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MAJOR ARTICLE

A Lipid/DNA Adjuvant–Inactivated Influenza Virus Vaccine Protects Rhesus Macaques From Uncontrolled Virus Replication After Heterosubtypic Influenza A Virus Challenge

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Background. Influenza A virus (IAV) vaccines offer little protection from mismatched viruses with antigenically distant hemagglutinin (HA) glycoproteins. We sought to determine if a cationic lipid/DNA complex (CLDC) adjuvant could induce heterosubtypic protection if added to a whole inactivated IAV vaccine (WIV).

Methods. Adult rhesus macaques (RMs) were vaccinated and at 2 weeks boosted with either an H1N1-WIV or an H3N2-WIV, with and without CLDC adjuvant. Four weeks postboost, animals were challenged with an H1N1 IAV matched to the H1N1-WIV vaccine.

Results. After challenge, viral RNA (vRNA) levels in the trachea of control RMs and RMs vaccinated with the unadjuvanted H1 or H3 WIV vaccines were similar. However, vRNA levels in the trachea of both the H1-WIV/CLDC– and the H3-WIV/CLDC–vaccinated RMs ($P < 0.01$ and $P < 0.05$, respectively) were significantly lower than in unvaccinated control RMs. Heterosubtypic protection in H3-WIV/CLDC RMs was associated with significantly higher levels of nucleoprotein (NP) and matrix-1–specific immunoglobulin G antibodies ($P < 0.05$) and NP-specific nonneutralizing antibody–dependent natural killer cell activation ($P < 0.01$) compared with unprotected H3-WIV RMs.

Conclusions. Addition of the CLDC adjuvant to a simple WIV elicited immunity to conserved virus structural proteins in RMs that correlate with protection from uncontrolled virus replication after heterosubtypic influenza virus challenge.

Keywords. cross-reactive; non-neutralizing antibodies; NK cell activation.

Seasonal influenza A virus (IAV) epidemics result in an estimated 3–5 million cases of severe respiratory illness worldwide, with 250 000–500 000 deaths annually [1]. Despite repeated natural exposure to IAV, most humans do not develop broad protective immunity to diverse IAVs [2]. The ability of novel IAVs to annually circumvent preexisting neutralizing antibodies (nAbs) is mostly attributed to accumulation of viral mutations in the hemagglutinin (HA) glycoprotein, or the introduction of an IAV strain with a novel HA subtype into the human population (reviewed in [3, 4]). Inactivated split-virion vaccines elicit strain-specific nAbs to the highly variable, immunodominant globular head of HA (HA1) [5, 6], and although they provide some protection in healthy adults, protection in the young, old, and immunocompromised is inconsistent [7, 8]. Moreover, none of these licensed IAV vaccines protect from disease caused by novel reassorted IAV strains that have been introduced into humans [9]. Thus, it is generally recognized that a universal IAV vaccine is needed that is broadly effective against IAV strains [2].

Whole inactivated IAV vaccines (WIVs) are mature virions containing the complete set of conserved structural proteins. While split IAV vaccines and WIVs elicit strain-specific HA-nAb responses [8, 10], WIVs can also elicit antibody [11–13] and cellular [14–17] responses to the abundant, immunogenic, and highly conserved M protein and nucleoprotein (NP). Natural IAV infection does not usually induce protective immune responses to these structural proteins. However, experimental IAV vaccines that produce immune responses to M and NP provide some protection against nonmatched IAV strains [18–20], suggesting that these responses are desirable in a universal IAV vaccine. Cationic lipid/DNA complex (CLDC) is an adjuvant composed of 1:1 molar ratio of cationic 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)

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imidazolinium chloride/cholesterol liposomes and noncoding plasmid DNA [21]. In mice and macaques, the addition of CLDC to influenza vaccines enhances virus-specific $CD4^+$ and $CD8^+$ T-cell responses and antibody responses [22–25]. The goal of this study was to determine if a CLDC adjuvant-H3N2 WIV could protect rhesus macaques from a heterosubtypic H1N1 IAV challenge.

MATERIALS AND METHODS

Animals

Adult rhesus macaques (RMs; *Macaca mulatta*) were housed at the California National Primate Research Center in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care International Standards. The Institutional Animal Use and Care Committee of the University of California, Davis approved these experiments. See the Supplementary Materials for additional details.

Virus Strains, CLDC Adjuvant, and Vaccine Preparation

The IAV A/Memphis/7/2001 (H1N1) stock, containing $10^{6.5}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL, used for all virus inoculations was grown in Madin-Darby canine kidney cells and has been previously described [26]. The whole inactivated A/Memphis/7/2001(H1-WIV) and A/Memphis/1/1990 (H3N2 [H3-WIV]) stocks were propagated in chicken eggs and sucrose gradient purified. The sucrose gradient–purified H1N1 contained $10^{7.8}$ $TCID_{50}$/mL, 0.25 mg H1N1/mL, and 1048 HA units/0.05 mL. The sucrose gradient–purified H3N2 contained $10^{4.0}$ $TCID_{50}$ /mL, 0.40 mg H3N2/mL, and 256 HA units/0.05 mL. Viral inactivation and protein sequence comparison are described in the Supplementary Methods. A sequence homology of H1-WIV and H3-WIV HA and neuraminidase is low (<41%; Supplementary Table 1). However, the M2 membrane and the internal NP, matrix-1 (M1), and PB2 proteins of H1-WIV and H3-WIV are 91%–96% homologous (Supplementary Table 1).

The CLDC adjuvant (JVRS-100, Colby Pharmaceutical) used in this study has been previously described [24]. The H1-WIV vaccines contained 5 μg of inactivated H1N1 (1048 HA units/0.05 mL) suspended in 0.5 mL 5% Dextrose in water (D5W) or 0.5 mL CLDC. The H3-WIV vaccines contained 5 μg inactivated H3N2 (HA units/0.05 mL) suspended in 0.5 mL D5W or 0.5 mL CLDC. See the Supplementary Materials for additional details.

Animal Immunization, Inoculation, and Sample Collection

Thirty-five adult monkeys were randomly assigned to 1 of 6 experimental groups (Table 1). All animals were immunized intramuscularly twice, 2 weeks apart. To test for protection against a matched IAV challenge, RMs were immunized with H1-WIV/CLDC (n = 5) or H1-WIV alone (n = 5). To test for protection against a heterosubtypic IAV challenge, RMs were immunized with H3-WIV/CLDC (n = 10) or H3-WIV alone (n = 5). Controls included RMs immunized with CLDC alone (n = 5) or unimmunized RMs (n = 5). However, as no differences in viral RNA (vRNA) levels were detected between control groups, data from these 2 groups were combined into a single WIV-naive control group (n = 10).

