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Induction of Broad-Based Immunity and Protective Efficacy by Self-amplifying mRNA Vaccines Encoding Influenza Virus Hemagglutinin

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ABSTRACT

Seasonal influenza is a vaccine-preventable disease that remains a major health problem worldwide, especially in immunocompromised populations. The impact of influenza disease is even greater when strains drift, and influenza pandemics can result when animal-derived influenza virus strains combine with seasonal strains. In this study, we used the SAM technology and characterized the immunogenicity and efficacy of a self-amplifying mRNA expressing influenza virus hemagglutinin (HA) antigen [SAM(HA)] formulated with a novel oil-in-water cationic nanoemulsion. We demonstrated that SAM(HA) was immunogenic in ferrets and facilitated containment of viral replication in the upper respiratory tract of influenza virus-infected animals. In mice, SAM(HA) induced potent functional neutralizing antibody and cellular immune responses, characterized by HA-specific CD4 T helper 1 and CD8 cytotoxic T cells. Furthermore, mice immunized with SAM(HA) derived from the influenza A virus A/California/7/2009 (H1N1) strain (Cal) were protected from a lethal challenge with the heterologous mouse-adapted A/PR/8/1934 (H1N1) virus strain (PR8). Sera derived from SAM(H1-Cal)-immunized animals were not cross-reactive with the PR8 virus, whereas cross-reactivity was observed for HA-specific CD4 and CD8 T cells. Finally, depletion of T cells demonstrated that T-cell responses were essential in mediating heterologous protection. If the SAM vaccine platform proves safe, well tolerated, and effective in humans, the fully synthetic SAM vaccine technology could provide a rapid response platform to control pandemic influenza.

IMPORTANCE

In this study, we describe protective immune responses in mice and ferrets after vaccination with a novel HA-based influenza vaccine. This novel type of vaccine elicits both humoral and cellular immune responses. Although vaccine-specific antibodies are the key players in mediating protection from homologous influenza virus infections, vaccine-specific T cells contribute to the control of heterologous infections. The rapid production capacity and the synthetic origin of the vaccine antigen make the SAM platform particularly exploitable in case of influenza pandemic.

Influenza is a viral infection that affects mainly nose, throat, bronchi and, occasionally, lungs. Most infected people recover within one to 2 weeks of infection without requiring hospitalization. However, in the very young, the elderly, and those with serious medical conditions, infection can lead to severe complications, including pneumonia and death. Vaccination is the best protection available against influenza. However, the constantly evolving nature of seasonal influenza viruses (antigenic drift) requires yearly review of vaccine strains and the sudden emergence of substantially different strains (antigenic shift) can lead to a pandemic. It was demonstrated in humans and in animal models that natural influenza virus infection confers protection against homologous and heterologous virus strains through CD4 and CD8 T cells mediated immunity (1–5). On the contrary, protective immunity induced by most inactivated influenza vaccines (IIV) has been correlated with antibodies directed to virion-expressed hemagglutinin (HA) (6–8). Finally, protection induced by live-attenuated influenza vaccines (LAIV) is not as well established but appears to correlate with several immune mechanisms, including cellular and mucosal immunity (3, 9–11), resulting in high level of heterosubtypic protection (12). Both IIV and LAIV require large-scale production of infectious virus, and the process of cultivation of the vaccine antigens in eggs (the source of the vast majority of vaccine) often alters the antigenic structure of the resulting vaccine. Production of novel influenza vaccines that avoid manufacturing constraints of current technologies is a recognized need. If

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these new vaccines were able to induce both antibody and cellular immunity, they could provide more effective protection against drifted variants of seasonal influenza viruses, and they could also reduce the impact of influenza virus pandemics. Adjuvanted IIVs that promote strong HA-specific CD4 T cell helper responses improve the cross-neutralization activity of HA-specific antibodies through the expansion of naive B cells with new specificities (for a review, see reference 13). In addition, memory CD4 T cells may also exert a direct effector function through the production of IFN-γ and perforin, and the activation of innate responses in influenza virus-infected tissues (14, 15). Finally, CD8 T-cell responses against influenza viruses are often generated toward conserved epitopes and contribute to heterosubtypic protection (16–18). Therefore, efforts are ongoing to generate new types of influenza vaccines able to induce protective antibodies against viral surface proteins, but also strong cellular immune responses essential at increasing the breath of protection in the case of an HA mismatch between the vaccine and circulating virus strains.

Influenza vaccines, based on live virus vectors such as poxvirus, adenovirus, or alphavirus (19–22), nucleic acid vaccines (23–27), or on virus-like particles (16, 28, 29) engineered to express influenza virus antigens induce cross-protective immune responses against different drifted strains of influenza. However, the potency of vectored vaccines may be limited by the concomitant induction of anti-vector immunity that interferes with subsequent vaccinations. Influenza vaccines based on noninfectious virus-like particles (VLPs) and produced both in insect and mammalian cells are immunogenic and protective in mice and ferrets (30–33). The VLPs technology exploits the self-assembly capacity and the budding properties of HA, neuraminidase (NA) and matrix protein (M1) antigens from the cell surface. Mimicking the virus structure makes VLPs highly immunogenic and able to activate both B- and T-cell responses (16, 34, 35), resulting in a very promising technology. However, there are still concerns related to the production capability, not only in terms of VLPs yield but also for the presence of cell-derived contaminants and vaccine purity (36).

Fully synthetic vaccines, based on nucleic acids such as DNA or RNA, are also being pursued. Plasmid DNA is stable, easy to produce and immunogenic in small animal models. However, the first DNA-based vaccines showed suboptimal potency in large animal models and humans. Recently, enhanced delivery technologies, such as electroporation (37, 38), and improved immunogenicity by triggering innate immune cells have increased the efficacy of DNA vaccines in clinical trials (39). Vaccines based on mRNA or RNA replicons are immunogenic in a variety of animal models, including nonhuman primates (40–44). RNA replicons are derived from the genomes of RNA viruses, such as alphaviruses, and have been engineered by eliminating the genes encoding the structural proteins and replacing them with genes of interest. Thus, RNA replicon-based vaccines are amplified in the cells of the vaccinated hosts, permitting the expression of the vaccine antigen, without generating virus particles. Furthermore, in the process of amplification of their genomes, RNA replicons engage pattern recognition receptors in the host cell, adjuvanting the responses to the encoded immunogen (45–47). Although both mRNA- and replicon RNA-based vaccines were shown to elicit antigen-specific antibody and cellular immune responses against several pathogens (44, 48–51), the self-amplifying nature of replicon-based vaccines is likely to result in higher levels of antigen expression and in a more effective engagement of innate immune responses than mRNA-based vaccine candidates.

We recently demonstrated that self-amplifying mRNA vaccines encoding influenza HA, and formulated with lipid nanoparticles were immunogenic in mice (43). In the present study, we demonstrate that SAM(HA) vaccine formulated with a novel cationic nanoemulsion (CNE) (50, 52) elicits broad and efficacious immune responses to influenza in mice and ferret models.

MATERIALS AND METHODS

RNA synthesis. RNA used for immunization was prepared as previously reported (48). Briefly, DNA plasmids encoding the self-amplifying RNAs were amplified in *Escherichia coli* and purified using Qiagen Plasmid Maxi kits (Qiagen). DNA was linearized immediately following the 3′ end of the self-amplifying RNA sequence by digestion with PmeI. Linearized DNA templates were transcribed into RNA using the MEGAscript T7 kit (Life Technologies) and purified by LiCl precipitation. RNA was then capped using the ScriptCap m^7G capping system (Cell Script) and purified by LiCl precipitation before formulation.

CNE/RNA formulation. CNE was prepared as previously described (50). RNA was prepared at a concentration of 300 μg/ml and was added to an equal volume of CNE, mixed, and allowed to complex on ice for 30 to 120 min. Prior to administration, formulations were diluted to dosing concentrations.

Influenza viruses. A/PR/8/1934 (H1N1) (PR8) (A. Wack, Francis Crick Institute, London, United Kingdom) and A/California/7/2009 (H1N1) (NYMC-X181) reassortant influenza virus (Cal) were grown on allantoic cavity of 10-day-embryonated chicken eggs. Viruses were stored at −80°C, titrated on MDCK cells and quantified as the 50% tissue culture infectious dose ($TCID_{50}$).

