lysate of the parental (wild-type, wt) VAK367 strain (Fig. 1**A**). This suggested that the recombinant yeasts expressed the $\mathrm{HA}_{\mathrm{PR8}}$ protein, but mainly as unprocessed HA0 precursor. Note that most of the recombinant protein remained insoluble in yeast pellet fractions (Fig. 1**A**). This was expected due to the known membrane localization of HA. In fact, immunofluorescence analysis of VAK952 protoplasts showed that the heterologously expressed $\mathrm{HA}_{\mathrm{PR8}}$ protein was mainly located in intracellular membranes and in the plasma membrane (SFig. 2).

During its synthesis in infected mammalian cells, the HA is glycosylated, and glycosylation was suggested to be an important immunogenic determinant [28,29]. To test for *N*-glycosylation of the expressed hemagglutinin, we subjected the whole cell lysate of VAK952 to digestion with peptide-*N*-glycosidase F (PNGase F). Interestingly, following PNGase F treatment, the molecular weight of the expressed $\mathrm{HA}_{\mathrm{PR8}}$ protein decreased from ~80 kDa to about 63 kDa corresponding to the calculated molecular size of the non-glycosylated HA0 (Fig. 1**B**). Hence, we concluded that the *K. lactis* expressed $\mathrm{HA}_{\mathrm{PR8}}$ precursor is *N*-glycosylated.

When we analyzed the growth behavior of VAK952 (see supplementary data and SFig. 3), the generation time of the recombinant strain was determined to be approximately 1 h longer than that of the wt yeast VAK367 (Fig. 1**C**). Interestingly, VAK952 started show-

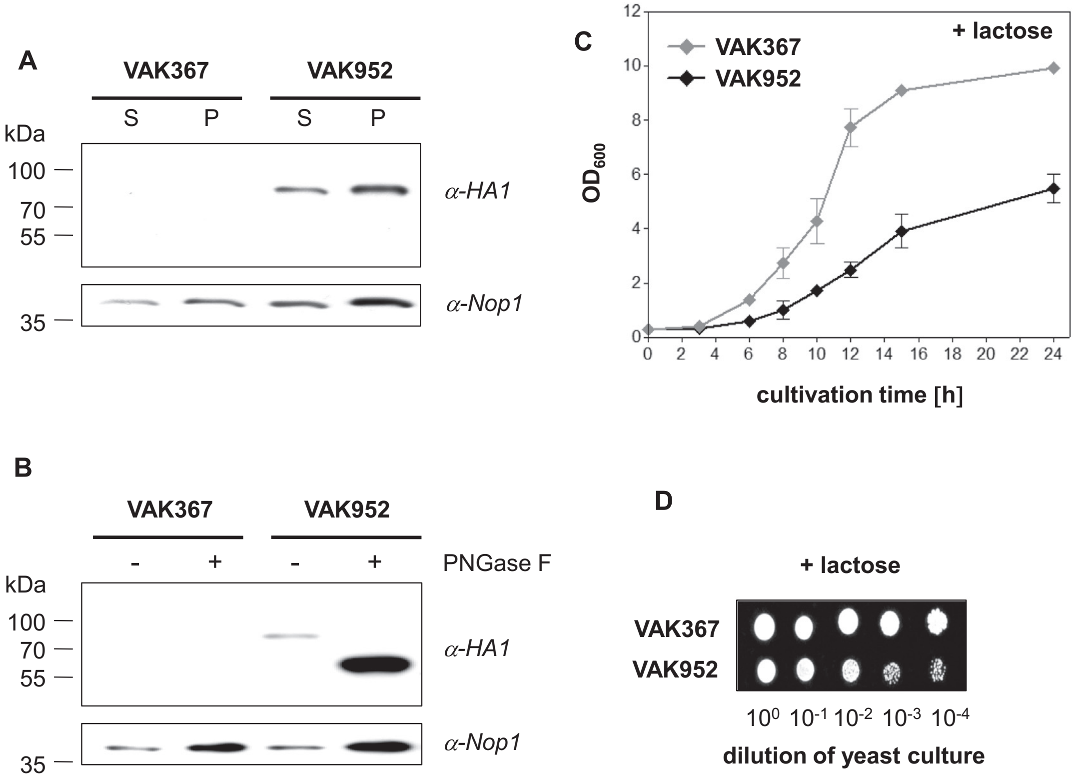


**Fig. 1. Characterization of the recombinant yeast strain VAK952.** (**A**) Tests for the presence of expressed hemagglutinin antigen. Strains VAK367 and VAK952 were grown following the standard protocol. 30 OD units of the liquid culture were lysed and 20 µg of yeast total protein of the soluble (S) and insoluble (P) fractions analyzed for the expression of the $\mathrm{HA}_{\mathrm{PR8}}$ antigen applying western-blot with a monoclonal antibody (CM1-1) directed against the 55 kDa HA1 domain. The house-keeping protein Nop1 was stained as a loading control. (**B**) Glycosylation of the recombinant $\mathrm{HA}_{\mathrm{PR8}}$-protein. Yeast strains VAK367 and VAK952 were grown in shaking flasks in YPD medium, shifted to YPL medium and induced for 10 h. 0.5 OD units of yeast material were harvested by pelleting, the pellet heat-denatured and incubated overnight at 37 °C with 500 U PNGase F. Subsequently, 0.125 OD units were analyzed by western-blot (see **A**). Note that for unknown reasons it was found that the blot signal was increased when samples were previously treated with PNGaseF. (**C**) Growth in culture. Yeast strains VAK367 and VAK952 were cultivated in shaking flasks with YPD medium, shifted to YPL medium and grown for 24 h. At the indicated time points, the $\mathrm{OD}_{600}$ was measured. The given data were averaged from at least three independent experiments. (**D**) Spot test. VAK367 and VAK952 were grown on YPD agar plates for 48 h at 30 °C. Starting with 1 OD unit, the yeast strains were serially diluted, dropped onto YPD/YPL agar plates and cultured for 48–72 h at 30 °C.

ing a significant reduction in the growth rate at 3 h post-induction, the time when $HA_{PR8}$ expression was first detectable (SFig. 3), which suggested that the accumulation of HA slows down yeast growth. Supporting this idea, we noticed a similar phenotype when we grew the yeast cells on rich medium agar plates: *i.e.*, on plates that contained the inducer lactose as carbon source, VAK952 showed a clear growth defect compared to VAK367 (Fig. 1**D**).

To estimate the amount of HA0 expressed by the VAK952 cells at 12 h post induction, *i.e.*, the time at which protein expression was maximal (SFig. 3), we prepared inactivated VAK952 cell material following a downstream processing protocol that included freeze-drying and heat inactivation (in the following determined as the "standard protocol"; see Materials and methods). After cell lysis, we analyzed the soluble and insoluble yeast cell fractions *via* western-blot in parallel with a protein standard consisting of the purified $HA_{PR8}$ ectodomain (aa 1–528). Thus, we determined a quantity of $0.5 \pm 0.02$ µg of expressed $HA_{PR8}$ per milligram of freeze dried yeast VAK952 (data not shown).

Taken together, in the recombinant *K. lactis* strain VAK952, which carried the IAV *HA* gene, expression of the HA was stable and reproducible over the time of cultivation. By choosing appropriate growth conditions, the VAK952 yeast could be grown without major restrictions, despite an apparent growth defect caused by the expression of the antigen. The protein was expressed as glycosylated precursor HA0.

### *2.2. Immunization with the K. lactis-based subunit vaccine VAK952 protects mice against a lethal IAV infection*

Next, we tested the capacity of the strain VAK952 to be used as a vaccine. VAK952 yeast induced for 12 h was prepared by the standard protocol and the $HA_{PR8}$ protein confirmed to be intact and to be synthesized at a quantity that was comparable to the earlier determined value of ca. 0.5 µg of protein per milligram of yeast (not shown). The heat-killed, powdered yeast was re-suspended in phosphate-buffered saline and the adjuvant AddaVax$^{TM}$ and was directly, *i.e.*, without further treatment, subcutaneously applied to standard laboratory BALB/c mice. AddaVax$^{TM}$ was known to be well tolerated by mice and to heighten significantly the immune response [30].