Blood samples were collected weekly preimmunization and postimmunization (PI). At week 6 PI, animals were challenged with the H1N1 A/Memphis/7/01 virus stock, and nasopharyngeal, tracheal secretions, and blood samples were collected on −7, −4, 1, 2, 3, 7, and 14 days after challenge, as previously described [27].

Viral RNA Quantitation

As previously described [26], the $\log_{10}$ concentration of influenza vRNA copies ($\log_{10}$ vRNA copies)/mL in respiratory secretions was quantified by reverse-transcription polymerase chain reaction, a method similar to that recommended by the World Health Organization (WHO) and the US Centers for Disease Control and Prevention for laboratory confirmation of human influenza infection [28, 29].

Influenza and HA Antibody Enzyme-Linked Immunosorbent Assay

Titers of anti-influenza antibodies against detergent disrupted A/Memphis/7/01 (H1N1) virions in plasma and respiratory secretions were determined by a 2-step screen and titration enzyme-linked immunosorbent assay method as previously described [27] (Supplementary Materials).

Hemagglutination Inhibition and Microneutralization Assays

H1 and H3 subtype-specific antibody hemagglutination inhibition (HI) and microneutralization (MN) titers were determined based on the methods of the WHO Global Influenza program [30]. The viral antigens used in these assays were the A/Memphis/7/01 (H1N1) and A/Memphis/1/90 (H3N2) stocks grown in 10-day-old embryonated chicken eggs (Charles River Laboratories).

Pseudotyped Neutralization Assay

The ability of serum antibody to prevent entry of a recombinant lentiviral vector expressing HA protein and luciferase reporter genes was tested as previously described [31, 32]. 293T cells were cotransfected with pseudotyped lentiviral vectors encoding 400 ng of either H1 HA (A/New Caledonia/20/99) or H3 HA(A/Beijing/352/1989) and a luciferase reporter gene that were first titrated by serial dilution. To ensure proteolytic activation of HA in the pseudoviruses, a human type II transmembrane serine protease TMPRSS2 gene was also transfected into the cells. Similar amounts of pseudovirus (p24 approximately 6.25 ng/mL) were then incubated with indicated amounts of rhesus plasma for 20 minutes at room temperature and added to 293A cells (10 000 cells/well in a 96-well plate) (50 μL/well, in triplicate). Plates were then washed and replaced

Table 1. Effect of Cationic Lipid/DNA Complex on Matched and Heterosubtypic Influenza-Specific Plasma Antibody Titers and Virus Replication

		vRNA in Tracheal Secretions		H1N1(A/Memphis/7/2001)–Specific Antibody Postimmunization					
				Plasma HI Titers[a]			Plasma IgG Titers[b]		
Animal Number	Sex	Peak, Log_{10}	Day of Peak	Week 0	Week 6[c]	Fold Increase[d]	Week 0	Week 6[c]	Fold Increase[d]
Whole inactivated A/Memphis/7/01 (H1N1) + CLDC									
32207	F	5.1	1	<4	64	16	800	51 200	64
33287	F	4.5	1	<4	256	64	800	25 600	32
35001	M	4.3	1	<4	64	16	800	25 600	32
34706	M	5.5	2	<4	32	8	800	12 800	16
33320	F	5.3	2	<4	128	32	800	102 400	128
Mean		4.9	1.4	<4	108.8	27.2	800	43 520	54
Whole inactivated A/Memphis/7/01 (H1N1)									
34235	F	6.3	1	<4	16	4	800	3200	4
34328	F	5.6	1	<4	32	8	800	12 800	16
34737	M	5.1	1	<4	32	8	800	12 800	16
34771	F	5.9	1	<4	16	4	800	6400	8
34955	M	5.7	2	<4	8	2	800	6400	8
Mean		5.7	1.2	<4	20.8	5.2	800	8320	10
Whole Inactivated A/Memphis/1/90 (H3N2) + CLDC [H3-WIV/CLDC]									
34865	M	4.8	1	<4	<4	1	800	51 200	64
35125	F	6.4	2	<4	<4	1	800	25 600	32
35496	M	5.8	1	<4	8	2	800	25 600	32
35177	M	5.3	1	<4	<4	1	800	25 600	32
36411	M	4.3	2	<4	<4	1	800	51 200	64
34823	F	4.6	1	<4	<4	1	800	12 800	16
36623	F	5.9	1	<4	<4	1	800	25 600	32
36778	F	6.7	3	<4	<4	1	800	25 600	32
37266	F	4.4	1	<4	<4	1	800	51 200	64
38455	F	4.8	1	<4	<4	1	800	51 200	64
Mean		5.3	1.4	<4	4.4	1.1	800	34 560	43
Whole inactivated A/Memphis/1/90 (H3N2)									
31139	F	6.3	1	<4	<4	1	800	6400	8
34781	M	5.9	2	<4	<4	1	800	6400	8
35503	M	5.7	3	<4	<4	1	800	3200	4
35581	M	5.6	2	<4	<4	1	800	12 800	16
36330	M	6.3	1	<4	<4	1	800	1600	2
Mean		6.0	1.8	<4	<4	1.0	800	6080	8
No immunization									
32698	F	5.4	1	…	<4	…	…	800	…
34380	F	5.6	1	…	<4	…	…	800	…
35290	F	5.6	1	…	<4	…	…	800	…
35733	F	6.6	2	…	<4	…	…	800	…
36317	M	6.5	1	…	<4	…	…	800	…
32322	F	5.7	1	…	<4	…	…	800	…
32787	F	6.8	1	…	<4	…	…	800	…
34511	F	6.5	1	…	<4	…	…	800	…
34952	F	5.5	2	…	<4	…	…	800	…
36547	F	5.8	1	…	<4	…	…	800	…
Mean		6.0	1.2	…	<4	…	…	800	…

Abbreviations: CLDC, cationic lipid/DNA complex; F, female; HI, hemagglutination inhibition; IgG, immunoglobulin G; M, male; vRNA, viral RNA.

[a]The lowest dilution tested was 1:4, and samples that were negative at this dilution are designated <4.

[b]The lowest dilution tested was 1:800, and some samples were positive at this dilution. Presumably the preimmunization samples are positive due to immunity to previous natural infections with unrelated strains of influenza A virus.

[c]Day of A/Memphis/7/2001 (H1N1) challenge.

[d]Titer at week 6 postimmunization (PI) (day of challenge) relative to the week 0 PI.

with fresh media 2 hours later, and luciferase activity was measured after 24 hours.