Animal *in vivo* studies. Ferret immunogenicity and efficacy studies were conducted at Lovelace Biomedical and Environmental Research Institute (Albuquerque, NM) in compliance with all applicable sections of the Final Rules of the Animal Welfare Act regulations. Groups of six ferrets (*Mustela putorius furo*) were immunized on study day 0 and 56 by intramuscular (i.m.) injection of SAM(H1)/CNE at 15 and 45 μg, H1N1 subunit (monovalent inactivated influenza vaccine [MIIV], 15 μg), MIIV+MF59 (15 μg), or CNE vehicle control. On day 84, all animals were challenged intranasally (i.n.) with a targeted dose of 8×10^5 to 2×10^6 PFU of influenza A/California/07/2009 (H1N1) virus/animal. Animals were observed twice per day for clinical signs of disease, from day 81 through day 98. Serum samples were collected on day 0, 28, and 70 and analyzed for functional antibodies.

Mouse immunogenicity and efficacy studies were conducted at Novartis Vaccines Animal Research Center in compliance with the ARRIVE guidelines and the current Italian legislation (legislative decree 116/92). Six-week-old female BALB/c mice were immunized i.m. (into both quadriceps, 50 μl per site) on days 0 and 56 with SAM(H1)/CNE (0.1 to 10 μg), MIIV (1 μg), MIIV+MF59 (1 μg), SAM(GFP) (10 μg), or saline as a negative control. Serum samples and spleens were collected on day 70 for immunological assessments. On day 84, mice were anesthetized and challenged i.n. with 20 $TCID_{50}$ of PR8 virus in 30 μl (15 μl/nostril). After infection, the mice were monitored daily and euthanized when they exhibited defined humane endpoints.

Determination of H1N1-specific serum antibody titers by ELISA. A two-step fully automated rapid enzyme-linked immunosorbent assay (ELISA; Hamilton Starlet System) was performed with individual sera to titrate antigen-specific IgG. Maxisorp plates (Nunc) were coated overnight at 4°C with 0.26 μg/well of monovalent egg-derived Cal/H1N1 antigen or PR8/H1 extracellular domain (Met_1-Gln_{528}) (Sino Biological, Inc.) and blocked for 1 h at 37°C with 200 μl of Smartblock solution (Candor Bioscience). Serum samples, serum standard, and controls were diluted in saline, 1% bovine serum albumin, and 0.05% Tween 20, transferred into coated-blocked plates, and 2-fold serially diluted. Antigen-specific total IgG and isotype antibodies were detected with alkaline phos-

phatase-conjugated goat anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2a (all from Sigma). The final titers were calculated as previously described (53).

HI assay. Hemagglutination inhibition (HI) assay for seasonal influenza strains was performed according to standard procedures. To inactivate nonspecific inhibitors, all serum samples were pretreated with receptor-destroying enzyme (Denka) according to the manufacturer's instructions. Sera were serially diluted 10- and 2-fold for ferrets and mice, respectively, and incubated with an equal volume of strain-specific influenza antigen (inactivated whole virus) for 60 min at room temperature. A 0.5% (vol/vol) suspension of turkey red blood cells was added, followed by incubation for another 60 min. The outcomes were determined by visual inspection. The HI titer was defined as the reciprocal of the serum dilution at which the last complete agglutination inhibition occurred.

VN assay. Virus neutralization (VN) was tested on pooled sera that had been heat-inactivated at 56°C for 30 min. Sera were serially diluted 2-fold in minimal essential medium with 1% penicillin-streptomycin-glutamine and 1:250 trypsin and were incubated 1 h at 37°C with 100 $TCID_{50}$ of Cal or PR8 virus. All samples were then incubated on MDCK monolayers in a 96-well plate (50,000 cells/well) for 18 h at 37°C. Cells were then washed with saline, fixed with 2% paraformaldehyde, and permeabilized with a solution of saline, 0.01% fetal calf serum (FCS) and 0.05% Tween. The expression of viral proteins was detected by ELISA with a monoclonal antibody (MAb) against matrix and nucleoprotein conjugated with fluorescein isothiocyanate (α-M/NP-FITC; Oxoid), followed by an anti-FITC polyclonal antibody conjugated with horseradish peroxidase (Roche). *o*-Phenylenediamine dihydrochloride (Sigma) was used as a substrate, and the absorbance was recorded at 450 nm. Serum titers were expressed as the reciprocal of the serum dilution that inhibited 50% of infection compared to control wells with virus alone. A titer of 10 was assigned to sera that gave a negative result at the first dilution tested (1:20).

Determination of virus titers. Tenfold serially diluted ferret nasal washes were added to MDCK cells previously seeded in flat-bottom 96-well plates to ≥90% of confluence and incubated at 37°C and 5% CO_2 for 60 min. The inoculum was then removed, 100 μl of methylcellulose overlay medium was added to the wells, and the plates were incubated at 37°C and 5% CO_2 for 24 h. The overlay was removed, and the cells were fixed for 30 min at 4°C with 4% paraformaldehyde, washed twice with saline, permeabilized with saline, 2% fetal bovine serum, and 0.05% saponin for 20 min at room temperature, washed twice, stained with an anti-influenza A NP antibody (Millipore) for 30 min at room temperature, washed, and incubated for 30 min at room temperature with alkaline phosphatase-conjugated goat α-mouse IgG secondary antibody (Millipore). The plaques were revealed with ELISA Vector blue stain (Vector Laboratories) and counted using a light microscope (Zeiss). Alternatively, whole mouse lungs were collected in Hanks balanced salt solution and homogenized using a Gentle MACS dissociator (Miltenyi). An aliquot was collected, centrifuged, and suspended in TRIzol (Life Technologies). Total RNA was extracted using phenol-chloroform, and cDNA generated using a ThermoScript RT-PCR system according to the manufacturer's instructions (Life Technologies). For the synthesis of cDNA, a universal primer (5′-AGCAAAAGCAGG-3′) specific for influenza A RNA segments was used in combination with random hexamers. The cDNA served as a template for the amplification of the influenza M1 gene and the eukaryotic *Hprt1* housekeeping gene by quantitative PCR (qPCR) using TaqMan gene expression assays (Applied Biosystems). The primer and probe set used was as follows: M1, forward (5′-AAGACCAATCCTGTCACCTCTGA-3′), reverse (5′-CAAAGCGTCTACGCTGCAGTCC-3′), and probe (FAM-5′-TTTGTGTTCACGCTCACCGT-3′-TAMRA); the *Hprt*-specific prime/probe set was obtained from Applied Biosystems. cDNA was denatured at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and then 60°C for 1 min. qPCR was performed with the LightCycler 480 system (Roche), and data were analyzed with LightCycler 480 software version 1.5.0 SP4 and Microsoft Excel Office 2010 (Microsoft). Relative gene expression was determined using the comparative $2^{\Delta\Delta CT}$ method (54).

Intracellular cytokine staining. Briefly, 1.5×10^6 splenocytes or 5×10^5 single-cell lung suspension were incubated for 4 h with Cal/H1 (JPT) or PR8/H1 (Department of Biochemistry, University of Lausanne, Lausanne, Switzerland) peptide pools in complete RPMI medium containing brefeldin A (Sigma) at 5 μg/ml and, when indicated, in the presence of anti-CD107a FITC (BD Biosciences). The cells were then stained with Live/Dead Yellow (Invitrogen), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and further incubated with anti-CD16/CD32 Fc-Block (BD Biosciences). T cells were stained with anti-CD3-PerCP-Cy5.5, anti-CD4-V500, anti-CD8-PE-Texas Red, anti-CD44-V450, anti-IFN-γ-PE Green, anti–IL-2-APC, and anti-TNF-Alexa 700 (all from BD Biosciences) and anti-IL-4-A488 and anti-IL-13-A488 (from eBioscience). Cells were then acquired on an LRSII special order flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

***In vivo* cytotoxicity.** BALB/c mice were immunized on day 0 and 56 with 10 μg of SAM(GFP), SAM(H1-Cal), and SAM(H1-PR8) delivered with CNE or preexposed to a sublethal dose of live PR8 virus. Ten days after the last immunization, all mice received i.v. a 1:1 mixture of syngeneic splenocytes pulsed with 5 μM cognate peptide IYSTVASSL ($HA_{533–542}$) and loaded with 5 μM carboxyfluorescein diacetate succinimidyl ester ($CFSE_{high}$) or pulsed with control peptide AMQMLKETI ($HIV_{197–205}$) and loaded with 0.5 μM CFSE ($CFSE_{low}$) (5×10^6 total splenocytes). After 20 h, the spleens were collected and analyzed by flow cytometry for CFSE-positive cells. The percentage of specific lysis (%SL) is calculated according to the following formula: $\%SL = [1- (R_{ctr}/R_{imm})]$, where $R = (CFSE_{low} + cells)/(CFSE_{high} + cells)$, ctr = control, and imm indicates immunized.