In the immunization scheme shown in Fig. 2, we vaccinated mice with 1, 2 or 5 mg of VAK952 and performed two subsequent boosts at 2-weeks-intervals with identical amounts of yeast. In two negative controls, the mice received either buffer or a 1:1 emulsion consisting of wt yeast (VAK367, 5 mg) and AddaVax$^{TM}$. Moreover, we evaluated the efficacy and quality of the serum antibody response induced by VAK952 in comparison with a vaccine control where mice were immunized with inactivated A/PR/8/34 virus. The induction and titers attained of neutralizing antibodies (nAb) were determined at defined time points of the vaccination scheme in a serum neutralization test (SNT).

As expected, no nAb reacting against A/PR/8/34 were detected in the sera of the mice that were treated with buffer or with the wt yeast. In contrast, nearly all other mice showed a seroconversion (Fig. 2). Interestingly, the measured seroconversion varied in close correlation with the amount of yeast vaccine used. When 1 mg of VAK952 was applied, 3 of 5 of the vaccinated mice developed nAb with titers of 5–9 $\log_2$. With 2 mg of VAK952, 8 of 10 mice showed titers that varied between 5 and 13 $\log_2$, and with 5 mg of applied VAK952, all tested mice revealed a seroconversion with titers of 9–15 $\log_2$. Importantly, the efficacy of the immune response that was induced by the application of 5 mg of VAK952

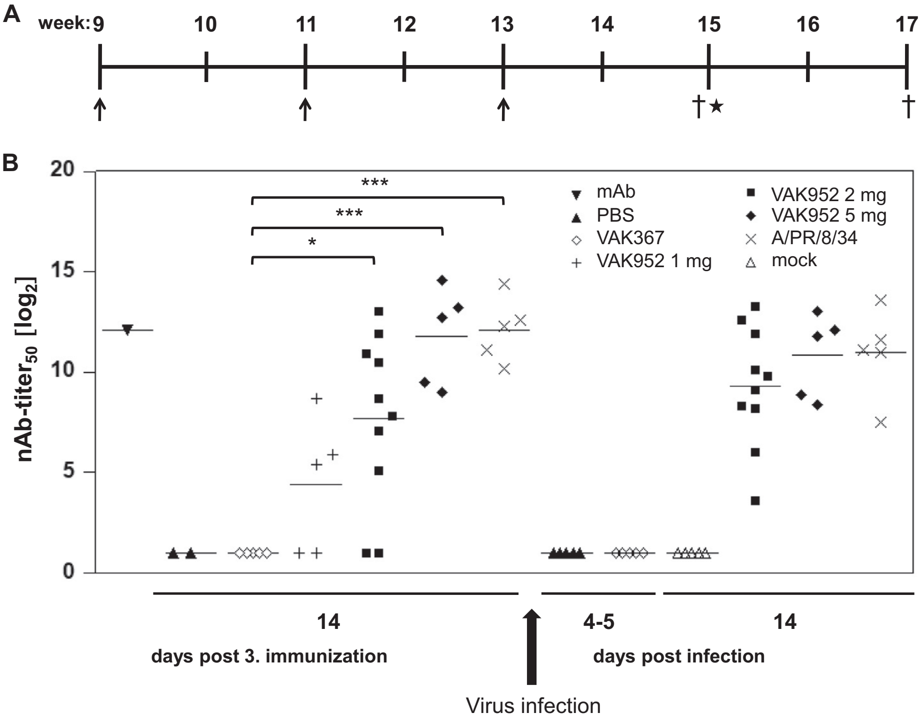


**Fig. 2. Antibody titers obtained after immunization and challenge of mice.** Yeast strains were pre-cultured in YPD, shifted to YPL and induced for 12 h. The cultures were prepared following the standard protocol, mixed with AddaVax$^{TM}$ and administered subcutaneously (arrows). Nine-weeks-old female BALB/c mice were immunized three times at two-week intervals (**A**) using VAK952 at the indicated concentrations, 5 mg of VAK367 and 10 µg (prime) and 5 µg (boost) of the vaccine control (formalin-inactivated A/PR/8/349), respectively. An additional group was immunized with PBS only. Two weeks after the last application, the animals were intra-nasally infected with the 5 × $MLD_{50}$ of influenza virus A/PR/8/34 (H1N1) (asterisk). At the indicated 4–5 and 14 days post infection († in **A**) and during the observation period (at day 14 after the 3. immunization), the sera of the animals were sampled and the virus-neutralizing antibody titers (nAb-titer$_{50}$) determined (**B**). To study significant differences between groups, the Student's *t*-test was used. Due to the logarithmic scale, serum samples without detectable antibodies were assigned the value $\log_2(2) = 1$. *: $0.01 < p \leq 0.05$; ***: $p < 0.001$; monoclonal antibody (mAb): assay control (anti-H1 (H37-66)); mock, animals mock-infected with PBS.

was similar to that obtained with 20 μg of whole inactivated virus vaccine (10–14 $\log_2$) (Fig. 2). These findings were confirmed in an independent vaccination experiment, in which we applied exclusively the high dose of 5 mg of VAK952 (SFig. 4). Herein, we compared also the reactivity-profile of the antibodies that were induced by vaccination with VAK952 or the virus vaccine control (Fig. 3). The obtained data showed that both, the VAK952 subunit vaccine and the inactivated A/PR8/34 virus, induced antibodies that reacted with the HA0 precursor and/or with the HA1 or HA2 hemagglutinin subunits (Fig. 3).

Since the application of 2 and 5 mg of VAK952 led to an effective induction of a humoral immune response, we decided to challenge mice vaccinated in this way (see Fig. 2) with infectious virus. Two weeks after the final boost, the mice were infected intranasally with the 5-fold $MLD_{50}$ of A/PR/8/34 (H1N1) and monitored for 14 days with respect to survival, body weight and clinical symptoms. In addition, we treated a group of mice solely by intranasal application of buffer (mock). As shown in Fig. 4, all mice of the negative control groups developed marked symptoms and reached the experimental endpoint (loss of body weight over 20%) within 4–5 days post infection. Accordingly, the challenge infection led to the induction of the disease, which in all non-vaccinated individuals was finally lethal. None of the control mice developed nAb (Fig. 2). In contrast, we observed that the nAb titers of all vaccinated mice were stable over the entire observation period of 14 days post infection (Fig. 2). In fact, the individuals that had been vaccinated with the full virus as well as those that had been vaccinated with either 2 mg or 5 mg of VAK952 were fully protected: all these mice survived the challenge with the cognate virus, and none developed symptoms (Fig. 4).

To further substantiate these data, we determined the viral load and lung etiology of animals vaccinated with the yeast vaccine. Hence, we vaccinated groups of mice with VAK952 (2 or 5 mg) and the A/PR/8/34 vaccine control, challenged with 5-fold $MLD_{50}$ of A/PR/8/34 and analyzed for viral titers in lung homogenates prepared four days post infection. Moreover, we calculated the lung index (ratio of organ to the final body weight) to monitor for potential pathological changes in the course of the immunization process. Following virus challenge, the lung tissue of the buffer- and VAK367-vaccinated mice displayed, as expected, high titers in the range of $10^6$–$10^7$ PFU of A/PR/8/34 per g tissue (Fig. 5). All mice of these groups showed an increased lung index and macroscopically visible pathological changes in the tissue such as lesions and pulmonary edema, both of which are recognizable signs of an influenza (Fig. 5**B**, and data not shown). While this was most obvious with the buffer-treated mice, the virus titers as well as the lung indices were slightly lower with the VAK367 wt yeast-treated animals. In contrast, the lungs of all mice that had been immunized with 5 mg VAK952 or with the vaccine control contained only spurious amounts of infectious virus, and the lung indices were inconspicuous (Fig. 5). In the experiments in which we vaccinated with the lower VAK952 dose (2 mg), we detected a minimal virus titer of 10 PFU per g of lung tissue in 2 out of 10 mice. However, as with the high dose VAK952-treated animals, the lungs of all the low dose-treated animals showed no apparent pathological abnormalities.

