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Preclinical evaluation of the saponin derivative GPI-0100 as an immunostimulating and dose-sparing adjuvant for pandemic influenza vaccines

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ABSTRACT

With the current global influenza vaccine production capacity the large demand for vaccines in case of a pandemic can only be fulfilled when antigen dose sparing strategies are employed. Here we used a murine challenge model to evaluate the potential of GPI-0100, a semi-synthetic saponin derivative, to serve as a dose-sparing adjuvant for influenza subunit vaccine. Balb/c mice were immunized with different doses of A/PR8 (H1N1) subunit antigen alone or in combination with varying doses of GPI-0100. The addition of GPI-0100 significantly stimulated antibody and cellular immune responses, especially of the Th1 phenotype. Furthermore, virus titers detected in the lungs of mice challenged one week after the second immunization were significantly reduced among the animals that received GPI-0100-adjuvanted vaccines. Remarkably, adjuvantation of subunit vaccine with GPI-0100 allowed a 25-fold reduction in hemagglutinin dose without compromising the protective potential of the vaccine.

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1. Introduction

Influenza pandemics are caused by the introduction of new influenza A virus subtypes in the human population. The viruses either circulated in animal reservoirs and enter the human population by zoönotic infections or they emerged by genetic reassortment between human and animal influenza A viruses [1]. The virus causing the outbreak of pandemic influenza A (H1N1) 2009 was the result of a series of reassortments among H1N1 swine influenza viruses, H1N1 avian influenza virus and H3N2 human influenza virus [2,3]. The reassorted virus crossed the species barrier from swine to humans and caused a severe disease outbreak partially due to a substantial antigenic drift of the swine H1 as compared to the H1 in the earlier circulating epidemic H1N1 virus. Generally, the human population is immunologically naïve to such zoönotic or reassorted strains. Accordingly, disease outbreaks usually affect large geographical areas involving many countries and can result in severe morbidity and mortality [4,5].

From both a public health and socio-economic point of view, vaccination stands as the primary strategy for the prevention and control of influenza virus infections [6]. Currently licensed influenza virus vaccines consist of whole inactivated virus or purified virus proteins derived from virus grown in embryonated chicken eggs. The manufacturing process is time-consuming and the production capacity is limited [7]. Incorporation of antigen-sparing adjuvant(s) into vaccine formulations is considered a promising solution for the supply problem, which is a serious concern during a pandemic outbreak [7]. Moreover, such adjuvants are required to stimulate protective antibody titers [8].

The bark extract of the Molina tree *Quillaia saponaria* contains triterpene saponins which have powerful adjuvant activity. In 1978, an enriched mixture of saponins called Quil A was identified and was used commercially in a veterinary foot-and-mouth disease vaccine [9]. However, its toxicity excludes its use in human vaccines. In order to lower the compound toxicity, immune-stimulating complexes (ISCOMs) containing cholesterol, saponin, phospholipid and viral envelope proteins were developed. Lethality studies in mice determined the lethal dose of ISCOM-incoporated Quil A to be 10–50 μg [10]. In another approach to lower the adjuvant's toxicity, RP-HPLC was used to purify the components of the heterogenous mixture of Quil A. Ten of the obtained fractions showed a similar level of adjuvant activity as Quil A itself with different levels of toxicity. Among those fractions, QS-21 (with a lethal dose of 500 μg) had low toxicity and QS-7 showed no lethality in the dose range studied. More recently, a novel semi-

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synthetic saponin adjuvant called GPI-0100 has been developed from QS-7. Lethality studies in mice showed that GPI-0100 (with a lethal dose of 5000 μg) is 10 times less toxic than QS21 and 100 times less toxic than ISCOM-incorporated Quil A. In addition, it shows increased stability in aqueous solution at physiological pH [11,12]. Preclinical studies of GPI-0100 adjuvant with ovalbumin (OVA), hemagglutinin B (HagB) antigen of *Porphyromonas gingivalis* and glycoprotein D (gD) of herpes simplex virus type-1 (HSV-1) have shown increased induction of antigen-specific antibodies in mice with a particular enhancement of the IgG2a isotype [11–18]. In addition, GPI-0100 induces antigen-specific cellular immune responses exemplified by lymphocyte proliferation, cytokine (IFN-γ and IL-2) secretion and CTL responses [11,12,17]. Furthermore, GPI-0100-adjuvanted HSV vaccines protect mice from virus challenge with significant reductions in virus titers, infected (lesion) areas and mortality rates [16].

Subunit influenza vaccines contain purified hemagglutinin antigens without the presence of natural immune modulators and often possess comparitively modest immunogenicity. Here we evaluate the adjuvant activity of GPI-0100 for A/PR8 (H1N1) influenza subunit vaccine in mice. We show that influenza-specific immune responses are strongly boosted by low doses of GPI-0100 and that, in the presence of GPI-0100, the antigen dose can be reduced substantially without loss of protective efficacy. We therefore consider GPI-0100 a promising candidate adjuvant for pandemic influenza vaccines.