***In vivo* depletion of T cells.** CD8 T cells were depleted *in vivo* by the intraperitoneal (i.p.) injection of 100 μg of rat MAb 2.43 (IgG2b; BioXCell), and CD4 T cells were depleted using 100 μg of rat MAb GK1.5 (IgG2b; BioXCell). A combination of GK1.5 and 2.43 depleted both CD4 and CD8 T cells. As a control, mice were treated with rat MAb LTF-2 (IgG2b). All antibodies treatments were given on days −3, −1, and 1 relative to the PR8 infection.

Passive transfer of sera. BALB/c mice ($n = 18$) were immunized on days 0 and 56 with saline, 10 μg of SAM(H1-Cal), or 10 μg of SAM(H1-PR8). Two weeks after the second immunization, sera were collected, pooled, heat inactivated, and injected i.p. in naive BALB/c mice ($n = 6$), with each mouse receiving 300 μl of pooled serum. At 24 h after i.p. injection, mice were infected i.n. with 20 $TCID_{50}$ of PR8 virus.

Lung cell recruitment and *ex vivo* staining. Lungs were digested in the presence of collagenase D (2 mg/ml) and DNase (80 U/ml), both from Roche Diagnostics, for 30 min at 37°C. Single-cell suspensions were prepared with GentleMACS processor, and debris were removed by filtering through a 70-μm-pore-size cell strainer (BD Biosciences). Live cells were identified by labeling with Live/Dead Yellow (Life Technologies) and staining, after Cytofix (BD Biosciences) fixation, with anti-Ly6C-FITC, anti-CD11b-PE-Cy7, anti-Ly6G-PE, anti-CD11c-APC, anti-CD8-V500, anti-CD3-PerCP-Cy5.5 (all from BD Biosciences) and anti-MHCII-A700, anti-F4/80-eFluor450, anti-CD4-APC-Cy7 (all from eBioscience) in phosphate buffered saline (PBS)/Fc-Block (BD Biosciences). To characterize the effector/memory profile of antigen-specific CD8 T cells recruited to the lungs, live cells were stained with $HA_{533–542}$-specific H-$2K^d$ pentamer (ProImmune) PE-labeled for 20 min in PBS and 2% FCS. A mixture of antibodies was then added containing anti-CD3-PerCP-Cy5.5, anti-CD4-V500, anti-CD8-PE-Texas Red, anti-CD44-V450, anti-CD62L-APC, and anti-CD127-AF700 (BD Biosciences). Samples were acquired on an LSRII special order flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Statistical analyses. Statistical analyses were performed using GraphPad Prism 6.04 software. Experiments involving animal survival were analyzed by Mantle-Cox log-rank test. Standard one-way analyses of variance, followed by Tukey's or Dunn's multiple-comparison tests, were

used for evaluations involving more than two treatment groups unless otherwise indicated. *P* values of <0.05 were considered significant.

RESULTS

Generation of SAM vaccines expressing influenza virus HA. HA antigens from A/California/07/2009 (H1N1) (Cal) and A/PR/8/34(H1N1) (PR8) virus strains were cloned in SAM vectors. To generate SAM(H1-Cal) and SAM(H1-PR8) vaccines, RNAs were transcribed *in vitro*, capped, and formulated with CNE as described in Materials and Methods. Protection of CNE-complexed RNA molecules from RNase degradation was confirmed by agarose gel electrophoresis (see Fig. S1A in the supplemental material) (48). Western blot analyses with HA strain-specific antibodies confirmed that both SAM(H1-Cal) and SAM(H1-PR8) vaccines launched specific HA protein expression in transfected BHK cells (see Fig. S1B in the supplemental material).

Immunogenicity and efficacy of SAM(H1-Cal)/CNE in ferrets. Ferrets are generally accepted as an excellent animal model of human influenza disease (55). Hence, the immunogenicity and efficacy of SAM(H1-Cal) vaccines formulated with CNE were evaluated in ferrets. Groups of six animals were vaccinated i.m. twice, 8 weeks apart, with 15 or 45 μg of SAM(H1-Cal), with 15 μg of H1N1-Cal monovalent inactivated influenza vaccine (MIIV), with 15 μg of MIIV+MF59, or with CNE alone (negative control). Serum samples were collected 4 weeks after the first immunization and 2 weeks after the second immunization and analyzed for their content in HA-specific functional antibody by HI and VN assays. A single dose of SAM(H1-Cal) induced partial seroconversion (titer greater than 80) with 2 of 6 and 3 of 6 animals showing HI titers above the detection limit in the 15- and 45-μg groups, respectively (Fig. 1A). Only one ferret in the MIIV group showed an HI titer above the detection limit after a single vaccination, whereas all ferrets immunized with MIIV+MF59 seroconverted after a single dose of vaccine. Similar results were obtained using the VN assay (Fig. 1B). All animals in the SAM(H1-Cal) groups were efficiently boosted after the second immunization (Fig. 1A and B), showing VN titers above 80. All animals immunized with CNE alone control were determined to be seronegative by HI and VN.

To assess SAM(H1-Cal) vaccine efficacy, ferrets were challenged i.n. with a nonlethal dose of 10^6 PFU of pandemic influenza Cal virus 4 weeks after the second immunization. Five days after challenge, nasal washes were collected and analyzed for the presence of infectious viral particles by plaque assay. The animals receiving SAM(H1-Cal) (15 and 45 μg) or MIIV (with or without MF59) had significantly lower virus titers than the control animals (Fig. 1C). Animals were also monitored for 14 days after challenge for clinical signs of influenza, among the others also for weight loss (see Fig. S2 in the supplemental material) (56). The infectious dose of influenza virus administered to the animals, while nonlethal, induced signs of morbidity, indicated by a drop in weight of ≥10% (57). One of six and two of six ferrets vaccinated with 15 and 45 μg of SAM(H1-Cal), respectively, showed a weight loss greater than 10% (84 and 67% of healthy animals, Fig. 1D). Similarly, two animals of six in the group vaccinated with MIIV+MF59 experienced a weight loss. The highest morbidity was observed in the MIIV and CNE control immunization groups, with five ferrets of six showing weight loss equal or higher than 10% (17% of healthy animals) in each of these groups. Taken together, these data demonstrated that ferrets immunized with SAM(H1-Cal) had lower viral loads in their nasal cavity and lower morbidity after influenza virus infection compared to animals receiving CNE alone.

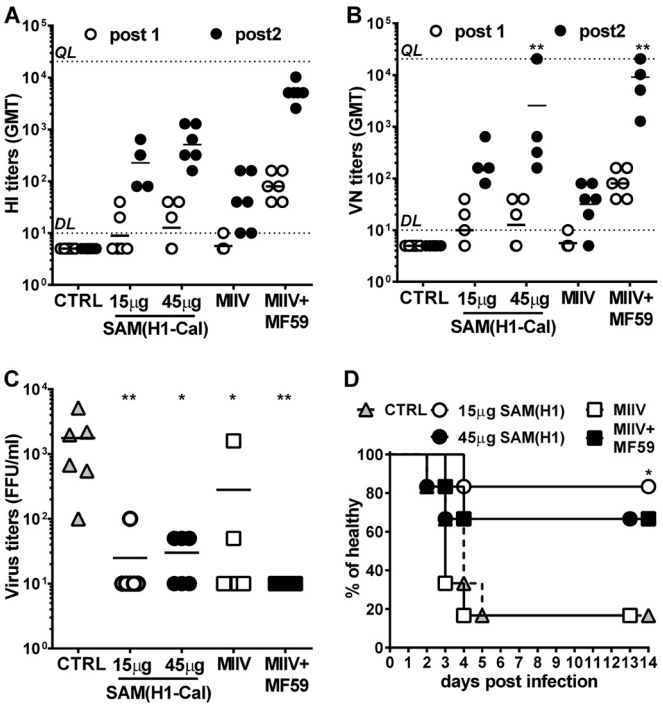

FIG 1 Immunogenicity and efficacy of SAM(H1-Cal)/CNE in ferrets. Ferrets ($n = 6$) were immunized i.m. on day 0 and day 56 with 15 or 45 μg SAM(H1-Cal)/CNE, 15 μg of MIIV, 15 μg of MIIV+MF59, or CNE alone (CTRL). Sera were collected on day 28 (post1) and day 80 (post2) and analyzed for HI (A) and VN (B) titers. *QL*, quantification limit; *DL*: detection limit. *, $P < 0.05$; **, $P < 0.01$ (compared to MIIV). (C) On day 94, ferrets were infected i.n. with 10^6 PFU of influenza Cal virus per animal. Five days after challenge, nasal washes were collected, and virus titers were determined as focus-forming units (FFU)/ml. *, $P < 0.05$; **, $P < 0.01$ (compared to CTRL). (D) Onset of illness as indicated by a loss of 10% of weight relative to the weight on the day of challenge. *, $P < 0.05$ (compared to CTRL).