Antibody-Mediated Natural Killer Cell Activation Assay

The ability of serum antibody to activate natural killer (NK) cells in the presence of influenza proteins was determined by modification of a previously described method [33] that is described in the Supplementary Methods. In brief, 96-well round-bottom plates were coated with 600 ng/well of inactivated H1N1 virus or HA/M1/NP proteins with homology to A/Memphis/7/01 in 1X phosphate-buffered saline and incubated with heat-inactivated RM plasma. Freshly isolated naive RM peripheral blood mononuclear cells (PBMCs) supplemented with 5 μg/mL Brefeldin A (Sigma) and 5 μg/mL GolgiStop (BD Biosciences) were added to each well and incubated for 5 hours at 37°C. Cells were washed, then incubated with surface staining antibodies (anti-CD3, anti-CD14, anti-CD107a, and anti-NKG2a). Cells were, fixed, permeabilized, and intracellularly stained for interferon gamma (IFN-γ). At least 200 000 lymphocytes events were collected on a FACSAria flow cytometer (BD Biosciences) and analyzed using FlowJo version 10.0.7 (Tree Star).

H1N1-Specific T-Cell Responses

For intracellular staining to detect influenza-specific T cells in PBMCs, previously reported methods were used [26, 34] and are described in the Supplementary Methods. Cryopreserved cells were stimulated with 1 μg/mL of whole inactivated H1N1 (A/New Caledonia/20/99). H1N1-stimulated PBMCs were surface stained for CD3, CD4, CD8, and a mixture of CD107a and CD107b. Cells were fixed, permeabilized, and intracellularly stained for IFN-γ, tumor necrosis factor, and interleukin 2. At least 100 000 events were collected on a FACSAria and analyzed using FlowJo software.

Statistical Analysis

Data are reported as the mean and the standard error of the mean for each animal group, using Prism 7.0a software (GraphPad, San Diego, California). Two groups were compared with a 2-tailed *t* test, and 3 groups were compared with a 1-way analysis of variance (ANOVA) with the Tukey–Kramer post hoc test. Area under the curve (AUC) was calculated by Prism using the trapezoid rule, $\Delta X \times (Y1 + Y2) / 2$, in which the area of a trapezoid under the curve is repeatedly calculated for a series of XY points with equally spaced X values.

RESULTS

CLDC Adjuvant Enhanced Protection From H1N1 Challenge in H1-WIV–Immunized RMs

All animals were immunized at week 0 and week 2 PI (Table 1). To determine WIV efficacy, RMs immunized with H1-WIV were challenged with intranasal and intratracheal inoculation of A/Memphis/7/01 (H1N1) at week 6 PI, and vRNA levels in respiratory secretions were determined [26]. Peak vRNA levels ($\log_{10}$ vRNA copies/mL) and the total level of vRNA shed over the 14-day postchallenge follow-up period were calculated by converting the influenza vRNA data from each RM into an AUC of the H1-WIV–immunized groups and compared to the unimmunized control group. Influenza RNA was detectable in the tracheal secretions of all H1-WIV–immunized and control RMs on days 1, 2, and 3 after challenge (Figure 1A). Although vRNA was readily detectable in tracheal secretions of the H1-WIV and unimmunized control RMs at day 7 after challenge, it was rarely detected in tracheal secretions of H1-WIV/CLDC RMs (Figure 1A). Furthermore, H1-WIV/CLDC RMs

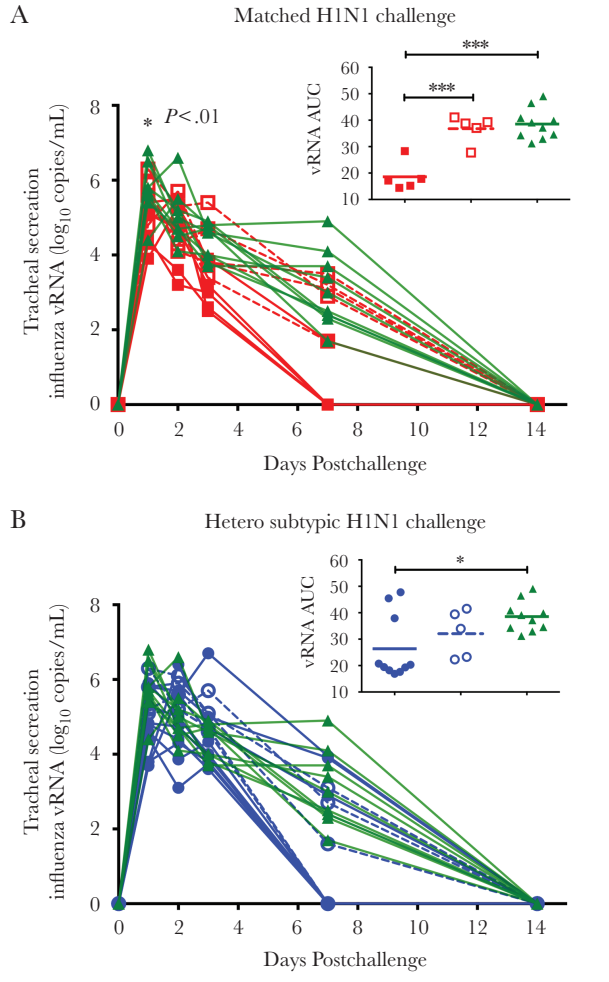

Figure 1. Virus replication in the lower respiratory tract after influenza A virus challenge. Mean viral RNA (vRNA) copy number in tracheal lavages of unimmunized control rhesus macaques (RMs) compared to RMs immunized with the whole inactivated H1N1 influenza vaccine (H1-WIV) ± cationic lipid/DNA complex (CLDC) (*A*), or RMs immunized with the inactivated H3N2 influenza vaccine (H3-WIV) ± CLDC (*B*). The inset scatter graphs compare vRNA area under the curve (AUC). Mean AUC values were compared using analysis of variance and Bonferroni post hoc test. $^*P < 0.05$; $^{***}P < 0.001$ ▲, Unimmunized control RMs; ■, H1-WIV/CLDC RMs (n = 5); □, H1-WIV RMs; ●, whole inactivated H3N2 influenza vaccine (H3-WIV)/CLDC RMs; ○, H3-WIV RMs.

had 10-fold lower mean peak vRNA compared with control RMs ($P < 0.01$, ANOVA). Based on AUC, total vRNA shed was about 2-fold lower in H1-WIV/CLDC RMs compared with H1-WIV and unimmunized control RMs ($P < 0.001$ for both, ANOVA; Figure 1A). Surprisingly, there was no evidence that the unadjuvanted H1-WIV had any effect on H1N1 challenge virus replication, as the mean peak vRNA levels and mean vRNA AUC value in H1-WIV and unimmunized RMs were not significantly different (Figure 1A).