2. Material and methods

2.1. GPI-0100

GPI-0100 was provided by Hawaii Biotech, Inc. (Aiea, HI, U.S.A.) as powder and was stored at 4 °C. A stock solution of GPI-0100 (5 mg/ml) was prepared in HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA, pH 7.4). After centrifugation through a Spin-X centrifuge tube filter (Corning, U.S.A.), the sterile stock solution was stored at 4 °C for use within one month.

2.2. Virus and subunit vaccine

A stock of A/PR8 (H1N1) influenza virus propagated on Madin–Darby canine kidney cells (MDCK) was kindly provided by Solvay Biologicals (Weesp, The Netherlands). The virus titer was determined by measuring the tissue culture infectious dose 50 ($TCID_{50}$). To this end serial twofold dilutions of virus suspension were inoculated on MDCK cells grown in serum-free medium. 1 h later TPCK trypsin (Sigma, Zwijdrecht, Netherlands) was added to a final concentration of 7.5 μg/ml. After 72 h, supernatants were collected and transferred to a round-bottom 96-well plate followed by the addition of 50 μl 1% guinea pig erythrocytes to each well. The plate was incubated for 2 h before reading. The titer was determined as the highest virus dilution at which hemagglutination was visible and the $TCID_{50}$ was calculated by the method of Reed and Muench [19].

For inactivation, the virus was incubated with freshly prepared 10% β-propiolactone in citrate buffer (125 mM sodium citrate, 150 mM sodium chloride, pH 8.2) at a final concentration of 0.1% β-propiolactone. Inactivation was carried out for 24 h at 4 °C under continuous stirring. After inactivation, the virus was dialyzed against phosphate-buffered saline (PBS) overnight at 4 c. Subunit vaccine was prepared by solubilizing the inactivated virus (0.8 mg virus protein/ml) in PBS containing Tween 80 (0.3 mg/ml) and hexadecyltrimethylammonium bromide (CTAB, 1.5 mg/ml) for 3 h at 4 °C under continuous stirring, and removal of the viral nucleocapsid from the preparation by ultracentrifugation for 30 min at 50,000 rpm in a TLA100.3 rotor at 4 c. Detergents were then removed by overnight absorption onto Biobeads SM2 (634 mg/ml, Bio-Rad, Hercules, CA) washed with methanol prior to use.

Protein content of the inactivated virus and subunit material was determined by a modified Lowry assay [20]. Hemagglutinin (HA) content was assumed to be one third of the total protein for whole inactivated virus (based on the known protein composition of influenza virus and the molecular weight of the viral proteins) and to be equal to the total protein for subunit material (based on silver-stained SDS polyacrylamide gels run under reducing and non-reducing condition) [21]. Vaccines were mixed at the indicated amounts of subunit and GPI-0100 just before immunization.

2.3. Animal handling

The protocol for the animal experiment described here was approved by the Ethics Committee on Animal Research of the University of Groningen. Female Balb/c mice (Harlan, The Netherlands) aged 8–10 weeks were grouped ($n = 6$ per group) and immunized intramuscularly (i.m.) with A/PR/8 subunit vaccine with or without GPI-0100 adjuvant in a two-dose immunization regimen (day 0 and day 20). Control mice were injected with HNE buffer. On day 27, mice were challenged intranasally with 1×10^4 $TCID_{50}$ A/PR/8 influenza virus in 50 μl of HNE buffer. The virus challenge was carried out under isoflurane anesthesia to ensure deposition of the virus into the lungs. Mice were monitored, twice a day at fixed time points, for clinical signs of illness including weight loss, changes in behavior and appearance. Mice were bled and sacrificed on day 30. Serum samples were collected for ELISA assay. Spleens were harvested and splenocytes were used for ELISPOT assay. The lung lobes were collected and stored in 1 ml PBS in a −80 °C freezer for later homogenization and lung virus titer detection.