SAM(HA)/CNE induces functional antibody responses in mice. To characterize humoral responses induced by SAM(HA)/CNE, BALB/c mice were immunized i.m. twice, 8 weeks apart, with SAM(H1-Cal)/CNE. As positive controls, mice were immunized with 1 μg of Cal/H1N1 MIIV or MIIV+MF59. As a negative control, mice were immunized with 10 μg of SAM(GFP)/CNE. Two weeks after the second immunization, sera were collected and analyzed for the presence of functional H1-specific IgG (Fig. 2).

SAM(H1-Cal) elicited H1-specific IgG titers in a dose-dependent manner (Fig. 2A). At the highest dose of SAM(H1-Cal), H1-specific IgG titers were comparable to that observed with MIIV. The functionality of the H1-specific IgG elicited by the vaccines was evaluated further by determining serum HI titers (Fig. 2B). As for IgG responses, SAM(H1-Cal) vaccine induced levels of functional antibodies comparable to MIIV.

SAM(HA)/CNE induces functional T-cell responses in mice. To characterize cellular immune responses induced by SAM(HA), BALB/c mice were immunized with 10 μg of SAM(H1-Cal) or with 1 μg of H1N1-Cal MIIV with or without MF59 adjuvant as benchmarks. Two weeks after the second immunization, splenocytes were collected and stimulated *in vitro* with a pooled peptide

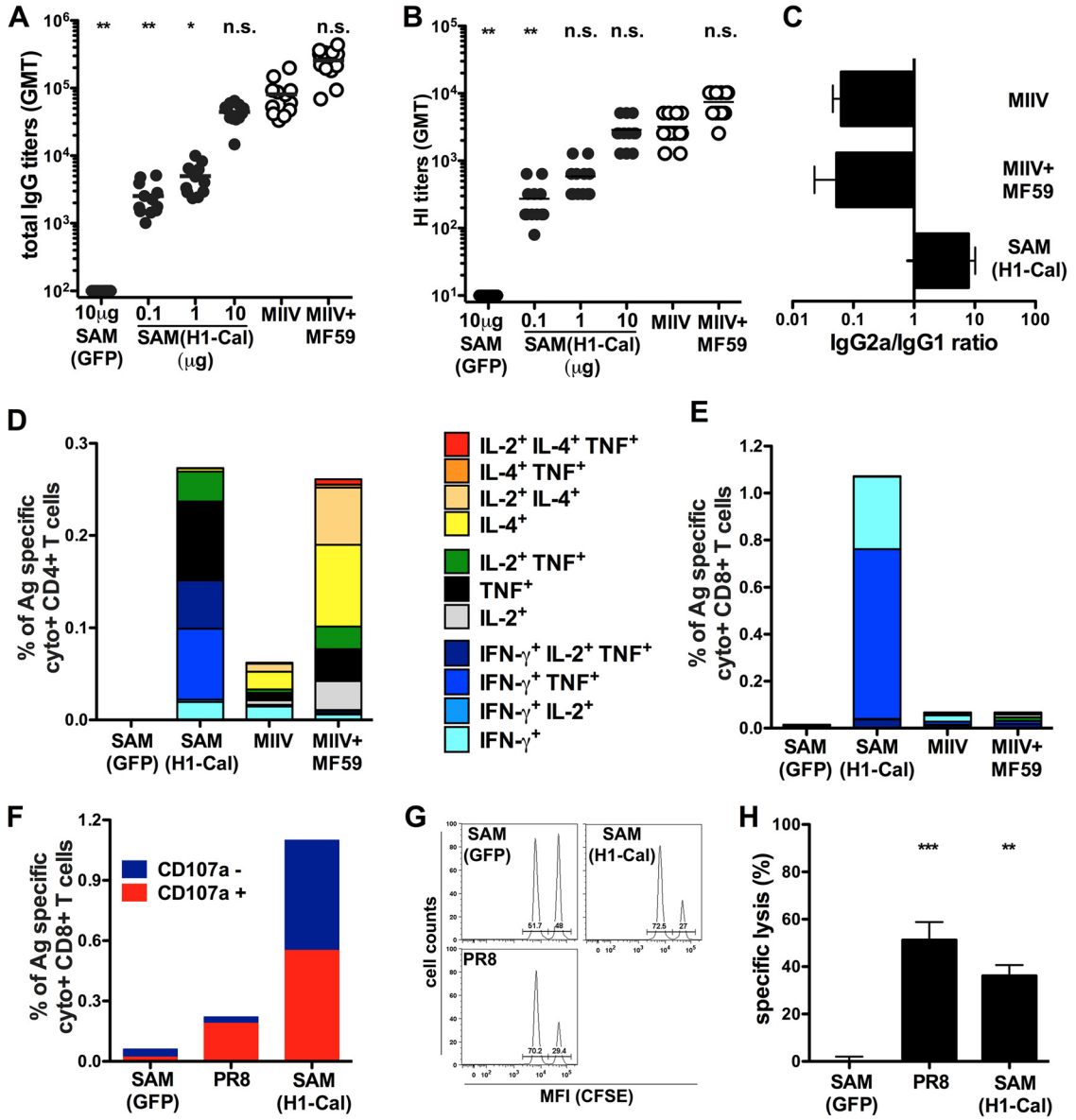

FIG 2 Immunogenicity of SAM(H1-Cal) in mice. Mice ($n = 12$) were immunized i.m. on day 0 and day 56 with SAM(GFP) at 10 μg, SAM(H1-Cal) at 0.1, 1, or 10 μg, MIIV at 1 μg, or MIIV+MF59 at 1 μg. Sera and spleens were collected 2 weeks after the second immunization. Sera were analyzed for H1N1-specific total IgG titers (A), HI titers (B), and IgG2a/IgG1 ratios (C). *, $P < 0.05$; **, $P < 0.01$ (compared to MIIV). (D to F) Splenocytes ($n = 4$) were stimulated *in vitro* with a H1-Cal peptide pool, and T cells were analyzed for cytokine production by flow cytometry (see Fig. S3A and B in the supplemental material for the gating strategy). The bars represent the cumulative frequency of H1-specific CD4 T cells (D) and CD8 T cells (E) expressing combinations of cytokines, as indicated in the graph. (F) CD107a expression by CD8 T cells. (G to H) *In vivo* cytotoxicity. CFSE-labeled $H1_{533–541}$ or HIV-$Gag_{107–205}$ -pulsed target cells were administered i.v. to mice previously immunized with SAM(GFP) or SAM(H1-Cal) or exposed to a sublethal dose of PR8 virus. Splenocytes were harvested 20 h later and analyzed for the presence of $CFSE^+$ target cells by flow cytometry, as described in Materials and Methods. (G) Representative histograms showing the percentage of $CFSE_{high}$ and $CFSE_{low}$ target cells recovered. (H) Percentage of specific target cell lysis. **, $P < 0.01$; ***, $P < 0.001$ [compared to SAM(GFP)]. The data shown are merged data from three independent experiments.

library spanning the entire Cal/H1 antigen sequence. The frequencies of Cal-H1-specific CD4 and CD8 T cells were determined by flow cytometry analysis, by measuring expression levels of intracellular cytokine (gamma interferon [IFN-γ], tumor necrosis factor [TNF], interleukin-2 [IL-2], IL-4, and IL-13) (Fig. 2D and E; see also Fig. S3 in the supplemental material).