CLDC Adjuvant Enhanced Protection After H1N1 Challenge of H3-WIV–Immunized RMs

Influenza vRNA was detectable in the tracheal secretions of all H3-WIV–immunized RMs on days 1–3 after H1N1 challenge (Figure 1B) and remained detectable at day 7 after challenge. Mean peak vRNA levels and mean vRNA AUC values in H3-WIV and unimmunized RMs were not significantly different from those of controls. A higher proportion of H3-WIV–immunized RMs shed vRNA at day 7 PI compared with H3-WIV/CLDC RMs (3/5 vs 3/10, respectively); although the mean peak vRNA level in the H3-WIV/CLDC RMs was lower than in control RMs, the difference was not significant. However, the mean vRNA AUC of H3-WIV/CLDC RMs was 1.5-fold lower compared with unimmunized RMs ($P < 0.05$, ANOVA; Figure 1B). Thus, the H3-WIV/CLDC vaccine protected a large proportion of immunized RMs from uncontrolled virus replication following heterosubtypic H1N1 challenge (Figure 1B).

CLDC Adjuvant Did Not Enhance H1N1-Specific T-Cell Responses Prior to Challenge

To determine if vaccine-elicited T-cell responses contributed to the observed heterosubtypic protection, we determined the fold-change from week 0 to week 6 PI in the H1N1-specific $CD4^+$ and $CD8^+$ T-cell responses of each RM, and the mean frequencies of specific T cells in the groups were compared. H1N1-specific $CD4^+$ and $CD8^+$ T-cell responses were not detected on the day of immunization in PBMCs from most of the RMs (Figure 2A). After 2 immunizations, a similar proportion of RMs immunized with the H1-WIV or H1-WIV/CLDC vaccines had modest increases in H1N1-specific $CD4^+$ T-cell responses, but limited or no H1N1-specific $CD8^+$ T-cell responses were detected (Figure 2B). The strength of H1N1-specific $CD4^+$ and $CD8^+$ T-cell responses in the H3-WIV and H3-WIV/CLDC RMs remained unchanged on the day of challenge relative to preimmunization (Figure 2B).

Following H1N1 challenge, the H1-WIV/CLDC RMs had the largest proportion of RMs with anti-H1N1 T-cell responses, and the H1N1-specific $CD4^+$ T-cell responses in this group were 30-fold higher than in H1-WIV–immunized RMs (Figure 2A). Similarly, compared to H3-WIV–immunized RMs, a higher proportion of H3-WIV/CLDC–immunized RMs produced anti-H1N1 $CD4^+$ T-cell responses that were also 16 times stronger (Figure 2A). After challenge, more H1-WIV/CLDC RMs and H3-WIV/CLDC RMs made anamnestic H1N1-specific $CD8^+$ T-cell responses, and the mean strength of the responses were higher than among H1-WIV RMs (Figure 2B). Thus, although vaccine elicited effector T cells present at the time of challenge did not correlate with the protection from H1N1 challenge (Supplementary Table 2), the WIV/CLDC vaccines did prime for strong anamnestic and cross-reactive T-cell responses after challenge.

CLDC Adjuvant Enhanced H1N1 Binding Antibody Responses to H1N1 Virions That Correlated With Protection

To compare the strength and durability of vaccine-induced antibody responses, longitudinal plasma antibody titers were converted into AUC values; and mean plasma and tracheal antibody titers in

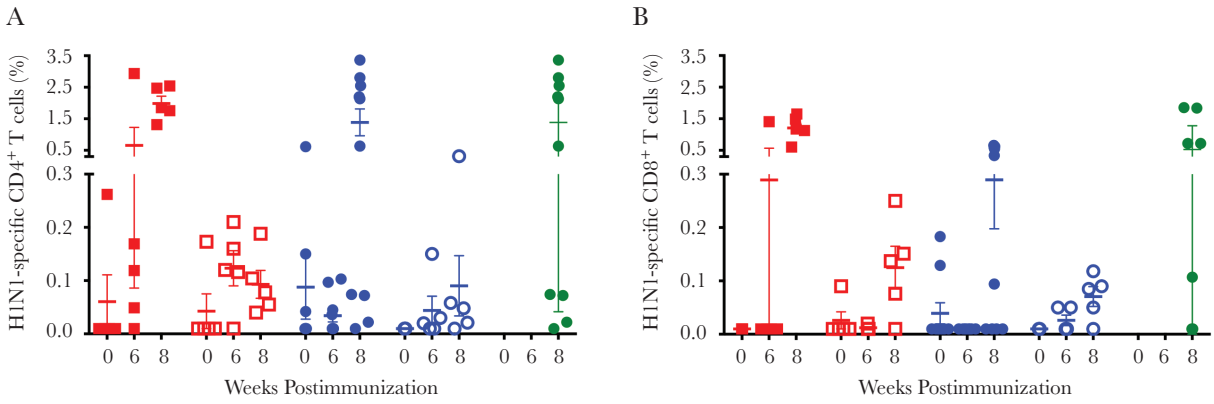

Figure 2. Vaccine-induced H1N1-specific $CD4^+$ and $CD8^+$ T-cell responses. The H1N1-specific T-cell frequencies reported are the sum of the different combinations of effector molecules (CD107ab, interleukin 2, interferon-γ, and tumor necrosis factor) in the $CD4^+$ or $CD8^+$ T-cell populations after H1N1 stimulation. *A*, Frequency of H1N1-specific $CD4^+$ T cells on day of immunization (week 0 postimmunization [PI]), day of virus challenge (week 6 PI), and day 7 after virus challenge (week 8). *B*, Frequency of H1N1-specific $CD4^+$ T cells on day of immunization (week 0 PI), day of virus challenge (week 6 PI), and day 14 after virus challenge (week 8). ■, Whole inactivated H1N1 influenza vaccine (H1-WIV)/cationic lipid/DNA complex (CLDC) rhesus macaques (RMs); □, H1-WIV RMs; ●, whole inactivated H3N2 influenza vaccine (H3-WIV)/CLDC RMs; ○, H3-WIV RMs; ●, unimmunized controls (only tested after virus challenge). Bars indicate the mean frequency in a group and the standard error of the mean.

samples collected close to the time of H1N1 challenge were compared. All immunized RMs developed plasma H1N1 immunoglobulin G (IgG) binding antibodies (Figure 3A) and the responses were stronger and more durable (AUC) in H1-WIV/CLDC vs H1-WIV RMs and H3-WIV/CLDC vs H3-WIV RMs (both $P < 0.01$, t test). By the day of challenge, the mean H1N1-specific IgG titers were 10-fold higher for both H1-WIV/CLDC vs H1-WIV RMs and H3-WIV/CLDC vs H3-WIV RMs (Figure 3A). In fact, at week 6 PI, plasma anti-H1N1 IgG titers in H1-WIV/CLDC RMs and H3-WIV/CLDC RMs were similar (Table 1). Plasma H1N1-specific immunoglobulin A (IgA) antibody levels followed a similar trend as the H1N1-specific IgG antibody responses, except H1N1-specific IgA AUC values in the H1-WIV and H3-WIV RMs were similar (Figure 3B). In tracheal secretions, H1N1-specific IgG titers were higher in the H1-WIV/CLDC vs H1-WIV RMs and H3-WIV/CLDC vs H3-WIV RMs ($P < 0.05$; Figure 3C).