2.4. IgG, IgG1 and IgG2a ELISAs

Influenza HA-specific antibody titers were determined by ELISA [21]. Briefly, ELISA plates (Greiner, Alphen a/d Rijn, Netherlands) were coated with 0.2 μg of PR8 influenza subunit antigen per well. Twofold serial dilutions of serum samples in PBST (0.05% Tween 20 in PBS) were applied to the wells in duplicate and incubated for 1.5 h. Horseradish peroxidase-conjugated goat antibody against mouse IgG-isotypes (Southern Biotechnologies) was added for the detection of bound H1N1-specific IgG, IgG1 or IgG2a antibodies. All incubations were carried out at 37 °C. The staining was performed with substrate buffer (50 mM phosphate buffer, pH 5.5, containing 0.04% o-phenylenediamine and 0.012% H_2O_2) and the absorbance at 492 nm (A_{492}) was measured using an ELISA reader (Bio-tek instruments, Inc., Vermont, U.S.A.). Titers (with the standard error of the means (S.E.M.)) are given as the ^{10}log of the reciprocal of the sample dilution calculated to correspond to an A_{492} of 0.2. For calculation purposes, sera with titers below detection limit were assigned an arbitrary ^{10}log titer corresponding to half of the detection limit.

Calibration plates for IgG1 and IgG2a assay were coated with 0.1 μg goat anti-mouse IgG (Southern Biotechnologies). Increasing concentrations of purified mouse IgG1 or IgG2a (Southern Biotechnologies) were added to the plates. Sample IgG1 and IgG2a titers were expressed as concentrations (μg/ml) of influenza HA-specific IgG1 and IgG2a ± S.E.M.

2.5. ELISPOT

ELISA plates were coated with purified rat IgG1 against mouse IFN-γ or IL-4 (Pharmingen, San Diego, CA) [21]. Freshly isolated splenocytes (500,000 cells per well) were added to the plates in triplicate in medium containing 5% fetal calf serum with or

without PR8 subunit (1 μg per well). After an overnight incubation at 37 °C, cells were lysed in ice-cold water and plates were washed. IFN-γ detection was carried out by 1 h incubation with biotinylated anti-mouse IFN-γ antibody followed by a subsequent incubation with streptavidin-alkaline phosphatase (Pharmingen) for 1 h. Spots were developed by adding 100 μl of substrate solution to each well. The substrate solution included 5-bromo-4-chloro-3-indolylphosphate in water containing 6 mg/ml agarose (Sigma), 9.2 mg/ml 2-amino-2-methyl-1-propanol (Sigma) and 0.08 μl/ml Triton X-405 at 1 mg/ml. The plates were further incubated for 3 h at 37 °C. Images of the plates were taken by an automated ELISA-spot assay video analysis system (A EL VIS, Hannover, Germany). Spots were counted manually. Spots observed in the wells without PR8 subunit (backgrounds) were subtracted from the spots observed in the stimulated wells. Results are presented as number of influenza-specific IFN-γ- or IL-4-secreting cells per 500,000 splenocytes.

2.6. Determination of virus titers in lungs of challenged mice

Lungs collected from the challenged mice were homogenized and the supernatants of lung extracts were collected and stored at −80 °C until use [21]. Virus titers were determined by inoculating serial dilutions of the supernatants on MDCK cells as described above (Section 2.2). The highest dilution that still resulted in hemagglutination was taken as the virus titer in the lungs. Results are presented as ^{10}log virus titer per gram of lung tissue.

2.7. Statistical analysis

The unpaired Student's *t*-test was used to determine if the differences in influenza-specific responses observed between groups of mice were significant. A *p* value of $p < 0.05$ was considered significant.

3. Results

3.1. GPI-0100 boosts influenza-specific humoral immune responses in a dose-dependent manner

To elucidate the adjuvant activity of GPI-0100 on antibody responses elicited by influenza subunit vaccine, mice were immunized twice on day 0 and day 20 with 1 μg HA with different doses of GPI-0100 (15, 50 or 150 μg). Blood samples were taken one week after the second immunization for evaluation of total influenza-specific IgG levels. The IgG levels were significantly increased upon GPI-0100 adjuvantation in a dose-dependent manner (Fig. 1A, $p < 0.0005$ for all tested adjuvant doses). The enhancing effects of GPI-0100 were observed for both IgG1 and IgG2a antibodies (Fig. 1B and C). In the group of mice receiving 1 μg unadjuvanted HA, influenza-specific IgG1 was found in all immunized mice but titers were low, while only 4 out of the 6 mice developed detectable IgG2a titers. GPI-0100-adjuvanted HA induced detectable levels of both IgG subtypes in all immunized mice in a dose-dependent manner. ($p \leq 0.001$ for IgG1 and $p < 0.05$ for IgG2a for all GPI-0100 doses tested).

Spleens from the immunized mice were harvested and spleen weights were determined (Fig. 2A). No changes in spleen weight were observed in mice receiving 15 μg GPI-0100-adjuvanted vaccines. However, significant increments in spleen weight were found in mice receiving vaccine adjuvanted with 50 μg or more GPI-0100 ($p < 0.005$). For the follow-up study 30 μg GPI-0100 adjuvantation was used with the aim of boosting sufficient immune responses without inducing splenomegaly. No significant changes in spleen weight were observed at this GPI-0100 dose (Fig. 2B).