SAM(H1-Cal) elicited H1-specific CD4 T-cell responses with frequencies comparable to those elicited by MIIV+MF59 and superior to MIIV (Fig. 2D). Furthermore, SAM(H1-Cal) shifted the T helper (Th) cell profile toward a Th0/Th1 phenotype, dominated by the production of Th1-associated IFN-γ and the combinations IFN-γ/TNF and IFN-γ/TNF/IL-2. In contrast, MIIV+MF59 elicited a Th0/Th2 profile dominated by the production of Th2-associated IL-4/IL-13 and the combination IL-4/IL-13/IL-2 (Fig. 2D). The polarization of the Th profile is also reflected in the IgG2a/IgG1 isotype ratio, where SAM(H1-Cal) strongly induced H1-specific IgG2a subclasses (Fig. 2C).

H1-specific CD8 T cells were observed in splenocytes of mice

immunized with SAM(H1-Cal), whereas they were not detected in MIIV- and MIIV+MF59-immunized mice (Fig. 2E). The majority of H1-specific CD8 T cells expressed IFN-γ and TNF, cytokines generally associated with an effector phenotype. In addition to intracellular cytokine expression, cell surface expression of CD107a, a specific marker for degranulation associated with cytotoxic activity, was also observed. Approximately half of the total H1-specific CD8 T cells induced by SAM(H1-Cal) stained positive for CD107a (Fig. 2F). To assess the cytotoxic potential of H1-specific CD8 T cells, we performed an *in vivo* cytotoxicity assay. CFSE-labeled naive BALB/c splenocytes were pulsed with H2-K^d-restricted $HA_{533–541}$ peptide (5 μM CFSE [$CFSE_{high}$]) or with HIV-$Gag_{197–205}$ peptide (0.5 μM CFSE [$CFSE_{low}$]) as a control. These two cell populations were mixed 1:1 and injected i.v. in mice previously immunized with SAM(H1-Cal) or sublethally infected with PR8 virus. The next day, the frequencies of $CFSE^+$ cells recovered in spleens were determined by flow cytometry and provided a measure for CD8 T cell lytic activity (Fig. 2G and H). SAM(H1-Cal)-immunized mice showed (49% ± 6%)-specific lysis activity, defined as lysis of target cells pulsed with $HA_{533–541}$ peptide relative to $Gag_{197–205}$ peptide-pulsed target cells. These frequencies were comparable to that observed in PR8 virus-infected animals and were consistent with the expression of CD107a observed *in vitro*.

BALB/c mice immunized with SAM(H1-PR8) displayed a similar immunogenicity profile as animals immunized with SAM(H1-Cal) (see Fig. S4 in the supplemental material). SAM(H1-PR8) at a 10-μg dose elicited functional antibodies titers comparable to a sublethal infection (see Fig. S4A and B in the supplemental material), whereas the soluble recombinant HA protein (rHA) was unable to elicit neutralizing antibodies in the absence of MF59. Similar to SAM(H1-Cal), SAM(H1-PR8) elicited antigen-specific CD4 and CD8 T cells (see Fig. S4C and D in the supplemental material). HA-specific CD4 T cells elicited by SAM(H1-PR8) were induced with frequencies comparable to rHA-PR8+MF59 but showed a cytokine profile similar to PR8 virus infection. HA-PR8-specific CD8 T cells expressed IFN-γ and TNF and were positive for CD107a (see Fig. S4D and E in the supplemental material). Consistent with their effector profile, HA-PR8-specific CD8 T cells were associated with a (41% ± 7%)-specific lysis activity *in vivo*.

SAM(HA)/CNE protects mice from a lethal dose of influenza virus. Since immunization with SAM(HA) controlled influenza virus infection in ferrets and was immunogenic in mice, we further assessed its efficacy in mouse models of homologous and heterologous influenza virus infection (Fig. 3).

For homologous challenge, BALB/c mice were immunized i.m. twice, 8 weeks apart, with 0.01 to 10 μg of SAM(H1-PR8), and challenged 4 weeks after the second immunization with a lethal dose of PR8 virus. Immunization with SAM(H1-PR8) protected mice from death (Fig. 3A) and weight loss (Fig. 3D) at all of the RNA doses tested. In contrast, all mice immunized with SAM(GFP) control vaccine died within 7 days after challenge.

For heterologous influenza virus infection, BALB/c mice were immunized with SAM(H1-Cal), as described above, and challenged with a lethal dose of PR8 virus. Immunization with SAM(H1-Cal) protected mice from death (Fig. 3B) and weight loss (Fig. 3E) after lethal heterologous challenge. At the highest RNA dose tested (10 μg), the survival rate was 87.5% and decreased in a dose-dependent manner to 10% in mice immunized with 0.01 μg of RNA. All animals showed signs of disease during the course of the observation, with transient weight loss peaking at day 4 postinfection in the surviving mice.

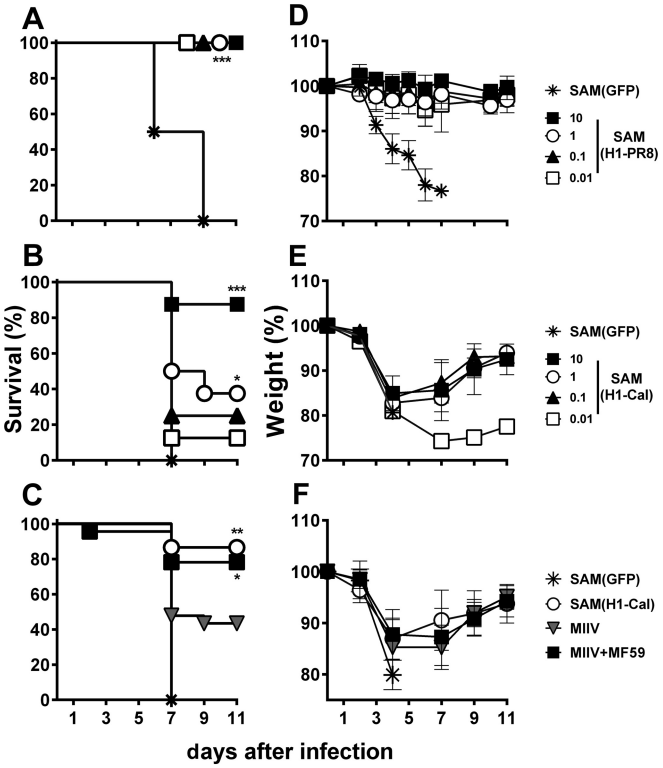

FIG 3 SAM(H1) protects mice from lethal infection. Mice ($n = 8$) were immunized i.m. on day 0 and day 56. Four weeks after the second immunization, mice were challenged with 20 $TCID_{50}$ of PR8 virus. Survival (A, B, and C) and body weight (D, E, and F) were monitored for 11 days postinfection. Immunizations were as follows: 10 μg of SAM(GFP) and SAM(HA-PR8) at the indicated doses (A and D), 10 μg of SAM(GFP) and SAM(HA-Cal) at the indicated doses (B and E), and 10 μg of SAM(GFP) or SAM(H1-Cal) or 1 μg of MIIV or MIIV+MF59 (C and F). The percent survival was determined based on humane endpoint criteria. Statistical analysis was performed using Mantel-Cox test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [compared to SAM(GFP) (A and B) or MIIV (C)]. The data shown are merged data from three independent experiments.

To compare the efficacy of SAM(H1-Cal) and MIIV vaccines in mediating protection against a heterologous influenza virus challenge, BALB/c mice were vaccinated twice i.m. with 10 μg of SAM(H1-Cal), 1 μg of MIIV, or 1 μg of MIIV+MF59. Four weeks after the second immunization, mice were infected with a lethal dose of PR8 virus. Mice immunized with SAM(H1-Cal) and MIIV+MF59 showed comparable survival rates (86 and 78%, respectively), while mice that had received MIIV showed a significantly lower survival rate (43.5%) (Fig. 3C). Weight loss peaked at day 4 after infection in all three influenza vaccine groups (Fig. 3F).

Viral load and cellular recruitment in the lungs after challenge with influenza virus. Since lungs are important sites of influenza virus infection, we characterized pulmonary viral loads and immune responses in mice vaccinated with SAM(H1-Cal) after mismatched heterologous challenge with PR8 virus.