High plasma H1N1-specific IgG antibody titers were associated with protection in both H1-WIV and H3-WIV RMs, as they inversely correlated with peak vRNA levels in tracheal secretions after H1N1 influenza virus challenge (Figure 3D; Supplementary Table 2). Furthermore, in H3-WIV RMs, H1N1-specific IgG binding antibodies in tracheal secretions inversely correlated with peak vRNA titers in tracheal secretions (Figure 3D; (Supplementary Table 2). These results suggest a role for both systemic and local antibody responses in the heterosubtypic protection observed in these animals.

CLDC Adjuvant Did Not Enhance Cross-Reactive Influenza A Virus H1-HA Binding Antibody or Neutralizing Antibody Responses

To determine if the H3-WIV elicited H1N1-specific binding antibodies and nAbs in the RMs, we characterized vaccine-induced H1-HA binding antibody titers, HI, MN, and

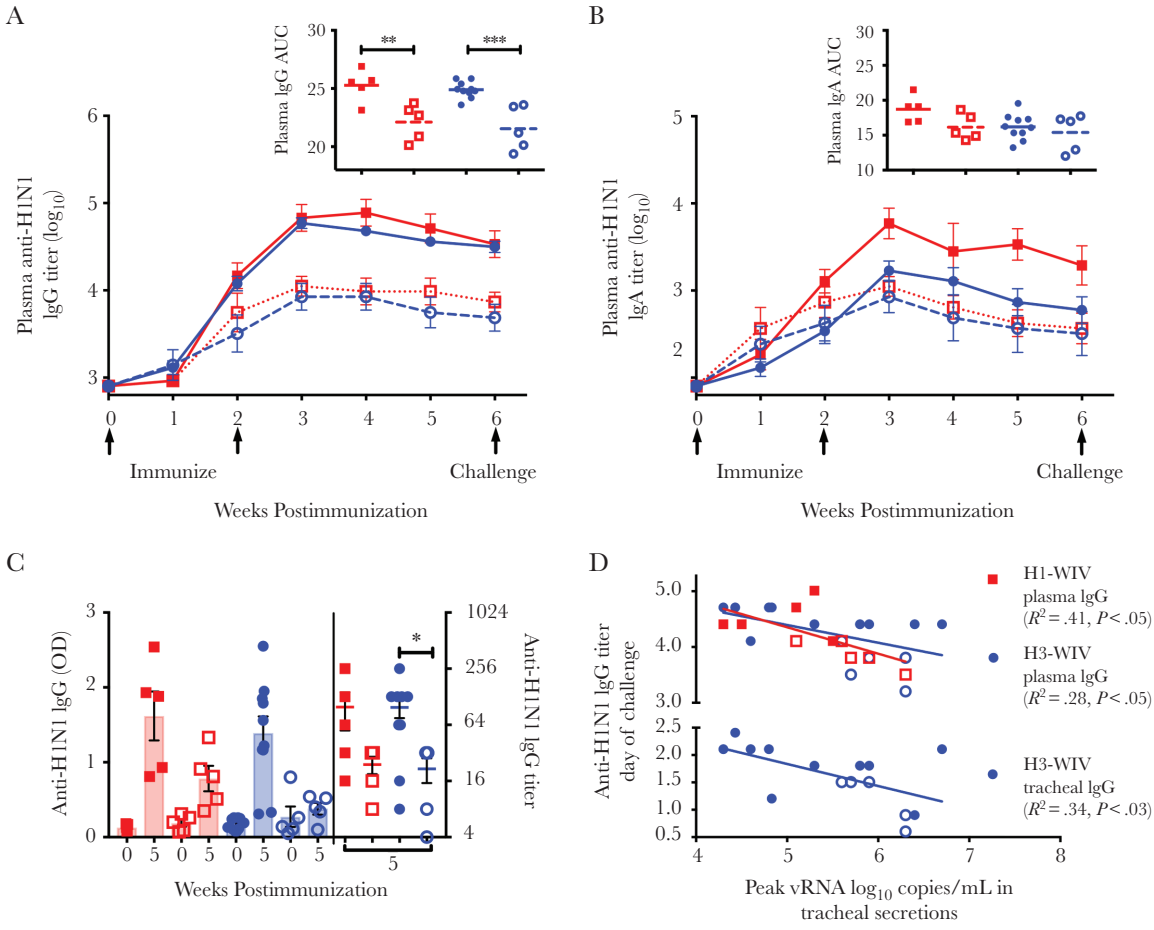

Figure 3. Vaccine-induced H1N1-specific antibody responses. Mean plasma H1N1-specific immunoglobulin G (IgG) antibody (*A*) and immunoglobulin A (IgA) antibody (*B*) enzyme-linked immunosorbent assay (ELISA) titers. *C*, H1N1-specific IgG antibody (optical density [OD]) responses in tracheal secretions collected before immunization (week 0) and 4 days prior to H1N1 challenge (week 5). The left side of the graph shows the results of the antibody screening ELISA on the week 0 and week 5 samples tested at a 1:4 dilution, and the right side of the graph indicates the titer of specific antibody at week 5. *D*, The relationship between the level of antibodies to whole-virion H1N1 in plasma and tracheal secretions and peak viral RNA (vRNA) in tracheal secretions. In the insets in panels *A–C*, mean area under the curve (AUC) values were compared using analysis of variance with Bonferroni post hoc test. *$P < 0.05$; **$P < 0.001$; ***$P < .001$. ■, Whole inactivated H1N1 influenza vaccine (H1-WIV)/cationic lipid/DNA complex (CLDC) rhesus macaques (RMs); □, H1-WIV RMs; ●, whole inactivated H3N2 influenza vaccine (H3-WIV)/CLDC RMs; ○, H3-WIV RMs. Arrows below the x-axis indicate timing of the immunizations and the subsequent challenge with H1N1 (A/Memphis/7/2001). Bars indicate the mean value in a group and the standard error of the mean.