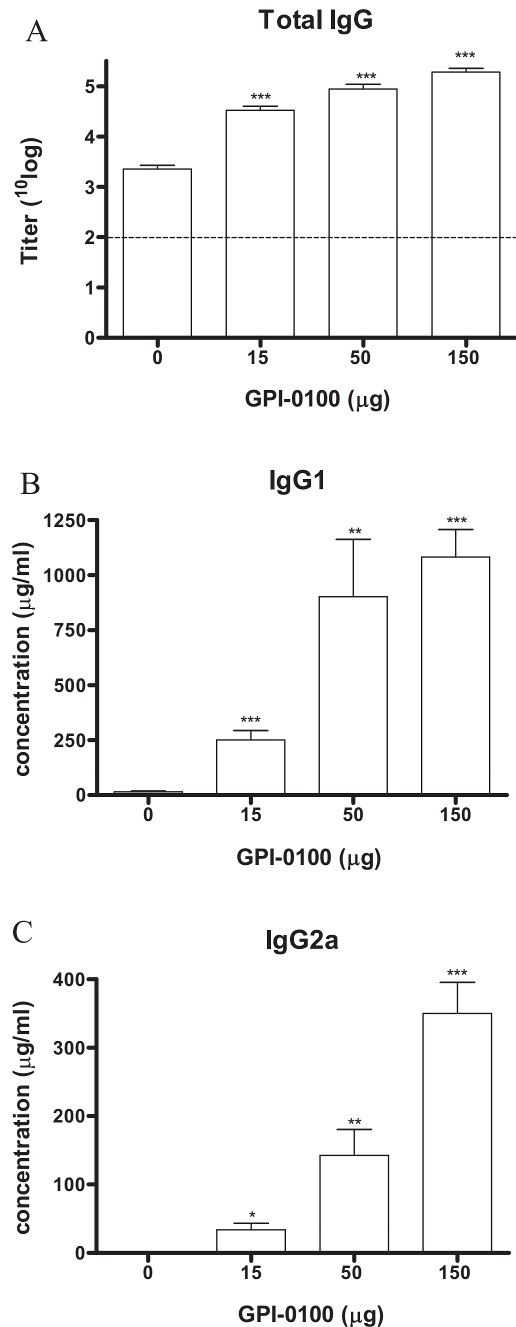

Fig. 1. Total IgG, IgG1 and IgG2a antibody responses one week after the second immunization. Mice were vaccinated on day 0 and day 20 with 1 μg A/PR/8 subunit vaccine without and with the indicated doses of GPI-0100 adjuvant. (A) Average ^{10}log IgG titers ± standard error of the mean (S.E.M.), $n = 6$ mice per group. The detection limit of the assay is represented by the dotted line. (B, C) Average quantities (μg/ml) of IgG1 and IgG2a ± S.E.M. Levels of significance are depicted as follows: $^{*}p < 0.05$, $^{**}p < 0.005$ and $^{***}p < 0.0005$, which refer to the differences between immunizations with and without GPI-0100.

3.2. GPI-0100-adjuvanted low-dose PR8 subunit vaccine induces high influenza-specific humoral immune responses

To evaluate dose-sparing effects of GPI-0100, mice were immunized twice with decreasing doses of A/PR/8 subunit vaccine (1, 0.2 and 0.04 μg HA) adjuvanted with 30 μg GPI-0100. Serum samples were taken one week after the second immunization. None of the mice receiving unadjuvanted 0.04 μg HA and only 2 out of 6 mice receiving 0.2 μg HA developed detectable influenza-specific IgG