Mice were immunized twice with saline, 10 μg of SAM(H1-Cal), or preexposed to a sublethal dose of PR8 live virus, and challenged 4 weeks later with a lethal dose of PR8 virus. Lungs

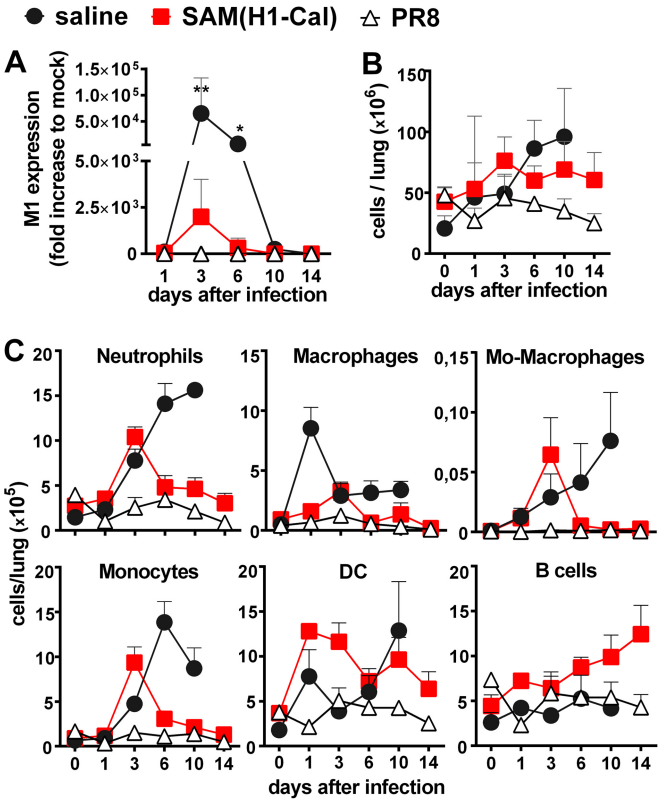

FIG 4 Immune cells are recruited in the lungs after influenza virus challenge. Mice were immunized i.m. on day 0 and day 56 with saline or 10 μg of SAM(H1-Cal) or were exposed to a sublethal dose of PR8 virus. Four weeks after the second immunization, mice were infected with 20 $TCID_{50}$ of PR8 virus. Lungs ($n = 3$) were collected at different time points after infection as indicated. (A) Viral loads expressed as the fold change compared to noninfected lungs. *, $P < 0.05$; **, $P < 0.01$ (compared to saline). (B) Total cell recruited in the lung. (C) Immune cell recruitment following the gating strategy described in Fig. S5A in the supplemental material. The data shown are merged data from two independent experiments.

were collected immediately before or at different time points after challenge, and organ homogenates were prepared to determine viral load by quantitative RT-PCR analysis using the influenza virus M1 target. Mice immunized with SAM(H1-Cal) limited virus replication after challenge and had an average titer increase in M1 expression of (2×10^3)-fold at day 3, with almost complete clearance of viral particles by day 6 (Fig. 4A). In contrast, saline-treated mice showed (5.8×10^4)- and (1.2×10^4)-fold increases in M1 expression on days 3 and 6 after challenge, respectively. No viral nucleic acids were detected in the lungs of mice that were challenged after preexposure to a sublethal dose of live PR8 virus.

To monitor the levels of inflammation and the type of immune cells recruited in response to viral challenge, lungs from mice immunized and infected as previously described were isolated at 1, 3, 6, 10, and 14 days postinfection and analyzed by flow cytometry (see Fig. S5A of the supplementary material for the gating strategy). In SAM(H1-Cal)-immunized mice, the recruitment of innate cells such as neutrophils, monocytes, monocyte-derived macrophages and macrophages, peaked on day 3, and returned to baseline on day 6 after challenge, whereas the number of dendritic cells peaked on day 1 (Fig. 4B and C). In contrast, in saline-treated mice, the number of all cell types, except macrophages, continued to increase between days 6 to 10. B cells remained stable until day 3 and then showed a continuous increase up to day 14, but only in mice immunized with SAM(H1-Cal).

The impact of SAM(H1-Cal) vaccination on the recruitment of CD4 and CD8 T cells upon infection was also assessed (Fig. 5A and D). CD4 T cells showed a peak on day 1, followed by a rapid decline back to the initial basal level. CD8 T cell recruitment was instead characterized by two distinct phases: a primary phase with a peak on day 1 and a secondary phase with a peak on day 10 after infection. Saline-treated mice showed a similar pattern of T cell recruitment postinfection.

To characterize further the functionality of H1-specific lung T cells, single-cell suspensions were stimulated *in vitro* with a pool of H1-Cal overlapping peptides, and T-cell frequency and cytokine production were evaluated by flow cytometry. In mice immunized with SAM(H1-Cal), the frequencies of H1-specific cytokine$^+$ CD4 T cells were very low preinfection and, 24 h postinfection, increased from day 3 to day 10 and decreased thereafter (Fig. 5B). The pattern of cytokine expression showed a gradual increase in TNF^+, IFN-γ^+, and IFN-γ/TNF^+ cells, a hallmark of terminal effector CD4 T cells, peaking on day 10 after challenge, and representing about two-thirds of the H1-specific CD4 T cells in the lungs. This population contracted by day 14 to represent about half of the H1-specific CD4 T cells in the lungs, whereas IFN-γ/TNF/IL-2^+ and TNF/IL-2^+ CD4 T cells composed the other half. Unlike CD4 T cells, H1-specific TNF^+, IFN-γ^+, and IFN-γ/TNF^+ effector CD8 T cells were readily detectable in the lungs of SAM(H1-Cal)-immunized mice preinfection (0.7% of CD8 T cells) and were maintained with similar frequencies until 3 days postinfection (Fig. 5E). A major increase in H1-specific CD8 T cells, characterized by the production of IFN-γ/TNF/IL-2, was observed starting on day 6 (5% of CD8 T cells), peaking on day 10 (15%) and decreasing by day 14 (12%). In addition to secreting cytokines upon stimulation, most H1-specific CD8 T cells in the lungs were $CD107a^+$ at all time points after infection, indicating a cytotoxic functional phenotype (Fig. 5F), whereas none of the H1-specific CD4 T cells expressed this degranulation marker (Fig. 5C). Despite that the number of lung T cells recruited in saline-treated mice was comparable to SAM(H1-Cal)-immunized animals, lungs of control mice showed low frequencies of H1-specific cytokine$^+$ CD4 and CD8 T cells only on day 10 postinfection (0.6% of the total CD4 cells and 2% of the total CD8 cells), with combinations of IL-2 and TNF and of IFN-γ and TNF, respectively. Lungs of PR8 preexposed animals did not show T cell recruitment or activation during the course of the observation (see Fig. S5B in the supplemental material).

Finally, CD8 T cells specific for the immunodominant H1-Cal epitope ($HA_{533–541}$) were identified by staining with a H-$2K^d$/$HA_{533–541}$ pentamer to characterize the memory phenotype of the CD8 T cells recruited in the lungs after PR8 virus infection. The number of pentamer$^+$ CD8 T cells gradually increased until day 10 postinfection in SAM(H1-Cal)-immunized mice (Fig. 5G). At the peak of recruitment, the phenotype of lung pentamer$^+$ CD8 T cells in SAM(H1-Cal)-immunized animals was mainly effector memory cells ($CD44^{hi}$ $CD62L^{lo}$ $CD127^{hi}$, TEM, 3×10^4 cells/lung), some effector cells ($CD44^{hi}$ $CD62L^{lo}$ $CD127^{lo}$, Teff, 0.5×10^4 cells/lung) and very few central memory cells ($CD44^{hi}$ $CD62L^{hi}$ $CD127^{hi}$, TCM, 0.1×10^4 cells/lung). Pentamer$^+$ CD8 T cells were detected in the lungs of saline-control mice on day 10