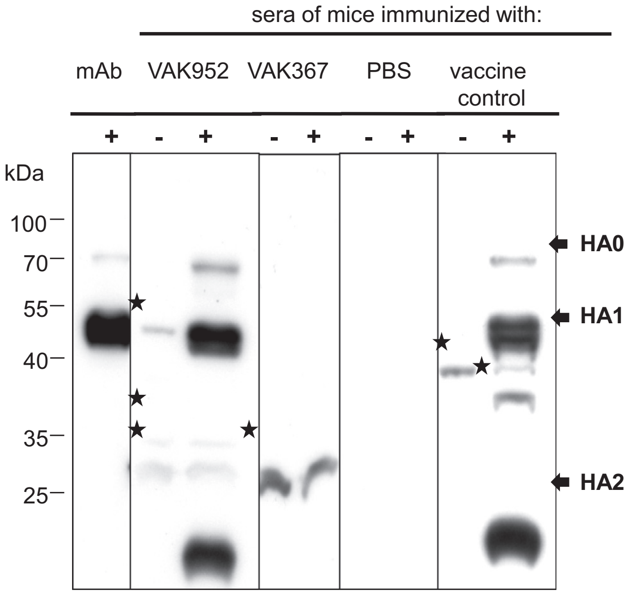


**Fig. 3. Detection of HA-specific antibodies in mice vaccinated with VAK952.** In a vaccination trial with VAK952, which was carried out in a similar way as the experiment shown in Fig. 2 but applied only the high dose of 5 mg of VAK952 (SFig. 4), the sera of the mice were taken at week 15 and examined for HA-specific antibodies using Western blot. Total protein samples of cytoplasmic extracts (10 μg per lane) of influenza virus A/PR/8/34 infected (+) or PBS mock-infected (−) MDCK cells were used as antigen sources and separated on a 10% SDS gel. Protein detection was performed with the murine antisera (dilution 1:8000) or anti-HA1 (CM1-1, mAb). $HA_{PR8}$-specific signals are marked by arrows, non-specific signals are marked by stars.

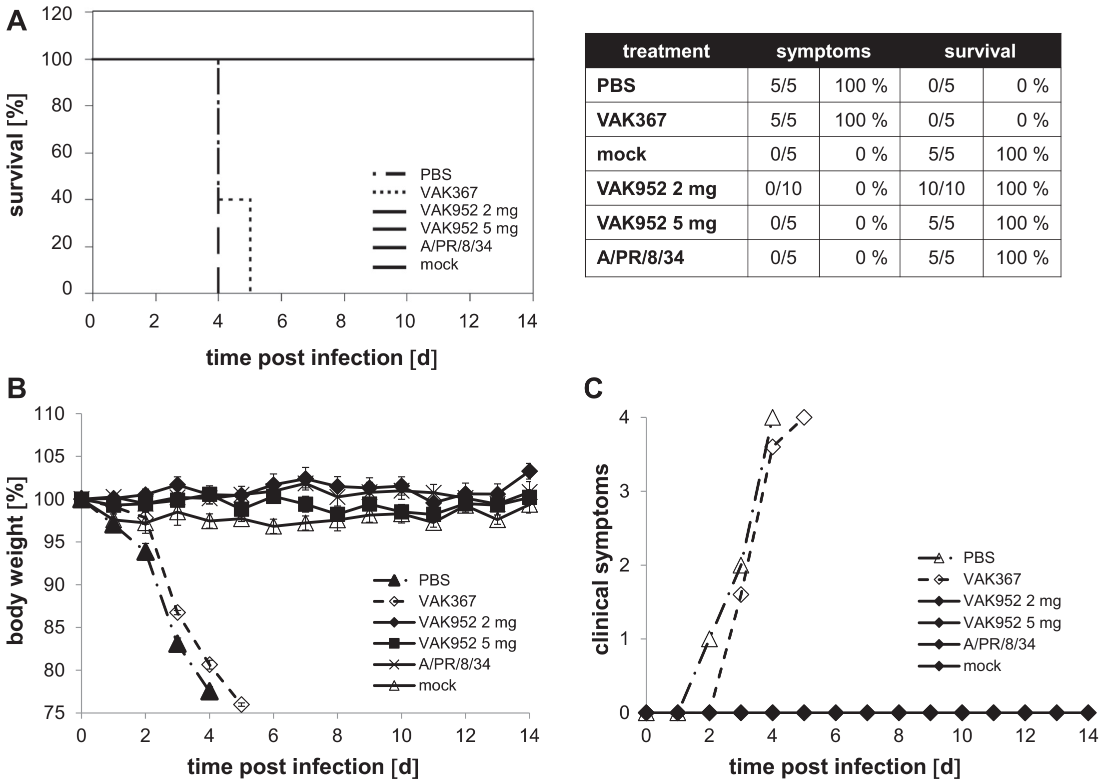
These data revealed an evident dose-dependency of a humoral protective response induced by the *K. lactis*-based VAK952 vaccine towards a lethal IAV infection. Our findings suggested 2 mg of VAK952 (containing ca. 1 μg of $HA_{PR8}$) to represent the minimal amount of VAK952 that had to be given to BALB/c mice to achieve a full protection against a subsequent infection and to attain a memory that enables a nearly complete virus clearance. In fact, when we challenged mice that were vaccinated with solely 1 mg of VAK952, we found the protection to be incomplete (not shown).

### *2.3. Generation and characterization of a K. lactis vaccine expressing the IAV hemagglutinin and the matrix protein 1*

While the subcutaneous injection of 5 or 2 mg of HA-expressing VAK952 per application were protective (see above), we asked whether it is possible to further improve the immunogenicity of the *K. lactis*-based vaccine. This was particularly important with regard to the objective of significantly simplifying the complex vaccination plan down from three to two or even one injection(s) (Fig. 2). For this purpose, we decided to insert a second *GOI* into a second, defined locus of the yeast genome and to co-express the $HA_{PR8}$ protein together with the A/PR/8/34 matrix protein 1 ($M1_{PR8}$). M1 was chosen because other studies suggested that co-expression of HA and M1 increases immunogenicity [31–34].

For the generation of a *K. lactis* strain that is capable of expressing two genes transgenically, we took advantage of the fact that strain VAK367-D4 displays an uracil auxotrophy, which is caused by a frame-shift mutation in the *KLURA3* gene. Thus, we developed a novel integration vector, KIpURA3, where the expression cassette of the *GOI*, which, in turn, is under the control of the *LAC4* promoter, is organized such that it enables integration upstream of the *KlURA3* gene (SFig. 1). The wild-type sequence of *KlURA3* thus is restored, and recombinant strains can be obtained by selection for uracil prototrophy.