pseudotyped neutralization (PN) plasma antibody responses to the H1N1 challenge virus (Figure 4). The PN assay is a useful tool for defining the specificity of neutralizing antibodies targeting influenza HA proteins [31, 32]. The assay can identify antibodies targeting conserved stem regions of HA, which the MN and HI assays fail to detect [35]. H1-HA binding plasma antibodies were not detected prior to immunization, but strong responses were elicited in H1-WIV and H1-WIV/CLDC RMs, but not in the H3-WIV CLDC RMs, by 6 weeks PI (Figure 4A). H1-specific HI antibodies were detected in plasma of the RMs that received the H1-WIV but not in those that received the H3-WIV (Figure 4B; Table 1). The H1-WIV/CLDC vaccine produced higher H1-specific HI titer AUC values compared with the unadjuvanted H1-WIV vaccine ($P < 0.02$, t test), and HI titers were higher in the H1-WIV/CLDC RMs compared with H1-WIV RMs at week 6 PI ($P < 0.01$, t test). Similarly, H1-specific MN antibodies were detectable only in RMs that received H1-WIV, and the titers in H1-WIV/CLDC RMs were significantly higher than in H1-WIV RMs at week 6 PI ($P < 0.02$, t test; Figure 4C). The RMs immunized with H3-WIV had no detectable H1-specific PN antibody activity (Figure 4D), but did generate robust H3-specific PN antibody activity (data not shown). Thus, H1-specific IgG binding or neutralizing antibodies were not associated with protection from H1N1 challenge in the H3-WIV–immunized RMs (Supplementary Table 2).

CLDC Adjuvant Enhances Antibody Responses to Conserved NP and M1 Influenza Proteins

To determine if immunization enhanced antibody responses targeting conserved internal proteins, we characterized the IgG antibody responses to NP and M1 from an H1N1 strain that is highly homologous to the H1N1 challenge virus. A few of the RMs had weak but detectable NP-specific and M1-specific plasma IgG antibodies prior to immunization (Figure 5).

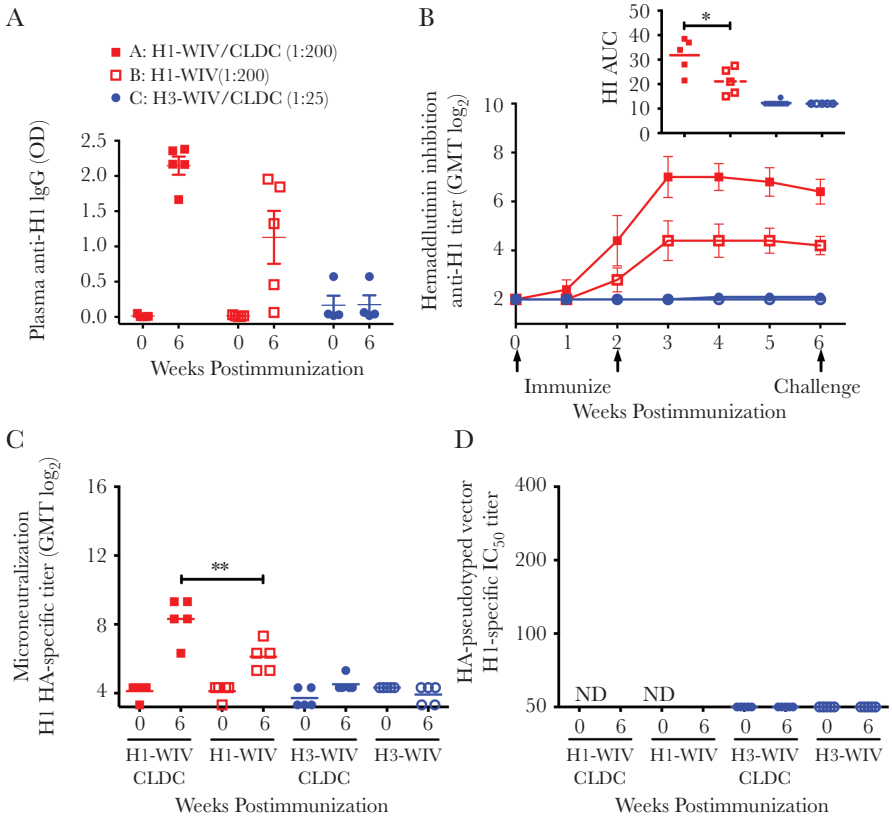

Figure 4. Vaccine-induced H1 hemagglutinin (HA)–specific antibody responses. *A*, Plasma H1-specific (A/New Caledonia/20/99) immunoglobulin G antibody responses before immunization (week 0) and prior to H1N1 challenge (week 6). The graph shows the results of the antibody screening enzyme-linked immunosorbent assay on the week 0 and week 6 samples from a subset of immunized rhesus macaques (RMs) tested at a 1:200 (whole inactivated H1 influenza vaccine [H1-WIV]) or 1:25 (whole inactivated H3N2 influenza vaccine [H3-WIV]) dilution. *B*, Mean plasma HA inhibition antibody titers against A/Memphis/7/2001. Arrows below x-axis indicate timing of the immunizations and the subsequent challenge with H1N1 (A/Memphis/7/2001) at week 6 postimmunization (PI). The lowest dilution tested in the hemagglutination inhibition assay was 1:4. *C*, H1N1-specific (A/Memphis/7/01) microneutralizing antibody responses at week 0 PI (day of immunization) and week 6 PI (day of challenge). *D*, H1-specific neutralization antibody activity measured using a luciferase reporter with 1999 New Caledonia H1 HA-pseudotype lentiviral vector. Mean area under the curve values were compared using analysis of variance and Bonferroni post hoc test. *$P < 0.05$; **0.01. ■, H1-WIV/cationic lipid/DNA complex (CLDC) RMs; □, H1-WIV RMs; ● H3-WIV/CLDC RMs; ○, H3-WIV RMs. Bars indicate the mean value in a group and the standard error of the mean. Abbreviations: AUC, area under the curve; CLDC, cationic lipid/DNA complex; GMT, geometric mean titer; HA, hemagglutinin; IC_{50}, 50% inhibitory concentration; IgG, immunoglobulin G; ND, not done; OD, optical density; WIV, whole inactivated influenza vaccine.