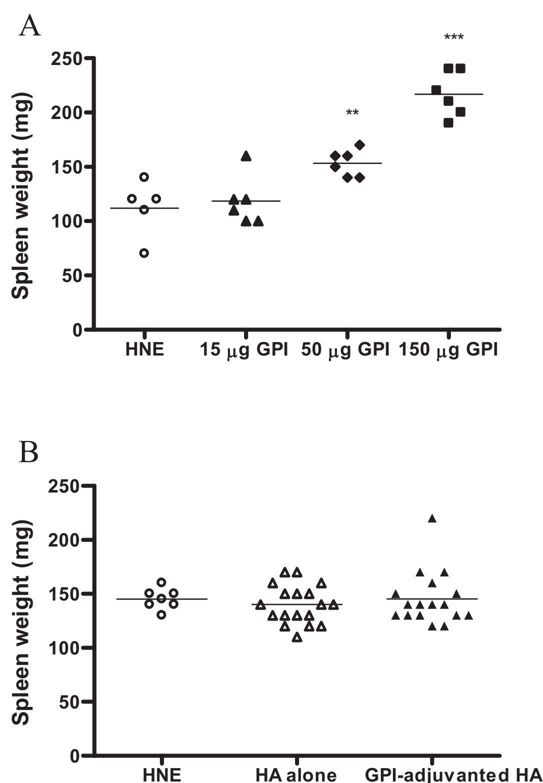

Fig. 2. Effect of GPI-0100 on the spleen weight of immunized mice. (A) Mice were immunized with HNE buffer (circles) or 1 μg A/PR/8 subunit vaccine adjuvanted with 15 μg (triangles), 50 μg (diamonds) or 150 μg (squares) GPI-0100. Spleens were harvested and weighed one week after the second immunization. Spleen weights of individual mice are depicted and the average per group is given as a black line. (B) Mice were immunizaed with HNE buffer (circles), unadjuvanted (open triangles) subunit vaccine, or with 30 μg GPI-0100 adjuvant combined with 0.04, 0.2 or 1 μg of antigen (filled triangles). Antigen dose itself had no effect on spleen weight.

titers (Fig. 3A). Unadjuvanted 1 μg HA induced IgG in 5 out of 6 mice with an average titer of 3.31 ^{10}log.Vaccines adjuvanted with 30 μg GPI-0100 induced IgG titers in all vaccinated animals and these were significantly higher than in the mice receiving unadjuvanted vaccines ($p < 0.005$ for all tested antigen doses.) Notably, IgG titers achieved with adjuvanted low dose antigen (0.04 μg) were about 1 log higher than those achieved with non-adjuvanted high-dose antigen (1 μg).

The GPI-0100 adjuvant significantly enhanced IgG1 titers at the low antigen doses (0.04 and 0.2 μg HA) and IgG2a titers at all tested antigen doses, respectively (Table 1, $p < 0.0001$ (0.04 μg HA) and <0.0005 (0.2 μg HA) for IgG1 and <0.005 for IgG2a (all HA doses)). Notably, mice receiving low antigen doses (0.04 and 0.2 μg HA) developed detectable IgG2a titers only in the presence of the GPI-0100 adjuvant. The adjuvant effects were especially pronounced for low antigen doses.

3.3. *GPI-0100-adjuvanted low-dose PR8 subunit vaccine enhances influenza-specific cellular immune responses*

To evaluate adjuvant activity of GPI-0100 on cellular immune responses elicited by A/PR/8 subunit vaccine, ELISPOT assays were performed to detect influenza-specific cytokine-producing T cells from the immunized and challenged mice (Fig. 3B). No influenza-specific IFN-γ-producing T cells were found in control animals injected with buffer and challenged with virus three days before sacrifice (data not shown). Unadjuvanted 0.04 and 0.2 μg HA barely induced detectable influenza-specific IFN-γ responses. At a dose of 1 μg, HA alone induced an average of 4 IFN-γ-producing cells