Applying this procedure, we generated a strain, designated VAK1283, which permanently contains the $M1_{PR8}$ and $HA_{PR8}$ genes. Protein expression was verified, *i.e.*, after lactose induction we could detect the 80 kDa ($HA_{PR8}$) and the 28 kDa ($M1_{PR8}$) in the pellet and supernatant fractions of VAK1283 cell lysates. Compared to the HA protein, M1 was found to be more enriched in the supernatant (Fig. 6**A**). Interestingly, the amount of $HA_{PR8}$ protein, which was detectable in VAK1283, was comparable to that found in VAK952. In line with the fact that M1 is a cytoplasmic protein [35,36], we obtained no evidence suggesting a glycosylation of M1.



| treatment | symptoms | | survival | |
|---|---|---|---|---|
| **PBS** | 5/5 | 100 % | 0/5 | 0 % |
| **VAK367** | 5/5 | 100 % | 0/5 | 0 % |
| **mock** | 0/5 | 0 % | 5/5 | 100 % |
| **VAK952 2 mg** | 0/10 | 0 % | 10/10 | 100 % |
| **VAK952 5 mg** | 0/5 | 0 % | 5/5 | 100 % |
| **A/PR/8/34** | 0/5 | 0 % | 5/5 | 100 % |

**Fig. 4. Symptoms and survival rates after virus infection.** Following infection, the indicated groups of mice were examined twice daily for a period of 14 days for (**A**) survival (given as the percentage of survivors/individuals showing symptoms of the total number of tested animals; summarized in the table), (**B**) body weight (original weight at day 0 set as 100%; variation of weight of representative animals of a vaccination group shown over time), and (**C**) clinical symptoms. Clinical symptoms were scored with 0–4 and the values averaged for each group (0: no abnormalities; 1: slightly rough fur, weight loss >5% <10%; 2: rough fur, reduced activity, weight loss 10–15%; 3: rough fur, loss of movement, hunched posture, weight loss of 15–20%; 4: rough fur, lack of movement, hunched posture, weight loss >20%). Mock: animals, mock-infected with PBS; vaccine control: inactivated A/PR/8/34.

Studies on the synthesis kinetics of the recombinant proteins in VAK1283 revealed that both the $HA_{PR8}$ and the $M1_{PR8}$ protein were first detectable at 3 h and up to a period of 15 h post induction (Fig. 6**B**). Concentration maxima for $HA_{PR8}$ and $M1_{PR8}$ were reached after 6 h (Fig. 6**B**). Despite the expression of an additional foreign protein, VAK1283 showed a slightly faster growth rate than VAK952 (Fig. 6**C**, D), which was explained by the fact that VAK1283 is an uracil prototrophic strain. Indeed, when we compared other uracil auxotrophic and prototrophic strains, we generally observed a growth advantage of the prototrophic strains (not shown).

Our approach thus yielded a *K. lactis* strain that expressed two different IAV antigens. Interestingly, the co-expression of $M1_{PR8}$ neither affected the accumulation of $HA_{PR8}$ in VAK1283 nor had a negative effect on the strain's growth behavior.

### *2.4. Protection of mice against IAV by K. lactis based vaccines in a one shot immunization scheme*

In the last test series, we applied VAK952 and VAK1283 to parallel vaccination programs. These were significantly simplified as compared to the previous experiments (Fig. 2 and SFig. 4). Along with the appropriate negative controls (see above), we immunized BALB/c mice with 2 mg of the heat-inactivated yeasts by using a classical prime-boost scheme, *i.e.*, following a first application, the animals were boosted after three weeks with the same amount of yeast vaccine. Alternatively, we applied a one-shot protocol, in which we immunized only once with 2 mg of VAK952 and VAK1283 or with 1 mg of VAK1283 (Fig. 7). Three (prime-boost) or six (one shot) weeks after the final immunization, we collected the sera and tested for nAb.

The SNT showed that after prime-boost immunization with VAK952, 4 out of 5 mice developed neutralizing antibody titers of 4–11 $\log_2$, while with VAK1283, 3 out of 5 mice developed titers of 9–11 $\log_2$. Following one shot immunization, all 5 mice that were immunized with VAK952 developed titers that were in the range of 2–12 $\log_2$; with VAK1283, 4 out of 5 mice were seropositive with titers of 5–14 $\log_2$. After one shot with 1 mg VAK1283, 3 out of 5 mice generated titers of 8–9 $\log_2$ (Fig. 7). Thus, both immunization schemes with both types of yeast vaccines led to the induction of considerable titers of nAb. The obtained titers were generally slightly higher with VAK1283; however, this was not statistically significant considering the small number of mice tested. Remarkably, even the one-shot immunization scheme with only 1 mg of VAK1283 yielded substantial titers of nAb (Fig. 7).

To assay for protection, we challenged all mice following the earlier described protocol (Fig. 8). Interestingly, we observed that each of the mice that had been vaccinated with 2 mg of VAK1283 by either the prime boost or the one-shot scheme were fully protected against the subsequent, lethal virus infection. With 1 mg VAK1283 applied in the one-shot scheme, one animal showed clinical signs of an infection and had to be euthanatized after 6 days. The same was observed with one animal that was subjected to the prime boost scheme with 2 mg of VAK952 (Fig. 8). Three mice that were vaccinated once with 2 mg of VAK952 showed a slight weight loss (>5% < 10%) on day 5–8 after challenge (data not shown). In contrast, the mice that were immunized once with 2 mg VAK1283 showed no remarkable body weight loss.

While both vaccines were highly protective, these data suggested a lower level of immunogenicity of VAK952 in comparison to VAK1283. This idea was further supported by the observation that in the VAK952-treated mice, after challenge, the measured

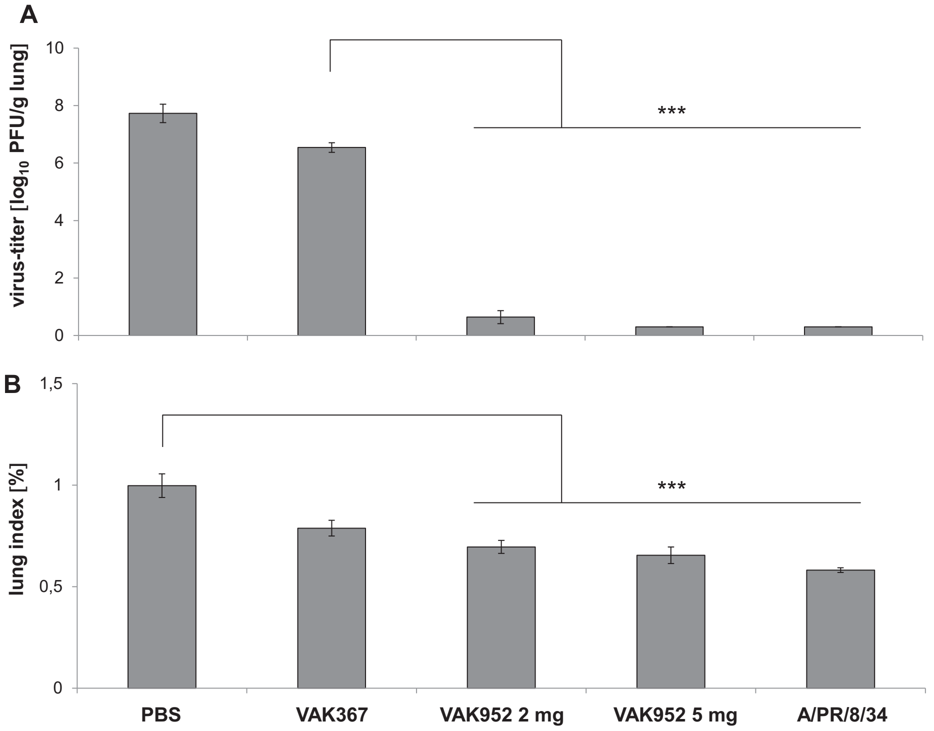


**Fig. 5. Viral load in lung tissue of immunized mice, four days after infection.** (**A**) Determination of virus titers. Immunization and viral infection were performed as described, and four days post infection, the mice were euthanized and the lungs removed. The lungs were weighted, disrupted and the tissue supernatant analyzed by plaque assay to determine the virus titer per g lung tissue. (**B**) Determination of lung index. Due to the logarithmic plot, samples in which no virus could be detected by plaque formation were assigned the value of $\log_{10}(2) = 0.3$. Shown are the means of 5–10 mice. To study significant differences, the Student's *t*-test was used. ***: $p < 0.001$.