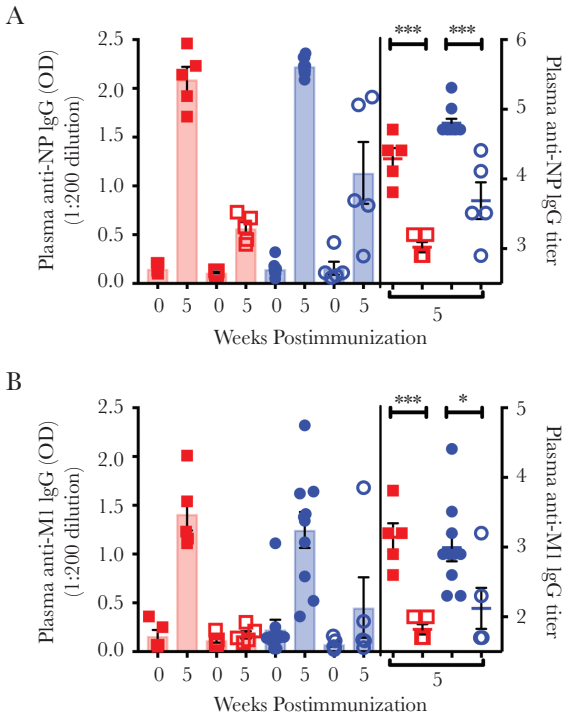

Figure 5. Vaccine-induced nucleoprotein (NP)– and M1-specific plasma antibody responses. Mean NP-specific (*A*) and M1-specific (*B*) immunoglobulin G (IgG) antibody (optical density [OD]) enzyme-linked immunosorbent assay (ELISA) titers prior to H1N1 (A/Memphis/7/01) challenge. The left side of the graphs show the results of the antibody screening ELISA on the week 0 and week 5 samples tested at a 1:200 dilution, and the right side of the graphs indicate the titer of specific antibody at week 5 (log10). The mean week 5 titers between the 2 matched (whole inactivated H1N1 influenza vaccine [H1-WIV]) and 2 heterosubtypic (whole inactivated H3N2 influenza vaccine [H3-WIV]) challenge groups were compared separately using a 2-tailed *t* test. $^{*}P < 0.05$; $^{***}P < 0.001$. ■, H1-WIV/cationic lipid/DNA complex (CLDC) rhesus macaques (RMs); □, H1-WIV RMs; ●, H3-WIV/CLDC RMs; ○, H3-WIV RMs. Bars indicate the mean value in a group and the standard error of the mean.

However, by week 6 PI, the mean NP-specific IgG titers for H1-WIV/CLDC and H3-WIV/CLDC were 20-fold and 8-fold higher than the H1-WIV and H3-WIV–immunized RMs, respectively (both $P < 0.001$, *t* test; Figure 5A). Similarly, mean M1-specific IgG titers for H1-WIV/CLDC and H3-WIV/CLDC RMs were 30- and 8-fold higher than the H1-WIV and H3-WIV–immunized RMs, respectively ($P < 0.001$, $P < 0.05$; *t* test; Figure 5B).

CLDC Adjuvant Enhances Cross-Reactive NP-Specific NK Cell–Activating Antibodies

To assess the effect of CLDC on induction of H1N1-specific Fc-functional antibodies, we determined whether plasma from H1-WIV or H3-WIV–immunized RMs, in the presence of inactivated H1N1 virus or influenza proteins (HA/M1/NP) that are homologous to the H1N1 challenge virus, activated $CD3^{-}CD14^{-}NKG2a^{+}$ NK cells from influenza-naive RM donors. The fold change in the relative frequency of NK cells expressing CD107a, a marker of degranulation, of each RM from week 0 to week 6 PI was calculated and the mean fold-change value of each group was compared (Figure 6A–D). All immunized RMs developed detectable H1N1-specific antibody-dependent cellular cytotoxicity (ADCC) antibodies (Figure 6A). However, H1-HA–specific ADCC antibodies were only detectable in the 2 groups of RMs immunized with H1-WIV (Figure 6B). Although NP- or M1-specific ADCC antibodies were detected in some RMs prior to immunization, vaccination enhanced the NP- and M1-specific ADCC antibodies in most H1-WIV– and H3-WIV–immunized RMs (Figure 6C and 6D).

Although we detected NP- and M1-specific NK cell–activating antibodies at 6 weeks PI at a 1:2 dilution, finely titrating these responses could reveal differences in the magnitude of the response. The mean M1-specific ADCC antibody levels were similar in the adjuvanted and unadjuvanted H3-WIV–immunized RM groups (Figure 6D). However, the mean NP-specific ADCC titer in H3-WIV/CLDC RMs was 8-fold higher than in H3-WIV–immunized RMs ($P < 0.01$, *t* test; Figure 6C). Finally, plasma H1N1 NP-specific ADCC antibodies inversely correlated with both the peak vRNA titers and vRNA AUC in tracheal secretions of the H3-WIV–immunized RMs (Figure 6C), suggesting that NP-specific ADCC-mediating antibodies play a role in the heterosubtypic protection seen in these animals.

DISCUSSION

Addition of a CLDC adjuvant to inactivated H3N2 virions resulted in a vaccine that protects RMs against heterosubtypic H1N1 virus challenge. While sterilizing immunity was not elicited, virus replication was suppressed. The goal of the candidate universal IAV vaccines that are furthest down the developmental pipeline is to elicit broadly cross-reactive antibodies to the conserved stem region of HA [36, 37]. However, the heterosubtypic protection elicited by the WIV/CLDC vaccine in this study was not associated with cross-reactive binding, neutralizing, or NK cell–activating HA-specific antibodies. Rather, cross-reactive H1N1-specific binding antibodies in plasma and tracheal secretions to virion proteins other than HA, and plasma NP-specific binding antibodies and NP-specific non-neutralizing ADCC activity, correlated with heterosubtypic protection. NP is expressed on the surface of infected cells, providing antibodies a target [38]. CLDC added to WIV also enhanced M1-specific IgG binding antibodies dramatically, however because M1 is not expressed on the surface of infected cells, it is not clear how anti-M1 antibodies could contribute to control of virus replication. The M1 response may simply track with the anti-NP antibody response as a marker of immune response to internal virus proteins in general. Thus, the adjuvant promoted antibody responses against conserved internal influenza proteins that were associated with heterosubtypic protection.