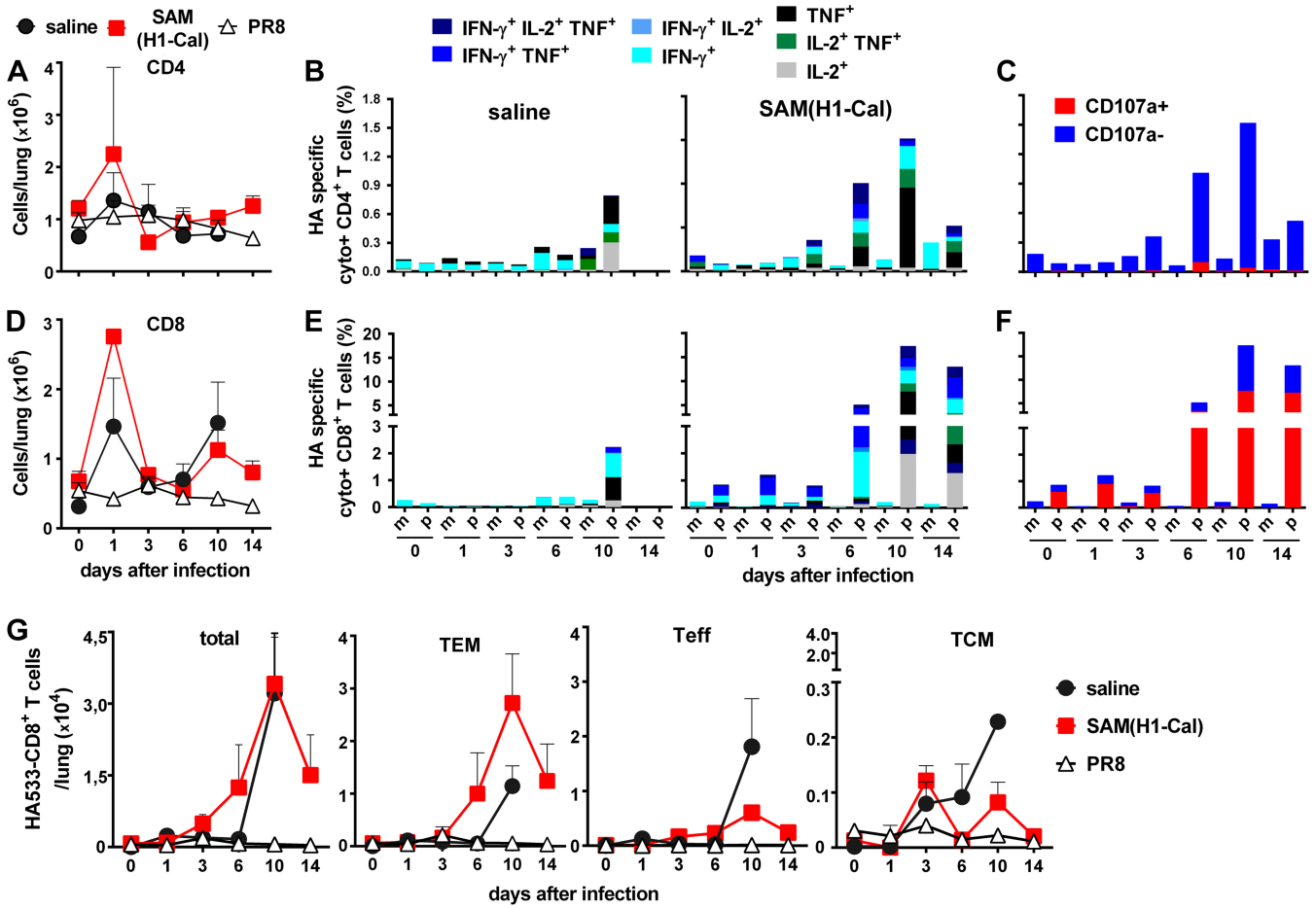

FIG 5 T-cell responses in lungs of infected mice. Mice were immunized i.m. on day 0 and day 56 with saline or 10 μg of SAM(H1-Cal) or exposed to a sublethal dose of PR8 virus. Four weeks after the second immunization, mice were infected with 20 $TCID_{50}$ of PR8 virus. Lungs ($n = 3$) were collected at different time points after infection and characterized for CD4 (A) and CD8 (D) T-cell recruitment. The frequencies of cytokine-producing (B and E) and $CD107a^+$ (C and F) T cells after *in vitro* stimulation with H1-Cal peptide pool (p) or medium (m) were also determined. (G) $H1_{533–541}$-specific CD8 T cells were identified *ex vivo* with PE-labeled H-$2K^d$/$HA_{533–541}$ pentamer and are characterized as T effectors (Teff, $CD44^{hi}$ $CD62L^{low}$ $CD127^{low}$), T effector memory (TEM, $CD44^{hi}$ $CD62L^{low}$ $CD127^{high}$), and T central memory (TCM, $CD44^{hi}$ $CD62L^{high}$ $CD127^{high}$). The data shown are merged data from two independent experiments.

postinfection and were mainly Teff and TEM cells (2×10^4 and 10^4, respectively).

Taken together, these results show that mice vaccinated with SAM(H1-Cal) and challenged with a mismatched influenza virus efficiently controlled viral replication with reduced recruitment of inflammatory cells and increased recruitment of polyfunctional CD4 Th1 and cytotoxic CD8 T cells in the lungs.

SAM(H1-Cal)-induced immunity contributes to protection against heterologous influenza virus challenge. Since HA-specific functional antibodies can prevent influenza virus from infecting cells of the host, sera from SAM(H1-PR8)- and SAM(H1-Cal)-immunized mice were evaluated for *in vitro* neutralizing activity against PR8 and Cal influenza viruses. Sera were completely strain specific, preventing infection only by the homologous virus strain and showing no cross-neutralization activity *in vitro* (Fig. 6A). To evaluate whether these sera could have other role in inhibiting influenza virus infection (e.g., through mechanisms of antibody-dependent phagocytosis or cytotoxicity), these sera were passively administered to naive mice 1 day before challenge with a lethal dose of PR8 virus. Sera from SAM(H1-PR8)- and SAM(H1-Cal)-immunized mice promoted the survival of 100 and 0% of the animals, respectively (Fig. 6B). These data suggest that sera from SAM(H1-PR8)-immunized mice are sufficient to control homologous virus infection. In contrast, sera from SAM(H1-Cal) did not cross-neutralize heterologous PR8 virus either *in vitro* or *in vivo*.

The cross-reactivity of CD4 and CD8 T cells against mismatched HA was assessed *in vitro* by stimulation of splenocytes from SAM(H1-Cal)-immunized mice with H1-Cal and H1-PR8 peptide pools and by quantification of the frequencies of cytokine-positive T cells by flow cytometry. CD4 and CD8 T cells responded to H1-Cal and H1-PR8 peptide pools, albeit to a lesser extent (Fig. 6C and D). These data suggest that some but not all T-cell epitopes are shared between the two viral strains. Finally, to confirm further the role of T cells in mediating protection against heterologous influenza virus infection, *in vivo* depletion studies were performed. Mice immunized with SAM(H1-Cal) were treated with anti-CD4, anti-CD8, anti-CD4+anti-CD8, or isotype control antibodies (see Fig. S6 in the supplemental material) prior to challenge with a lethal dose of A/PR/8/1934 (H1N1) virus. The survival rates 2 weeks after the infection were 53, 83, and 78% in the depleted groups, respectively, compared to 94% in the control