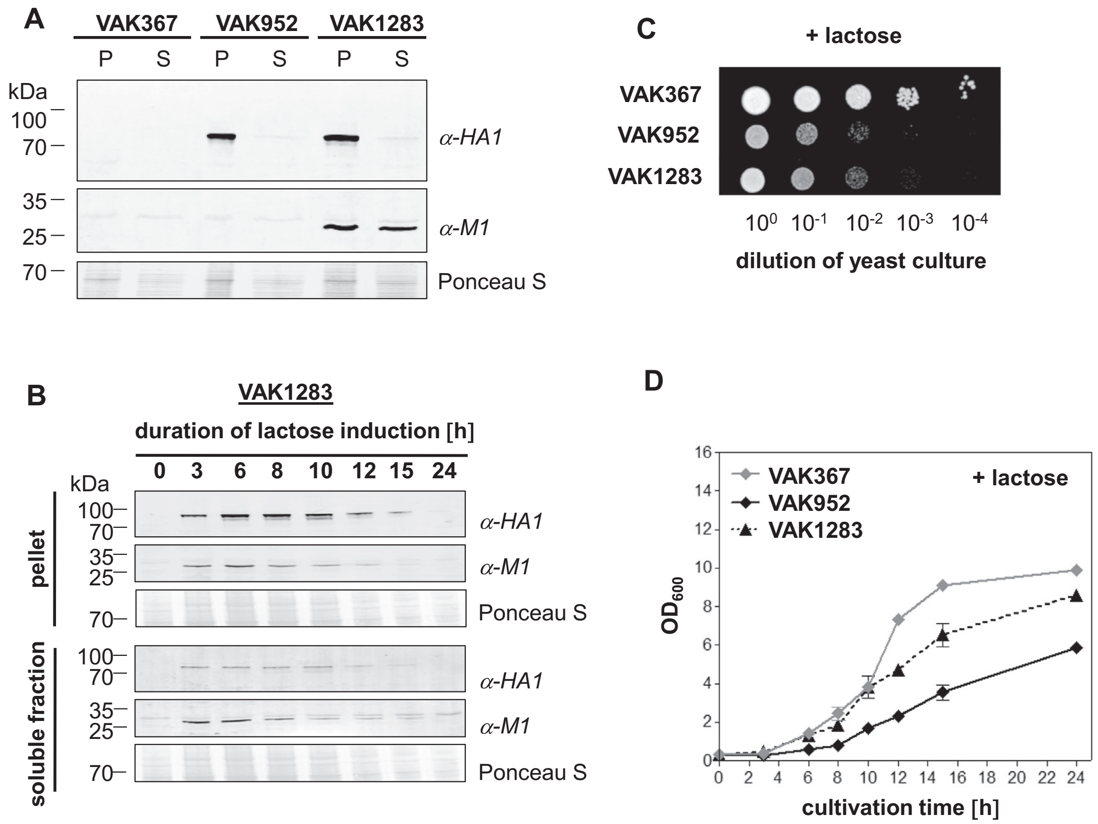


**Fig. 6. Characterization of VAK1283.** The VAK1283 strain was tested in parallel with the wt VAK367 and the VAK952 strains for (**A**) antigen expression, (**B**) expression of $HA_{PR8}$ and $M1_{PR8}$ monitored at different (indicated) times of growth, (**C**) growth by spot test, (**D**) growth in culture flasks as described in Fig. 1.

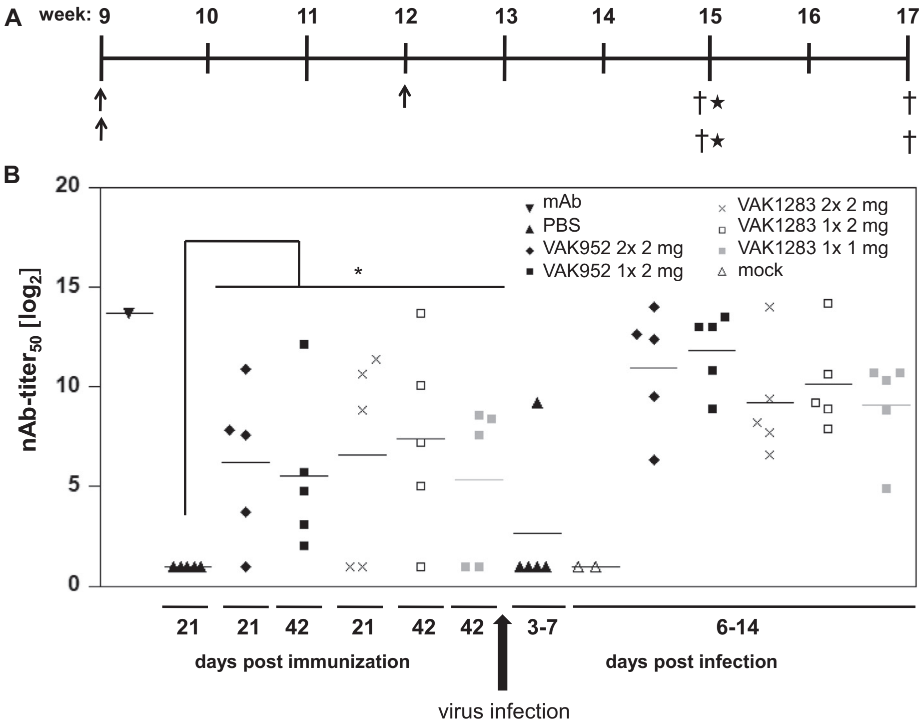
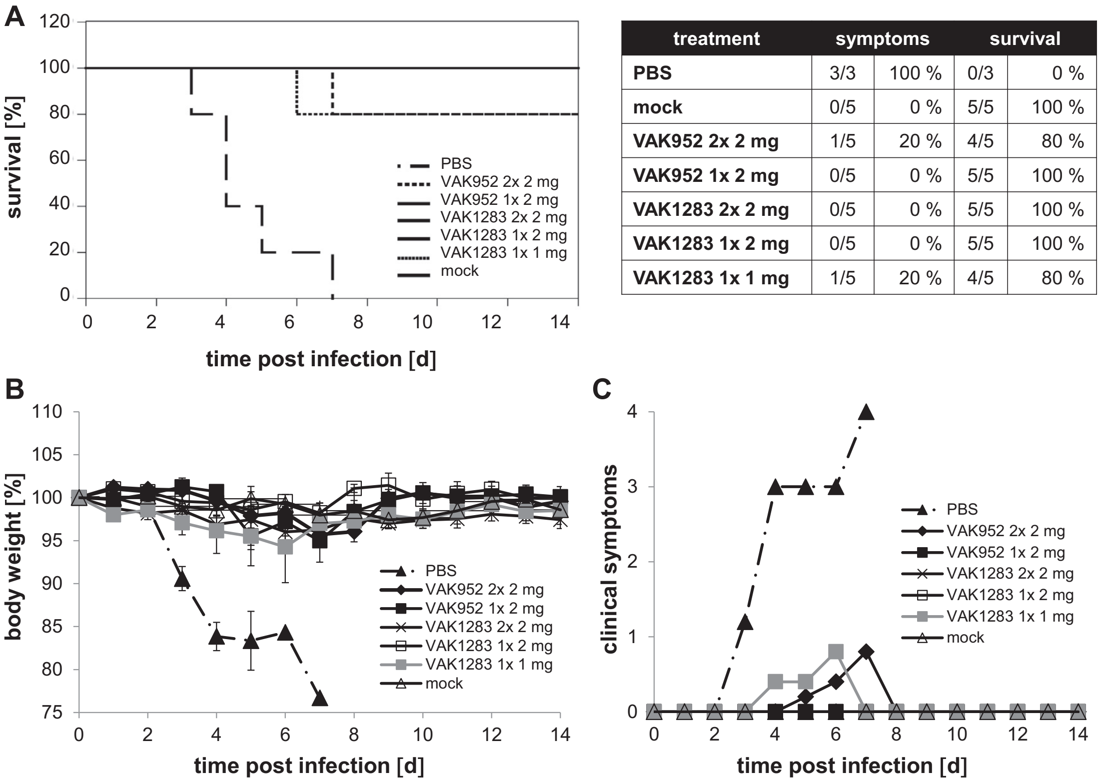