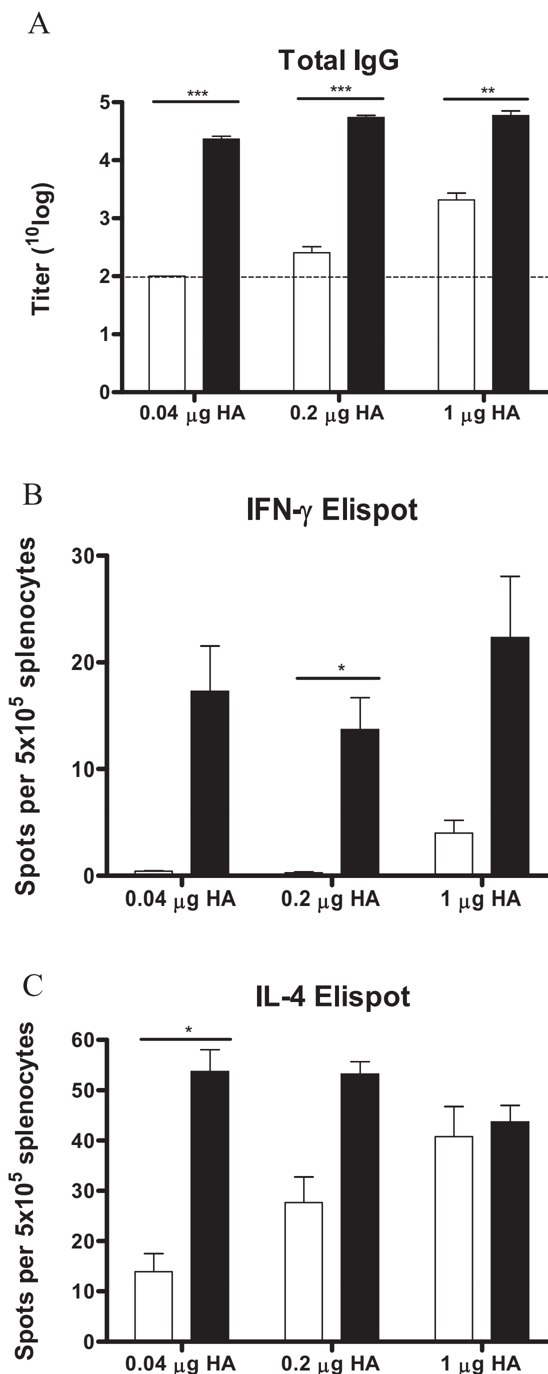

Fig. 3. Antigen dose-dependent pre-challenge IgG and post-challenge cytokine-producing T cell responses. Mice were immunized on day 0 and on day 20 with the indicated doses of A/PR/8 subunit vaccine without (white bars) or with (black bars) 30 μg GPI-0100. (A) Sera were collected one week after the second immunization. Average ^{10}log IgG titers ± S.E.M. are depicted ($n = 6$ mice per group). The detection limit of the assay is represented by the dotted line. (B, C) An intranasal infection with 1×10^4 $TCID_{50}$ live A/PR/8 virus was performed directly after serum collection. Spleens were harvested three days after the virus challenge upon termination. Splenocytes were isolated and stimulated overnight with PR8 subunit. Average numbers of cytokine-producing splenocytes per 5×10^5 cells ± S.E.M. are depicted.

per 5×10^5 splenocytes in 3 out of 6 mice. GPI-0100 enhanced the IFN-γ responses at all tested antigen doses. However, due to the large variation in the number of IFN-γ-producing T cells within the experimental groups, significance of the differences between unadjuvanted and adjuvanted vaccines was achieved

Table 1
Phenotype of the pre-challenge influenza-specific antibody responses.

μg HA	30 μg GPI-0100	Average influenza HA-specific titer (^{10}log ± S.E.M.)		IgG2a/IgG1 ± S.E.M.[a]
		IgG1	IgG2a	
0.04	–	1.79 ± 0.09[b]	N.D.[c]	N.A.
	+	4.63 ± 0.14[d]	2.87 ± 0.30[d]	0.62 ± 0.06
0.20	–	2.51 ± 0.50[e]	N.D.[c]	N.A.
	+	4.80 ± 0.08[d]	3.63 ± 0.40[d]	0.76 ± 0.08
1.00	–	3.61 ± 0.28	2.10 ± 0.26[e]	0.59 ± 0.07
	+	4.48 ± 0.58[f]	3.56 ± 0.32[d]	0.87 ± 0.12

[a] Ratios were calculated per mouse. The average mean ratio and S.E.M. per experimental group are presented.
[b] One out of six samples had a detectable serum antibody level. For calculation purposes, non-responding sera were assigned an arbitrary titer of 1.7 which corresponds to half of the detection limit.
[c] Titer below detection limit.
[d] Significantly higher than without GPI-0100 ($p < 0.05$).
[e] Two out of six samples had a detectable serum antibody level. For calculation purposes, non-responding sera were assigned an arbitrary titer of 1.7 as explained under footnote[b].
[f] One sample had a serum antibody level below the detection limit. For calculation purposes, the non-responding serum was assigned an arbitrary titer of 1.7 as explained under footnote[b].

only for the animals that received 0.2 μg HA ($p < 0.05$). Low numbers of influenza-specific IL-4-producing T cells were found three days after infection of control animals (data not shown). Similar low numbers were observed in mice immunized with 0.04 μg unadjuvanted vaccines, but numbers increased in an antigen dose-dependent manner (Fig. 3C). GPI-0100 induced an increase in the number of IL-4-producing cells at all tested antigen doses, yet the difference was significant only for the lowest antigen dose ($p < 0.05$).

Thus, the GPI-0100 adjuvant enhanced the number of influenza-specific cytokine-producing cells to a similar level at all antigen doses tested. The effect of GPI-0100 on IFN-γ responses was stronger than that on IL-4 responses. The phenotype of the cellular immune responses was further analyzed by calculating IFN-γ/IL-4 ratios per individual mouse (Table 2). GPI-0100 adjuvantation did not change the Th2 dominance of the response to PR8 subunit vaccines, but significantly enhanced Th1 responses leading to a more balanced immune phenotype.

