Extensive preparation is underway to mitigate the next pandemic influenza outbreak. New vaccine technologies intended to supplant egg-based production methods are being developed, with recombinant hemagglutinin (rHa) as the most advanced program for preventing seasonal and avian H5N1 Influenza. Increased efforts are being focused on adjuvants that can broaden vaccine immunogenicity against emerging viruses and maximize vaccine supply on a worldwide scale. Here, we test protection against avian flu by using H5N1-derived rHa and GLA-SE, a two-part adjuvant system containing glucopyranosyl lipid adjuvant (GLA), a formulated synthetic Toll-like receptor 4 agonist, and a stable emulsion (SE) of oil in water, which is similar to the best-in-class adjuvants being developed for pandemic flu. Notably, a single sub-microgram dose of rH5 adjuvanted with GLA-SE protects mice and ferrets against a high titer challenge with H5N1 virus. GLA-SE, relative to emulsion alone, accelerated induction of the primary immune response and broadened its durability against heterosubtypic H5N1 virus challenge. Mechanistically, GLA-SE augments protection via induction of a Th1-mediated antibody response. Inmate signaling pathways that amplify priming of Th1 CD4+ T cells will likely improve vaccine performance against future outbreaks of lethal pandemic flu.

H5N1 is a highly pathogenic avian influenza virus that can cause severe disease and death in humans, and world health authorities agree that the potential for pandemic H5N1 infection is high. Vaccination remains the most effective mechanism for preventing influenza, but there are complex challenges in implementing a pandemic preparedness plan, including: an inability to rapidly deploy the vast numbers of safe and effective doses needed on a worldwide scale; the fact that the immunogenicity of current non-adjuvanted H5N1 vaccines are relatively weak and require large antigen doses; and the potency of stockpiled pre-pandemic vaccines may be severely limited given the anticipated antigenic shift associated with the emergence of a novel strain of pandemic H5N1.

The US government has outlined provisions for new technologies that maximize immunogenicity and manufacturing capacity of vaccines for influenza, including the use of recombinant protein-based vaccines and adjuvants, which augment immunity and dose-sparing capability. The most advanced egg-free flu vaccine candidate is a recombinant multimeric H5 hemagglutinin protein (rH5) produced by using a baculovirus expression vector system in SF9 insect cells (1, 2). Previous clinical studies suggested that two 90-μg doses of rH5 induced modest responses equivalent to conventional subvirion-based H5N1 vaccines (3, 4). This finding has prompted efforts to test rH5 with an adjuvant. Currently, the leading H5N1 vaccine adjuvants are oil-in-water (o/w) emulsions, which augment neutralizing antibody titers, increase the breadth of cross-reactive antibodies, and possess significant dose-sparing activity (5, 6). Importantly, these adjuvants are particularly effective in priming naïve individuals in the absence of preexisting memory.

Vaccine adjuvants regulate adaptive immunity by stimulating dendritic cell maturation and antigen presentation (7, 8). A leading adjuvant target on DC is the family of innate Toll-like receptors, particularly the LPS receptor, Toll-like receptor 4 (TLR4). Glucopyranosyl lipid adjuvant (GLA) is a formulated form of the synthetic TLR4 agonist PHAD (Avanti Polar Lipids), which is analogous to the detoxified LPS derivative monophosphoryl lipid A (MPL), a component of the human papillomavirus vaccine Cervarix (9). Experimental vaccines containing GLA demonstrate enhanced immunogenicity in a variety of disease models (8), and in the context of influenza, GLA formulated in a stable emulsion (GLA-SE) improved Fluzone-dependent antibody titers in mice and nonhuman primates, relative to an emulsion alone (10–13). Given the critical importance of immunological priming for pandemic vaccine preparedness, we set out to test whether adjuvanting a recombinant H5 antigen with GLA-SE would broaden protective immunity against H5N1.