**Fig. 7. Antibody titers obtained after immunization and challenge of mice.** The applied yeast strains were produced according to the standard protocol (induction of protein expression: VA952-12 h, VAK1283-6 h) and administered subcutaneously as described in the text and Fig. 2 to nine week-old female BALB/c mice using two immunization schemes (prime/boost) and one-shot/single-dose (**A**) (arrows). Three or six weeks after the last application, the animals were infected intra-nasally with the 5 × $MLD_{50}$ of influenza A/PR/8/34 (H1N1), indicated by an asterisk. Mock, animals mock-infected with PBS. At the indicated 4–5 and 14 days post infection († in **A**) and during the observation period (at day 14 after the 3. immunization), the sera of the animals were sampled and the nAb-$titer_{50}$ determined as described above (**B**). To study differences between groups, the Student's *t*-test was used (see also Fig. 2). *: $0.01 < p \leq 0.05$; mAb: assay control (anti-H1 (H37-66)).



| treatment | symptoms | | survival | |
|---|---|---|---|---|
| **PBS** | 3/3 | 100 % | 0/3 | 0 % |
| **mock** | 0/5 | 0 % | 5/5 | 100 % |
| **VAK952 2x 2 mg** | 1/5 | 20 % | 4/5 | 80 % |
| **VAK952 1x 2 mg** | 0/5 | 0 % | 5/5 | 100 % |
| **VAK1283 2x 2 mg** | 0/5 | 0 % | 5/5 | 100 % |
| **VAK1283 1x 2 mg** | 0/5 | 0 % | 5/5 | 100 % |
| **VAK1283 1x 1 mg** | 1/5 | 20 % | 4/5 | 80 % |

**Fig. 8. Symptoms and survival rates after virus infection.** After infection with the 5×$MLD_{50}$ of influenza A/PR/8/34 (H1N1), mice were examined for (**A**) survival (given as the percentage of survivors/individuals showing symptoms of the total number of tested animals; summarized in the table), (**B**) body weight (original weight at day 0 set as 100%; variation of weight of representative animals of a vaccination group shown over time), and (**C**) clinical symptoms twice daily for a period of 14 days. Clinical signs were scored using scores of 0–4 (see above). Mock, animals mock-infected with PBS; vaccine control, inactivated A/PR/8/34.

antibody titers continued to increase in a more pronounced manner than was the case with the VAK1283-treated animals. Note that a stronger increase of the antibody level after challenge points to a lower level of memory cell-generated nAb after vaccination.

In conclusion, these experiments showed that the generated *K. lactis*-based subunit vaccines are capable of protecting mice in prime boost and even one-shot vaccination schemes against a massive, lethal IAV infection. In addition, these data suggested a higher efficacy of the HA plus M1 vaccine in inducing a humoral immune response than the HA vaccine.

## 3. Discussion

The development of novel types of vaccination approaches is of central importance. This applies particularly to the prevention of diseases caused by rapidly evolving viral pathogens such as influenza virus.

In this context, the possibility to use transgenic, subunit-expressing strains of the yeast *K. lactis* as vaccines in mice and chicken and to directly apply complete, killed yeast material for inoculation [17] was an important finding. While these earlier studies of ours were performed with sub-optimally developed yeast strains and required a cumbersome triple vaccination program, they already revealed the benefits of *K. lactis*-based vaccines. Besides the general advantages of subunit vaccines offering pathogen-free vaccination and DIVA [37], these include short generation and evaluation times of recombinant vaccine strains, high biomass yields during fermentation enabled by the Crabtree-negative character of *K. lactis* [38–40], and a particular vitality of the applied VAK367-D4 strain at high cell densities [17]. Expression of the heterologous antigen can be straightforwardly induced and, due to the genomic insertion of one copy of the heterologous gene, protein levels are constant and reproducible, and vaccination does not require antigen purification. Finally, the dried, heat-killed yeast is thermostable and has a high shelf life (e.g., six months at 37 °C; data not shown). Hence, together with the fact that *K. lactis* uses the cheap and abundant sugar lactose as sole carbon source [41], this implies that these vaccines are cost-effective and may be stored and/or transported in variable temperature ranges.

Here, we further improved the technology by using codon-optimized genes, which considerably enhanced the protein expression levels in the yeast cells (data not shown) and by combining the yeast material with the potent, safe adjuvant AddaVax™ [42]. Moreover, we developed the *K. lactis* platform such that it enables the expression of more than one antigen. Thus, we generated HA only and HA plus M1-expressing vaccine strains, and, by using defined cultivation protocols, we could produce appropriate amounts of material with both yeasts, although the expression of the HA protein causes a significant growth defect (Fig. 1).

In the murine model of influenza, our study convincingly demonstrated the high protective efficacy of both *K. lactis* vaccine strains. That is, the induced titers of neutralizing antibodies (Figs. 2 and 7), the recognition pattern of these antibodies (Fig. 3) and the protective effects of VAK952 and VAK1283 (Figs. 4 and 8) were readily comparable to those obtained with the inactivated virus vaccine control. Strikingly, we obtained protection against a lethal influenza virus infection in prime/boost and even one-shot/single-dose regimes showing for the first time that the *K. lactis*-based subunit vaccines can be used in practical, competitive vaccination schemes (Figs. 4 and 8). Similar to previous reports [23,43], we observed a dose-response, *i.e.* an evident correlation between the amount of yeast/virus antigen used and the efficacy of protection. Additionally, we found the quality of the yeast vaccine played a role in the induction of the immune response. With comparable amounts of HA subunit expressed (Fig. 6), our data suggested the vaccine VAK1283 expressing HA plus M1 to be more effective than the vaccine VAK952 expressing solely HA: *i.e.*, even with the low quantity of 1 mg of applied VAK1283, we found 80% of the animals were protected (Fig. 8).

Several hypotheses are conceivable, which may explain the remarkably high immunogenicity of the *K. lactis* subunit vaccines. Important hints came from the observations that HA is expressed as the HA0 precursor and is glycosylated (Fig. 1). These data suggest that the protein is correctly folded and that it may form glycosylated trimers in the yeast cell [23,44]. The fact that the HA0 is not processed in the yeast supports the notion that the precursor, which essentially has the fold of the proteolytically cleaved HA1/HA2 [45], displays the highly immunogenic pre-fusion/open conformation of HA [46]. Similarly, the higher immunogenicity of the VAK1283 vaccine observed in the experiment in Fig. 8 may be explained by the formation of higher order protein complexes from HA and M1 [31,32,47–49].

Importantly, the yeast quantities applied subcutaneously here were well tolerated by the vaccinated mice. With 5 mg of inoculated yeast, we observed a slight swelling at the injection site for about one week, with 2 mg, the time of swelling was reduced to one day. Importantly, with both dosages we did not find any incompatibilities, not even with three consecutive yeast applications, suggesting a rather moderate immune response against the *K. lactis* platform. These observations are in close agreement with earlier studies [17] and fit the GRAS status (generally regarded as safe) that was suggested for the use of several heterologously expressed *K. lactis* proteins [50,51].

Our finding that the *K. lactis* subunit vaccines also protect in a one-shot scheme promotes the idea that the subcutaneously applied yeast material may act as a depot, which enables long-term but well-tolerated stimulation of antigen-presenting cells (APCs). A similar scenario has been earlier proposed to be the case with subcutaneously applied *S. cerevisae* vaccines, which were shown to induce a cytotoxic T cell (CTL) response against the expressed subunit protein [52]. A depot effect and the induction of a massive, virus-clearing humoral and cellular response may also explain that the applied amounts of hemagglutinin antigen, corresponding to approximately 2.5 μg HA with 5 mg yeast material or 1 μg HA with 2 mg yeast material per shot, were very low compared to the amounts of HA present in commercial inactivated whole virus vaccines (ca. 15 μg HA per shot). Alternative subunit vaccine approaches in the mouse model had to apply even 10–50 μg of purified HA-protein per inoculation to achieve a comparable protection [23,43].