3.4. GPI-0100 is required for lung protection against virus challenge at low antigen dose

Four weeks after the first immunization mice were challenged by intranasal administration of live A/PR/8 virus. During the first two days after challenge little effect of the virus infection was seen. By day three animals started to loose weight. This weight loss was higher in the mice which had been immunized with adjuvanted vaccines than in non-immunized mice and in mice which received unadjuvanted vaccines. Weight loss correlated with the strength of the induced immune responses but not with GPI-0100 dose. Three days after virus challenge, the animals were sacrificed and virus titers were determined in lung homogenates to evaluate protection elicited by the vaccines. The HNE buffer group showed an average lung virus titer of 6.45 ^{10}log (Fig. 4). The average titer in lungs of mice receiving a low dose of unadjuvated HA (0.04 and 0.2 μg) was not statistically different from that of the buffer group. Only mice receiving 1 μg unadjuvanted HA showed a statistically significant reduction in lung virus titers ($p < 0.05$). Immunization with GPI-0100-adjuvanted vaccine resulted in significantly decreased lung virus titer at all tested antigen doses (p values between buffer and the adjuvanted vaccines were ≤0.01 for all antigen doses tested, p values between unadjuvanted and adjuvanted vaccines were ≤0.05 or ≤0.01, at HA doses of 1 μg or 0.04 and 0.2 μg, respectively). The result shows that GPI-0100 improves vaccine-elicited protection against influenza virus infection even at an extremely low antigen dose of 0.04 μg HA.

Table 2
Phenotype of the post-challenge influenza-specific cytokine responses.

μg HA	30 μg GPI-0100	IFN-γ/IL-4 ± S.E.M.[a]
0.04	–	0.01 ± 0.01
	+	0.52 ± 0.36
0.20	–	0.01 ± 0.01
	+	0.21 ± 0.11
1.00	–	0.14 ± 0.12
	+	0.35 ± 0.22

[a] Ratios were calculated per mouse. The average mean ratio and S.E.M. per experimental group are presented.

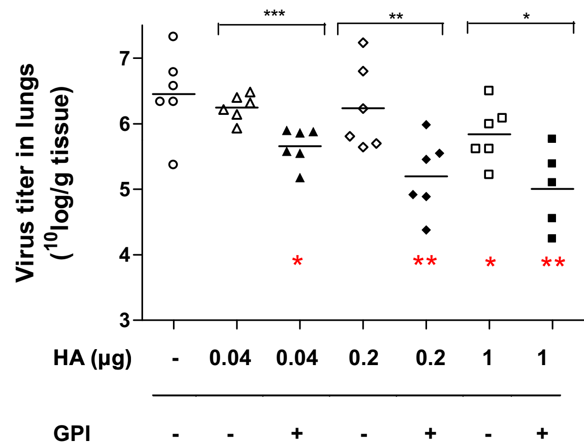

Fig. 4. Effects of unadjuvanted and GPI-0100-adjuvanted subunit vaccine on lung virus titers. Mice described in the legend to Fig. 3, immunized with HNE buffer (circles), 0.04 μg (triangles), 0.2 μg (diamonds) or 1 μg (squares) A/PR/8 subunit vaccine without (open symbols) or with (filled symbols) 30 μg GPI-0100 were infected with live A/PR/8 virus 1 week after the second immunization. Lung samples were collected three days later upon termination. Virus titer is expressed as the ^{10}log virus titer per gram of lung tissue of individual mice. The black line represents the geometric mean virus titer per group. Small stars indicate significant differences between mice receiving unadjuvanted and adjuvanted vaccine at the indicated antigen dose. Large stars indicate significant differences between immunized groups and the HNE control group.

4. Discussion

GPI-0100 is a stable semi-synthetic saponin derivative, which has been demonstrated to stimulate both the humoral and the cellular arm of the immune system [10–12,17,22]. In the present study we evaluated the immunogenicity and protective efficacy of GPI-0100-adjuvanted A/PR8 influenza subunit vaccine in mice. The results show that GPI-0100 boosts influenza-specific antibody responses of the IgG1 and especially the IgG2a subtype in a dose-dependent manner. There was also a trend towards higher numbers of influenza-specific cytokine-producing T cells in mice immunized with GPI-0100 adjuvanted vaccine though differences were not significant for all antigen doses studied. Furthermore, GPI-0100-enhanced immune responses provided better protection against influenza virus infection as demonstrated by reduced lung virus titers after challenge. Remarkably, an adjuvanted 0.04 μg HA dose presented a better formulation than an unadjuvanted 1 μg HA dose for all immune parameters studied.

In line with earlier studies using OVA, HagB antigen of *P. gingivalis* and gD antigen of HSV-1, here we confirm that GPI-0100 boosts antigen-specific antibody responses with a Th1 IgG isotype

profile in a dose-dependent manner [11,12,14,16]. High levels of antigen-specific IgG2a titers were induced in addition to IgG1 titers, resulting in a more balanced Th1/Th2 antibody response. In addition, we observed that GPI-0100 stimulates antigen-specific IFN-γ responses, which has also been reported previously in OVA studies [11]. Structural analysis has identified the C4 aldehyde group of GPI-0100 as a feature essential for inducing Th1 immunity. The aldehyde group has been suggested to form an imino linkage with amino groups on certain T cell surface receptors. This may generate co-stimulatory signals similar to those provided by activated antigen-presenting cells [10,12].