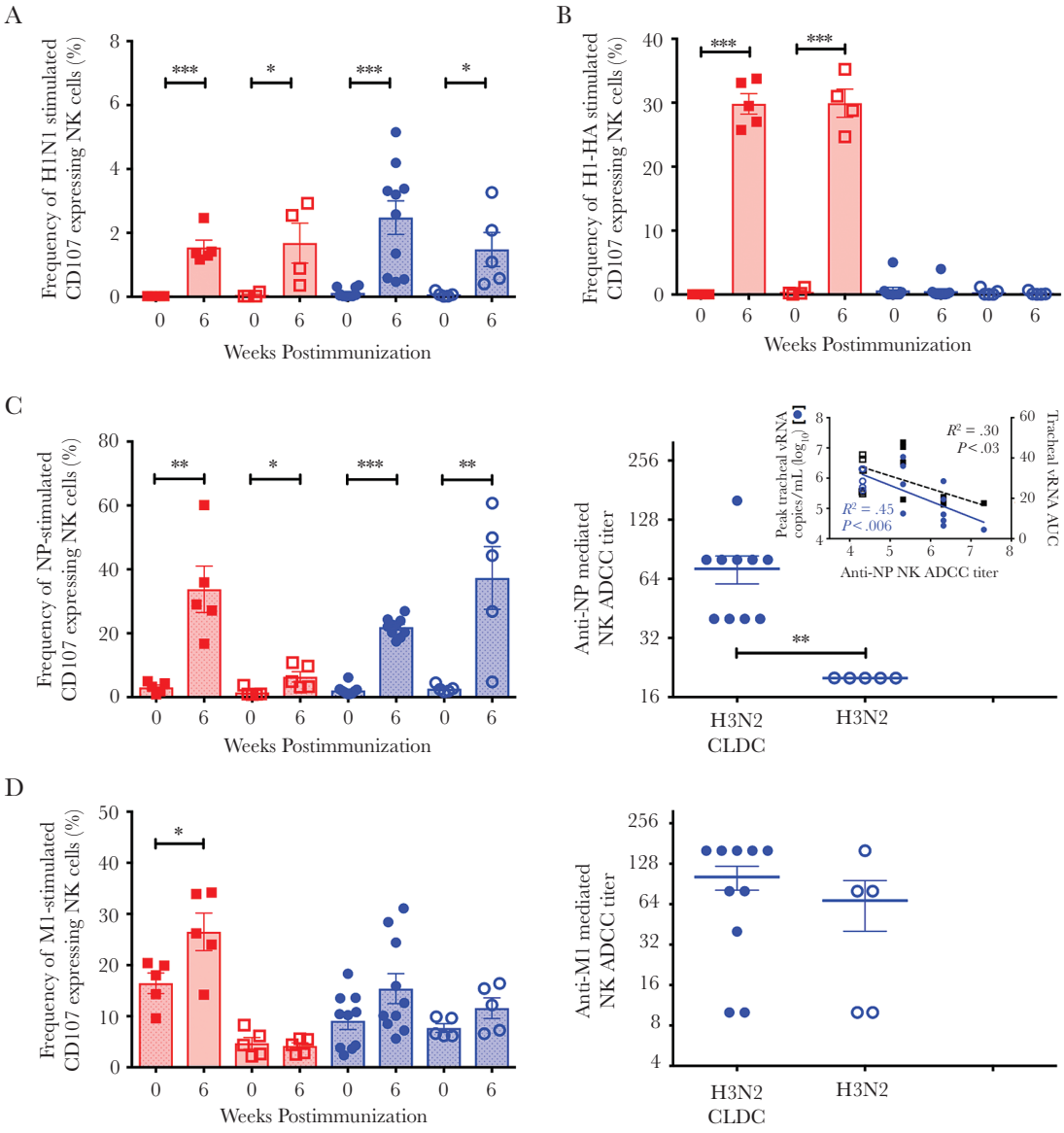

Figure 6. Vaccine-induced antibody -dependent cellular cytotoxicity (ADCC)–mediated natural killer (NK) activation to H1N1. Week 0 postimmunization (PI) (day of immunization) and week 6 PI (day of challenge) frequency of $CD14^{-}CD3^{-}NKG2a^{+}$ NK cells expressing CD107a in response to whole H1N1 virions (A/New Caledonia/20/99) (*A*); H1 hemagglutinin (A/New Caledonia/20/99) (*B*); nucleoprotein (NP) (A/Ann Arbor/6/1960) (*C*); and M (A/PR8/Mt Sinai) (*D*) influenza viral proteins. ADCC antibody endpoint titers to NP (A/Ann Arbor/6/1960) and M1 (A/PR8/Mt Sinai) were determined by 2-fold dilutions of rhesus macaque (RM) plasma samples from week 6 PI, where a threshold of 2-times background (plasma but without viral protein) was used to determine endpoint. *C*, inset: Correlation between the frequency of $CD107^{+}$ NK cells after stimulation with NP and peak viral RNA (vRNA) area under the curve (AUC) in tracheal secretions. Mean values between the matched (whole inactivated H1N1 influenza vaccine [H1-WIV]) and heterosubtypic (whole inactivated H3N2 influenza vaccine [H3-WIV]) challenge groups were compared using a 2-tailed *t* test. *P < 0.05; **p<0.01; ***$P < 0.001$. ■, H1-WIV/cationic lipid/DNA (CLDC) RMs; □, H1-WIV RMs; ●, H3-WIV/CLDC RMs; ○, H3-WIV RMs. Bars indicate the mean frequency in a group and the standard error of the mean.

We did not determine why the unadjuvanted H1-WIV RMs were not protected against homologous virus challenge; however, CLDC improved antibody responses to both the surface glycoproteins and the internal structural proteins. With CLDC added to H1-WIV, viral replication was reduced by 100- to 1000-fold 7 days after challenge, and the protection correlated with plasma H1-specific HA antibody titers at time of challenge. Moreover, the degree of protection from the matched challenge virus in the H1-WIV/CLDC RMs was greater compared to the protection from heterosubtypic challenge. Compared to control animals, the peak levels of vRNA were significantly lower after H1-WIV/CLDC vaccination, but not after mismatched H3-WIV/CLDC vaccination. For comparison, the decrease in peak and AUC vRNA levels was similar to that found in RMs challenged with the same H1N1 influenza virus stock after treatment with the antiviral drug oseltamivir [26]. While the H3-WIV/CLDC RMs did not have lower peak vRNA levels, the vaccine did shorten the duration on virus replication to the

same degree as adjuvanted matched Fluzone and better than unadjuvanted matched Fluzone [27]. Thus, the reduction in virus replication in H3-WIV/CLDC RMs after heterosubtypic virus challenge exceeded the reduction in RMs vaccinated with a unadjuvanted licensed vaccine matched to the same challenge virus used in the study reported here.

Vaccine-elicited T cell-mediated immunity to M2 and NP antigens can significantly reduce heterosubtypic challenge virus replication in mice and ferrets [39, 40]. Furthermore, passive transfer of anti-NP antibodies and vaccine-elicited antibodies against the extracellular domain of M2 (M2e) protects mice from lethal influenza challenge [41–43]. How anti-NP antibody mediates protection remains unclear, but NP is found on the surface of influenza-infected cells in vitro [38, 44]. IAV infection induces robust ADCC antibodies in humans and nonhuman primates (reviewed in [45]). In humans, high levels of antibodies that mediate ADCC correlate with decreased IAV replication and disease despite the absence of detectable nAbs [46]. Thus, NP and M2e may provide highly conserved targets for ADCC (reviewed in [12]) that could be included in a universal IAV vaccine. These studies support the conclusion that nonneutralizing antibody responses to conserved internal proteins contributed to protection from heterosubtypic IAV challenge in H3-WIV/CLDC–vaccinated RMs. Testing and development of vaccines that target conserved internal structural proteins, as well as vaccines targeting the HA stem in the pursuit of a universal IAV vaccine, may be prudent.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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