In summary, *K. lactis*-based vaccines thus may bypass major handicaps of subunit vaccination such as expensive antigen processing and unsatisfactory immunogenicity. For rapidly evolving pathogens such as influenza virus, future vaccines should certainly aim to offer effective cross-protection, for example by co-expression of several unrelated HA subunits and/or conserved antigens [13,47,53]. Inactive subunit *K. lactis* vaccines, applied in competitive vaccination schemes, could therefore be attractive alternatives to virus-based vaccines. This applies specifically to veterinary applications that require low manufacturing and inoculation costs as well as DIVA properties.

## 4. Materials and methods

### 4.1. Synthetic genes

cDNAs encoding hemagglutinin HA, type H1 ($HA_{PR8}$, NCBI: NP_040980.1) or the matrix protein M1 ($M1_{PR8}$, NCBI: NP_040978.1) of Influenza A/Puerto Rico/8/1934 (H1N1) were synthesized in a codon-optimized form (GeneScript, USA).

### 4.2. Yeast manufacturing

Yeasts were grown at 30 °C with YPD and, for protein expression, shifted to YPL (1% w/v bacto yeast extract, 2% w/v peptone, 2% w/v glucose (YPD) or 2% w/v lactose (YPL)). Note that the induction times varied between the yeast strains (see text). Following protein expression, the cells were pelleted and freeze-dried. Finally, the yeast was heat-killed for 2 h at 90 °C [17]. The described seedling and inactivation method is hereinafter referred to as the "standard protocol".

### 4.3. Vaccination of mice

Ethics statement: all experiments with mice were performed along the guidelines and by approval of the Animal Care and Use Committee of the State Administration Office (LVA) Saxony-Anhalt, Germany and in accordance with the EU Directive 2010/63/EU for animal experiments (Permit Number 42502-2-1088MLUG). The immunization experiments were performed with nine-week old female mice (BALB/c; Charles River) in groups of up to ten individuals. For subcutaneous immunization, the yeast was re-suspended in PBS and mixed 1:1 (v/v) with the adjuvant AddaVax$^{TM}$ (InvivoGen, 5% squalene, 0.5% Tween 80, 0.5% Span 85). Per immunization/boost step, 100 μL emulsion (containing 1, 2 or 5 mg of yeast) were injected per mouse. A control group of mice were vaccinated with a final concentration of 20 μg inactivated A/PR/8/34 (H1N1) virus (Charles River). Six weeks after the initial immunization or two or three weeks after the last subcutaneous application, the mice were euthanized with 1.5% isoflurane, and blood samples were taken from the *vena cava caudalis*.

### 4.4. Determining the MLD$_{50}$

The median mice lethal dose (MLD$_{50}$) of the influenza A/PR/8/34 (H1N1) virus stock was determined in groups of four 15 week-old female BALB/c mice. The animals were anesthetized with isoflurane followed by intranasal infection with virus doses of $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ PFU in a total volume of 20 μL PBS. Body weight and clinical symptoms were monitored several times daily for 14 days. All mice, which showed a weight loss of ≥20% of their starting body weight (day of challenge infection) within a short time (*i.e.* days) after the infection and/or unacceptable disease symptoms [54] were immediately euthanized. A detailed score classification of clinical symptoms is given in the virus challenge section described below. The MLD$_{50}$ was calculated according to the method of Reed and Muench [55].

### 4.5. Virus challenge

The infection tests were carried out in the animal facilities of the Biozentrum Halle according to the safety classification of the used agent (A/Puerto Rico/8/1934 (H1N1); risk group 2 according to the TRBA 462 protection guideline 2000/54/EC). The LVA Saxony-Anhalt approved the necessary measures for the implementation. Groups of 15 weeks old, female BALB/c mice that had been vaccinated or mock-vaccinated were intra-nasally infected with the 5-fold MLD$_{50}$ (see above). Body weight and clinical symptoms were monitored several times daily for a period of 14 days. All mice, which showed a weight loss of ≥20% of their starting body weight (day of challenge infection) within a short time (*i.e.* days) after the infection and/or unacceptable disease symptoms [54] were euthanized. For the assessment of the clinical symptoms, a clinical score of 0–4 was determined (0: no abnormalities; 1: slightly rough fur, weight loss >5% < 10%; 2: rough fur, reduced activity, weight loss 10–15%; 3: rough fur, loss of movement, hunched posture, weight loss of 15–20%; 4: rough fur, lack of movement, hunched posture, weight loss > 20%).

### 4.6. Serum neutralization test/plaque assay

The serum neutralization test (SNT) followed the protocol of Matrosovich et al. [56].

### 4.7. Viral load

The lungs of tested mice were removed, weighed and 0.1 g lung tissue per mL PBS homogenized for 2 min on ice and centrifuged twice (5 min, 3700*g* and 9500*g* at 4 °C). Following mechanical disruption, the supernatant of the lung tissue remnants was analyzed *via* plaque assay to determine the PFU per g of lung tissue. The virus titer was calculated in PFU per g lung tissue using the formula [PFU/g lung = PFU/mL × (volume tissue supernatant lung/lung weight)] and given as $\log_{10}$ of the calculated value. Due to the logarithmic scale, samples in which no viruses (plaque formation) could be detected, were assigned the value $\log_{10}(2) = 0.3$. To determine the ratio of the organ to the final body weight, the lung index was calculated using the formula [lung index (%) = (lung weight/final body weight mouse) × 100%].

For further details and other Materials and methods, see supplementary data.

## Declaration of Competing Interest

None.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.094.

## References

[1] Short KR, Richard M, Verhagen JH, van Riel D, Schrauwen EJA, van den Brand JMA, et al. One health, multiple challenges. The inter-species transmission of influenza A virus. One health (Amsterdam, Netherlands) 2015;1:1–13.

[2] World Health Organization. 2018. Influenza (Seasonal), Fact Sheet No. 211. http://www.who.int/mediacentre/factsheets/fs211/en/.

[3] VanderWaal K, Deen J. Global trends in infectious diseases of swine. Proc Natl Acad Sci USA 2018;115:11495–500.

[4] Vincent AL, Perez DR, Rajao D, Anderson TK, Abente EJ, Walia RR, et al. Influenza A virus vaccines for swine. Vet Microbiol 2017;206:35–44.

[5] Shaw ML, Palese P. Orthomyxoviridae. In: Fields virology. Philadelphia: Lippincott Williams & Wilkins; 2013. p. 1151–85.

[6] Neumann G, Kawaoka Y. Transmission of influenza A viruses. Virology 2015;479–480:234–46.

[7] Ozawa M, Kawaoka Y. Cross talk between animal and human influenza viruses. Ann Rev Anim Biosci 2013;1:21–42.

[8] Nogales A, Martínez-Sobrido L. Reverse genetics approaches for the development of influenza vaccines. Int J Mol Sci 2016;18:20.

[9] Rajão DS, Pérez DR. Universal vaccines and vaccine platforms to protect against influenza viruses in humans and agriculture. Front Microbiol 2018;9:123.

[10] Rahn J, Hoffmann D, Harder TC, Beer M. Vaccines against influenza A viruses in poultry and swine. Status and future developments. Vaccine 2015;33:2414–24.