Results
We have established an assay for adjuvant-dependent priming of a protective immune response. The kinetics of this response were measured by immunizing BALB/c mice once with 50 ng of rH5 derived from A/Vietnam/1203/04 (H5VN) protein adjuvanted with GLA-SE or SE and then challenging animals after increasing the number of days with a 100 LD50 (500 pfu) dose of A/Vietnam/1203/04 virus. As indicated in Fig. 1A, only mice injected with antigen plus adjuvant survived; corresponding protective responses were induced in 60% of animals immunized with rH5 + GLA-SE for 4 d before challenge, and all mice receiving rH5 in SE or GLA-SE survived high-dose viral challenge when the vaccine was delivered six or more days before challenge. The suggestion that GLA accelerates protection relative to SE was reflected in measurements of body weight (Fig. 1B), general health (Fig. S1A), and viral clearance (Fig. S1B). Animals immunized with rH5 + GLA-SE 6 d before high-dose virus challenge recovered more quickly compared with mice immunized with rH5 + SE alone, and the kinetics were identical in mice challenged 14 d after immunization. Thus, the relative responses mediated by GLA-SE and SE become fixed within the first 6 d after vaccination. To test whether adjuvant activity is influenced by antigen dose, mice were injected once with increasing amounts of rH5 alone or with adjuvant and then challenged with a 1,000 LD50 (5,000 pfu) dose of virus (Fig. 1C). Again, only mice receiving adjuvanted rH5 survived, and although the 50-fold increase in antigen dose improved recovery rates, the relative differences in adjuvant activity observed previously with GLA-SE and SE were maintained. As a result, SE requires significantly more antigen than GLA-SE to prevent equivalent weight loss. One mechanism by which GLA-SE appears to improve protection is antibody mediated, as indicated by the antigen-dependent

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In a separate experiment, we asked whether GLA-SE signaling might protect animals systemically from a site distal to antigen administration. However, no mice (0/25) survived high-dose viral challenge after a split-dose vaccination involving rH5 (50 ng) delivery in one flank and increasing amounts of GLA-SE (0, 1, 5, 20, or 50 \( \mu \)g GLA) in the other flank, whereas all mice (25/25) survived coadministration of antigen plus these adjuvants (Table S1). This result strengthens the argument that TLR4-mediated signals enhance priming of an antigen-dependent immune response within the local microenvironment.

We tested these vaccine components in a second genetic background by using C57BL/6 mice (Fig. 3). As before, only animals receiving rH5VN (50 ng) combined with SE or GLA-SE survived a high titer (1,000 LD\(_{50}\)) virus challenge and GLA-SE remained the superior adjuvant as measured by weight loss (Fig. 3A). We have observed that immunized C57BL/6 mice recover from virus challenge more quickly than BALB/c mice (compare Figs. 1A and 3A). This difference is likely due to the fact that C57BL/6 mice express significantly higher day 14 antibody titers than BALB/c mice.
BALB/c mice (Fig. 3B). Again, GLA-SE outperformed SE in stimulating neutralizing antibodies directed against the vaccine strain of H1 as well as drifted strains of H5N1 (Fig. S2), and as indicated by the relative changes in IgG2c and IgG1 expression, GLA induced a significant shift from a Th2 to Th1 type antibody response. To investigate this response further, we assayed mice for antigen-specific CD4+ T cells on days 7, 10, and 13 after immunization and confirmed that GLA-SE stimulated the induction of IFN-γ+ Th1 cells within 7 d (Fig. 4A), whereas SE induced a smaller frequency of IL-5+ Th2 cells that peaked on day 10 (Fig. 4B). These T-cell responses are required for protection because treatment of mice with a CD4 T-cell lineage-depleting MAb (GK1.5) eliminated vaccine-induced antibody responses (Fig. 4C) and protection (Fig. 4D).

CD4 T cells play a central role in priming heterosubtypic immune responses to influenza (15, 16). To assess adjuvant-mediated protection against a heterologous virus, we immunized C57BL/6 mice with 50 ng of rH5Indo protein cloned from the Clade 2.3 A/Indonesia/2005 virus and then challenged 14 d later with Clade 1 A/Vietnam/1203/04. Control antigens included rH5VN, rHA derived from the swine A/California/04/2009 (H1N1) virus (rSH1), and a nonrelevant HSV-2 recombinant capsid protein (Table 1). In sharp contrast to the homologous virus challenge, GLA-SE stimulated the induction of IFN-γ+ Th1 cells that peaked on day 10 to nearly all rH5Indo (Fig. 4C) and protection (Fig. 4D). Although antibody responses were undetectable before challenge, survivors in the GLA-SE group expressed larger antibody titers than ferrets treated with rH5 + SE and demonstrated improved tolerance to infection as measured by minimal to no weight loss, reduced spike in body temperature, and faster clearance of virus in nasal washes. In addition, GLA-SE appeared to perform better than SE in two heterologous challenge experiments. In the first, we used the same regimen as before with ferrets (4 per group) receiving one injection of 0.5 μg of rH5Indo followed by a challenge on day 28 with A/H5N1/Vietnam/1203/04. Survival in this experiment was as follows: naive (0/4), GLA-SE alone (0/4), rH5 alone (2/4), rH5 + SE (1/4), and rH5 + GLA-SE (3/4). As indicated in Fig. 6 A and B, GLA-SE induced a consistent cross-reactive antibody response directed against the challenge virus and demonstrated minimal to no weight loss in three of four ferrets. For the second heterologous challenge (Fig. 6C), animals received less antigen (0.3 μg of rH5Indo) and were challenged sooner on day 14 after immunization. Survival in this experiment was as follows: naive (0/4), GLA-SE alone (1/4), rH5 alone (0/4), rH5 + SE (2/4), and rH5 + GLA-SE (3/4). Although antibody responses were undetectable before challenge, survivors in the GLA-SE group appeared to retain normal body weight, but the group sizes were too small to measure significant differences. Collectively, these results demonstrate that a single submicrogram dose of adjuvanted rH5 will effectively protect ferrets against lethal H5N1 and, as with mice, GLA-SE improves vaccine-mediated protection against a homologous virus and augments immunological priming against a drifted strain of H5N1.