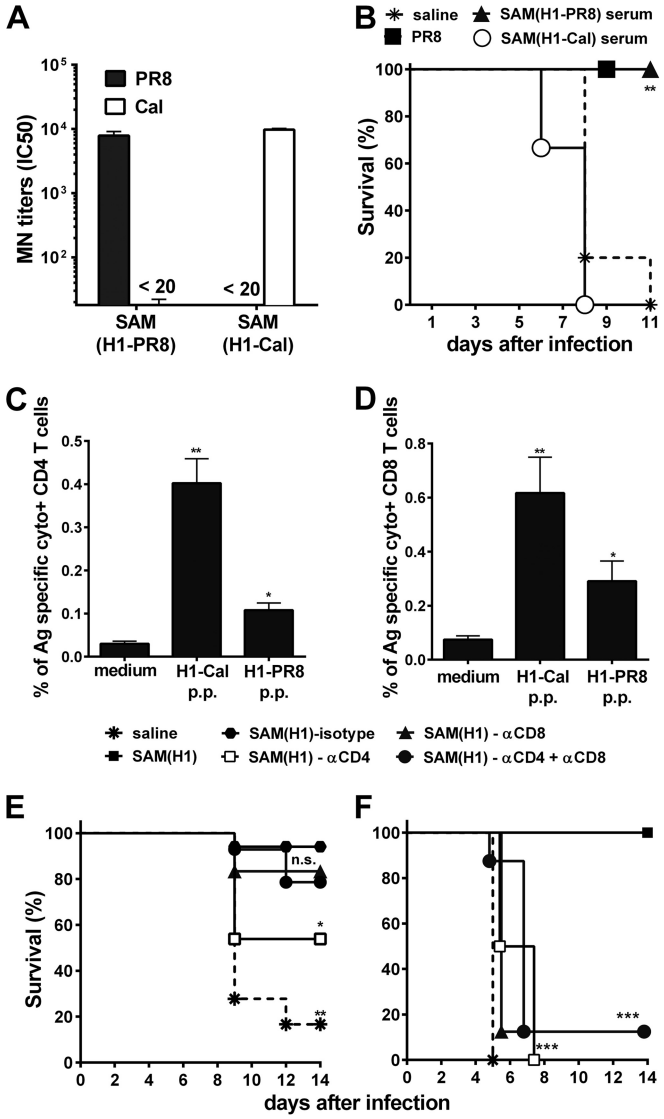

FIG 6 Role of T cells in mediating protection against lethal heterologous influenza virus challenge. Mice ($n = 8$) were immunized i.m. on day 0 and day 56 with 10 μg of SAM(H1-PR8) or SAM(H1-Cal). (A) Serum neutralization titers, corresponding to a 50% inhibition of infection (IC_{50}) by PR8 or Cal influenza viruses. The data show the means ± the standard deviations and are representative of four independent experiments. (B) Survival of mice after passive transfer of sera from saline-, SAM(H1-Cal)-, or SAM(H1-PR8)-immunized mice or mice exposed to a sublethal dose of PR8 virus prior to challenge with 20 $TCID_{50}$ of PR8 virus. **, $P < 0.01$ (compared to saline). (C and D) Frequency of H1-Cal/H1-PR8 cross-reactive cytokine$^+$ CD4 (C) and CD8 (D) T cells in splenocytes from SAM(H1-Cal)-immunized mice. The data show means ± the standard deviations and are cumulative of four independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared to medium). (E and F) Survival of SAM(H1-Cal)-immunized mice after depletion of CD4 and/or CD8 T cells and challenged with PR8 virus at 20 $TCID_{50}$ (E) or 100 $TCID_{50}$ (F). The percent survival was determined based on humane endpoint criteria. The data are cumulative for two independent experiments ($n = 16$). *, $P < 0.05$; ***, $P < 0.001$ (compared to treatment with an isotype control).

group (Fig. 6E). At this virus dose, only CD4-depleted mice showed a significant difference compared to control ($P < 0.05$).

To more rigorously test this model, the amount of virus challenge was increased 5-fold (Fig. 6F). Under these conditions, no CD4-depleted animals survived and only 12% in the CD8- and CD4/CD8-depleted mice. Altogether, these data suggest that SAM(H1-Cal)-induced T cell immunity contributes to protection against heterologous influenza virus infection.

DISCUSSION

We recently demonstrated that SAM vaccines encoding H1 and H7 influenza virus HA molecules delivered by lipid nanoparticles were immunogenic in mice (43). In the present study, we have extended those observations and shown that SAM(H1-Cal) formulated with CNE, a second-generation RNA delivery technology (50), elicited broad-based immunity sufficient to confer protection against both homologous and heterologous influenza viruses.

Numerous studies have reported the protective efficacy induced by HA-based vaccines in ferrets (24, 31, 58, 59). The majority of these studies, however, focused on pandemic HA for which the severity and lethality of the infection allow for a clear determination of vaccine efficacy. Recently, Petsch et al. demonstrated that three doses of 250 μg of mRNA encoding HA from a A/California/07/2009 (H1N1) clinical isolate were immunogenic in ferrets, resulting in seroconversion and HI titers comparable to licensed MIIV (44). In the present study, we demonstrate that all ferrets immunized with two doses of 45 μg of SAM(H1-Cal)/CNE seroconverted, with HI and VN titers significantly higher than MIIV. The data also confirmed previous observations that infection of ferrets with A/California/07/2009 (H1N1) virus induces a mild disease and that MIIV was not sufficient to limit viral shedding from the nose (60), whereas MF59-adjuvanted MIIV induced strong protection against the development of lesions and disease caused by influenza A/California/07/2009 (H1N1) virus infection (61). In addition, we showed that SAM(H1-Cal) vaccine limited viral replication in the nose and decreased the impact of influenza virus infection on animal health, similarly to MF59-adjuvanted MIIV. These data show that the SAM vaccine technology compares favorably with conventional influenza vaccines licensed for human use.

We utilized a mouse model of influenza virus infection to characterize the mechanisms of protection induced by SAM(HA) and address the role of H1-specific antibodies and T cells. In serum transfer experiments, humoral responses provided complete protection against homologous but not heterologous virus infection. The lack of cross-reactivity of antibodies induced by SAM(HA) vaccines was not surprising and was comparable to observations obtained with MIIV. The lack of cross-reactivity of MIIV in mice is consistent with the necessity for annual update of seasonal influenza vaccines. Progress toward improved cross-neutralizing antibody responses is being made using prime-boost immunization regimens involving different types of vaccines, such as DNA, viral vectors, and inactivated vaccines (62–66).

Despite a lack of cross-neutralizing antibodies, immunization with SAM(H1-Cal) provided protection from heterologous PR8 virus infection through the contribution of CD4 Th1 and CD8 cytotoxic T cells. These observations are in agreement with previous reports showing that cellular-mediated immunity elicited by vaccination with adjuvanted seasonal antigens (61) or by primary infection with influenza viruses mediated protection from influenza virus infection in mice and ferrets (67–69). The frequency of HA-specific CD4 T cells in humans was also associated with vaccine efficacy in humans (70).

A subset of H1-specific CD4 and CD8 T cells induced by SAM(H1-Cal) immunization was cross-reactive with H1-PR8, suggesting that these cross-reactive T cells might be part of the protective immune mechanism, as already demonstrated in humans and linked to reducing the severity of the disease (71). Indeed, H1-specific cross-reactive CD4 T cells might provide the necessary help to B cells (72) for faster production of H1-PR8-specific antibodies in response to viral infection that in turn may play a role in rapid containment of viral replication. Furthermore, antigen-specific CD4 T cells are known to be directly involved in helping the effector function of CD8 T cells (73, 74) and may also have a direct role in mediating viral clearance in the lungs through a variety of mechanisms, including the production of IFN-γ to create a cytokine environment inhibitory to viral replication (75). In effect, the control of influenza virus infection in mice has been associated with Th1 CD4 T cells secreting IFN-γ and IL-2 (76). SAM(HA) elicited CD4 T cells that are cross-reactive toward both H1-Cal and H1-PR8 at frequencies comparable to MF59-adjuvanted MIIV, and with a Th1 profile associated with increased antiviral functions, equivalent to the profile induced by a sublethal dose of PR8 virus. The role of Th1-polarized CD4 T cells has been highlighted also in the human population where preexisting influenza virus-specific Th1 CD4 T cells have been associated with a reduced disease severity (77) and the expansion of HA-specific IFN-γ^+ CD4 T cells correlated with increased neutralizing antibody titers (78). Finally, IFN-γ production and Th1 polarization have been shown to be crucial in containing influenza virus infection in the elderly (79). In our experimental settings, H1-specific multifunctional CD4 T cells were quickly recruited in infected lungs. Depletion experiments showed that, in the absence of CD4 T cells, the protection from heterologous infection was substantially reduced, confirming previous reports associating the presence of multifunctional cross-reactive memory CD4 T cells with heterologous immunity (70, 80).

H1-specific CD8 T cells induced by SAM(HA) are cross-reactive, multifunctional, with an effector cytokine profile and cytotoxic activity *in vivo*. H1-specific CD8 T cells were present in the lung after immunization and rapidly increased after viral infection compared to saline-treated mice. Moreover, the majority of H1-specific CD8 T cells recruited after viral infection in the lungs of SAM(HA)-vaccinated mice had an effector memory phenotype that may play a major role in viral clearance in the lungs (81). Depletion of CD8 T cells in SAM(HA)-immunized mice reduced survival rate upon lethal viral challenge, confirming previous observations by others regarding the functional contribution of HA-specific CD8 T cells in mediating viral clearance and conferring protection against influenza virus infection (16–18, 20, 82).

In summary, the SAM vaccine technology is an effective strategy for induction of broad T and B cell immune responses conferring protection in animal models. The combination of strain-specific neutralizing antibodies and cross-reactive T-cell responses could contribute to more effective vaccines to prevent pandemic and seasonal influenza, respectively, by protecting against infection by shifted and drifted strains of influenza virus.

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