[11] van Reeth K, Ma W. Swine influenza virus vaccines. To change or not to change-that's the question. Curr Top Microbiol Immunol 2013;370:173–200.
[12] Kumru OS, Joshi SB, Smith DE, Russell Middaugh C, Prusik T, Volkin DB. Vaccine instability in the cold chain. Mechanisms, analysis and formulation strategies. Biologicals 2014;42:237–59.
[13] Soema PC, Kompier R, Amorij JP, Kersten GFA. Eur J Pharm Biopharm 2015;94:251–63.
[14] Milián E, Kamen AA. Current and emerging cell culture manufacturing technologies for influenza vaccines. Biomed Res Int 2015;2015:504831.
[15] Palese P. Making better influenza virus vaccines? Emerg Infect Dis 2006;12:61–5.
[16] Krijger JJ, Baumann J, Wagner M, Schulze K, Reinsch C, Klose T, et al. A novel, lactase-based selection and strain improvement strategy for recombinant protein expression in Kluyveromyces lactis. Microb Cell Fact 2012;11:112.
[17] Arnold M, Durairaj V, Mundt E, Schulze K, Breunig KD, Behrens SE. Protective vaccination against infectious bursal disease virus with whole recombinant Kluyveromyces lactis yeast expressing the viral VP2 subunit. PLoS ONE 2012;7:e42870.
[18] Hernandez LA, Miller CL, Vaughn EM. Particle and subunit-based hemagglutinin vaccines provide protective efficacy against H1N1 influenza in pigs. Vet Microbiol 2016;191:35–43.
[19] Lin SC, Liu WC, Jan JT, Wu SC. Glycan masking of hemagglutinin for adenovirus vector and recombinant protein immunizations elicits broadly neutralizing antibodies against H5N1 avian influenza viruses. PLoS ONE 2014;9:e92822.
[20] Mallajosyula JK, Hiatt E, Hume S, Johnson A, Jeevan T, Chikwamba R, et al. Single-dose monomeric HA subunit vaccine generates full protection from influenza challenge. Hum Vaccin Immunother 2014;10:586–95.
[21] Bayne AC, Boltz D, Owen C, Betz Y, Maia G, Azadi P, et al. Vaccination against influenza with recombinant hemagglutinin expressed by Schizochytrium sp. confers protective immunity. PLoS ONE 2013;8:e61790.
[22] Liu G, Zhang F, Shi J, Tian G, Chen H, Yu K, et al. A subunit vaccine candidate derived from a classic H5N1 avian influenza virus in China protects fowls and BALB/c mice from lethal challenge. Vaccine 2013;31:5398–404.
[23] Athmaram TN, Saraswat S, Santhosh SR, Singh AK, Suryanarayana VVS, Priya R, et al. Yeast expressed recombinant Hemagglutinin protein of novel H1N1 elicits neutralising antibodies in rabbits and mice. Virol J 2011;8:524.
[24] Tsai SM, Chiang YC, Chin LT, Liu HJ, Wang CY. Novel post-translational modifications of the hemagglutinin and neuraminidase proteins of avian influenza virus expressed by Kluyveromyces lactis. J Virol Methods 2011;175:175–81.
[25] Wood JM, Robertson JS. From lethal virus to life-saving. Nat Rev Microbiol 2004;2:842–7.
[26] Rodríguez-Limas WA, Tyo KEJ, Nielsen J, Ramírez OT, Palomares LA. Molecular and process design for rotavirus-like particle production in Saccharomyces cerevisiae. Microb Cell Fact 2011;10:33.
[27] Chianese-Bullock KA, Russell HI, Moller C, Gerhard W, Monaco JJ, Eisenlohr LC. Antigen processing of two H2-IEd-restricted epitopes is differentially influenced by the structural changes in a viral glycoprotein. J Immunol 1998;161:1599–607.
[28] Eggink D, Goff PH, Palese P. Guiding the immune response against influenza virus hemagglutinin toward the conserved stalk domain by hyperglycosylation of the globular head domain. J Virol 2014;88:699–704.
[29] Macioła AK, Pietrzak MA, Kosson P, Czarnocki-Cieciura M, Śmietanka K, Minta Z, et al. The length of N-glycans of recombinant H5N1 hemagglutinin influences the oligomerization and immunogenicity of vaccine antigen. Front Immunol 2017;8:444.
[30] Stelzner J, Behrens M, Behrens SE, Mäder K. Squalene containing solid lipid nanoparticles, a promising adjuvant system for yeast vaccines. Vaccine 2018;36:2314–20.
[31] López-Macías C. Virus-like particle (VLP)-based vaccines for pandemic influenza. Performance of a VLP vaccine during the 2009 influenza pandemic. Hum Vaccin Immunother 2012;8:411–4.
[32] Galarza JM, Latham T, Albert Cupo. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol 2005;18:244–51.
[33] Quan FS, Lee YT, Kim KH, Kim MC, Kang SM. Progress in developing virus-like particle influenza vaccines. Expert Rev Vaccines 2016;15:1281–93.
[34] Luo Y, Mohan T, Zhu W, Wang C, Deng L, Wang BZ. Sequential Immunizations with heterosubtypic virus-like particles elicit cross protection against divergent influenza A viruses in mice. Sci Rep 2018;8:4577.
[35] Braciale TJ. Immunologic recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells. J Exp Med 1977;146:673–89.
[36] Cao S, Liu X, Yu M, Li J, Jia X, Bi Y, et al. A nuclear export signal in the matrix protein of Influenza A virus is required for efficient virus replication. J Virol 2012;86:4883–91.
[37] Hasan NH, Ignjatovic J, Peaston A, Hemmatzadeh F. Avian influenza virus and DIVA strategies. Viral Immunol 2016;29:198–211.
[38] Donnini C, Farina F, Neglia B, Compagno MC, Uccelletti D, Goffrini P, et al. Improved production of heterologous proteins by a glucose repression-defective mutant of Kluyveromyces lactis. Appl Environ Microbiol 2004;70:2632–8.
[39] Breunig KD, Bolotin-Fukuhara M, Bianchi MM, Bourgarel D, Falcone C, Ferrero I, et al. Regulation of primary carbon metabolism in Kluyveromyces lactis. Enzyme Microb Technol 2000;26:771–80.
[40] Mulder W, Scholten IH, Grivell LA. Carbon catabolite regulation of transcription of nuclear genes coding for mitochondrial proteins in the yeast Kluyveromyces lactis. Curr Genet 1995;28:267–73.
[41] Rodicio R, Heinisch JJ. Yeast on the milky way. Genetics, physiology and biotechnology of Kluyveromyces lactis. Yeast 2013;30:165–77.
[42] Calabro S, Tritto E, Pezzotti A, Taccone M, Muzzi A, Bertholet S, et al. The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect. Vaccine 2013;31:3363–9.
[43] Cornelissen LA, de Vries RP, de Boer-Luijtze EA, Rigter A, Rottier PJ, de Haan CA. A single immunization with soluble recombinant trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1. PLoS ONE 2010;5:e10645.
[44] Schulze IT. Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. J Infect Dis 1997;176:24–8.
[45] Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell 1998;95:409–17.
[46] Rey FA, Lok SM. Common features of enveloped viruses and implications for immunogen design for next-generation vaccines. Cell 2018;172:1319–34.
[47] Kang SM, Kim MC, Compans RW. Virus-like particles as universal influenza vaccines. Expert Rev Vaccines 2012;11:995–1007.
[48] Thompson CM, Petiot E, Lennaertz A, Henry O, Kamen A. Analytical technologies for influenza virus-like particle candidate vaccines. Challenges and emerging approaches. Virol J 2013;10:141.
[49] Wang M, Jiang S, Wang Y. Recent advances in the production of recombinant subunit vaccines in Pichia pastoris. Bioengineered 2016;7:155–65.
[50] van Ooyen AJJ, Dekker P, Huang M, Olsthoorn MMA, Jacobs DI, Colussi PA, et al. Heterologous protein production in the yeast Kluyveromyces lactis. FEMS Yeast Res 2006;6:381–92.
[51] Spohner SC, Schaum V, Quitmann H, Czermak P. Kluyveromyces lactis. An emerging tool in biotechnology. J Biotechnol 2016;222:104–16.
[52] Ardiani A, Higgins JP, Hodge JW. Vaccines based on whole recombinant Saccharomyces cerevisiae cells. FEMS Yeast Res 2010;10:1060–9.
[53] Berlanda Scorza F, Tsvetnitsky V, Donnelly JJ. Universal influenza vaccines. Shifting to better vaccines. Vaccine 2016;34:2926–33.
[54] Morton D, Griffiths P. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet Rec 1985;116:431–6.
[55] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938;27:493–7.
[56] Matrosovich M, Matrosovich T, Garten W, Klenk HD. New low-viscosity overlay medium for viral plaque assays. Virol J 2006;3:63.