Discussion

We tested whether combination of two clinically validated adjuvant strategies would strengthen priming of a recombinant subunit vaccine. Oil-in-water emulsions, comprised of squalene nanodroplets (~160 nM), successfully augment influenza vaccines in humans (5, 6, 15). These adjuvants stimulate local inflammatory responses at the injection site, which lead to leukocyte recruitment, antigen transport to draining lymph nodes, and APC activation (17). In preclinical models, adjuvants like MF59 appear to stimulate lymphocyte priming through a TLR-independent MyD88-dependent pathway (18) and subsequent Th2-mediated antibody responses (19), whereas the majority of helper CD4 T cells induced in people are biased to a Th0/Th1 phenotype (20, 21). A separate strategy for inducing anti-viral Th1 responses involves activation of TLR4, which is unique among the TLR family in its ability to activate MyD88 and IFN production through the TRIF pathway (8). To date, the only licensed TLR4 agonist is the detoxified LPS derivative, MPL (9).
Protective immune responses in rH5-vaccinated mice were induced within 4–6 d, kinetics similar to seasonal influenza mouse models (22). Neither antigen nor adjuvant alone was protective against H5N1 challenge, and survival required codeelivery of each at the site of injection. A single small dose (50 ng) of rH5 adjuvanted with either SE or GLA-SE was effective in priming protective immunity against a high-titer H5N1 challenge, although GLA-SE accelerated survival onset and the rate of recovery over a 50-fold range of rH5, and induced greater HI and total IgG titers in two mouse strains. In addition, GLA-SE was more effective than SE in priming cross-clade antibody responses (Fig. S2; refs. 12 and 13) and protecting vaccinated mice against a heterosubtypic strain of virus. This trait for an increased breadth of response will be important for preventing infection against newly emerging H5N1 viruses. Another distinguishing feature of GLA-SE is its ability to amplify a Th1 CD4 T-cell response; animals immunized with GLA-SE produced >30-fold more IFN-γ+ CD4 T cells than the number of IL-5+ CD4 T cells stimulated with SE, which led to a significant IgG1 to IgG2c shift in antibody expression. Mechanistically, antibody responses are critical for anti-influenza protection (23, 24). Consistent with this fact, we showed that B cells were required for rH5-induced protection and that conditions that increased antibody production improved recovery from infection. This relationship was observed by modifying the vaccine with more antigen or using GLA-SE, and by comparing responses between mouse strains where C57BL/6 mice expressed detectable day 14 antibody titers at lower antigen doses and recovered faster from virus than BALB/c mice. Undoubtedly, CD4 helper T cells are critical in this regard because depletion of this lineage during rH5 priming eliminated antibody production (25, 26). Protection may also have been influenced by the induction of cytotoxic CD4 T cells or after CD4-mediated help of killer CD8 T cells (27), although antigen-specific CD8 T cells were not observed in peptide stimulated splenocytes before challenge.

Ferrets are considered the best animal model for investigating influenza pathobiology and vaccine development (28) and have been used to characterize adjuvant-induced gene activity and TLR4 expression (29). Similar to our findings in mice, adjuvants significantly improved priming of rH5-dependent protection against a homologous and heterologous virus in outbred ferrets, and GLA-SE outperformed SE in mediating survival and reducing the severity of infection as measured by changes in body weight, temperature, and viral clearance. Also similar to mice, GLA-SE induced a greater antibody response in ferrets before viral challenge, which most likely improved protection afforded by the vaccine.