In our study, the enhanced immunogenicity elicited by subunit vaccine containing 50 μg or more GPI-0100 was accompanied by spleen enlargement and increased spleen weights in vaccinated mice. However, neither significant increase in splenocyte number nor any change in the relative frequency of B cells, CD4 and CD8 T cells was found. Therefore, it is unlikely that the observed effects are due to hyper immune-stimulation. Some saponin adjuvants are known to possess an angiogenic effect and the spleen enlargement may thus be caused by increased blood supply [23,24]. Earlier lethality studies and toxicology tests analyzing serum creatinine kinase (CK) and aspartate aminotransferase (AST) levels (as indicator for muscle and liver damage, respectively) showed that GPI-0100 under 1000 μg has little to no effect in mice, a species reported to be sensitive to saponin compounds [10,12]. Moreover, a clinical study with GPI-0100-adjuvanted prostate cancer vaccines showed high induction of antigen-specific IgM and IgG (IgG1 and IgG3) titers in the cancer patients without serious side effects at an ajuvant dose of 3000 μg [15].

Many adjuvants have been tested in animal models yet aluminum-based adjuvants have long been the only licensed adjuvants for use in human vaccines [25,26]. In recent years, squalene-based adjuvants like MF59 and AS03 were also licensed in Europe as adjuvants for influenza vaccines, and a vaccine against human papilloma virus containing monophosphoryl lipid (MPL) A was registered in the U.S. and around the world [27–29]. Clinical trials with aluminum-based adjuvants in combination with pandemic influenza virus vaccines did not provide evidence for a significant immunostimulating effect of aluminum compounds on influenza-specific responses [30–32]. On the other hand, MF59 and AS03 do enhance antibody responses to pandemic influenza virus vaccines and allow antigen dose reduction [28,33–38]. An MF59-adjuvanted seasonal influenza vaccine is registered in Europe for use in elderly. Moreover, MF59 and AS03 were both used as adjuvants for H1N1 vaccines during the 2009 A/H1N1 pandemic. Clinical trials on MPLA-adjuvanted influenza virus vaccines are yet to be done.

In our experiments, GPI-0100 enhanced influenza-specific IgG titers to A/PR/8 subunit vaccine by a factor of 30-230 with the greatest enhancement seen at low antigen doses. Moreover, GPI-0100 adjuvantation especially stimulated Th1-related immune responses (IgG2a and IFN-γ-producing T cells) and significantly improved the protective potential of influenza subunit vaccine. In contrast, adjuvantation with aluminum hydroxide enhanced the IgG titer to A/PR8 subunit vaccine by a factor of 15 but increased mainly antibodies of the IgG1 subtype and did not improve protection against challenge with live virus [21]. Mouse studies have shown that the MF59 adjuvant can stimulate influenza-specific IgG titers up to 120-fold [27,39,40]. The enhancements were observed in both IgG1 and IgG2a subtypes, with a bias to IgG1, and correlated with better lung protection. AS03-adjuvanted influenza vaccines have been studied in ferrets but no data in mice are available for comparison [41]. Thus, with respect to enhancement of antibody titers (at least in mice) GPI-0100 performs as well or better as adjuvants currently used in clinical influenza vaccines.

Despite the boosting effects on humoral immune responses, both aluminum-based adjuvants and MF59 have minimal effects on antigen-specific IFN-γ production and cellular immunogenicity, which are important in controlling influenza virus in the lungs and are crucial for immune memory formation and long-term vaccine protection [21,39,42–44]. GPI-0100, on the other hand, does show adjuvant effects on cellular immunogenicity especially on IFN-γ- but also on IL-4-responses.

In conclusion, we show that GPI-0100 has the capacity to function as a potent adjuvant for influenza subunit vaccines. In the murine model system the immune-enhancing effects of GPI-0100 are stronger than those observed in previous studies using aluminum-based adjuvants or MF59 [21,27,39,40]. Furthermore, GPI-0100 boosts both Th1 (IgG2a and IFN-γ) and Th2 (IgG1 and IL-4) responses. Th1 responses are particularly stimulated resulting in skewing to a desirable immune phenotype that leads to better protection against influenza virus infection [21,45,46]. Notably, when adjuvanted with GPI-0100, a very low dose of subunit vaccine (0.04 μg HA) remains immunogenic and provides protection from virus growth in the lungs. In order to achieve a similar level of protection 1 μg unadjuvanted HA, a 25-fold higher dose, was required. Therefore, GPI-0100 is a promising candidate adjuvant for stimulating influenza-specific immune responses and for antigen sparing in case of an influenza pandemic.

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