Large numbers of safe and effective vaccine doses are needed to protect the world against novel strains of pandemic H5N1 Influenza. A variety of egg-independent vaccine strategies designed to accelerate manufacturing capacity and improve immunogenicity are being developed (30). We have shown that one submicrogram injection of rH5 produced in insect cells is protective against H5N1 challenge, and survival required codeelivery of each at the site of injection. A single small dose (50 ng) of rH5 adjuvanted with either SE or GLA-SE was effective in priming protective immunity against a high-titer H5N1 challenge, although GLA-SE accelerated survival onset and the rate of recovery over a 50-fold range of rH5, and induced greater HI and total IgG titers in two mouse strains. In addition, GLA-SE was more effective than SE in priming cross-clade antibody responses (Fig. S2; refs. 12 and 13) and protecting vaccinated mice against a heterosubtypic strain of virus. This trait for an increased breadth of response will be important for preventing infection against newly emerging H5N1 viruses. Another distinguishing feature of GLA-SE is its ability to amplify a Th1 CD4 T-cell response; animals immunized with GLA-SE produced >30-fold more IFN-γ+ CD4 T cells than the number of IL-5+ CD4 T cells stimulated with SE, which led to a significant IgG1 to IgG2c shift in antibody expression. Mechanistically, antibody responses are critical for anti-influenza protection (23, 24). Consistent with this fact, we showed that B cells were required for rH5-induced protection and that conditions that increased antibody production improved recovery from infection. This relationship was observed by modifying the vaccine with more antigen or using GLA-SE, and by comparing responses between mouse strains where C57BL/6 mice expressed detectable day 14 antibody titers at lower antigen doses and recovered faster from virus than BALB/c mice. Undoubtedly, CD4 helper T cells are critical in this regard because depletion of this lineage during rH5 priming eliminated antibody production (25, 26). Protection may also have been influenced by the induction of cytotoxic CD4 T cells or after CD4-mediated help of killer CD8 T cells (27), although antigen-specific CD8 T cells were not observed in peptide stimulated splenocytes before challenge.

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this adjuvant may serve an important public health role in saving lives.

Materials and Methods

Animals, Immunization, and Virus Challenge. Female C57BL/6, BALB/c mice (Jackson Laboratories), and BALB/c JmO1D Thy1.1 mice (Tacconi) were maintained under pathogen-free conditions, and all experimental protocols were conducted in accordance with the guidelines of the Influenza Disease Research Institute and Colorado State University Animal Care and Use Committees. Male fitch ferrets (Triple F Farms) were maintained at the Rocky Mountain Regional Biocontainment Laboratory, Colorado State University. Recombinant H5N1 hemagglutinin protein (Protein Sciences Corporation) was mixed on the day of immunization with the following adjuvants; PBS, SE [10% oil-in-water SE], or GLA-SE (20% GLA) as previously described (10).

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specific peptides, and anti-CD3 plus anti-CD28 mAb were included in each assay as negative and positive controls.

**Statistical Analysis.** Comparisons between vaccinated groups were performed by using a nonparametric one-way ANOVA with the Tukey multiple comparison test and Fisher's exact test, and survival data were performed by using the Log-rank (Mantel–Cox) test. The analyses were performed by using GraphPad Prism version 5.00 for Windows (GraphPad Software). P values of <0.05 were considered to be significant.

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**Supporting Information**

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**Fig. S1.** GLA-SE accelerates recovery from infection. (A) BALB/c mice (5 per group) were injected with 50 ng of rH5VN alone or with adjuvant (SE, GLA-SE) and challenged 6 d or 14 d after immunization with 100 LD$_{50}$ A/H5N1/Vietnam/1203/04. Mice were then monitored for general health. Each point reports the mean clinical score ($\pm$SEM) by using a six-point grading system: 0, normal; 1, questionable illness; 2, mild illness; 3, moderate illness; 4, moribund (euthanized); 5, found dead. (B) Kinetics of virus clearance in BALB/c mice (12 per group) immunized with 50 ng of rH5VN alone or with adjuvant (SE, GLA-SE) and then challenged 14 d later with 1,000 LD$_{50}$ A/H5N1/Vietnam/1203/04. Each point is the average virus titer measured in mouse lung homogenate ($n = 3$), where 1 mL represents $\sim$20% of lung volume. The asterisk on day 5 indicates a significant difference in titer ($P = 0.014$) between mice immunized with GLA-SE and SE.

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**Fig. S2.** GLA-SE broadens H5N1 antibody responses. C57BL/6 mice (7 per group) were injected with rH5VN (100 ng) either alone or with adjuvant (SE, GLA-SE) and day 14 sera were assayed for anti-H5VN HI (Fig. 3B) and the indicated strains of drifted clade 1 and clade 2 viruses. The bar within each group reports the geometric mean titer and asterisk denotes statistical differences ($P < 0.05$) between groups adjuvanted with SE and GLA-SE.
Table S1. GLA augments antigen priming at the injection site

<table>
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C57B6 mice (5 per group) were vaccinated one time. For combined dosing, mice received antigen + adjuvant in each flank (50 μL) and for split dosing the antigen and adjuvant were injected in separate flanks (50 μL).

*The final amount of GLA injected in 2% stable emulsion.

†Mice were challenged after 14 days with H5N1 A/Vietnam/1203/2004 (1,000 LD₅₀) and monitored for percent survival.